A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family

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We have isolated a cold-sensitive mutant of *Saccharomyces cerevisiae* in which the first step of mRNA splicing is inhibited. The growth and splicing defects are recessive and cosegregate, thus defining a single essential gene (*PRP28*). The wild-type *PRP28* gene was cloned, and sequence analysis reveals extensive homology to a family of proteins that are thought to function as ATP-dependent RNA helicases. The cold sensitivity is caused by a glycine-to-glutamic acid change in a conserved sequence motif. Interestingly, double mutants containing conditional alleles of *PRP28* and *PRP24*, which encodes a U6 snRNA-binding protein, are inviable. In addition, a suppressor of *prp28-1* is a mutant allele of *PRP8*, which encodes a U5 protein, thus linking *PRP28* with U5. These data are consistent with a scenario in which *PRP28* acts to unwind the U4/U6 base-pairing interaction in the U4/U6/U5 snRNP, facilitating the first covalent step of splicing.

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major goal in understanding splicing is elucidating the mechanisms of conformational rearrangements during assembly of the spliceosome.

We set out to identify new trans-acting factors involved in mRNA splicing in _S. cerevisiae_. In particular, we were interested in generating cold-sensitive splicing mutants for two reasons. First, macromolecular assembly processes are likely to be particularly sensitive to cold. It has been argued on thermodynamic grounds that in general, mutational defects affecting assembly processes may be intensified at low temperatures due to the largely hydrophobic nature of the interactions driving these reactions (Cantor and Schimmel 1980). This predisposition to cold sensitivity has been borne out for the biopolymers tubulin and actin (Huffaker and Botstein 1988; Novick et al. 1989), as well as phage head structures (Jarvik and Botstein 1975) and ribosomal proteins (Guthrie et al. 1969; Tai et al. 1969), for which many cold-sensitive alleles have been found. Second, some gene products are inherently more likely to mutate to cold rather than heat sensitivity. There are some genes for which multiple cold-sensitive alleles, but no temperature-sensitive alleles (and vice versa), have been isolated. The cell division cycle (cdc) class of mutants illustrate this point (Moir et al. 1982). Therefore, the genetic target set for cold sensitivity is likely to differ from that for temperature sensitivity. We thus hoped to find cold-sensitive splicing mutants defective in spliceosome assembly that would shed light on functional interactions between the components of the splicing machinery.

Here, we report the isolation of a cold-sensitive splicing mutant that defines a member of a putative ATP-dependent RNA helicase gene family. These proteins are thought to unwind RNA in an ATP-dependent fashion, on the basis of the in vitro activity of the founding member elf4A (Rozen et al. 1990). As a first step toward determining the function of this protein, we looked for genetic interactions with other splicing factors. The results from this analysis point to a functional relationship between this protein and the U4/U6/U5 snRNP. This protein is an excellent candidate for catalyzing the destabilization of U4/U6, thus facilitating the first covalent step of splicing.

**Results**

**Isolation of a cold-sensitive splicing mutant**

Wild-type haploid yeast cells were UV-mutagenized to 1% survival. Following growth at 25°C, the colonies formed by survivors were replica-plated and incubated at 16°C and 37°C. Isolates with conditional growth phenotypes were identified: Of 3000 candidates, 18 were cold-sensitive and 31 were temperature-sensitive.

To test whether any of these conditional mutants had splicing defects, primer extension analysis was carried out on RNA obtained after growth to mid-log phase at 25°C, followed by a shift to the nonpermissive temperature for one to two generations. We used a primer specific to the intron-containing _MATα1_ gene that enabled us to detect pre-mRNA, mRNA, and splicing intermediates. One cold-sensitive strain and three of the temperature-sensitive strains accumulated pre-mRNA relative to a wild-type control, while one temperature-sensitive candidate accumulated lariat intermediate (data not shown).

We subsequently carried out primer extension analysis using primers to two other spliced genes to determine the generality of the splicing defect. The results for the cold-sensitive mutant (called _cs1_) and, for comparison, two of the temperature-sensitive mutants, are shown in Figure 1. The ratio of pre-mRNA/mRNA increases (relative to a wild-type strain) for _CYH2_ and _RP73_, as well as for _MATα1_ (data not shown and Fig. 2). This result indicates that the mutations reside in genes encoding elements of the generic splicing apparatus.

To determine whether the growth and splicing defects of _cs1_ were due to the same mutation, we analyzed a cross between _cs1_ and a wild-type strain. The heterozygous diploid was cold-insensitive and displayed no splicing defect, demonstrating that both phenotypes are recessive. Of 18 four-spored tetrads all 18 yielded two cold-sensitive and two _cs+_ spores, indicating the segregation of a single gene. We have named this locus _PRP28_ (see below). RNA from nine random spores that were grown under the same conditions as the original mutant was

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**Figure 1.** Primer extension of RNA from mutant and wild-type strains. (Left) _CYH2_; (right) _RP73_. Cells were grown as stated in text. Positions of precursor and mature mRNAs are indicated. (Lanes 1 and 6) Wild-type, 16°C; (lanes 2 and 7) _cs2_ (a cold-sensitive strain that does not exhibit a splicing phenotype), 16°C; (lanes 3 and 8) _ts1_, 37°C; (lanes 4 and 9) _ts2_, 37°C; (lanes 5 and 10) _cs1_, 16°C.
assayed by primer extension analysis. The two cold-sensitive spores accumulated pre-mRNA while the seven cs+ spores were wild-type for splicing. These results suggest that a single mutant gene \( prp28-1 \) gives rise to both the splicing and growth phenotypes. Biochemical linkage between cold sensitivity and splicing was established by comparing primer extensions of RNA extracted from cs1 grown under permissive conditions with RNA obtained from the cold-shifted cultures (Fig. 2). At 25°C, a temperature at which cs1 strains are slightly impaired for growth relative to wild type, splicing is somewhat inhibited (cf. lanes 1 and 2). However, after incubation at 16°C there is a substantial increase in the ratio of pre-mRNA/mRNA (lane 3). This result suggests that the cold sensitivity reflects the block in splicing. Only cs1 splices inefficiently: Cold-sensitive growth does not generally result in aberrant splicing (e.g., Fig. 1, lanes 2 and 7 are primer extensions of RNA extracted from a cold-sensitive strain, cs2, that is inviable at 16°C but splices efficiently).

To determine the kinetics of onset of the \( prp28-1 \) growth defect, we monitored the growth rate after a shift to the nonpermissive temperature. In general, shutting off the synthesis of essential, relatively stable proteins and snRNAs results in a lag before growth rate slows. For example, a strain in which the synthesis of U5 RNA is arrested grows unhindered for six generations (Patterson and Guthrie 1987). Similarly, arresting synthesis of PRP16, a protein directly involved in splicing (Schwer and Guthrie 1991), results in impaired growth beginning only after 10 generations (S. Burgess and C. Guthrie, unpubl.). In contrast, conditionally lethal mutations in the coding region of U5 or \( PRP16 \) inhibit growth within one generation of the shift to the nonpermissive temperature (D. Frank and C. Guthrie; S. Burgess and C. Guthrie, both unpubl.). As shown in Figure 3, the doubling times for cs1 and a wild-type strain are indistinguishable at 30°C but diverge almost immediately after shifting to 17°C. Within two generations cs1 exhibits its terminal growth phenotype: The doubling time is eight times that of wild type. The observed kinetics are consistent with the interpretation that the \( prp28-1 \) mutation confers a direct splicing defect. Alternatively, the mutation may interfere with the synthesis, modification, or folding of a very short-lived splicing factor.

**PRP28 defines a new splicing gene**

As described in the introductory section, temperature-sensitive mutations in factors involved in splicing define 18 complementation groups (Hartwell et al. 1970, Vijayaraghavan et al. 1989). We carried out complementation and allelism tests between cs1 and \( prp2-\) \( prp8 \) and \( prp11 \) strains. In all cases, we obtained temperature-sensitive/cold-sensitive double mutant spores, as well as each single mutant and wild-type spores. This indicates that the mutations segregate independently and thus reside in different genes. We then asked for rescue of the cold sensitivity with wild-type clones for snRNA genes \( SNR19 [U1] \), \( SNR20 [U2] \), \( SNR14 [U4] \), \( SNR7 [U5] \), and \( SNR6 [U6] \). All transformants remained cold-sensitive, indicating that the wild-type snRNAs do not complement the cs1 lesion. Finally, the wild-type \( PRP28 \) clone (see below) was transformed into \( prp16-\) \( prp24 \) and \( prp27 \) strains and tested for ability to rescue the conditional lethal phenotype of these strains. None of the transfor-

**Figure 2.** Primer extension of \( MAT\alpha_1 \) RNA from cs1 grown at different temperatures. (Lane 1) Wild-type, 16°C; (lane 2) cs1, 25°C; (lane 3) cs1, 16°C.

**Figure 3.** Growth curve of cs1 (□) and wild-type strains (■). Cultures were grown at 30°C to early log phase and shifted to 17°C (indicated by arrow).
Figure 4. Nucleotide and predicted protein sequence of 2.5-kb complementing fragment. The prp28-1 mutation is shown above nucleotide 1019 (\textit{\text{*A*}}), and the predicted amino acid change is shown below amino acid 297 (\textit{\text{*E*}}). Restriction sites referred to in the text are indicated.

**PRP28 encodes a putative ATP-dependent helicase**

We cloned the wild-type PRP28 gene by complementing the cold sensitivity with yeast genomic DNA plasmid libraries and obtained three plasmid-dependent cold-insensitive survivors, one from a low-copy-number CEN bank (Burgess et al. 1990) and two from a high-copy-number 2\mu bank (Carlson and Botstein 1982). All three clones contained the same complementing piece of DNA (data not shown). Subsequent analysis was carried out with the CEN plasmid.

We identified a 2.5-kb EcoRI fragment capable of complementing \textit{prp28-1} cold sensitivity. Sequence analysis (Fig. 4) revealed an open reading frame of 588 amino acids, encoding a protein with a predicted molecular mass of 67 kD. \textit{PRP28} contains a 300-amino-acid domain with motifs shared by a family of proteins that are thought to function as ATP-dependent RNA helicases (Fig. 5). Though many sequence elements in this class of proteins are conserved from \textit{Escherichia coli} to mammals, the precise function of most is unknown. The crystal structures of several ATP- and GTP-binding proteins show that the conserved G/AX4GKT motif, characteristic of NTP-binding proteins, forms a turn between a \beta-strand and an \alpha-helix (de Vos et al. 1988; Drevsicke et al. 1989) and is conserved among a huge number of helicase homologs. We therefore propose that \textit{PRP28} encodes a family of \textit{PRP28} family helicases.

**PRP28 contains all of the signature sequences of the ATP-dependent RNA helicase gene family, as seen in Figure 5. With the exception of elf4a, which is thought to unravel mRNA secondary structure during translation initiation (Rozen et al. 1990), little is known about the specific biological function of these proteins. Notably, however, this family includes two other proteins involved in RNA splicing, \textit{MS816} and \textit{PRP5} (Seraphin et al. 1989; Dalbadie-McFarland and Abelson 1990). Two other members, \textit{SRM8} and \textit{SPB4}, function in ribosome
Cold-sensitive splicing mutant is putative helicase

Figure 5. Alignment of DEAD-box proteins. Conserved motifs are indicated in Consensus. The putative NTP-binding site begins with the first A in the third block of sequence and ends with the T of GKT. The DEAD box, which gives this family of proteins their name, appears near the middle of the fourth block of sequence. The site of the prp28-1 mutation is marked by an asterisk [*]. [eIF4A] Nielsen et al. (1985); [PRPS] Dalbadie-McFarland and Abelson (1990); [MSS16] Seraphin et al. (1989); [Vasa] Hay et al. (1988); [p68] R. Lago (pers. comm.), see also Hloch et al. (1990), [SrnmB] Nishi et al. (1988, 1989); [SL10] Leroy et al. (1989), [SPB4] Sachs and Davis (1990); [DEDI] B. Rahe and J. Pringle (pers. comm.) [TIF1/2] Linder and Slonimski (1989).
assembly [Nishi et al. 1988, 1989; Sachs and Davis 1990]. Five genes have been isolated in a direct screen for this class of proteins using polymerase chain reaction (PCR) technology, one of which is identical to PRP28 [Chang et al. 1990].

PRP28 is essential

To test whether PRP28 is an essential gene, we made a null allele in which all but 38 amino acids of PRP28 are replaced with TRP1. We transformed a diploid strain and selected for Trp⁺ transformants. Insertion at the PRP28 locus was confirmed by Southern analysis (data not shown). Tetrads from this strain yielded only two viable spores, and none of these were Trp auxotrophs. We therefore conclude that PRP28 is an essential gene.

We mapped the 5' end of the PRP28 message using primer extension and S1 nuclease protection analysis. There are three transcription initiation sites, all beginning within 27-34 nucleotides upstream of the first AUG (data not shown). These are used with approximately equivalent efficiencies. We therefore assume that the 5'-most AUG is the start codon.

Cold sensitivity results from a single amino acid change in a conserved domain

To identify the mutation responsible for the cold-sensitive growth and splicing defect, we used the method of gap repair [Orr-Weaver et al. 1981]. Plasmids bearing the wild-type gene were linearized with either SacI or EcoRV, which each cut once in the coding region of the gene. These linearized plasmids were then used to transform a haploid cs1 strain, and Ura⁺ transformants were selected. To remain stably in the cell, the plasmid must be repaired using the chromosomal copy of the gene as a template [Orr-Weaver et al. 1981, 1988]. At a frequency dependent on the distance between the cut and the mutation, the plasmid will be “repaired” to the mutant sequence. These cells will be cold-sensitive, because they now contain two copies of the mutated gene. Fifty Ura⁺ transformants from each transformation were streaked on Ura⁻ plates and incubated at 18°C. Six cold-sensitive isolates were identified, four from the SacI- and two from the EcoRV-digested DNA. Plasmids were isolated and partially sequenced.

A G-to-A mutation at position 1019 was identified that results in replacement of a conserved glycine with a glutamic acid (Figs. 4 and 5). To demonstrate that this mutation is necessary and sufficient for cold-sensitive growth, we exchanged a 223-nucleotide restriction fragment [BstEII-MscI; see Fig. 4 and legend to Fig. 6] containing the mutation with the same fragment from the wild-type clone. As shown in Figure 6, these cells are cold-sensitive.

prp28, prp24 double mutants are inviable

As a first step in determining the specific function of PRP28, we have employed genetics to identify factors with which the protein may interact. Of particular interest is the substrate for the putative helicase activity, one obvious candidate is the U4/U6 complex. To investigate this possibility, we first tested whether prp28-1 strains are hypersensitive to mutations in U4- or U6-associated proteins.

PRP24 is a protein identified genetically by mutations that suppress the cold-sensitive phenotype of the U4-G14C mutation. Antibodies to PRP24 coimmunoprecipitate U6 snRNA in wild-type strains, and U4 as well as U6 in U4-G14C strains [Shannon and Guthrie 1991]. Tetrads from a diploid heterozygous for both the temperature-sensitive allele prp24-1 (for summary of growth phenotypes of strains discussed in this paper, see Table 1A) and prp28-1 were dissected and incubated at 25°C, a temperature permissive for each single mutant (see Table 1A). Two noninteracting, unlinked mutations are expected to generate equal numbers of each phenotypic class: wild type, cold sensitive, temperature sensitive, and cold sensitive/temperature sensitive. However, we recovered only one (extremely sick) cold-sensitive/temperature-sensitive double mutant [prp24-1, prp28-1] of

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Figure 6. Growth phenotypes of cs1 containing wild-type and mutant plasmids. (Left) 30°C; (Right) 18°C. {WT} Wild-type PRP28 clone (pHD20); {gap repair} original gap-repaired clone {Sac7}; {WT swap} 223-nucleotide restriction fragment [BstEII-MscI] of pHD20 cloned into the same sites of Sac7; {gap repair swap} 223-nucleotide restriction fragment [BstEII-MscI] of Sac7 (containing G-to-A mutation at position 1019) cloned into the same sites of pHD20.
A suppressor of prp28 is prp8

A standard method for identifying genetically interacting gene products is to seek second site suppressors. Of particular interest are suppressors that also exhibit an independent phenotype, because this greatly facilitates the assignment of complementation groups and assessment of the phenotype of the suppressor locus. Ten independent cultures of prp28-1 cells were grown to saturation at 30°C, and 10^7 cells were plated on YPD. These plates were incubated at 30°C, and eight colonies from each plate were purified. The revertants were then scored to see if they grew at 37°C. We obtained five independent temperature-sensitive suppressors of prp28-1, which (spp281-1) proved to be particularly interesting because this suppression phenotype of spp281-1 is not temperature-sensitive in the wild-type strain (PRP28) but is temperature-sensitive in a wild-type PRP24 strain. The suppressor function is semidominant because diploids homozygous for prp28-1 and heterozygous for the suppressor grow nearly as well as the original haploid revertant at 18°C. The spp281-1 mutation does not reside in the PRP28 gene because a plasmid bearing the wild-type PRP28 gene cannot rescue the recessive temperature-sensitive growth defect.

To determine whether the five temperature-sensitive suppressors correspond to known splicing factors, we carried out complementation tests with the temperature-sensitive strains prp2- prp11, prp17- prp24, and prp27. The spp281-1 strain failed to complement the temperature-sensitive defect of a prp8-1 strain, though diploids from the other crosses grew at 37°C, demonstrating that the temperature-sensitive phenotype of spp281-1 is recessive. We investigated whether the wild-type PRP8 gene could rescue the temperature-sensitive defect conferred by spp281-1. spp281-1 cells were transformed with a low-copy plasmid bearing PRP8 or with

likely that the synthetic lethality is due to a nonspecific additive effect of the two mutants because it is specific to this pair of splicing mutants; haploid cells containing prp28-1 in combination with prp2- prp8 and prp11 are viable (see Table 1B).

Because PRP5 also encodes a putative RNA helicase involved in splicing, it was of interest to determine whether (1) the synthetically lethal interaction with prp24-1 is specific to prp28-1, and (2) the helicases might carry out overlapping functions, in which case prp28-1 cells might be hypersensitive to mutations in PRP5. We therefore analyzed progeny from prp5-1, prp28-1, and prp5-1, prp24-1 crosses. In both cases, viable double mutant spores were obtained [Table 1B]. The simplest interpretation of these results is that PRP28 and PRP5 normally play functionally distinct roles.
the vector alone. Transformants containing PRP8 were able to grow at 37°C, indicating that spp28-1 is a mutant allele of PRP8. We have thus renamed the suppressor locus prp8-9. Interestingly, PRP8 is a known U5 protein [Lossky et al. 1987]. The prp8-9 allele thus appears to identify a genetic interaction between PRP28 and the U5 snRNP.

Discussion

PRP28 defines a new splicing factor

We have isolated a cold-sensitive splicing mutant that defines a complementation group distinct from those of previously identified temperature-sensitive splicing mutants. Strains harboring this mutation accumulate pre-mRNA after a shift to the nonpermissive temperature. The growth and splicing defects of the mutant cosegregate, defining a single allele, prp28-1. The lethal phenotype of a null allele (prp28::TRP1) demonstrates that as for all other genes encoding splicing factors that have been analyzed to date, the PRP28 gene product is essential for growth.

The prp28-1 mutation lies in a motif highly conserved among members of the ATP-dependent RNA helicase gene family

We cloned the wild-type PRP28 gene by complementing the cold-sensitive phenotype with a wild-type, single-copy yeast bank (construction described in Burgess et al. 1990). Sequence analysis of the smallest complementing fragment revealed a single open reading frame of 588 amino acids, which should encode a protein with a predicted molecular mass of 67 kD. As shown in Figure 5, this sequence contains matches to seven of the seven motifs common to a family of 10 proteins believed to function as ATP-dependent RNA helicases [the "DEAD-box" family, named after this conserved tetrapeptide] [Linder et al. 1989], on the basis of the in vitro activities of two of its members, p68 and eIF4A [Hirling et al. 1989; Rozen et al. 1990]. With the exception of eIF4A, which is thought to unwind secondary structure in mRNA during translation initiation, the biological functions of the genes in this family are unknown.

The mutation responsible for the cold sensitivity in prp28-1 was isolated and found to be a single nucleotide change at position 1019 (G to A), which results in conversion of a highly conserved glycine to glutamic acid (see Fig. 5). This is one of two adjacent glycines that appears in all members of this family except SPB4, ~20 amino acids downstream from the PTREL and 40 amino acids upstream from the VLD/DEAD sequences. The presence of this residue in all of these proteins implies a conserved function.

PRP28 displays genetic interactions with the U4/U6 and U5 snRNPs

The most obvious candidate substrate for an RNA helicase involved in splicing is the U4/U6 snRNP. As detailed in the introductory section, disruption of this extensively base-paired particle prior to the first nucleolytic cleavage is the most striking conformational change known in the splicing pathway.

We used a genetic approach to identify interactions between PRP28 and genes implicated in U4/U6 function. prp28-1 and prp24-1 mutations are synthetically lethal: Haploid cells containing both of these mutations are inviable under conditions in which each single mutant is viable. This observation is interesting because several mutant alleles of PRP24 were isolated as suppressors of a U4 mutation, and PRP24 has been shown to be a U6-associated protein [Shannon and Guthrie 1991]. These results are consistent with the hypothesis that the PRP28 and PRP24 gene products functionally associate during splicing, perhaps during the unwinding of the U4/U6 snRNP. Synthetic lethal interactions have been identified for alleles of a number of proteins that are known to interact physically: actin and the SAC6 gene product [Adams and Botstein 1989; Adams et al. 1989], α- and β-tubulin [T. Stearns, pers. comm.], and SEC61p, SEC62p, and SEC63p [Rothblatt et al. 1989; R. Deshaies, pers. comm.].

We identified a second genetic interaction by isolating a suppressor of prp28-1, spp28-1, which has a temperature-sensitive phenotype on its own. Intriguingly, the temperature-sensitive growth phenotype conferred by spp28-1 fails to complement that of prp8-1. The wild-type PRP8 gene [supplied on a low-copy plasmid] alleviates the temperature sensitivity in a spp28-1 strain. Therefore, spp28-1 defines an allele of PRP8, prp8-9.

A model for PRP28 function

Until biochemical data are available, the precise role of PRP28 in splicing cannot be known. However, the genetic interactions we have identified are consistent with a simple model for PRP28 function as a helicase that unwinds U4 and U6 in the context of the U4/U6/U5 snRNP in the spliceosome; this is diagramed in Figure 7. In this model PRP24 protein facilitates the unwinding of U4/U6 by binding to and thus stabilizing the unwound form of U6. PRP24 contains three regions homologous to the RNP consensus domain conserved among many RNA-binding proteins and has been shown to bind tightly to U6 snRNA by immunoprecipitation studies [Shannon and Guthrie 1991]. The substrate U4/U6 in this scenario is associated with a U5 particle containing the PRP8 protein, which would act to stabilize the base-paired form. The synthetic lethality and suppression results could then be explained by recessive, loss-of-function mutations in PRP24, PRP28, and PRP8. The dominant suppression of the prp28-1 cold-sensitive phenotype by prp8-9 would be due to the ability of the U5 particle containing the spp28-1 product to assemble into the spliceosome. Once there, it would augment the diminished helicase activity of prp28-1 by stabilizing the tightly associated form of U4/U6 less well than its wild-type counterpart would, making the RNA duplex easier to unwind.
Cold-sensitive splicing mutant is putative helicase

Obviously, other possibilities for PRP28 function exist; there are also other putative helicases implicated in splicing that could unwind the U4/U6 snRNP (see below). We present this model because the genetic results are consistent with a role for PRP28 in the event in splicing that most obviously points to the requirement for RNA helicase activity. Experiments to test the biochemical predictions of this model are under way.

Seven putative ATPases are implicated in nuclear mRNA splicing

PRP5, a protein required for mRNA splicing in vivo and in vitro, also contains all of the motifs characteristic of the eIF4A family (see Fig. 5; Dalbadie-McFarland and Abelson 1990). Interestingly, genes for two additional DEAD-box proteins that have been implicated in mRNA splicing interact genetically with U5. SPP81 [also called DED1] is a cold-sensitive suppressor of a temperature-sensitive allele of the U5 protein, prp8-1, and DBP1 was identified as a suppressor of the cold sensitivity of SPP81; each contains the consensus sequences shown in Figure 5 (D. Jamieson and J. Beggs, pers. comm.).

Interestingly, the four DEAD-box proteins implicated in splicing constitute only one of two classes of putative ATPases involved in this process. The second class of proteins [PRP2, PRP16, PRP22] contain sequence motifs that are clearly related to the DEAD-box family, however, there are significant variations (e.g., DEAH vs. DED) (Burgess et al. 1990; Chen and Lin 1990; Comer et al. 1991; SPP81; DED1).

As described in the introductory section, the splicing pathway requires ATP at many steps. This probably reflects various uses for ATP hydrolysis. It has been suggested that PRP16 plays a role in maintaining fidelity by coupling ATP binding and/or hydrolysis to accurate branchpoint recognition (Burgess et al. 1990). Another possible role for ATP hydrolysis is as an energy source to drive the reaction forward through a very precise path-

**Materials and methods**

Yeast media were prepared as described by Rose et al. (1989). AMV reverse transcriptase was obtained from Life Sciences. S1 nuclease was obtained from Boehringer Mannheim. Restriction enzymes were obtained from New England Biolabs, BRL, Boehringer Mannheim, and U.S. Biochemicals. T4 polynucleotide kinase was obtained from U.S. Biochemicals. dATP labeled with 32P was obtained from Amersham, and [32P]ATP was obtained from ICN. Oligonucleotides were synthesized by the Biomedical Resource Center at University of California, San Francisco.

Oligonucleotides

The following oligonucleotides were employed in this work:

**Strains**

The following strains were employed in this work:

**Y[C13], the parent strain used for mutagenesis:** MATa, ura3, his4, trp1, leu2

**TR3**, the wild-type strain used for outcrossing (obtained from Phil Hieter): MATa ura3 lys2 his3 ade2 trp1

**YPB67:** MATa prp6 prp2 ura3-52 leu2 his ade tyl

**RL92:** MATa prp2 ura3-52 leu2-3 leu2-112

**naa3-1-3A:** MATa prp3 leu ade

**SP13.33:** MATa prp3 ura3-52 leu2 lys2 his3

**YPF90:** MATa prp4 ura3-52 leu2 lys2 his7 ade

**YPF70:** MATa prp4 ura3-52 leu2 lys2 ade

**naa4-1:** MATa prp4 ura3-52 leu2 his7 ade ade2 ade2 tyr1 gal1

**YBP71:** MATa prp5 ura3-52 leu2 his7

**YBP72:** MATa prp6 ura3-52 lys2 his3

**naa6-1:** MATa prp6 ura3-52 lys2 his7 ade ade2 ade2 tyr1 gal1

**YBP73:** MATa prp7 leu2 lys2 his

**naa8 [AH]:** MATa prp8 ura1 ade arg trp

**naa8-1:** MATa prp8 ura1 lys2 his7 ade ade2 tyr1\n
**YBP80:** MATa prp8 lys2 his3 ade2 ade2 trp1

**SPP102:** MATa prp11 ura leu lys his ade tyr

**YBP74:** MATa prp11 ura3-52 leu2 his4-512

**YDL15:** MATa ura3-52 prp16 ura3-52 his2 ade2 ade2 tyr1

**pp17-prp17** strains were the kind gift of Usha Vijayraghavan, Mahshid Company, and John Abelson.

**YJC52**, used to construct the gene replacement was the kind gift of John Chant: MATa/MATa, trp1/trp1, ura3-52/ura3-52, his4/ his4.
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Genetic methods

All genetic manipulations, including yeast transformations, were done as described by Rose et al. (1989).

Mutagenesis

YJC13 was grown to saturation, washed with water, and UV-irradiated for 30 or 45 sec. (This treatment had been calibrated previously to give 0.4–4% survival with this strain.) Cells were plated and incubated at 25°C. Survivors were tested for viability at 16°C and 37°C.

DNA libraries and isolation of PRP28 clones

Two DNA libraries were used: The CEN bank was obtained from Joe Couto (Burgess et al. 1990), and the 2μ bank was obtained from Marian Carlson (Carlson and Botstein 1982). Subcloning was achieved by inserting restriction fragments into a CEN, URA3 yeast vector (pSE360) and testing for ability to complement the cold sensitivity conferred by prp28-1 (see Plasmids below, pHD1, pHD4, pHD14, and pHD20).

Plasmids

Plasmids used for sequencing are as follows:

- pBJ1: 3.2-kb SacI fragment of pHD4 cloned into the SacI site of Bluescript.
- pBJ2: 2.3-kb SacI fragment of pHD4 cloned into the SacI site of Bluescript.
- pBJ3-2: 2.5-kb EcoRI fragment of pHD14 cloned into the EcoRI site of Bluescript.
- pBJ4: 530-bp PsI site fragment of pHDJ3-2 cloned into the PsI site of Bluescript.
- pBJ5: 800-bp EcoRV fragment of pHDJ3-2 cloned into the EcoRV site of Bluescript.
- pBJ6: 950-bp EcoRV-SacI fragment of pHDJ3-2 cloned into the EcoRV/SacI sites of Bluescript.
- pBJ8: 450-bp RsaI-SacI fragment of pHDJ3-2 cloned into the EcoRV/SacI sites of Bluescript.
- pBJ9: 300-bp EcoRV-BglII fragment of pHDJ3-2 cloned into the EcoRV site of Bluescript.
- pBJ10: 500-bp EcoRV-BglII fragment of pHDJ3-2 cloned into the EcoRV site of Bluescript.

Plasmids used for gene replacement are as follows:

- pHPH336: pBR322 containing TRP1.
- pBJ13-11: 2.5-kb EcoRI fragment of pHDJ3-2 cloned into the EcoRI site of pUC19.
- pBJ16-5: TRP1 BglII fragment of pHPH336 cloned into 3.4-kb BglII fragment of pHDJ3-11.

Other plasmids

- pSE360 was constructed and provided by Steve Elledge (Elledge and Davis 1988). It contains the yeast ARS1, CEN4, Amp', URA3, and a polylinker region in E. coli lacZ'.
- pHD1: 14-kb complementing fragment in TRP1 CEN vector, original clone from CEN bank (Burgess et al. 1990).
- pHD2: 10-kb complementing fragment in URA3 2μ vector, original clone from high copy (2μ) bank (Carlson and Botstein 1982).
- pHD3: 12-kb complementing fragment in URA3 2μ vector, original clone from high copy (2μ) bank (Carlson and Botstein 1982).
- pHD4: 7.5-kb BamHI-SmaI complementing fragment of pHDJ1 cloned into SmaI site of pSE360.
- pHD14: 5.5-kb Nael-SmaI complementing fragment of pHDJ4 cloned into SmaI site of pSE360.
- pHDJ20: 2.5-kb fragment containing PRP28 cloned into EcoRI site of pSE360.
- Sac7: One of the original gap-repaired plasmids obtained by digesting pHDJ20 with SacI prior to transformation (see Results).

Plasmids containing SNR19, SNR20, SNR14, SNR7, and SNR6 used in the complementation analysis are described in Siliciano et al. (1987a), Shuster and Guthrie (1988), Siliciano et al. (1987b), Patterson and Guthrie (1987), and Brow and Guthrie (1988), respectively.

- pY8500, a plasmid containing PRP8 in yCP50 (Jackson et al. 1988), was provided by Jean Beggs.

Primer extension and S1 analysis

RNA extraction was achieved by the method described in Vijayraghavan et al. (1989). Primer extensions were done according to Bensi et al. (1985). Methods to map the 5' end of the PRP28 transcript were carried out on wild-type RNA. Oligonucleotides cs1-seq and cs1-5' end were annealed for 20 min at 45°C, and the oligonucleotide–RNA hybrid was ethanol-precipitated prior to the extension reactions. Ends were mapped by comparing mobilities of primer-extended products with those of DNA size markers or of a DNA sequencing reaction (using pBJ3-2 as a template), with end-labeled cs1-5' end as a primer. S1 analysis was done according to Maniatis et al. (1982), using an empirically determined amount of S1 nuclease (5 units) on 20 μg of RNA. A double-stranded 833-nucleotide BstEII–BamHI DNA fragment was gel-purified by using Geneclean (Bio101) prior to end-labeling. Following this, the probe was incubated with RNA for 3 hr at 50°C.

Sequencing and sequence analysis

DNA sequencing was carried out using the dideoxy method (Sanger et al. 1977) with the Sequenase kit (U.S. Biochemicals). Fragments to be sequenced were cloned into Bluescript plasmids (Stratagene). These are listed as the pBJ series under plasmids. Sequences not covered by this method were sequenced by using oligonucleotides that anneal to sequenced regions.

Construction of gene replacement

The prp28::TRP1 allele was created by cloning the 2.5-kb EcoRI fragment containing PRP28 into the EcoRI site of pUC19 to generate pBJ13-11. This plasmid was then digested with BglII, and the 3.4-kb fragment containing pUC19 and the 5' and 3' ends of the complementing EcoRI fragment was gel-purified by using Geneclean (Bio101). A 1.7-kb BglII fragment of DNA containing the TRP1 gene was purified from pHPH336 (using Geneclean) and cloned into the 3.4-kb pUC19/PRP28 vector prepared as described above. Integration of this allele into the chromosome was achieved by cutting the plasmid [pBJ16-5] with EcoRI prior to transformation.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession number X56934.

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