Title
Antagonistic control of RNA base-pairing in the spliceosome cycle

Author
Raghunathan, Pratima Lalita

Publication Date
1997

Peer reviewed|Thesis/dissertation

ANTAGONISTIC CONTROL OF RNA BASE-PAIRING IN THE SPLICEOSOME CYCLE

by

PRATIMA LALITA RAGHUNATHAN

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: . . . . . .

To my family:

Mom

Dad

Vijay

Kurt

ACKNOWLEDGMENTS

The body of this thesis captures perhaps one-tenth of my graduate school experience. Somewhat unnaturally, this short section expresses the remainder. Even more than the scientific training I received, I value the people in the departmental community who have both supported and challenged me. and the bonds with my family that have been . I have also been fortunate to have a wonderful network of family

Kurt Zingler deserves first mention as the greatest of these friends: he has been my unswerving advocate, loving partner, and toughest critic. Kurt has boundless reserves of patience and good cheer, which he liberally expended on me during my gloomiest days. He has brought much laughter and light into my life, and his buoyancy is a constant source of inspiration. It is no exaggeration to say that I would not have finished this thesis without his reassurance and resourcefulness (not to mention his laundry service and considerable figure-making skills).

Tanya Awabdy was the first classmate I met during orientation to graduate school, and we have been stuck like glue ever since. After spending our first year doing nearly everything together -- studying in S980, eating at Moffitt Cafeteria, vacationing to Mexico -- we had no choice but to join the same lab. Tanya's delightful silliness has kept me laughing throughout the years, and her warmth and compassion as a listener will keep me grounded for many years to come.

My parents have given me life and the liberty to pursue happiness. My mother, in particular, has opened doors for me that had been closed to her, and has taught me the value of perseverance. My father was the first scientist to whom I was exposed, and he is probably responsible for infecting me with his fascination for science and natural curiosity about the world.

My brother, Vijay Iyer, has turned from the well-travelled path to dedicate his life to his passion, music. His courageous choice has inspired me to rethink my own priorities. Vijay has been an unusually close confidant for a younger brother. Perhaps one day he will forgive me for insisting we share a single towel while travelling together in Europe.

My advisor, Christine Guthrie, has impressed me with her incisive intelligence, her poise, and ... Dear Christine, please forgive me as this is where my strength left me at 1AM on this 29th day of August, 1997. I shall include a proper acknowledgments when I get just a little more sleep.

Antagonistic Control of RNA Base-Pairing In the Spliceosome Cycle

Pratima Lalita Raghunathan

ABSTRACT

The mechanism of pre-messenger RNA (pre-mRNA) splicing is surprisingly dynamic. The pre-mRNA substrate and small nuclear RNAs (snRNAs) undergo a series of helical exchanges to promote intron removal within the spliceosome, a ribonucleoprotein (RNP) complex. Activation of the spliceosome requires the disruption of extensive intermolecular base-pairing in the U4/U6 small nuclear ribonucleoprotein particle (snRNP). This highly conspicuous yet uncharacterized RNA rearrangement poses a further reassembly problem. If the separated U4 and U6 snRNPs are to participate in later rounds of splicing, the U4/U6 RNA duplex must be re-formed. In this thesis work, I identify two factors, Brr2 and Prp24, that exert antagonistic effects on U4/U6 base-pairing during each spliceosome cycle.

A long-standing hypothesis has been that spliceosomal RNA helices would be dissociated by members of the DEXH/DEAD-box superfamily of putative RNA helicases. Here, I show that the ATP-dependent disruption of the U4/U6 duplex in isolated snRNPs demands the function of Brr2, a spliceosomal DEXH-box protein. A mutation in the helicase-like domain (*brr2-1*) prevents this rearrangement. This is the first demonstration of helical unwinding within an

RNP that depends on the activity of a DEXH-box protein. Brr2 is also proposed to be required for the ATP-dependent separation of U4 from U6 on the spliceosome.

An important unanswered question is how the U4/U6 helices are re-formed for new splicing events. Here, the yeast RNA binding protein Prp24 is shown to reanneal U4 and U6 to promote recycling of spliceosomal snRNPs. In the absence of Prp24, initial rounds of splicing proceed normally. With time, snRNPs containing unpaired U4 and U6 snRNAs accumulate and splicing is inhibited. Addition of purified Prp24 regenerates the U4/U6 snRNP and restores splicing. Interestingly, Prp24 reanneals native U4 and U6 snRNPs much more efficiently than deproteinized snRNAs.

Brr2 and Prp24 are likely to disrupt and reanneal U4/U6 base-pairs at different times, reinforcing the notion of the spliceosome cycle. These studies demonstrate that proteins can govern helical exchanges, and thus are key regulators of spliceosomal dynamics.

Christine Guthrie

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Epilogue

PROLOGUE

In eukaryotes, genes are interrupted by introns, which are removed from pre-messenger RNAs (pre-mRNAs) by an elaborate splicing machinery. For each splicing event, small nuclear ribonucleoprotein particles (snRNPs) and proteins coalesce on pre-messenger RNA, forming a large ribonucleoprotein complex called the spliceosome (reviewed in (Guthrie, 1991; Rymond and Rosbash, 1992; Moore et al., 1993)). This spliceosome catalyzes two transesterification reactions that lead to intron excision. The chemistry of the pre-mRNA splicing reaction recalls that of the self-splicing group II introns, and has led to the proposal that pre-mRNA splicing catalysis may be similarly RNA-based (Cech, 1986). The prevailing view is that the spliceosomal active site may therefore be composed of RNA (Madhani and Guthrie, 1994a; Nilsen, 1994; Ares and Weiser, 1995).

However, in addition to the small nuclear RNAs (snRNAs), the spliceosome consists of numerous proteins of unknown function in both yeast (Guthrie, 1991; Ruby and Abelson, 1991) and mammalian systems (Krämer, 1996; Will and Lührmann, 1997). The mechanism of spliceosome assembly offers some clues to what proteins' roles could be. Like other large nucleoprotein complexes that participate in gene expression, the spliceosome undergoes sequential conformational rearrangements during its assembly and disassembly. The salient and perhaps most distinctive feature of the spliceosome is that its structural transitions require breaking and building of RNA base-pairs. When I began this work, intron recognition was well documented to require the correct timing and placement of helices between the U1 snRNP and the 5' splice site (Séraphin et al., 1988; Siliciano and Guthrie, 1988), and the U2 snRNP and the branchpoint (Parker et al., 1987). The disruption of extensive intermolecular base-pairing between snRNAs

also figured prominently in spliceosome assembly. The duplex U4/U6 snRNP, joined by two long stems with a melting temperature of ~55°C (in yeast) (Brow and Guthrie, 1988), was inferred to undergo complete unwinding on the spliceosome prior to catalysis (Konarska and Sharp, 1987). Interestingly, U6 remained an integral component of the spliceosome, while stripping of U4 did not impede splicing (Yean and Lin, 1991). A provocative speculation at the time postulated that U6 snRNA was the catalytic element of the spliceosome, and that U4 snRNA served as its "antisense negative regulator" or protective shield prior to catalysis (Guthrie and Patterson, 1988). This view of U6 snRNA was bolstered by an explosion of information during my early years as a graduate student. New U2/U6 and U6/pre-mRNA helices were defined through compensatory base-pair analysis (Madhani and Guthrie, 1992; Lesser and Guthrie, 1993) and UV crosslinking studies (Wassarman and Steitz, 1992). These base-pairing interactions were mutually exclusive with helices thought to occur earlier in the assembly process. Consequently, these results reinforced the concept of the dynamic spliceosome, in which RNA duplexes were formed and dissolved with remarkable fluidity. Such RNA rearrangements were thought to drive the construction of an intricate RNA network that executes the chemical steps of exon ligation, the spliceosome's catalytic core (Madhani and Guthrie, 1994a; Nilsen, 1994; Ares and Weiser, 1995).

An unexamined question was how these critical helical transactions were controlled: did proteins regulate unwinding and annealing of these RNAs? Since ATP was required for multiple steps in the in vitro splicing, it was plausible that spliceosomal rearrangements were transduced by proteins that consumed ATP (Ruby and Abelson, 1991). Splicing proteins that bore

homology to the DEAD/DEAH-box families of putative RNA helicases and ATPases were excellent candidates for destabilizing RNA duplexes on the spliceosome (Wassarman and Steitz, 1991). When I began this work, the prototype DEAH-box splicing factor was Prp16, which hydrolyzed ATP to promote an ill-defined conformational change on the spliceosome before the second step of splicing (Schwer and Guthrie, 1991; Schwer, 1992). Parallel analyses suggested that Prp16 coupled its ATPase activity to a mechanism to maintain fidelity at the second step (Burgess et al., 1990; Burgess and Guthrie, 1993). Neither of these experimental approaches provided evidence for RNA unwinding, nor hints of specific RNA substrates. Analyses of the DEAH-box splicing factors Prp2 (Kim and Lin, 1993) and Prp22 (Company et al., 1991) also failed to link splicing function to specific helical exchanges. Since these proteins did not appear to exhibit strand displacement activity with synthetic RNAs in vitro (Kim et al., 1992; Strauss, 1992); (B. Schwer and C. Guthrie, unpublished), it was possible that these proteins did not unwind RNA at all. Thus while base-pairing dynamics were well-established in the spliceosome, the driving forces behind these rearrangements were murky.

All of my thesis work has revolved around the helical dynamics of the U4/U6 snRNP, although I did not initially plan this. The U4/U6 duplex contains the longest contiguous stretch of base-pairing known to be unwound during pre-mRNA splicing, and searching for the responsible agent seemed a bit like looking for the holy grail -- far beyond the reach of a naive young graduate student, at any rate. My first set of experiments was much more limited in scope. I attempted to shed light on the function of DEAD-box proteins in splicing by studying one example, Prp28. In the course of these efforts, I despaired that Prp28 did not seem illustrative of anything at all in

splicing; but I did learn the pitfalls of centering my studies around a protein, rather than a physiological event. Once I began to use the U4/U6 cycle as a clear jumping-off point to define the biological problems, the answers also crystallized.

Chapter 1 describes my investigation of Prp28, an early candidate for the U4/U6 helicase. I identified multiple genetic interactions between *prp28* and alleles of U4 and U6 snRNAs, supporting the idea that Prp28 functions closely with the U4/U6 snRNP. However, my attempts to describe Prp28 function biochemically led to unforeseen results. Prp28 was selectively removed from whole cell extract using two methods: in vivo depletion of the protein from metabolizing cells; and immunodepletion of the protein from otherwise wildtype extract in vitro. These two depletion regimes had divergent effects on in vitro splicing, which substantially weakened the hypothesis that Prp28 unwinds U4/U6 on the spliceosome. But a plausible interpretation of these data is that splicing-related RNA rearrangements may occur outside the assembling spliceosome, during the "invisible" part of the splicing cycle that is opaque to conventional in vitro analyses.

In reconsidering the spliceosome cycle, I was drawn towards a previously neglected aspect of splicing that was ripe for study. The splicing field has focused intensely on RNA rearrangements during spliceosome assembly, but an equally interesting (and unexplored) question is how these helical transactions are reversed during spliceosome disassembly to regenerate active splicing components. The U4/U6 snRNP provided a useful lens through which to view this spliceosomal recycling problem. This duplex is most conspicuously disrupted during activation of the spliceosome; thus

some mechanism must ensure the reassociation of the snRNAs for reuse in new rounds of splicing. The RNA binding protein Prp24 presented a likely U4/U6 RNA annealing factor, since elegant genetic analysis by Shannon and Guthrie (1991) implicated it in forming the U4/U6 snRNP. In the second chapter, I demonstrate that Prp24 is a spliceosomal recycling factor that reanneals U4 and U6 snRNPs. Surprisingly, Prp24 is not required for initial rounds of splicing in vitro. Although Prp24 appears to operate entirely off the spliceosome, this protein promotes a helical transition critical for new rounds of spliceosome assembly: re-formation of the U4/U6 duplex.

While I studied reannealing of the U4 and U6 snRNPs, I found myself unexpectedly returning to the question of how the U4/U6 helices are separated. My experiments with extracts lacking Prp24 revealed an intriguing phenomenon: when I added ATP, free U4 snRNP appeared. It was not clear how to exploit this biochemical assay for U4/U6 disruption until I attended the 1996 RNA Processing Meeting in Madison, Wisconsin. There I learned that a helicase-like protein, Snu246, was likely to be tightly associated with U4/U6 in the triple snRNP (Lauber et al., 1996); and that our lab had an available allele in *brr2-1*, isolated by Suzanne Noble during her graduate work (Noble and Guthrie, 1996a). Thus, Brr2/Snu246 seemed a good candidate for the spliceosomal ATPase responsible for disrupting the U4/U6 helices. The third chapter describes how I tested this hypothesis by determining whether the mutant Brr2-1 protein blocked U4/U6 disruption. I initially used fractionated extract in the assay developed during studies with Prp24, but then I extended the analyses to isolated snRNP complexes. These results advance the hypothesis that proteins of the DEAD-box superfamily can govern helical dissociation within native RNPs.

In the epilogue, I discuss the picture of the spliceosome cycle that emerges from this thesis work. A key finding is that splicing proteins can regulate helical dynamics of native RNPs. In contrast to the mysterious functions of most splicing proteins, Brr2 and Prp24 exert antagonistic control over U4/U6 base-pairing dynamics. These helical unwinding and reannealing events highlight the cyclic nature of spliceosomal rearrangements, a property that is shared with other large nucleoprotein machines.

CHAPTER 1

In Vitro and In Vivo Analyses of Prp28 Function in Pre-mRNA Splicing

ABSTRACT

To determine the role of the DEAD-box family member Prp28 in pre-mRNA splicing, two methods have been used to remove the protein from cell extracts. Immunodepletion of Prp28-3HA from otherwise wildtype extract has surprisingly little effect on in vitro splicing. In contrast, in vitro splicing is inhibited in genetically depleted extract (prepared from metabolizing cells in which Prp28-3HA synthesis is repressed). Splicing activity in genetically depleted extract can be restored by the addition of purified Prp28 protein, or by the addition of a snRNP-enriched fraction devoid of Prp28. The latter complementing activity is sensitive to micrococcal nuclease, suggesting that snRNPs in genetically depleted extract are defective. Since numerous alleles of U4 and U6 snRNAs (but not U2 snRNA) display synthetic lethality with *prp28-1*, Prp28 activity appears sensitive to U4/U6 status in the cell. Together, these results indicate that Prp28 may function outside the spliceosome, perhaps in generating splicing-competent U4/U6.U5 snRNP.

INTRODUCTION

During nuclear pre-mRNA splicing, nascent transcripts undergo the removal of intervening sequences in an ribonucleoprotein (RNP) complex termed the spliceosome. Within this particle, the two-step splicing reaction takes place. Sequential transesterifications produce, in the first step, free 5'exon and intron-lariat joined to 3'exon; and the second step results in covalently bonded exons and the characteristic excised intron lariat. Prior to catalysis, however, the five snRNAs (U1, U2, U5, and U4/U6) and 50-100 splicing proteins must assemble into a structure that promotes these reactions (Guthrie, 1991; Rymond and Rosbash, 1992; Moore et al., 1993). This elaborate macromolecular RNP assembles through an ordered series of RNA-RNA and protein-RNA interactions that require ATP. The exogenous energy source is believed to fuel the rearrangements that create a catalytic conformation of the spliceosome.

Splicing in yeast utilizes six members of a class of RNA-dependent ATPases named the "DEAD-box" family after the common glu-asp-ala-glu motif (Wassarman and Steitz, 1991; Schmid and Linder, 1992; Gorbalenya and Koonin, 1993). Prp5 and Prp28 exhibit the closest homology to the DEAD-box prototype eIF4A, while Prp2, Prp16, and Prp22 share deviations significant enought to warrant their inclusion in a "DEAH-box" subfamily. Brr2/Snu246, the subject of the second chapter of this thesis, is a member of the DEXH-box subfamily (Lauber et al., 1996; Noble and Guthrie, 1996a). Translation initiation factor eIF4A displays ATP-dependent RNA helicase activity in vitro (Rozen et al., 1990). Because strand displacement requires a large molar excess of protein versus RNA, it is not clear whether this in vitro

activity reflects a biological role. Nevertheless, this observation has prompted speculation that these DEAD-box splicing factors function to unwind base-paired RNA on the spliceosome.

Whether or not they unwind RNA directly, a likely role of these DEAD- and DEAH-box proteins is to effect conformational transitions of the spliceosome through ATP hydrolysis. Prp5, Prp2, and Prp16 are required for distinct ATP-dependent steps in the splicing pathway (Schwer and Guthrie, 1991; Kim and Lin, 1993; Ruby et al., 1993). The current model of spliceosome assembly, which has been illuminated by studies of yeast mutants blocked at different stages, is replete with alterations in snRNP/snRNP and snRNP/precursor interactions (Moore et al., 1993). In an ATP-independent "commitment" step, U1 snRNP base-pairs with the conserved intron sequence at the 5' splice site, while simultaneously inspecting the integrity of the intron branchpoint sequence (Ruby and Abelson, 1988; Seraphin and Rosbash, 1991). The association of U2 snRNP with the branchpoint sequence requires ATP and possibly Prp5 protein (Parker et al., 1987; Ruby et al., 1993; O'Day et al., 1996). In order to bind the spliceosome, U5 joins with U4/U6 to create a "triple snRNP," which is believed to be a functional intermediate in assembly (Seraphin et al., 1991; Utans et al., 1992). After the triple snRNP engages the spliceosome, U4 is destabilized by some unknown process, indicating that its intermolecular base-pairs with U6 have been unwound (Cheng and Abelson, 1987; Konarska and Sharp, 1987). U6 has been hypothesized to be the catalytic moiety of the spliceosome, and this event is thought to expose U6 residues implicated in catalysis (Guthrie, 1991). Consistent with the proposed role of U4 as the antisense negative regulator of U6 (Guthrie and Patterson, 1988), the presence of U4 has been shown to be

unnecessary for the chemical steps of splicing (Yean and Lin, 1991). The transition to an active spliceosome occurs when Prp2 and ATP promote the first catalytic step of splicing, resulting in the formation of splicing intermediates (Kim and Lin, 1993). The second step is coupled to ATP hydrolysis by Prp16 and a concomitant conformational change (Schwer, 1992). Finally, Prp22 is necessary to release mature mRNA from the spliceosome, and presumably to liberate splicing components for new rounds of assembly (Company et al., 1991).

Genetic suppression and biochemical crosslinking experiments provide evidence for many more RNA-RNA interactions during splicing. Notably, conserved residues in U6 have been proven to engage in base-pairing with nucleotides in U2; these helices are mutually exclusive with the U4/U6 pairing and with an intramolecular U2 stem (Madhani and Guthrie, 1992; Field and Friesen, 1996). U6 has also been observed to interact with intron sequences within the 5' splice site, implying the destabilization of the U1-5' splice site interaction (Wassarman and Steitz, 1992; Lesser and Guthrie, 1993). The driving forces behind these documented rearrangements have yet to be identified.

We have attempted to elucidate a role for Prp28 in the spliceosome. Several pieces of evidence previously supported its requirement at or prior to the first step of splicing. The original *prp28-1* allele, isolated in a screen for cold-sensitive splicing mutants, accumulated precursor rather than intermediate mRNAs when shifted to the nonpermissive temperature (Strauss and Guthrie, 1991). Immunodepletion of Prp28 protein from splicing extract inhibited the first step of splicing, consistent with a role in spliceosome

assembly (Strauss and Guthrie, 1994). Genetic interactions with an allele of *PRP8*, a U5 snRNP protein, and *prp24-1*, which encodes a U6-binding protein, suggested that Prp28 may destabilize the U4/U6 interaction on the spliceosome (Strauss and Guthrie, 1991).

We have studied the effects of in vivo and in vitro removal of Prp28 protein on pre-mRNA splicing. To our surprise, these two depletion regimes produced different results: in vivo removal caused spliceosome assembly to arrest at an early stage, while in vitro removal did not markedly interfere with spliceosome assembly. Further genetic analysis revealed that *prp28-1* exhibits synthetic lethality in combination with many U4 and U6 alleles predicted to destabilize U4/U6 base-pairing. These results suggest that U4/U6 stability may be important for Prp28 function, and point to a role for Prp28 in generating splicing-competent U4/U6.U5 snRNPs off the spliceosome.

RESULTS

Construction of a galactose-regulated, epitope-tagged *GAL-PRP28-3HA* gene

Prp28 has been previously reported to be required for the first transesterification reaction in vitro(Strauss and Guthrie, 1994). This result was obtained by immunodepleting Prp28 from whole cell splicing extracts with anti-Prp28 polyclonal antibodies. To gain further insight into Prp28 function, we wished to determine which specific stage of spliceosome assembly was blocked by the absence of Prp28 protein. Our attempts to reproduce this immunodepletion protocol were hampered by lack of reproducibility and difficulty estimating residual Prp28 levels. Therefore, we decided to use two different techniques to remove Prp28 from whole cell extracts. We inserted a triple hemagglutinin (3HA) epitope into the *PRP28* coding sequence to allow us to immunodeplete the protein with anti-HA antibodies in vitro. In addition, we placed the *PRP28-3HA* gene under the control of the *GAL1-10* upstream activating sequence, so that we could repress synthesis of the protein in vivo by transferring cells from galactose to glucose.

PRP28 is an essential gene(Strauss and Guthrie, 1991), and the *PRP28-3HA* gene complemented the *PRP28::TRP1* gene disruption in a similar fashion to the untagged version (Table 1). Surprisingly, the *GAL*-regulated *PRP28-3HA* and *GAL-PRP28* constructs did not behave identically in this assay. In glucose-containing media, cells harboring *GAL-PRP28* as their sole source of Prp28 were viable, while those bearing *GAL-PRP28-3HA* were inviable (Table 1). In both instances, Prp28 expression was regulated in response to carbon source, as determined by Western blotting (data not shown). A low level of untagged Prp28 is synthesized in glucose-grown *GAL-*

PRP28 cells (data not shown), which is apparently sufficient to support growth. In contrast, Western blotting revealed that Prp28-3HA expression appears fully repressed in glucose (see Figure 1B). The introduction of the epitope tag appears to have altered the expression levels, most likely by decreasing protein stability. In support of this explanation, no Prp28-3HA protein is detectable within an hour after shifting cells from galactose to glucose (see Figure 1B), under which conditions untagged Prp28 is still abundant (data not shown). The *GAL-PRP28-3HA* construct also conferred cold-sensitivity on galactose media, indicating that the introduction of the 3HA tag compromised Prp28 function at low temperatures (Table 1).

Genetic depletion of Prp28-3HA inhibits the first step of splicing in vivo and in vitro

Despite this shortcoming, the *GAL-PRP28-3HA* construct proved a powerful reagent to study the impact of in vivo versus in vitro depletion of Prp28-3HA protein on pre-mRNA splicing. To eliminate Prp28-3HA from metabolizing cells, cells containing the *GAL-PRP28-3HA* plasmid were grown in galactose, and then transferred to glucose to repress transcription of *PRP28*. As shown by the growth curves in Figure 1A, cultures treated in this fashion ceased growth within six hours after the shift to glucose (about two generations). However, depletion of Prp28-3HA protein and inhibition of in vivo pre-mRNA splicing occurred more rapidly. No Prp28-3HA protein was observed after one hour of shift, as judged by Western blotting (Figure 1B, lane 4). Moreover, this depletion correlated with the onset of accumulation of pre-U3 RNA (Figure 1C). These results demonstrate that Prp28 can be depleted metabolically, and is essential for cell growth. Since endogenous precursor RNAs are not spliced when Prp28-3HA is absent, Prp28 is required

for the first step of pre-mRNA splicing in vivo (as observed previously by (Strauss and Guthrie, 1991)).

To characterize the specific role of Prp28 during in vitro splicing, we prepared extract from cells metabolically depleted of Prp28-3HA after 5.5 hours of growth in glucose (GLU extract). We simultaneously prepared a companion extract from cells grown in galactose to serve as the normal Prp28-3HA control (GAL extract). Standard in vitro splicing reactions were performed in GAL and GLU extracts, in some cases supplemented with purified recombinant Prp28 protein (a generous gift of L. Stewart). GLU extract by itself exhibited very low levels of in vitro splicing activity compared to GAL extract (Figure 2A, compare lanes 2-4 with 9-11). When GLU extract was complemented with Prp28 protein, splicing activity was restored (Figure 2A, lanes 5-7 vs. lanes 2-4). Thus, the absence of Prp28 (and not any other concurrently depleted factors) caused the splicing defect in GLU extract. Prp28 protein also increased the amount of splicing in GAL extract (Figure 2A, lanes 12-14 vs. lanes 9-11). These results showed that as expected, Prp28 is required for the first step of in vitro splicing in genetically depleted extract.

To determine why splicing is impeded in the absence of Prp28, we monitored the progress of spliceosome assembly using native gel electrophoresis (Pikielny et al., 1986). In GAL extract, complex III (the U2-containing pre-spliceosome) appears early and is quickly converted to complex I (with U2, U4, U5, and U6 snRNAs) and subsequently to complex II, the active spliceosome with splicing intermediates and U2, U5, and U6 snRNAs. (Figure 2B, lanes 1-4; complexes I and II are not well-resolved in this experiment.) In contrast, GLU extract splicing reactions exhibited

drastically reduced complex formation (Figure 2B, lanes 5-8). No particular complex accumulated at the expense of the others. However, addition of purified Prp28 protein restored complex I/II formation, with very little complex III observed (Figure 2B, lane 9). These results suggest that spliceosome assembly is blocked at a very early step in extract genetically depleted for Prp28, perhaps prior to of U2 snRNP addition.

Immunodepletion of Prp28-3HA does not arrest in vitro splicing

We wished to confirm this conclusion using a different method to remove Prp28 from extract. Therefore, we immunodepleted Prp28-3HA from GAL extract with antibodies against the HA epitope (Δ28 GAL); a control mock-depleted extract was prepared simultaneously (MOCK Δ GAL). Immunodepletion and genetic depletion reduced Prp28-3HA amounts similarly, as revealed by comparison with serial dilutions of GAL extract on Western blots (Figure 3A). Since the residual amount of Prp28-3HA in Δ28 GAL extract (Figure 3A, lane 8) appeared equivalent to that seen in a sixteen-fold dilution of GAL extract (lane 6), we estimate that ~95% of Prp28-3HA is depleted in Δ28 GAL extract. In GLU extract, the remaining Prp28-3HA appeared less than the eight-fold dilution of GAL extract (Figure 3A, lanes 2 versu 5); therefore, GLU extract is ~90% depleted of Prp28-3HA.

Next, we performed in vitro splicing in mock- and immunodepleted extracts in the presence or absence of recombinant Prp28 protein. Even though immunodepleted extract lacks 95% of its Prp28-3HA protein, both steps of splicing were observed (Figure 3B, lanes 11-14), and spliceosome assembly was unimpeded (Figure 3C, lanes 11-14). Thus, while in vivo depletion blocks splicing, in vitro depletion of Prp28 has minimal effects on

splicing. Why do these two depletion regimes produce different results? One possibility is that Prp28 remodels RNA/protein interactions off the spliceosome, and immunodepleted extract contains abundant targets that have been previously modified by Prp28. Therefore, spliceosomes can assemble de novo when very little Prp28 is present. In contrast, during cell growth in the absence of Prp28, factors previously modified by Prp28 may be used up while immature substrates accumulate. Consequently, genetically depleted extract may exhibit low levels of splicing activity.

Genetically depleted extract contains inactive snRNPs

If this theory is correct, then genetically depleted extract is predicted to harbor inactive splicing factors that have not been converted by Prp28. Furthermore, Prp28 should not be strictly required for in vitro splicing, as long as the reaction contains sufficient targets previously activated by Prp28. To test these ideas, we attempted to restore splicing activity to genetically depleted extract with cellular fractions lacking Prp28. Fraction I is a snRNP-rich ammonium sulfate precipitate of whole cell extract; GAL Fraction I preparations were further immunodepleted of Prp28-3HA (data not shown). Interestingly, all of these Fraction I preparations rescued splicing of GLU extract, indicating that the complementing activity could be separated from Prp28 protein (Figure 4A, lanes 3-6 vs. lanes 1-2). We suspected that snRNPs might be the target of Prp28 action; if true, then the complementing activity should be sensitive to micrococcal nuclease. In fact, when Fraction I lacking Prp28 was pre-treated with micrococcal nuclease, complementation was abolished (Figure 4A, lanes 7-8). However, if Prp28 was present, micrococcal nuclease had no effect on the complementing activity (Figure 4B, lanes 7-8 vs. 9-10). Thus, genetically depleted extract can be complemented either by the

addition of snRNPs, or by the addition of Prp28. These results suggest that when Prp28 is depleted in metabolizing cells, snRNPs are rendered inactive.

prp28-1 is synthetically lethal with multiple alleles of U4 and U6 snRNAs, but not with mutant U2 snRNAs

To determine which snRNPs might be Prp28's targets, we sought genetic interactions between *prp28-1* and various alleles of U2, U4, and U6 snRNAs. We reasoned that if *prp28-1* encodes a crippled helicase, then mutations that destabilize its target helix might suppress the cold-sensitive phenotype; conversely, mutations that hyperstabilize the target helix might produce synthetic lethality in combination with the mutant helicase. We introduced the *prp28-1* lesion into the *SNR20* (U2), *SNR14* (U4), and *SNR6* (U6) deletion strains. Since all splicing snRNA genes are essential, these strains require the presence of the wildtype snRNA gene on a plasmid, which is marked by *URA3*. By introducing plasmid-borne mutant snRNA genes into these strains and plasmid shuffling on 5-fluoroorotic acid (5-FOA), we determined the phenotypes of several *prp28-1 snr* double mutant combinations in comparison to *PRP28 snr* strains. The results of these directed screens for genetic interactions are summarized in Tables 2-6, and in Figures 5B and 5C. No genetic interactions were observed with mutations in the stem-loop Ia, stem IIa, loop IIa, or stem IIb regions of U2 (Ares and Igel, 1990): all of the U2 snRNA alleles tested behaved similarly in *prp28-1* and *PRP28* cells (Table 2). Many of these mutations cause substantial misfolding of U2 snRNA in vivo (Zavanelli and Ares, 1991), so the absence of synthetic lethality with *prp28-1* (a defective splicing factor) is particularly striking.

In contrast, many alleles of U4 (Table 3) and U6 (Table 4) snRNAs were inviable in combination with *prp28-1*, but not with *PRP28*. All of these alleles are predicted to disrupt intermolecular U4/U6 base-pairing in stems I and II (Brow and Guthrie, 1988). Interestingly, synthetic lethality was detected on both sides of the helix in two U4/U6 base-pairs: changing either nucleotide in the U4/U6 base pairs G58/C61 or G9/C72 was not tolerated with *prp28-1* (Tables 3, 4, 5). In the case of the G58/C61 base pair, simple compensatory base-pair changes did not alleviate the synthetic lethality (Table 5). Therefore, mutations in U6 position C61 must create problems in addition to faulty pairing with U4 snRNA. In contrast, the U6 C72A *prp28-1* double mutant strain grew substantially better in the presence of an additional copy of U4 snRNA, even when the U4/U6 base-pairing was not restored (Table 5). In this instance, impaired U4/U6 interaction causes synthetic lethality, and can be overcome by increasing the amount of U4 snRNA in the cell. In summary, contrary to our simple expectations about helicases, mutations that destabilize helices produced synthetic lethality with *prp28-1*. Synthetic lethality appeared snRNA- and allele-specific, since only a subset of mutant U4 and U6 snRNAs and not any mutant U2 snRNAs were inviable with *prp28-1*. These results suggest that while defective base-pairing is not the sole cause of synthetic lethality with *prp28-1*, the integrity of the U4/U6 helices might be important for Prp28 function.

To determine if these synthetic lethal alleles were coincidentally located in U4/U6 base-pairing regions, we extended this screen for genetic interactions by using a library of alleles mutagenized along the entire length of U6 snRNA (Madhani et al., 1990) (Figure 5A). We transformed this library into the *snr6::LEU2 prp28-1* strain, and 10,000 transformants were screened for

inviability on 5-FOA at 30°C and suppression of cold-sensitivity at 16°C. No suppressors were recovered, but ~750 candidates for synthetic lethality were selected. The majority of these 5-FOA-sensitive isolates were likely to contain null alleles of U6 snRNA that would be lethal in the presence of either wildtype *PRP28* or *prp28-1*. We employed a replica mating strategy to eliminate these strains (Figure 5A), and isolated 51 recessive synthetic lethal candidates. We recovered the mutant U6 plasmids from these strains, and transformed them into fresh *snr6::LEU2 prp28-1* and *snr6::LEU2 PRP28* cells. When combined with *prp28-1*, forty-nine plasmids exhibited synthetic phenotypes of varying severity: reduced growth, drastically reduced growth, or inviability (Table 6). By inspecting the mutant sequences, we identified three overlapping categories of synthetic lethal U6 alleles (Table 6). Many mutations are predicted to interfere with the U6 intramolecular stem; most of these disrupt U4/U6 stems I and II, as well, confirming the analysis of the site-directed mutants (Tables 3, 4, 5). A second class of mutations was isolated in nucleotides 40-44 and 92-97 of U6 snRNA; these regions have been implicated in binding the U6 snRNP protein Prp24 (Shannon and Guthrie, 1991; Jandrositz and Guthrie, 1995). Finally, a third class of U6 alleles alters residues in positions 30-32, a region which has no known base-pairing or protein-binding potential. Most of the synthetic lethal alleles contain multiple mutations that affect more than one region of U6. Interestingly, only residues within the three categories seem to have been targeted: only one mutation lies outside these regions (U6 G50C). The screen was likely to have saturated the entire U6 gene, since we repeatedly isolated different allelic combinations of the same mutations. Therefore, cells with impaired Prp28 function are especially sensitive to mutation in three regions of U6, including the U4/U6 helices. We conclude that Prp28 is likely to act closely

DISCUSSION

These experiments were initiated in order to determine the function of Prp28, a DEAD-box protein involved in pre-mRNA splicing. An early hypothesis based on genetic evidence suggested that Prp28 might be responsible for unwinding the U4/U6 helices during catalytic activation of the spliceosome (Strauss and Guthrie, 1991). We asked whether the removal of Prp28 from splicing reactions produced a phenotype consistent with this role. Surprisingly, we found that the requirement for Prp28 during in vitro splicing was less stringent than previously observed for other DEAD/DEAH-box splicing factors. While our genetic results substantiate Prp28's connection with the U4/U6 snRNP, our biochemical data support a function that differs from initial expectations.

Is Prp28 the U4/U6 helicase?

The dissociation of U4 from U6 snRNA begins a series of helical isomerizations that are thought to form the catalytic reaction center of the spliceosome (Madhani and Guthrie, 1994a; Ares and Weiser, 1995); DEAD-box proteins have been hypothesized to control these RNA rearrangements (Wassarman and Steitz, 1991). If such a factor were removed, splicing should be completely blocked before the first transesterification; and spliceosome assembly should stall prior to U4 release. Since depletion of Prp28 achieves neither of these outcomes, Prp28 is unlikely to unwind U4/U6 on the spliceosome. We did not observe an absolute requirement for Prp28 in splicing reactions; instead, we noted differential effects on in vitro splicing depending on the method used to remove the protein. Under our immunodepletion conditions, ~95% of Prp28 could be removed (Figure 3A),

yet in vitro splicing was not arrested (Figure 3B). In contrast, extracts prepared from genetically depleted cells lacked ~90% of Prp28 protein (Figure 3A), yet exhibited low splicing activity unless supplemented with purified Prp28 (Figure 2A). Moreover, spliceosome assembly stalled at a very early stage in genetically depleted extract, apparently before U2 snRNP addition (Figure 2B). These results are inconsistent with a role for Prp28 in a late rearrangement crucial for splicing catalysis such as U4/U6 unwinding.

An indirect role for Prp28 in spliceosome assembly?

Since Prp28 is dispensable during in vitro splicing, this DEAD-box protein may play an indirect role in spliceosome assembly, perhaps by modifying inactive splicing factors. Any hypothesis about Prp28 function must address why the two depletion regimes produced different in vitro splicing results. One possible explanation for this discrepancy is that Prp28 is necessary to activate splicing components in vivo. We speculate that when Prp28 is removed from metabolizing cells, its normal substrates accumulate in inactive form, causing inhibition of the first step of splicing in vivo (Figure 1C). Extracts prepared from these cells may be unable to support in vitro splicing for two reasons: because they contain inactive factors that do not assemble onto spliceosomes, and because they lack Prp28 protein that can render these factors competent for splicing. Thus, splicing can be restored by providing either Prp28 protein or the active factors (Figures 4A, B). At the same time, if these active factors are normally abundant in wildtype extract, Prp28 may be superfluous under conventional in vitro splicing conditions (Figure 3B). Removal of Prp28 from extract by immunodepletion should therefore have minimal effect on splicing, since the activated targets are still available. This hypothesis frames Prp28 function as extrinsic to the

spliceosome, unique among all the known DEAD/DEAH-box splicing proteins. In support of this idea, Prp28 does not coimmunoprecipitate with spliceosomal snRNAs or pre-mRNA from in vitro splicing reactions (data not shown).

U4/U6.U5 snRNPs are plausible targets of Prp28

Several lines of evidence point to the U4/U6 or U4/U6.U5 snRNPs as the likely targets of Prp28 action. The splicing defect of genetically depleted extract can be restored by Fraction I (Figure 4), which contains significant amounts of U4/U6.U5 snRNPs but not U2 or free U6 snRNPs (data not shown). This complementing activity is distinct from Prp28 protein and contains nucleic acid, because immunodepletion of Prp28 does not abolish activity, but micrococcal nuclease treatment does (Figures 4A, B). Because snRNPs appear to restore splicing activity to genetically depleted extract, we conclude that the snRNPs endogenous to GLU extract are nonfunctional for in vitro splicing. Removing Prp28 from growing cells seems to inactivate some population of snRNPs; therefore, snRNPs are a likely target of Prp28 action. This notion is consistent with the minor effects of in vitro removal of Prp28 (Figure 3B): immunodepletion of Prp28 from idle extract may not inactivate the abundant snRNP population, and thus in vitro splicing may proceed in the absence of Prp28.

Additional experiments not presented in this chapter suggest that GLU extract contains aberrant snRNPs. When snRNPs from GAL and GLU extracts were compared by native gel electrophoresis, a broad, slow-migrating band that hybridized to U2, U4, U5, and U6 probes was noticeably enriched in GLU extract (data not shown). This putative complex did not dissociate or

change mobility upon addition of Prp28 protein, although the amount of U6 signal was variably enhanced (data not shown). We suggest that this snRNP "complex" observed on native RNP gels may represent the inactive splicing factors postulated to accumulate in genetically depleted extract. This complex may sequester U2, U4, U5, and U6 snRNPs away from the productive splicing pathway, thus inhibiting spliceosome assembly prior to U2 binding in GLU extract (Figure 2B). In summary, the biochemical data support the hypothesis that Prp28 is necessary to activate snRNPs for splicing. Unfortunately, definitive proof is still lacking. We have been unable to detect Prp28-dependent structural alterations of snRNAs in GLU extract by psoralen crosslinking (data not shown). Finally, we have not observed physical associations between Prp28 and snRNPs under a variety of conditions (data not shown).

While splicing biochemistry implicates Prp28 in general snRNP activity, genetic interactions establish a specific link between *PRP28* and U4 and U6 snRNAs. Neither suppression nor synthetic lethality was observed between *prp28-1* and multiple alleles of U2 snRNA that disrupted stem-loops I and II (Table 2). In contrast, numerous alleles of U4 and U6 snRNAs were synthetically lethal in combination with *prp28-1* (Tables 3-6, Figure 5B). Many of these mutations are predicted to inhibit U4/U6 formation directly or indirectly. For example, all the synthetically lethal U4 alleles and a large number of the U6 alleles disrupt U4/U6 base-pairing in stems I or II (Figure 5B). But other U6 alleles are predicted to destabilize elements in the free U6 snRNP that facilitate U4/U6 annealing. The RRM (RNA recognition motif) protein Prp24 is likely to promote U4/U6 annealing, and it presumably binds to nucleotides 38-43 and 90-95 in the free U6 snRNP (Shannon and Guthrie,

1991; Jandrositz and Guthrie, 1995) (possibly in an unproven pseudoknot structure: (Brow and Vidaver, 1995)). Both Prp24/U6 snRNP association (Shannon and Guthrie, 1991; Jandrositz and Guthrie, 1995) and the integrity of the intramolecular U6 stem (Fortner et al., 1994) are likely to be required for efficient U4/U6 formation. Since many of the synthetic lethal alleles would interfere with these regions, deficient U4/U6 assembly does not appear to be tolerated when Prp28 function is impaired. We do not believe that Prp28 facilitates U4/U6 annealing, because Prp28 is not required for U4/U6 association in extract (data not shown). Instead, we suggest that Prp28 may be sensitive to U4/U6 helical stability because it acts directly on U4/U6 containing snRNPs. For example, Prp28 may modify U4/U6.U5 snRNPs to help them achieve a splicing-competent conformation. This may be part of post-spliceosomal snRNP recycling, or may be a necessary step in the U4/U6.U5 snRNP maturation process. Furthermore, such an activity could in principle occur on snRNPs before or after they assemble onto the spliceosome. Alternatively, Prp28 may be required to resolve the structures of misfolded snRNPs that accumulate in its absence. In this view, Prp28 may behave as an RNA (or RNP) chaperone (Herschlag, 1995). Future experiments should investigate whether Prp28 causes subtle changes in U4/U6.U5 snRNPs that correlate with assembly on the spliceosome.

MATERIALS AND METHODS

Yeast methods and molecular biology

We employed standard techniques for yeast genetics (Guthrie and Fink, 1991), plasmid construction (Sambrook et al., 1989), and analysis of RNA and proteins from yeast (Guthrie Lab Protocol Book).

Oligonucleotides

GLAS	5' CCG AAT TCG TCG ACA AAA ATC ATC GCT TCG CTG A 3'
5' GAL UAS	5' CCG AAT TCG AGC CCC ATT ATC TTA GCC 3'
5'CLAHA	5' CCA TCG ATA TCT TTT ACC CAT ACG ATG TTC CT 3'
3'CLAHA	5' CCA TCG ATC TGA GCA GCG TAA TCT GGA ACG TC 3'

Yeast strains

PRY30	*MAT alpha ura3 his3 trp1 leu2 ade2 prp28Δ::TRP1 pSE360-PRP28* (*PRP28 URA3 CEN ARS* plasmid); aka "E9B"
PRY49	*prp28-1 MAT a ura3 his3 trp1 lys2 leu2 snr14Δ::TRP1 yCP50-SNR14* (*U4 URA3 CEN ARS* plasmid); sister spore of PRY50; created by crossing YKS1 (Shannon and Guthrie, 1991) to the outcrossed *prp28-1* strain PRY36

PRY50 *PRP28 MATalpha ura3his3 trp1 lys2 leu2 snr14Δ::TRP1 yCP50-SNR14* (*U4 URA3 CEN ARS* plasmid); sister spore of PRY49; created by crossing YKS1 (Shannon and Guthrie, 1991) to the outcrossed *prp28-1* strain PRY36

PRY66 *prp28-1MATa ura3 his3 lys2 trp1 leu2 snr6Δ::LEU2 yCP50-SNR6* (*U6 URA3 CEN ARS* plasmid); derived by integrating *prp28-1* into YHM1 (Madhani et al., 1990)

PRY67 *PRP28 MATa ura3 his3 lys2 trp1 leu2 snr6Δ::LEU2 yCP50-SNR6* (*U6 URA3 CEN ARS* plasmid); derived as Cs^+ isolate during integration of *prp28-1* into YHM1 (Madhani et al., 1990)

PRY88 5-FOA derivative of PRY30 transformed with pGAL-PRP28-3HA as the sole source of Prp28; grows only in media containing galactose

PRY94 *PRP28 MAT alpha leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101 ura3-52 snr20Δ::LYS2* (*U2 URA3 CEN ARS* plasmid); backcrossed twice to YPH399; sister spore of PRY95

PRY95 *prp28-1MAT alpha leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101 ura3-52 snr20Δ::LYS2* (*U2 URA3 CEN ARS* plasmid); backcrossed twice to YPH399; sister spore of PRY94

Construction of *pGAL-PRP28* and *pGAL-PRP28-3HA*

A 2.4 kb EcoRI-DraI fragment spanning the *PRP28* gene was excised from pHD25 (E. J. Strauss, unpublished) and ligated to SphI linkers. The resulting EcoRI-SphI fragment containing *PRP28* was subcloned into pSE362 to create the plasmid pTERS (Twenty-Eight/RI/SphI). To make the pGAL-PRP28 construct, a PCR-amplified GAL UAS was inserted into the unique EcoRI site

upstream of *PRP28* in pTERS. (The 390 bp GAL UAS region encompassed the Sau3AI/DdeI fragment of the GAL1-10 UAS; we used the 5' GAL UAS and 3' GLAS primers for PCR to add EcoRI sites at both ends.) In the final step to create the pGAL-PRP28-3HA construct, we inserted a 110 bp PCR fragment encoding the triple hemagglutinin epitope (3HA) (Tyers and Futcher, 1993) into the ClaI site of pGAL-PRP28 (12 base-pairs downstream from the predicted start codon of *PRP28*). The 3HA DNA was amplified with ClaI ends using the primers 5'CLAHA and 3'CLAHA.

Primer extension analysis

Primer extension analysis of total yeast RNAs was performed using the standard protocol (Patterson and Guthrie, 1991) as described in (Noble and Guthrie, 1996a).

In vitro splicing, spliceosome assembly, micrococcal nuclease treatment

PRY88 cells were harvested 5.5 hours after shifting to galactose and glucose. Whole cell splicing extracts (GAL and GLU, respectively) were prepared using the liquid nitrogen method (Umen and Guthrie, 1995), and Fraction I was prepared according to (Cheng and Abelson, 1986). In vitro splicing reactions were incubated for various times at 25°C or 15°C under standard conditions (Lin et al., 1985). Where indicated, 100 ng of recombinant Prp28 (generously provided by Leslie Stewart) was included in the reaction. Spliceosome assembly was monitored on native gels using the technique of (Pikielny et al., 1986). For micrococcal nuclease treatment of Fraction I, 2μL of mock-depleted or Δ28 GAL Fraction I was incubated with 1 μL 4 mM $CaCl_2$ and 1 μL 1

mg/mL micrococcal nuclease (Pharmacia) at 25°C for 45 minutes, and then 0.5 µL of 60 mM EGTA was added (for a final concentration of 5 mM EGTA in the splicing reaction). For mock-nuclease treatment of Fraction I, enzyme was replaced with water.

Immunodepletion of Prp28-3HA

40 µL of GAL extract was incubated with 4 µL of PBS (= MOCK ΔGAL) or 4 µL (~ 4 µg) of 12CA5 anti-HA antibody (BAbCo) (= Δ28 GAL) on ice for one hour, and then added to 40µL protein A Sepharose beads washed previously with phosphate-buffered saline (Sambrook et al., 1989). Samples were placed on a nutator in the cold room for one hour, and then the supernatants were carefully collected for in vitro splicing reactions. Similar conditions were used to immunodeplete Prp28-3HA from Fraction I.

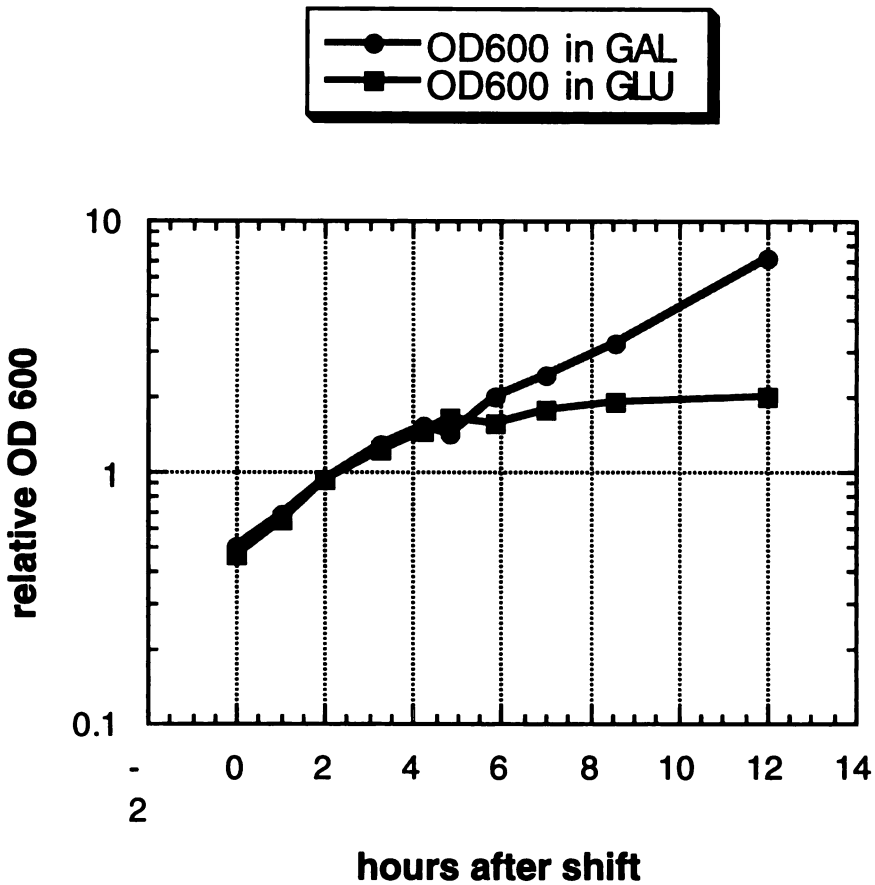


Figure 1A. *GAL-PRP28-3HA* cells arrest growth six hours after shift to glucose, as shown by growth curves. Cells grown in galactose were transferred to galactose (circles) or glucose (squares) at time 0, and culture density was monitored over time by absorbance at 600 nm (relative optical density is plotted). Doubling time in galactose is approximately 3 hours at 30°C. Thus, genetic depletion of *PRP28-3HA* rapidly arrests growth within two generations.

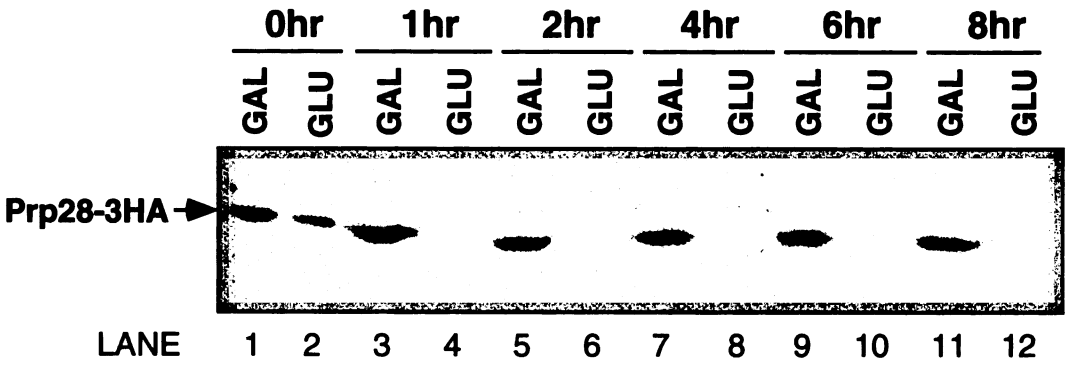


Figure 1B. Prp28-3HA protein is genetically depleted after one hour in glucose. Western blot to detect Prp28-3HA in whole cell lysates prepared from cells shifted to galactose (GAL) or glucose (GLU) for the indicated amounts of time. Prp28-3HA protein is no longer detectable in cells after one hour of shift to glucose (compare lanes 3 and 4).

Figure 1C. Genetic depletion of Prp28-3HA results in the rapid accumulation of unspliced precursors. A severe in vivo splicing defect is observed after one hour in glucose. Primer extension analysis of in vivo pre-U3 RNA splicing in *GAL-PRP28-3HA* cells shifted to galactose (GAL) and glucose (GLU) for the indicated amounts of time. Pre-U3 RNA accumulates with respect to mature U3 RNA within one hour of shift to glucose (compare lanes 3 and 4). As an internal control for loading, primer extension of U5 snRNA was performed simultaneously. The two pre-U3 bands reflect transcripts from two different U3 genes.

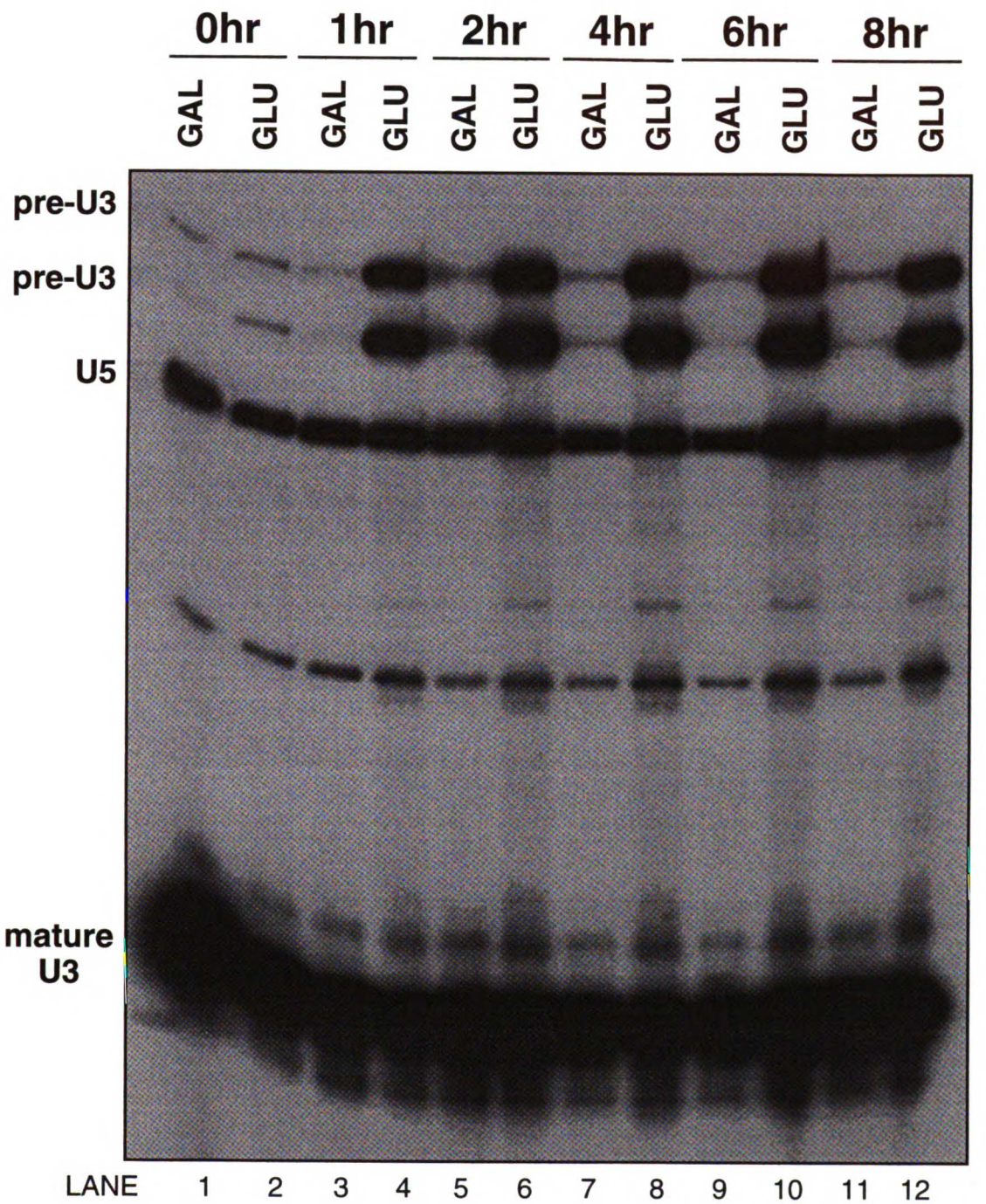

0hr
1hr
2hr
4hr
6hr
8hr
GAL
GLU
pre-U3
pre-U3
U5
mature U3
LANE
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2. Extracts genetically depleted of Prp28-3HA support low levels of in vitro splicing, because spliceosome assembly is inhibited at an early step.

A. In vitro splicing activity is low in GLU extract genetically depleted of Prp28-3HA (lane 4), but is complemented by the addition of recombinant Prp28 protein (lane 7). In vitro splicing reactions were performed in GAL (lanes 8-14) and GLU (lanes 1-7) extracts with (lanes 2-7, 9-14) or without ATP (lanes 1, 8) for the indicated amounts of time at 25°C. Reactions in lanes 1, 5-8, and 12-14 were supplemented with purified recombinant Prp28 protein.

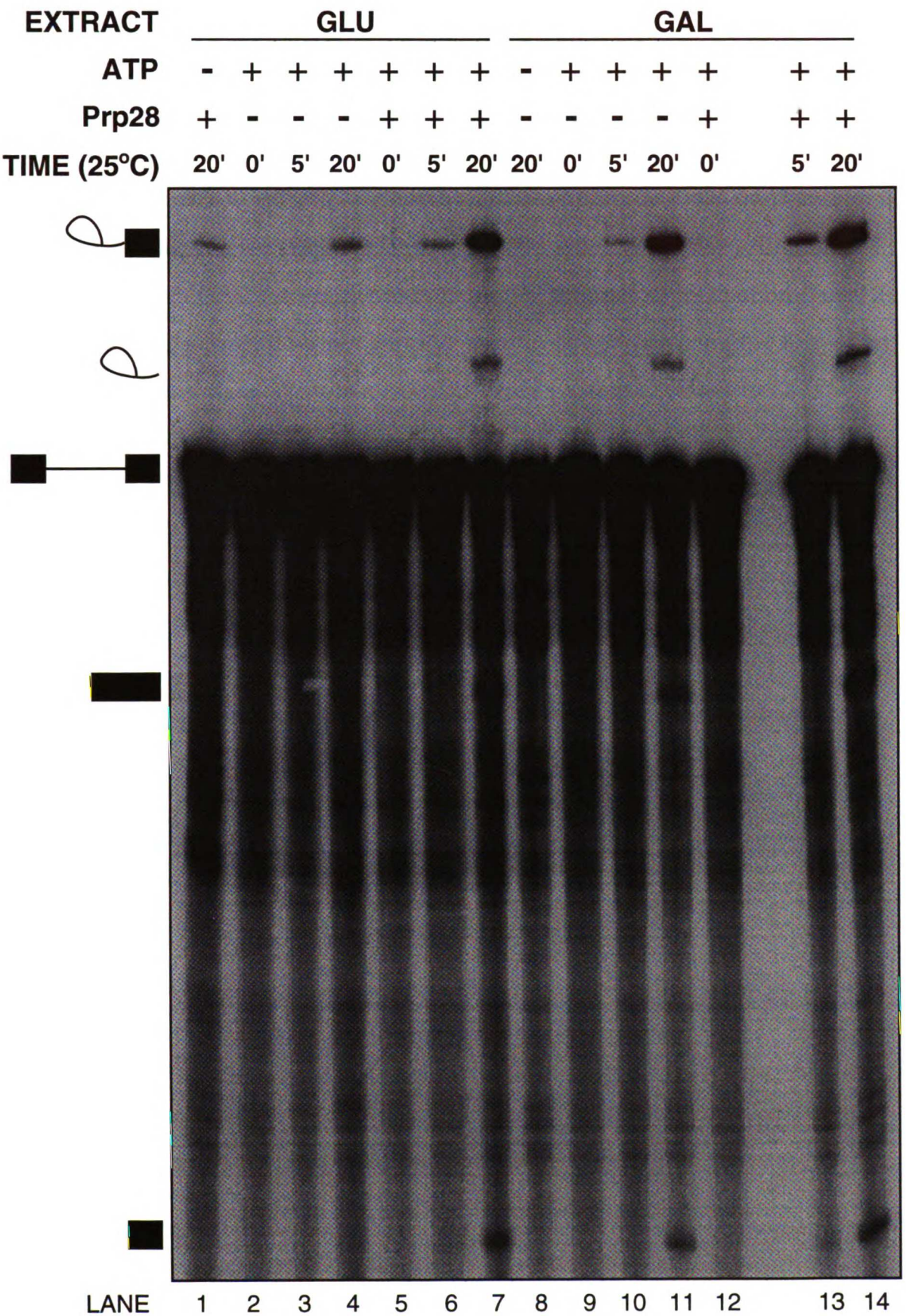
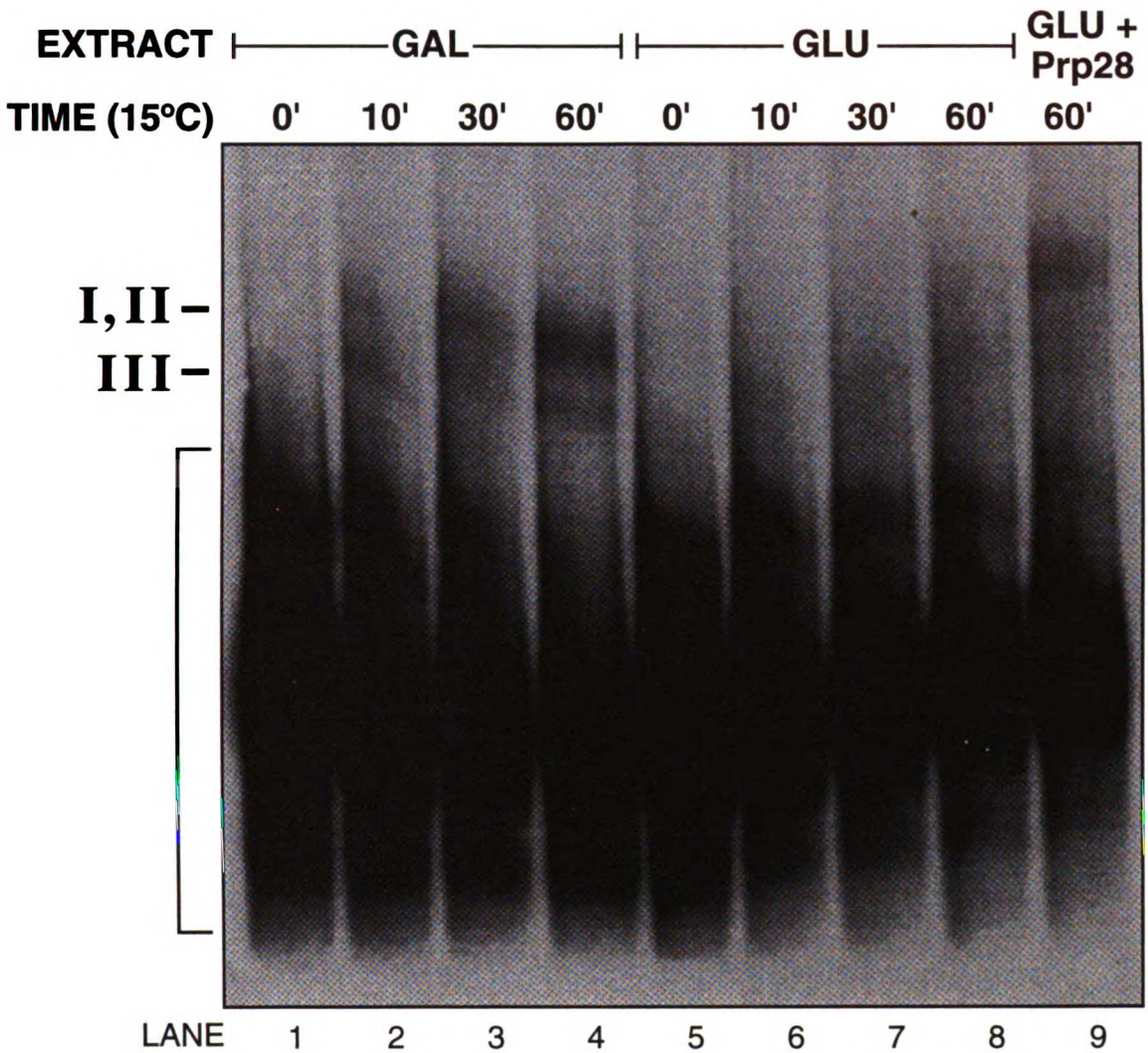

EXTRACT
GLU
GAL
ATP - + + + + + + - + + + + + + +
Prp28 + - - - + + + - - - - + + +
TIME (25°C) 20' 0' 5' 20' 0' 5' 20' 20' 0' 5' 20' 0' 5' 20'
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 2. Extracts genetically depleted of Prp28-3HA support low levels of in vitro splicing, because spliceosome assembly is inhibited at an early step.

B. Splicing complex formation is impeded at an early step in extract genetically depleted of Prp28-3HA. Spliceosome assembly in GAL (lanes 1-4) and GLU (lanes 5-9) extracts was monitored after various times on native RNP gels using the technique of (Pikielny et al., 1986). Complexes III and I/II are apparent in GAL extract between 10 and 30 minutes of incubation (lanes 2-3), while GLU extract does not support significant splicing complex formation after 60 minutes (lane 8). When purified Prp28 protein is added to GLU extract, some complex I/II is formed (lane 9). The bracketed bands represent non-splicing specific complexes.


EXTRACT
GAL
GLU
GLU + Prp28
TIME (15°C)
0'
10'
30'
60'
0'
10'
30'
60'
60'
I, II
III
LANE
1
2
3
4
5
6
7
8
9

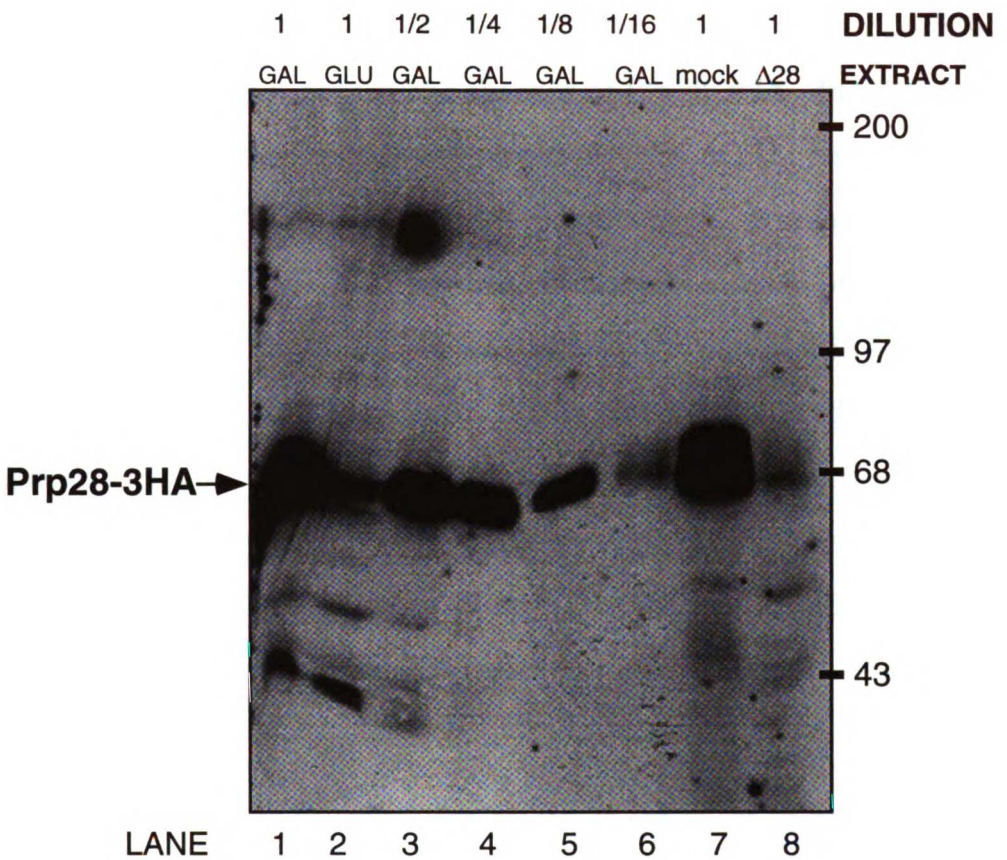


Figure 3A. Two different methods -- immunodepletion and genetic depletion -- remove greater than 90% of Prp28-3HA from extracts. Overexposed Western blot (probed with 12CA5 anti-HA antibodies) comparing Prp28-3HA levels in GAL (lane 1), GLU (lane 2), mock- (lane 7) and immunodepleted (lane 8) GAL extracts alongside twofold serial dilutions of total GAL extract (lanes 3-6). Positions of molecular weight markers (in kilodaltons) are indicated on the right; the predicted molecular weight of Prp28-3HA is 71 kD. Prp28-3HA is ~90% depleted in GLU extract and ~95% depleted in Δ28 GAL extract.

Figure 3. Immunodepletion of Prp28-3HA to <5% of normal levels does not produce a significant block to in vitro splicing.

B. Extract immunodepleted of ~95% of Prp28-3HA supports both steps of splicing in vitro, despite the absence of Prp28-3HA. In vitro splicing reactions were performed in mock-depleted (MOCK ΔGAL, lanes 1-9) or immunodepleted (Δ28 GAL, lanes 10-18) extracts in the presence (lanes 2-9, 11-18) or absence (lanes 1, 10) of ATP for the indicated amounts of time at 25°C. Purified recombinant Prp28 protein was included in samples in lanes 6-9 and 15-18. The amount of splicing intermediates and mRNA produced in Δ28 GAL extract is slightly lower than in MOCK ΔGAL extract (compare lanes 5 and 14); this slight impairment of splicing disappears with the addition of recombinant Prp28 (compare lanes 14 and 18). Although immunodepleted extract lacks 95% of Prp28-3HA, splicing can proceed.

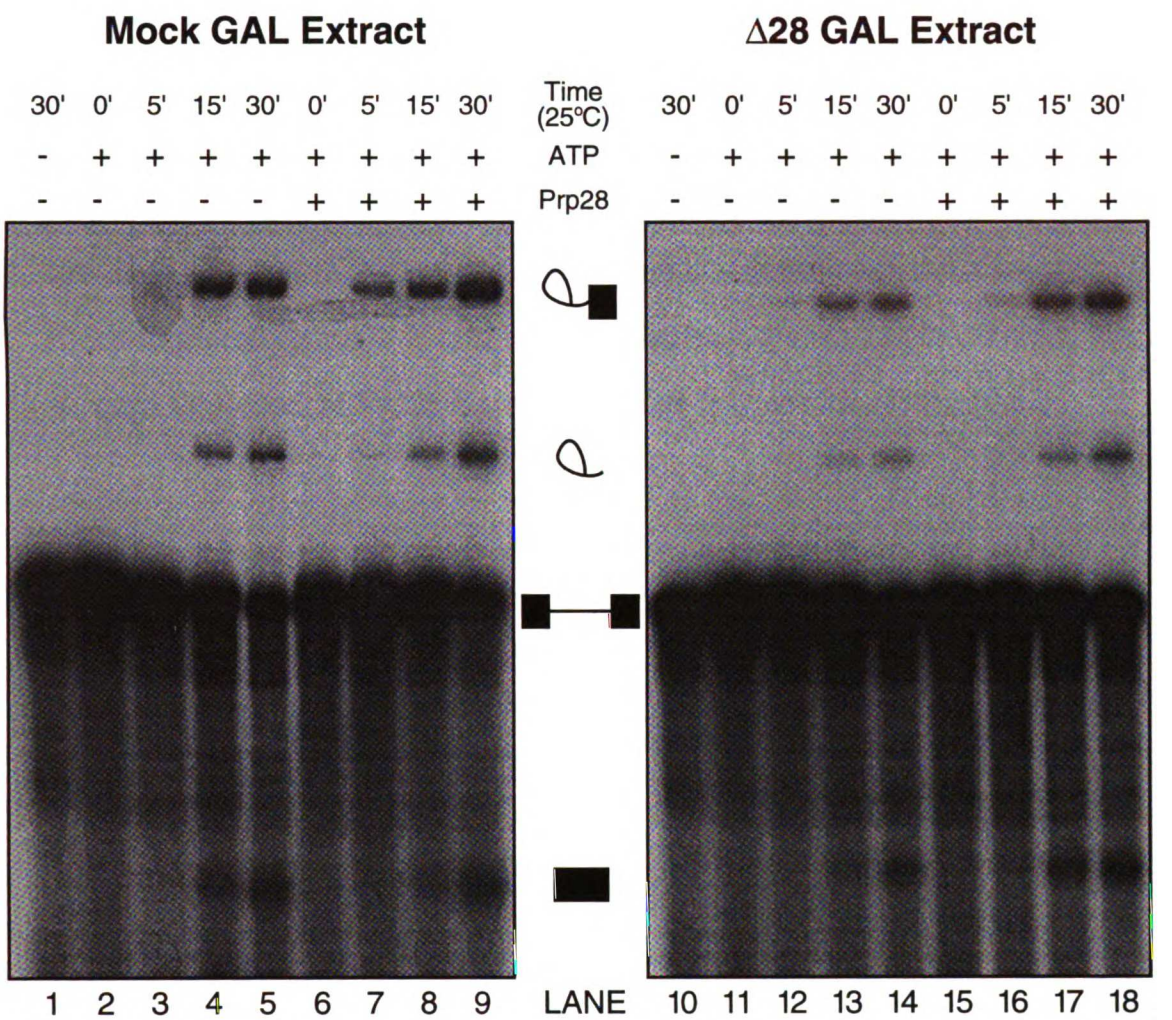

Mock GAL Extract
Δ28 GAL Extract
30' 0' 5' 15' 30' 0' 5' 15' 30'
Time (25°C)
- + + + + + + + +
ATP
- - - - - + + + +
Prp28
1 2 3 4 5 6 7 8 9
LANE
10 11 12 13 14 15 16 17 18

Figure 3. Immunodepletion of Prp28-3HA to <5% of normal levels does not produce a significant block to in vitro splicing.

C. Spliceosome assembly occurs without impediment in immunodepleted extract. Splicing reactions from Figure 3B were analyzed by native gel electrophoresis (Pikielny et al., 1986). Splicing complexes (III, I, II) are designated as in Figure 2B. Spliceosome assembly proceeds similarly in mock-depleted (lanes 1-9) and immunodepleted (lanes 10-18) extracts. Thus in vitro depletion of Prp28 does not inhibit spliceosome assembly.

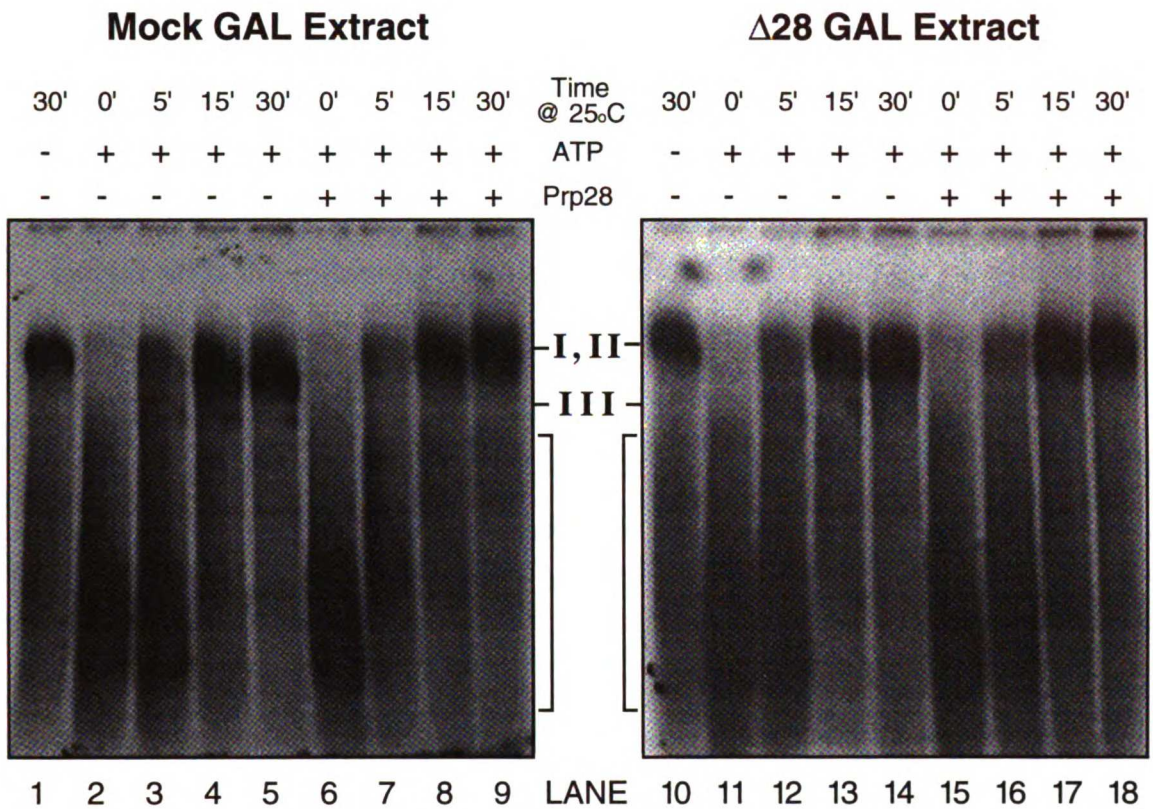

Mock GAL Extract
Δ28 GAL Extract
30' 0' 5' 15' 30' 0' 5' 15' 30'
Time @ 25oC
30' 0' 5' 15' 30' 0' 5' 15' 30'
- + + + + + + + + ATP
- - - - - + + + + Prp28
I, II
III
1 2 3 4 5 6 7 8 9 LANE 10 11 12 13 14 15 16 17 18

Figure 4. Splicing activity in genetically depleted extract can be restored by a snRNP-enriched fraction devoid of Prp28. The complementing activity is sensitive to micrococcal nuclease.

A. GLU extract can be complemented by Prp28, or by a micrococcal nuclease-sensitive activity from Fraction I. In vitro splicing reactions in GLU extract with the following additions: buffer (lane 1); 100 ng recombinant Prp28 (lane 2); 1 or 2 μL of mock-depleted Fraction I (lanes 3-4); 1 or 2 μL of Fraction I immunodepleted of Prp28-3HA (lanes 5-6); 2 μL of Fraction I immunodepleted of Prp28-3HA that has been treated with (lane 8) or without (lane 7) micrococcal nuclease. Levels of lariat-intermediate (top band) are a measure of splicing activity.

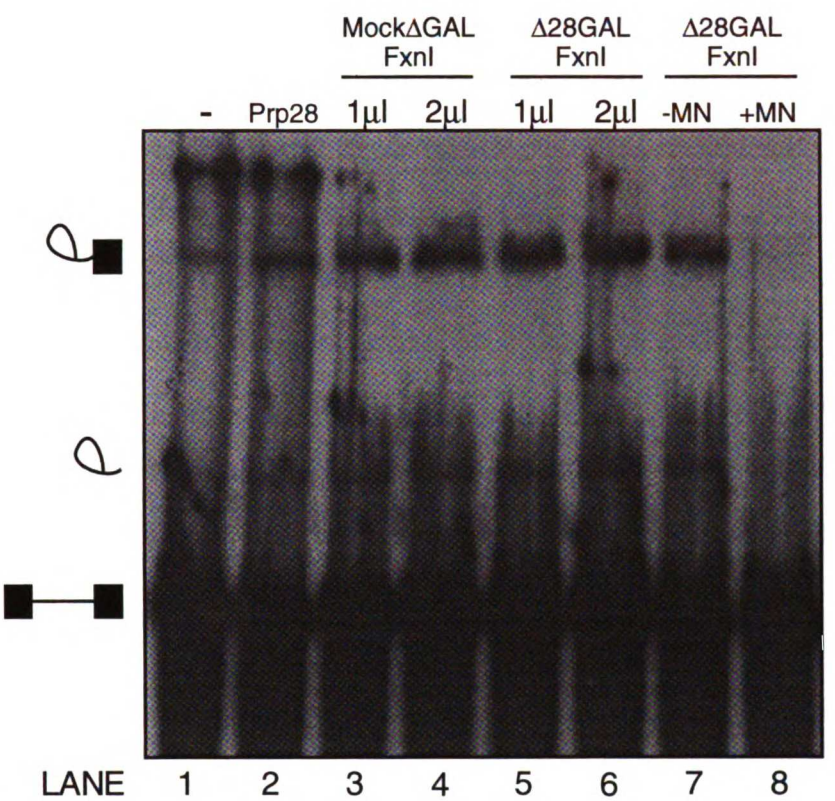

MockΔGAL FxnI
Δ28GAL FxnI
Δ28GAL FxnI
-
Prp28
1µl
2µl
1µl
2µl
-MN
+MN
LANE
1
2
3
4
5
6
7
8

Figure 4. Splicing activity in genetically depleted extract can be restored by a snRNP-enriched fraction devoid of Prp28. The complementing activity is sensitive to micrococcal nuclease.

B. Fraction I contains two sources of complementing activity: Prp28, and a micrococcal nuclease-sensitive factor. In vitro splicing reactions in GLU extract with the additions of Prp28 or Fraction I as indicated. Levels of lariat-intermediate (top band) are a measure of splicing activity. Fraction I was either mock-depleted (mockΔ, lanes 3, 5, 7, 8) or immunodepleted of Prp28 (Δ28, lanes 4, 6, 9, 10). Also, in lanes 7-10, Fraction I was treated with (lanes 8, 10) or without (lanes 7, 9) micrococcal nuclease. Nuclease treatment diminishes complementing activity of Fraction I lacking Prp28 (lanes 9-10), but does not affect the activity in Fraction I containing Prp28 (lanes 7-8).

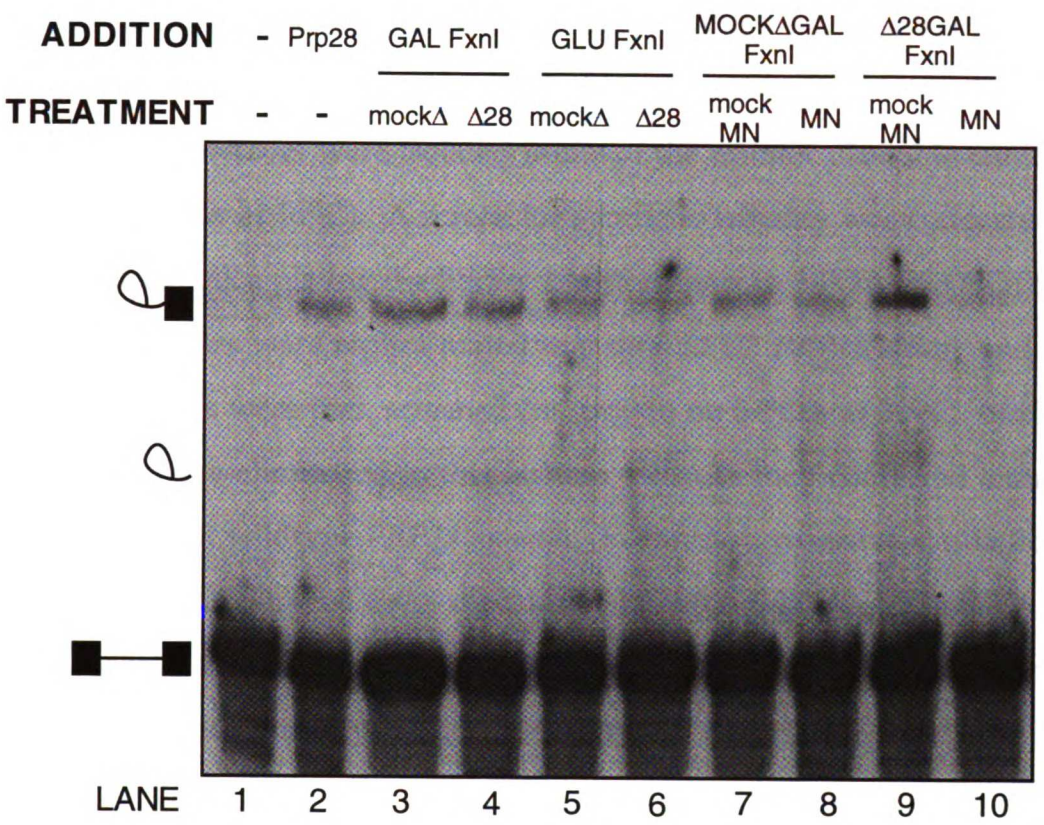
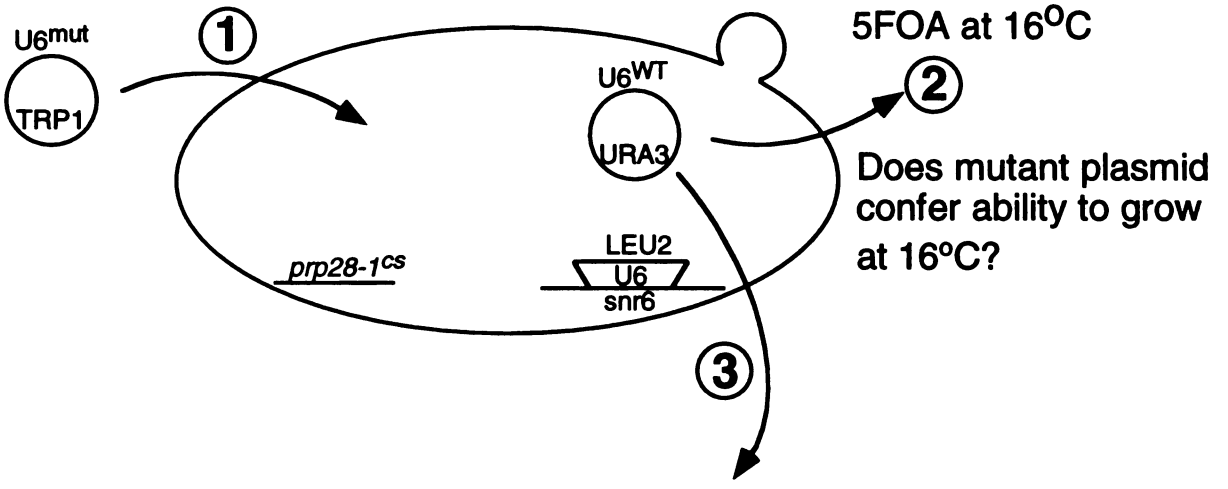


Figure 5A. Genetic strategy to identify mutant snRNAs that suppress (2) or exhibit synthetic lethality with (3)*prp28-1*. A mutagenized library of U6 snRNA alleles was transformed (1) into a cold-sensitive *snr6::LEU2 prp28-1* strain. Transformants were replica plated to 5-FOA at 16°C to select for suppressors of the cold-sensitive *prp28-1* phenotype (2). As described in the text, no U6 suppressors were isolated (none of the mutant plasmids conferred the ability to grow at 16°C). A screen for synthetic lethality was concurrently conducted by replica plating the library transformants to 5-FOA at 30°C (3). Inviable candidates were replica mated to a *snr6::LEU2 PRP28* strain, and the resulting diploids were then screened for growth on 5-FOA at 30°C. Null alleles of U6 were eliminated in this fashion, because they conferred lethality in the presence of wildtype *PRP28* as well. In contrast, recessive synthetic lethal alleles of U6 allowed these diploids to grow on 5-FOA. Mutant U6 snRNA plasmids from these candidates were recovered in order to confirm the synthetic lethal phenotype and to identify the responsible mutations.

Transform bank of mutant snRNA genes

Select for suppressors of cold-sensitive *prp28-1*

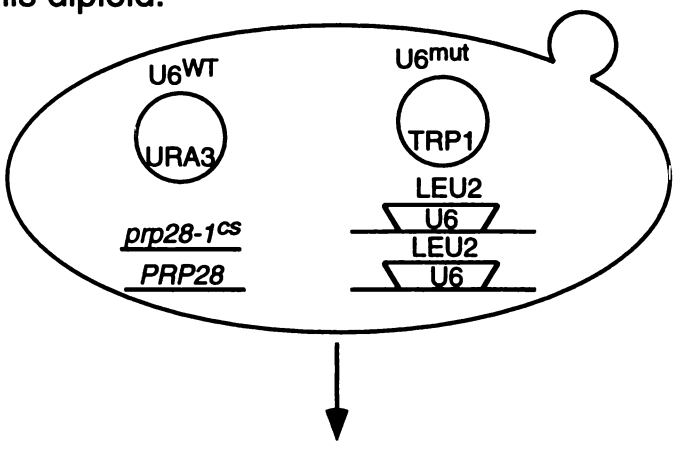


Screen for synthetic lethality with *prp28-1^{cs}*

Is cell dead in presence of mutant plasmid alone?
If yes, then mutant allele is either

a) null or
b) synthetically lethal with *prp28-1^{cs}*

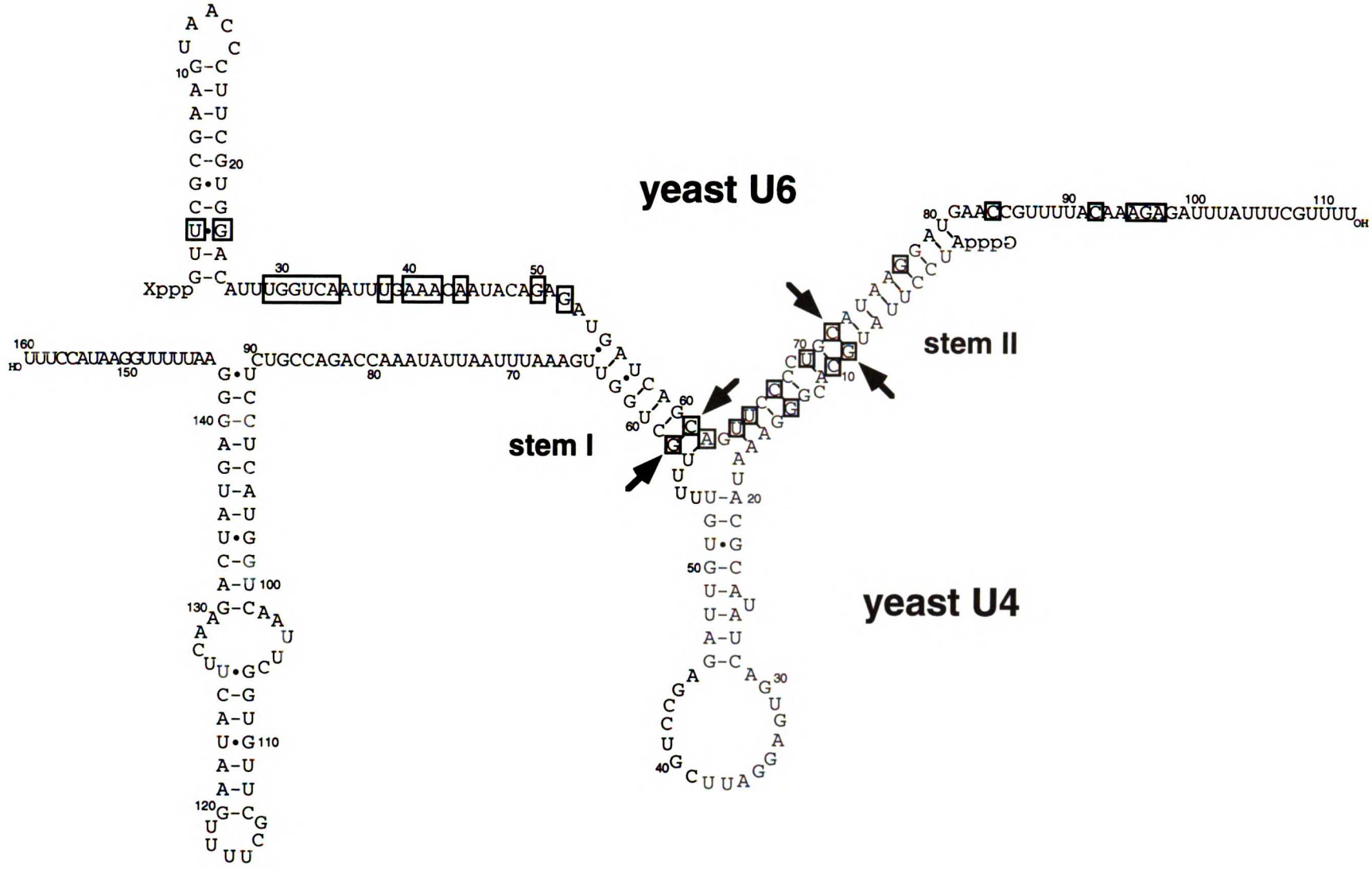
To distinguish, replica mate to form this diploid:



5FOA at 30°C : Dead or Alive?

If dead, then U6^{mut} is a null allele.
If alive, then U6^{mut} is a recessive synthetic lethal allele.

Figure 5B. Secondary structure of duplex U4/U6 snRNAs. Boxes indicate positions of U4 and U6 alleles synthetically lethal with *prp28-1*. Arrows indicate U4/U6 base-pairs in which mutations on either side of the helix display synthetic lethality (see Table 5).


yeast U6
stem I
stem II
yeast U4

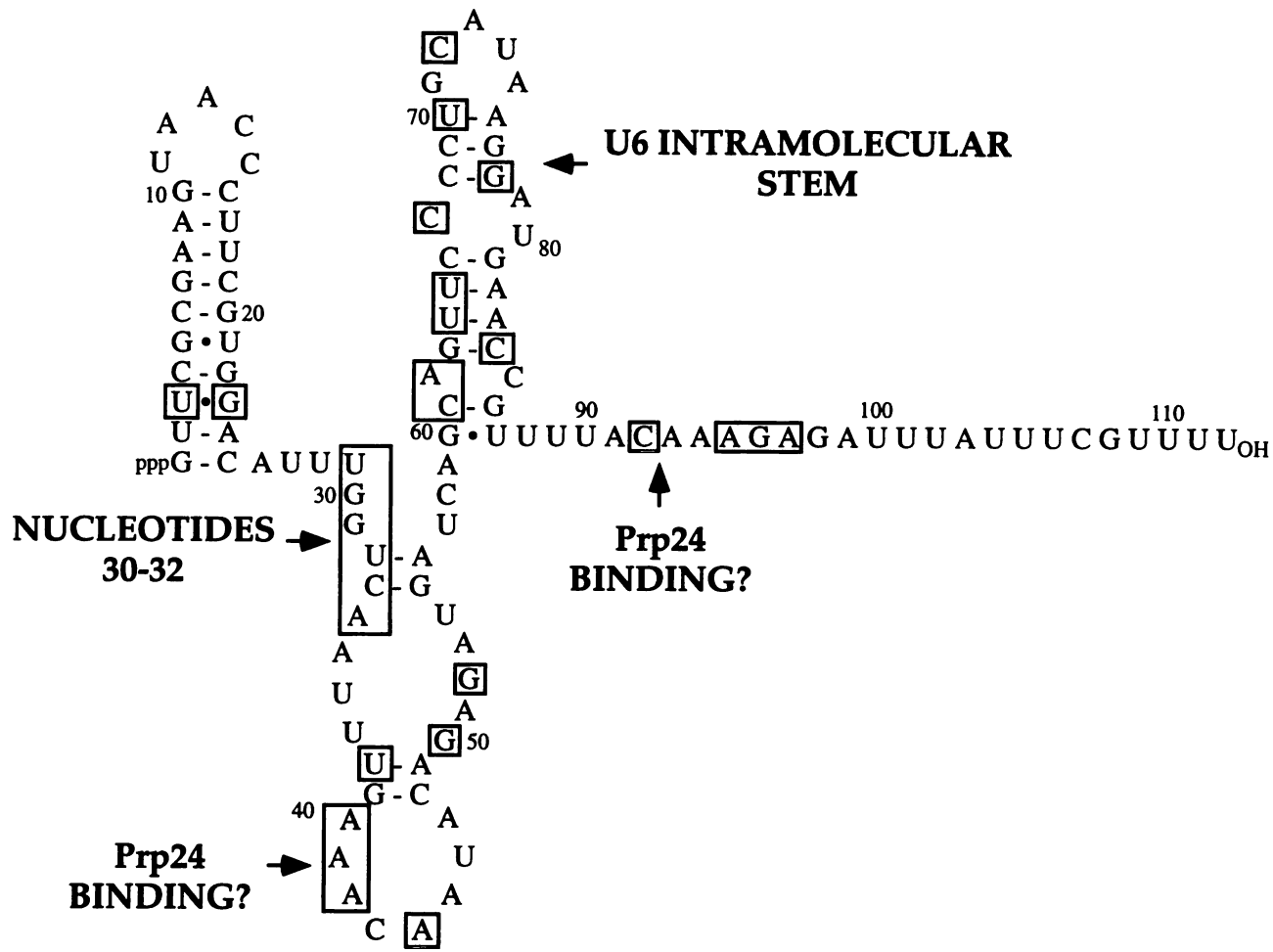

U6 INTRAMOLECULAR STEM
NUCLEOTIDES 30-32
Prp24 BINDING?
Prp24 BINDING?
pppG - C A U U
10
20
30
40
50
60
70
80
90
100
110
G • U U U U A C A A A G A G A U U U A U U U C G U U U U OH

Table 1. *prp28::TRP1* strains (PRY30) bearing the indicated construct as the sole source of Prp28 were streaked onto YEP-galactose (GAL) or YEP-glucose (GLU). Growth was monitored after 4 days at 30°C or 7 days at 18°C.

CONSTRUCT	GAL, 30°C	GLU, 30°C	GAL, 18°C	GLU, 18°C
PRP28	++	++	++	++
GAL-PRP28	++	++	++	++
GAL-PRP28-3HA	++	-	-	-

Table 2. U2 snRNA alleles (generously provided by Manny Ares) were transformed into isogenic *snr20::LYS2 PRP28* (PRY94) or *snr20::LYS2 prp28-1* (PRY95) strains. The growth of these strains was compared on 5-FOA at 18°C, 25°C, 30°C, and 37°C. All of the mutant U2 snRNAs exhibited similar growth patterns in *prp28-1* and *PRP28* strains; these phenotypes are described in the third column. (abbreviations: Cs = cold-sensitive, Ts = temperature-sensitive) As indicated in the second column, these mutations are predicted to alter stem loops Ia, IIa, and IIb in U2 snRNA; and U2/U6 helices Ia and Ib. Therefore, aberrations in U2 snRNA structure are tolerated by cells with impaired Prp28 function.

U2 allele	altered snRNA structures	growth in *prp28-1* and *PRP28* strains
wildtype U2	none	viable at all temps
U49A	U2 stem IIa	viable at all temps
A66U	U2 stem IIa	viable at all temps
U49A, A66U	U2 stem IIa	viable at all temps
A65G	U2 stem IIa	viable at all temps
G53A	U2 stem IIa	Cs
G53C	U2 stem IIa	sick, Cs, Ts
C62G	U2 stem IIa	lethal
C62U	U2 stem IIa	Cs
G53A, C62U	U2 stem IIa	viable at all temps
G53C, C62G	U2 stem IIa	viable at all temps
U56C	U2 loop IIa	viable at all temps
U56G	U2 loop IIa	viable at all temps
A57C	U2 loop IIa	viable at all temps
A57G	U2 loop IIa	viable at all temps
C59U	U2 loop IIa	viable at all temps
50 AUG 52 (tmA)	U2 stem IIa	lethal
63 CAU 65 (tmA')	U2 stem IIa	lethal
tmA + tmA'(smA)	U2 stem IIa	viable at all temps
68 ACAG 71 (tmC)	U2 stem IIb	viable at all temps
81 CCUG 85 (tmC')	U2 stem IIb	slightly Cs
tmC + tmC' (smC)	U2 stem IIb	viable at all temps
U23C	U2 stem-loop Ia; U2/U6 helix Ib	viable at all temps
U23G	U2 stem-loop Ia; U2/U6 helix Ib	viable at all temps
G26A	U2 stem-loop Ia; U2/U6 helix Ia	Cs, Ts
G26U	U2 stem-loop Ia; U2/U6 helix Ia	lethal

Table 3. Analysis of genetic interactions between *prp28-1* and mutant U4 snRNA alleles. U4 snRNA alleles (Shannon and Guthrie, 1991)were transformed into *snr14::TRP1 PRP28* (PRY50) and *snr14::TRP1 prp28-1* (PRY49) strains, and the growth of these strains was compared on 5FOA at 30°C (third and fourth columns). The alleles indicated in bold type displayed slow growth or inviability in combination with *prp28-1* (fifth column). In all cases, the synthetic phenotype was recessive, because growth in the *prp28-1* strain could be restored by an additional copy of the wildtype *PRP28* gene. (This control also indicates that synthetic lethality is due to *prp28-1*, and not any other fortuitous mutation in the PRY49 strain background.) Many of these synthetic lethal alleles display severe growth defects in the presence of wildtype *PRP28*, as well (compare last two columns).

U4 snRNA allele	altered snRNA structures	growth in *prp28-1* strain, 5FOA, 30°C	growth in *PRP28* strain, 5FOA, 30°C	severity of synthetic lethality	growth phenotype in *PRP28* strain
wildtype U4	none	++	++		wildtype
U4 G9A	U4/U6 stem II	+/-	++	**slow growth**	Cs
U4 G9C	U4/U6 stem II	-	+	**inviable**	severe Cs
U4 G9U	U4/U6 stem II	-	+	**inviable**	severe Cs
U4 C10A	U4/U6 stem II	++	++		Cs
U4 C10G	U4/U6 stem II	+/-	++	**slow growth**	Cs
U4 C10U	U4/U6 stem II	++	++		wildtype
U4 G13A	U4/U6 stem II	++	++		Cs
U4 G13C	U4/U6 stem II	++	++		Cs
U4 G13U	U4/U6 stem II	++	++		mild Cs
U4 G14A	U4/U6 stem II	+	++	**slow growth**	severe Cs
U4 G14C	U4/U6 stem II	-	+	**inviable**	severe Cs
U4 G14U	U4/U6 stem II	+/-	++	**slow growth**	severe Cs
U4 G58A	U4/U6 stem I	-	++	**inviable**	Ts
U4 G58C	U4/U6 stem I	-	+	**inviable**	sick/Ts
U4 G58U	U4/U6 stem I	-	+	**inviable**	severe Ts
U4 U60A	U4/U6 stem I	++	++		wildtype
U4 U60C	U4/U6 stem I	++	++		wildtype

Table 3.

Table 4. Analysis of genetic interactions between *prp28-1* and pre-existing alleles of U6 snRNA. U6 snRNA alleles, previously generated to disrupt stems I and II of U4/U6 (Madhani et al., 1990; Shannon and Guthrie, 1991), were transformed into *snr6::LEU2 prp28-1* (PRY66) and *snr6::LEU2 PRP28* (PRY67) strains, and the growth of these strains was compared on 5-FOA at 16°C (or 18°C for the first four alleles listed in the table), 25°C, 30°C, 33°C, 35°C, and 37°C. The alleles indicated in bold type exhibit synthetic lethality in combination with *prp28-1* (see adjacent columns to compare growth at each temperature in *prp28-1* vs. *PRP28* strains). U6 position C61 engages in multiple helical interactions during splicing: U4/U6 stem I, U2/U6 helix Ib, and the U6 intramolecular helix. U6 position C72 is base-paired in U4/U6 stem II and is a key residue in the loop atop the U6 intramolecular structure.

U6 snRNA allele	16°C *prp28-1*	16°C *PRP28*	25°C *prp28-1*	25°C *PRP28*	30°C *prp28-1*	30°C *PRP28*	33°C *prp28-1*	33°C *PRP28*	35°C *prp28-1*	35°C *PRP28*	37°C *prp28-1*	37°C *PRP28*
C58A	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
C58U	+/-	++	++	++	++	++	++	++	+	+	-	-
A59C	+/-	+/-	+	++	++	++	+/-	++	+/-	++	-	-
A59G	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
C50U	-	++	+/-	+/-	-	+/-	-	-	-	-	-	-
G52U C68U	-	++	+/-	++	++	++	-	-	-	-	-	-
C58U C67G	-	++	++	++	++	++	-	-	-	-	-	-
C61G	-	+/-	+/-	++	-	++	-	-	-	-	-	-
C61U	-	++	+/-	++	+/-	++	-	++	-	++	-	++
C61U C72U	-	++	+/-	++	-	++	-	-	-	-	-	-
G60A	-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
G60U	-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
C67A	-	+/-	+/-	+/-	+/-	++	++	++	++	++	++	++
C67G	-	-	+/-	+/-	++	++	++	++	++	++	++	++
C67U	-	++	++	++	++	++	++	++	++	++	++	++
C72A	-	+/-	+/-	+/-	-	+	+/-	++	+	++	+/-	++
C72G	-	++	+	++	++	++	++	++	++	++	++	++
C72U	-	++	++	++	++	++	++	++	++	++	++	++
wildtype U6	-	++	++	++	++	++	++	++	++	++	++	++

Table 5. Restoration of U4/U6 base-pairing with synthetically lethal U6 snRNA alleles. PRY66 (*snr6::LEU2 prp28-1*) was co-transformed with synthetically lethal U6 alleles (first column) along with an additional copy of U4 snRNA bearing all changes at the cognate position (second column), one of which restored base-pairing (third column). Growth of the resulting strains was monitored on 5-FOA at nonpermissive temperatures [C61G, 30°C; C61U, 33°C; C61U C72U, 30°C; C72A, 33°C] (fourth column). Compensatory mutations of the the U4 G58 / U6 C61 base pair did not allow growth in the presence of *prp28-1*, indicating that disruption of base-pairing was not the sole cause of synthetic lethality. In contrast, an extra copy of U4 snRNA suppressed synthetic lethality of U6 C72A, even when base-pairing was not restored. Plasmid-borne *PRP28* also alleviated growth defects of the U6 alleles in these strains, indicating that the synthetic lethality arose from the *prp28-1* lesion and no other fortuitous mutation in PRY66 (data not shown).

Table 5.

U6 snRNA allele	additional U4 snRNA allele	Watson-Crick base-pairing?	growth in *prp28-1* strain
U6 C61G	none	no	-
U6 C61G	wildtype U4 G58	no	-
U6 C61G	U4 G58A	no	-
U6 C61G	U4 G58C	yes (58C/61G)	-
U6 C61G	U4 G58U	no	-
U6 C61U	none	no	-
U6 C61U	wildtype U4 G58	no	-
U6 C61U	U4 G58A	yes (58A/61U)	-
U6 C61U	U4 G58C	no	-
U6 C61U	U4 G58U	no	-
U6 C61U C72U	none	no	-
U6 C61U C72U	wildtype U4 G58	no	-
U6 C61U C72U	U4 G58A	yes (58A/61U)	-
U6 C61U C72U	U4 G58C	no	-
U6 C61U C72U	U4 G58U	no	-
U6 C72A	none	no	-
U6 C72A	wildtype U4	no	++
U6 C72A	U4 G9A	no	++
U6 C72A	U4 G9C	no	++
U6 C72A	U4 G9U	yes	++

Table 6. U6 snRNA alleles that are synthetically lethal with *prp28-1*: cross-classification by phenotypes (columns) and genotypes (rows). Each mutant U6 snRNA allele recovered from the screen depicted in Figure 5A displayed a characteristic degree of synthetic lethality (severity of growth phenotype increases from left to right). Several alleles contain multiple mutations that affect more than one region of the snRNA, and thus are listed under more than one of the genotypic categories. In each category, the relevant mutation is in bold type, and other affected regions are denoted by abbreviations in brackets. [abbreviations: B = intramolecular U6 stem; I, II = U4/U6 stems I or II; Ib = U2/U6 helix Ib; P = regions of U6 implicated in Prp24 binding; G = U6 nucleotides 30-32] Alleles that were isolated multiple times are followed by parentheses: e.g. C61U (5).

Table 6.

	GROWTH PHENOTYPE in *prp28-1* STRAIN (compared to *PRP28* strain)		
CATEGORY of U6 snRNA ALLELE	**SLOW**	**VERY SLOW**	**INVIABLE**
U6 intramolecular stem (aka Brow stem) **[B]** **[I,II] = U4/U6 stem** **[Ib] = U2/U6 helix**	**C61U** (5) [I, Ib] A26C **C61U** [I, Ib] G30A **U64G U65A** [II, G] G10A **ΔC67 C72U** [II] **ΔU70** (3) [II] G31A **C72U** [II, G] G31A **ΔC84** [G]	**C61A** [I, Ib] **C61U** A97G [I, Ib, P] G30U **C67U** [II, G] A42C **C84U** [P]	G31A **C61U** [I, Ib, G] **C61U C67U** [I, II, Ib] **C61U** G96A [I, Ib, P] **ΔA62** [I] +CCCC after **C69** [II] G30A **U70G** [II, G] G31U ΔU38 **C84U** [G] after **A83**, insert duplication of +35-end
U6 regions implicated in Prp24 binding **[P]**	**A42C** (3) U32C **C92G A95C** [G] U32C **A40C +C** after **C43** [G]	U3A **A42C** **A42C** C84U [B] C61U **A97G** [I, Ib, P]	**A41G A44C** G31C **A95C** [G] ΔG30 **ΔG96** [G] C61U **G96A** [I, Ib, P]

U6 nucleotides 30-32 **[G]**	**G30A** U64G U65A [II, B] **G31A** (8) **G31A ΔU32** **G31A** C72U [II, B] **G31A** ΔC84 [B] **U32C** C92G A95C [P] **U32C**, A40C, +C after C43 [P]	**Δ30-32** **G30U** C67U [II, B]	**Δ29-34** **ΔG30** ΔG96 [P] **G30A** G52U **G30A** U70G [II, B] **G31U** **G31A** C61U [I, Ib] **G31C** A95C [P] **G31U** ΔU38 C84U [B]
misfit			**G50C**

CHAPTER 2

A Spliceosomal Recycling Factor that Reanneals U4 and U6 snRNPs

ABSTRACT

An extensive base-pairing interaction between U4 and U6 snRNAs is disrupted during activation of the spliceosome. An important unanswered question is how these helices are re-formed for the next round of splicing. Here, we show that the yeast RNA-binding protein Prp24 reanneals U4 and U6 to promote recycling of spliceosomal snRNPs. In the absence of Prp24, initial rounds of splicing proceed normally. With time, snRNPs containing unpaired U4 and U6 snRNAs accumulate and splicing is inhibited. Addition of purified Prp24 regenerates the U4/U6 snRNP and restores splicing. Strikingly, Prp24 reanneals native U4 and U6 snRNPs much more efficiently than deproteinized snRNAs.

An unexpected finding from the genetic and biochemical analysis of pre-messenger RNA (pre-mRNA) splicing is that the process is highly dynamic. The pre-mRNA substrate and small nuclear RNAs (snRNAs: U1, U2, U4/U6, U5) undergo a series of helical exchanges to promote intron removal within the spliceosome, a large ribonucleoprotein (RNP) complex (1, 2). The most conspicuous RNA rearrangement involves the disruption of an extensive base-pairing interaction between U4 and U6 snRNAs (3). This RNA duplex is packaged with proteins within the U4/U6 small nuclear ribonucleoprotein particle (snRNP), and joins the spliceosome in a U4/U6.U5 triple snRNP (4). The ATP-dependent displacement of U4 from the spliceosome allows U6 snRNA to adopt new configurations that are believed to create the active site for splicing catalysis (5, 6). In contrast, U4 snRNA is not required for the chemical steps of splicing (7). Each round of splicing demands the separation of the U4/U6 duplex. However, snRNPs are thought to be re-used for multiple splicing events because they are long-lived (1). The regeneration of U4/U6 snRNPs would presumably entail reannealing the RNAs within the free U4 and U6 snRNPs, a reversal of their unwinding on the spliceosome. Such spliceosomal recycling pathways have not been previously examined.

The protein encoded by the essential yeast gene *PRP24* initially emerged as a candidate U4/U6 recycling factor from genetic suppression studies (8). Prp24 is a U6 snRNP protein that harbors three RNA recognition motifs (RRMs). These conserved structural folds contact RNA directly in many proteins (9), some of which promote annealing of complementary RNAs in vitro (10 , 11). The genetic analysis underscored this sequence homology, and led to the proposal that Prp24 anneals free U6 and U4 liberated

from spliceosomes (8). In support of this model, Ghetti et al. demonstrated that recombinant Prp24 anneals synthetic U4 and U6 RNAs in vitro, albeit inefficiently (12). This activity could be relevant only for newly manufactured U4 and U6 snRNPs during biogenesis, however, because reannealing of U4 and U6 snRNPs has not been established as an integral part of the splicing pathway.

We have tested the hypothesis that Prp24 reanneals U4 and U6 snRNPs in its role as a spliceosomal recycling factor. We predicted that removal of Prp24 from a splicing reaction would result in the accumulation of free U4 and free U6 snRNPs. Therefore, we immunodepleted greater than 97% of epitope-tagged Prp24 (13, 14) from yeast cell extract (ΔPrp24 extract) (15) (Fig. 1A). snRNP populations in mock-depleted and ΔPrp24 extracts undergoing splicing were visualized by native gel electrophoresis (16). Prp24 protein purified from yeast (17 , 18) (Fig. 1B) was also added to some of the reactions. From this analysis, Prp24 is indispensable for recycling spliceosomal snRNPs. Splicing in ΔPrp24 extract generated large amounts of free U4 snRNP and U6*, a fast-migrating precursor to free U6 snRNP that apparently lacks Prp24 (Fig. 1C, lanes 10 vs. 4 and 6). Meanwhile, virtually all U4/U6.U5 and U4/U6 snRNPs disappeared (Fig. 1C, lanes 10 vs. 4). This snRNP imbalance resulted from splicing, because depletion of U4/U6.U5 required intact 5' splice site and branchpoint signals in pre-mRNA, incubation at 23°C ((PR,)19), and ATP (Fig. 1C, lanes 10 vs. 9). A normal snRNP profile was recovered with the addition of Prp24 protein: free U4 was diminished, U6* was abolished, and U4/U6 and U4/U6.U5 snRNPs were restored (Fig. 1C, lanes 14 vs. 10). Splicing in wildtype extract also produced some free U4 and U6*, which decreased in the presence of purified Prp24 (Fig. 1C, lanes 4 and 6). From

these observations, we conclude that splicing consumes U4/U6.U5 and liberates free U4 and U6* snRNPs. Prp24 is necessary and sufficient to transform free U4 and U6* to U4/U6 snRNPs, facilitating the regeneration of U4/U6.U5 snRNPs. No other protein in the extract substitutes for these activities.

Surprisingly, adding ATP to extract caused snRNP rearrangements even in the absence of exogenous pre-mRNA. ATP addition to ΔPrp24 extract resulted in the appearance of free U4 snRNP and U6*; mock-depleted extract produced U4/U6 instead (Fig. 1C, lanes 7-8 vs. 1-2). Purified Prp24 protein restored U4/U6 formation in ΔPrp24 extract (Fig. 1C, lanes 7-8 vs. 11-12, 1-2). From these and other results (Fig. 3B, 3C, 4A), we infer that ATP normally disrupts some U4/U6.U5 snRNPs into free U4, U6*, and U5 snRNPs without added pre-mRNA, and indirectly induces Prp24-dependent snRNP recycling.

These data demonstrate that U4 and U6 snRNPs become separated during splicing, and that Prp24 activity is required for their recycling. If the sole function of Prp24 is to replenish U4/U6.U5 snRNPs for spliceosome assembly, then Prp24 should be unnecessary for in vitro splicing as long as U4/U6.U5 levels are adequate. In fact, standard in vitro splicing reactions (20) proceeded normally in ΔPrp24 extract (Fig. 2A). Such behavior is unprecedented, since all other snRNP proteins that are essential for splicing in vivo are similarly essential for splicing in vitro ((Banroques and Abelson, 1989; Vijayraghavan et al., 1989; Brown and Beggs, 1992)21). However, Prp24's primary recycling function may be obscured by the standard in vitro splicing assay. In a typical reaction, the molar ratio of active U4/U6.U5 snRNPs to input pre-mRNA molecules may be large (22), and mRNA may be produced

even if snRNPs are not regenerated. In order for in vitro splicing efficiency to reflect snRNP recycling capability, U4/U6.U5 snRNPs in the extract must be exhausted.

We devised an in vitro splicing assay that we predicted would be sensitive to a recycling defect (Fig 2B). Unlabelled pre-mRNA was added to splicing reactions in mock- and immunodepleted extracts for a short period in order to force most U4/U6.U5 snRNPs through a round of splicing. Then, later splicing events were monitored by adding labelled pre-mRNA in a second incubation. When Prp24 was present, even a tenfold excess of unlabelled pre-mRNA did not fully impede later rounds of splicing (Fig 2C, lane 4). In contrast, prior incubation of ΔPrp24 extract with twofold excess unlabelled pre-mRNA largely blocked subsequent rounds of splicing (Fig 2C, lane 9). In this protocol, Prp24 is essential for later rounds of in vitro splicing. To ascertain that Prp24 was the critical component (and not other missing U6 snRNP proteins), we showed that purified Prp24 partially complemented the ΔPrp24-3HA splicing defect, confirming that this splicing assay responds to Prp24 activity (Fig. 2D). Therefore, although Prp24 is not required for conventional in vitro splicing, Prp24 is necessary under conditions in which splicing is likely to rely heavily on snRNP recycling.

These results establish a role for Prp24 in U4/U6 snRNP recycling during splicing, and suggest that Prp24/snRNP interactions are likely to be dynamic. Therefore, we assessed whether Prp24's associations with snRNPs changed during an in vitro splicing reaction. To determine which snRNPs bind Prp24, we attempted to detect a shift in snRNP mobilities on native gels with antibodies against epitope-tagged Prp24. Two specific supershifts of free

U6 snRNP were observed (Fig. 3A, lane 3) (23), corroborating previous data (8). Interestingly, under splicing conditions (when both ATP and pre-mRNA are present), these supershifted species were diminished, suggesting that the free U6/Prp24 complex was depleted by splicing (Fig. 3A, lane 4). As an independent and more sensitive probe of Prp24's physical interactions, we monitored snRNAs that immunoprecipitated with tagged Prp24 (24). Consistent with its presence in the free U6 snRNP, tagged Prp24 associated predominantly with unpaired U6 snRNA in the absence of ATP (Fig. 3B, 3C, lanes 3). Interestingly, when ATP was added, Prp24 coimmunoprecipitated base-paired U4/U6, as well as free U6 and small amounts of free U4 RNAs (Fig. 3B, 3C, lanes 4). Inclusion of pre-mRNA in the reaction did not change the spectrum of snRNAs that coprecipitated with Prp24, suggesting that Prp24 does not assemble onto the spliceosome (Fig. 3B, 3C, lanes 3-4 vs. 5-6). In summary, as predicted for a protein implicated in U4/U6 annealing, Prp24 is complexed with both free U6 and duplex U4/U6 snRNPs.

Notably, the association of Prp24 with U4/U6 required ATP. A plausible explanation for this result arises from snRNP gel analysis (Fig. 1C): ATP induces a redistribution of snRNPs in the extract, making U4 available to anneal to the free U6/Prp24 complex. Specifically, U4/U6.U5 has been previously observed to dissociate in the presence of ATP ((Cheng and Abelson, 1987; Konarska and Sharp, 1987)5). We sought to determine whether Prp24 catalyzes the reannealing of native U4 and U6 snRNPs released by ATP from higher-order snRNP complexes. We took advantage of antibodies against epitope-tagged Brr2/Snu246, a reported U4/U6.U5 snRNP component (25), to immunopurify complexes that contained U4/U6 on beads (26). The U4/U6 duplex bound to the beads was disrupted when ATP was

added, and unpaired U4 and U6 were released into the supernatant (Fig. 4A). We tested whether Prp24 protein promoted base-pairing between these RNAs. In fact, Prp24 rapidly converted freed U4 and U6 to a duplex U4/U6 species with a melting temperature of ~55°C, identical to native U4/U6 (Fig. 4B, data not shown). Reannealing of native snRNPs by Prp24 was complete within five minutes; however, when the released U4 and U6 snRNPs were deproteinized prior to incubation with Prp24, annealing was markedly slowed (Fig. 4B). Moreover, efficient reannealing of native U4 and U6 snRNPs occurred with 0.4 to 2 nM Prp24, while deproteinized snRNPs were poorly reannealed by 2 to 50 nM Prp24 (Fig. 4C). These data suggest that other proteins in the supernatants contribute to annealing efficiency. The absence of these proteins may explain why lengthy incubations (1-3 hours) with higher concentrations of Prp24 (80 nM) are necessary to anneal synthetic U4 and U6 RNAs (12). A plausible hypothesis is that the U4 and U6 RNAs are uniquely structured in the released snRNP particles. Indeed, properly packaged RNPs may be the optimal substrates for hnRNP A1 and other proteins with only modest RNA annealing activity in vitro (11).

We have presented evidence that Prp24 is a recycling factor that reanneals U4 and U6 snRNPs liberated by splicing. The following model is consistent with our observations (Fig. 5). When the U4/U6 helices are disrupted on spliceosomes (or in U4/U6.U5 snRNPs) through the action of ATP, pre-existing Prp24/free U6 snRNP captures the released free U4 snRNP. Prp24 converts free snRNPs into duplex U4/U6 snRNPs by reannealing the RNAs. U4/U6 snRNPs are then reincorporated into U4/U6.U5 snRNPs, which are used in new rounds of spliceosome assembly. Since neither Prp24 protein nor U4/U6 annealing activity has been detected in the U4/U6.U5

snRNP or on spliceosomes (27), Prp24 is likely to leave U4/U6 when U5 joins. Spliceosome disassembly and U4/U6.U5 dissociation liberate U6*, which may then bind Prp24 to replenish the Prp24/free U6 snRNP. Although Prp24 specifically restores U4/U6, other proteins may govern recycling of U1, U2, and U5 snRNPs if they are reconfigured by splicing.

Prp24 catalyzes a specific RNA annealing step necessary for spliceosomal recycling. Other dynamic RNPs in the spliceosome (28) and ribosome (29) may use similar annealing proteins to assist their structural transitions. Dissection of how Prp24 restructures U4 and U6 snRNAs promises to yield much-needed insights into the physiological mechanisms of RNA folding (30).

REFERENCES and NOTES

1. M. J. Moore, C. C. Query, P. A. Sharp, in *The RNA World*, R. F. Gesteland, J. F. Atkins, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993) pp. 303-358; B. Rymond and M. Rosbash, in *The Molecular and Cellular Biology of the Yeast Saccharomyces*, E. W. Jones, J. R. Pringle and J. R Broach, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992) pp. 143-192.

2. H. D. Madhani, C. Guthrie, *Ann. Rev. of Genetics* **28**, 1-26 (1994); M. Ares, B. Weiser, *Progress in Nucleic Acid Research and Molecular Biology* **50**, 131-159 (1995).

3. D. A. Brow, C. Guthrie, *Nature* **334**, 213-218 (1988).

4. S.-E. Behrens, R. Lührmann, *Genes and Dev.*. **5**, (1991).

5. M. M. Konarska, P. A. Sharp, *Cell* **49**, 763-774 (1987); S.-C. Cheng, J. Abelson, *Genes and Dev.* **1**, 1014-1027 (1987).

6. H. Madhani, C. Guthrie, *Cell* **71**, 803-817 (1992); T. Wolff, A. Bindereif, *Genes and Dev.* **7**, 1377-1389 (1993); D. M. Fortner, R. G. Troy, D. A. Brow, *Genes and Dev.* **8**, 221-233 (1994).

7. S.-L. Yean, R.-J. Lin, *Mol. and Cell. Biol.* **11**, 5571-5577 (1991).

8. K. W. Shannon, C. Guthrie, *Genes & Dev.* **5**, 773-785 (1991).

9. D. J. Kenan, C. C. Query, J. D. Keene, *TIBS* **16**, 214-220 (1991).

10. B. W. Pontius, P. Berg, *PNAS* **87**, 8403-8407 (1990).

11. D. S. Portman, G. Dreyfuss, *EMBO J.* **13**, 213-221 (1994).

12. A. Ghetti, M. Company, J. Abelson, *RNA* **1**, 132-145 (1995).

13. A NotI site was introduced immediately upstream of the stop codon of the *PRP24* gene by polymerase chain reaction (PCR); no mutations were found in the amplified region. The 121 bp triple-hemagglutinin (3HA) tag [M. Tyers, B. Futcher, *Mol. Cell. Biol.* **13**, 5659 (1993)] and 54 bp polyoma (Pya) tag (31) were separately inserted into the NotI site, and the resulting *PRP24-3HA* and *PRP24-Pya* fragments were subcloned into pUN90 [S. J. Elledge, R. W. Davis, *Gene* **70**, 303-312]. These plasmids were transformed into PRY98 (*MATα his3Δ trp1Δ lys2-801 ura3-52 ade2-101 PRP24::LYS2 pUN50-PRP24*) and were shown to complement the *PRP24::LYS2* deletion by plasmid shuffling on 5-fluoroorotic acid [C. Guthrie and G. R. Fink (eds), *Guide to Yeast Genetics and Molecular Biology*, Academic Press, San Diego, CA (1991)]. The resulting *PRP24-3HA* strain (PRY112, or PRY98 with *pUN90-PRP24-3HA*) was used for biochemical analysis.

14. 100 µL aliquots of Prp24-3HA extract were incubated with 5 µg 12CA5 antibody (ΔPrp24) or 5 µL phosphate-buffered saline (mock-depleted) on ice for one hour, and then nutated one hour with ~20 µL protein A Sepharose beads (Pharmacia) washed with buffer D (20 mM HEPES pH 7.9, 0.2 mM

EDTA, 50 mM KCl, 20% vol/vol glycerol, 0.5 mM DTT). The depleted supernatants lacked Prp24 and free U6 snRNP (see Fig. 1C, lanes 7-10).

15. Yeast whole cell extracts were prepared using the liquid nitrogen method [J. G. Umen and C. Guthrie, *Genes and Dev.* **9**, 855 (1995)] with modifications [A. Ansari and B. Schwer, *EMBO J.* **14**, 4001 (1995)].

16. Native gel analysis (without heparin) was modified from M. M. Konarska and P. A. Sharp, Cell **49**, 763 (1987). 5 µl samples contained 40% extract, 2 mM ATP, 2.5 mM MgCl2, 3% PEG 8000, 60 mM potassium phosphate pH 7, 1 mM spermidine, and 4 nM actin pre-mRNA unless otherwise stated. Reactions were incubated 30 minutes at 23-25°C, and loaded on a pre-run, 4% polyacrylamide (80:1) gel (15 x 15 x .15 cm) made up in TGM buffer (50 mM Tris base, 50 mM glycine, 2 mM $MgCl_2$). Electrophoresis was for 6-7 hours at 160V in TGM buffer at 4°C. Northern analysis was conducted as described (33). To supershift snRNP complexes formed during 23°C incubation, samples were incubated with 1 µg 12CA5 antibody for one hour on ice prior to loading.

17. The first 220 bp of the *PRP24* gene were PCR amplified as a BamHI/ApaI fragment (using an internal ApaI site and a primer with a BamHI site just upstream of the start codon). This fragment was inserted downstream of the GPD promoter of pG1 [M. Schena, D. Picard, K. R. Yamamoto, Meth. Enzymol. **194**, 389 (1991)] along with either a 1.6 kb ApaI/SalI *PRP24* fragment or a 1.4 kb ApaI/SalI *PRP24-Pya* fragment. Both constructs overexpressed Prp24p when transformed into PRY98 as determined by Western blotting with anti-Prp24 polyclonal antibodies.

18. Three liters of each strain PRY115 (PRY98 with *pG1-PRP24* as sole *PRP24* gene) and PRY116 (PRY98 with *pG1-PRP24-Pya* as sole *PRP24* gene) were harvested in late log phase. Whole cell extract was prepared (15), and 150 mg of each was subjected to 30-55% ammonium sulfate precipitation. The precipitates were resuspended in 40 mL AGK 200 [10 mM HEPES pH 7.9, 200 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 μg/mL pepstatin A, 1 μg/mL leupeptin], and incubated with 150 μL protein G Sepharose (washed in AGK 200) on a nutator at 4°C for 30 minutes. (This step removed proteins that bound nonspecifically to the resin.) The supernatants were added to 1 mL protein G Sepharose coupled to anti-Polyoma antibodies (beads: AGK 200 = 1:1) as in (31). After 1.5 hours nutating at 4°C, the supernatants were removed, and the beads were washed 5 x 20 mL AGK 700 (AGK buffer with 700 mM KCl), 1 x 15 mL AGK 200, 1 x 15 mL AGK 50 (AGK buffer with 50 mM KCl). The washed beads were eluted twice by nutating at 23°C for 10 minutes with 2 x 400 μL 1 mg/mL peptide [EYMPME; (31)] in AGK 50. Each eluate was dialyzed against 2 x 2 L of buffer D at 4°C for 3.5 hours total. The dialysates were microcentrifuged 10 minutes, and the supernatants were collected. Approximately 15 μg of Prp24 was recovered, and no snRNAs copurified under these conditions (i.e. with a sensitivity to ~1 fmol U6, no U6 was detected in 1 pmol Prp24 protein).

19. P. L. R. and C. G., unpublished results.

20. R. J. Lin et al, *J. Biol. Chem.* **260**, 14780-14792 (1985).

21. U. Vijayraghavan, M. Company, J. Abelson, *Genes and Dev.* **3**, 1206-1216 (1989); J. Banroques, J. N. Abelson, *Mol. and Cell. Biol.* **9**, 3710-3719 (1989); J. D. Brown, J. D. Beggs, *EMBO J.*. **11**, 3721-9 (1992); F. Galisson, P. Legrain, *Nucl. Acids Res.* **21**, 1555-1562 (1993); S. W. Ruby, T.-H. Chang, J. Abelson, *Genes and Dev.* **7**, 1909-1925 (1993).

22. A typical splicing reaction may contain ~3 nM U4 and ~6 nM U6 snRNAs, compared to 0.2-0.5 nM pre-mRNA. (It is not known what fraction of the snRNAs is competent for splicing.) Levels of U6 snRNA are constitutively lower in PRY112 (see Fig. 3B), so sensitivity to snRNP recycling defects may be enhanced in this extract.

23. Supershifted free U6 snRNP did not hybridize to U4; appeared in Prp24-3HA extract and not in untagged Prp24 extract; and increased with antibody concentration. Only the faster-migrating, diffuse supershift was observed at lower antibody concentrations, suggesting that multiple antibody molecules may bind the 3HA epitope (20).

24. 80 µL splicing reactions (21) containing extract, ATP and unlabelled actin pre-mRNA as noted were incubated 30 minutes at 25°C. After nutating for one hour at 4°C with 3.2 µg 12CA5 antibody and 400 µL NET 50 (50 mM Tris pH 7.4, 50 mM NaCl, 0.05% Nonidet-P40), antibody complexes were mixed with protein A Sepharose (Pharmacia) for one hour at 4°C. The bound complexes were washed three times with 800 µL NET 50, phenol/chloroform extracted on ice twice, and ethanol precipitated to isolate RNAs.

25. S. M. Noble, C. Guthrie, *Genetics* **143**, 67-80 (1996); J. Lauber, et al., *EMBO J.* **15**, 4001-4015 (1996).

26. P. L. R. and C. G., manuscript in prep.

27. On a glycerol gradient, the vast majority of Prp24-3HA comigrates with U6 and U4/U6 snRNPs, and away from triple snRNPs and spliceosomes. Also, Prp24 is not detected in Prp8-3HA immunoprecipitates that contain U4, U6, and U5 snRNAs. Thus, we have no evidence indicating that Prp24 is a component of U4/U6.U5 snRNPs or of the spliceosome.

28. J. Valcárcel et al., *Science* **273**, 1706-1709 (1996).

29. M. Altmann, B. Wittmer, N. Méthot, N. Sonenberg, H. Trachsel, *EMBO J.* **14**, 3820-3827 (1995).

30. D. Herschlag, *J.. Biol. Chem.* **270**, 20871-4 (1995).

31. K. R. Schneider, R. L. Smith, E. K. O'Shea, *Science* **266**, 122 (1994)

32. S. M. Noble, C. Guthrie, *EMBO J.* **15**, 4368-79 (1996); R. Bordonné, J. Banroques, J. Abelson, C. Guthrie, *Genes and Dev.* **4**, 1185-1196 (1990).

33. Blots were stripped for one hour at 65°C in 6X SSC, 0.2% SDS to remove probes. Subsequent probings were performed as described (32).

34. Z. Li, D. A. Brow, *Nucl. Acids Res.* **21**, 4645-6 (1993).

35. U4/U6 snRNPs were coimmunoprecipitated with polyoma-tagged Brr2 (26). The bound complexes were washed 3 x 500 µL NET 50, and then incubated for 10 minutes at 23°C with 150 µL buffer (40% buffer D, 2.5 mM $MgCl_2$, 3% PEG 8000, 60 mM potassium phosphate, ± 2 mM ATP). The supernatants were separated from the beads, and the beads were washed 1 x 500 µL NET 50. For Fig. 4A, the samples were directly deproteinized for nondenaturing gel analysis of U4 and U6 snRNAs. For Fig. 4B and 4C, 1 µL of purified Prp24 was added to 11 µL of +ATP supernatant (~ 0.4 nM each of U4 and U6) and incubated for 2 minutes at 23°C, unless otherwise stated. Reactions were stopped by the addition of 200 µL 0.3 M sodium acetate, 6 mM EDTA, 0.5% SDS on ice. RNAs were isolated by proteinase K digestion (3) or phenol/chloroform extraction and ethanol precipitation. To deproteinize U4 and U6 prior to annealing, 100 µL +ATP supernatant was incubated at 23°C for 30 minutes with 10 mg proteinase K beads (Sigma) washed in buffer D. The deproteinized supernatant was carefully collected for annealing reactions.

36. We thank Marc Lenburg, Betsy O'Neill, and Erin O'Shea for helpful advice on immunoaffinity purification; Jeremy Brown for sharing the anti-polyoma hybridoma line; past and present members of the Guthrie lab for constructive criticism during the course of experiments; and Liz Blackburn, Cathy Collins, Alan Frankel, Hiten Madhani, Stephen Rader, Chris Siebel, Jon Staley, Olke Uhlenbeck, and Kurt Zingler for comments on the manuscript. We are indebted to Lucita Esperas, Carol Pudlow, and Heli Roiha for matchless technical assistance. P. R. was supported by a Howard Hughes Medical Institute predoctoral fellowship. C. G. is an American Cancer Society Research Professor of Molecular Genetics.

Figure 1A. Estimation of extent of immunodepletion in ΔPrp24 extract by Western blot. Epitope-tagged Prp24-3HA extract (13) was subjected to mock- and immunodepletion (ΔPrp24) (15). Two-fold serial dilutions of Prp24-3HA extract were compared to samples of mock-depleted and ΔPrp24 extract on a Western blot probed with 12CA5 antibodies (BAbCo). Since the residual Prp24-3HA in 10 µl of ΔPrp24 extract is less than the 1/16 dilution of 5 µl Prp24-3HA extract, ΔPrp24 extract is >97% depleted of Prp24-3HA.

EXTRACT	tagged Prp24			mock	ΔPrp24
DILUTION	1/8	1/16	1/32	1	1
μL LOADED	5	5	5	10	10

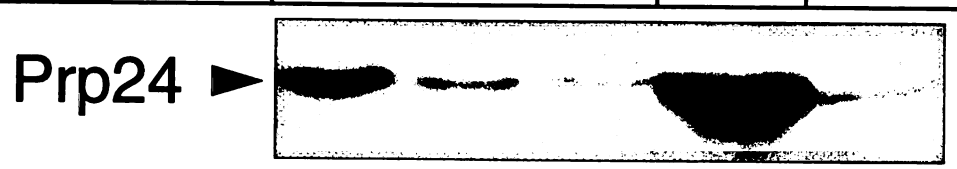


Figure 1B. Silver-stained gel of purified Prp24 protein. Polyoma-tagged Prp24 was overexpressed in yeast (17), and immunoaffinity purified (18). Molecular weight standards (MW) are in kilodaltons. Lane 1 shows 1% of a control eluate prepared from yeast overexpressing untagged Prp24; lane 2 shows 1% of the tagged Prp24 eluate (~150 ng). Tagged Prp24 migrates as a single 53 kDa polypeptide.

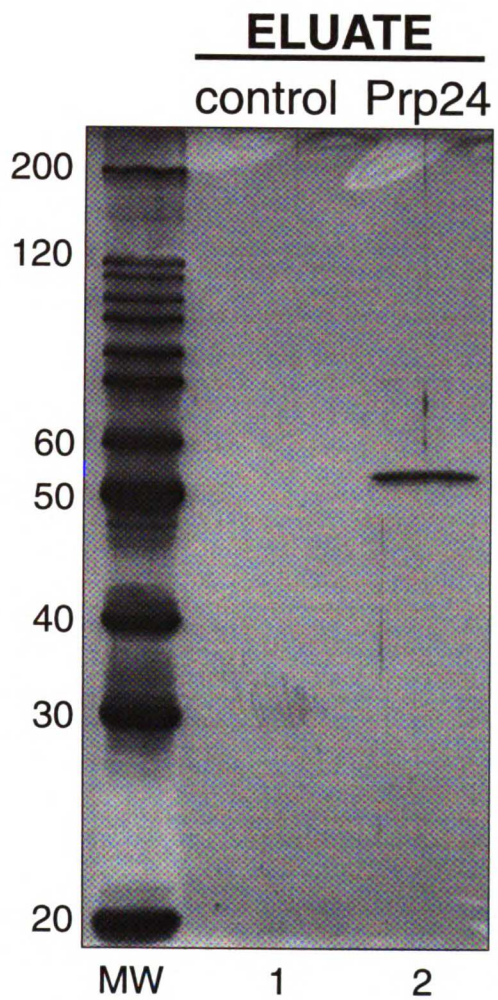

ELUATE
control Prp24
200
120
60
50
40
30
20
MW
1
2

Figure 1C. Splicing in ΔPrp24 extract causes aberrant snRNP distributions, which can be rescued by the addition of purified Prp24 protein. Mock-depleted and ΔPrp24 extracts were incubated ± ATP, pre-mRNA, and purified Prp24 and electrophoresed on a native gel (16) to resolve snRNPs. After Northern blotting (32), the individual snRNAs were sequentially detected by stripping and reprobing (33). Only blots probed for U4 (left) and U6 (right) snRNAs showed significant differences ± Prp24.

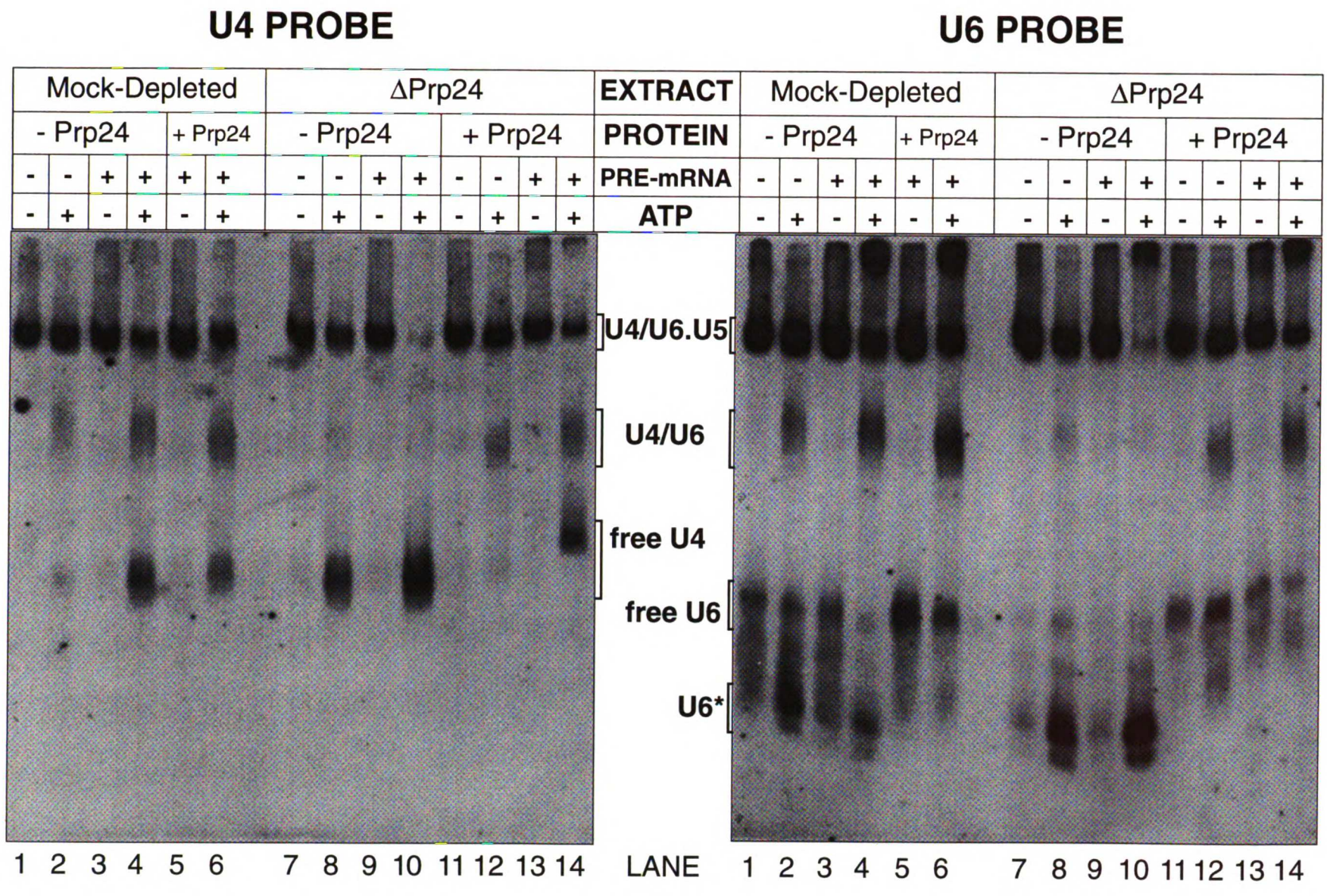

U4 PROBE
U6 PROBE
Mock-Depleted
ΔPrp24
EXTRACT
- Prp24
+ Prp24
PROTEIN
PRE-mRNA
ATP
U4/U6.U5
U4/U6
free U4
free U6
U6*
1 2 3 4 5 6 7 8 9 10 11 12 13 14
LANE

Figure 2A. Immunodepletion of Prp24 does not inhibit conventional in vitro splicing. Time course of standard in vitro splicing reactions in mock-depleted and ΔPrp24 extract. In vitro splicing was performed and analyzed as described (20), except that reactions contained 50% extract. Intermediates and products of splicing reaction depicted from top to bottom: lariat-3'exon, excised lariat intron, pre-mRNA, mature mRNA.

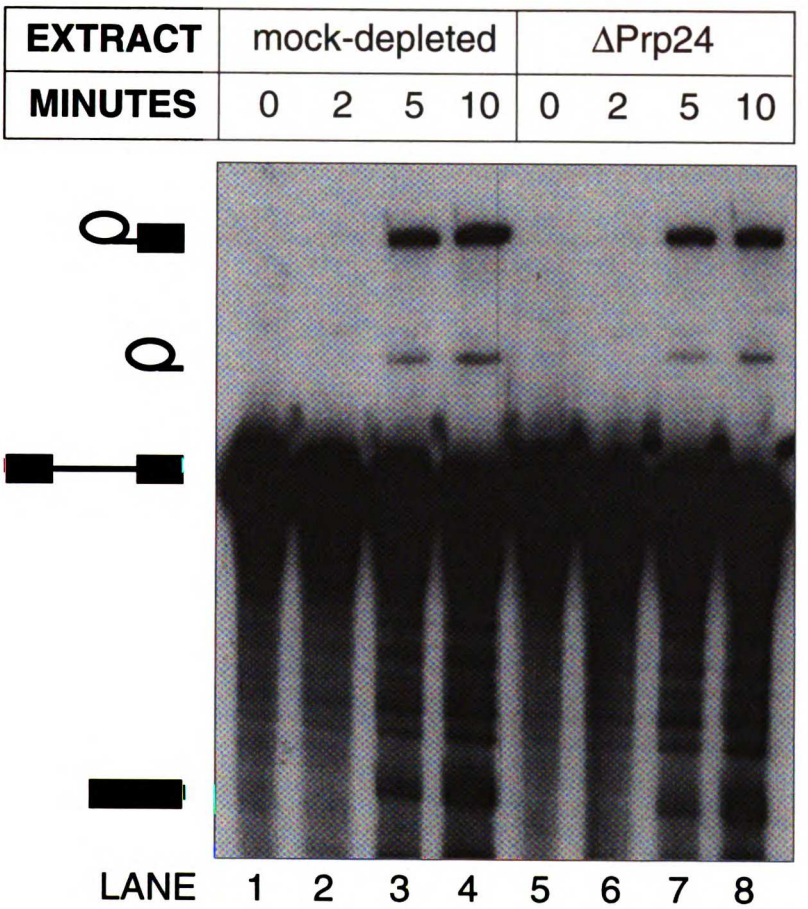

EXTRACT
mock-depleted
ΔPrp24
MINUTES
0 2 5 10 0 2 5 10
LANE 1 2 3 4 5 6 7 8

Figure 2B. Schematic of in vitro splicing assay sensitized to snRNP recycling defects. Splicing reactions as in (A) contained varying amounts of very low specific activity ("cold") actin pre-mRNA in the first incubation, and were supplemented with 0.3 nM high specific activity actin pre-mRNA in the second incubation. Reactions were analyzed as in (A).

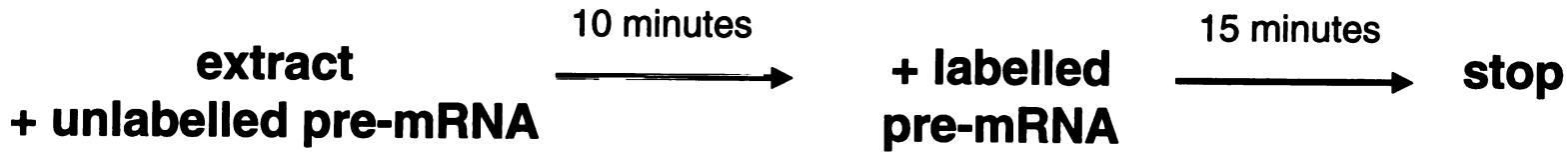

extract
+ unlabelled pre-mRNA
10 minutes
+ labelled
pre-mRNA
15 minutes
stop

Figure 2C. Immunodepletion of Prp24 impedes later rounds of splicing. Sensitized in vitro splicing in mock-depleted and ΔPrp24 extracts. Concentrations of cold pre-mRNA in reactions are indicated as fold excess over 0.3 nM labelled pre-mRNA. When labelled and unlabelled pre-mRNAs were added simultaneously, splicing efficiency in mock and ΔPrp24 extracts was similar, indicating that pre-incubation is necessary for the ΔPrp24 splicing defect (19). Moreover, the pre-mRNA in the initial incubation required intact intron consensus sequences to inhibit later rounds of splicing in ΔPrp24 extract (19).

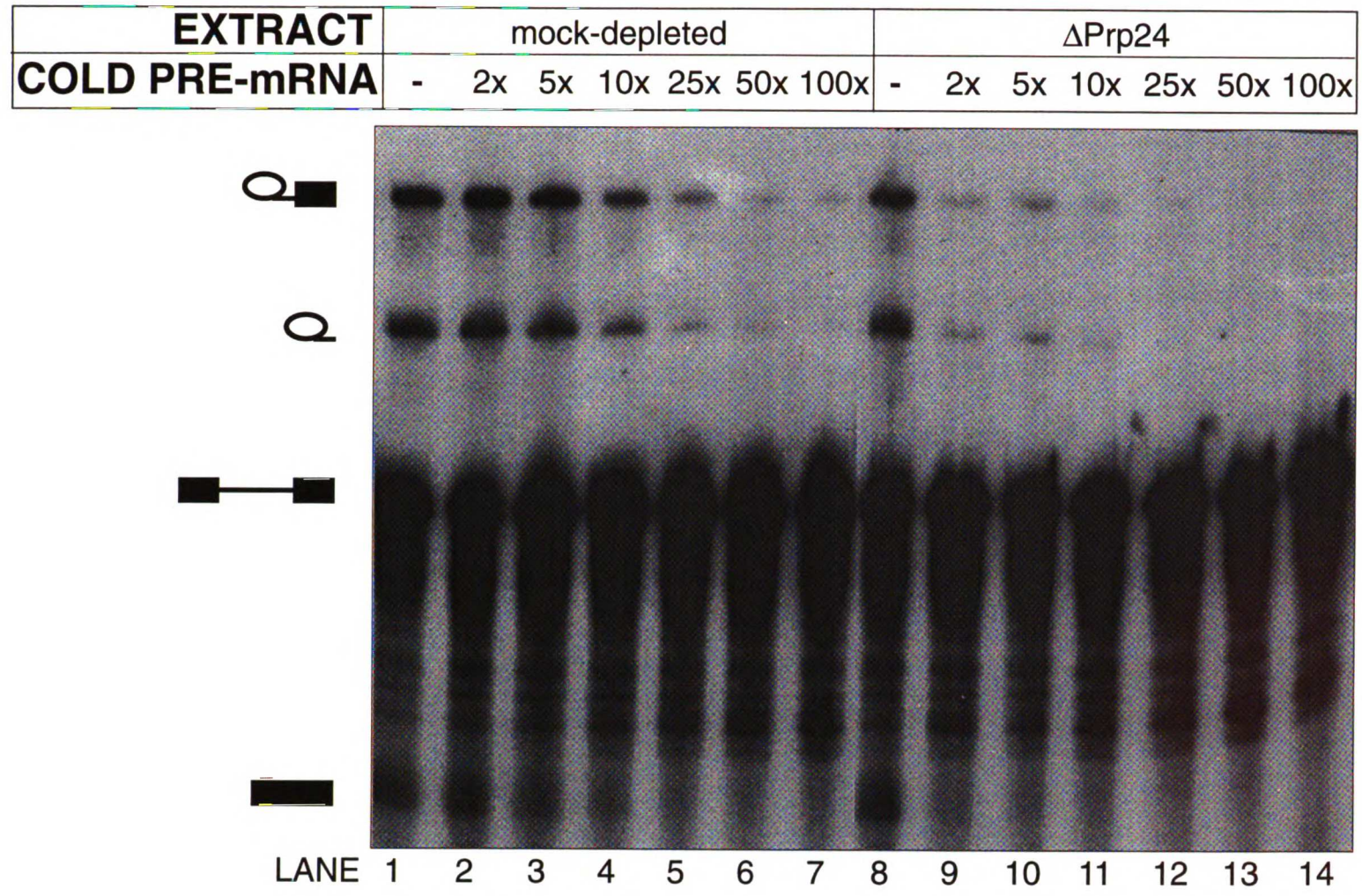

EXTRACT
mock-depleted
ΔPrp24
COLD PRE-mRNA
- 2x 5x 10x 25x 50x 100x
- 2x 5x 10x 25x 50x 100x
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 2D. Complementation of ΔPrp24 extract splicing defect with 4 nM purified Prp24 protein (lanes 7-12). Concentrations of cold pre-mRNA in reactions are as in (C).

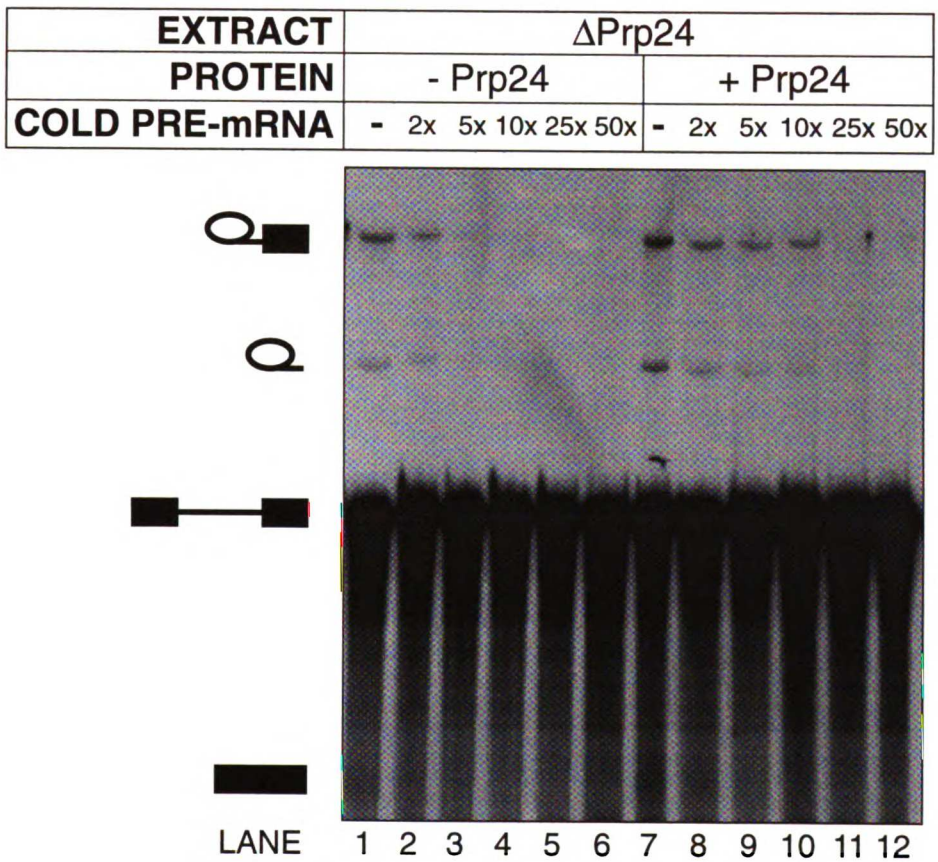

EXTRACT
ΔPrp24
PROTEIN
- Prp24
+ Prp24
COLD PRE-mRNA
- 2x 5x 10x 25x 50x
- 2x 5x 10x 25x 50x
LANE
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3A. Native gel analysis of supershifted snRNPs. Samples of epitope-tagged Prp24-3HA extract were incubated with ATP, ± pre-mRNA and anti-HA antibodies (12CA5; BAbCo). Reactions were then electrophoresed on a native gel to separate snRNPs as in Fig. 1C. Free U6 snRNPs bound to antibodies display decreased mobility ("supershift") (23). U4/U6 supershifts are not detected, either because they are too faint or because they comigrate with U4/U6.U5 snRNPs.

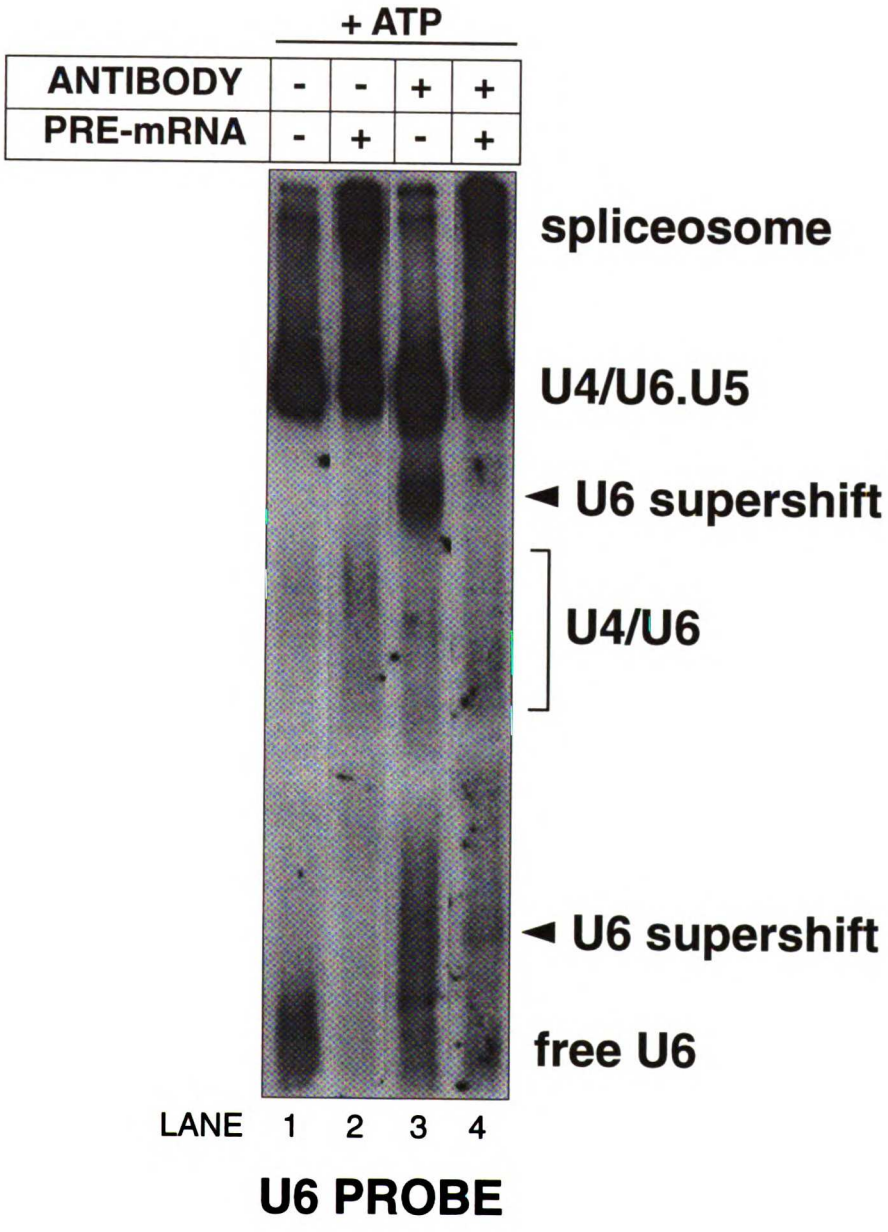

+ ATP
ANTIBODY - - + +
PRE-mRNA - + - +
spliceosome
U4/U6.U5
U6 supershift
U4/U6
U6 supershift
free U6
LANE 1 2 3 4
U6 PROBE

Figure 3B. Denaturing gel analysis of snRNAs coimmunoprecipitated with Prp24. Epitope-tagged Prp24-3HA was immunoprecipitated (24) from samples of whole cell extract incubated ± ATP and pre-mRNA. The coprecipitated RNAs from one-half of each sample were subjected to Northern analysis on a denaturing gel (32). In untagged extract or in tagged extract without antibody, no snRNAs are coimmunoprecipitated.

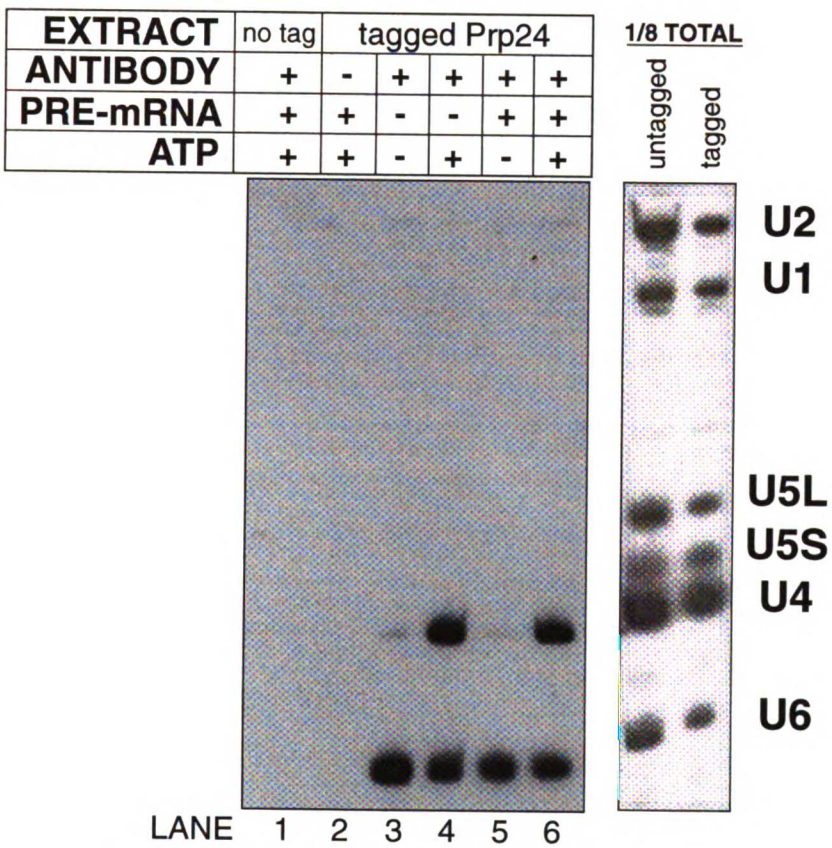

EXTRACT
no tag
tagged Prp24
ANTIBODY
+ - + + + +
PRE-mRNA
+ + - - + +
ATP
+ + - + - +
1/8 TOTAL
untagged
tagged
U2
U1
U5L
U5S
U4
U6
LANE 1 2 3 4 5 6

Figure 3C. Prp24 associates dynamically with free U6, free U4, and duplex U4/U6 snRNAs. Nondenaturing gel analysis of snRNAs coimmunoprecipitated with Prp24. One-quarter of each RNA sample from (B) was hybridized in solution to a ^{32}P-labelled oligo complementary to U6 or U4, and electrophoresed on a nondenaturing polyacrylamide gel to assess base-pairing status (34). Duplex U4/U6 migrates more slowly than free U6 or free U4 RNAs.

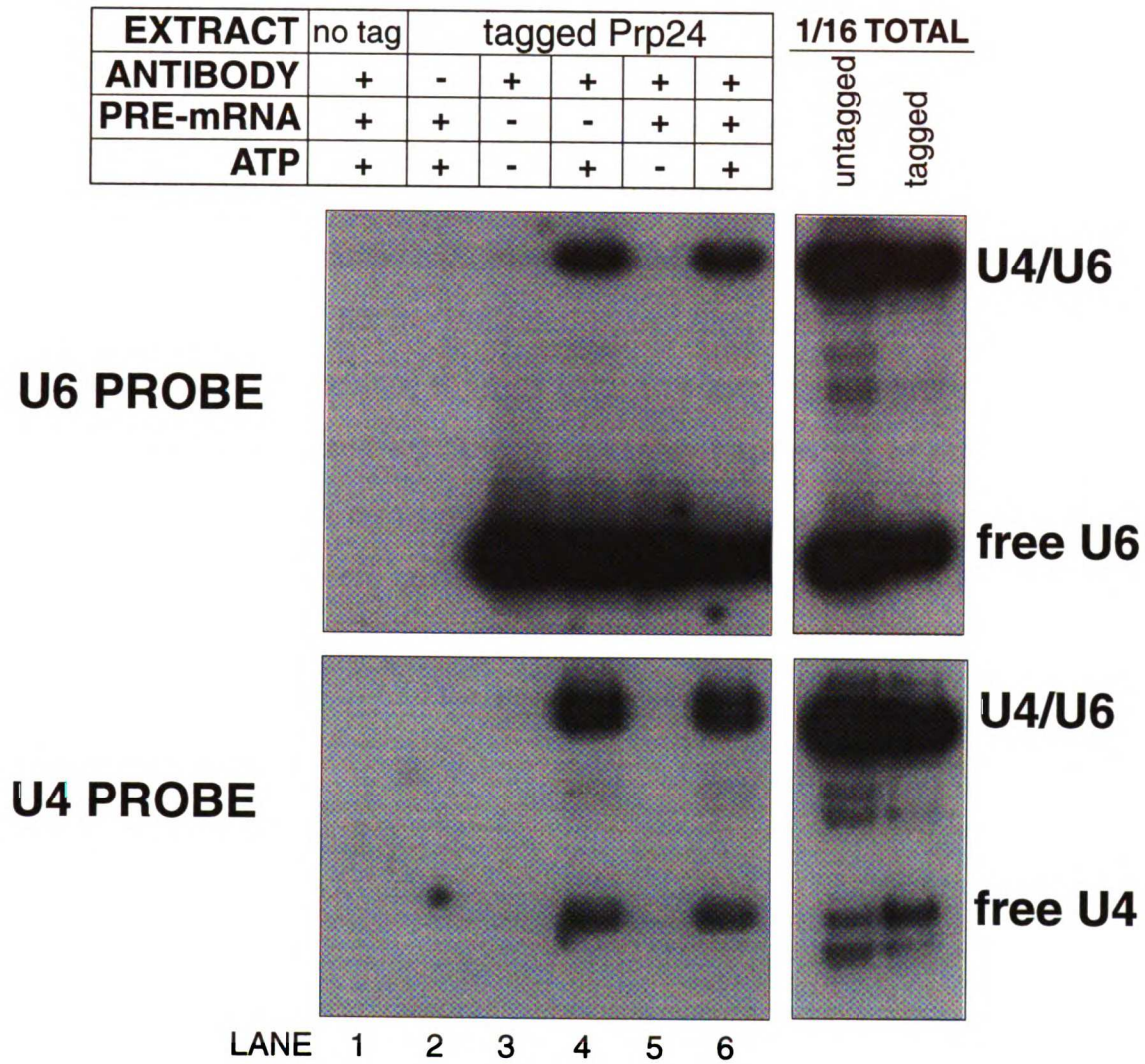
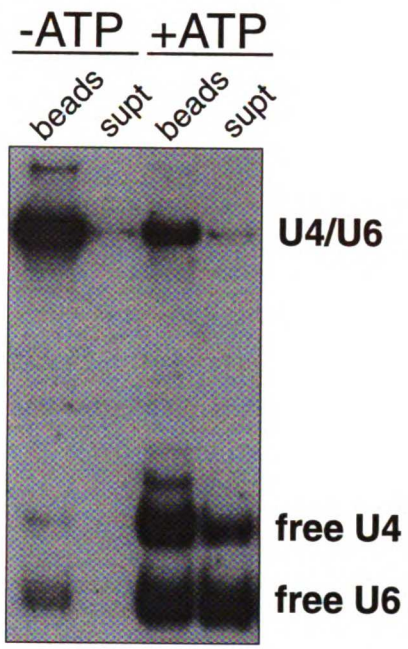

EXTRACT
no tag
tagged Prp24
ANTIBODY
PRE-mRNA
ATP
1/16 TOTAL
untagged
tagged
U6 PROBE
U4/U6
free U6
U4 PROBE
U4/U6
free U4
LANE 1 2 3 4 5 6

Figure 4A. Immunopurified Brr2 snRNP complexes bound to beads contain U4/U6 duplex. When ATP is added, the U4/U6 helices are disrupted, and free U4 and free U6 are released into the supernatant (supt) (35). Deproteinized RNAs were analyzed on nondenaturing gels as in Fig. 3C.

Figure 4B. Purified Prp24 rapidly reanneals free U4 and free U6 released from duplex U4/U6. Time course of Prp24-catalyzed reannealing. 2 nM Prp24 was added to supernatants containing ~0.4 nM free U4 and free U6 from (A), and incubated at 23°C for the indicated time. Alternatively, supernatants were treated with proteinase K beads prior to annealing ("deproteinized snRNPs"). Deproteinized snRNPs do not inhibit annealing of native snRNPs ("mix"), indicating that no proteinase K remains in the annealing reactions. Deproteinized RNAs were analyzed on nondenaturing gels as in Fig. 3C.

A

U4/U6 DISSOCIATION



B

ANNEALING TIME COURSE

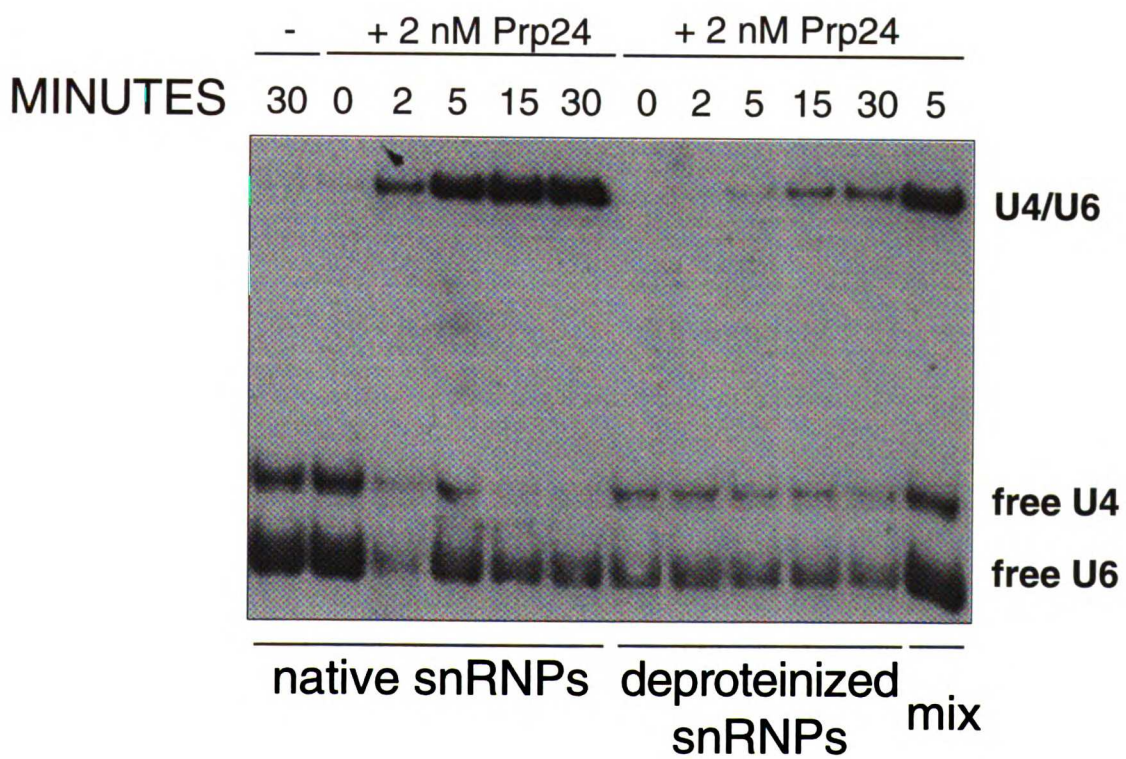


Figure 4C. Titration of Prp24 protein in annealing reactions. Annealing reactions as in (B) were incubated for 2 minutes at 23°C with the stated amount of Prp24. Deproteinized RNAs were analyzed on nondenaturing gels as in Fig. 3C.

C

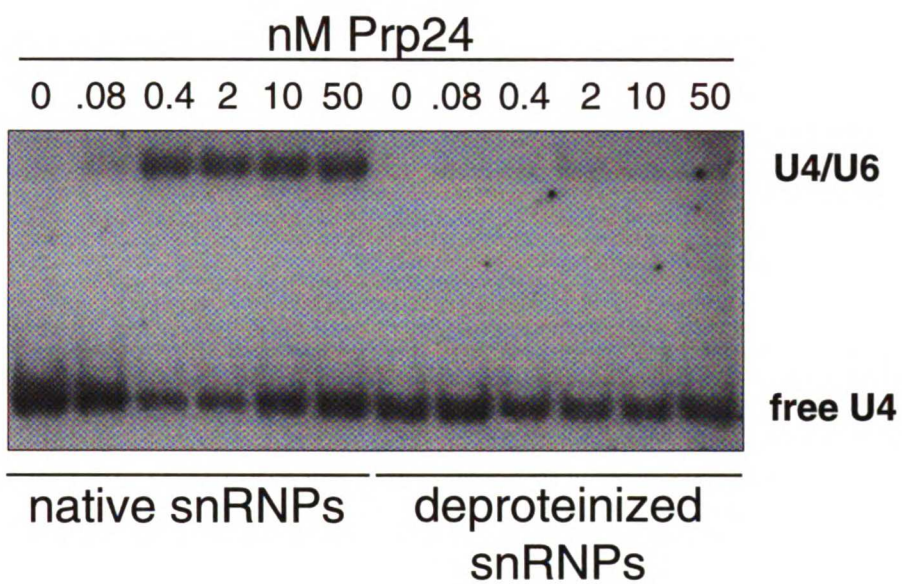
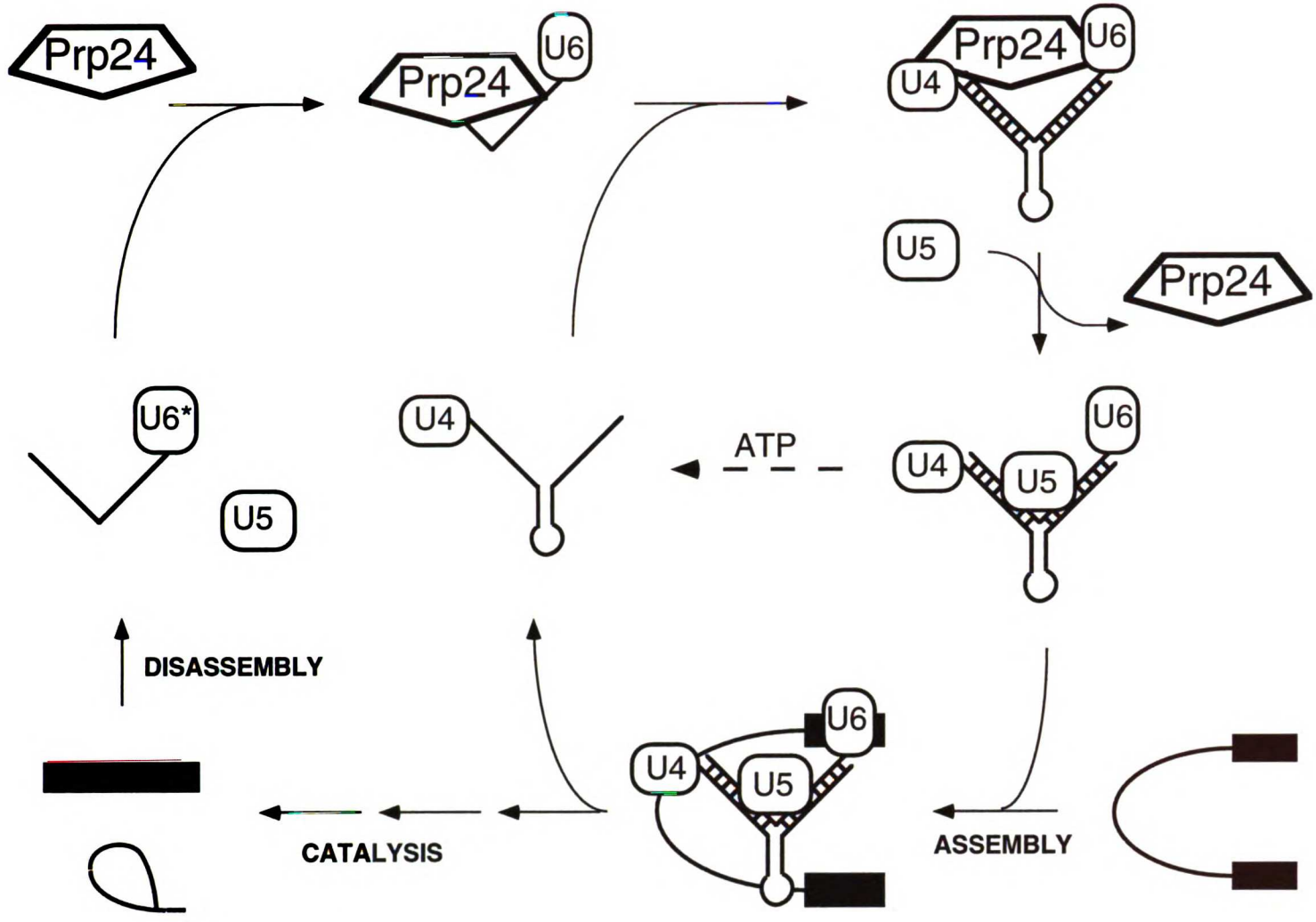

nM Prp24
0 .08 0.4 2 10 50 0 .08 0.4 2 10 50
U4/U6
free U4
native snRNPs
deproteinized snRNPs

Figure 5. Model depicting the proposed recycling role of Prp24 in pre-mRNA splicing. For simplicity, only U4, U6, and U5 snRNPs are depicted. Prp24 reanneals U6* and free U4 released from spliceosomes, facilitating the regeneration of U4/U6.U5 snRNP. Some U4/U6.U5 snRNP dissociates in the presence of ATP; the resulting free U4 and U6* are similarly recycled by Prp24.


Prp24
Prp24
U6
Prp24
U6
U4
U5
Prp24
U6*
U5
U4
ATP
U4
U5
U6
DISASSEMBLY
U6
U4
U5
CATALYSIS
ASSEMBLY

CHAPTER 3

Disruption of RNA Base-Pairing in U4/U6 snRNPs Requires ATP Hydrolysis and Brr2, a DEXH-box Helicase-Like Protein

ABSTRACT

An extensive base-pairing interaction between U4 and U6 snRNAs is disrupted during catalytic activation of the spliceosome. We have tested the long-standing hypothesis that an RNA-dependent ATPase of the DEAD-box superfamily promotes this rearrangement. A cold-sensitive mutation (*brr2-1*) in the RNA helicase-like domain of Brr2 blocks splicing before the first chemical step. Both wildtype and mutant proteins are found in endogenous spliceosome-like complexes containing base-paired U4/U6. Intriguingly, particles associated with Brr2 release unpaired U4 and U6 snRNPs when incubated with ATP. In striking contrast, ATP fails to dissolve the U4/U6 duplex within Brr2-1 complexes. We propose that Brr2 is required for the ATP-dependent dissociation of the U4/U6 helices on the spliceosome.

INTRODUCTION

Introns are removed from pre-messenger RNAs (pre-mRNAs) by spliceosomes, large ribonucleoprotein complexes consisting of five small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4/U6, U5) and a large cast of proteins that coalesce on pre-mRNA (Rymond and Rosbash, 1992; Moore et al., 1993). The spliceosome executes the two chemical steps of the splicing reaction using active sites which consist, at least in part, of intricately structured RNAs (Nilsen, 1994). Construction of this catalytic core demands the precise positioning of RNA helices in time and space. Thus, the progress of spliceosome assembly is marked by the sequential formation and dissolution of RNA duplexes (Madhani and Guthrie, 1994a; Ares and Weiser, 1995). Base pairs form first between U1 snRNP and the 5' splice site, and then between U2 snRNP and the intron branch point sequence. Next, the U4/U6.U5 triple snRNP ushers the U5 snRNP and the hybrid U4/U6 snRNP to the spliceosome. U4 and U6 snRNAs engage in extensive intermolecular base-pairing within the U4/U6 snRNP; in yeast, the remarkably stable U4/U6 snRNA duplex melts in vitro at 55°C (Brow and Guthrie, 1988). Shortly after U4/U6.U5 contacts the pre-mRNA, a major rearrangement occurs. U4 is no longer tightly bound to the spliceosome, and critical residues of U6 snRNA are juxtaposed with the 5' splice site and to U2 snRNA. These newly created U2/U6 and U6/5' splice site helices are thought to constitute the chemical reaction center of the spliceosome.

Because U4/U6 base-pairing is mutually exclusive with the U2/U6 interaction, the disruption of the U4/U6 duplex is necessary to activate the spliceosome for catalysis. Regulation of this event is likely to be crucial to

ensure proper alignment of U6 with other active site RNAs. Destabilization of U4 from the spliceosome displays a stringent requirement for ATP in vitro, suggesting active unwinding and/or displacement processes (Cheng and Abelson, 1987; Konarska and Sharp, 1987). However, the two known factors involved in this key transition, Prp19 and the U4 snRNP protein Prp4, do not contain signature ATPase motifs, and their specific roles are unclear (Tarn et al., 1993a; Ayadi et al., 1997).

Six yeast splicing factors have been identified that bear homology to the DEAD- and DEXH-box families of RNA-dependent ATPases and putative RNA helicases, and thus are attractive candidates for governing such helical exchanges on the spliceosome (Rymond and Rosbash, 1992; Moore et al., 1993). Four of these proteins (Prp5, Prp2, Prp16, and Prp22) are required at distinct steps in the splicing pathway, and four possess RNA-dependent ATPase activity (Prp5, Prp2, Prp16, Brr2/Snu246) (Company et al., 1991; Schwer and Guthrie, 1991; Kim et al., 1992; Kim and Lin, 1993; O'Day et al., 1996; Xu et al., 1996). Despite intensive scrutiny, none of these splicing factors has been shown to unwind generic RNA duplexes in vitro. One possible explanation is that these proteins are not conventional processive helicases, but they function instead on short, specific spliceosomal targets that cannot be easily reproduced outside the physiological context. Indirect assays suggest that Prp16 and Prp2 hydrolyze ATP to promote conformational changes on the spliceosome (Schwer, 1992; Kim and Lin, 1996), but detecting specific helical displacements has proven surprisingly difficult. Consequently, these helicase homologs have eluded assignment to spliceosomal RNA substrates or unwinding events, including U4/U6 dissociation.

Our work has focused on the DEXH-box ATPase Brr2 as the most promising candidate for a U4/U6 helicase. *brr2-1* was identified in our laboratory as a cold-sensitive yeast mutant defective for pre-mRNA splicing in vivo (Noble and Guthrie, 1996a). The *BRR2* gene was independently isolated by three other groups as *SNU246* (Lauber et al., 1996), *SLT22* (Xu et al., 1996), and *RSS1* (Lin and Rossi, 1996). These analyses revealed that Brr2 is likely to be an RNA-dependent ATPase (Xu et al., 1996) within the U4/U6.U5 triple snRNP (Lauber et al., 1996). Because of this physical association, we speculated that Brr2's putative RNA helicase activity might be responsible for the disruption of U4/U6 base-pairing on the spliceosome. We have now tested this hypothesis using the cold-sensitive *brr2-1* allele (Noble and Guthrie, 1996a). We have found that both Brr2 and Brr2-1 associate with a snRNP complex that contains base-paired U4/U6. Strikingly, only the wildtype Brr2 complex releases unpaired U4 and U6 in response to ATP. Studies of in vitro splicing in *brr2-1* extract point to a dual role for Brr2 in U4/U6.U5 assembly, as well as in U4/U6 disruption during spliceosomal activation.

RESULTS

Cold-sensitivity of *brr2-1* is conferred by a mutation in the first putative helicase domain

The Brr2 protein is unique among the DEXH/DEAD-box splicing factors because it has two predicted helicase domains, as revealed by comparison with the sequence of its human homolog, U5 200K(Lauber et al., 1996) (Figure 1). To determine whether the cold-sensitive *brr2-1* lesion resided in these regions, we transferred the chromosomal *brr2-1* allele to a plasmid bearing the wildtype *BRR2* gene by gap repair(Rothstein, 1991). A single mutation within a 447 bp ApaLI/StuI fragment from the first DEXH-box domain was sufficient to confer cold-sensitivity when substituted into the wildtype gene (data not shown). This mutation, A2417G, converts a glutamic acid to a glycine within a putative helicase motif absolutely conserved between the yeast and human homologs (Figure 1). Other DEAD/DEXH-box proteins with mutations in the motifs depicted in Figure 1 display impaired ATPase/helicase activities(Pause and Sonenberg, 1992) and splicing deficits(Plumpton et al., 1994). We therefore tested the prediction that the mutant Brr2-1 protein would prevent U4/U6 disruption.

ATP-dependent displacement of free U4 snRNP is blocked in *brr2-1* extracts

We first sought U4/U6 unwinding activity in U4/U6.U5 snRNPs, which contain Brr2(Lauber et al., 1996) and base-paired U4/U6 (see Figure 4B; data not shown). We and others(Cheng and Abelson, 1987) have noticed that ATP dissociates the U4/U6.U5 triple snRNP into free U4, free U6, and free U5 snRNPs. These individual snRNPs rapidly reassociate, so this ATP-induced U4/U6.U5 disintegration is noticed only when U4/U6 reannealing is

prevented. This can be accomplished by removing the snRNP recycling factor Prp24 (P. L. R. and C. G., manuscript submitted), or by preparing a 40% ammonium sulfate precipitate from the extract known as Fraction I ((Cheng and Abelson, 1986); P. L. R. and C. G., unpublished results). Dissociation of U4/U6.U5 appears mechanistically similar to the disruption of U4/U6 on the spliceosome, since both are ATP-dependent processes that separate the U4/U6 helices. We reasoned that if helix displacement requires Brr2 function, then Brr2-1 should block triple snRNP dissociation in Fraction I. Addition of ATP to wildtype Fraction I caused the appearance of a free U4 snRNP species (Figure 2A, lanes 1-2). ATPγS could not substitute (lane 3), suggesting that ATP hydrolysis is required for U4 release. In contrast, U4/U6.U5 snRNPs from *brr2-1* Fraction I did not separate into free snRNPs in the presence of ATP (Figure 2A, lanes 4-5). Therefore, snRNPs from *brr2-1* cells fail to undergo an ATP-dependent conformational rearrangement observed with wildtype snRNPs.

We next examined whether similar abnormalities could be detected in *brr2-1* whole cell extract. In a wildtype extract visualized on a native gel, U4/U6.U5 and free U6 snRNPs were typically abundant (Figure 2B, lanes 1, 5, 9). Diffusely migrating U4/U6 snRNPs were more apparent when ATP was added, because ATP produces free U4 and U6 which are efficiently reannealed (Figure 2B, lanes 2, 6; P. L. R. and C. G., manuscript submitted). The mobilities of free U5 snRNPs and free U2 snRNPs were also heterogeneous (Figure 2B, lanes 9-10, 13-14, thin bars). In *brr2-1* extract, by contrast, no U4/U6 snRNPs were observed in the presence of ATP, and the overall level of U4/U6.U5 snRNPs was very low (Figure 2B, lanes 3-4, 7-8, 11-12). Free U5 and free U2 snRNPs were also diminished in the mutant extract (Figure 2B, lanes

11-12, 15-16, thin bars). These deficits appear significant, because comparable amounts of free U6 snRNP were detected in wildtype and mutant extracts, and equivalent amounts of protein were loaded. Also, a very slow-migrating species that hybridized to U4, U6, U5 and U2 was noticeable in mutant extract, but not in wildtype extract (Figure 2B, lanes 3-4, 7-8, 11-12, 15-16, thick bars). In summary, *brr2-1* extract displays an aberrant snRNP distribution, with few U4/U6.U5 snRNPs and no U4/U6 snRNPs. Like the snRNPs in *brr2-1* Fraction I, snRNPs in *brr2-1* extract are minimally changed by ATP.

Brr2 and Brr2-1 associate with snRNP complexes containing U2, U4, U5 and U6 snRNAs

Although Brr2 is part of U4/U6.U5 snRNPs(Lauber et al., 1996), the mutant Brr2-1 protein might fail to associate with triple snRNPs, and thus indirectly prevent U4 displacement. To test this possibility, we created gene fusions (*BRR2(Pya)*, *brr2-1(Pya)*) that allowed us to perform immunoprecipitations of wildtype and mutant proteins with antibodies against the polyoma epitope tag (abbreviated Pya)(Schneider et al., 1994). The Pya-tagged and untagged versions of *BRR2* and *brr2-1* behave identically in complementing the *BRR2::LEU2* gene disruption (data not shown). Wildtype and mutant Pya-tagged proteins were immunoprecipitated from whole cell extracts at different salt concentrations, and the bound snRNAs were monitored by Northern analysis. Brr2(Pya) associated with U2, U4, U5, and U6 snRNAs from 50 to 250 mM NaCl (U1 snRNA also precipitated at 50 mM NaCl) (Figure 3A, lanes 5-8). Importantly, the same spectrum of snRNAs coprecipitated with tagged Brr2-1 through 350 mM NaCl. These interactions are specific, because no snRNAs were precipitated from untagged extracts (Figure 3A, lanes 1-3), tagged extracts without antibody (lanes 4 and 9), or from

tagged extracts with competitor peptide encoding the polyoma epitope (lanes 14-15). Our results are in general agreement with Lauber et al. (1996), who report that Brr2/Snu246 is a component of the U4/U6.U5 snRNP; however, we note the reproducible association with U2 as well. Moreover, Brr2-1, like Brr2, efficiently associates with U2, U4, U5, and U6 snRNAs in a salt-resistant manner.

To determine whether Brr2 and the snRNPs are present in a single large complex, we sedimented wildtype and mutant extracts on 15-40% glycerol gradients, and performed immunoprecipitations from alternate fractions. In wildtype extract, most U1, U2, U4, and U5 snRNAs resided in the densest third of the gradient, peaking in fractions 17-23 (Figure 3B). Brr2 immunoprecipitated with U1, U2, U4, U5, and U6 snRNAs within this narrow peak (Figure 3B, fractions 18-22; data not shown). The *brr2-1* snRNP profile differed: U4 and U6 were especially enriched in the densest fractions (Figure 3C, fractions 25-29), while U1, U2, and U5 snRNPs were broadly distributed (Figure 3C, fractions 11-29). Brr2-1 associated with U1, U2, U4, U5, and U6 snRNPs in the lower half of the gradient (Figure 3C, fractions 16-28), and with a separate but overlapping peak of U5 snRNPs (Figure 2E, fractions 14-20). In summary, Brr2, like Brr2-1, comigrates with all five snRNAs in the same gradient fractions. The Brr2/snRNP peak is tight and compact; the Brr2-1/snRNP peak is more heterogeneous. Both Brr2 and Brr2-1 appear to coexist with these snRNPs in large complexes (of possibly different composition; see Discussion).

When ATP was added prior to sedimentation, the gradient profile of snRNPs in wildtype extract changed noticeably: most of the U4 migrated in

the top third of the gradient (data not shown). On the other hand, sedimentation of snRNPs in the *brr2-1* extract did not appear markedly affected by the inclusion of ATP (data not shown). These results were consistent with the previously observed inhibition of ATP-dependent U4 snRNP rearrangement in *brr2-1* extract (Figures 2A, 2B).

Brr2 and ATP are required to disrupt U4/U6 base-pairing

We next tested whether snRNP complexes containing Brr2 or Brr2-1 could dissociate U4/U6 snRNAs in response to ATP. Brr2(Pya) and Brr2-1(Pya) snRNP complexes from whole cell extracts were immunopurified on beads, washed extensively, and incubated with or without ATP. The supernatants were separated from the beads, and the snRNAs were analyzed on a denaturing gel by Northern blotting. When isolated Brr2 snRNP complexes were exposed to ATP, U4 and U6 snRNAs were physically released into the supernatant (Figure 4A, compare lanes 5-6 and 7-8). ATPγS and AMP-PNP did not support this discharge of U4 and U6, suggesting that restructuring of the Brr2 particle requires ATP hydrolysis by an intrinsic component (data not shown). In striking contrast, Brr2-1 complexes failed to undergo this ATP-dependent snRNP rearrangement: in the presence of ATP, all snRNAs remained with Brr2-1(Pya) on the beads (Figure 4A, lanes 11-14). Therefore, wildtype Brr2 and ATP hydrolysis are required to displace U4 and U6 snRNAs from this complex.

An important question is whether ATP affects U4/U6 base-pairing in these Brr2 and Brr2-1 complexes. The deproteinized RNA samples from the previous experiment were subjected to nondenaturing gel electrophoresis to resolve duplex U4/U6 RNA from the free species. In the absence of ATP,

Brr2(Pya) associated with mostly duplex U4/U6 on the beads (Figure 4B, lanes 3). When ATP was added, the majority of U4/U6 was disrupted, and unpaired U4 and U6 RNAs were released into the supernatant (Figure 3B, lanes 5-6). Notably, the U4/U6 duplex remained intact within the Brr2-1 complex in the presence of ATP (Figure 3B, lanes 9-12). These results demonstrate that wildtype Brr2 function is necessary for the ATP-dependent separation of the U4/U6 helices in these snRNPs.

In vitro splicing in *brr2-1* extract is unconditionally blocked, but can be rescued by a micrococcal nuclease-sensitive activity from Fraction I

Thus far, we have shown that Brr2 is required to dissociate U4/U6 snRNPs in extract in the absence of exogenous pre-mRNA. If Brr2 functions similarly on the spliceosome, in vitro splicing in *brr2-1* extract is predicted to be inhibited before the first step due to a defect in U4 release. We expected that in vitro reactions might be impeded at low temperatures by analogy to the cold-sensitivity conferred by *brr2-1* in vivo. Surprisingly, in vitro splicing was blocked at all temperatures tested in three independent preparations of *brr2-1* extract (Figure 5A and data not shown). While wildtype extract produced lariat intermediate and mature mRNA within 2 and 5 minutes at 25°C, respectively (Figure 5A, lanes 3-4), *brr2-1* extract yielded no spliced products even after 15 minutes at 25°C (Figure 5A, lanes 9-12). Similar results were obtained with reactions incubated at 15°C, 30°C, or 33°C (data not shown). The *brr2-1* in vitro splicing defect could be rescued by adding Fraction I prepared from wildtype cells, but not from *brr2-1* cells (Figure 5A, lanes 13-14). This complementing activity was sensitive to micrococcal nuclease (Figure 5C, lanes 2-3), indicating that Brr2 requires intact nucleic acid to be active for splicing. Since Brr2 associates with snRNPs, one

interpretation is that wildtype Brr2 protein cannot exchange onto snRNP particles in the *brr2-1* extract. This nuclease-sensitive complementation is consistent with the hypothesis that snRNPs in the *brr2-1* extract are fundamentally defective for splicing due to their inability to dissociate U4/U6.

To determine what step in spliceosome assembly is blocked in *brr2-1* extract, we assessed the kinetics of splicing complex formation on labelled pre-mRNA using native gel electrophoresis (Cheng and Abelson, 1987). The empirically observed order of assembly intermediates on this gel system is B, A2-1, A1, and finally A2-2 (which comigrates with A2-1). These bands correlate with U2 snRNP association, U4/U6.U5 assembly, U4 dissociation, and splicing catalysis, respectively. In wildtype extract, very little B complex accumulated; instead, A2 and A1 spliceosomes predominated, consistent with the early abundance of spliced products (Figure 5B, lanes 2-5). The converse was true in *brr2-1* extract: almost no A complexes were observed, but B complex increased with time, even without added ATP (Figure 5B, lanes 8-12). Wildtype fraction I appeared to restore A complex formation, but *brr2-1* fraction I did not (Figure 5B, lanes 13-14). To identify the snRNAs in these stalled splicing complexes, identical reactions with unlabelled pre-mRNA were separated on a native gel, and analyzed by Northerns. The B complex in *brr2-1* extract did indeed hybridize to U2, and not to U4, U5, or U6 snRNA probes (data not shown). For further confirmation, we also examined the snRNAs assembled on biotinylated pre-mRNA in *brr2-1* extract. Again, U4, U6, and U5 snRNAs were poorly represented, while U2 and U1 associated efficiently with pre-mRNA (data not shown). These results were puzzling: we expected *brr2-1* spliceosomes to retain U4, since Brr2-1 inhibits U4/U6 disruption in snRNPs. Instead, two independent assays revealed that

U4/U6.U5 snRNPs load poorly onto pre-mRNAs in *brr2-1* extract. This may be due to an apparent dearth of U4/U6.U5 snRNPs competent for spliceosome assembly in *brr2-1* extract (Figure 2B; see Discussion).

The Northern blot of the spliceosome assembly gel revealed an unusual species in *brr2-1* extract. A complex that hybridized to U2, U4, U5, and U6 remained unchanged over the course of the reaction, and did not require incubation or ATP to form (data not shown). Interestingly, this complex had similar mobility to A1, the spliceosomal intermediate with the same snRNP content (data not shown). It seems likely that this is the endogenous Brr2-1/U2/U4/U5/U6 complex observed in many of the previous experiments (Figures 2B, 3A, 3B, 3C). This surprisingly stable species may account for the lack of U4/U6.U5 snRNPs in *brr2-1* extract (see Discussion).

Brr2 is an integral component of active spliceosomes, and Brr2-1 associates with nonproductive splicing complexes

If Brr2 unwinds U4/U6 during spliceosome assembly, then Brr2 should associate with splicing complexes. To test this, we performed immunoprecipitations from splicing reactions in Brr2(Pya) extract. Brr2(Pya) specifically coimmunoprecipitated pre-mRNA, splicing intermediates, and products; lariat-intermediate and excised lariat seemed to be preferentially selected (Figure 6A, lanes 11 and 5). Coprecipitation of pre-mRNA was negligible in untagged extract or in tagged extract without antibody, attesting to specificity (Figure 6A, lanes 7-9). Brr2(Pya) also inefficiently associated with pre-mRNA in the absence of ATP (Figure 6A, lane 10), conditions under which some U4/U6.U5 assembles onto pre-mRNA (Figure 5C, lane 1). Thus,

Brr2 is an integral component of the spliceosome, initially associating as part of the U4/U6.U5 snRNP.

Similar experiments in *brr2-1* extract show that although most spliceosomes do not proceed beyond U2 snRNP assembly, some apparently bind Brr2-1. Brr2-1 coimmunoprecipitated with a small but significant fraction of input pre-mRNA in the presence or absence of ATP (Figure 6B, lanes 10-11). Untagged Fraction I partly restored splicing, but Brr2-1 persistently precipitated with unreacted pre-mRNA and not with lariat intermediate (Figure 6B, lane 12). This observation suggests that splicing complexes bound to Brr2-1 are not functional. In fact, when complexes were preassembled in *brr2-1* extract and then chased with Fraction I, only a small portion of B complex was converted to active spliceosomes (data not shown). This result is consistent with the hypothesis that some spliceosomes formed in *brr2-1* extract are nonproductive.

DISCUSSION

We have tested the hypothesis that the DEXH-box RNA-dependent ATPase Brr2 is required to dissociate the U4/U6 snRNA duplex on the spliceosome. Using *brr2-1*, which harbors a mutation in a conserved helicase motif, we have found that wildtype Brr2 function and ATP hydrolysis are necessary to disrupt the U4/U6 helices within a multi-snRNP complex. Here, we address whether Brr2 is in fact the spliceosomal U4/U6 helicase.

Is Brr2 an RNA helicase?

We have demonstrated that Brr2 is a component of a U2/U4/U6/U5 RNP which releases unpaired U4 and U6 snRNPs in the presence of ATP. The U4/U6 helices in this complex are disrupted in a manner that requires Brr2 activity and ATP hydrolysis. This ATP-dependent U4/U6 dissociation differs significantly from the conventional RNA helicase activity exhibited by other DEAD-box proteins in vitro (Pause and Sonenberg, 1992). Under certain conditions, these purified putative helicases can be shown to dissociate artificial RNA duplexes in the presence of ATP. Typically, however, these reactions require a large molar excess of protein to RNA, and the enzymes display no sequence specificity with synthetic substrates. Thus, it is difficult to evaluate how these proteins' in vitro activities relate to their physiological functions. In contrast, we have characterized the disruption of an authentic helical substrate within snRNPs, the U4/U6 duplex. The Brr2-1 protein does not support dissociation of the U4/U6 snRNP in the presence of ATP. These experiments establish, for the first time, that a DEXH-box putative helicase is required to dissociate RNA helices within snRNPs.

This case represents a partial fulfillment of the prediction that RNA-dependent ATPases of the DEAD- and DEXH-box classes govern helical transactions. It is likely that the Brr2-1 protein is defective in some part of its ATPase cycle, since the *brr2-1* mutation resides in the domain crucial for ATP-driven activities in other proteins. Thus, the ATPase activity of Brr2 is likely to induce conformational changes that lead directly or indirectly to U4/U6 dissociation. But because our assay employs native RNP complexes and not defined RNA and protein components, we must emphasize that these results do not prove that Brr2 is the helicase that unwinds the U4/U6 helices. The simplest scenario is that Brr2 physically binds and unwinds the U4/U6 helices. Alternatively, Brr2 could reposition a protein (or a domain of Brr2) that binds preferentially to unpaired U4 or U6 snRNA. In the least direct case, ATP hydrolysis by Brr2 could activate a neighboring helicase to unwind U4/U6. The latter two possibilities do not demand that Brr2 possess traditional helicase activity (that is, a motor that translocates along and unwinds double-stranded nucleic acid) (Lohman, 1993). Further experiments are needed to clarify the mechanism by which Brr2 transforms snRNP structures.

Is Brr2 required for U4/U6 dissociation on the spliceosome?

We have also shown that wildtype Brr2 activity is required for in vitro splicing, because unsupplemented *brr2-1* extract does not process pre-mRNA. Our data support two roles for Brr2 in splicing: in U4/U6.U5 assembly onto pre-mRNA (Figure 5B), and in U4/U6 dissociation on the spliceosome (Figure 6B). Brr2 is an integral component of the U4/U6.U5 snRNP (Lauber et al., 1996), which *brr2-1* extract apparently lacks (Figure 2B). In *brr2-1* extract, a substantial portion of U4, U6, and U5 snRNPs are sequestered in the

nonfunctional Brr2-1 complex, which fails to dissociate in the presence of ATP (Figure 4A,B). We believe that the disassembly of this complex normally serves to regenerate U4/U6.U5 snRNPs for splicing. Because U4, U6, and U5 snRNPs trapped in the Brr2-1 particle cannot undergo this event, *brr2-1* extract contains an inadequate supply of triple snRNPs. Thus, spliceosome assembly stalls prior to U4/U6.U5 addition (Figure 5B). In support of this interpretation, a snRNP-rich fraction from wildtype (but not *brr2-1*) cells could restore splicing complex formation and splicing in *brr2-1* extract (Figure 5C). This complementing activity was sensitive to micrococcal nuclease (Figure 5C), suggesting that wildtype Brr2 protein is functional for splicing only in intact snRNPs. Moreover, these data imply that snRNPs in the *brr2-1* extract are not competent for splicing. Despite our observation of a Brr2-1/U2/U4/U6.U5 complex (see below), we note that U2 bound efficiently to pre-mRNA in *brr2-1* extract, while U4, U5, and U6 did not. Brr2 activity is clearly required for proper U4/U6.U5 addition onto spliceosomes, most likely because it is a triple snRNP constituent.

Since Brr2-1 prevented U4/U6 disruption in snRNPs (Figure 4A,B), we initially expected spliceosome assembly in *brr2-1* extract to stall prior to U4 release. Instead, we observed that U4/U6.U5 addition was inhibited on biotinylated pre-mRNAs (data not shown) or when monitored by native gel electrophoresis (Figure 5B). We suggest that the *brr2-1* extract does not clearly display the predicted spliceosomal block to U4/U6 dissociation because an upstream event (namely, U4/U6.U5 loading) is also impeded. However, close scrutiny of the experiments with *brr2-1* spliceosomes reveals hints of a later defect in U4/U6 disruption. Although most splicing complexes formed in *brr2-1* extract did not harbor U4/U6.U5 when assayed in the presence of

heparin on a native gel, evidence from gentler assays (immunoprecipitation, biotin-streptavidin precipitation) supports the existence of these complexes. We observed a small amount of U4, U6, and U5 snRNAs specifically bound to biotinylated pre-mRNAs incubated in *brr2-1* extract, indicating that some U4/U6.U5 snRNPs are competent for assembly in the mutant extract. Interestingly, we also noted that a small fraction of input pre-mRNA immunoprecipitated with Brr2-1, consistent with the assembly of a limited number of mutant Brr2-1/U4/U6.U5 snRNPs onto pre-mRNA. However, Brr2-1 never associated with splicing intermediates, even when the splicing reaction was complemented with wildtype fraction I. Together, these data imply that a few U4/U6.U5/Brr2-1 snRNPs can assemble onto spliceosomes, but can progress no further. These dead-end complexes may be blocked due to the inability of Brr2-1 to induce U4/U6 dissociation. In light of the suggestive splicing data and the convincing snRNP activity, we propose that Brr2 may be necessary for the ATP-dependent disruption of U4/U6 on the spliceosome. Nevertheless, we cannot exclude the possibility that Brr2 activity in the snRNP particle does not recapitulate its spliceosomal function, especially given the mysterious origins of these complexes (see below).

What are the Brr2 and Brr2-1 snRNP complexes?

Brr2 and Brr2-1 reproducibly immunoprecipitated with U1, U2, U4, U5, and U6 snRNAs under low salt concentrations (Figure 3A). This finding differs slightly from the previously observed association of Brr2 with U5 snRNA at high salt concentrations (Lauber et al., 1996). To reconcile these results, the Brr2/U5 snRNP may be viewed as the platform on which salt-sensitive snRNP interactions are built (e.g. the triple snRNP). There may be a physical interaction between Brr2 and the U2 snRNP, since in contrast to U4

and U6 snRNPs, U2 and U5 snRNPs remain bound to the Brr2 complex in the presence of ATP (Figure 4A). This notion is lent further credence by the recent observation of a two-hybrid interaction (Fromont-Racine et al., 1997) between Brr2 and Cus1, a likely component of the yeast U2 snRNP (Wells et al., 1996). Interestingly, Brr2 also interacts with the U1 70kD snRNP protein Snp1 in the two-hybrid assay (Fromont-Racine et al., 1997), consistent with our observation of a salt-sensitive coimmunoprecipitation of U1 snRNA (Figure 3A).

Brr2 and Brr2-1 each coexist with U2, U4/U6, and U5 snRNPs in large complexes in the absence of exogenously added pre-mRNA; such unusual complexes have not been previously described in yeast. However, a review of the literature reveals several experiments in which U2, U4, U5, and U6 snRNAs were coimmunoprecipitated with antibodies to individual snRNP proteins at low to moderate salt concentrations (Banroques and Abelson, 1989; Arenas and Abelson, 1993; Galisson and Legrain, 1993; Horowitz and Abelson, 1993; Ruby et al., 1993). In these reports, the interactions were interpreted as "background" or "nonspecific" binding. Assuming that we observed the same phenomena, our findings do not support this conclusion, since all of these coimmunoprecipitable snRNAs cosediment on a gradient (Figure 3B,C). These complexes may represent spliceosome precursors, dead-end spliceosomes, or recycling intermediates. Moreover, each "complex" may consist of several heterogeneous species which we cannot distinguish. Finally, the wildtype and mutant complexes may derive from different sources, although superficially they contain the same spectrum of snRNAs. Here, we speculate about the origins of the Brr2-1 and Brr2 complexes.

The Brr2-1 complex appears distinct from the wildtype complex. Unlike the wildtype case, the Brr2-1 complex is stable to electrophoresis on native spliceosome assembly gels (Figure 5, data not shown) and native snRNP gels (Figure 1A and data not shown). Its electrophoretic migration and sedimentation properties resemble those of spliceosomes. We suggest that the Brr2-1 complex represents endogenous spliceosomes stalled by the failure to disrupt U4/U6. The accumulation of these dead-end complexes prevents recycling of U4/U6.U5 snRNPs, and as a result de novo splicing is impeded in *brr2-1* extract. This hypothesis is testable, because it predicts the presence of endogenous pre-mRNAs and spliceosome-specific proteins (e.g. Prp31 (Weidenhammer et al., 1997)) in the Brr2-1 complex.

The Brr2 complex is more mystifying. Despite repeated efforts, we have no direct evidence that the Brr2/snRNP complex is a functional splicing intermediate. Attempts to chase Brr2/snRNP complexes into spliceosomes, or to splice pre-mRNA with the Brr2 complex alone, were unsuccessful, possibly for technical reasons (P. L. R. and C. G., unpublished results). Moreover, isolated Brr2 and Brr2-1 complexes do not associate specifically with pre-mRNA containing wildtype intron consensus sequences (P. L. R. and C. G., unpublished results). We suspect that the wildtype Brr2 snRNP complex may be heterogeneous, because, in the presence of ATP, not all U4/U6 is disrupted (Figure 4B, lane 5), and not all free U4 and free U6 snRNPs are released (Figure 4B, lane 6). Some of these Brr2-containing species may be recycling intermediates that must be dissociated (through the action of Brr2 and ATP) in order to replenish U4/U6.U5 snRNPs. Other complexes may arise from endogenous spliceosomes; or from the intrinsic affinities of snRNPs for one another. To shed some light on this question,

one could analyze whether the Brr2 complex contains endogenous pre-mRNAs, or attempt more elaborate measures to reconstitute splicing activity with the Brr2/snRNP complex.

Other potential regulators of U4/U6 base-pairing

Paradoxically, *brr2-1* cells are viable (but cold-sensitive) despite their severe in vitro splicing deficits. Therefore, the Brr2-1 protein must be able to function minimally for splicing in vivo. Perhaps other factors that facilitate this spliceosomal transition in vivo are missing or less active in vitro. Thus far, only two other splicing proteins, Prp4 and Prp19, are known to be involved in the release of U4 from the spliceosome. However, it is not clear in either case whether these proteins are necessary to disrupt U4/U6 base-pairing as well. If so, then they would likely act in close concert with Brr2. Prp19, along with other proteins in a large, non-snRNP complex, associates with the spliceosome during the A2-1 to A1 transition (Tarn et al., 1993b; Tarn et al., 1994). Some of these factors may escort U4 off the spliceosome subsequent to Brr2 action. If Brr2 directly displaces U4, then it may contact the U4 snRNP protein Prp4, since thermosensitive *prp4* mutants prevent U4 destabilization (Ayadi et al., 1997). Other proteins may negatively regulate U4/U6 dissociation. Genetic experiments have suggested that the highly conserved U5 snRNP protein Prp8 stabilizes the U4/U6 interaction (Strauss and Guthrie, 1991). Given its proximal location in the U4/U6.U5 snRNP, Brr2 may have to counteract Prp8 function. We have shown elsewhere that the U6 snRNP protein and spliceosomal recycling factor Prp24 reverses Brr2's action, because it reanneals U4 and U6 snRNPs liberated from Brr2 complexes by ATP (P. L. R. and C. G., manuscript submitted). Thus, Prp24 and Brr2 are key antagonists in the control of U4/U6 base-pairing dynamics.

Multiple roles for Brr2 during pre-mRNA splicing

Other DEAD- and DEXH-box proteins described to date appear to occupy the spliceosome transiently. In contrast, Brr2 coimmunoprecipitates with pre-mRNA, splicing intermediates, and lariat intron. This persistent association with active spliceosomes may arise because Brr2, like its human homolog, is a U5 snRNP constituent (Lauber et al., 1996). Consequently, Brr2 may be uniquely situated to induce rearrangements at multiple steps in the splicing pathway. Two independently isolated alleles of *BRR2* , *slt22-1* and *rss1-1*, support this interpretation. Like *brr2-1*, these mutations alter residues in the first DEXH-box domain(Lin and Rossi, 1996; Xu et al., 1996). In *slt22-1* mutant extracts, nonfunctional spliceosomes form that contain U2 and U6 but not U5 or U4 snRNAs (Xu et al., 1996). A plausible explanation is that this mutant protein allows U4/U6.U5 assembly, U4/U6 disruption, and U2/U6 interaction, but fails to anchor U5 tightly to the spliceosome. *slt22-1* is also synthetically lethal with mutations in stem-loop Ia of U2 snRNA, suggesting that Brr2 may control rearrangements that lead to U2/U6 base-pairing. If so, an interesting question is whether Brr2 couples U4/U6 dissociation to U2/U6 formation. The relative order of these events has been inferred because U2/U6 helix I is mutually exclusive with U4/U6 stem I. In principle, stem I of U4/U6 could be independently disrupted, and U2/U6 helix I could coexist with U4/U6 stem II in a U4/U6/U2 triply base-paired complex. Thus, further studies of Brr2 will illuminate the mechanism of U4/U6 disruption and its connection to subsequent helical transactions on the spliceosome.

Finally, a number of lines of evidence suggest that Brr2 plays an important role in the second chemical step of splicing. The *rss1-1* allele of

BRR2 suppresses a step two block created by the introduction of a stable stem-loop structure at the 3' splice site (Lin and Rossi, 1996). Antibodies to the human U5 200 kD homolog of Brr2 specifically inhibit the second step of splicing in vitro (Lauber et al., 1996). We note that Ansari and Schwer (1995) partially purified a ~200 kD factor that promotes mRNA formation in gradient-purified spliceosomes lacking Prp16; an intriguing possibility is that Brr2 could be this SSF1 activity. Mutagenesis of *BRR2* will undoubtedly elucidate its multiple functions on the spliceosome.

MATERIALS AND METHODS

Yeast Methods and Strains

Yeast genetic manipulations were performed using standard methods (Guthrie and Fink, 1991). The four yeast strains employed in this work were created by plasmid shuffling in PRY118 (*MAT a brr2::LEU2 ade2 lys2 his3 ura3 leu2 pSE360-BRR2*). [PRY118 was a haploid segregant of the heterozygous *BRR2/brr2::LEU2* diploid strain YSN404 transformed with pSN108 (Noble and Guthrie, 1996).] Therefore, these strains differ only in their plasmid-borne *BRR2* genes:

STRAIN	*BRR2* GENOTYPE	PLASMID
PRY122	*BRR2*	pPR130
PRY123	*brr2-1*	pPR133
PRY132	*BRR2(Pya)*	pPR150
PRY135	*brr2-1(Pya)*	pPR151

Plasmid Construction

Standard molecular biological techniques were used (Sambrook et al., 1989) in the *HIS3 CEN ARS* vector pSE362 (=pUN90; (Elledge and Davis, 1988)) or in pBluescript KS$^-$ (Stratagene). 7 kb SacI *BRR2* and *brr2-1* fragments were inserted into pSE362 to create pPR130 and pPR133, respectively. To place the polyoma (Pya) epitope tag at the C-terminus of each of these genes, we used the Bluescript subclone pSN125, which contains an 860 bp SalI fragment encompassing the final 653 bp of *BRR2* (Noble and Guthrie, unpublished data). A NotI site was introduced by polymerase chain reaction (PCR) immediately upstream of the stop codon to generate pPR149; the amplified *BRR2* sequence was confirmed by sequencing. Annealed, kinased oligos

encoding the polyoma epitope were inserted into the NotI site (5' Not Pya: 5' GGCCGCATGGAATATATGCCAATGGAAATGGAATATATGCCAATGGA AGGC 3'; 3' Not Pya: GGCCGCCTTCCATTGGCATATATTCCATTTC-CATTGGCATATATTCCATGC 3'). The resulting SalI fragment with a *BRR2-Pya* C-terminal fusion was swapped into pPR130 and pPR133 to create plasmids pPR150 and pPR151, respectively.

Identification of the*brr2-1* Mutation

The *brr2-1* allele was recovered by gap repair onto plasmid pSN123, which contains the *BRR2* gene on the 2μ *URA3* vector pRS426 (Noble and Guthrie, unpublished data; (Sikorski and Hieter, 1989)). Briefly, this plasmid was linearized with BglII (which excises a 2.5 kb fragment spanning the first putative helicase domain of *BRR2*) and transformed into the *brr2-1* strain YSN405 (Noble and Guthrie, 1996a). Plasmids were rescued from Ura$^+$, cold-sensitive transformants. One plasmid (pPR137) conferred cold-sensitivity when transformed into the *BRR2* disruption strain PRY118; this plasmid was used to create pPR133. The 2.5 kb BglII fragment from pPR137 was subcloned into pBluescript KS$^+$ (Stratagene) for sequencing. Within this construct, the 447 bp ApaLI-StuI fragment was fully sequenced on both strands, and only the A2417G mutation was discovered. This fragment was replaced into pPR130, and found to confer cold-sensitivity after plasmid shuffling in PRY118.

Extract Preparation

Yeast whole cell extract was prepared from PRY122 (untagged *BRR2*), PRY123 (untagged *brr2-1*), PRY132 (Pya tagged *BRR2*), and PRY135 (Pya tagged *brr2-1*) using the liquid nitrogen method (Umen and Guthrie, 1995) with

modifications (Ansari and Schwer, 1995). Fraction I was prepared from PRY122 and PRY123 as described (Cheng and Abelson, 1986).

snRNP Gels

Native snRNP gel analysis without heparin was modified from (Konarska and Sharp, 1987). 5 µl samples contained 40% extract, 2 mM ATP (or ATPγS), 2.5 mM $MgCl_2$, 3% PEG 8000, 60 mM potassium phosphate pH 7, and 1 mM spermidine unless otherwise stated. Reactions were incubated 30 minutes at 23-25°C, and loaded on a pre-run, 4% polyacrylamide (80:1) gel (15 x 15 x .15 cm) made up in TGM buffer (50 mM Tris base, 50 mM glycine, 2 mM $MgCl_2$). Electrophoresis was for 6-7 hours at 160V in TGM buffer at 4°C, and the gel was electroblotted onto Hybond-N membrane at 25-30 V for 12-16 hours in 0.1 M sodium phosphate, pH 6.5. The blot was sequentially probed with kinased oligos complementary to the snRNAs (Noble and Guthrie, 1996b).

Immunoprecipitations

Protein G Sepharose beads were coupled to anti-polyoma antibodies as described (Schneider et al., 1994) and washed with NET50 buffer (50 mM Tris pH 7.4, 0.05% Nonidet-P40, 50 mM NaCl). For coimmunoprecipitations of snRNPs, 20 µL whole cell extract, 200 µL NET with the indicated NaCl concentration, and 10 µL settled beads were nutated 1 hour at 4°C. Some samples contained 1 mM competitor peptide encoding the polyoma epitope (EYMPME) to demonstrate specificity. For immunoprecipitations from glycerol gradient fractions, 10 µL settled beads were nutated with 400 µL fractions 1 hour at 4°C. The beads were washed with 3 x 500 µL NET50, and the associated RNAs were isolated by phenol/chloroform extraction at 4°C followed by ethanol precipitation. RNA samples were separated on 6%

polyacrylamide 7 M urea denaturing gels and subjected to Northern analysis as described (Noble and Guthrie, 1996b). For immunoprecipitations from splicing reactions, 35 µL splicing reactions were incubated 10 minutes at 25°C. 5 µL aliquots were removed (=1/6 total), from which RNAs were isolated. The remaining 30 µL was nutated with 300 µL NET 50 and 15 µL settled beads for one hour at 4°C. The beads were washed and processed for associated RNAs (=IP) as for snRNPs. The RNAs were electrophoresed on denaturing polyacrylamide gels, which were directly exposed to film at -80°C with intensifying screens.

Glycerol Gradient Sedimentation

200 µL reactions containing 50% whole cell extract, 0 or 2 mM ATP, 2.5 mM $MgCl_2$, 3% PEG, 60 mM potassium phosphate pH 7.2 were incubated 10 minutes at 25°C, and then subjected to glycerol gradient sedimentation as described (Ansari and Schwer, 1995). 400 µL fractions were removed from the top of the gradient. Even fractions were subjected to immunoprecipitations, and odd fractions were subjected to denaturing Northern analysis for total snRNA content (Noble and Guthrie, 1996b).

snRNP Release Assay

snRNPs were immunoprecipitated with Brr2(Pya) or Brr2-1(Pya) as described above. The beads were washed 3 x 500 µL NET50, and then incubated with 30 µL of splicing buffer ±ATP (2 mM ATP, 2.5 mM $MgCl_2$, 3% PEG, 60 mM potassium phosphate pH 7.2, 40% buffer D (Lin et al., 1985)) for 5 minutes at 23-25°C. After centrifugation, the supernatants were carefully collected and the beads were washed 1 x 500 µL NET50. RNAs were extracted and split into three portions. To determine snRNA content, one-half of each sample was

electrophoresed on a denaturing gel and subjected to Northern analysis (Noble and Guthrie, 1996b). To determine base-pairing status of U4 and U6 snRNAs, one-quarter of each sample was hybridized in solution to an oligo probe for either U4 or U6 snRNAs, and electrophoresed on a nondenaturing gel (Li and Brow, 1993).

In Vitro Splicing Reactions

In vitro splicing was performed according to (Lin et al., 1985), except that reactions contained 50% extract, 60 mM potassium phosphate, pH 7.2, and ~0.3 nM labelled actin pre-mRNA. The indicated samples also contained 1 µL of *BRR2* or *brr2-1* Fraction I. For Figure 5C, *BRR2* Fraction I was mixed with 1 mM $CaCl_2$ and 0.33 or 0.16 mg/mL micrococcal nuclease (lanes 2-3 vs. 4-5). In lanes 2 and 4, 2.5 mM EGTA was present during the 10 minute preincubation at 25°C, inhibiting nuclease activity. In lanes 3 and 5, 2.5 mM EGTA was added at the conclusion of the preincubation to stop digestion. The treated Fraction I samples were then added to standard splicing reactions in *brr2-1(Pya)* extract.

Spliceosome Assembly Gels

Analysis of spliceosome assembly on native gels was conducted as described in (Cheng and Abelson, 1987) with the following modifications. The gel and electrophoresis buffer was 40 mM Tris-acetate, 1 mM EDTA, pH 7.2 at 23°C. Electrophoresis was at 4°C for 5.5 hours at 200V with constant buffer recirculation.

Figure 1. The *brr2-1* mutation lies in a highly conserved motif in the first helicase-like domain of *BRR2*..

A. Schematic of the Brr2 protein, which contains two helicase-like DEXH-box domains.

B. Alignment shows eight signature motifs from each DEXH-box domain (I and II) of *Saccharomyces cerevisiae* Brr2 and the human U5 200K homolog (Lauber et al., 1996) compared to the consensus sequences of the DEAD, DEAH, and DEXH-box families (Pause and Sonenberg, 1992; Schmid and Linder, 1992). A period denotes a gap, and dashes indicate nonconserved residues in the consensus sequences. The second helicase-like domain of Brr2 diverges more from the DEXH-box consensus than the first. The *brr2-1* mutation converts the glutamic acid at residue 610 in DEXH-box domain I to a glycine (indicated with a black box and arrow).

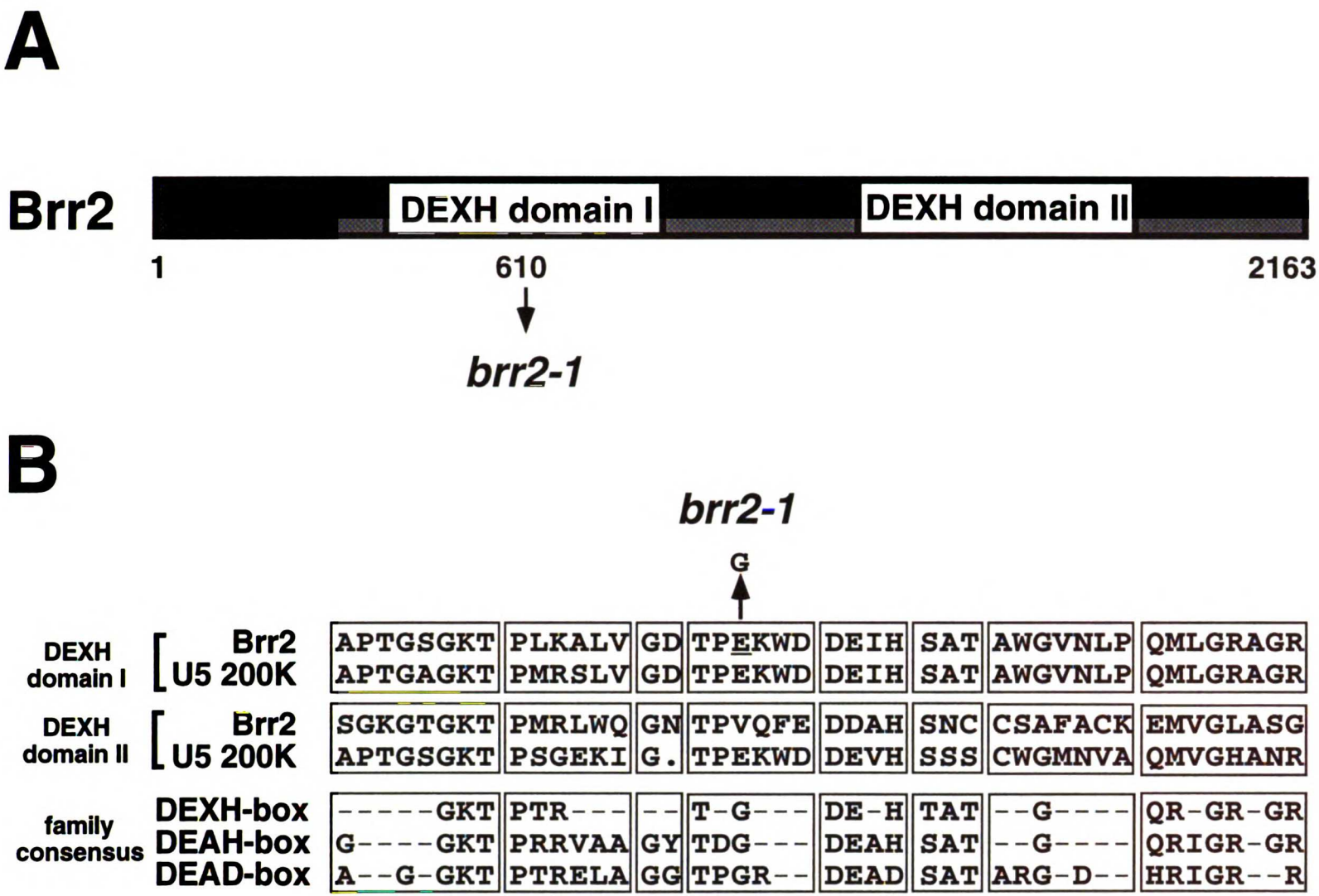

A
Brr2
DEXH domain I
DEXH domain II
1
610
2163
brr2-1
B
brr2-1
G
DEXH domain I
Brr2 APTGSGKT PLKALV GD TPEKWD DEIH SAT AWGVNLP QMLGRAGR
U5 200K APTGAGKT PMRSLV GD TPEKWD DEIH SAT AWGVNLP QMLGRAGR
DEXH domain II
Brr2 SGKGTGKT PMRLWQ GN TPVQFE DDAH SNC CSAFACK EMVGLASG
U5 200K APTGSGKT PSGEKI G. TPEKWD DEVH SSS CWGMNVA QMVGHANR
family consensus
DEXH-box -----GKT PTR--- -- T-G--- DE-H TAT --G---- QR-GR-GR
DEAH-box G----GKT PRRVAA GY TDG--- DEAH SAT --G---- QRIGR-GR
DEAD-box A--G-GKT PTRELA GG TPGR-- DEAD SAT ARG-D-- HRIGR--R

Figure 2A. ATP-dependent displacement of U4 is blocked in snRNP-rich Fraction I from *brr2-1* cells. Fraction I prepared from wildtype *BRR2* (lanes 1-3) or mutant *brr2-1* cells (lanes 4-6) was incubated in the presence or absence of ATP or ATPγS. The snRNPs were resolved on a native gel, subjected to Northern analysis, and probed for U4 snRNA. Sequential stripping and reprobing confirmed the identity of U4/U6.U5 (not shown); the mobility of this complex appears to change when ATP is present (see also Figure 2B). ATP hydrolysis is necessary for the generation of free U4 in *BRR2* Fraction I (lanes 2-3), but no free U4 is produced in *brr2-1* Fraction I (lane 5).


FRACTION I
BRR2
brr2-1
ATP
- + γS - + γS
U4/U6.U5
free U4
LANE 1 2 3 4 5 6

Figure 2B. *brr2-1* extract contains an aberrant snRNP distribution. Whole cell extract from wildtype *BRR2* or mutant *brr2-1* cells was incubated with or without ATP, electrophoresed on a native gel to resolve snRNPs, and subjected to Northern analysis with the indicated snRNA probes. Free snRNP species are marked with thin bars to the left of each panel, and the U2/U4/U6.U5 multi-snRNP species is marked with a thick bar to the right of each panel. U4/U6.U5, U4/U6, and U5 snRNPs are less abundant in *brr2-1* extract.

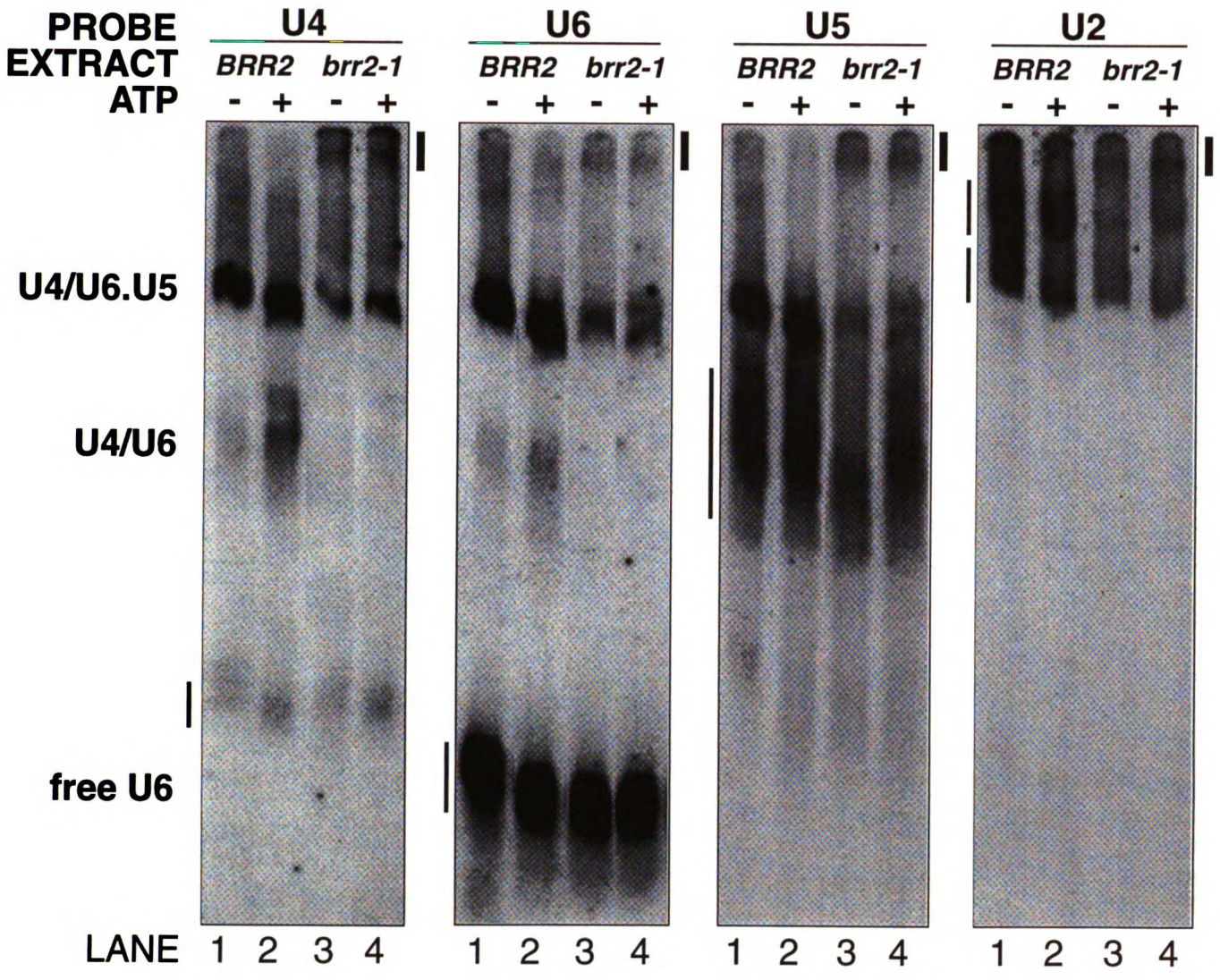

PROBE
EXTRACT
ATP
U4
U6
U5
U2
BRR2
brr2-1
- + - +
U4/U6.U5
U4/U6
free U6
LANE
1 2 3 4

Figure 3A. Brr2 and Brr2-1 coimmunoprecipitate U2, U4, U5, and U6 snRNAs from extract in a salt-resistant manner. Extracts from untagged *BRR2*, polyoma-tagged *BRR2(Pya)*, or *brr2-1(Pya)* cells were incubated with protein G Sepharose coupled to anti-polyoma antibodies (lanes 1-3, 5-8, 10-15), or to protein G Sepharose alone (lanes 4, 9) in the presence of the indicated amount of salt. After washing, the bound RNAs were isolated, resolved on a denaturing gel, and subjected to Northern analysis for U1, U2, U4, U5, and U6 snRNAs. Peptide encoding the polyoma epitope competed for immunoprecipitation of these snRNAs, demonstrating specificity (lanes 14-15). The lanes marked "1/10 total RNA" contain deproteinized extract equivalent to one-tenth of each immunoprecipitation sample in lanes 4-8 ("*WT*") and lanes 9-13 ("*brr2*").

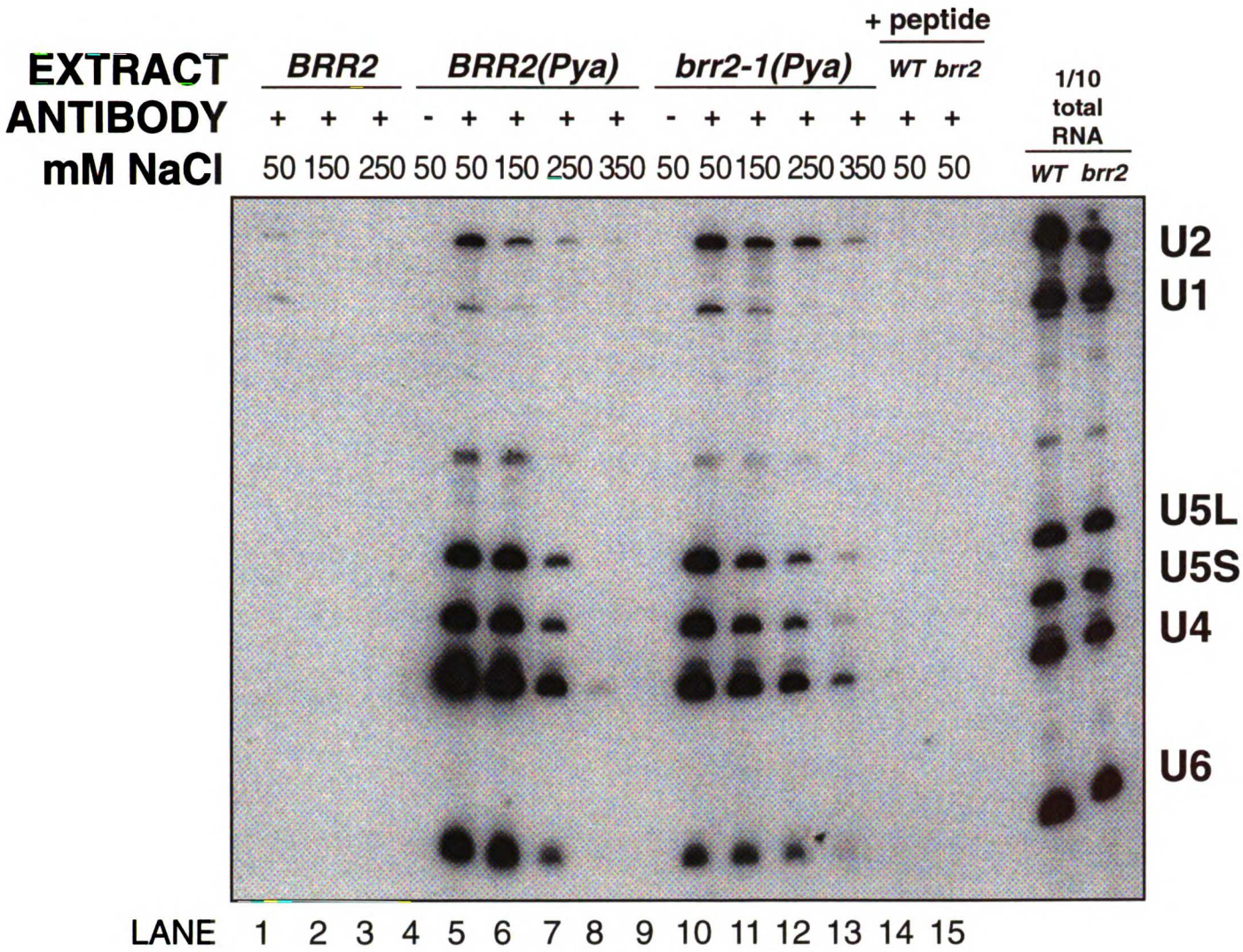

+ peptide
EXTRACT
BRR2
BRR2(Pya)
brr2-1(Pya)
WT brr2
ANTIBODY
+ + + - + + + + - + + + + + + +
mM NaCl
50 150 250 50 50 150 250 350 50 50 150 250 350 50 50
1/10 total RNA
WT brr2
U2
U1
U5L
U5S
U4
U6
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3B. Brr2 coimmunoprecipitates with U1, U2, U4, U5, and U6 snRNAs in a large complex. Extract from wildtype *BRR2(Pya)* cells was sedimented on a 15-40% glycerol gradient, and fractions were collected from the top. Odd numbered fractions were analyzed for total snRNA content (left), and immunoprecipitations were performed in even numbered fractions (right). Northern analysis of snRNAs was conducted as in (A).

BRR2 glycerol gradient

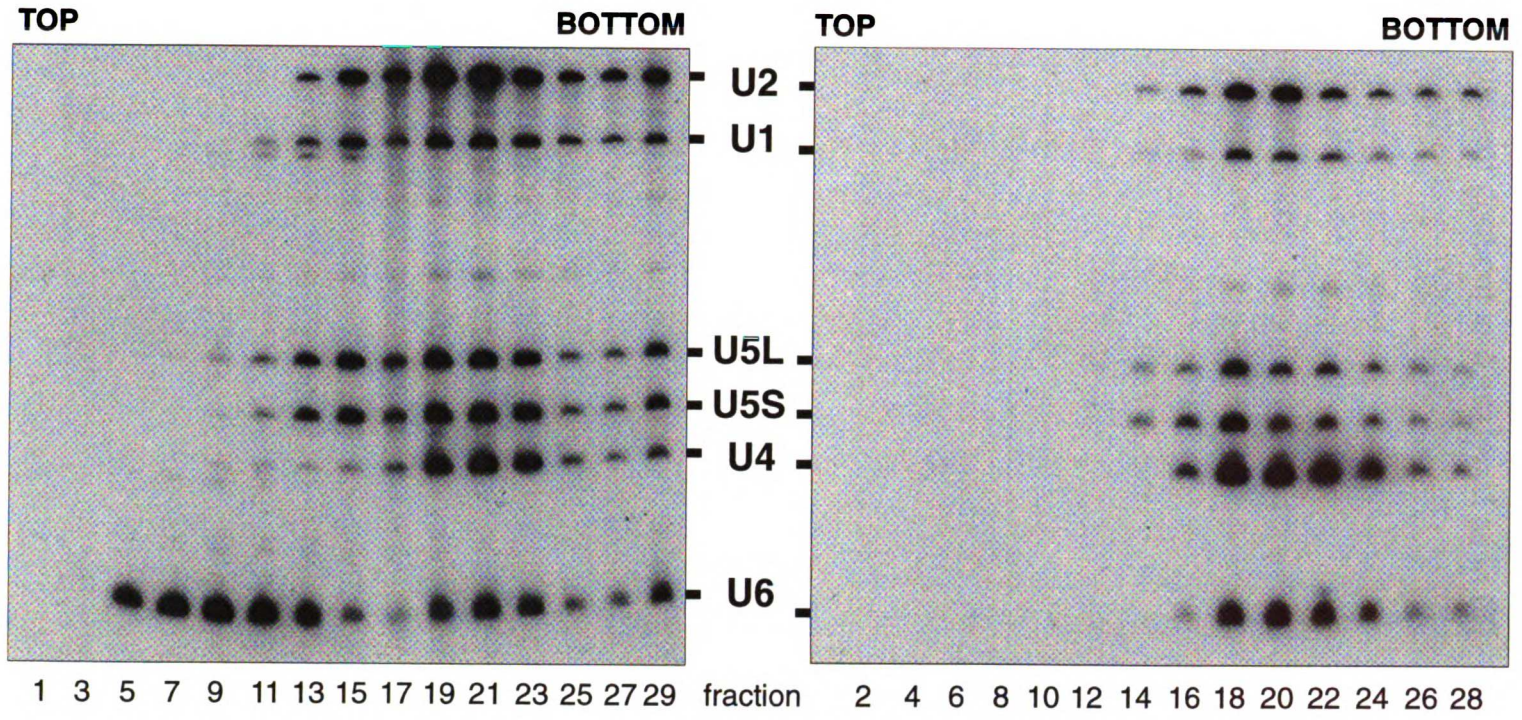
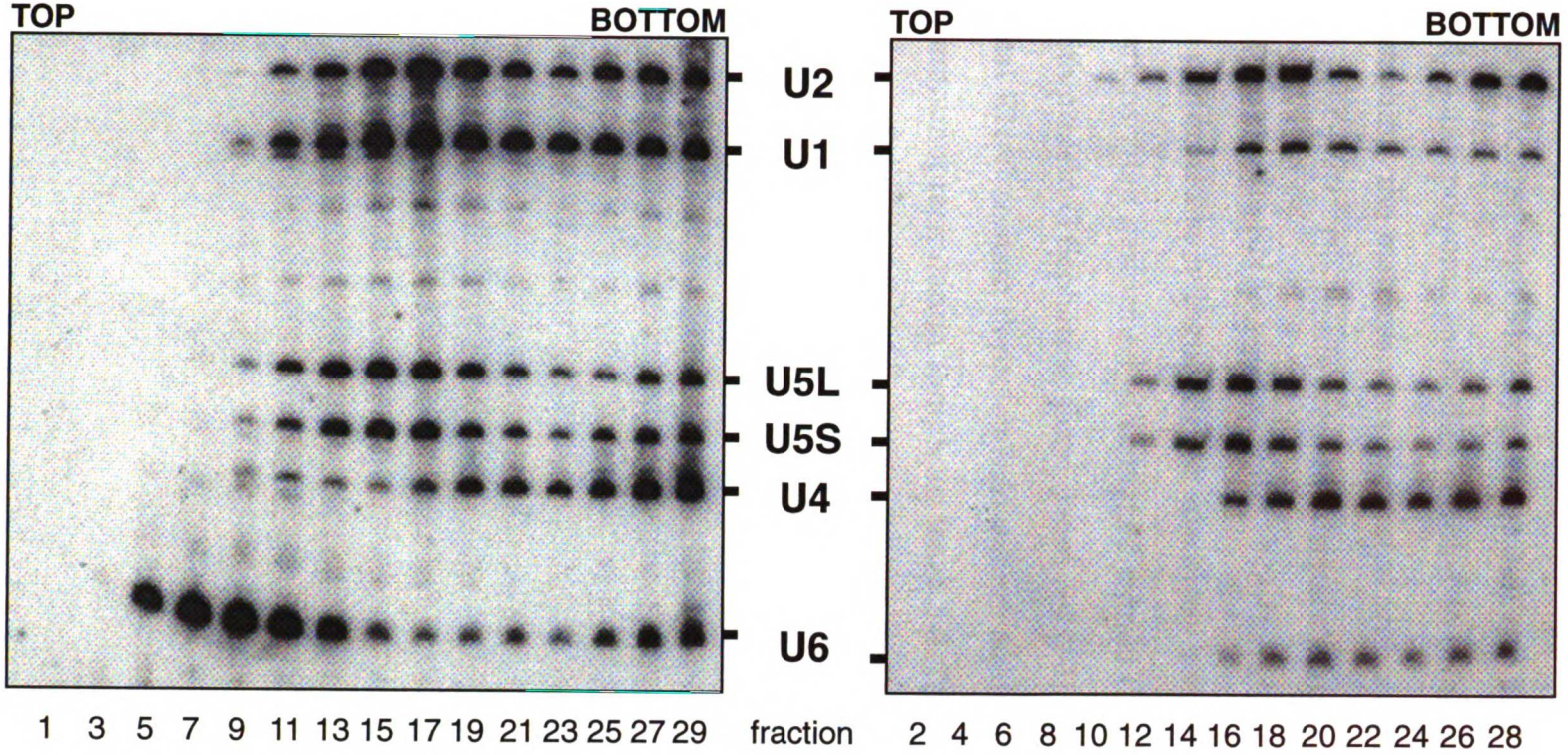


Figure 3C. Brr2-1 immunoprecipitates with U1, U2, U4, U5, and U6 snRNAs in large complexes. Extract from mutant *brr2-1(Pya)* cells was fractionated on a 15-40% glycerol gradient and analyzed as in (B).

brr2-1 glycerol gradient



total fractions

Brr2-1 coimmunoprecipitations

Figure 4A. The Brr2/snRNP complex physically releases U4 and U6 snRNAs in response to ATP, but the Brr2-1/snRNP complex does not. snRNP complexes associated with Brr2(Pya) (lanes 3-8) and Brr2-1(Pya) (lanes 9-14) were bound to anti-polyoma antibodies coupled to protein G Sepharose. After extensive washing, the matrices were incubated with splicing buffer containing 0 or 2 mM ATP. The beads were then separated from the supernatants (supt), and the RNAs were isolated. Northern analysis to detect the snRNAs was performed as in Figure 3. As specificity controls, immunoprecipitations were performed in untagged extract (lanes 1-2) or in tagged extract with protein G Sepharose alone (lanes 3-4, 9-10). The lanes marked "1/40 total RNA" contain deproteinized extract equivalent to one-fortieth of each immunoprecipitation sample in lanes 3-8 ("*BRR2*") and lanes 9-14 ("*brr2-1*").

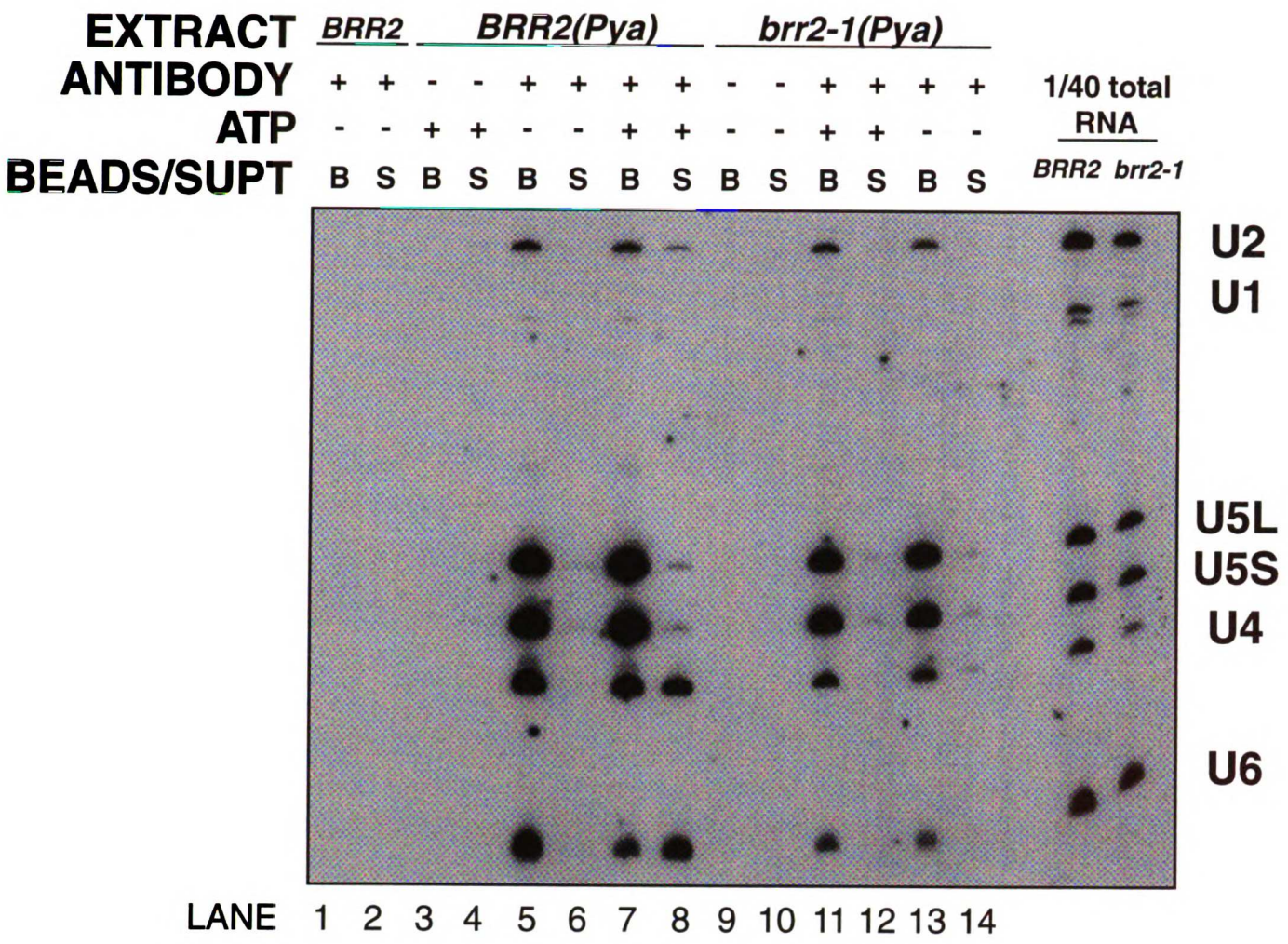

EXTRACT
BRR2
BRR2(Pya)
brr2-1(Pya)
ANTIBODY + + - - + + + + - - + + + +
ATP - - + + - - + + - - + + - -
BEADS/SUPT B S B S B S B S B S B S B S
1/40 total RNA
BRR2 brr2-1
U2
U1
U5L
U5S
U4
U6
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4B. The U4/U6 snRNA duplex is disrupted in the presence of ATP in the Brr2/snRNP complex, but not in the Brr2-1/snRNP complex. RNA samples from Figure 4A were hybridized in solution to labelled oligos specific for U4 (top) or U6 (bottom) snRNAs, and electrophoresed on nondenaturing gels to distinguish slow-migrating U4/U6 duplex from the free snRNAs (Li and Brow, 1993). The lanes marked "1/80 total RNA" contain deproteinized extract equivalent to one-eightieth of each immunoprecipitation sample in lanes 1-6 ("*BRR2*") and lanes 7-12 ("*brr2-1*"). In the Brr2 complexes exposed to ATP, the U4/U6 helices are separated, and free U4 and free U6 snRNA are released into the supernatant (lanes 5-6). The Brr2-1 complexes fail to disrupt U4/U6 in response to ATP (lanes 11-12).

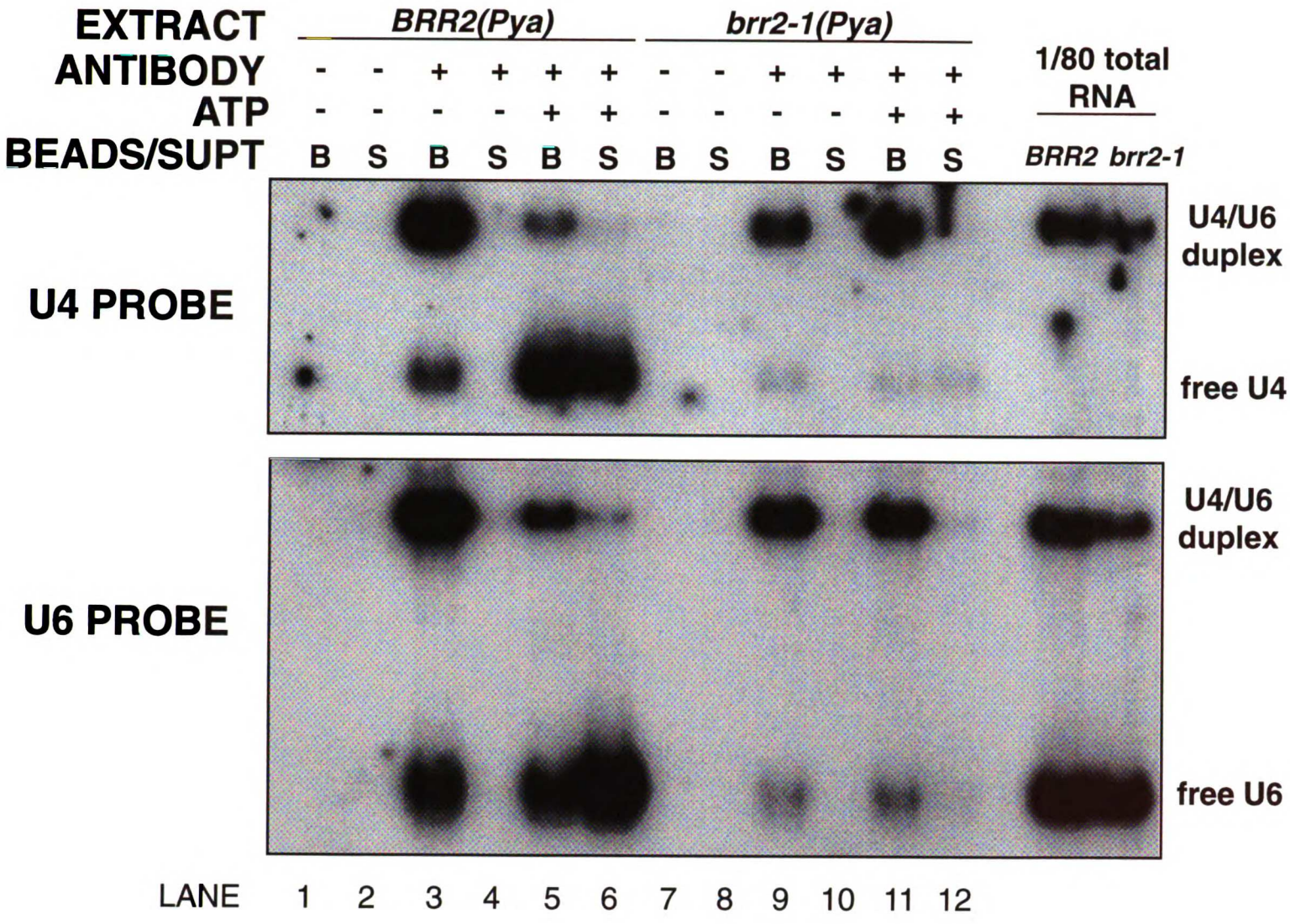

EXTRACT
BRR2(Pya)
brr2-1(Pya)
ANTIBODY
ATP
BEADS/SUPT
B S B S B S B S B S B S
1/80 total RNA
BRR2 brr2-1
U4 PROBE
U4/U6 duplex
free U4
U6 PROBE
U4/U6 duplex
free U6
LANE 1 2 3 4 5 6 7 8 9 10 11 12

Figure 5A. In vitro splicing in *brr2-1* extract is blocked before the first step. Time courses of in vitro splicing reactions in wildtype *BRR2* and mutant *brr2-1* extracts are depicted. Splicing is restored in *brr2-1* extract with the addition of Fraction I from wildtype cells, but not from *brr2-1* mutant cells (lanes 13-14). The labelled RNA species are, from top to bottom, lariat-intermediate, excised intron lariat, pre-mRNA, mRNA, and 5' exon.

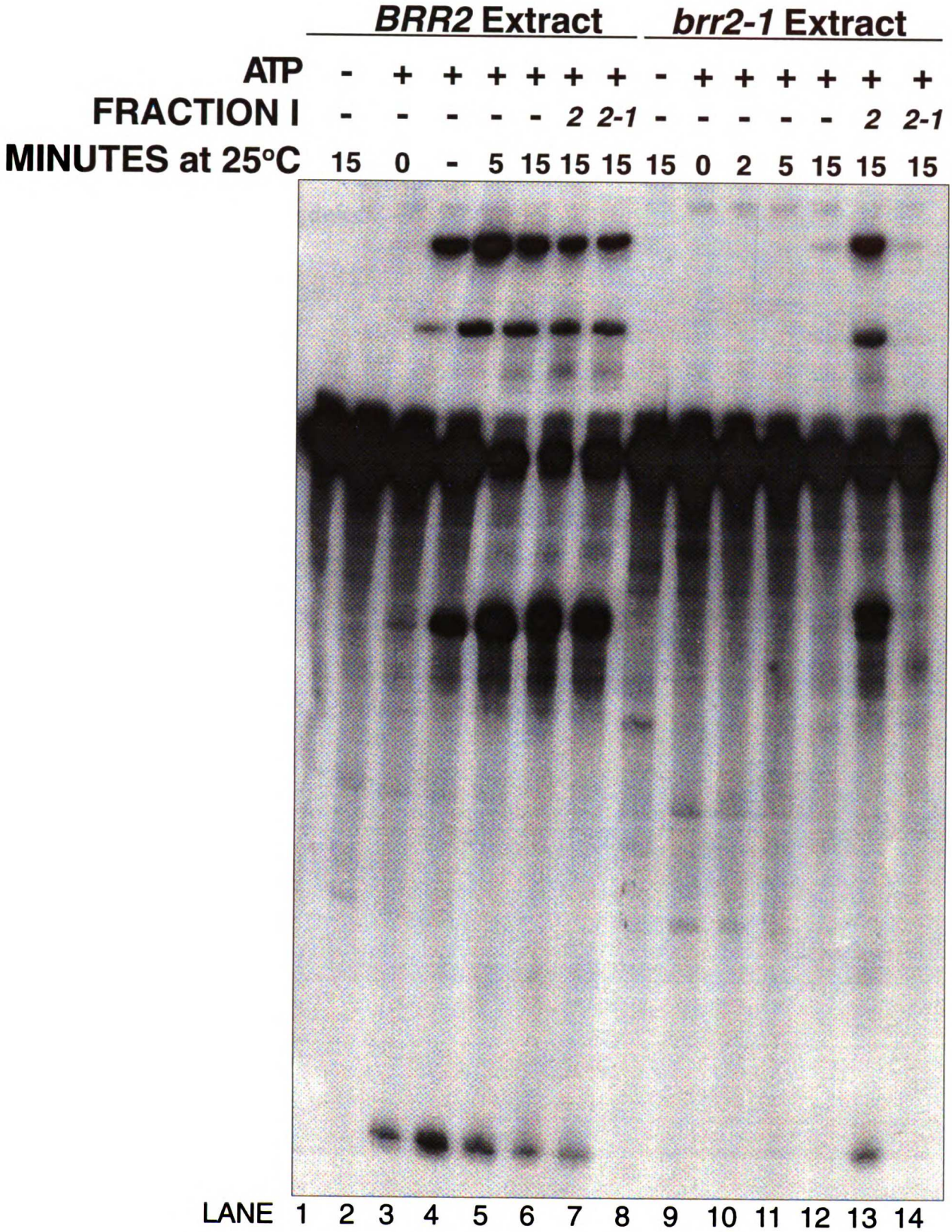

BRR2 Extract
brr2-1 Extract
ATP - + + + + + + - + + + + + +
FRACTION I - - - - - 2 2-1 - - - - - 2 2-1
MINUTES at 25°C 15 0 - 5 15 15 15 15 0 2 5 15 15 15
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 5B. *brr2-1* extract displays a defect in U4/U6.U5 addition during spliceosome assembly. In vitro splicing reactions performed in wildtype *BRR2* and mutant *brr2-1* extracts were electrophoresed on a native gel to resolve splicing complexes B, A2, and A1. A large amount of complex B and a small amount of an A-like complex accumulate in *brr2-1* extract, indicative of a U4/U6.U5 loading defect.

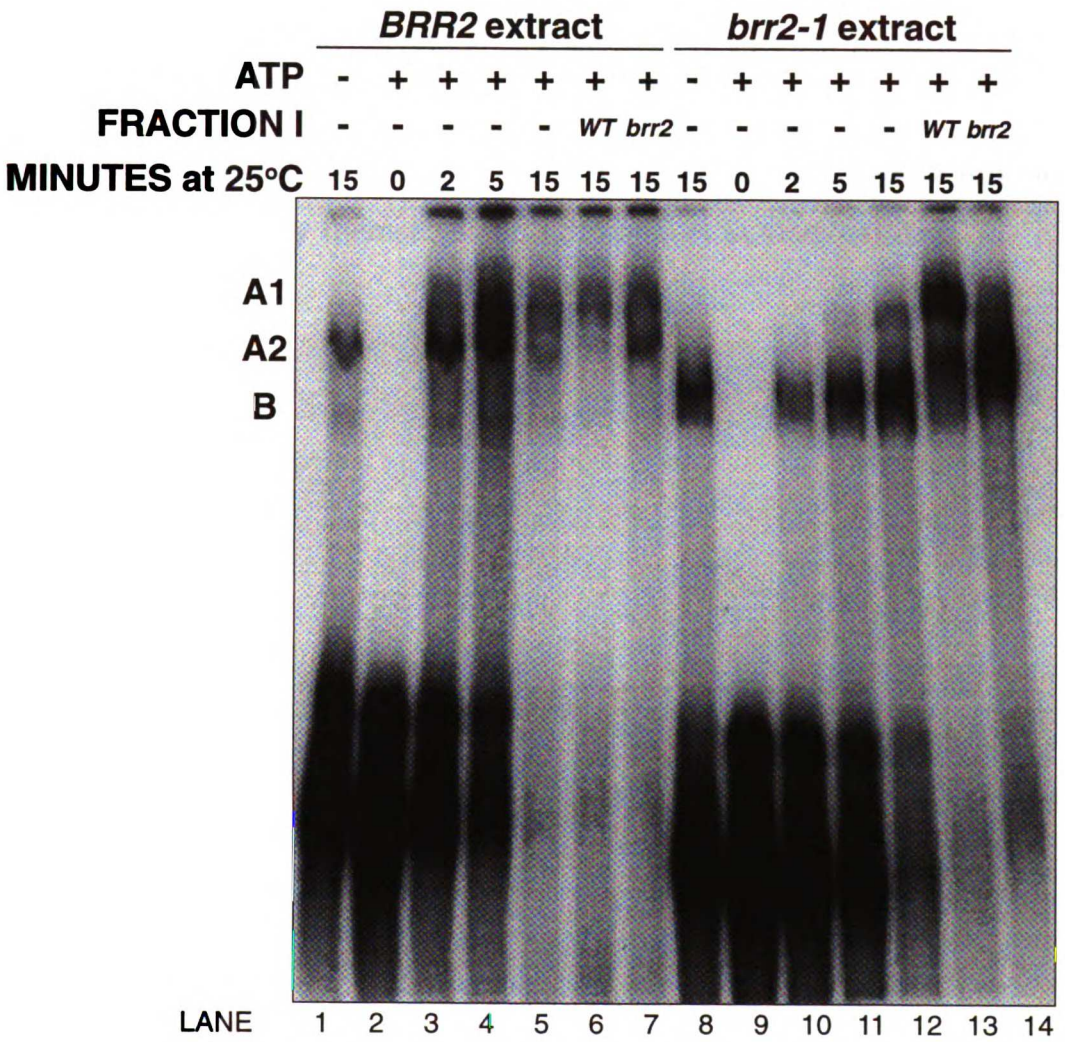

BRR2 extract
brr2-1 extract
ATP - + + + + + + - + + + + + +
FRACTION I - - - - - WT brr2 - - - - - WT brr2
MINUTES at 25°C 15 0 2 5 15 15 15 15 0 2 5 15 15 15
A1
A2
B
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 5C. In vitro splicing in *brr2-1* extract is complemented by a micrococcal nuclease-sensitive activity from wildtype Fraction I. Wildtype fraction I was incubated with micrococcal nuclease and $CaCl_2$ to digest nucleic acids, and then EGTA was added to inactivate the nuclease (lanes 3, 5). Alternatively, EGTA was present from the beginning of the incubation for mock-digested controls (lanes 2, 4). The treated Fraction I samples were added to in vitro splicing reactions in *brr2-1* extract. Samples from lanes 4 and 5 contain one-and-a-half as much Fraction I, and half as much micrococcal nuclease, as those from lanes 2 and 3. *brr2-1* extract (lane 1) and untreated Fraction I (lane 6) by themselves do not support in vitro splicing .

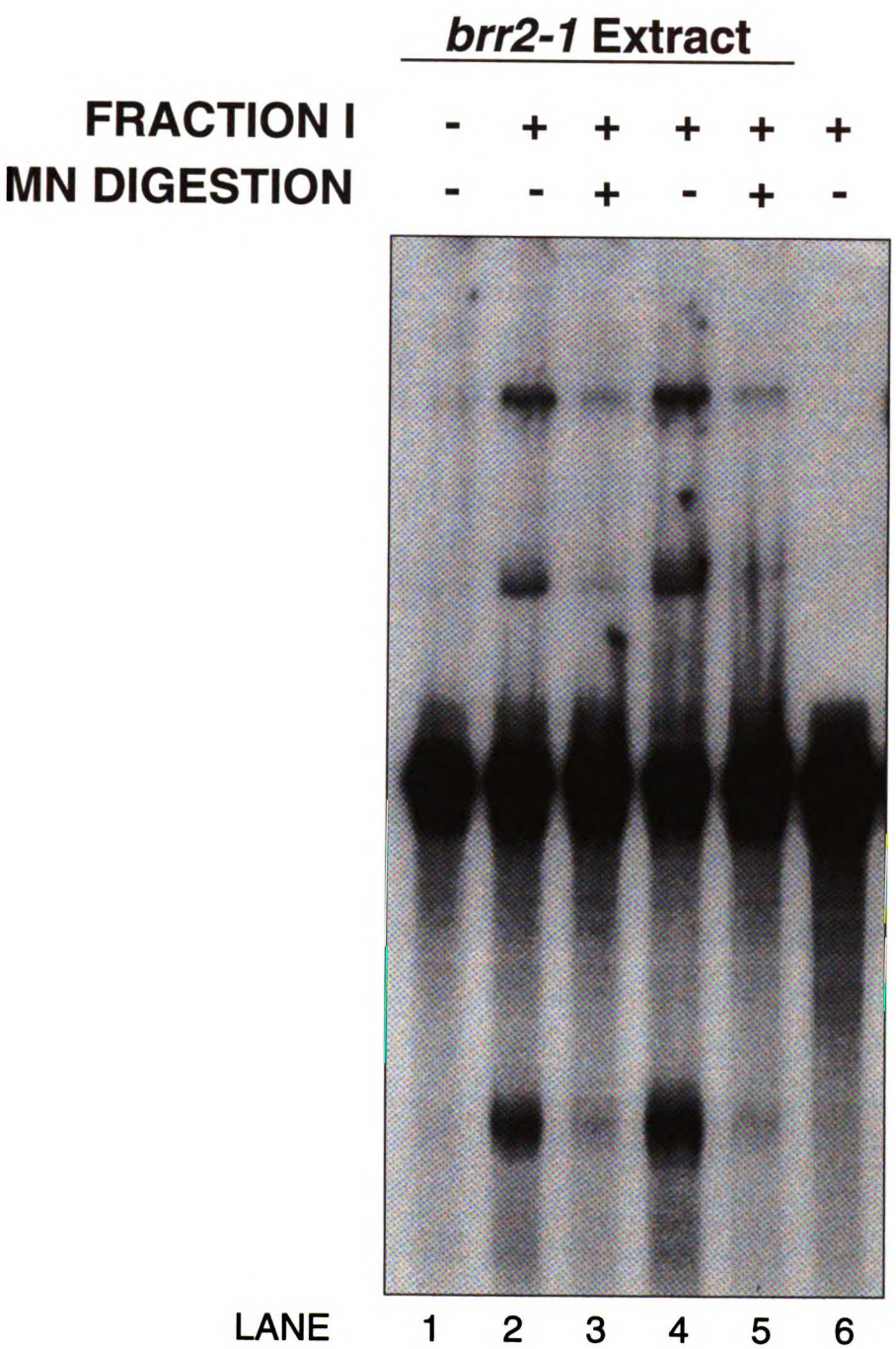

brr2-1 Extract
FRACTION I - + + + + +
MN DIGESTION - - + - + -
LANE 1 2 3 4 5 6

Figure 6A. Brr2 is an integral component of active spliceosomes. Brr2 coimmunoprecipitates with pre-mRNA, splicing intermediates, mRNA, and excised lariat intron. Large scale splicing reactions were performed in untagged or tagged *BRR2* extract in the presence or absence of ATP and Fraction I as indicated, and aliquots were removed for analysis (lanes 1-6). The remainder of the samples were subjected to immunoprecipitation on protein G Sepharose alone (lanes 7, 9) or protein G Sepharose coupled to anti-polyoma antibodies (lanes 8, 10-12). Lanes 7-12 correspond to the bound RNAs collected from samples in lanes 1-6, respectively.

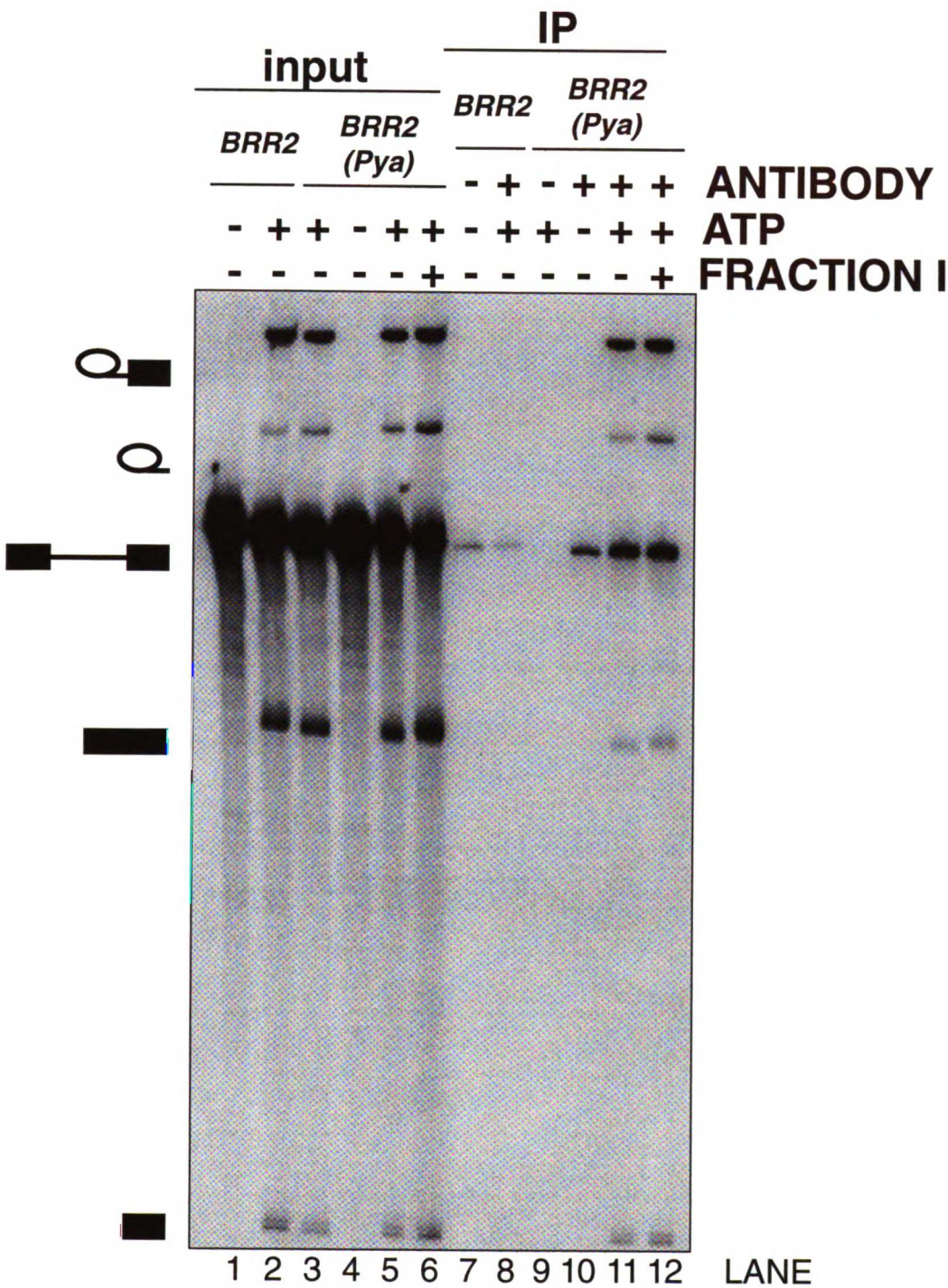
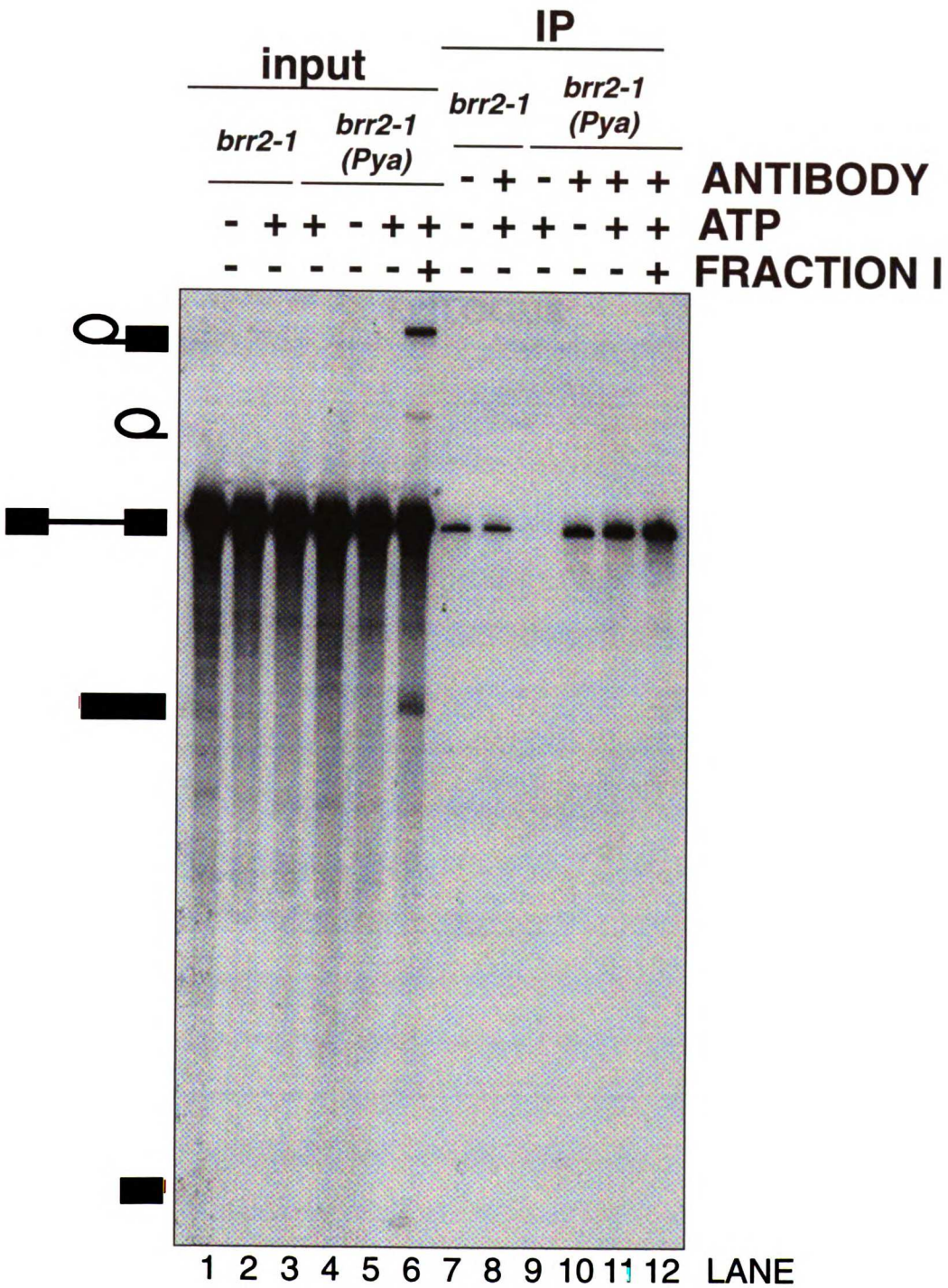

IP
input
BRR2
BRR2 (Pya)
BRR2
BRR2 (Pya)
- + - + + + ANTIBODY
- + + - + + - + + - + + ATP
- - - - - + - - - - - + FRACTION I
Q
Q
1 2 3 4 5 6 7 8 9 10 11 12 LANE

Figure 6B. Brr2-1 coimmunoprecipitates with pre-mRNA. Experiments identical to those described in Figure 6A were performed with untagged or tagged *brr2-1* extract. Even when splicing proceeds due to the inclusion of untagged wildtype fraction I (lane 6), Brr2-1 remains bound to unproductive splicing complexes that fail to progress through the first step (lane 12).


IP
input
brr2-1
brr2-1 (Pya)
brr2-1
brr2-1 (Pya)
- + - + + + ANTIBODY
- + + - + + - + + - + + ATP
- - - - - + - - - - - + FRACTION I
1 2 3 4 5 6 7 8 9 10 11 12 LANE

EPILOGUE

I. U4/U6 Base-Pairing Dynamics in the Spliceosome Cycle

This thesis work analyzes two opposing transitions that occur during each round of splicing: unwinding and reannealing of the U4/U6 duplex. I have identified two splicing factors, Brr2 and Prp24, that exert antagonistic effects on U4/U6 base-pairing. Because these proteins are required at different points in the splicing pathway, they reinforce the notion of the spliceosome as a cycle. Moreover, these studies demonstrate that proteins can govern helical exchanges, and thus are key regulators of spliceosomal dynamics.

Recycling is part of the spliceosome cycle

The study of pre-mRNA splicing has greatly benefited from the development of in vitro systems comprised of extract (prepared from whole yeast cells (Lin et al., 1985) or HeLa nuclei (Padgett et al., 1984; Ruskin et al., 1984)), radioactively labelled pre-mRNA, and ATP. Because this assay monitors conversion of the input pre-mRNA to lariat intermediates to mature mRNA, the splicing reaction appears to conform to a linear pathway. In fact, this superficial view neglects the contribution of snRNPs and proteins of the spliceosome, the presumed catalysts of the reaction. If we consider the splicing reaction from the perspective of the "enzyme" rather than the pre-mRNA substrate, the enzyme must dissociate from the product in order to fulfill its catalytic potential. In other words, if the spliceosome is a catalyst, its components must be regenerated in order to promote multiple rounds of intron excision. An *a priori* definition of the spliceosomal recycling pathway excludes assembly and catalysis on the pre-mRNA, and encompasses the dissociation and reassociation events necessary to renew splicing factors. Ruby and Abelson (1991) indirectly acknowledged the importance of recycling

in their depiction of yeast spliceosome assembly, catalysis, and disassembly as segments of a cycle that propels pre-mRNA splicing. While several assembly intermediates had been characterized (Cheng and Abelson, 1987), nothing was known about the fate of spliceosome components after release of the mature mRNA. Moore et al. (1993) also postulated that snRNPs reassemble subsequent to catalysis, since the notable stability of snRNAs in vivo implies that they participate in more than one splicing reaction. Despite these expectations, spliceosomal recycling has not been subjected to rigorous biochemical examination.

In the intervening years, our understanding of the helical transactions driving spliceosome assembly has advanced significantly. These details strongly reinforce the prediction that an active spliceosomal recycling process must exist. During assembly and catalysis, the snRNPs exchange base-pairing partners and undergo massive structural transformations; if they are not reconfigured during disassembly, post-splicing snRNPs will presumbably be incompetent to load onto new spliceosomes. A plausible model of U4/U6 reassembly was hypothesized from genetic analysis of suppressors of U4 snRNA, implicating the RNA binding protein Prp24 in re-formation of the U4/U6 duplex (Shannon and Guthrie, 1991). Chapter 2 describes a test of this model for Prp24 function during in vitro splicing. These results establish that U4 and U6 snRNPs must be reannealed in order to promote subsequent rounds of splicing, providing the first biochemical evidence for a spliceosomal recycling pathway. The striking helical transitions of the U4/U6 snRNP will provide useful benchmarks for characterizing structural changes of other RNAs during the spliceosome cycle.

Proteins regulate helical exchanges during the spliceosome cycle

Helical dynamics drive spliceosome assembly; a recycling pathway is necessary to reverse these RNA rearrangements. The spliceosome cycle demands fluid helical transitions that are well-timed and precisely placed. When I began this thesis work, it was assumed that these transformations of RNA structure were regulated by proteins; but specific factors and mechanisms had yet to be demonstrated. Since ATP is required for many steps in spliceosome assembly, putative RNA dependent ATPases of the DEAD/DEAH/DEXH-box families were believed to be the likely consumers. In fact, Schwer and Guthrie (1991) convincingly demonstrated that ATP hydrolysis by the DEAH-box splicing factors Prp16 was required to promote second chemical steps; similar analyses established an analogous role for Prp2 at the first step (Kim and Lin, 1993). Furthermore, because this class of proteins bears homology to RNA helicases, these factors were presumed to promote directional RNA unwinding on the spliceosome. As Chapter 1 illustrates for the case of Prp28, this prediction has not been borne out for the first five DEAD-box proteins discovered: no direct links have been established between Prp5, Prp28, Prp2, Prp16, or Prp22 and helical exchanges in the spliceosome cycle.

The transitions catalyzed by these putative RNA helicases have been subjected to considerable scrutiny, but only subtle structural changes in RNPs have been detected. For example, the 3' splice site becomes resistant to oligonucleotide-directed RNAase H cleavage through a conformational change of the spliceosome caused by Prp16 (Schwer, 1992). In contrast, RNAase H cleavage of U2 snRNP in extract is enhanced by the presence of Prp5 (O'Day et al., 1996). A plausible hypothesis is that Prp5 activates U2

snRNP for assembly onto the spliceosome, perhaps by making U2 snRNA's branchpoint region available for base-pairing (Wiest et al., 1996). Finally, the structure of U6 snRNA on the spliceosome is influenced by Prp2, as detected by an alteration in accessibility to dimethyl sulfate (Yean and Lin, 1996). The failure to observe clearcut helical transitions on the spliceosome may arise from the inadequacy of our biochemical assays. Helices are short within the spliceosome's postulated catalytic core; a meagre three base-pairs have been shown to form between U6 snRNA and the 5' splice site (through genetic techniques) (Lesser and Guthrie, 1993). Such duplexes may exist transiently, or in a complex quaternary structure with stabilizing proteins. Thus they may be difficult to detect by conventional methods that rely on deproteinization, such as nondenaturing gel electrophoresis.

In Chapters 2 and 3, these difficulties were circumvented by monitoring the dynamics of an abundant, unusually stable duplex in a native RNP. I have characterized two critical helical exchanges of the U4/U6 snRNP that occur at different points in the spliceosome cycle, and discovered a functional requirement for proteins during both unwinding and reassociation. Notably, a putative RNA helicase, Brr2, is required for the ATP-dependent dissociation of the U4/U6 duplex in isolated snRNP complexes. Xu et al. (1996) presented data indicating that the Brr2 protein is an RNA-dependent ATPase. The current work is the first to establish that DEAD-box superfamily members can indeed control RNA unwinding in native complexes such as snRNPs, in contrast to the vaguely defined structural changes seen previously . In Chapter 3 and elsewhere (P. L. R. and C. G., manuscript in preparation), we propose based on strong circumstantial evidence that Brr2 disrupts the U4/U6 duplex on the spliceosome, as well. If

this assignment of Brr2 function is correct, then a critical role of this ATPase would be to regulate U4/U6 unwinding. For example, inappropriate disruption of U4/U6 might inactivate the assembling spliceosome. A new challenge is to determine the constraints on U4/U6 dissociation: how does Brr2 delay ATP-dependent unwinding? Moreover, once the strands are separated, what mechanism prevents their reannealing? Since we have identified a key regulator of U4/U6 disruption within its normal RNP context, we are poised to gain insights into the physiological mechanisms of RNA unwinding.

As described in Chapter 2, a protein with three RNA recognition motifs (RRMs), Prp24, was found to reverse this reaction and reanneal the unpaired U4 and U6 snRNPs. Other RRM-containing proteins, including many hnRNPs, can facilitate hybrid formation with synthetic RNAs in vitro (Portman and Dreyfuss, 1994). Since hnRNPs have not been demonstrated to be necessary for annealing in physiological contexts, it is not clear whether these in vitro activities reflect in vivo functions. In contrast, another RRM protein, U2AF-65, has been shown to promote base-pairing between U2 snRNA and the pre-mRNA during an important transition in the spliceosome cycle (Valcárcel et al., 1996). Somewhat surprisingly, the region encoding the RRM of U2AF-65 is not directly responsible for annealing; rather, its arginine-serine-rich domain (RS domain) is necessary for this activity. Psoralen crosslinking between U2 snRNA and the branchpoint (indicative of base-pairing) is observed with high concentrations (~720 nM) of U2AF-65, providing a salient difference with the highly effective Prp24-catalyzed U4/U6 reannealing. Since Prp24 lacks an RS domain, and appears more potent than U2AF-65, its mode of action is likely to be quite different

from the only other known regulator of RNA-RNA association in the spliceosome cycle. Because of the ease with which U4/U6 base-pairing can be detected, the detailed mechanism of Prp24 in this reaction is ripe for investigation.

A working model of the spliceosome cycle

In Figure 1, I present an updated model of the spliceosome cycle incorporating the findings from this thesis work (revised from (Ruby and Abelson, 1991; Moore et al., 1993)). I have highlighted the ATP-dependent spliceosomal transitions likely to be controlled by the known DEAD-box splicing factors, with the caveat that some of these assignments have not been rigorously demonstrated. Thus this should be viewed as a conceptual framework rather than a definitive description of the spliceosome cycle.

The data in chapter 2 validate the model of triple snRNP formation first proposed by Shannon and Guthrie (1991). In addition, reassembly of the U4/U6.U5 snRNP has been placed within the context of the spliceosome cycle; red solid lines in Figure 1 depict this recycling pathway. Splicing consumes U4/U6.U5 snRNPs and generates free U4 and free U6 snRNPs. Normally, Prp24 binds to the U6 snRNP released from spliceosomes, and captures the free U4 snRNP. The re-formation of the U4/U6 duplex occurs in a U4/U6.Prp24 complex. Subsequently, free U5 snRNP associates to create the U4/U6.U5 snRNP, and Prp24 presumably leaves. One key finding is that Prp24 is not required for spliceosome assembly per se, but rather defines a new class of spliceosomal recycling factors required to regenerate splicing components. Also, chapter 2 shows definitively that Prp24 reanneals the RNAs within the U4 and U6 snRNPs. The spliceosome probably liberates U4

and U6 snRNPs at different times. Free U4 snRNP is likely to be ejected before catalysis is completed: when splicing in ΔPrp24 extract is blocked prior to the second step (due to the 3' splice site mutation G303C, G305C), free U4 is still liberated (P. L. R., unpublished results). In contrast, free U6 does not accumulate under the same circumstances, suggesting that it is sequestered in the stalled spliceosome (P. L. R., unpublished results). It is completely unknown how U6, U2, and U5 snRNPs are discharged from the spliced mRNA and lariat intron products into the recycling pathway.

In chapter 3, I have shown that Brr2 associates with duplex U4/U6 in a large complex containing U4, U6, and U5 snRNPs. In the presence of ATP, the U4/U6 helices are separated, and unpaired U4 and U6 snRNPs are physically released from the complex. This activity in the Brr2/snRNP particle is likely to be identical to a phenomenon characterized in chapter 2: when Prp24 is depleted, a subset of "U4/U6.U5" snRNPs disintegrates into individual snRNPs in the presence of the ATP. In both cases, U4/U6.U5 snRNPs are dissociated by ATP in the absence of added pre-mRNA. After Brr2 disrupts the U4/U6 helices in the Brr2 snRNP complex, Prp24 is capable of reannealing the unpaired snRNPs (chapter 2). A pictoral interpretation of these results is presented in Figure 1 (red dashed lines). A population of U4/U6.U5 snRNPs, when exposed to ATP, releases free U4, U6, and U5 snRNPs. These individual snRNPs are rapidly reincorporated into U4/U6 and U4/U6.U5 snRNPs through the action of Prp24.

What is the significance of this apparently futile cycle of dissociation and reassociation? This process does not require pre-mRNA, but it does require the antagonists Brr2 and Prp24. snRNP complexes bearing the Brr2-1

protein fail to dissociate in response to ATP (chapter 3). On the other hand, if Prp24 is missing, free U4 and U6 snRNPs accumulate at the expense of U4/U6 and U4/U6.U5 snRNPs (chapter 2). Under these in vitro conditions, one protein undoes the action of the other: Brr2 is required for the ATP-dependent disruption of the duplex U4/U6 snRNP, and the resulting unpaired U4 and U6 snRNPs are reannealed by Prp24 (Figure 2). It is not clear what purpose this ATP-dependent snRNP recycling serves during in vivo pre-mRNA splicing. One possibility is that the dynamic behavior of snRNPs allows rapid regulation in response to environmental stresses, such as heat shock. If snRNPs traverse this cycle rapidly and continuously, splicing can be shut down nearly instantaneously by preventing reassociation. In fact, heat shock inhibits pre-mRNA splicing in HeLa cells by inactivating a factor that promotes triple snRNP formation (Utans et al., 1992). Thus, external sensing systems may govern pre-mRNA splicing through the triple snRNP assembly pathway.

Alternatively, this ATP-driven cycling may activate snRNPs for splicing. Brr2 also appears necessary to assemble wildtype amounts of U4/U6.U5 snRNPs, because *brr2-1* extract harbors low levels of this species, and does not promote U4/U6.U5 addition onto pre-mRNA (chapter 3). Moreover, a large, static Brr2-1/snRNP complex accumulates in the mutant extract that fails to dissociate in response to ATP. These data suggest that ATP hydrolysis by Brr2 is required to convert an inactive snRNP complex (designated by "inactive [U4/U6.U5]" in Figure 1) to splicing-competent U4/U6.U5 snRNP. Reassembly may reconfigure the triple snRNP so that it can load onto spliceosomes.

Brr2 is also proposed to be required for U4/U6 dissociation on the spliceosome based on several pieces of circumstantial evidence. ATP-dependent unwinding of U4/U6 does not occur in snRNP complexes containing Brr2-1, illustrating this event's contingency on wildtype Brr2 function. U4/U6 unwinding is necessary on the spliceosome, and is likely to be reflected by this snRNP-based activity. Furthermore, Brr2 is present on spliceosomes stalled prior to U4 release by low concentrations of ATP (P. L. R., unpublished results), consistent with a role in this key transition. For these reasons, Figure 1 shows that U4/U6 dissociation, which precedes Prp2 action, requires Brr2 and ATP (blue solid lines).

Finally, Figure 1 provides two alternative roles for Prp28 in the spliceosome cycle, which are not mutually exclusive. Prp28 may activate the U4/U6.U5 snRNP before it binds the spliceosome (red solid lines in Figure 1), as suggested by chapter 1. These data show that Prp28 per se is not required for spliceosome assembly in immunodepleted extract, while a nuclease-sensitive component can substitute for it in genetically depleted extract. Normal levels of U4/U6.U5 snRNPs have been observed in the latter extract (P. L. R., unpublished results), but these snRNPs do not appear competent to associate with pre-mRNA. Furthermore, genetic interactions with U4 and U6 snRNAs raise the possibility that Prp28 causes rearrangements involving these snRNPs. Intriguingly, the apparent human homolog of Prp28 has recently been isolated as a component of the U4/U6.U5 triple snRNP (R. Lührmann, personal communication). Although numerous experiments have failed to demonstrate a physical interaction between Prp28 and snRNPs in yeast (P. L. R., unpublished results), it is quite possible that the yeast

protein's functional interaction with the U4/U6.U5 snRNP is conserved between systems.

During the late stages of the work presented in chapter 1, a synthetic lethal interaction was discovered between *prp28-1* and a hyperstabilized U1 snRNA/5' splice site helix (J. P. Staley, personal communication). Spliceosomes assembled in *prp28-1* extract appear to have all five snRNAs bound (J. P. Staley, personal communication). These and other results are consistent with the hypothesis that Prp28 governs the exchange of U1 for U6 snRNA at the 5' splice site (blue solid lines in Figure 1). Both sets of data can be accommodated by positing that Prp28 modifies U4/U6.U5 snRNPs to facilitate U6 snRNA/5' splice site pairing. In this view, unmodified triple snRNPs in *prp28-1* extract may associate weakly with spliceosomes, but then Prp28 may act directly on these complexes to promote tight binding and progression through catalysis. The superficial contradictions observed between experiments in *prp28-1* and depleted extracts may be due to methodological differences. For example, different native gel assays were used to monitor spliceosome assembly in GLU and *prp28-1* extracts, and biotinylated pre-mRNA "pull-down" assays were successfully performed only in *prp28-1* extracts (J. P. Staley, personal communication). Also, in vitro splicing may be differentially affected by the presence of the mutant Prp28-1 protein (in *prp28-1* extract) versus the removal of Prp28 protein (in GLU and Δ28 GAL extracts). For example, immunodepletion of Prp22 inhibits step two of splicing (B. Schwer, personal communication) while temperature inactivation of the *prp22-1* mutant produces a downstream block to mRNA release (Company et al., 1991). In summary, Prp28 may be capable of modifying U4/U6.U5 snRNPs before and/or after they load onto

spliceosomes. This model draws an interesting analogy between the functions of Prp28 and its most closely related DEAD-box splicing factor Prp5, which has been proposed to activate U2 snRNP for spliceosome binding (Wiest et al., 1996).

II. The Spliceosome Cycle: Future Directions

This thesis work raises many questions about the roles of Prp28, Brr2, and Prp24 in the spliceosome cycle. Here, I describe what I regard as particularly fruitful avenues for future investigation.

Dissecting the recycling pathway

Currently, there are no biochemical assays to monitor the disassembly of the spliceosome following the second catalytic step. As a result, studies of the recycling pathway have been entirely neglected. To remedy this situation, an in vitro assay can be developed to demonstrate formally that snRNPs are recycled between spliceosomes. One such procedure would entail using donor spliceosomes, known to have progressed through splicing catalysis, as the sole source of U6 snRNPs for newly assembling spliceosomes. For example, as donor spliceosomes, one could use glycerol gradient-purified ΔPrp16 spliceosomes assembled on unlabelled pre-mRNA. These could complement a splicing reaction lacking U6 snRNPs comprised as follows: ΔPrp16 ΔU6 extract (pre-treated with antisense U6 oligo and RNAase H to ablate the U6 snRNP), ATP, purified Prp16, and labelled pre-mRNA. When the donor spliceosomes are added to the extract in the presence of Prp16, they will complete the second step and begin disassembly. If these post-splicing U6 snRNPs are regenerated and reassemble on the labelled pre-mRNA, lariat

intermediates and mature mRNA should be observed. An important control is to observe a complete dependence of new splicing on the presence of Prp16. Donor spliceosomes could be contaminated with free snRNPs, or the snRNPs on ΔPrp16 spliceosomes might dissociate through a discard pathway without continuing through catalysis. Thus, true recycling demands that splicing of the labelled pre-mRNA be dependent on the completion of splicing in the donor spliceosome, which can only occur when Prp16 is present. If this assay works, one could begin to determine which factors are necessary to recycle U6 snRNPs between spliceosomes. For example, if Prp24 is depleted from the reaction, is splicing impeded? Is Prp28 required as well? This assay depends on de novo spliceosome assembly on labelled precursor as well as disassembly from unlabelled mRNA. Thus, the main drawback is that one can test only those factors which are not also required for spliceosome assembly.

Another approach would be to characterize the disassembly pathway from a known entry point. For example, splicing could be initiated in a heat-inactivated *prp22-1* extract with a pre-mRNA tagged with biotin on its 3' end (e.g. via splint labelling, oligo hybridization, etc.). The stalled spliceosomes could be purified from a gradient to achieve a more homogeneous population, and then isolated on streptavidin beads. If purified Prp22 protein and ATP are added to the washed complexes, can differences be observed in snRNP content or structure? Is the lariat released from the beads? Are the snRNPs released as well? If Prp22 protein is not sufficient to produce rearrangements, snRNP-free extract could also be added in order to achieve some measurable changes (i.e. does chemical modification of snRNPs reveal structural alterations +/- Prp22?). If a snRNP/lariat complex is ejected from the beads through the action of Prp22, its disassembly kinetics might be

monitored by native gel electrophoresis. By depleting factors or fractionating the extract, one could begin to identify intermediates in the snRNP disassembly pathway. It is possible that a post-splicing U2/U6/U5 multi-snRNP might serve as a platform for snRNP reassembly, and perhaps Prp24-facilitated reannealing. Wassarman and Steitz (1992) have reported U4/U6/U2 snRNPs that pre-exist in extract in the absence of added pre-mRNA. Chapter 3 describes the existence of large multi-snRNP complexes of mysterious origin, which could be recycling intermediates. A genetic interaction has been observed between *prp24-1* and *prp21-2*, which encodes a U2 snRNP protein; perhaps the functional interaction between these two proteins occurs in the "post-spliceosome."

At least one other DEAH-box protein (Prp40, or JA1) displays temperature-sensitive splicing inhibition in vivo, but its immunodepletion does not impede splicing in vitro (Company et al., 1991) (J. Arenas and J. Abelson, personal communication). Such proteins are ideal candidates for recycling factors, and should be pursued in the assays described above. For example, they may catalyze unwinding of the U2/U6 structure expected for the post-spliceosome. In order to identify further components of this pathway, one could screen for synthetic lethality in combination with *prp24* or *prp22* alleles.

Prp24 and U4/U6 reannealing

Prp24 is the first protein that has been shown to hybridize RNAs within native RNP complexes. This in vitro annealing assay should provide an ideal opportunity to analyze the mechanism of RNA annealing within a physiologically relevant context. The basic dilemma is how this one protein

associates with 3 different RNPs: free U6 snRNP, free U4 snRNP, and duplex U4/U6 snRNP. Does Prp24 bind directly to U6 snRNA in the free U6 snRNP? If so, what U6 snRNA elements are necessary for recognition by Prp24? Chemical modification and crosslinking techniques can be used to assess whether the Prp24 binding site on U6 snRNP resembles the proposed pseudoknot structure (Brow and Vidaver, 1995). Likewise, chemical probing of the free U4 snRNP will reveal the structural constraints of this normally fleeting intermediate. To determine which RNA residues within each snRNP are critical for annealing, a chemical modification/interference assay could be developed. This technique would require purifying separate pools of free U6 and free U4 snRNPs, and modifying each snRNP with one chemical hit per molecule. After the modified snRNPs are allowed to anneal in the presence of Prp24, the RNA duplexes should be selected and examined for the absence of modification on particular residues. These regions can be inferred to be required for proper annealing. A similar approach was used to identify U6 snRNA residues necessary for U4/U6 assembly in HeLa extract, and a kissing loop mechanism was proposed (Wolff and Bindereif, 1993). The advantage of the present system is that both U4 and U6 snRNAs can be studied. In a similar vein, the annealing assay lends itself well to structure/function analyses of the Prp24 protein. Site-directed mutagenesis, domain deletions, and truncations can establish whether Prp24's three RRM domains are necessary and/or sufficient for annealing activity.

Kinetic analyses of annealing will also help determine whether Prp24 stabilizes a partially duplex intermediate, as is the case with Rom and the RNA I/RNA II stem-loops (Eguchi and Tomizawa, 1990) (see end of epilogue, section III). Another way to trap intermediates in the annealing process

might be to mix mutant U4 snRNPs with wildtype U6 snRNPs (or vice versa). To isolate mutant snRNPs, polyclonal anti-Prp8 antibodies could be used to immunoprecipitate U4/U6.U5 snRNPs from extracts bearing mutant snRNAs. Incubation of the washed bound snRNP complexes with ATP should release these mutant free U4 and U6 snRNPs into the supernatants. The U4 G14C mutant is an obvious first candidate with which to attempt annealing, since this mutation inhibits U4/U6 formation in vivo (Shannon and Guthrie, 1991; Jandrositz and Guthrie, 1995). Since the cold-sensitive phenotype of U4 G14C is suppressed in vivo by mutations in the third RRM of Prp24 (Shannon and Guthrie, 1991), it would be interesting to see whether this suppression can be reproduced in the in vitro annealing assay. Mutations in the intramolecular loop of U6 snRNA (nt 71-75, especially nt 72) are also likely to inhibit annealing (Fortner et al., 1994), if the "kissing-loop" mechanism is at work.

In chapter 2, deproteinization of the unpaired snRNPs slowed the annealing reaction catalyzed by Prp24. Do proteins on one or both snRNPs contribute to annealing efficiency? For example, does deproteinization of U6 snRNP inhibit annealing as much as deproteinization of U4 snRNP? snRNP proteins may maintain the proper structural conformation of the RNP, or they may provide binding sites for Prp24. These possibilities might be distinguished by comparing snRNA structures and dissociation constants for Prp24 binding before and after deproteinization. Are there other soluble protein factors required for rapid annealing? To determine what proteins are present on each snRNP, purified snRNP proteins can be probed with antibodies against suspected constituents. Sm proteins (Rymond, 1993; Séraphin, 1995) and Prp4 (Banroques and Abelson, 1989) might be expected to

associate with U4 snRNP, and Uss1 (Cooper et al., 1995) and U6-specific Sm-like proteins (Séraphin, 1995) might be observed on the U6 snRNP.

A long-term goal should be to achieve reconstitution of splicing-competent U4/U6.U5 snRNP from defined components. U4/U6 snRNPs annealed by Prp24 provide the ideal starting reagents for such experiments. Towards this aim, a central question is whether Prp24 dissociates from the duplex U4/U6 snRNP, and how it does so. Is Prp24 bound to all freshly annealed duplex U4/U6? If so, what happens when U5 snRNP (perhaps immunoaffinity purified from Prp8-3HA extract) is included in the reaction? It is tempting to speculate that NTPase activity of a U5 snRNP constituent (e.g. Brr2?) could be responsible for ejecting Prp24. A different way to approach this issue is to determine whether Prp24 protein can reanneal unpaired U4 and U6 snRNPs that remain bound to the Brr2 snRNP complex on beads (chapter 3). This experiment demands the use of Prp24 protein that is not polyoma-tagged, in order to avoid confusion with polyoma-tagged Brr2 snRNP complexes. If Prp24 can re-form the U4/U6 duplex within the larger snRNP complex, what prevents Prp24 from reannealing these snRNPs on the spliceosome? Perhaps the physical ejection of U4 snRNP on the spliceosome ensures that helical disruption is irreversible. If Brr2 and Prp24 antagonistically control U4/U6 base-pairing in vivo, their activities may need to be segregated in time and space during the spliceosome cycle.

Finally, two unresolved biological issues are worth mentioning. While Prp24 reanneals pre-existing snRNPs after a round of splicing, it is not clear whether Prp24 or some other factor is necessary to anneal newly manufactured U4 and U6 snRNPs during biogenesis. To test the requirement

for Prp24 in vivo, a temperature-sensitive *prp24* strain could be constructed with a galactose-inducible "tagged" U4 snRNA gene that can be distinguished from the wildtype U4 (Noble and Guthrie, 1996b). The strain should be simultaneously shifted to the nonpermissive temperature (to inactivate Prp24) and to galactose media (to induce synthesis of tagged U4 snRNA). At various times thereafter, total yeast RNA should be isolated, and monitored by nondenaturing gel electrophoresis for the presence of tagged U4/U6 duplexes. If Prp24 is required to anneal U4 and U6 during biogenesis, newly synthesized U4 snRNAs will not be incorporated into U4/U6 duplexes under these conditions. Tagged U4 snRNA and tagged U6 snRNA genes can also be used to develop an exchange assay, to examine whether U4 snRNA changes pairing partners with each round of splicing. Under splicing conditions, two extracts could be mixed: one containing tagged U4 but wildtype U6 snRNAs, and the other with wildtype U4, but tagged U6 snRNAs. If "hybrid" tagged U4/tagged U6 and wildtype U4/wildtype U6 duplexes can subsequently be detected, then promiscuous pairing has occurred. One could also test whether this exchange of pairing partners was dependent on ATP, pre-mRNA, and/or Prp24.

Brr2 and U4/U6 unwinding

Brr2 is likely to play more than one role in the spliceosome cycle, since it remains bound to the pre-mRNA throughout catalysis (chapter 3), and the *brr2-1*, *slt22-1*, and *rss1-1* alleles display different splicing phenotypes (Lin and Rossi, 1996; Xu et al., 1996). This work describes the involvement of Brr2 in U4/U6 unwinding in snRNPs, but falls short of establishing Brr2 as the spliceosomal U4/U6 helicase due to idiosyncrasies with the *brr2-1* allele. To solidify the connection between Brr2 and U4/U6 dissociation on the

spliceosome, a different allele may be more revealing. I engineered a GKT to GET mutation predicted to abolish ATP-binding in the first DEXH-box ATPase domain of Brr2; as expected, this allele fails to complement the *brr2::LEU2* disruption at all temperatures (P. L. R., unpublished results). When expressed from a high-copy 2-micron plasmid in an otherwise wildtype strain, *BRR2-GET* exhibits a mild dominant cold-sensitive growth phenotype (P. L. R., unpublished results). Under similar conditions, overexpression of *brr2-1* does not produce any dominant negative phenotype (P. L. R., unpublished results). A priority is to investigate whether this Brr2-GET protein associates with spliceosomes stalled prior to U4/U6 disruption. This can be done by preparing companion extracts in which either wildtype Brr2(Pya) or Brr2-GET(Pya) is overexpressed, and analyzing the spliceosomes that immunoprecipitate with these tagged proteins. The Brr2-GET protein may also block splicing at an entirely novel step. Therefore, one might need to screen PCR- or hydroxylamine-mutagenized libraries of *BRR2* plasmids for different dominant negative alleles (perhaps in a *brr2-1* background, where the phenotypes might be more evident).

To gather more evidence that Brr2 disrupts U4 and U6 snRNAs, one could screen for genetic interactions between *brr2-1* and mutagenized libraries of the snRNAs, as done for *prp28-1* in chapter 1. In fact, this technique was subsequently used to search for suppressors of another cold-sensitive mutant helicase, *prp16-302*. (Madhani and Guthrie, 1994b). In this case, U6 snRNA suppressors were successfully isolated, proving that the technique is replicable and can be informative.

While ATP releases some unpaired U4 and U6 snRNPs from the Brr2 complex, a large amount of free U4 and free U6 remains bound to Brr2 (chapter 3). Does Brr2 associate with different populations of snRNPs? For example, is it possible that ATP dissociates one subset of snRNPs, and activates another subset for spliceosome assembly? I previously determined that the Brr2 snRNP complex did not bind specifically to pre-mRNA with correct intron consensus sequences (P. L. R., unpublished results); perhaps these snRNPs are inactive for loading because they have not been reorganized by ATP. To test this idea, one could expose the Brr2 snRNP complex to ATP, wash away the released (presumably inactive) snRNPs, and incubate the remaining bound snRNPs with pre-mRNAs containing wildtype and mutant intron consensus sequences. Does the addition of ATP facilitate specific binding of the Brr2/snRNP complex to pre-mRNA? If not, does the coordinate addition of ATP and Prp24 complete the snRNP activation process, and alter pre-mRNA binding?

The Brr2 snRNP complex should be further characterized. If the snRNP complex consists of spliceosomes formed in vivo, endogenous pre-mRNA species may be detectable within it. One could probe the immunoprecipitated RNAs for those highly expressed endogenous transcripts that contain introns (and for highly expressed intronless mRNAs as controls); these mRNAs have been identified by analysis of the yeast transcriptome (Velculescu et al., 1997). Moreover, the requirements for ATP-dependent U4/U6 unwinding within this complex could be further defined. For example, does disruption of the U4/U6 helices depend on the integrity of the U2 snRNP? It is possible that U4/U6 dissociation is coupled to the formation of U2/U6 helices on the spliceosome, and perhaps within this

snRNP particle as well. To address these questions, one could cleave U2 snRNP with an oligonucleotide and RNAase H, and determine whether a) U4/U6 dissociation and b) release of freed snRNPs occurs in the Brr2 complex in the absence of functional U2 snRNP. To delve into the order of these helical transitions in more detail, one must be able to monitor the status of each individual helix using assays which have yet to be developed (perhaps through psoralen crosslinking?). Such techniques will distinguish whether U2/U6 helix Ia coexists with U4/U6 stem II, and whether disruption of U4/U6 stems I and II occur independently. Perhaps the other existing mutant versions of Brr2, when tested for ATP-dependent U4/U6 unwinding, will reveal such notable intermediates in the snRNP complex.

The relevant spliceosomal question is whether U4/U6 dissociation substantially precedes the release of U4 snRNP. Previously, no one has been able to examine such intermediates in U4/U6 disruption on the spliceosome. One approach that does not rely on Brr2 would be to assemble spliceosomes on biotinylated pre-mRNA with low concentrations of ATP, and then bind the complexes to streptavidin beads. After washing away unbound material, the status of the U4/U6 duplex could be monitored in the presence or absence of additional ATP. Is U4 snRNP liberated from the spliceosome under these conditions? Is the U4/U6 duplex partially disrupted prior to release? Could this bound spliceosome catalyze the first step if supplied with Prp2 and Spp2? These admittedly difficult experiments are likely to enrich our understanding of the mechanism of U4/U6 unwinding on the spliceosome.

The role of Prp28 in splicing

If Prp28 activates the U4/U6.U5 snRNP for spliceosome binding, one might expect to observe a structural alteration in the RNP after Prp28's action. To see such effects, I would chemically modify the U4/U6.U5 snRNPs purified from GLU extract in the presence and absence of Prp28 (with dimethyl sulfate, kethoxal, etc.). I would first focus on differences in protection patterns of the U6 snRNP, since the genetic experiments predict that it functions closely with Prp28 (chapter 1). In fact, J. P. Staley has observed a Prp28-dependent change in the sensitivity of U6 snRNA to RNAase H cleavage (J.P.S., personal communication). If such gross structural transitions in the triple snRNP correlate with its ability to bind the spliceosome, the function of Prp28 may at long last begin to be defined.

III. Paradigms: Helices and Cycles

Other regulated RNA unwinding and annealing events

The dynamics of the U4/U6 snRNP demonstrate that dramatic changes in RNA base-pairing can be regulated during the spliceosome cycle. These results may have broader relevance to the regulation of RNA unwinding and annealing in other biological contexts, too. Coordination of RNA rearrangements is likely to be a recurring problem for most cellular transactions that involve RNPs. For example, during ribosomal biogenesis, precursors to ribosomal RNAs (pre-rRNAs) are transcribed and processed through a complex series of cleavage reactions (Maxwell and Fournier, 1995). Processing occurs with the assistance of a set of small nucleolar RNAs (snoRNAs) that are complementary to short regions of the pre-rRNAs. These short snoRNA/pre-rRNA helices are thought to contribute to pre-rRNA processing and 2' O methylation; controlling the formation of these duplexes

may be critical for proper ribosome synthesis (Bachellerie et al., 1995). In fact, at least three putative helicases of the DEAD-box family are required for efficient ribosomal biogenesis in yeast: Spb4 (Sachs and Davis, 1990), Drs1 (Ripmaster et al., 1992), and Dbp3. Dbp3 is a nucleolar DEAD-box protein required for the RNase MRP-mediated cleavage of the 35S precursor rRNA (Weaver et al., 1997). Plausible regulatory roles for Dbp3 include transforming pre-rRNA structure, inducing a conformational change of RNase MRP structure, or modulating snoRNA/pre-rRNA helices. It is likely that the distinct pre-rRNA processing and pre-mRNA splicing machineries share common strategies to regulate RNA secondary structure. These parallels will undoubtedly emerge with further research.

The ribosome is another dynamic RNP machine that may undergo a series of RNA transitions to catalyze protein synthesis, with some intriguing analogies to this thesis work. Initiation of translation in eukaryotes requires the cooperation of an ATP-dependent RNA helicase (the DEAD-box protein eIF4A) and an RRM protein (eIF4B) (Hershey, 1991). Together these factors have been proposed to promote some aspect of ribosome/mRNA interaction, perhaps by scanning along and unwinding secondary structure in mRNA. In fact, synthetic RNA duplexes are dissociated when both eIF4A and eIF4B are added (Rozen et al., 1990). Surprisingly, eIF4B alone anneals complementary RNAs (Altmann et al., 1995). Furthermore, the yeast homolog of eIF4B, Tif3, enhances binding of ribosomes to mRNAs in cell-free translation systems. Altman et al. (1995) speculated that antagonistic actions of eIF4A and eIF4B may facilitate ribosome recognition of the appropriate initiator AUG codon on the mRNA. For example, eIF4B could promote intermolecular RNA base-pairing between rRNA in the 40S ribosomal subunit and regions flanking the

AUG of the mRNA. If the 40S/mRNA complex were not sufficiently stable, ATP hydrolysis by eIF4A could disrupt the interaction and force the 40S ribosomal subunit to translocate further along the mRNA. Thus in order for the ribosome to identify the correct initiator codon on the mRNA, repeated cycles of eIF4B-mediated RNA annealing and eIF4A/4B-mediated RNA unwinding may be necessary. Here, a DEAD-box protein may regulate RNA annealing as well, since in principle a helicase could destabilize inhibitory secondary structure. This model has yet to be tested, but provides a possible example of antagonistic modulation of RNA base-pairing in another RNP.

Conformational rearrrangements of RNPs may also be important for localized translation of mRNAs in Drosophila oocytes. Expression of *nanos* is necessary to determine the longitudinal body axis during Drosophila development (Johnston and Nüsslein-Volhard, 1992). *nanos* mRNA is found throughout the oocyte with increasing concentrations towards the posterior pole, yet the nanos protein is made exclusively in the oocyte posterior (Gavis and Lehmann, 1992; Gavis and Lehmann, 1994). Repression of translation in the anterior requires a conserved stem-loop in the 3' untranslated region of the mRNA. This element is likely to bind an unknown repressor protein. Derepression of translation in the posterior requires the DEAD-box protein vasa, which is postulated to inactivate the repressor/stem-loop RNP (Gavis et al., 1996). These clues strongly suggest that mRNA expression is regulated by the restructuring of an RNA-protein complex.

Spatial restriction of another mRNA in Drosophila oocytes may depend on the formation of intra- and intermolecular RNA base-pairs. The

bicoid (*bcd*) mRNA is packaged in a large RNP with the staufen protein, which is necessary for *bcd* mRNA transport and localization to the oocyte anterior (Johnston and Nüsslein-Volhard, 1992). Interestingly, the staufen protein contains five double-stranded RNA binding motifs (Johnston et al., 1992), and binds the 3' untranslated region of *bcd* mRNA in vitro. This transport control element in the *bcd* 3' UTR is also predicted to form a stable stem-loop structure (Ferrandon et al., 1997). In order for staufen to bind this region, the *bcd* 3' UTR must form an intramolecular helix as well as an intermolecular helix: the loops of two *bcd* mRNAs must be base-paired. When intermolecular loop-loop pairing is destabilized, the transport granule (the *bcd* mRNA/staufen large RNP complex) is neither built nor localized properly in vivo (Ferrandon et al., 1997). Thus, the formation of a large, transport-competent RNP is likely to require intermolecular RNA helices, which ultimately regulate mRNA expression.

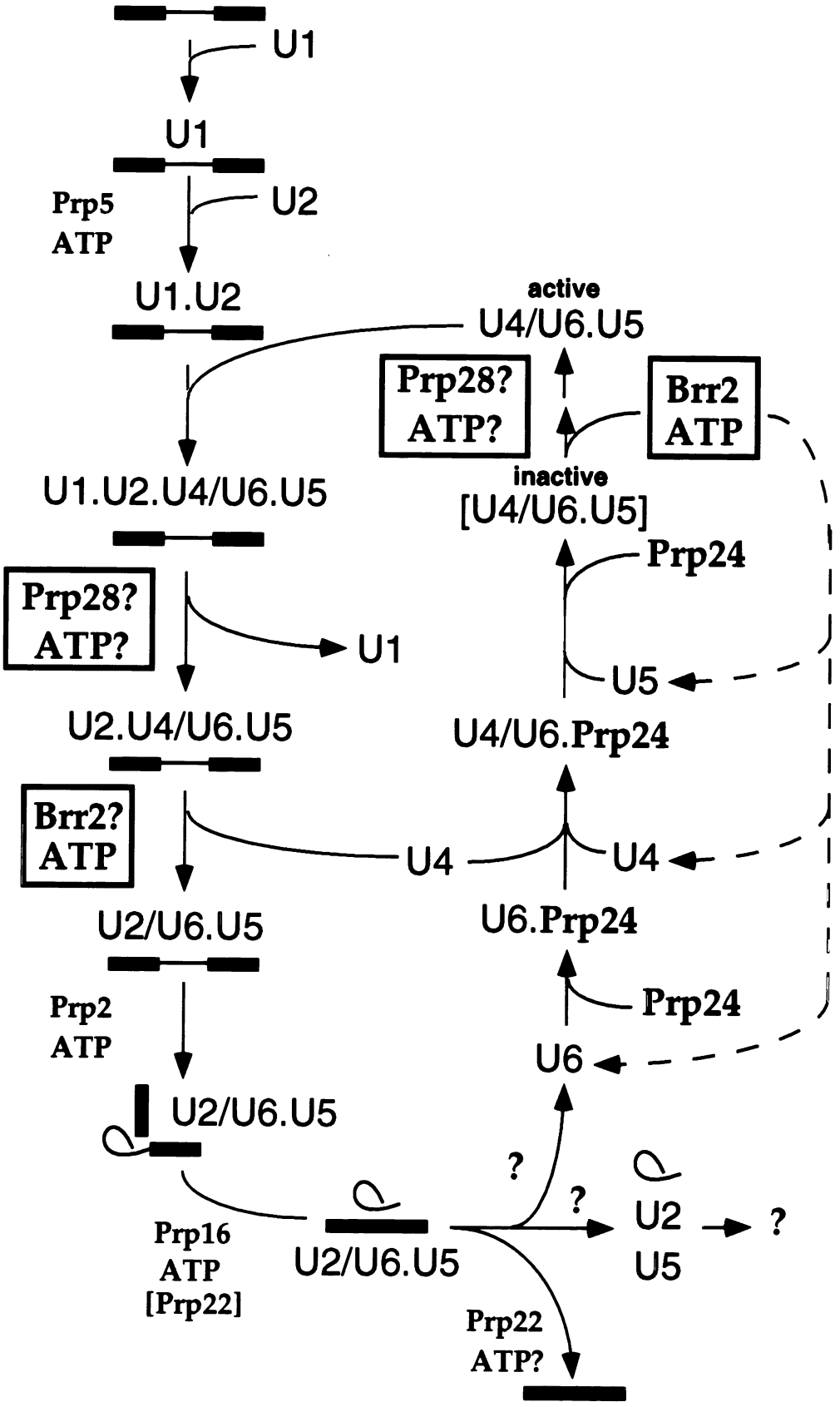
A well-studied regulated RNA annealing event controls copy number of the ColE1 plasmid in *E. coli.* RNA II is the primer for ColE1 DNA replication, but it cannot function if it is hybridized to the complementary RNA I instead. This intermolecular RNA interaction is transiently initiated when the complementary loops of the two hairpin structures come into contact. This "kissing loop" structure is stabilized by the Rom protein (also known as Rop), which prevents its dissociation (Eguchi and Tomizawa, 1990). Rom apparently binds this RNA I/RNA II complex without sequence specificity, but recognizes the unusual structure of the base-paired loops (Predki et al., 1995). A simple three component in vitro reaction has been useful for kinetic analyses of Rom/RNA binding (Eguchi and Tomizawa, 1990; Eguchi and Tomizawa, 1991). Moreover, the structure of the Rom

protein has been solved (Banner et al., 1987; Eberle et al., 1990). Thus studies of the Rom/RNA I/RNA II complex provide a useful paradigm for further experiments with Prp24, U4, and U6 snRNAs.

Cycling of nucleoprotein machines

The nucleoprotein machines involved in gene expression -- the DNA replication machinery, the transcription apparatus, the spliceosome, the ribosome -- undergo cycles of assembly, catalysis, and disassembly. These cycles are characterized by conformational changes driven by nucleotide hydrolysis. The spliceosome is unusual in its reliance on RNA helical transitions as its mechanistic principle. However, because pairing partners are often exchanged and RNAs are completely restructured, these reactions must evidently be reversed for new rounds of splicing. If we accept the paradigm of the cycle, then studies of disassembly and assembly of nucleoprotein machines deserve equal emphasis and attention. Recycling processes have begun to be investigated in the generation of Okazaki fragments by DNA polymerase III (Stukenberg et al., 1994), the re-initiation at promoters by RNA polymerase II and III (Hawley and Roeder, 1987; Corden, 1993; Zawel et al., 1995; Dieci and Sentenac, 1996), and multiple rounds of translation by ribosomes (Pavlov et al., 1997). In some of these systems, the detailed rates of reactions are known, thus facilitating the analysis of parameters that affect the time per catalyzed round. Guided by the advances in these disparate systems, similar approaches may ultimately be extended to the spliceosome cycle.

Figure 1. A working model of the spliceosome cycle. Blue lines delimit spliceosome assembly and catalysis, and red lines denote the recycling pathway. Red dashed lines indicate pre-mRNA-independent, ATP-dependent snRNP dissociation.


U1
U1
Prp5
ATP
U2
U1.U2
active
U4/U6.U5
Prp28?
ATP?
Brr2
ATP
inactive
[U4/U6.U5]
U1.U2.U4/U6.U5
Prp24
Prp28?
ATP?
U1
U5
U2.U4/U6.U5
U4/U6.Prp24
Brr2?
ATP
U4
U4
U2/U6.U5
U6.Prp24
Prp24
Prp2
ATP
U6
U2/U6.U5
?
?
U2
U5
?
Prp16
ATP
[Prp22]
U2/U6.U5
Prp22
ATP?

U6
U4
Brr2
ATP
Prp24
U6
U4

REFERENCES

Altmann, M., Wittmer, B., Méthot, N., Sonenberg, N. and Trachsel, H. (1995). The Saccharomyces cerevisiae translation initiation factor Tif3 and its mammalian homologue, eIF-4B, have RNA annealing activity. EMBO Journal *14*, 3820-3827.

Ansari, A. and Schwer, B. (1995). SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing. Embo Journal *14*, 4001-9.

Arenas, J.E. and Abelson, J.N. (1993). The *Saccharomyces cerevisiae PRP21* gene product is an integral component of the prespliceosome. Proceedings of the National Academy of Sciences *90*, 6771-6775.

Ares, M. and Igel, A.H. (1990). Lethal and temperature-sensitive mutations and their suppressors identify an essential structural element in U2 small nuclear RNA. Genes & Development *4*, 2132-2145.

Ares, M. and Weiser, B. (1995). Rearrangement of snRNA structure during assembly and function of the spliceosome. Progress in Nucleic Acid Research and Molecular Biology *50*, 131-159.

Ayadi, L., Miller, M. and Banroques, J. (1997). Mutations within the yeast U4/U6 snRNP protein Prp4 affect a late stage of spliceosome assembly. RNA *3*, 197-209.

Bachellerie, J.-P., Michot, B., Nicoloso, M., Balakin, A., Ni, J. and Fournier, M.J. (1995). Antisense snoRNAs: a family of nucleolar RNAs with long complementarities to rRNA. Trends in Biochemical Sciences *20*, 261-264.

Banner, D.W., Kokkinidis, M. and Tsernoglou, D. (1987). Structure of the ColE1 rop protein at 1.7 angstrom resolution. Journal of Molecular Biology *196*, 657-675.

Banroques, J. and Abelson, J.N. (1989). PRP4: a protein of the yeast U4/U6 small nuclear ribonucleoprotein particle. Molecular and Cellular Biology *9*, 3710-3719.

Bordonné, R., Banroques, J., Abelson, J. and Guthrie, C. (1990). Domains of yeast U4 spliceosomal RNA required for PRP4 protein binding, snRNP-snRNP interactions, and pre-mRNA splicing in vivo. Genes and Development *4*, 1185-1196.

Brow, D.A. and Guthrie, C. (1988). Spliceosomal U6 RNA is remarkably conserved from yeast to mammals. Nature *334*, 213-218.

Brow, D.A. and Vidaver, R.M. (1995). An element in human U6 RNA destabilizes the U4/U6 spliceosomal RNA complex. RNA *1*, 122-131.

Brown, J.D. and Beggs, J.D. (1992). Roles of PRP8 protein in the assembly of splicing complexes. Embo Journal *11*, 3721-9.

Burgess, S., Couto, J.R. and Guthrie, C. (1990). A putative ATP-binding protein influences the fidelity of branchpoint recognition in yeast splicing. Cell *60*, 705-717.

Burgess, S.M. and Guthrie, C. (1993). A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates. Cell *73*, 1377-1391.

Cech, T.R. (1986). The generality of self-splicing RNA: relationship to nuclear mRNA splicing. Cell *44*, 207-210.

Cheng, S.-C. and Abelson, J. (1986). Fractionation and characterization of a yeast mRNA splicing extract. Proceedings of the National Academy of Sciences *83*, 2387-2391.

Cheng, S.-C. and Abelson, J. (1987). Spliceosome assembly in yeast. Genes and Development *1*, 1014-1027.

Company, M., Arenas, J. and Abelson, J. (1991). Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. Nature *349*, 487-493.

Cooper, M., Johnston, L.H. and Beggs, J.D. (1995). Identification and characterization of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins. EMBO Journal *14*, 2066-2075.

Corden, J.L. (1993). RNA polymerase II transcription cycles. Current Opinion in Genetics and Development *3*, 213-218.

Dieci, G. and Sentenac, A. (1996). Facilitated recycling pathway for RNA polymerase III. Cell *84*, 245-252.

Eberle, W., Klaus, W., Cesareni, G., Sander, C. and Rosch, P. (1990). Proton nuclear magnetic resonance assignments and secondary structure determination of the ColE1 rop (rom) protein. Biochemistry *29*, 7402-7407.

Eguchi, Y. and Tomizawa, J.-i. (1990). Complex formed by complementary RNA stem-loops and its stabilization by a protein: function of ColE1 Rom protein. Cell *60*, 199-209.

Eguchi, Y. and Tomizawa, J.-i. (1991). Complexes formed by complementary RNA stem-loops: their formations, structures and interaction with ColE1 Rom protein. Journal of Molecular Biology *220*, 831-842.

Elledge, S.J. and Davis, R.W. (1988). A family of versatile centromeric vectors for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene *70*, 303-312.

Ferrandon, D., Koch, I., Westhof, E. and Nüsslein-Volhard, C. (1997). RNA-RNA interaction is required for the formation of specific bicoid mRNA 3' UTR-STAUFEN ribonucleoprotein particles. EMBO Journal *16*, 1751-1758.

Field, D.J. and Friesen, J.D. (1996). Functionally redundant interactions between U2 and U6 spliceosomal snRNAs. Genes & Development *10*, 489-501.

Fortner, D.M., Troy, R.G. and Brow, D.A. (1994). A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. Genes and Development *8*, 221-233.

Fromont-Racine, M., Rain, J.-C. and Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nature Genetics *16*, 277-82.

Galisson, F. and Legrain, P. (1993). The biochemical defects of *prp4-1* and *prp6-1* yeast splicing mutants reveal that the PRP6 protein is required for the accumulation of the [U4/U6.U5] tri-snRNP. Nucleic Acids Research *21*, 1555-1562.

Gavis, E.R. and Lehmann, R. (1992). Localization of nanos RNA controls embryonic polarity. Cell *71*, 301-313.

Gavis, E.R. and Lehmann, R. (1994). Translational regulation of nanos by RNA localization. Nature *369*, 315-318.

Gavis, E.R., Lunsford, L., Bergsten, S.E. and Lehmann, R. (1996). A conserved 90 nucleotide element mediates translational repression of nanos RNA. Development *122*, 2791-2800.

Ghetti, A., Company, M. and Abelson, J. (1995). Specificity of Prp24 binding to RNA: A role for Prp24 in the dynamic interaction of U4 and U6 snRNAs. RNA *1*, 132-145.

Gorbalenya, A.E. and Koonin, E.V. (1993). Helicases: amino acid sequence comparisons and structure-function relationships. Current Biology *3*, 419-429.

Guthrie, C. (1991). Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science *253*, 157-163.

Guthrie, C. and Fink, G.R. (1991). Guide to yeast genetics and molecular biology. (Academic Press, San Diego, 1991).

Guthrie, C. and Patterson, B. (1988). Spliceosomal snRNAs. Annual Review of Genetics *22*, 387-419.

Hawley, D.K. and Roeder, R.G. (1987). Functional steps in transcription initiation and reinitiation from the major late promoter in HeLa nuclear extract. Journal of Biological Chemistry *262*, 3452-3461.

Herschlag, D. (1995). RNA chaperones and the RNA folding problem. Journal of Biological Chemistry *270*, 20871-4.

Hershey, J.W.B. (1991). Translational control in mammalian cells. Annual Review of Biochemistry *60*, 717-755.

Horowitz, D.S. and Abelson, J. (1993). A U5 small nuclear ribonucleoprotein particle protein involved only in the second step of pre-mRNA splicing in *Saccharomyces cerevisiae*. Molecular and Cellular Biology *13*, 2959-2970.

Jandrositz, A. and Guthrie, C. (1995). Evidence for a Prp24 binding site in U6 snRNA and in a putative intermediate in the annealing of U6 and U4 snRNAs. EMBO Journal *14*, 820-832.

Johnston, D.S., Brown, N.H., Gall, J.G. and Jantsch, M. (1992). A conserved double-stranded RNA binding domain. Proceedings of the National Academy of Sciences *89*, 10979-10983.

Johnston, D.S. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. Cell *68*, 201-219.

Kenan, D.J., Query, C.C. and Keene, J.D. (1991). RNA recognition: towards identifying determinants of specificity. TIBS *16*, 214-220.

Kim, S.-H. and Lin, R.-J. (1993). Pre-mRNA splicing within an assembled yeast spliceosome requires an RNA-dependent ATPase and ATP hydrolysis. Proceedings of the National Academy of Sciences *90*, 888-892.

Kim, S.-H. and Lin, R.-J. (1996). Spliceosome Activation by PRP2 ATPase prior to the First Transesterification Reaction of Pre-mRNA Splicing. Molecular and Cellular Biology *16*, 6810-6819.

Kim, S.-H., Smith, J., Claude, A. and Lin, R.-J. (1992). The purified yeast pre-mRNA splicing factor PRP2 is an RNA-dependent NTPase. EMBO Journal *11*, 2319-2326.

Konarska, M.M. and Sharp, P.A. (1987). Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. Cell *49*, 763-774.

Krämer, A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. in Annual Review of Biochemistry. Richardson, C.C., Abelson, J.N. and Raetz, C.R.H., Eds. (Annual Reviews Inc., Palo Alto) pp 367-409.

Lauber, J., Fabrizio, P., Teigelkamp, S., Lane, W.S., Hartmann, E. and Lührmann, R. (1996). The HeLa 200 kDa U5 snRNP-specific protein and its homologue in *Saccharomyces cerevisiae* are members of the DEXH-box protein family of putative RNA helicases. EMBO Journal *15*, 4001-4015.

Lesser, C.F. and Guthrie, C. (1993). Mutations in U6 snRNA that alter splice site specificity: implications for the active site. Science *262*, 1982-1988.
Li, Z. and Brow, D.A. (1993). A rapid assay for quantitative detection of specific RNAs. Nucleic Acids Research *21*, 4645-6.

Lin, J. and Rossi, J.J. (1996). Identification and characterization of yeast mutants that overcome an experimentally introduced block to splicing at the 3' splice site. RNA *2*, 835-848.

Lin, R.-J., Newman, A.J., Cheng, S.-C. and Abelson, J. (1985). Yeast mRNA Splicing *in Vitro*. J. Biol. Chem. *260*, 14780-14792.

Lohman, T.M. (1993). Helicase-catalyzed DNA unwinding. Journal of Biological Chemistry *268*, 2269-2272.

Madhani, H. and Guthrie, C. (1992). A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. Cell *71*, 803-817.

Madhani, H.D., Bordonné, R. and Guthrie, C. (1990). Multiple roles for U6 snRNA in the splicing pathway. Genes and Development *4*, 2264-2277.

Madhani, H.D. and Guthrie, C. (1994a). Dynamic RNA-RNA interactions in the spliceosome. Annual Review of Genetics *28*, 1-26.

Madhani, H.D. and Guthrie, C. (1994b). Genetic interactions between the yeast RNA helicase homolog Prp16 and spliceosomal snRNAs identify candidate ligands for the Prp16 RNA-dependent ATPase. Genetics *137*, 677-687.

Maxwell, E.S. and Fournier, M.J. (1995). The small nucleolar RNAs. in Annual Review of Biochemistry. Richardson, C.C., Abelson, J.N., Meister, A. and Walsh, C.T., Eds. (Annual Reviews, Inc, Palo Alto) pp 897-934.

Moore, M.J., Query, C.C. and Sharp, P.A. (1993). Splicing of precursor to mRNA by the spliceosome. in The RNA World. Gesteland, R.F. and Atkins,

J.F., Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) pp 303-358.

Nilsen, T.W. (1994). RNA-RNA Interactions in the Spliceosome: Unraveling the Ties That Bind. Cell *78*, 1-4.

Noble, S.M. and Guthrie, C. (1996a). Identification of Novel Genes Required for Yeast Pre-mRNA Splicing by Means of Cold-Sensitive Mutations. Genetics *143*, 67-80.

Noble, S.M. and Guthrie, C. (1996b). Transcriptional pulse-chase analysis reveals a role for a novel snRNP-associated protein in the manufacture of spliceosomal snRNPs. Embo Journal *15*, 4368-79.

O'Day, C.L., Dalbadie-McFarland, G. and Abelson, J. (1996). The Saccharomyces cerevisiae Prp5 protein has RNA-dependent ATPase activity with specificity for U2 small nuclear RNA. Journal of Biological Chemistry *271*, 33261-33267.

Padgett, R.A., Konarska, M.M., Grabowski, P.J., Hardy, S.F. and Sharp, P.A. (1984). Lariat RNAs as intermediates and products in the splicing of messenger RNA precursors. Science *225*, 898-903.

Parker, R.G., Siliciano, P.G. and Guthrie, C. (1987). Recognition of the TACTAAC box during mRNA splicing in yeast involves base-pairing to the U2-like snRNA. Cell *49*, 229-239.

Patterson, B. and Guthrie, C. (1991). A U-rich tract enhances usage of an alternative 3' splice site in yeast. Cell *64*, 181-187.

Pause, A. and Sonenberg, N. (1992). Mutational Analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. The EMBO Journal *11*, 2643-2654.

Pavlov, M.Y., Freistroffer, D.V., MacDougall, J., Buckingham, R.H. and Ehrenberg, M. (1997). Fast recycling of Escherichia coli ribosomes requires both ribosome recycling factor (RRF) and release factor RF3. EMBO Journal *16*, 4134-4141.

Pikielny, C.W., Rymond, B.C. and Rosbash, M. (1986). Electrophoresis of ribonucleoproteins reveals an ordered assembly pathway of yeast splicing complexes. Nature *324*, 341-345.

Plumpton, M., McGarvey, M. and Beggs, J.D. (1994). A dominant negative mutation in the conserved RNA helicase motif 'SAT' causes splicing factor Prp2 to stall in spliceosomes. The EMBO Journal *13*, 879-887.

Pontius, B.W. and Berg, P. (1990). Proceedings of the National Academy of Sciences *87*, 8403-8407.

Portman, D.S. and Dreyfuss, G. (1994). RNA annealing activities in HeLa nuclei. The EMBO Journal *13*, 213-221.

Predki, P.F., Nayak, L.M., Gottlieb, M.B.C. and Regan, L. (1995). Dissecting RNA-protein interactions: RNA-RNA recognition by Rop. Cell *80*, 41-50.

Ripmaster, T.L., Vaughn, G.P. and Woolford, J.L. (1992). A putative ATP-dependent RNA helicase involved in *Saccharomyces cerevisiae* ribosome assembly. Proceedings of the National Academy of Sciences *89*, 11131-11135.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. in Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology. Guthrie, C. and Fink, G.R., Eds. (Academic Press, San Diego) pp 281-301.

Rozen, F., Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C. and Sonenberg, N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Molecular and Cellular Biology *10*, 1134-1144.

Ruby, S.W. and Abelson, J. (1988). An early hierarchic role of U1 in spliceosome assembly. Science *242*, 1028-1034.

Ruby, S.W. and Abelson, J. (1991). Pre-mRNA splicing in yeast. Trends in Genetics *7*, 79-85.

Ruby, S.W., Chang, T.-H. and Abelson, J. (1993). Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA. Genes and Development *7*, 1909-1925.

Ruskin, B., Krainer, A.R., Maniatis, T. and Green, M.R. (1984). Excision of an intact intron as a novel lariat structure during pre-mRNA splicing in vitro. Cell *38*, 317-334.

Rymond, B. and Rosbash, M. (1992). Yeast pre-mRNA splicing. in The Molecular and Cellular Biology of the Yeast Saccharomyces. Jones, E.W., Pringle, J.R. and Broach, J.R., Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) pp 143-192.

Rymond, B.C. (1993). Convergent transcripts of the yeast PRP38-SMD1 locus encode two essential splicing factors, including the D1 core polypeptide of small nuclear ribonucleoprotein particles. Proceedings of the National Academy of Sciences *90*, 848-852.

Sachs, A.B. and Davis, R.W. (1990). Translation initiation and ribosomal biogenesis: involvement of a putative rRNA helicase and *RPL46*. Science *247*, 1077-1079.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Second Edition. (Cold Spring Harbor Laboratory Press, Plainview, NY).

Schmid, S.R. and Linder, P. (1992). D-E-A-D protein family of putative RNA helicases. Molecular Microbiology *6*, 283-292.

Schneider, K.R., Smith, R.L. and O'Shea, E.K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. Science *266*, 122-126.

Schwer, B. and Guthrie, C. (1992). A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. EMBO Journal *11*, 5033-5039.

Schwer, B. and Guthrie, C. (1991). PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature *349*, 494-499.

Séraphin, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. EMBO Journal *14*, 2089-2098.

Seraphin, B., Abovich, N. and Rosbash, M. (1991). Genetic depletion indicates a late role for U5 snRNP during in vitro spliceosome assembly. Nucleic Acids Research *19*, 3857-3860.

Séraphin, B., Kretzner, L. and Rosbash, M. (1988). A U1 snRNA-premRNA base-pairing interaction is required early in spliceosome assembly but does not uniquely define the 5' splice site. EMBO Journal *7*, 2533-2538.

Seraphin, B. and Rosbash, M. (1991). The yeast branchpoint sequence is not required for the formation of a stable U1 snRNP pre-mRNA complex and is recognized in the absence of U2 snRNA. EMBO Journal *19*, 1209-1216.

Shannon, K.W. and Guthrie, C. (1991). Suppressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-binding motifs. Genes & Development *5*, 773-785.

Sikorski, R.S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19-27.

Siliciano, P.G. and Guthrie, C. (1988). 5' splice site selection in yeast: genetic alterations in base-pairing with U1 reveal additional requirements. Genes & Development *2*, 1258-1267.

Strauss, E.J. (1992) Prp28: A DEAD-box protein involved in nuclear pre-mRNA splicing (Thesis, University of California, San Francisco).

Strauss, E.J. and Guthrie, C. (1991). A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family. Genes and Development *5*, 629-641.

Strauss, E.J. and Guthrie, C. (1994). PRP28, a 'DEAD-box' protein, is required for the first step of mRNA splicing in vitro. Nucleic Acids Research 22, 3187-3193.

Stukenberg, P.T., Turner, J. and O'Donnell, M. (1994). An explanation for lagging strand replication: polymerase hopping among DNA sliding clamps. Cell *78*, 877-887.

Tarn, W.-Y., Hsu, C.-H., Huang, K.-T., Chen, H.-R., Kao, H.-Y., Lee, K.-R. and Cheng, S.-C. (1994). Functional association of essential splicing factor(s) with PRP19 in a protein complex. The EMBO Journal *13*, 2421-2431.

Tarn, W.-Y., Lee, K.-R. and Cheng, S.-C. (1993a). Yeast precursor mRNA processing protein PRP19 associates with the spliceosome concomitant with or just after dissociation of U4 small nuclear RNA. Proceedings of the National Academy of Sciences, USA *90*, 10821-10825.

Tarn, W.-Y., Lee, K.R. and Cheng, S.-C. (1993b). The yeast PRP19 protein is not tightly associated with small nuclear RNAs, but appears to associate with the spliceosome after binding of U2 to the pre-mRNA and prior to formation of the functional spliceosome. Molecular and Cellular Biology *13*, 1882-1891.

Tyers, M. and Futcher, B. (1993). Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. Molecular and Cellular Biology *13*, 5659-69.

Umen, J.G. and Guthrie, C. (1995). A novel role for a U5 snRNP protein in 3' splice site selection. Genes and Development *9*, 855-68.

Utans, U., Behrens, S.-E., Lührmann, R., Kole, R. and Krämer, A. (1992). A splicing factor that is inactivated during in vivo heat shock is functionally equivalent to the [U4/U6.U5] triple snRNP-specific proteins. Genes and Development *6*, 631-641.

Valcárcel, J., Gaur, R.K., Singh, R. and Green, M.R. (1996). Interaction of U2AF65 RS Region with Pre-mRNA Branch Point and Promotion of Base-Pairing with U2 snRNA. Science *273*, 1706-1709.

Velculescu, V.E., L, L.Z., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett, D.E., Hieter, P., Vogelstein, B. and Kinzler, K.W. (1997). Characterization of the yeast transcriptome. Cell *88*, 243-51.

Vijayraghavan, U., Company, M. and Abelson, J. (1989). Isolation and characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*. Genes and Development *3*, 1206-1216.

Wassarman, D.A. and Steitz, J.A. (1991). Alive with DEAD proteins. Nature *349*, 463-464.

Wassarman, D.A. and Steitz, J.A. (1992). Interactions of small nuclear RNA's with precursor messenger RNA during in vitro splicing. Science *257*, 1918-1925.

Weaver, P.L., Sun, C. and Chang, T.-H. (1997). Dbp3p, a putative RNA helicase in Saccharomyces cerevisiae, is required for efficient pre-rRNA processing predominantly at site A3. Molecular and Cellular Biology *17*, 1354-1365.

Weidenhammer, E.M., Ruiz-Ortega, M., and Woolford, J.L. (1997). Prp31p promotes the association of the U4/U6.U5 tri-snRNP with prespliceosomes to

form spliceosomes in *Saccharomyces cerevisiae.* Molecular and Cellular Biology *17*, 3580-3588.

Wells, S.E., Neville, M., Haynes, M., Wang, J., Igel, H. and Ares, M. (1996). CUS1, a suppressor of cold-sensitive U2 snRNA mutations, is a novel yeast splicing factor homologous to human SAP145. Genes and Development *10*, 220-232.

Wiest, D.K., O'Day, C.L. and Abelson, J. (1996). In vitro studies of the Prp9.Prp11.Prp21 complex indicate a pathway for U2 small nuclear ribonucleoprotein activation. Journal of Biological Chemistry *271*, 33268-33276.

Will, C.L. and Lührmann, R. (1997). Protein functions in pre-mRNA splicing. Current Opinion in Cell Biology *9*, 320-328.

Wolff, T. and Bindereif, A. (1993). Conformational changes of U6 RNA druing the spliceosome cycle: an intramolecular helix is essential both for initiating the U4/U6 interaction and for the first step of splicing. Genes and Development *7*, 1377-1389.

Xu, D., Nouraini, S., Field, D., Tang, S.-J. and Friesen, J.D. (1996). An RNA-dependent ATPase associated with U2/U6 snRNAs in pre-mRNA splicing. Nature *381*, 709-713.

Yean, S.-L. and Lin, R.-J. (1991). U4 small nuclear RNA dissociates from a yeast spliceosome and does not participate in the subsequent splicing reaction. Molecular and Cellular Biology *11*, 5571-5577.

Yean, S.-L. and Lin, R.-J. (1996). Analysis of small nuclear RNAs in a precatalytic spliceosome. Gene Expression *5*, 301-313.

Zavanelli, M.I. and Ares, M. (1991). Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. Genes & Development *5*, 2521-2533.

Zawel, L., Kumar, K.P. and Reinberg, D. (1995). Recycling of the general transcription factors during RNA polymerase II transcription. Genes and Development *9*, 1479-1490.