SPlicing of RNA is the removal of introns from messenger RNA precursors (pre-mRNAs) by two successive trans-esterification reactions. It is a highly dynamic process that is catalyzed by a multi-component complex, the spliceosome. During spliceosome formation and subsequent catalysis, conformational rearrangements of RNA-RNA, protein-protein, and protein-RNA interactions occur (reviewed in Staley and Guthrie 1998). The formation of a prespliceosomal complex is characterized by the ordered assembly of two small nucleoprotein (snRNP) particles, U1 and U2, on the pre-mRNA. U1 snRNA anneals to the intron at the junction with the first exon, the 5’ splice site. Mud2p and Msl5p are thought to direct the addition of the U2 snRNP (Abovich and Rosbash 1997; Fromont-Racine et al. 1997), which results in binding of U2 snRNA to the branchpoint sequence within the intron. Subsequent addition of the U4/U6.U5 tri-snRNP, preassembled from the U5 snRNP and the U4/U6 di-snRNP, is accompanied by rearrangements that lead to formation of the catalytic center needed for the first trans-esterification reaction.

During or just after U4/U6.U5 tri-snRNP recruitment to the prespliceosome the U4/U6 base pairing is disrupted by the U5 snRNP-associated RNA unwindase Brt2p (Slt22p/Snu246p/Rss1p/Prp44p; Lauber et al. 1996; Lin and Ross 1996; Noble and Guthrie 1996; Xu et al. 1996; Kim and Ross 1999) accompanied by ATP hydrolysis (Raghunathan and Guthrie 1998; Kim and Ross 1999). Another U5 snRNP protein, Prp8p, that contacts U6 snRNA in U4/U6.U5 tri-snRNPs (Vidal et al. 1999) is proposed to regulate unwinding of the U4/U6 duplex (Kuhn et al. 1999) and to anchor the first exon in the spliceosome (Newman and Norman 1992; Teigelkamp et al. 1995; Dix et al. 1998). The rearranged U6 snRNA displaces U1 snRNA from the 5’ splice site in an ATP-dependent step facilitated by Prp28p (Staley and Guthrie 1999) and a more 3’ region of U6 snRNA binds to U2 snRNA to form the catalytic center of the spliceosome for the first step of splicing. Prp2p, an RNA-dependent NTPase like Brt2p, is required to activate the spliceosome but dissociates from it after ATP hydrolysis, when the first trans-esterification reaction takes place (Kim and Lin 1996).

Upon completion of the first catalytic step, Prp16p, another RNA-dependent NTPase, joins the spliceosome, interacts with the 3’ splice site (the junction between intron and exon 2), and drives further rearrangements in the spliceosome (Umen and Guthrie 1995b; Wang and Guthrie 1998). Subsequently, Prp8p, Shu7p, Prp17, Prp22p, and Prp18p are required for completion of the second step (Umen and Guthrie 1995b;
SCHWER and GROSS 1998; McPHEETERS et al. 2000). Prp8p and U5 snRNA hold together the ends of the exons that are to be joined (NEWMAN and NORMAN 1992; TEIGELKAMP et al. 1995; UMEM and SCHWER 1995a; DIX et al. 1998), whereas Slu7p, Prp18p, and Prp22p are essential in vitro for the removal of introns with long branchpoint-3′ splice site distances (BRYNS and SCHWER 1996; ZHANG and SCHWER 1997; SCHWER and GROSS 1998). At this stage, U2 snRNA is in close proximity to U5 snRNA and the 3′ splice site (NEWMAN et al. 1995; XU et al. 1998). How the catalytic center for the second trans-esterification reaction is formed is under debate, but the activity of another U5 snRN protein, Snu114p, may be needed to locate the 3′ splice site (FABRIZIO et al. 1997; STALEY and GUTHRIE 1998) so that both exons can be precisely aligned by U5 snRNA (O’KEEFE and NEWMAN 1998).

After the second splicing reaction, ATP hydrolysis by Prp22p releases the mature mRNA from the spliceosome, the spliceosome dissociates, and the components are recycled. The DEAD-box protein Prp22p is structurally related to the DExH/DEAD-box proteins Prp2p, Prp16p, Prp28p, and Brr2p. RNA unwindase activity has been demonstrated in vivo and in vitro for Br2p (RAGHUNATHAN and GUTHRIE 1998; KIM and ROSSI 1999) and in vitro for Prp16p (WANG et al. 1998) and Prp22p (SCHWER and GROSS 1998; WAGNER et al. 1998).

All these proteins share a central region containing conserved sequence elements implicated in ATP binding and hydrolysis and in RNA binding. Intragenic suppressors of conditional mutations in Prp28p suggest a close proximity between some of these elements (CHANG et al. 1997). Brr2p contains a second, more C-terminal helicase-like domain with less conserved elements (LAUBER et al. 1996; XU et al. 1996). This domain tolerates mutations that in the upstream helicase domain confer a dominant negative phenotype (KIM and ROSSI 1999), possibly indicating a different function.

To fully understand the interactions that take place within the spliceosome all the components have to be identified and their interactions mapped. Recent purification of subcomplexes and microsequencing of the component proteins increased the number of known yeast splicing factors over those previously identified by genetics and sequence homology (e.g., GOTTSCHALK et al. 1999; STEVENS and ABELOS 1999). Furthermore, six new splicing factors were identified in 16 exhaustive two-hybrid screens (FROMONT-RACINE et al. 1997; DIX et al. 1999). Although two-hybrid screens might not reveal all possible functional interactors (VIDAL and LEGRAIN 1999), they provide information about putative interacting domains whose function can be analyzed further by other approaches. Furthermore, the sensitivity of this technique allows detection of short-lived interactions that can be expected within the dynamic context of a rearranging spliceosome. We therefore undertook exhaustive two-hybrid screening according to FROMONT-RACINE et al. (1997), using their complex yeast genomic library (FRYL). As baits we used the U5 snRN proteins Prp8p and Brr2p, the second-step factors Prp18p and Slu7p, and Snu66p, a protein found in Brr2p screens. Snu66p was recently identified as a component of the U4/U5.U6 tri-snRN (GOTTSCHALK et al. 1999; STEVENS and ABELOS 1999) and, as we show below, is essential for splicing in vivo at low temperatures. We observe that Brr2p specifically interacts with Prp2p, Prp8p, Prp16p, Snp1p, Slu7p, and Snu66p. The two-hybrid interactions between Prp16p, Prp8p, Snu66p, Snp1p, and Brr2p were confirmed by co-immunoprecipitation in vitro and we conclude that Prp16p recruitment to the spliceosome (WANG and GUTHRIE 1998) is mediated by Brr2p.

We present in vivo evidence for the hypothesis that the prp8-1 allele causes a splicing defect by disrupting the interaction with Brr2p, an interaction that in human cells was found to be independent of U5 snRNA (ACHSEL et al. 1998). The broad range of Brr2p interactions confirms its central role in spliceosomes and suggests a particular function for the C-terminal half, including the second helicase-like domain. This brings forward the hypothesis that, apart from U4/U6 duplex unwinding, Brr2p catalyzes other RNA rearrangements, acting in a synergistic manner with other spliceosomal proteins, including the structurally related proteins Prp2p and Prp16p. Such a cooperation of unwindases, although not previously reported to occur in pre-mRNA splicing, is a common feature of helicases that are part of viral, bacterial, or eukaryotic DNA replication machines. Overall, our combination of exhaustive, iterative two-hybrid screening with functional analyses provides novel information concerning the dynamic control of the order of events in spliceosomes.

**MATERIALS AND METHODS**

**General procedures:** Preparation of splicing extracts, immunoprecipitation of proteins and snRNAs, Western detection, isolation of RNA, and hybridization of Northern blots with snRNA probes were as described previously (TEIGELKAMP et al. 1995; DIX et al. 1999; MAYES et al. 1999). Antibodies were from Santa Cruz Biotechnology, Santa Cruz, California [polyclonal rabbit anti-α-haemagglutinin (anti-HA); polyclonal rabbit anti-c-MYC), Boehringer Mannheim (Indianapolis; 3F10 anti-HA), kind gifts of J. Lewis and D. Xu (12CA5 monoclonal mouse anti-HA), or described previously (anti-Prp8.6 in TEIGELKAMP et al. 1995). For identification of yeast DNA sequences by BLAST search or design of primers the Saccharomyces Genomic Database (http://genome-www.stanford.edu/Saccharomyces/) was used. Homologous proteins were aligned using algorithms Clustal W, Pima, or Mapmaker.

Oligonucleotides sequences are 5′ to 3′: 5′ ACT-PCR (GGCGTTTGGATCTACGAGGAGTG), 3′ ACT-PCR (GAAATTGAGATATGGTGCACGATGCAC), TNT-ACT (AAACUGCAGTATACGAGTCACTATAGGGAGGCACATGCTGCTCTGGTCGTGTC).

**Two-hybrid screens and direct analysis of two-hybrid interactions:** The two-hybrid bait vectors were pASAD (Gal4 DNA-binding domain), pBTM116 (LexA DNA-binding domain),
or their derivatives with +1 or −1 frameshifts in the multiple cloning site. Prey constructs were made in pACTII-stop (Gal4 activation domain; Fromont-Racine et al. 1997) or in a +1 derivative. The open reading frames (ORFs) for Slu7p, Prp18p, Prp16p, Prp22p, and Prp2p were cloned directly. The Snu66p baits were made by recombination of prey Snu2 and Snu5 (Table 1). Full-length PRP8 and BRR2 baits and prey, as well as slu22-1 and prp16-1 baits, were constructed by gap repair in yeast. JDY4, a Sc266 derivative (MATα, ade-2-101; his3Δ200, leu2Δ1, trypΔ94, ura3Δ99, ααΔ; a kind gift of J. D. Brown). Linearized plasmids containing both the N and C terminus of each open reading frame were cotransformed with DNA fragments bridging the gap. Successful gap repair of prp16-1 bait plasmid was scored by the growth defect at 16°C for PRP16/prp16-1 strains. Full-length bait and prey fusions of Prp8p complemented prpΔ strains. Stable expression of prey peptides was verified by Western blotting using 12CA5 anti-HA antibodies. Two-hybrid screens were done by mating (Fromont-Racine et al. 1997) or by sequential transformation (Parchaliuk et al. 1999) using the FYR1 genomic library (Fromont-Racine et al. 1997). Direct two-hybrid analyses were done with either mated diploids (in the Gal4-system; Maves et al. 1999) or cotransformed haploid cells (in the LexA-system), which were tested by β-galactosidase assays and on media selecting for expression of the HIS3 reporter gene. The plates contained different concentrations of the HIS3 inhibitor triaminotriazolone to evaluate the strength of two-hybrid interactions.

Isolation and in vivo analysis of ePrp8p mutations that affect two-hybrid interaction with Br2p: A pool of PCR fragments, generated under mutagenic conditions with primers 5′-PCR and 3′-ACT-PCR on prey plasmid E3 DNA (Figure 3A), was recombined into gapped pACTII-stop in yeast L40 cells expressing the LexAbrr2p bait. After 1-hr recovery in dropout medium free of tryptophan and leucine, the transformation mixture was plated on histidine-free medium and incubated at 25°C. Dot-sized colonies were replica-plated onto histidine-free medium with different triaminotriazolone (3AT) concentrations (incubated at 30°C) or without 3AT (and incubated at 16°C, 25°C, 30°C, and 36°C). Comparison of plates yielded candidates for temperature-sensitive, 3AT-sensitive, or 3AT-resistant Br2p interactions. Rescued prey plasmids were verified to cause the growth phenotype and their ePrp8p inserts were sequenced. Fragments with mutations responsible for the phenotype were identified after recloning into gapped E3-plasmid. Two mutations, prp8-29 (clone E3-N) and prp8-32 (clone E3-H), showed the strongest temperature sensitivity and 3AT resistance, respectively. These mutations as well as allele prp8-1 were introduced into PRRP8 on either pRS315 (ARS/CEN, LEU2, a gift from D. Xu) or pJJ225 (2µ; TRP1; UME6 and Guthrie 1996). The alleles were tested for their ability to replace the pRS316 (ARS/CEN, URA3)/PRP8 maintenance plasmid from strains YDX216 and YDX225, derivatives of yeast W305 (ade-2, his3-11, leu2-3, -112, trp1-1, ura3-1, can1-100), with a disrupted PRP8 locus (prp8Δ::HIS3; kindly provided by D. Xu). YDX225 differs from YDX216 in mating type (MATα) and the slt22-1 allele (Xu et al. 1996). The prp8-1, −28, and −52 plasmids were also tested in yeast JYU75 with a prp8Δ::L52S disruption (Umen and Guthrie 1996). The maintenance plasmid was lost by streaking transformants on solid 5-fluoroorotic acid medium, also selective for the genomic PRP8 gene disruption as well as the vector with the tested prp8 allele. Yeast colonies that grew at 25–30°C were streaked on uracil-containing medium and incubated at different temperatures.

Epitope tagging of genes: Three different haploid strains were made with BRR2 placed under control of the conditional MET3 promoter that stimulates strong gene expression but is tightly repressed by methionine (Mountain et al. 1991). By targeted integration of an HIS3P-MYC3 cassette (with flanking sequences homologous to the 5′ untranslated region and the extreme N terminus of BRR2) into JDY5 (as JDY4, see above, but MATα), a strain that produced cMYC-tagged Br2p was obtained. The second strain, a JDY4 derivative, had a PACT-controlled, HA-tagged BRR2 gene separated from a truncated BRR2 gene (with the native promoter) by pUC18 DNA and the TRP1 gene (see Figure 2B). In the third strain Br2p was placed under PACT control without a tag. In an analogous way JDY4 derivatives were obtained in which SNU66, YMR102C, or ECM5 were N-terminally tagged with a double HA-epitope.

Immunoﬂuorescence: Yeast cells grown to OD600 ~ 0.5 were fixed with 1/10 volume 37% formaldehyde for 15–30 min, collected, washed with 0.1 M KPO4, pH 7.5/0.7 M sorbitol, and resuspended in 1 ml 250 µg/ml zymolyase 20,000 for 20 min at 30°C to digest the cell walls. Cells were collected and resuspended in PBS/0.7 M sorbitol. Fixed cells were immobilized on microscope slides, blocked with 4% milk/0.1% Tween/PBS, and stained overnight at 4°C with 3F10 anti-HA antibodies (diluted 1:200) in a humid chamber. After three washes with PBS, cells were incubated with FITC-conjugated Alexa 488 secondary antibody (Molecular Probes, Eugene, OR) for 1 hr at 20°C in the dark and examined on an Axiosplan2 (Zeiss) microscope attached with CCD camera (Hamamatsu, Bridgewater, NJ).

Gene disruptions and growth analysis: In yeast strain JDY6 (diploid of JDY4 and JDY5, see above) open reading frames YMR102C, YOR308C, YNL099C, and YPL064C were entirely substituted by the HIS3 gene. Correct integration was verified by PCR analysis. Dissected tetrads from diploids with disrupted genes were analyzed at 14°C, 16°C, 25°C, 30°C, and 37°C on plates with rich medium (YPD) or complete synthetic medium lacking histidine and testing glucose, galactose, or lactate as sole carbon source. Growth of SNU66 and snu66Δ::HIS3 haploids (derived from the same tetrad) was monitored over time in liquid cultures. Cells collected from a preculture incubated overnight at 24°C were divided in two, diluted with precooled or prewarmed liquid medium, and further incubated at either 16°C or 30°C. At particular time points samples of 50 OD units of cells were taken and stored at –80°C before RNA was isolated from the cell pellets. Synthetic lethality between snu66Δ::HIS3 and slt22-1 was tested using the strain YDX22100 (MATα, ade-2-1, his3-11, leu2-3-112, trp1-1, ura3-1, can1-100, slt22-1, kindly provided by D. Xu) that was mated to haploid snu66Δ::HIS3. Dissected spores were allowed to germinate at 25–30°C prior to testing for temperature sensitivity at 30°C and cold sensitivity at 14°C.

Analysis of splicing efficiency by means of an in vivo reporter assay: Wild-type and gene-disrupted haploid transformants with either reporter plasmid pJC51 (Rain and Legrain 1997) or pLACZ (Dix et al. 1999) were grown in triplicate to mid-log phase (OD600 between 0.5 and 1) in noninducing liquid medium at 20°C or 30°C. Two cultures were then induced with galactose and one was repressed with glucose before incubation was continued for 3 hr (at 20°C) or for ½ hr (at 30°C). β-Galactosidase activity in each culture was assayed in triplicate according to Rain and Legrain (1997).

Immunoprecipitation of in vitro-synthesized peptides: 35S-labeled peptides were produced in vitro using Promega’s (Madison, WI) T7- system and [35S]methionine (Amersham, Buckinghamshire, UK) with transcription template PCR products amplified from two-hybrid prey plasmids using primers 5′-ACT-PCR and TNT-ACT. Aliquots of cMYC-tagged BRR2, splicing extract were depleted of ATP (and thus splicing activity) by endogenous hexokinase at 24°C for 20 min after adding glucose and mixed with 35S-labeled peptides and polyclonal anti-
### Details of prey fragments isolated in two-hybrid screens and of other constructs used in this work

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<td>131–733</td>
</tr>
<tr>
<td></td>
<td>Tn1</td>
<td>Prey</td>
<td>5–437</td>
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Like Fromont-Racine et al. (1997) we obtained out-of-frame prey fusions (indicated by underlining) for which readthrough fusion products were detected on Western blots (data not shown). Furthermore, an out-of-frame fusion of a Snu66p fragment (clone Su6) was functional in a complementation assay. The Snu66p residues (aa 272–447) that are required for strong Brr2p interaction (resistant to 10–20 mM 3AT; data not shown) or essential (but not necessarily sufficient) for full complementation of snu66Δ at 14°C (aa 448–458; data not shown), lie in between the N- and C-terminal regions most highly conserved between Snu66p homologs from yeast (YOR308C), human (NCBI protein accession no. BAA24056.1), worm (NCBI protein accession no. AAB52287.1), or fission yeast (GenBank accession no. SPAC167.03C).

cMYC antibodies in conditions of 150 mM NaCl. After a half-hour incubation on ice protein, A-Sepharose (Sigma, St. Louis) was added and the mixtures were rotated at 4°C for 2 hr. The beads were washed three times with IP buffer containing 150 mM NaCl, after which the attached proteins were eluted with loading buffer at 60°C. Eluted proteins and supernates (50%) were separated on 10% SDS polyacrylamide and visualized by fluorography on Kodak Biomax MR film after fixing the gel in 10% glacial acetic acid/30% methanol.

RESULTS

Two-hybrid screens: The characteristics of our exhaustive two-hybrid screens and of the proteins found as prey are presented in Figure 1 (classified according to A1, A2/A3, A4, and B prey categories; FROMONT-RACINE et al. 1997). The parameters of functionally interesting prey fusions are listed in Table 1. The discussion focuses predominantly on interactions between splicing factors and the validation of these interactions by other approaches; however, the A1, A2, and A3 category prey are considered to be potentially interesting and the analysis of these continues.

We started with Prp18p and Slu7p as bait fusions. The Prp18p screen was highly selective, the main prey (of the A1 category) being Slu7p. Ten different Slu7p fragments (54 clones in total) share a region of overlap (residues 170–249) that was sufficient for the interaction (M. Albers, R. W. van Nues and J. D. Beggs, unpublished results; also implicated by data reported by Zhang and Schwer 1997). As bait, Slu7p fished the C-terminal regions of Prp18p (clone Et2) and Brr2p (clone B1), as well as the DEAH-box region of Prp22p (see Table 1 for details). As Prp18p and Slu7p are known to interact genetically (see references in Umen and Guthrie 1995b) and physically (Zhang and Schwer 1997), the specificity of these screens gave us a high level of confidence in the quality of the genomic library and the effectiveness of the technique (FROMONT-RACINE et al. 1997).

Screens with the full-length U5 snRNP protein Brr2p were also highly informative. Brr2p retrieved the C terminal of another U5 snRNP protein, Prp8p, in three different fusions (prey Et3, Et4*, and Et5*), both termini of Prp16p (St1–3 and St5–10), the middle of Snu66p (Su1–Su10), and almost complete Snl1p (Sp1 and Sp2; see Table 1 for details of all these prey fusions). Furthermore, there are some statistically significant interactions of Brr2p with proteins not directly implicated in splicing, such as the finding of a cyclophilin of unusual length, Ypl064p (Table 1). Mammalian cyclophilins have been described that co-localize with splicing factors (Mortillaro and Berezney 1998) and tri-snRNPs (discussed in Gottschalk et al. 1999; Stevens and Abelson 1999) but no yeast cyclophilins were reported to be stably associated with the yeast U4/U6/U5 tri-snRNP (Gottschalk et al. 1999; Stevens and Abelson 1999). Similarly, Brr2p interacted with WD40 fragments of the uncharacterized Ymr102p, which might conceivably be the yeast orthologue of the 40-kD human U5 snRNP protein (Achsel et al. 1998), although this was not found in purified yeast tri-snRNPs (Gottschalk et al. 1999; Stevens and Abelson 1999). Selection of the N terminus of Fab1p as prey of Brr2p remains to be evaluated.
A

Figure 1.—(A) Results of two-hybrid screens with full-length or truncated U5 snRNP-associated splicing proteins as bait. (Top) Distribution profiles of prey proteins, which are classified according to A1, A2/A3, A4, and B categories (Fromont-Racine et al. 1997) and partitioned with respect to the number of clones from the same genomic locus. Multiple fragments overlapping a protein interaction domain constitute prey of category A1 and indicate a significant two-hybrid interaction. Interaction domains that start within 50 amino acids of the N terminus of the protein (category A2) or that require >333 amino acids to be present in the prey fusion (category A3) will be underrepresented and therefore are statistically relevant even if found only as a single clone. Other coding prey fusions fall within category A4, whereas category B consists of noncoding intergenic, or antisense, fusions or of prey derived from mitochondrial or ribosomal DNA (see inset). Known splicing factors (in ovals), previously described loci (in italics; Fromont-Racine et al. 1997; Dix et al. 1999), or open reading frames with statistically significant occurrence are printed alongside each column. Weakly interacting prey fusions are within parentheses. Total number of prey clones analyzed is indicated above each column. Note the threefold larger scale for the Brr2p column. Zigzags in the Prp8p-B class signify that 40% of cut segments are shown. (Bottom) Characteristics of the two-hybrid screens. Total number of potential interactions tested with each bait (i.e., cells with both bait and prey plasmid) is shown under the columns, which are divided according to the different experiments done (see inset and MATERIALS AND METHODS). The arrow indicates the practical threshold of 45 million potential interactions tested to reach exhaustive coverage of the FRYL yeast genomic library (Fromont-Racine et al. 1997, 2000). (B) Overlap with previously described two-hybrid screens using splicing factors as bait (Fromont-Racine et al. 1997; Dix et al. 1998, 1999).
but, provocatively, a Brr2p fragment was retrieved in a Fab1p screen (R. H. Michell, R. W. van Nues, J. D. Beggs and S. K. Dove, unpublished results; Table 1), and the finding that PIP5-kinases like Fab1p are not only vacuolar but also co-localize with splicing factors in nuclear speckles (Boronenkov et al. 1998) suggests this may be a functional protein interaction. In a partial screen with a truncated Brr2p lacking the first helicase domain (clone Brr2ΔM; Table 1), the most abundant A1 prey were Dbp9p, Fab1p, and Pet127 (data not shown), while Prp16p was found once (clone S4; Table 1).

Full-length Prp8p as bait did not produce such obviously specific interactions (although the bait fusion protein proved to be functional by complementation of prp8Δ strains; data not shown). The only known splicing factor to be found as prey was the U1 snRNP protein Prp39p, of which one A2/A3 clone with the N-terminal tetratricopeptide repeat (TPR) region was found twice. As Prp8p is very large (280 kD), we also performed screens with several Prp8p fragments that have been successfully used to raise antibodies (Jackson et al. 1988) or that have been implicated in regulation of 3′ splice site fidelity (“mPrp8p,” amino acids 1649–2115; Figure 3A; Umen and Guthrie 1995a). These had mixed success as baits. The N-terminal region of Prp8p, encompassing proline-rich repeats and a basic segment (“nPrp8p,” residues 1–263; Figure 3A), fished a C-terminal fragment of Brr2p (clone B1, Table 1). The nPrp8 region also turned out to form the main contact with the fragment of Prp39p isolated in the full-length Prp8p screen (data not shown). This seems to be a specific interaction, as nPrp8p did not associate with the TPR-containing Prp6p fragment found by Snu66p, and the Prp39p prey did not interact with Snu66p (data not shown). Intriguingly, in every screen with a Prp8p fragment as bait we retrieved fragments with the middle region of the uncharacterized protein Ydr213p. Kuhn and Brow (2000) also performed two-hybrid screens with fragments of Prp8p, one of which corresponded to an N-terminal region. None of the prey identified in our screens with Prp8p baits coincided with theirs, but they did not find two-hybrid interactions with any known splicing factors.

A screen with Snu66p, isolated as one of the main prey with Brr2p, yielded the C terminus of Brr2p (clones B1 and B3; Table 1), a large fragment of Prp6p with TPR repeats (clone S1), as well as Ynl099p and Yli101p. Ynl099p is a nonessential protein (our data, see below, and Saiz et al. 1999) with a phosphatase-like domain, present in the prey fragments, that is supposed to interact with phosphoserine, -threonine, or -tyrosine residues (Wishart and Dixon 1998). Ynr053p was found as an A2/A3 category prey in screens with Snu66p, Brr2p, and Prp8p and has been found by others in screens with five other splicing factors (Prp9p, Prp11p, Prp21p, Smb1p, and Lsm8p; Fromont-Racine et al. 1997). Ynr053p is similar to a human breast tumor-associated auto-antigen. Interestingly, the human homologue of SNU66, hSART1 (NCBI protein accession no. BAA24056.1), has also been isolated as a tumor antigen (Kikuchi et al. 1999), whereas the Snu66p-interacting fragments of Yli101p show high homology with human proteins hY22 and hOS4, the genes of which are deleted or amplified in carcinoma cell lines (Ishikawa et al. 1997; Su et al. 1997). In all these experiments we screened the complete FRY1 yeast genomic library (Fromont-Racine et al. 1997; see Figure 1). Prp8p and Brr2p screens with different two-hybrid systems (Gal4 vs. LexA) or different methods (mating the yeast strains that carry bait or prey plasmids vs. sequential transformation of the library and bait plasmids) yielded comparable results that were characteristic for each bait protein. Figure 1B presents a schematic overview of interactions detected between known splicing factors. This highlights the central position of Brr2p as both bait and prey in the network of interactions and shows the overlap with results of two-hybrid screens described previously (Fromont-Racine et al. 1997; Dix et al. 1998, 1999).

In subsequent experiments we sought evidence for the functional significance of these two-hybrid interactions through genetic analyses, localization studies, and co-immunoprecipitation experiments.

Genetic analysis of uncharacterized open reading frames: We further analyzed five open reading frames that were uncharacterized at the time. YOR308C (SNU66), YMR102C, and ECM5 were epoite tagged by adding a double HA epitope to the N termini (materials and methods) and the YOR308C, YPL064C, YMR102C, and YNL099C ORFs were disrupted by replacing the entire open reading frame with HIS3 (materials and methods). Growth and localization studies allowed elucidation of gene function only in the case of YOR308C (SNU66; the second most abundant open reading frame found in our Brr2p screens). None of the other disrupted genes was essential for growth under the various conditions tested (materials and methods). However, although haploid snu66Δ::HIS3 cells showed no apparent growth defect at 30°, they did not grow at 14° (Figure 2A) and grew only very slowly at 16°. The growth defect at 14° was specific for the disruption of SNU66 as demonstrated by complementation of the defect by full-length Snu66p or some of the Snu66 fragments fished in Brr2p screens (data not shown). Deletion of SNU66 is not synthetic lethal with the BRR2 allele slt22-1.

Nuclear localization of Brr2p and Snu66p: To evaluate the role of YOR308C we tested the cellular localization of the gene product using strains in which the BRR2 or SNU66 gene was N terminally tagged with a double HA epitope (Figure 2B). By addition of methionine to the medium the MET3 promoter that controlled expression of tagged SNU66 (Pmet3:HA2:SNU66) was effectively repressed as observed by the strong decrease of HA-specific Western signals, although cell growth was not
Figure 2.—Snu66p localizes to the nucleus, whereas disruption of SNU66 causes a cold-sensitive growth and splicing phenotype. (A) Tetrad dissection of sporulated snu66D::HIS3/ snu66D::HIS3 (tetrad 1) or SNU66/snu66D::HIS3 (tetrads 2 and 3) diploid cells. Haploid snu66Δ cells are not viable at 14°C and at 16°C have twice the doubling time of wild-type cells (data not shown). (B) The chromosomal SNU66 and BRR2 genes were put under control of the conditional MET3 promoter by insertion of a TRP1-PMET3 cassette that, in the case of N-terminally tagged proteins, also included a double haemagglutinin (HA2) sequence. (C) As shown by indirect immunofluorescence, tagged Brr2p and tagged Snu66p display nuclear localization, as for DNA stained with DAPI. Cells containing untagged protein or with the MET3 promoter repressed by addition of 0.5 mM methionine to the growth medium did not stain with anti-HA antibodies. (D) Northern analysis of total RNA isolated from wild-type or snu66Δ cultures incubated at 30°C or 16°C. Samples were taken at the indicated time points after the temperature shift from 24°C (see materials and methods). Pre-U3 snoRNA accumulated in the snu66Δ strain at 16°C and was even detectable at 30°C. In contrast, the levels of U1 snRNA (as well as U2, U4, U5, and U6, not shown) did not increase at 30°C, although at 16°C some accumulation was observed.

affected (see also Figure 2C). Under these conditions PMET3;HA2:BRR2 cells stopped growing, since BRR2 is an essential gene (Lauber et al. 1996; Lin and Rossi 1996; Noble and Guthrie 1996; Xu et al. 1996).

By indirect immunofluorescence we found that, as expected for a pre-mRNA splicing factor, tagged Brr2p localized in the nucleus (Figure 2C): the 4′,6-diamidino-2-phenylindole (DAPI) staining of DNA merged with the immunostaining obtained with anti-HA primary antibodies. HA2-Snu66p displayed clear nuclear accumulation in a protein-specific manner, as upon repression of the MET3 promoter only background signals were observed (Figure 2C). The localization of Snu66p was analyzed more precisely by immunoprecipitation experiments with anti-Prp8p and anti-HA antibodies. HA2-Snu66p was present in Prp8p-containing complexes and Northern analysis of RNAs isolated from anti-HA antibody precipitates showed that U5, U4, and U6 snRNAs associated specifically with tagged Snu66p under non-splicing conditions, whereas U1 and U2 snRNAs and U3 snoRNA did not (data not shown, but see Gottschalk et al. 1999).

Snu66p is a splicing factor required for efficient splicing: Snu66p was previously shown to copurify with U4/
ura only on media with 5-fluoroorotic acid, which permits growth of two-hybrid interaction in both BrR2p and sLT22-1p baits (data transformed with plasmids carrying prp8 genomic locus and wild-type PRP8 Prp8 the Gal4 two-hybrid system. (B) mutation was not synthetic lethal with sLT22-1 caused synthetic lethality in sLT22-1 (mutant by a plasmid shuffle experiment. Otherwise isogenic shown) but it prevented the growth of prp8-28 (clone E3-D) or the in vivo prp8-1 (clone E3-H (prp8-52)). Furthermore, contrary to wild type (clone E3), the prp8-28 (clone E3-N) or the E3-H prp8-52) interaction between BrR2p bait and the Prp8p fragments is indicated in terms of growth on plates containing different levels of triaminotriazole (mM 3AT); i.e., >20 mM (+++ + + + +), 20 mM (++ + + + + +), 10 mM (+ + + +), 5–10 mM [++ (+ +)], 5 mM (++), 2 mM (+ +), and 0 mM 3AT (±); ng means no growth on medium lacking histidine (in absence of 3AT). Mutants marked with a gray arrow were used in subsequent experiments. In the LexA two-hybrid system full-length BrR2p interacted strongly with the last 400 amino acids of Prp8p. This interaction is reduced progressively upon extension of the interacting segment into regions upstream of residue 1200. Full-length Prp8p did not bind to BrR2p in two-hybrid assays. Furthermore, contrary to wild type (clone E3), the prp8-1 allele (clone E3-D) or the prp8-28 (clone E3-N) mutation generated by PCR mutagenesis resulted in a BrR2p interaction that was weakened at 30°C and abolished at 37°C (temperature sensitive). At 37°C wild-type Prp8p fragments but not the E3-H mutant (prp8-52) lost the interaction with sLT22-1p (mutant BrR2p) in the Gal4 two-hybrid system. (B) Prp8p mutations were analyzed in vivo by a plasmid shuffle experiment. Otherwise isogenic sLT22-1 (mutant brR2) or BrR2 strains with a disrupted PRP8 genomic locus and wild-type PRP8 on a URA3 plasmid were transformed with plasmids carrying prp8 alleles. Incubation on media with 5-fluoroorotic acid, which permits growth on only ura - (plasmid-free) cells, showed that the prp8-1 allele caused synthetic lethality in sLT22-1 strains (right). The prp8-28 mutation was not synthetic lethal with sLT22-1 (data not shown) but it prevented the growth of BrR2 strains at 36°C (left). Furthermore, the PCR-generated prp8-52 mutation did not show a phenotype in vivo nor did it suppress the temperature sensitivity of sLT22-1 strains despite its strengthening of two-hybrid interaction in both BrR2p and sLT22-1p baits (data not shown). For all alleles tested the plasmid shuffle was complete, because afterward the strains needed uracil in the medium for growth (data not shown).
(clone E3Δ) greatly reduced the affinity for Brr2p (Figure 3A) but did not affect the stability or expression of the prey peptides as checked by Western analysis (data not shown). This suggests that the Brr2p interaction with cPrp8p is weakened by the presence of Prp8p regions implicated in 3′ splice site recognition (UMEN and GUTHRIE 1996), crosslinking to the 5′ splice site (REVES et al. 1999), U4c5 suppression (KUHN et al. 1999), and U2 synthetic lethality (XU et al. 1998; D. Xu, personal communication)—upstream of residue 2010.

We searched by reverse two-hybrid analysis (VIDAL and LEGRAIN 1999) for cPRP8 point mutations that affect interaction with Brr2p in growth assays at different temperatures. The strength of the interaction was measured as the level of resistance to 3AT and was confirmed by measuring the β-galactosidase activities resulting from expression of the LACZ reporter gene. As a candidate for such a mutation that might affect the interaction, the prp8-1 allele was tested directly. Interestingly, Brr2p interaction was severely affected by this mutation at 30° and abolished at 36° (clone E3-D; Figure 3A). A screen for interaction mutants yielded five cPrp8p clones with reduced and temperature-sensitive interaction with Brr2p, two mutants with reduced interaction, and five mutants that interacted more strongly. The prp8-28 temperature-sensitive mutation that changed the semiconserved isoleucine-2259 to asparagine was located near the prp8-1 allele (G2347D; HODGES et al. 1995) but had a less detrimental effect on Brr2p interaction. Comparison of clones E5* and E4* suggests that Prp8p amino acids 2033–2067 are not essential for, but enhance the strength of, two-hybrid interaction with Brr2p. We isolated mutations (e.g., prp8-52 in clone E3-H) in this region that enhance the affinity for Brr2p. The prp8-52 allele also suppressed the heat-sensitive growth defect caused by the slt22-1 mutation (in the first helicase region of Brr2p) at 37°, indicating that the prp8-52 mutation affects a functionally important interaction with Brr2p. The prp8-52 allele contains two amino acid changes (Y2037H and I2051T) of which the tyrosine to histidine substitution appears to be the most important because it was found in another clone that displayed the same phenotype but also carried a second mutation (K2018E).

Full-length prp8 genes carrying these mutations were constructed for further analysis in vivo. BRR2 and slt22-1 strains that contain a chromosomal prp8Δ plus a plasmid-borne prp8-1, prp8-28, or prp8-52 allele were produced by a plasmid-shuffle system (kindly provided by D. Xu; MATERIALS AND METHODS). In contrast to the prp8-52 allele that behaved like wild-type PRP8, the prp8-28 allele did not support growth at elevated temperatures in BRR2 cells. At 36° these cells grew very slowly (Figure 3B, left) and at 37° they were dead. The prp8-1 allele had a more severe effect; it caused a mild growth defect in BRR2 cells at 30°, whereas the prp8-1, slt22-1 double mutant was not viable at this temperature (Figure 3B, right). The prp8-28 and prp8-52 alleles did not cause such synthetic lethality. Thus, mutations of Prp8p that affect its interaction with Brr2p caused growth defects comparable to the reduced affinity for Brr2p.

Co-immunoprecipitation experiments validate two-hybrid interactions of Brr2p with Prp16p, Snplp, and Snu66p: As an independent means of testing the Brr2p interactions detected in the two-hybrid screens, and to check the stability and strength of these interactions, we did co-immunoprecipitation experiments using in vitro 35S-labeled prey peptides and yeast extract containing triple cMYC-tagged Brr2p. We also tested the effects of the prp8-1, prp8-28, and prp8-52 mutations. As shown in Figure 4A, the wild-type Prp8 peptide (E3) and the one containing the prp8-52 mutation (E3-H) precipitated with cMYC-tagged Brr2p, confirming that Brr2p and Prp8p associate, directly or indirectly. In contrast, none of the mutant peptides E3-D, E3-N, and E3Δ that had displayed reduced two-hybrid interaction with Brr2p was detectably co-immunoprecipitated with Brr2p. These results also demonstrate the specificity of the cMYC antibodies (e.g., that they do not stick to the peptide derived from pACTII sequences that were amplified with each DNA template used for the in vitro transcription/translation reaction; see MATERIALS AND METHODS). Thus, the two-hybrid interactions accurately indicate the ability of these two splicing factors to associate (directly or indirectly).

Similarly, we observed specific coprecipitation of Snplp (Sp1), Snu66p (Su2), and Nprp16p (St1, St2), cPrp16p (St10) fragments (Figure 4, B and C) that strongly supports the two-hybrid results. In contrast, the weakly interacting, but frequently found, Ypl064p prey fusions did not coprecipitate with cMYC-Brr2p under our stringent test conditions (Figure 4B). Interestingly, ~20-fold more of the 35S-labeled cPrp8 protein precipitated with cMYC-Brr2p (fragment E3; Figure 4A) than did nSnu66p (fragment Su2; Figure 4B; note that both prey peptides contain nine methionines). Also in the two-hybrid assay we found that the interaction of Brr2p with cPrp8p appears to be stronger than with Snup66p. When abundance of prey peptides was a limiting factor for interaction (e.g., in the case of prey fusions requiring a frameshift for complete expression), the interaction of Snu66p (clones Su1* or Su6*; Table 1) with Brr2p was markedly down (i.e., lost after addition of 3AT to the medium) compared to cPrp8p (clones E4* and E5*, resistant to 20 and 5 mM 3AT, respectively; Figure 3A).

Both the N- and C-terminal fragments of Prp16p that interacted with Brr2p correspond to regions of Prp16p that have been reported to bind the spliceosome (WANG and GUTHRIE 1998). The N-terminal region of Prp16p most frequently found in our Brr2p screens (Table 1 and Figure 1) forms the primary contact during Prp16p recruitment under splicing conditions (WANG and GUTHRIE 1998). To directly test for interaction between Brr2p and nPrp16p in yeast cell extracts, ATP-depleted
Interactions of Splicing Proteins

Figure 4.—Analysis of interactions between Brr2p and (A) the C-terminal region of Prp8p; (B) Snu66p, Snp1p; and (C) the N- and C-terminal regions of Prp16p by co-immunoprecipitation experiments with yeast extract containing MYC-tagged Brr2p and [35S]methionine-labeled peptides produced in vitro. Mock precipitations without antibodies were included as a negative control as well as luciferase (Luc. in A). Although Ypl064p-fragments were frequently found in Brr2p screens, their two-hybrid interaction with Brr2p was weak and no co-immunoprecipitation with tagged Brr2p was observed (B). Compared to A, the precipitates in B and C are relatively overexposed. (D) MYC-tagged Brr2p co-immunoprecipitated with nPrp16p prey fusions pulled down with anti-HA antibodies. (E) Under these conditions no snRNAs were detected in the anti-HA precipitates.

(to prevent spliceosome formation) extracts from cMYC-BRR2 strains carrying an nPrp16 prey plasmid (clone St1) were treated with anti-HA antibodies directed to the internal HA epitope of the nPrp16p fusion peptide. In this setup, tagged Brr2p and the nPrp16p fusion peptide are relatively overproduced so that both could be titrated away from other spliceosomal components in the case of a direct interaction. Western blotting using anti-cMYC antibodies (Figure 4D) demonstrated that cMYC-tagged Brr2p co-immunoprecipitated specifically with the fusion of the N-terminal Prp16p fragment without detectable association of any spliceosomal snRNAs (Figure 4E). In parallel experiments, full-length Prp16p also co-immunoprecipitated with tagged Brr2p (data not shown).

A network of contacts between splicing proteins: In our screens we observed interactions between Brr2p as bait and splicing factors Prp16p, Snu66p, Prp8p, or Snp1p on one hand and between Slu7p, Snu66p, or nPrp8p as bait with Brr2p on the other (Figure 1). To delineate the Brr2p portion responsible for each interaction, we tested two-hybrid interactions between all these proteins and prey peptides. Brr2p deletion variants, slt22-1p, truncated Prp16p, prp16-1p and other DEAH-box proteins, Prp22p (prey of Slu7p), and Prp2p were also included (see Figure 5A and Table 1 for definition of clones). The results of these direct two-hybrid tests (Figure 5B) not only confirmed the specificity of our two-hybrid screens but also showed two-hybrid interactions of Brr2p with Prp2p and of Prp16p with Prp22p.

Interestingly, a near-exhaustive screen with Prp16p as bait retrieved, as the sole prey, two different Prp22p fragments (with fusions starting at residues 390 and 647; A. Colley and J. D. Beggs, unpublished results), which limits the Prp16p-interacting region to Prp22p residues 647–806. Note that the termini of Prp16p (e.g., St1p or St6p as prey) interact with Brr2p but not with Prp22p, whereas deletion of the internal GKT-DEAH region from Prp16p (bait 16ΔM) reduced the Prp22p interaction but did not affect the contact with Brr2p. Similarly, the prp16-1 allele (Y386D), located within the DEAH-box region, affected the interaction with Brr2p only slightly at 30°C (more so at 14°C) but made the interaction with Prp22p temperature sensitive (as the interaction of Prp16p with clone T2 at 36°C was disrupted by prp16-1). Thus, the interactions of Prp16p with Brr2p and with Prp22p appear to be independent.

In contrast to Prp16p that interacted through either terminus with Brr2p, PRP2 clone T1 (the C-terminal half, starting at amino acid 460) did not interact, whereas full-length Prp2p interacted with Brr2p. The failure to retrieve Prp2p in Brr2p screens may therefore
be explained by a lack of sufficiently long inserts in the FYRL yeast genomic library (Fromont-Racine et al. 1997).

The C terminus of Brr2p is a protein interaction domain, but is not sufficient for cPrp8p interaction: Deletion analysis of Brr2p showed that the C-terminal Brr2p...
region with the second helicase-like domain was responsible for most Br2 interactions. Prp2p, nPrp8p, Prp16p, Snu66p, and Slu7p appear to interact differently with the C-terminal Br2p fragments pulled out in two-hybrid screens (clones B1, B2, and B3). The region that is common to these Br2p fragments (clone B123) was not sufficient for these interactions, whereas the extreme C terminus by itself interacts strongly with Prp2p, Prp16p, and Slu1p (compare Br2 clones wild type, ΔM, and C with clone ΔC as bait fusions, and compare Br2 clones wild type, ΔN, and ΔNΔM with ΔMΔC as prey fusions). In contrast, the interaction with the C-terminal region (clone C or E3) of Prp8p requires the M region of Br2p (compare Br2 clones wild type and ΔN with clone ΔNΔM as prey fusions and compare Br2 clone wild type with clone ΔM as bait fusions). Thus, as for the strength of the interaction (as concluded from the co-immunoprecipitation experiments), the Br2p region responsible for the interactions with Prp2p, Prp16p, Slu1p, and Snu66p also differs from that responsible for cPrp8p.

Note, in particular, that full-length Prp8p did not show any interactions but that its N terminus and C terminus interacted in a mutually exclusive way with Br2p: cPrp8p does not interact with truncated Br2p baits lacking the first helicase domain (i.e., all ΔM variants). In contrast, nPrp8p, like full-length Slu7p, interacted only with such truncated Br2p fragments (the proline repeats in the extreme N terminus of Prp8p are not required for this interaction). This suggests that different regions of Br2p interact with the Prp8p termini and also that in spliceosomes particular conformations of either protein might be required for their association. As a summary, the network of splicing protein associations emerging from our protein interaction studies is shown in Figure 5C, indicating how these relate to kinetic events in spliceosome assembly and function.

**DISCUSSION**

The data presented in this article provide strong evidence for functional interactions between Br2p (Slu22p/Snu246p/Rss1p/Prp44p) and the splicing factors Prp8p, Prp16p, Snu1p, and Snu66p. Furthermore, we observed other specific two-hybrid interactions that suggest functional links of Br2p with Snu1p and Prp2p and of Prp16p with Prp22p. Intriguingly, and reminiscent of helicases involved in DNA replication, the interaction of different DExH/DEAD-box proteins might be instrumental during pre-RNA splicing for obtaining specific and optimal unwindase activities. Overall, our data will help to formulate a more accurate model of protein dynamics (and of the central role of Br2p therein) during spliceosome assembly and subsequent activation of the first and second catalytic steps.

Apart from most Prp8p screens, all two-hybrid screens presented in this article were highly specific and sensitive according to the criteria of Vidal and Legrain (1999). We found interactions with a number of proteins that were previously found as prey of other splicing factors, thus extending the network of protein interactions, identified through two-hybrid screens, that are highly relevant for pre-mRNA splicing (Fromont-Racine et al. 1997; Dix et al. 1999; Figure 1).

**Interactions of Prp8p:** Prp8p is a very large protein (280 kD, 2413 amino acids) and different regions have been shown to interact with Snu114p (Dix et al. 1998), Snu1p (S. Ruby et al., personal communication), and the yeast homologue of U1C (P. Legrain, personal communication). In a pairwise two-hybrid test, Abovich and Rosbash (1997) found that the N terminus of Prp8p interacted with Prp40p, whereas we found that the Prp8p N terminus associated with Br2p and Prp39p. Prp8p has been demonstrated to bind to the 5′ splice site region of pre-mRNAs (where U1 snRNPs also associate) in spliceosomes (Tegelkamp et al. 1995; Reyes et al. 1999), and as the U1 snRNP proteins Snu1p, Prp39p, Prp40p, and yU1C copurify with crude U4/U6.U5 tri-snRNP fractions (Gottschalk et al. 1999), their interaction with Prp8p is feasible, even without spliceosomes. Additionally, mutations that affect U4/U6 unwinding in spliceosomes were recently identified in an N-terminal segment of Prp8p (amino acids 193–388; Kuhn and Brow 2000). Thus, taken together, these interactions provide physical evidence to support the proposed role...
of Prp8p in the Brr2p-controlled unwinding of U4/U6 and displacement of U1 snRNA from the 5′ splice site, as suggested by genetic data (Kuhn et al. 1999).

Recently, genetic analyses and sequence comparisons implicated particular regions of Prp8p in recognizing the uridine tract near the 3′ splice site, in controlling the fidelity of 3′ splice site, selection (Umen and Guthrie 1995a,b; Collins and Guthrie 1999), in cross-linking to the 5′ splice site (Reyes et al. 1999), and in genetic interaction with Prp16p, Prp17p, Prp18p, and Shu7p (Umen and Guthrie 1995b; Ben-Yehuda et al. 2000) as well as with the U2 (Xu et al. 1998) and U4 snRNAs (Kuhn et al. 1999). However, the region of Prp8p that was selected with Brr2p in two-hybrid screens was distinct and more C-terminal to all these regions (prey fusions E3, E4*, and E5*; Figure 3A). In fact, extension of the cPrp8p prey fragment into the upstream segment involved in 3′ splice site interactions abolished the Brr2p interaction (construct E1; Figure 3A). In addition, the upstream segment that inhibited the Brr2p interaction slightly overlaps with part of a region of Prp8p (amino acids 1626–1651) shown by Kuhn and Brow to interact genetically with U4 snRNA (Kuhn and Brow 2000). This is one of two regions of Prp8p proposed by Kuhn and Brow to interact and cause an allosteric change that might initiate spliceosome activation, in part by affecting the activity of Brr2p/Prp44p. As we show here that a distinct, more C-terminal region of Prp8p interacts with Brr2p, conceivably this proposed intramolecular interaction modulates the Prp8p:Brr2p interaction indirectly.

Four further observations support the functional significance of the Prp8p:Brr2p interaction observed here. First, in contrast to regions more upstream, the C terminus of Prp8p implicated in Brr2p interaction is not highly conserved, and when this region of yeast Prp8p was replaced by the plant Arabidopsis thaliana counterpart the two-hybrid interaction with Brr2p was lost, and hybrid yeast/plant Prp8p proteins were not functional in vivo (J. Hamilton, R. W. van Nues, J. D. Beggs and J. W. S. Brown, unpublished results). Second, in this region the cPrp8p mutation (prp8-28) that caused the interaction with Brr2p to be temperature sensitive (Figure 3A) also led to a conditional phenotype in vivo (Figure 3B). Third, other mutations, like prp8-52, could be isolated that increase the affinity for Brr2p and for the temperature-sensitive slt22-1p (Figure 3A) that has a mutation within the first helicase domain of Brr2p affecting ATP hydrolysis (Xu et al. 1996). Mutant prp8-52p, however, did not complement the slt22-1 allele in vivo, presumably because other Brr2p functions that are affected by this allele (Xu et al. 1996, 1998) were not suppressed. Fourth, the prp8-1 allele (which maps to the C-terminal Brr2p-interacting region) was synthetic lethal with slt22-1 (Figure 3B), whereas no synthetic lethality of prp8-1 with alleles of other second-step factors has been found (Umen and Guthrie 1995b). The reduced affinity of mutant prp8-1p for Brr2p, as found by two-hybrid analysis (Figure 3A) and co-immunoprecipitation experiments (Figure 4A), could explain the unstable association of prp8-1p with the U5 particle and the diminished assembly of U4/U6.U5 tri-snRNPs observed in heat-treated prp8-1 extracts (Brown and Beggs 1992). Thus, the strong and specific interaction between Brr2p and the C terminus of Prp8p agrees well with the purification from Hela cells of an RNA-free p220/p200/p116/p40 complex that contains the counterparts of yeast Prp8p, Brr2p, and Snu114p (Achsel et al. 1998).

In summary, we propose that the N- and C-terminal regions of Prp8p have distinct interactions that are likely to contribute to the regulation of Brr2p. On the one hand, interaction of nPrp8p with the C terminus of Brr2p may affect the activity of Brr2p in assembling spliceosomes (unwinding the U4/U6 duplex and/or disrupting the U1/5′ splice site interaction), while, on the other hand, interaction of cPrp8p with Brr2p may affect the assembly and/or stability of U5 snRNPs and/or U4/U6.U5 tri-snRNPs. The mutually exclusive nature of these interactions in the two-hybrid assay suggests that Prp8p undergoes conformational rearrangements that could modify the function of Brr2p (and likely other splicing factors also) in the spliceosome.

**Interactions of Brr2p:** In contrast to Prp8p, screens with the comparably long Brr2p (246 kD; 2163 amino acids) worked well and yielded enough information to delineate interaction domains of Prp8p (as discussed above), Prp16p, and Snu66p. Brr2p, in turn, was fished in screens with nPrp8p (see above), Shu7p, and Snu66p as bait (Figure 1 and Table 1). Apart from interactions with splicing factors, the screens with Brr2p also suggested contacts with proteins involved in other cellular processes such as signal transduction (Fabl1p, Cdc25p), cell division (Dbi9p), or gene transcription (Met28p). Although beyond the scope of this article, further analysis of these links might provide insight into the relation between the regulation of the pre-mRNA splicing machinery and that of other cellular events.

**Specific association of RNA unwindases:** Among the most interesting outcomes of our analyses are the interactions between RNA unwindases; Brr2p with Prp16p and Prp2p on one hand and Prp16p with Prp22p on the other. Because we tested all these proteins against each other using either full-length or truncated forms, we can exclude that these interactions are due to a general stickiness of the conserved helicase domains or that ATP or RNA molecules specifically mediate these interactions (Figure 5 and data not shown). The statistically most relevant two-hybrid interaction of this kind was observed between Brr2p and the N terminus of Prp16p. This interaction, like that between Prp22p and Prp16p (A. Colley and J. D. Beggs, unpublished results), could be reproduced in vitro by co-immunoprecipitation assays under nonsplicing conditions. We were
able to pull down Brr2p with nPrp16p without detectable coprecipitation of snRNAs, indicating that no spliceosomal complexes were present and suggesting a direct physical interaction. Both ends of Prp16p that were pulled out as Al prey of Brr2p are involved in recruitment of Prp16p to the spliceosome (Wang and Guthrie 1998). In contrast, Prp22p needs the internal helicase domain of Prp16p for interaction and acts later in the splicing process (Schier and Gross 1998), depending on the presence of Prp16p (McPheeters et al. 2000). Therefore, our data indicate that Brr2p is the first spliceosomal partner of Prp16p and may act as its receptor in the spliceosome.

The interactions between the four DExH box proteins Brr2p, Prp2p, Prp16p, and Prp22p suggest that during pre-mRNA splicing associations of different unwindases occur, which might be important for their functionality. Interestingly, another DExH-box protein, NS3h from the hepatitis C virus, is active as an (unstable) oligomer (Levin and Patel 1999). NS3h has a helicase domain structure similar to that of bacterial DNA helicases that also function as oligomers (West 1996). Further parallels exist with the proteins of the minichromosome maintenance (MCM) complex that are essential for eukaryotic DNA replication. MCM proteins need to associate to obtain DNA helicase activity (Chong et al. 2000), but can also form different complexes with altered functionalities (Lee and Hurwitz 2000), implying different states of the replication machinery or how its action is controlled.

**Does the C-terminal helicase-like domain regulate Brr2p function through protein interactions?** Our deletion analysis to delineate the protein-binding regions of Brr2p showed that the interaction with the C terminus of Prp8p required the presence of the first helicase domain of Brr2p. In contrast, for all the other interactions analyzed, the C-terminal Brr2p region with the second helicase-like domain was predominantly responsible (Figure 5). This second domain is well conserved between human (Lauber et al. 1996), yeast, and plants (GenBank accession no. AC002561) but, in contrast to the first helicase domain, does not seem to be involved in ATP hydrolysis. Mutations in the GKT and DDAH boxes do not cause a phenotype in vivo (Kim and Rossit 1999), which further indicates that the protein interactions of this domain, if vital, are not dependent on the altered residues.

Multiple unwindases are implicated in the splicing process (Staley and Guthrie 1998) but little progress has been made in identifying the targets/substrates of their activity. Conceivably, some of these unwindases may work cooperatively on common targets. There is strong evidence that Brr2p recognizes as its substrate not a primary but a particular secondary RNA structure (Lin and Rossit 1996). This secondary structure resembles the U4/U6 snRNA duplex that is unwound by Brr2p (Raghunathan and Guthrie 1998; Kim and Rossit 1999) and the U2/U6 snRNA duplex within which certain U2 snRNA mutants are synthetic lethal with mutations in Brr2p (Xu et al. 1996, 1998). It is possible that Brr2p has alternative substrate specificities controlled by its association with different proteins (such as Prp2p or Prp16p) during splicing. Furthermore, in contrast to the transiently associated Prp2p and Prp16p, Brr2p is an integral snRNP protein thought to act at multiple points in the splicing pathway (Staley and Guthrie 1998; see Lauber et al. 1996; Xu et al. 1996; Raghunathan and Guthrie 1998). If so, multiple interactions will probably contribute to the temporal control of its function. Our data support such a model and suggest that the C-terminal half with the second helicase-like domain might regulate the activity of Brr2p through distinct, sequential protein-protein interactions.

**Conclusions:** The protein interaction data presented here are compatible with and augment the current model of splicing events (Figure 5C). The strong interaction between Brr2p and the C terminus of Prp8p is important for the assembly and/or stability of the U5 snRNP. Being important for the first step of splicing (Gottschalk et al. 1999), Snu66p might facilitate tri-snRNP formation by linking the U5 snRNP and the U4/U6 di-snRNP through its associations with Brr2p and Prp6p. Once the U4/U6.U5 tri-snRNP enters the spliceosome, Snlp1p and the N terminus of Prp8p could affect the Snu66p/Brr2p interaction during U1 displacement, with Brr2p being activated to unwind U4/U6 (Kühn et al. 1999). Upon recruitment to the spliceosome Prp2p might redirect the Brr2p activity from the U4/U6 dimer to the U6/U2 snRNA duplex II, which is thought to unwind just before the first catalytic step (discussed in Xu et al. 1998). Subsequently, Prp16p could displace Prp2p from Brr2p and induce a conformational change in the spliceosome that exposes the catalytic center to the 3' splice site. This event further involves Prp8p, Snu7p, and Prp22p (Umen and Guthrie 1995b; Schier and Gross 1998; McPheeters et al. 2000), which might be directly responsible for the release of Prp16p from the 3' splice site and the spliceosome. The interaction between Prp16p and Brr2p could be weakened upon association of Prp22p with Prp16p and disrupted by Snu7p displacing Prp16p from Brr2p. Experiments to test this proposed order of Brr2p interactions in more detail are beyond the scope of this article, but hopefully we have furthered our understanding of dynamic protein interactions that drive spliceosome function.

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