Identification of Novel Genes Required for Yeast Pre-mRNA Splicing by Means of Cold-Sensitive Mutations

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ABSTRACT

Genetic approaches in *Saccharomyces cerevisiae* have identified 38 genes required for efficient RNA splicing. The majority have been found by screening (high) temperature-sensitive (ts) mutants for those defective in splicing, an approach limited by the presence of ts hotspots and by the fact that many essential genes rarely mutate to the ts phenotype. To identify novel genes, we screened a collection of 340 cold-sensitive (cs) mutants for those that exhibited diminished splicing of several pre-mRNAs. We isolated 12 mutants in nine complementation groups. Four of these affected known genes (*PRP8, PRP16, PRP22, PRP28*), three of which encode RNA helicase homologues. Five genes are novel (*BRR1, BRR2, BRR3, BRR4, BRR5*; Bad Response to Refrigeration); mutations in these genes inhibited splicing before the first chemical step of the reaction. Analysis of *BRR2* revealed it to encode an essential member of a new class of RNA helicase-like proteins that includes the yeast antiviral protein Ski2. These data validate the use of cs mutants in genetic screens and raise the possibility that RNA helicase family members are particularly prone to mutation to cold sensitivity.

THE removal of intervening sequences from mRNA precursors is a fundamental event of eukaryotic gene expression. Splicing occurs via two successive transesterification reactions (RUSKIN *et al.* 1984; KONARSKA *et al.* 1985; MOORE and SHARP 1993). Despite its simple chemistry and the fact that some Group II organellar introns are able to complete the reaction *in vitro* without the aid of proteins, nuclear splicing requires a complex machinery of five small nuclear RNAs (snRNAs) and an estimated 50–100 proteins (reviewed by RYMOND and ROSBASH 1992; MOORE *et al.* 1993). Work in recent years suggests that the active site of the spliceosome is built as a network of RNA-RNA interactions that involve the U2, U5, and U6 snRNAs as well as the intron containing substrate (reviewed by MADHANI and GUTHRIE 1994). Several of these interactions require disruption of RNA secondary structures and the formation of mutually exclusive alternative structures. It has been proposed that the roles of the protein elements are to assure that spliceosome assembly is ordered and precise, to correctly orient and rearrange the *trans*-acting snRNAs within the spliceosome, and, in some cases, to mediate alternative splicing choices (see GUTHRIE 1991). Determination of such functions requires the isolation of the protein factors.

A number of proteins required for splicing have been identified. In vertebrate cells, biochemical strategies have been used to isolate and clone the genes for all eight of the core (Sm) snRNP proteins, as well as a number of snRNP-specific and *trans*-acting proteins (reviewed by MOORE *et al.* 1993; see also BENNETT and REED 1993; BROSI *et al.* 1993; PATTON *et al.* 1993; CHAMPOIN-ARNAUD and REED 1994; CHIARA *et al.* 1994; KRÄMER *et al.* 1994, 1995; HERMANN *et al.* 1995); however, this approach is made challenging by the low abundance of many splicing proteins. In yeast, tractable genetics and genome sequencing efforts have permitted the identification of ~40 splicing proteins in a "gene-first" manner. Many have been identified by means of genetic suppression (COUTO *et al.* 1987; LAST *et al.* 1987), synthetic lethality (FRANK *et al.* 1992; LIAO *et al.* 1993; ABOVICH *et al.* 1994), and homology to known mammalian splicing factors (SMITH and BARRELL 1991; KAO and SILICIANO 1992; RYMOND 1993; COOPER *et al.* 1995; ROY *et al.* 1995; SÉRAPHIN 1995). Virtually all other published mRNA splicing components have been discovered by screening collections of temperature-sensitive (ts) lethal mutants for those that fail to efficiently splice endogenous pre-mRNAs after a shift to the nonpermissive temperature (HARTWELL *et al.* 1970, these were first screened for failure to synthesize total RNA and subsequently shown to affect RNA splicing; ROSBASH *et al.* 1981; VIJAYRAGHAVAN *et al.* 1989; BLANTON *et al.* 1992; LOCKHART and RYMOND 1994; B. RYMOND, personal communication; J. WOOLFORD, personal communication). Twenty-eight genes have been identified by this method, which in aggregate has involved screening >2000 ts mutants.

Despite the success of screens of ts mutants in identifying novel splicing factors, there exist serious obstacles to identifying the remainder by the same means. First, studies of *Saccharomyces cerevisiae* chromosome *I* demon-

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strate that only a minority (~30%) of essential genes mutate detectably to temperature sensitivity, at least when the target of the mutagen is the intact cell (DIEHL and PRINGLE 1991; HARRIS and PRINGLE 1991). If true in general, these data suggest that many splicing proteins probably cannot be detected in screens that rely upon ts mutants. Second, although screens for ts splicing mutants have not been saturating, the existence of mutational hotspots makes the detection of additional splicing factors a case of diminishing returns (HARTWELL *et al.* 1970; VIJAYRAGHAVAN *et al.* 1989). For instance, mutations in the *PRP2* and *PRP3* genes compose 24 and 16%, respectively, of the mutants isolated in the aforementioned screens (HARTWELL *et al.* 1970; VIJAYRAGHAVAN *et al.* 1989; J. WOOLFORD, personal communication; B. RYMOND, personal communication).

A possible solution to this quandary is to select for mutants based on a different conditional phenotype, such as cold sensitivity (cs). Early studies of bacteriophage mutants indicated that cs and ts mutants tend to fall in different subsets of genes (SCOTTI 1968; COX and STRACK 1971; JARVIK and BOTSTEIN 1975). Moreover, GUTHRIE *et al.* (1969a,b) and TAI *et al.* (1969) demonstrated that, while extensive searches for ts mutants affecting prokaryotic ribosomal assembly had proven fruitless, cs mutants are remarkably easy to obtain. In yeast, comparative data exist from genetic analysis of the cell division cycle (cdc). MOIR *et al.* (1982) generated and screened a collection of 350 cs mutants for those that arrest with morphologies characteristic of a cdc defect. Whereas their rationale in choosing cs mutants had been to use them for pseudoreversion analysis, the screen proved to be successful in another way, yielding at least six novel complementation groups that had not been identified in a previous screen of 1500 ts yeast strains (HARTWELL *et al.* 1973). Only one complementation group from the cs screen had been previously identified (MOIR *et al.* 1982), consistent with the idea that, for *cdc* mutations, ts and cs alleles occur in largely nonoverlapping sets of genes. We reasoned that a strategy employing a collection of cs yeast strains might be equally useful when applied to studies of mRNA splicing. In support of this idea, STRAUSS and GUTHRIE (1991) identified a novel splicing factor, *PRP28*, in a pilot screen of only 18 cs mutants.

We examined the cs collection produced by MOIR *et al.* (1982) for strains with defects in the splicing of endogenous pre-mRNAs. Genetic analysis of the initial candidates revealed nine complementation groups in which the cs phenotype was associated with the RNA splicing defect. To identify which of these genes were novel, complementation analysis was performed with the previously identified *prp* strains. Four of the complementation groups were found to correspond to previously identified genes (*PRP8, PRP16, PRP22, PRP28*), whereas five had not been described previously (*BRR1, BRR2, BRR3, BRR4, BRR5*; Bad Response to Refrigeration). Nucleotide sequence and gene disruption analysis of *BRR2* demonstrate it to encode an essential member of a novel family of RNA helicase-like proteins that includes the product of the yeast antiviral gene *SKI2*. As *PRP16, PRP22,* and *PRP28* also encode RNA helicase homologues, it is possible that genes for helicase-like proteins are preferentially mutable to cold sensitivity. Like the previous findings of MOIR *et al.* (1982), our results demonstrate that screening mutants of a different conditional phenotype can be an efficient method for identifying novel genes involved in an essential cellular process.

MATERIALS AND METHODS

Yeast and molecular biology methods: Cultivation and genetic manipulation of *S. cerevisiae* were performed by standard methods (GUTHRIE and FINK 1991). Molecular biological methods are described in SAMBROOK *et al.* (1989).

Temperature shift of cs collection and Northern analysis of *CRY1* mRNA: Of the 350 cs strains described by MOIR *et al.* (1982; Table 1), 340 were recovered from storage at −80°. Each of these, as well as the wild-type parental strain (DBY473), was inoculated into 25 ml of YEPD liquid medium and grown to mid-log phase at 30°. The cultures were next moved to a shaking water bath at 16°, where they were incubated for 10 hr. Subsequently, the cells were pelleted by centrifugation, frozen, and total cellular RNA was prepared.

For Northern analysis, 20 μg of RNA from each mutant as well as the wild-type parent and a positive control mutant (*prp18-1*^{ts}, shifted to 37° for 4 hr) was electrophoresed overnight at 30 V on 1.5% agarose/6% formaldehyde gels. The resolved material was electroblotted onto a Hybond-N (Amersham) nylon membrane for 3 hr at 40 V. The blots were crosslinked with ultraviolet light using a Stratalinker (Stratagene) on the automatic setting. Hybridization was performed overnight at room temperature using standard methods and ^{32}P 5′end-labeled oligonucleotides complementary to *CRY1* second exon sequences and to U5 snRNA (see below for oligonucleotide sequences). Autoradiograms were scanned visually for candidates that displayed differences from the wild-type control in the ratio of *CRY1* mRNA to the U5 snRNA internal standard or of *CRY1* pre-mRNA or lariat intermediate to *CRY1* mRNA.

Oligonucleotides used in this study: Name/Sequence: CRY1 exon 2/GTATCGTTGAAAGAAGCG; U5-7wtsmnr/AAGTTCCAAAAAATATGGCAAGC; U3 exon 2/CCAAGTTGGATTCAGTGGCTC; RP51A exon 2/CGCTTGACGGTCTTGGTTC; U1-19K/CAATGACTTCAATGAACAATTAT; RP51A intron/GTATGACTTTATTGCGCATGTCGACTC.

Primer extension analysis of transcripts from the *SNR17A, SNR17B,* and *RP51A* genes: The 98 candidates identified by Northern analysis of *CRY1* message were rescreened for splicing of U3 pre-snoRNA by means of an abbreviated primer extension protocol (LESSER and GUTHRIE 1993). ^{32}P-labeled oligonucleotide U3 exon 2 was used to detect the U3 precursor and mature species; oligonucleotide U5wtsmnr was used to detect U5 snRNA, which served as the internal control for RNA loading.

The candidates were assessed for splicing of pre-*RP51A* using our standard primer extension protocol (PATTERSON and GUTHRIE 1991) and primers RP51A exon 2 (for visualization of pre-*RP51A*, mature *RP51A*, and lariat intermediate) or RP51A intron (for visualization of pre-*RP51A* and lariat intermediate/lariat intron). In these primer extensions, primer U5-7wtsmnr was used to visualize the internal control, U5 snRNA.

Complementation analysis among the cs mutants: Cs mu-

TABLE 1

***S. cerevisiae* strains used in this study**

Name	Relevant genotype	Background genotype	Source
DBY4157-4482	Cold-sensitive mutants	Derived from mutagenized DBY473 (see below)	D. BOTSTEIN
DBY4483-4497	Cold-sensitive mutants	Derived from mutagenized DBY640 (see below)	D. BOTSTEIN
DBY473	Wild-type strain	(S288C background) *MAT*α *gal*$^-$ mal$^-$ *his4-619*	D. BOTSTEIN
DBY640	Wild type strain	(S288C background) *MAT***a** *gal*$^-$ *mal*$^-$ *ade2*	D. BOTSTEIN
YGS1	Wild type strain	(S288C background) *MAT***a** *ade2-100° ura3-52 lys2-801^a his3-Δ200 leu2-Δ1*	I. HERSKOWITZ
YGS5	*prp28-1^{cs}* on plasmid pHD26 (*HIS3, CEN*)	*MAT*α *prp28:Δ::TRP1 trp1 ura3 his3 leu2*	This laboratory
YS111	*prp16-101^{cs}* on plasmid A7.5 (*TRP1, CEN*)	*MAT***a** *ade2-101 trp1 ura3-52 leu2 lys2-801 prp16Δ::LYS2 cup1Δ::URA3-52*	This laboratory
YEJS 35	*prp23-1 (=prp16-2)*	*MAT***a** *ade2-101 his3Δ200 ura3-52 tyr1*	J. ABELSON
yEJS7	*prp2*	*MAT*α *ade2-1 his3-532 trp1-289 ura3-1 ura3-2*	J. ABELSON
yEJS8	*prp3*	*MAT***a** *his3 leu2 lys2 ura3-52*	J. ABELSON
YEJS45	*prp4*	*MAT*α *ade ura3-52 his leu2 lys2*	J. ABELSON
YSR5.1a	*prp5-1*	*MAT***a** *ade2-101 his3-Δ200 tyr1*	S. RUBY
YEJS14	*prp6*	*MAT*α *his3 lys2 ura3-52*	J. ABELSON
YEJS75	*prp8-7*	*MAT*α *leu2-3 leu2-112 tyr1 his ura3-52*	J. BEGGS
YSR9-2	*prp9-1*	*ade2-101 his3-Δ200 tyr1 ura3-52*	S. RUBY
YBP74	*prp11*	*MAT***α** *ura3-52 leu2 his-4-512*	This laboratory
YEJS23	*prp17*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS24	*prp18*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS26	*prp19*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS29	*prp20*	*MAT*α *ade2-101 his3Δ200 ura3-52 tyr1*	J. ABELSON
YEJS31	*prp21*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS33	*prp22*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS34	*prp23=prp16*	*MAT*α *ade2-101 his3Δ200 ura3-52 tyr1 lys2-801*	J. ABELSON
YEJS36	*prp24*	*MAT***a** *ade2-101 his3Δ200 ura3-52 lys2-801*	J. ABELSON
YEJS41	*prp27*	*MAT*α *ade2-101 his3Δ200 ura3-52 lys2-801 rho*$^-$	J. ABELSON
JWY2439	*prp30-1*	*MAT***a** *his4 leu2-3,112 lys2-801 ura3-52*	J. WOOLFORD
JWY2431	*prp31-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. WOOLFORD
JWY2433	*prp32-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. WOOLFORD
JWY2435	*prp33-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. WOOLFORD
JWY2437	*prp34-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. WOOLFORD
YSN4A	*slu1-1*	*MAT***a** *ura3 his3 trp1 lys2 ade2*	This laboratory
YBP97	*slu2-1*	*MAT***a** *ura3-52 his3Δ trp1Δ lys2-801^a ade2-101°*	This laboratory
YDF50	*slu7-1*	*MAT***a** *trp1Δ63 his3Δ200 ura3-52 ade2-101 lys2-801 leu2Δ1*	This laboratory
YSN113	*prp28-2*	(from DBY4234) *MAT***a** *leu2-Δ1 ade2-100° his3-Δ200 lys2-801^a*	This study
YSN150	*prp28-4*	(from DBY4489) *MAT***a** *his3-Δ200 lys2-801^a*	This study
YSN97	*prp22-2*	(from DBY4172) *MAT*α *ura3-52 leu2-Δ1 ade2-100° his3-Δ200*	This study
YSN160	*prp8-8*	(from DBY4490) *MAT*α *ura3-52 leu2-Δ1 ade2-100° lys2-801^a*	This study
YSN142	*brr1-1*	(from DBY4483) *MAT***a** *ura3-52 leu2-Δ1 ade2-100° lys2-801^a*	This study
YSN239	*brr2-1*	(from DBY4340) *MAT***a** *ura3-52 leu2-Δ1 ade2-100° lys2-801^a*	This study
YSN405	*brr2-1*	(from DBY4340) *MAT*α *ura3-52 leu2-Δ1 ade2-100° his3-Δ200 lys2-801^a*	This study
YSN103	*brr3-1*	(from DBY4217) *MAT***a** *ura3-52 ade2-100° his3-Δ200*	This study
YSN137	*brr4-1*	(from DBY4475) *MAT***a** *ura3-52 leu2-Δ1 ade2-100°*	This study
YSN402	*brr5-1*	(from DBY4236) *MAT*α *ura3-52 leu2-Δ1 lys2-801^a*	This study
JO226	Wild type	(S288C background) *MAT***a**/α *his3Δ/his3Δ ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*	I. HERSKOWITZ
YSN404	*brr2Δ::LEU2/BRR2*	(S288C background) *MAT***a**/α *his3Δ/his3Δ ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*	This study

TABLE 2

Complementing plasmids

Gene	Plasmid name	Original DBY strain	Mutant strain used for plasmid isolation
BRR1	pSN19	DBY4483	YSN142
BRR2	pSN21	DBY4340	YSN239
BRR3	pSN17	DBY4217	YSN103
BRR4	pSN24	DBY4475	YSN137
BRR5	pSN100	DBY4276	YSN402
PRP8	pSN25	DBY4490	YSN160
PRP22	pSN16	DBY4172	YSN97

Listed are genomic DNA clones isolated from a YCp50 library (ROSE *et al.* 1987) by complementation of the indicated cs mutants.

tant candidates were crossed to wild-type strains of similar S288C genetic background (either DBY640 or DBY473 for the first cross and YGS1 in subsequent matings; Table 1). The resulting diploids, heterozygous for the cs mutation, were sporulated and the resulting ascospores dissected. **a** and α spores from each mutant were mated by cross-stamping patches of spore progeny on YEPD. After incubation at 30° for 1 day, the plates were replica-plated to prechilled YEPD plates and incubated at 17° for 2 days. Outcrossed descendants of strains DBY4234, 4236, and 4489 formed a single complementation group, as did descendants of strains DBY4172 and 4423. The DBY4172 and 4423 progeny were crossed to each other and dissected. In 19 four-spore tetrads, the cs phenotype segregated in a pattern of 4:0 (cs^- to cs^+) indicating that the two mutants are allelic. Descendants of strains DBY4234 and 4489 were subsequently shown to be linked to a previously identified *prp* mutation (see below), indicating that they are also allelic to each other.

Linkage analysis of cs growth and splicing defects: The progeny of five tetrads from each cross (described in the section above) were assessed for growth at 16° and also for splicing defects. The latter was accomplished by performing a temperature shift similar to the one described above. Total cellular RNA was prepared and analyzed for splicing of pre-U3 snRNA by our standard primer extension protocol.

Cloning of genomic complementing plasmids: Complementing clones for DBY4483 (*BRR1*), DBY4340 (*BRR2*), DBY4217 (*BRR3*), DBY4475 (*BRR4*), DBY4490 (*PRP8*), and DBY4172 (*PRP22*) were obtained in the following manner: cs splicing mutants that had been outcrossed one to four times were transformed with DNA from the Rose library; this library consists of wild-type yeast genomic DNA carried in the *URA3*-marked, centromeric vector, YCp50 (ROSE *et al.* 1987). Transformation reactions were plated directly on pre-chilled plates lacking uracil and incubated at 17° for 4–6 days. Plasmids recovered from strains in which the CS^+ phenotype was plasmid linked were retransformed into the original cs mutant strains and scored once again for their ability to confer growth at 17°.

BRR5 could not be cloned by direct selection at the nonpermissive temperature because of a relatively high reversion frequency. Thus, *brr5-1* transformants were plated first on plates lacking uracil and incubated at the permissive temperature of 30°, followed by replica-plating to prechilled plates at 17°. The remainder of the cloning strategy was the same as for the other mutants. A list of the complementing plasmids appears in Table 2.

Determination of allelism with previously identified splicing factors: At least one cs mutant from each of the nine complementation groups for which there was association between the splicing and cs growth phenotypes was crossed to cs alleles of *PRP16* (*prp16-101*; strain YS111), *PRP28* (*prp28-1*; strain YGS5) and *SLU7* (*slu7-1*, strain YDF50). As failure to complement was observed between the outcrossed progeny of DBY4406 and *prp16-101*, as well as between progeny of DBY4234, DBY4236, DBY4489 and *prp28-1*, further crosses were made for linkage analysis. A DBY4406 descendant was crossed to a temperature-sensitive strain containing the *prp16-2* mutation, YEJS35 (Table 1). In six of six tetrads, 2:2 segregation of the cs^-:ts^- phenotypes was observed, indicating that the two mutations are allelic. Likewise, descendants of DBY4234 (YSN113) and DBY4489 (YSN150), both members of the same complementation group, were crossed to a cs strain containing the *prp28-1* mutation (YGS5; Table 1). Of 12 and nine four-spore tetrads, respectively, 4:0 segregation of the cs^-:cs^+ phenotypes was observed for each of the tetrads, again indicating allelism of the mutations.

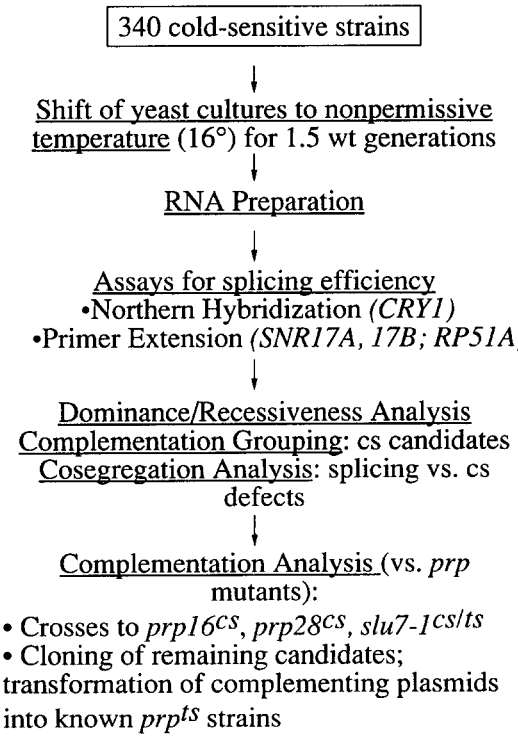


FIGURE 1.—Screen for cs mutants defective in pre-mRNA splicing.

TABLE 3

Cold-sensitive RNA splicing mutants isolated in this study

Complementation group	Gene name	DBY strain	*CRY1* phenotype	U3 phenotype	*RP51A* phenotype	
					Exon II primer	Intron primer
1	*PRP28*	4234	↓M	↑P	↑P	↑P
		4236	↓M	↑P	↑P	↑P
		4489	↑P	↑P	↑P; ↓M	↑P
2	*PRP22*	4172	↓M	↑P	↑P; ↑LI	↑P; ↑LI/L
		4423	↓M	↑P	↑P; ↑LI	↑P; ↑LI/L
3	*PRP16*	4406	↓M	↑P	↑P; ↑LI	↑P; ↑LI/L
4	*PRP8*	4490	↑P	↑P	↑P	↑P
5	*BRR1*	4483	↓U5 snRNA	↑P	↑P	↑P (weak)
6	*BRR2*	4340	↓M	↑P	↑P	↑P
7	*BRR3*	4217	↓M	↑P	↑P	↑P
8	*BRR4*	4475	↓M (weak)	↑P	↑P (weak); ↓M	↑P (weak)
9	*BRR5*	4276	↓M	↑P	↑P	↑P

In describing the phenotypes of the various mutants, the following symbols are used: ↓M, loss of mature *CRY1* RNA relative to the U5 snRNA internal standard; ↑P, an increase in intron-containing precursor relative to an internal control (U1 snRNA in the U3 primer extensions; U5 snRNA in the *RP51A* primer extensions); ↑LI, for an increase in the lariat intermediate relative to an internal control; ↑LI/L, an increase in the lariat intermediate and/or excised lariat intron species in the *RP51A* intron primer extensions.

Only ts alleles were available for 22 previously identified pre-mRNA processing mutants, as well as for several unpublished splicing mutants (kindly provided by J. WOOLFORD; Table 1). Complementation analysis of the ts splicing mutants was performed by transformation with the wild-type clones for the remaining candidates from this screen. Transformants were streaked on YEPD plates, and growth at 37° was compared between transformants with wild-type clones *vs.* a vector control. As the clone for DBY4172/4423 complemented *prp22*, and that for DBY4490 complemented *prp8*, their inserts were examined further. Restriction fragment analysis of both clones as well as PCR analysis of the *PRP22* clone confirmed the identity of the complementing DNAs.

Molecular analysis of *BRR2*: To better delineate the *BRR2* gene within the 12 kb insert of pSN21, we prepared a library of size-fractionated fragments of pSN21, which had been digested only partially with *Sau*IIIA. Fragments were isolated in the following size classes: 1–3.5 kb, 3–5 kb, and >5 kb; these were ligated to *Bam*HI-digested pSE360, a centromere, ARS-containing vector (pSE360 is identical to pUN50; ELLEDGE and DAVIS 1988).

Complementing clones were obtained by transforming the three minilibraries in parallel into YSN405 (a *brr2-1* strain that has been outcrossed four times; Table 1) and plating directly to prechilled plates lacking uracil, as described above. Plasmids that were able to confer wild-type growth at 17° to YSN405 cells upon retransformation were compared for the size of their inserts. The smallest complementing plasmid had a 7.1-kb insert (pSN108) and was selected for further analysis. Sequencing reactions were performed with primers homologous to regions of the vector that flank the insert. Using this sequence information, a search of the database was performed, and an exact match was located on chromosome *V* (Swiss Protein accession number P32639).

Linkage of the cloned *BRR2* gene to *brr2-1*: To determine whether the minimal complementing plasmid corresponded to the wild-type version of *brr2-1* (rather than a suppressor), we created a plasmid that could be used to target the *URA3* selectable marker to the chromosomal locus of the cloned gene. A 877-bp *Sal*I-*Sal*I fragment from pSN108, which includes the C-terminus of the cloned gene, was subcloned into the integrating vector, pRS306 (*URA3*; SIKORSKI and HIETER 1989). This construct was linearized with *Bst*XI, which cuts within the 877-bp insert, and transformed into the wild-type strain, YGS1, which is auxotrophic for uracil. Uracil prototrophs were screened using a PCR method for integration to the correct locus (*i.e.*, to chromosomal sequences homologous to the cloned gene). One of the correct integrants was crossed to a *ura3, brr2-1* strain and the meiotic progeny scored for segregation of the cs and uracil auxotrophic phenotypes.

Disruption of the *BRR2* gene: A disrupted allele of *BRR2* was created by replacing a 2493-bp *Bgl*II-*Bgl*II fragment corresponding to the middle of the Brr2 ORF with the *LEU2* gene to yield pSN211. The *Bgl*II-*Bgl*II fragment encodes almost the entire helicase homology region of Brr2. pSN211 was digested with *Sal*I to liberate the *LEU2* gene flanked by 1660 and 3143 bp of *BRR2* flanking sequences. This restriction fragment was gel-purified and transformed into the wild-type diploid strain, JO226, in a one-step gene replacement procedure. Integration at the correct locus was diagnosed by screening leucine prototrophs by a whole yeast PCR procedure, using primers against the *LEU2* gene and sequences adjacent to the *BRR2* gene. Tetrads from two diploids heterozygous for the gene disruption were dissected onto YEPD plates, which were incubated at room temperature.

RESULTS

Identification of cs mutants defective in RNA splicing: We obtained the bank of 350 cs mutants of *S. cerevisiae* that had been generated by MOIR *et al.* (1982) for the isolation of cdc mutants; this collection had not been rescreened subsequently for any other defect (J. MULHOLLAND and D. BOTSTEIN, personal communication). To identify mutants that are cs because of defects in splicing, we shifted log phase cultures of each of the mutants from the permissive temperature of 30° to the nonpermissive temperature of 16° (see Figure 1). The

duration of this temperature shift was 10 hr, ~1.5 doublings for the wild-type parental strain at this temperature. This interval was similar in terms of generation time used previously in a similar screen of ts mutants (VIJAYRAGHAVAN *et al.* 1989); our hope was that a relatively short shift to the nonpermissive temperature would enrich the detection of genes whose products participate directly in splicing and whose inactivation therefore results in a rapid decrease in splicing efficiency. We next prepared total cellular RNA and evaluated the splicing efficiency of each of the mutants by Northern hybridization analysis, using oligonucleotide probes against the *CRY1* pre-mRNA (LARKIN and WOOLFORD 1983), which encodes a ribosomal protein, and U5 snRNA (PATTERSON and GUTHRIE 1987), which was used as an internal control for RNA loading. *CRY1* was chosen because of its sensitivity to RNA splicing defects in known mutants (S. NOBLE, unpublished observations).

We examined the resulting Northern blots for mutants with altered RNA profiles. The ratio of spliced mRNA to unspliced pre-mRNA is thought to provide the most reliable measure of splicing efficiency (PIKIELNY and ROSBASH 1985); however, the unspliced pre-*CRY1* species in wild-type cells and in some *prp* mutants is undetectable in our assay. Therefore, we focused on mutants that exhibited either a decrease in *CRY1* mRNA relative to the internal control (U5) or an increase in unspliced pre-mRNA or lariat intermediate (which have similar electrophoretic mobilities in this assay) relative to mRNA. Ninety-eight candidates were identified that exhibited a difference in these ratios (data not shown). Eighty-two candidates exhibited a decrease in the ratio of mature *CRY1* mRNA to U5—with no apparent change in pre-mRNA levels—while 14 exhibited an increase in the ratio of pre-mRNA to mRNA. Two candidates demonstrated an increase in the ratio of mature *CRY1* mRNA to U5 snRNA, but the absolute level of U5 snRNA appeared to be very low. This pattern, while not indicative of a defect in splicing efficiency *per se,* is consistent with a defect in U5 synthesis or stability.

To determine which of these mutants exhibited general defects in splicing, we examined three other pre-mRNAs: *SNR17A, SNR17B,* and *RP51A. SNR17A* encodes the nucleolar U3 snoRNA, as does *SNR17B.* The two genes differ in the size and sequence of the introns that interrupt the coding sequence (MYSLINSKI *et al.* 1990). By examining the splicing of a number of pre-mRNAs in known mutants, we have found that the levels of *SNR17A* and *SNR17B* pre-snoRNAs are highly sensitive indicators of defects in the first chemical step of splicing (S. NOBLE, unpublished data). Likewise, the level of lariat intermediate for the *RP51A* transcript, which encodes a ribosomal protein (TEEM and ROSBASH 1983), is a sensitive indicator for defects in the second chemical step of splicing (S. NOBLE, unpublished observations). Using a primer extension assay, we analyzed the 98 candidates for their ability to splice *SNR17A, SNR17B,* and *RP51A.* Twenty candidates exhibited an increase in the levels of precursor or splicing intermediates for all three gene transcripts; those meeting certain additional criteria (described below) are represented in Table 3. Seventeen of these exhibited first-step defects with the three substrates (accumulation of precursor), whereas three exhibited a second-step defect (accumulation of the *RP51A* lariat intermediate; these mutants also accumulated pre-mRNA). An example of this analysis for 12 candidate mutants, as well as wild-type and *prp18-1* negative and positive controls, is shown in Figure 2. Several of these mutants (DBY4172, 4217, 4234, 4236, 4276), show accumulation of *RP51A* pre-mRNA, while DBY4172 demonstrates in addition the accumulation of lariat intermediate. (Note that the fraction of mutants with splicing defects was uncharacteristically high on this gel, which represents some of the candidates with the strongest phenotypes with *CRY1*

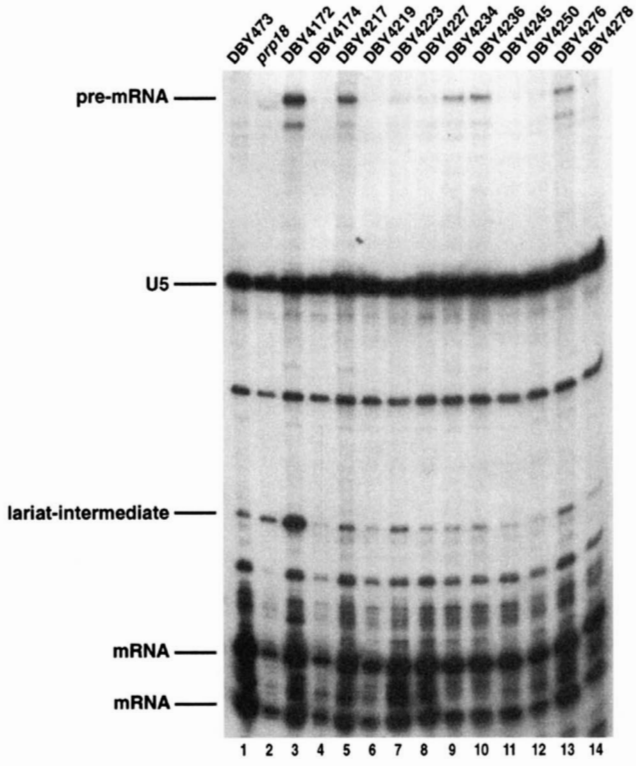


FIGURE 2.—RNA profiles of candidate mutants. Total RNA was extracted from the indicated cs mutants after a shift to 16° (see MATERIALS AND METHODS) and analyzed by a primer-extension method using an oligonucleotide complementary to the second exon of the yeast *RP51A* gene. The positions of fully unspliced pre-mRNA, lariat intermediate, and mRNA are indicated. The two *RP51A* mRNA species result from transcription initiation at more than one site. Also shown are levels of U5 snRNA, which was analyzed as a control for RNA loading in the same reactions. DBY473 is the parental wild-type strain and illustrates the normal ratios among splicing intermediates; *prp18-1* was included as a standard for a mutant that affects the second step of splicing, resulting in an increase in the ratio of lariat intermediate to mature mRNA.

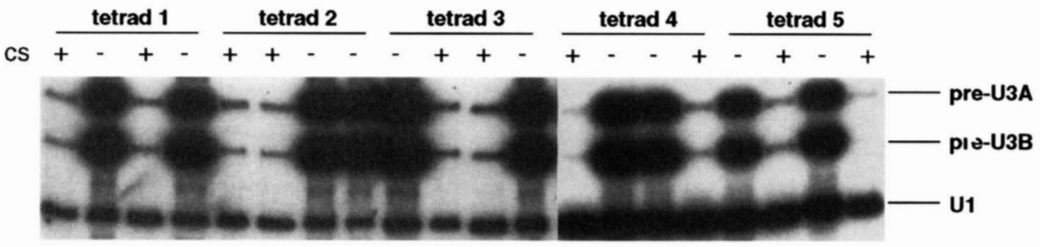


FIGURE 3.—Cosegregation analysis of DBY4340 (*brr2-1*). Shown is primer-extension analysis of RNA splicing efficiency among five tetrads derived from a cross of candidate DBY4340 to a wild-type strain. Bands corresponding to unspliced RNA from the *SNR17A* and *SNR17B* genes (encoding U3 snoRNA) are shown. The levels of U1 snRNA, which was analyzed in the same reactions as a control for RNA loading, is also shown. Indicated above each lane is the ability of the corresponding spore progeny to grow at 16° (−, cold sensitivity; +, cold resistance).

pre-mRNA.) In addition to these, 11 candidates displayed a decrease in mature *RP51A* message with no concomitant increase in precursor or intermediates (*e.g.*, DBY4174 in Figure 2); as this phenotype could result from mutation of factors that stabilize mRNA in addition to *bona fide* splicing factors, these candidates were not pursued further.

At least one ts RNA splicing mutant, *prp22*, has been reported to accumulate the excised lariat intron (VIJAYRAGHAVAN *et al.* 1989). Because the primer extension assays performed above were not able to detect the accumulation of this species (as the primers were directed against the second exon of *RP51A*), RNAs from the 20 candidates were examined by primer extension with a primer directed against the intron of *RP51A*. Using this primer, extension products from lariat intron comigrate with those from lariat intermediate. We found that only the three candidates already shown (above) to accumulate lariat intermediate also accumulate this lariat intermediate and/or lariat intron band (Table 3). Therefore, none of the mutants accumulate the lariat intron either in isolation or in combination with pre-mRNA.

Genetic analysis reveals nine complementation groups in which the cs growth defect is associated with the RNA splicing defect: To determine whether the cs alleles of the remaining candidates were dominant or recessive to wild type, the 20 strains were crossed to a wild-type strain of similar genetic background, and the diploids were examined for growth at 16°. All of the heterozygous diploids grew as well as a wild-type diploid, demonstrating that the cs mutants are recessive. One candidate was discarded because of its failure to mate.

To determine the number of genes represented by the remaining 19 mutants, the heterozygous diploids were sporulated, the resulting ascospores dissected, and *MAT***a** and *MAT*α progeny sorted for complementation analysis. Certain candidates were backcrossed to the wild-type strain multiple times at this stage, either because of poor spore viability or the presence of multiple cs$^-$ mutations; in cases of multiple independent cs$^-$ mutations, splicing was assessed for each of the segreg-

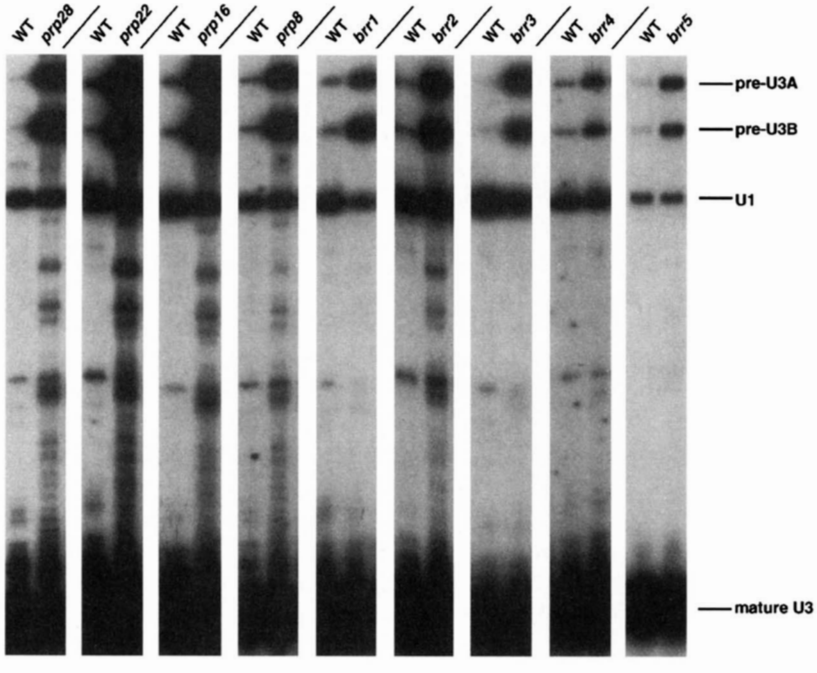
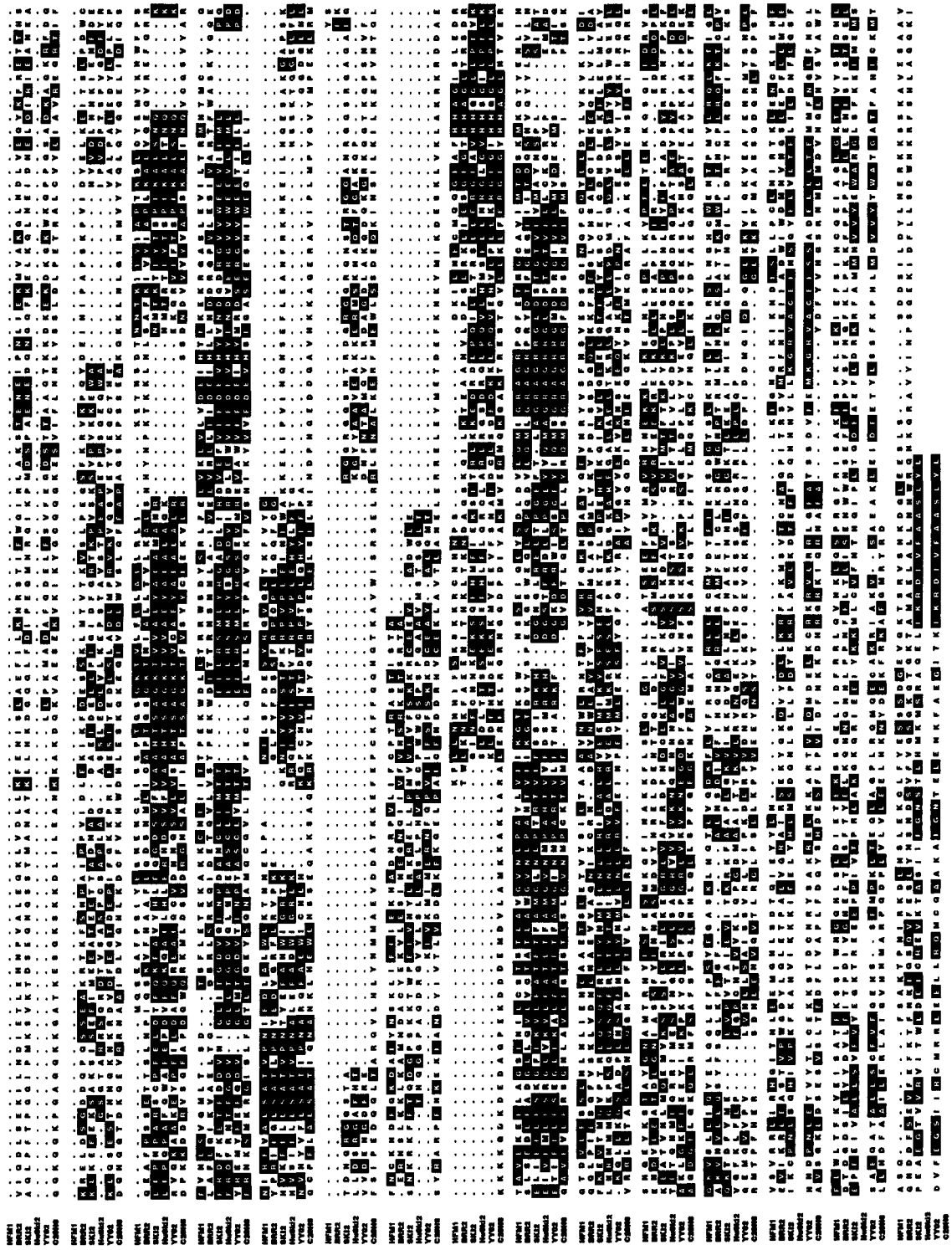


FIGURE 4.—Splicing defects among the nine cs complementation groups. A representative of each of the complementation groups identified in the screen as well as a wild-type sister spore were shifted to 16°. Total RNA was prepared and primer extension was used to visualize the splicing of pre-U3 snoRNA, as in Figure 4. Bands corresponding to pre-U3A and pre-U3B, mature U3 snoRNA (which is overexposed in these autoradiograms to allow visualization of the pre-snoRNA species), and the U1 snRNA internal control are indicated. While only single alleles of *PRP28* (*prp28-2*) and *PRP22* (*prp22-2*) are shown, the other alleles of these genes were found to exhibit similar splicing defects.


A.

B.

	MOTIF						
SUBFAMILY	I	IA	II	III	IV	V	VI
DEAD	AXTGXGKT	PTRELA	VLDEADXML	XSATXP	FXXT	TXVXARGXDXXV	HRIGRXGR
DEAH	GETGSGKT	PRRVAA	MIDEAHERT	TSATMN	FLTG	TSLTIDGIRYVI	QRXGRAGR
DE(I/V)H	AXTSAGKT	PXKALX	IFDE(I/V)HYI	LSATVP	FSXG	TETXAMGVNXPA	QMXGRAGR

FIGURE 5.—Brr2 belongs to a novel subfamily of RNA helicase-related proteins. (A) Alignment of Brr2 with related proteins. Shown is an alignment among the homologous sequences of Brr2; the yeast proteins, Hfm1 and Ski2; the hypothetical *C. elegans* protein, C28H8.3; and the two human proteins, HuSki2 and YY02. Amino acid identities are shown as filled boxes. The Brr2 amino acid sequence depicted begins at position 308 of the predicted full-length protein; the Hfm1, YY02, and HuSki2 sequences begin at the first amino acid; and the *C. elegans* sequence begins at amino acid 601. The sequence identity between Brr2 and Hfm1 is 21%, with a similarity of 46%. The sequence identity between Brr2 and Ski2 is 19%, with a similarity of 41%. These values were calculated using the Bestfit program in the GCG Wisconsin Sequence Analysis Package, using a gap penalty of 6 and a gap extension penalty of 0.1. Accession numbers for these sequences are as follows: Brr2 (SwisProt P32639), Hfm1 (GenBank U22156), Ski2 (SwissProt 35207), HuSki2 (GenBank U09877), YY02 (SwissProt P42285), and C28H8.3 (SwissProt Q09475). (B) Conserved helicase motifs. The conserved helicase motifs described by GORBALENYA *et al.* (1989) as they occur in Brr2 [DE(I/V)H] and in the DEAD and DEAH subfamilies are shown.

ants of a 4:0 cs^-:cs^+ tetrad. A complete matrix of crosses among the mutants, with testing of each diploid for growth at 16°, revealed the presence of 16 cs complementation groups.

Although these mutants were identified because of their defects in RNA splicing at the nonpermissive temperature, it remained possible that the cs growth defect was not associated with the RNA splicing defect. We therefore performed linkage analysis. Five tetrads from a heterozygous diploid (mutant crossed to wild type) representing each complementation group were examined. As in the initial screen, cells were shifted to the nonpermissive temperature and examined for splicing of U3 pre-snoRNA. Nine of the candidates exhibited perfect 2:2 cosegregation of the cs growth and splicing defects, indicating linkage between the two traits with 95% certainty; these are the mutants described in Table 3. Cosegregation analysis for such a mutant (derived from DBY4340) is shown in Figure 3. In three of the candidates, the two phenotypes were unlinked, and in four candidates, the splicing defect was not reproduced; these seven mutants were not characterized further.

Four of the complementation groups correspond to *PRP8, PRP16, PRP22,* and *PRP28:* Cs alleles existed for three previously identified splicing genes, *prp16-1, prp28-1,* and *slu7-1*(COUTO *et al.* 1987; STRAUSS and GUTHRIE 1991; FRANK *et al.* 1992). These were crossed to members of each of the nine remaining complementation groups, and the resulting diploids were tested for growth at 16°. *prp28-1* (YGS5) failed to complement cs strains derived initially from DBY4234, DBY4236 and DBY4489. *prp16-1* (YS111) failed to complement a descendant of DBY4406. Linkage of these mutations was confirmed by cosegregation analysis of growth phenotypes among the meiotic progeny.

As the remainder of previously identified *prp* mutants existed only as ts alleles, we could not determine potential allelism by assessing the cold sensitivity of the heterozygous diploids. We therefore isolated wild-type complementing plasmids for the seven remaining, unidentified candidate splicing factors and tested for their ability to complement the known ts splicing mutants (see MATERIALS AND METHODS). The mutants tested (Table 1) were *prp2–9, prp10/11* (HARTWELL *et al.* 1970; ROSBASH *et al.* 1981); *prp17–22, prp24, prp27* (VIJAYRAGHAVEN *et al.* 1989); *slu1, slu2*(FRANK *et al.* 1992); *prp38, prp39* (BLANTON *et al.* 1992; LOCKHART and RYMOND 1994); and *prp30–34* (J. WOOLFORD, personal communication). Two mutants were complemented by clones from the screen: *prp8-7,* by the plasmid corresponding to the strain DBY4490; and *prp22-1,* by that corresponding to DBY4172 (Table 3).

Complementing clones for candidates derived from the strains DBY4483, DBY4340, DBY4217, DBY4475, and DBY4276 failed to complement any of the previously identified splicing mutants, suggesting that they correspond to novel genes. These were named *BRR1, BRR2, BRR3, BRR4,* and *BRR5,* respectively (Bad Response to Refrigeration; Table 3). Figure 4 displays the splicing defects of each of these mutants with respect to pre-U3 snoRNA. The defects of the previously identified splicing factors as well as of *brr2-1* and *brr3-1* are quite strong; those of *brr1-1* and *brr5-1* are moderate; and that of *brr4-1* is the weakest.

***BRR2* encodes a helicase-like protein:** As an initial step toward the molecular characterization of the *BRR* genes, we analyzed *BRR2,* mutation of which leads to a strong defect before the first chemical step of splicing. The 12-kb insert of the original complementing clone was trimmed to a 7.1-kb minimal complementing fragment as described in MATERIALS AND METHODS. Partial sequencing of this region revealed that *BRR2* is located on chromosome *V* (Swiss Protein database accession number P32639). A long open reading frame of 2163 amino acids spans almost the entire length of the minimal complementing fragment.

To determine whether the gene that complements *brr2-1*corresponds to *bona fide BRR2* (rather than being a low-copy suppressor), we created a *URA3*-marked version of the gene, which was integrated at its chromosomal locus in a wild-type strain. This strain was crossed to a cs *brr2-1* strain, followed by sporulation of the diploid, dissection of ascospores, and assessment of the cs and uracil auxotrophic phenotypes among the meiotic progeny. Among 11 four-spore tetrads, all cs spore progeny were also uracil auxotrophs, and all cold-resistant progeny were uracil prototrophs, confirming that the cloned gene that complements the *brr2-1* mutation is genetically linked to the *BRR2* locus.

Searches of the database with the Brr2 amino acid sequence identified two other yeast proteins with significant sequence homology, as well as two sequences from humans and one from *Caernorhabditis elegans* (Figure 5A). The two yeast proteins are Hfm1 and Ski2. One of the human genes encodes a close homologue of Ski2, while the other human sequence and the worm sequence appear not to be close homologues of either yeast protein. Each of these proteins contains a 400 amino acid region more distantly related to the eIF4A family of RNA helicases, such that variants of the six motifs characteristic of the RNA helicase superfamily can be identified (Figure 5B; GORBALENYA *et al.* 1989). Interestingly, the ~1000 amino acid region of homology among the six proteins extends both upstream and downstream of this helicase-related domain (Figure 5A).

***BRR2* is essential for viability:** Because RNA splicing is essential for cell growth, we sought to determine whether the *BRR2* gene was required for cell viability. A disrupted allele was created in the cloned gene by replacing an 831 amino acid region of *BRR2,* including the entire helicase-like domain, with *LEU2* (Figure 6A). The *brr2::LEU2* fragment was liberated from vector sequences and used to transform a wild-type diploid strain, JO226, in a one-step gene replacement procedure. The resulting strain, which was heterozygous for the disruption, was sporulated and 20 tetrads were dissected. The spore progeny germinated to yield 2:0 (13 cases) 1:0 (four cases) and 0:0 (three cases) segregation of viability:inviability (Figure 6B). All surviving progeny were leucine auxotrophs (Figure 6B), indicating that *BRR2* is essential for viability.

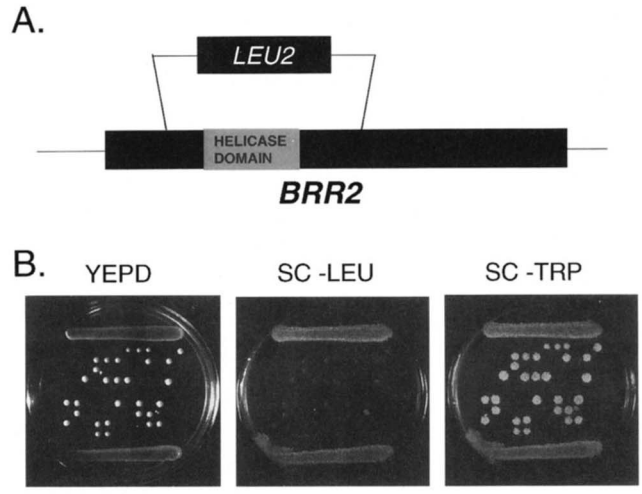


FIGURE 6.—*BRR2* is an essential gene. (A) Disrupted allele. A fragment of the *BRR2* gene that encodes amino acids 352–1185 was replaced with the yeast *LEU2* gene. The deleted portion includes the helicase-related domain, as well as other conserved sequences. (B) Analysis of gene disruption. The disrupted gene depicted in A was used to replace one of two endogenous copies of the *BRR2* gene in a wild-type diploid strain (see MATERIALS AND METHODS); this strain is normally auxotrophic for leucine, prototrophic for tryptophan. The heterozygous strain was sporulated and the resulting ascospores were dissected onto a YEPD plate. Spore progeny were subsequently replica-plated to synthetic complete plates lacking either leucine (SC-LEU) or tryptophan (SC-TRP).

DISCUSSION

Cold sensitivity as a route to novel RNA splicing genes: Previous efforts to identify genes involved in RNA splicing have relied heavily on screens of collections of ts yeast strains. This approach has been highly successful; however, the existence of genetic hotspots for temperature sensitivity as well as the apparent inability of many essential genes to mutate to temperature sensitivity (DIEHL and PRINGLE 1991; HARRIS and PRINGLE 1991) suggests that a large number of splicing genes have been overlooked by this method. Reasoning that screens for splicing mutants based on a different conditional phenotype might circumvent these problems, we examined a collection of cs mutants (MOIR *et al.* 1982). We chose for study those mutants that exhibited defects in the splicing of four cellular pre-mRNAs. We identified nine complementation groups in which such a biochemical splicing defect was genetically linked to the cs growth defect. Four of these correspond to *bona fide* splicing genes that have been identified previously (*PRP8, PRP16, PRP22, PRP28*), validating the specificity of the screen. The remaining five genes (*BRR1, BRR2, BRR3, BRR4,*and *BRR5*) fail to complement the ts *prp* mutants; moreover, sequence analysis of *BRR1* (S. NOBLE and C. GUTHRIE, unpublished data), *BRR2* (this manuscript), *BRR3* (T. AWABDY and C. GUTHRIE, unpublished data), and *BRR5* (S. NOBLE, unpublished data) reveals them to be novel genes. Finally, *in vitro* characterization of the *BRR1* gene product has demonstrated it to associate specifically with each of the spliceosomal snRNPs (S. NOBLE and C. GUTHRIE, unpublished observations), suggesting a direct role in splicing for at least one of the novel genes.

These results compare favorably with the studies of VIJAYRAGHAVAN *et al.* (1989), who screened 1000 ts mutants by Northern hybridization and identified ts alleles of eight genes that had not been identified previously. Our screen involved approximately one-third the number of mutants and identified five new genes. Our suc-

cess in obtaining the *BRR* genes suggests that screening cs mutants is an efficient alternative method of identifying novel genes involved in RNA splicing. These results parallel those of MOIR *et al.* (1982) who screened for cs *cdc* mutants. Like us, they identified nine complementation groups. Six (*CDC44, CDC45, CDC48, CDC49, CDC50, CDC51*) proved to be genes not identified previously in a screen of 1500 ts mutants by HARTWELL *et al.* (1973), whereas one had been identified previously as *CDC11*. The other two complementation groups were not analyzed with respect to the ts collection of HARTWELL *et al.*

Alleles of certain genes were represented in our screen more than once, the most frequent being the three alleles of *PRP28*. As mentioned above, *PRP28* was identified previously by a single mutation in a pilot screen of 18 cs strains (STRAUSS and GUTHRIE 1991). Interestingly, no alleles of *PRP28* have been isolated in subsequent screens of ts collections (VIJAYRAGHAVAN *et al.* 1989; B. RYMOND, personal communication; J. WOOLFORD, personal communication), despite the fact that this gene is essential for growth at all temperatures (STRAUSS and GUTHRIE 1991). These data are consistent with the idea that hotspots (which reflect the propensity of a gene to mutate to a given phenotype) differ for ts and cs mutations.

In both our screen for cs splicing-defective mutants and previous screens of ts mutants, the majority of genes were represented as single alleles, demonstrating that the screens are far from saturating (Tables 3 and 4; VIJAYRAGHAVAN *et al.* 1989; B. RYMOND, personal communication; J. WOOLFORD, personal communication). Although we lack precise estimates for the number of genes involved in splicing, the fact that more than half of the genes identified in our screen were previously unknown suggests that a large number of genes required for splicing have yet to be discovered. This notion reinforces the need to identify new and effective strategies for locating the remaining genes.

***BRR2* encodes an essential member of a novel class of helicase-related proteins:** A number of factors involved in RNA splicing are related in sequence. For instance, Prp2, Prp16 and Prp22 define a subfamily of helicase-related proteins (the so-called DEAH subfamily) that are required before the first chemical step of splicing, the second chemical step, and the spliceosome dissassembly reaction, respectively (reviewed by SCHMID and LINDER 1992). Two members of a different subfamily (the DEAD subfamily), Prp5 and Prp28, are also required for splicing. Analysis of the *BRR2* gene revealed it to encode a large protein related to the RNA helicase superfamily; however, closer inspection of its sequence exposed differences with the DEAD and DEAH families (Figure 5B). These are most readily apparent in the conserved RNA helicase motifs described by GORBALENYA *et al.* (1989). For example, helicase motif II (for which the DEAD family was named) consists in the Brr2 family of the consensus IFDE(I/V)HYI (Figure 5B), whereas the consensus is VLDEADML for the DEAD family. In addition, the homology within the Brr2 family extends both upstream and downstream of the 400 amino acid domain related to RNA helicases, such that the region of amino acid similarity spans ~1000 amino acids (Figure 5A). Taken together, these results demonstrate that Brr2 belongs to a novel subfamily of RNA helicase-like proteins that contain extensive sequence homology in regions unrelated to other RNA helicase family members.

Do the functions of the other genes in this family provide clues about the possible function of Brr2? Mutations in the *SKI2* gene reduce the resistance of *S. cerevisiae* to double-stranded RNA killer viruses (RIDLEY *et al.* 1984). It has been proposed that Ski2 normally functions in concert with the ribosome to discriminate against translation of uncapped cytoplasmic RNAs, such as cellular mRNAs that have been targeted for degradation as well as viral genomic RNAs (WIDNER and WICKNER 1993; JOHNSON and KOLODNER 1995; MASISON *et al.* 1995). Human Ski2 has been demonstrated to possess a small amount of ATPase activity (DANGEL *et al.* 1995); RNA-dependent ATPase activity is a known feature of several of the helicase-like factors required for splicing. Hfm1, the second yeast protein with homology to Brr2, was identified as a DNA binding protein with affinity for *GAL1-GAL10* operator sequences (R. W. WEST, unpublished observations). Unlike most canonical splicing factors, neither Hfm1 nor Ski2 is essential for viability. Thus, it seems likely that Brr2, Ski2, and Hfm1 are members of a helicase-like subfamily that participates in diverse cellular processes.

In the case of Brr2, the existence of dynamic RNA-RNA interactions in the spliceosome may be relevant to its function. With the important caveat that none of the six helicase-like factors (including Brr2) required for splicing has been demonstrated to possess helicase activity *in vitro*, the large number of dynamic spliceosomal RNA duplexes is certainly intriguing (reviewed by MADHANI and GUTHRIE 1994). Of 12 such RNA-RNA duplexes known to exist among splicing components, at least four must be remodeled for the splicing reaction to occur, and the remainder must be disrupted if snRNPs are to be recycled. Brr2 is the largest helicase-like factor yet identified in the splicing pathway, with large N- and C-terminal domains unrelated to other proteins. Because the *brr2-1* mutant inhibits splicing before the first step, the Brr2 protein may function in one of the rearrangements that occur at this time, such as the destabilization of the extensive U4-U6 interaction and the formation of the putative catalytic core of the spliceosome, involving U2 and U6 snRNAs (MADHANI and GUTHRIE 1992). It is therefore interesting that *BRR2* has been identified independently by another group as a mutant that is synthetically lethal with a mutant U2 snRNA (D. XU and J. FRIESEN, personal com-

TABLE 4

Summary of previous screens for temperature-sensitive splicing mutants

Screen	Mutagen	No. of screened, conditional phenotype	Assay	Percentage defective in splicing[a]	Genes identified in screen[b]	No. of alleles
HARTWELL *et al.* (1970)	Nitrosoguanidine	400, ts	^{14}C pulse-labeling of RNA synthesis	5.8	*PRP2*	6
					PRP3	5
					PRP4	4
					PRP5	2
					PRP6	1
					PRP7	1
					PRP8	1
					PRP9	1
					PRP10 = *PRP11*	2
VIJAYRAGHAVAN *et al.* (1989)	Ethylmethane sulfonate	1000, ts	Northern blot (*ACT1, CRY1*); primer extension (*RP51A*)	3.3	*PRP2*	6
					PRP3	7
					PRP6	6
					PRP9	2
					PRP17	2
					PRP18	3
					PRP19	1
					PRP20	1
					PRP21	1
					PRP22	1
					PRP16/23	1
					PRP24	1
					PRP25[c]	1
					PRP26[c]	3
					PRP27	1
J. MADDOCK, J. ROY, and J. WOOLFORD (personal communication)	Ethylmethane sulfonate	426, ts	Northern blot (*CRY1, ACT1*)	4.5	*PRP2*	5
					PRP3	2
					PRP6	1
					PRP16/23	1
					PRP18	2
					PRP19	1
					PRP26[c]	1
					PRP29	1
					PRP30	1
					PRP31	1
					PRP32	1
					PRP33	1
					PRP34	2
BLANTON *et al.* (1992); LOCKHART and RYMOND (1994); S. SCHULTE, A. SRINIVASAN, S. LOCKHART, and B. RYMOND (unpublished data)	Ethylmethane sulfonate	345, ts	Northern blot (*RP51A*)	4.1	*PRP2*	4
					PRP4	1
					PRP16	2
					PRP22	1
					PRP26[c]	1
					PRP38	1
					PRP39	1
					Uncharacterized	2

[a] Percentage represents those mutants that accumulate pre-mRNA or splicing intermediates at the nonpermissive temperature (as opposed to loss of mRNA only) and which exhibit linkage between their splicing and ts growth defects.

[b] Mutants identified for the first time are underlined.

[c] The alleles of *prp25* and *prp26* are unlinked to the ts mutations.

munication). Determining whether Brr2 functions as a helicase involved in spliceosomal RNA rearrangements will require its characterization *in vitro*.

Of the nine genes identified in this screen of ~300 cs mutants, four are homologous to RNA helicases. Previous screens of ts collections, involving >2000 strains, had also identified four factors sharing this domain. The enrichment for this class of factors in the cs screen suggests that helicase-like proteins may be particularly mutable to the cold-sensitive phenotype. In principle, a predisposition to cold sensitivity could reflect an inherent property of certain classes of proteins (for instance, the ability to be trapped in one conformation, such as a particular nucleotide-bound state) or of the process on which they act (for example, helix unwinding, which is more difficult at low temperatures). Regardless of the exact mechanism, screens of cs mutants might target processes that, in some cases, may be resistant to mutation to temperature sensitivity. In addition, cs mutants can potentially be used as bio-

chemical tools to trap structures such as the spliceosome in states corresponding to previously unrecognized intermediates. Precedents for this possibility come from studies of ribosome assembly and the U2 snRNP, in which cs mutants (affecting 16S rRNA and U2 snRNA, respectively) have been shown to result in the stabilization of conserved, alternative RNA structures (ZAVANELLI and ARES 1991; DAMMEL and NOLLER 1993, 1995; ZAVANELLI *et al.* 1994).

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LITERATURE CITED

ABOVICH, N., X. C. LIAO and M. ROSBASH, 1994 The yeast MUD2 protein: an interaction with PRP11 defines a bridge between commitment complexes and U2 snRNP addition. Genes Dev. **8:** 843–854.

BENNETT, M., and R. REED, 1993 Correspondence between a mammalian spliceosome component and an essential yeast splicing factor. Science **262:** 105–108.

BLANTON, S., A. SRINIVASAN and B. C. RYMOND, 1992 PRP38 encodes a yeast protein required for pre-mRNA splicing and maintenance of stable U6 small nuclear RNA levels. Mol. Cell. Biol. **12:** 3939–3947.

BROSI, R., K. KRONING, S. E. BEHRENS, R. LÜHRMANN, and A. KRÄMER, 1993 Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. Science **262:** 102–105.

CHAMPION-ARNAUD, P., and R. REED, 1994 The prespliceosome components SAP 49 and SAP 145 interact in a complex implicated in tethering U2 snRNP to the branch site. Genes Dev. **8:** 1974–1983.

CHIARA, M. D., P. CHAMPION-ARNAUD, M. BUVOLI, B. NADAL-GINARD and R. REED, 1994 Specific protein-protein interactions between the essential mammalian spliceosome-associated proteins SAP 61 and SAP 114. Proc. Natl. Acad. Sci. USA **91:** 6403–6407.

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

COUTO, J. R., J. TAMM, R. PARKER and C. GUTHRIE, 1987 A trans-acting suppressor restores splicing of a yeast intron with a branch point mutation. Genes Dev. **1:** 445–455.

COX, J. H., and H. B. STRACK, 1971 Cold-sensitive mutants of bacteriophage lambda. Genetics **67:** 5–17.

DAMMEL, C. S., and H. F. NOLLER, 1993 A cold-sensitive mutation in 16S rRNA provides evidence for helical switching in ribosome assembly. Genes Dev. **7:** 660–670.

DAMMEL, C. S., and H. F. NOLLER, 1995 Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. Genes Dev. **9:** 626–637.

DANGEL, A. W., L. SHEN, A. R. MENDOZA, L. C. WU and C. Y. YU, 1995 Human helicase gene SKI2W in the HLA class III region exhibits striking structural similarities to the yeast antiviral gene SKI2 and to the human gene KIAA0052: emergence of a new gene family. Nucleic Acids Res. **23:** 2120–2126.

DIEHL, B. E., and J. R. PRINGLE, 1991 Molecular analysis of Saccharomyces cerevisiae chromosome I: identification of additional transcribed regions and demonstration that some encode essential functions. Genetics **127:** 287–298.

ELLEDGE, S. J., and R. W. DAVIS, 1988 A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in Saccharomyces cerevisiae. Gene **70:** 303–312.

FRANK, D., B. PATTERSON and C. GUTHRIE, 1992 Synthetic lethal mutations suggest interactions between U5 small nuclear RNA and four proteins required for the second step of splicing. Mol. Cell. Biol. **12:** 5197–5205.

GORBALENYA, A. E., E. V. KOONIN, A. P. DONCHENKO and V. M. BLINOV, 1989 Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. **17:** 4713–4730.

GUTHRIE, C., 1991 Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science **253:** 157–163.

GUTHRIE, C., and G. R. FINK, 1991 *Guide to Yeast Genetics and Molecular Biology.* Academic Press, San Diego.

GUTHRIE, C., H. NASHIMOTO and M. NOMURA, 1969a Structure and function of E. coli ribosomes. 8. Cold-sensitive mutants defective in ribosome assembly. Proc. Natl. Acad. Sci. USA **63:** 384–391.

GUTHRIE, C., H. NASHIMOTO and M. NOMURA, 1969b Studies on the assembly of ribosomes in vivo. Cold Spring Harbor Symp. Quant. Biol. **34:** 69–75.

HARRIS, S. D., and J. R. PRINGLE, 1991 Genetic analysis of Saccharomyces cerevisiae chromosome I: on the role of mutagen specificity in delimiting the set of genes identifiable using temperature-sensitive-lethal mutations. Genetics **127:** 279–285.

HARTWELL, L. H., C. S. MCLAUGHLIN and J. R. WARNER, 1970 Identification of ten genes that control ribosome formation in yeast. Mol. Gen. Genet. **109:** 42–56.

HARTWELL, L. H., R. K. MORTIMER, J. CULOTTI, and M. CULOTTI 1973 Genetic control of the cell division cycle in yeast. V. Genetic analysis of the mutants. Genetics **74:** 267–286.

HERMANN, H., P. FABRIZIO, V. A. RAKER, K. FOULAKI, H. HORNIG *et al.*, 1995 snRNP Sm proteins share two evolutionarily conserved sequence motifs which are involved in Sm protein-protein interactions. EMBO J. **14:** 2076–2088.

JARVIK, J., and D. BOTSTEIN, 1975 Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. Proc. Natl. Acad. Sci. USA **72:** 2738–2742.

JOHNSON, A. W., and R. D. KOLODNER, 1995 Synthetic lethality of sep1 (xrn1) ski2 and sep1 (xrn1) ski3 mutants of Saccharomyces cerevisiae is independent of killer virus and suggests a general role for these genes in translation control. Mol. Cell. Biol. **15:** 2719–2727.

KAO, H. Y., and P. G. SILICIANO, 1992 The yeast homolog of the U1 snRNP protein 70K is encoded by the SNP1 gene. Nucleic Acids Res. **20:** 4009–4013.

KONARSKA, M. M., P. J. GRABOWSKI, R. A. PADGETT and P. A. SHARP, 1985 Characterization of the branch site in lariat RNAs produced by splicing of mRNA precursors. Nature **313:** 552–557.

KRÄMER, A., P. LEGRAIN, F. MULHAUSER, K. GRÖNING, R. BROSI *et al.*, 1994 Splicing factor SF3a60 is the mammalian homologue of PRP9 of *S.cerevisiae*: the conserved zinc finger-like motif is functionally exchangeable *in vivo*. Nucleic Acids Res. **22:** 5223–5228.

KRÄMER, A., F. MULHAUSER, C. WERSIG, K. GRÖNING and G. BILBE, 1995 Mammalian splicing factor SF320 represents a new member of the SURP family of proteins and is homologous to the essential splicing factor PRP21p of *Saccharomyces cerevisiae*. RNA **1:** 260–272.

LARKIN, J. C., and J. J. WOOLFORD, 1983 Molecular cloning and analysis of the CRY1 gene: a yeast ribosomal protein gene. Nucleic Acids Res. **11:** 403–420.

LAST, R. L., J. R. MADDOCK and J. L. WOOLFORD, JR., 1987 Evidence for related functions of the RNA genes of Saccharomyces cerevisiae. Genetics **117:** 619–631.

LESSER, C. F., and C. GUTHRIE, 1993 Mutational analysis of pre-mRNA splicing in Saccharomyces cerevisiae using a sensitive new reporter gene, CUP1. Genetics **133:** 851–863.

LIAO, X. C., J. TANG and M. ROSBASH, 1993 An enhancer screen identifies a gene that encodes the yeast U1 snRNP A protein: implications for snRNP protein function in pre-mRNA splicing. Genes Dev. **7:** 419–428.

LOCKHART, S. R., and B. C. RYMOND, 1994 Commitment of yeast

pre-mRNA to the splicing pathway requires a novel U1 small nuclear ribonucleoprotein polypeptide, Prp39p. Mol. Cell. Biol. **14:** 3623–3633.

MADHANI, H. D., and C. GUTHRIE, 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., and C. GUTHRIE, 1994 Dynamic RNA-RNA interactions in the spliceosome. Annu. Rev. Genet. **28:** 1–26.

MASISON, D. C., A. BLANC, J. C. RIBAS, K. CARROLL, N. SONENBERG *et al.*, 1995 Decoying the cap-mRNA degradation system by a double-stranded RNA virus and poly(A)-mRNA surveillance by a yeast antiviral system. Mol. Cell. Biol. **15:** 2763–2771.

MOIR, D., S. E. STEWART, B. C. OSMOND and D. BOTSTEIN, 1982 Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics **100:** 547–563.

MOORE, M. J., and P. A. SHARP, 1993 Evidence for two active sites in the spliceosome provided by stereochemistry of pre-mRNA splicing. Nature **365:** 364–368.

MOORE, M. J., C. C. QUERY and P. A. SHARP, 1993 Splicing of precursors to mRNA by the spliceosome, pp. 303–358 in *The RNA World,* edited by R. F. GESTELAND and J. F. ATKINS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MYSLINSKI, E., V. SEGAULT and C. BRANLANT, 1990 An intron in the genes for U3 small nucleolar RNAs of the yeast Saccharomyces cerevisiae. Science **247:** 1213–1216.

PATTERSON, B., and C. GUTHRIE, 1987 An essential yeast snRNA with a U5-like domain is required for splicing *in vivo.* Cell **49:** 613–624.

PATTERSON, B., and C. GUTHRIE, 1991 A U-rich tract enhances usage of an alternative 3′ splice site in yeast. Cell **64:** 181–187.

PATTON, J. G., E. B. PORRO, J. GALCERAN, P. TEMPST and B. NADAL-GINARD, 1993 Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev. **7:** 393–406.

PIKIELNY, C. W., and M. ROSBASH, 1985 mRNA splicing efficiency in yeast and the contribution of nonconserved sequences. Cell **41:** 119–126.

RIDLEY, S. P., S. S. SOMMER and R. B. WICKNER, 1984 Superkiller mutations in Saccharomyces cerevisiae suppress exclusion of M2 double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and L-A-HN. Mol. Cell. Biol. **4:** 761–770.

ROSBASH, M., P. K. HARRIS, J. J. WOOLFORD and J. L. TEEM, 1981 The effect of temperature-sensitive RNA mutants on the transcription products from cloned ribosomal protein genes of yeast. Cell **24:** 679–686.

ROSE, M. D., P. NOVICK, J. H. THOMAS, D. BOTSTEIN and G. R. FINK, 1987 A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene **60:** 237–243.

ROY, J., B. ZHENG, B. C. RYMOND and J. J. WOOLFORD, 1995 Structurally related but functionally distinct yeast Sm D core small nuclear ribonucleoprotein particle proteins. Mol. Cell. Biol. **15:** 445–455.

RUSKIN, B., A. R. KRAINER, T. MANIATIS and M. R. GREEN, 1984 Excision of an intact intron as a novel lariat structure during pre-mRNA splicing *in vitro.* Cell **38:** 317–331.

RYMOND, B., and M. ROSBASH, 1992 Yeast pre-mRNA splicing, pp. 143–192 in *The Molecular and Cellular Biology of the Yeast Saccharomyces,* edited by E. W. JONES, J. R. PRINGLE and J. R. BROACH. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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. Proc. Natl. Acad. Sci. USA **90:** 848–852.

SAMBROOK, J., E. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SCHMID, S. R., and P. LINDER, 1992 D-E-A-D protein family of putative RNA helicases. Mol. Microbiol. **6:** 283–291.

SCOTTI, P. D., 1968 A new class of temperature conditional lethal mutants of bacteriophage T4D. Mutat. Res. **6:** 1–14.

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 J. **14:** 2089–2098.

SIKORSKI, R. S., and P. HIETER, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122:** 19–27.

SMITH, V., and B. G. BARRELL, 1991 Cloning of a yeast U1 snRNP 70K protein homologue: functional conservation of an RNA-binding domain between humans and yeast. EMBO J. **10:** 2627–2634.

STRAUSS, E. J., and C. GUTHRIE, 1991 A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family. Genes Dev. **5:** 629–641.

TAI, P. C., D. P. KESSLER and J. INGRAHAM, 1969 Cold-sensitive mutations in Salmonella typhimurium which affect ribosome synthesis. J. Bacteriol. **97:** 1298–1304.

TEEM, J. L., and M. ROSBASH, 1983 Expression of a beta-galactosidase gene containing the ribosomal protein 51 intron is sensitive to the rna2 mutation of yeast. Proc. Natl. Acad. Sci. USA **80:** 4403–4407.

VIJAYRAGHAVAN, U., M. COMPANY and J. ABELSON, 1989 Isolation and characterization of pre-mRNA splicing mutants of Saccharomyces cerevisiae. Genes Dev. **3:** 1206–1216.

WIDNER, W. R., and R. B. WICKNER, 1993 Evidence that the SKI antiviral system of Saccharomyces cerevisiae acts by blocking expression of viral mRNA. Mol. Cell. Biol. **13:** 4331–4341.

ZAVANELLI, M. I., and M. J. ARES, 1991 Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. Genes Dev. **5:** 2521–2533.

ZAVANELLI, M. I. , J. S. BRITTON, A. H. IGEL and M. J. ARES, 1994 Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. Mol. Cell. Biol. **14:** 1689–1697.

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