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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The removal of introns from nuclear mRNA precursors proceeds via two consecutive *trans*-esterification reactions. In the first step, the 5' splice site is cleaved, and a lariat intron-3' exon intermediate is formed. This is followed by 3' splice site cleavage and exon ligation, with the release of the intron in lariat form (for review, see Green 1986; Padgett et al. 1986). The reaction requires five U snRNAs (for review, see Maniatis and Reed 1987; Sharp 1987; Guthrie and Patterson 1988; Steitz et al. 1988) and an as yet undetermined number of proteins, some of which are integral small nuclear ribonucleoprotein (snRNP) components. In yeast, at least 20 of these proteins have been identified by genetic means (pre-mRNA processing mutants: *prp2-prp9*, *prp11*, *prp16-prp27*) (Hartwell 1967; Hartwell et al. 1970; Vijayraghavan et al. 1989).

mRNA splicing occurs in a large complex called the spliceosome (Brody and Abelson 1985; Frendewey and Keller 1985; Grabowski et al. 1985; for review, see Green 1986; Padgett et al. 1986). Spliceosome assembly is a dynamic process, involving the ordered formation and severing of multiple interactions between snRNPs, proteins, and the pre-mRNA. In yeast, the first detectable event is the formation of a committed complex that requires the U1 snRNP and intact 5' splice site and branchpoint sequences (Ruby and Abelson 1988; Seraphin and Rosbash 1989). After U1 associates with the pre-mRNA, U2 binds to the branchpoint, followed by addition of U4/U6 and U5 (Pikielny et al. 1986; Cheng and Abelson 1987). It has been shown that the U4/U6 and U5 snRNPs

associate stably with each other: They comigrate on glycerol gradients and in native gel electrophoresis (Binderif and Green 1987; Cheng and Abelson 1987; Konarska and Sharp 1987); and in *Saccharomyces cerevisiae*, immunoprecipitation of a U5 protein, PRP8, coprecipitates U4 and U6 in an ATP-dependent fashion (Lossky et al. 1987). It is thought that U4, U5, and U6 join the spliceosome as a tripartite complex. Formation of the tri-snRNP complex requires the U4-associated protein PRP4 (Banroques and Abelson 1989; Xu et al. 1990) and appears to be an essential intermediate in the splicing pathway (Bordonné et al. 1990).

The U4/U6 snRNP is held together by two intermolecular helices, totaling 24 bp (Bringmann et al. 1984; Hashimoto et al. 1984; Siliciano et al. 1987b; Brow and Guthrie 1988). Prior to the first nucleolytic step of splicing, the particle undergoes a major conformational change, such that U4 is no longer associated with the spliceosome upon electrophoresis under nondenaturing conditions (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988). Given that the native U4/U6 snRNP in yeast has a melting temperature of 53°C (Brow and Guthrie 1988), the destabilization is presumed to require an active process. Notably, ATP is required at most steps in the *in vitro* pathway (Brody and Abelson 1985; Lin et al. 1987; Sawa et al. 1988; Vijayraghavan and Abelson 1990; Schwer and Guthrie 1991), and a number of splicing proteins have recently been shown to contain putative ATP-binding domains (Burgess et al. 1990; Dalbadie-McFarland and Abelson 1990). Thus, a

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 eIF4A (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 *MATa1* 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 *MATa1* (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

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.

Figure 2. Primer extension of *MATa1* RNA from *cs1* grown at different temperatures. (Lane 1) Wild-type, 16°C; (lane 2) *cs1*, 25°C; (lane 3) *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; Vijayraghavan 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 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).

AATTCGGAGTTAATCGCGATGATCCGTTTTTACATATTCGAAGCTTACAATCGAGAGAAT 60
 TACCAACGCAATAGAAATTTTGGACAAAGGTCATAGGAGCCGAAGAATTAACAT 120
 ACAGGACGATGGCAAGACCAATCGATGTTAGTCAGCTGATTTGGTGAATAAACAAGAAA 180
 M A R P I D V S Q L I A G I N K K 17
 AAAGGACTAGATGAAAATACATCAGGTAATCAGCAAGCCTAGGTTTTTGAATAAACA 240
 K G L D E N T S G K I S K P R F L N K Q 37
 BglII
 GAAAGATCTAAGCAAGAACGTTTGAAGGAGAAGAGAGTTTGACGCTACGCAAGT 300
 E R S K Q E R L K E N E E S L T P T Q S 57
 GATAGCGCTAAGGTGGAGATTAAGGTAACCTAGAGATGACAGTTTTCATGAA 360
 D S A K V E I K P V N S R K D D S F F N E 77
 ACAATGATAAGAGAGAAATCCATCAAGCAAAATGGCTTAAATTTCACTTTTCTGG 420
 T N D K K R N P S K Q N G S K F H F S W 97
 AATGAAAGCGAAGATACTCTGAGCGGATATGATCCTATCGTTTCACTAGAGCTATAGAT 480
 N E S E D T L S G Y D P I V S T R A I D 117
 PstI
 TTGCTATGGAAGGGAAGACTCCTAAAAATGCTGCAAAATCTCATATATGGGAAGCAT 540
 L L W K G K T P K N A A E S S Y M G K H 137
 TGGACTGAGAAGTCTTGACGAAATGAATGAGAGAGATTGGAGGATACTAAAAGAAGAC 600
 W T E K S L H E M N E R D W R I L K E D 157
 SacI
 TACGCTATTGTTACAAAAGGTGGGACAGTAGAAAAATCCATTGAGAAATGGGAAGAGCTC 660
 Y A L I V T K G G T P V N S R K D D S F F N E 177
 AATATAATACCAAGGACCTTGTACGTGTTATTATACAGGAGCTGCGATTTCCTTACCA 720
 N I I P R D L L R V I I Q E L R F P S P 197
 ACTCCTATTCAAAGGATTACTATTCCAAACGTTTGAATATGAACAATATAGAGATTTT 780
 T P I Q R I T I P N V C N M K Q Y R D F 217
 CTGTGTTGCTCTACAGGTTCCGGTGAAGACTTTGGCCTTTGTAATACCGATACTAATC 840
 L G V A S T G S G K T L A F V I P I L I 237
 BstEII
 AAGTAGTAGTACCTCAAGGCCACCATCTTTGAAGATTATTGATGGCCCCAAGCG 900
 K M S R S P P R P P V N S R K D D S F F N E 257
 TTAATTTTAGCTCCCAAGGGAATAGTGCAGCAATCAAAAAGAAACACAAAAAGTT 960
 L I L A P T R E L V Q Q I Q K E T Q K V 277
 A
 ACAAAAATATGGTCCAAAGAGAGCAACTATGACTGCAAAATTTCTATTGTTGGTGA 1020
 T K I W S K E S N Y D C K V I S I V G G 297
 E
 MscI
 CACTCCTTGGAGGAAATTTCTCTCTCGGAGGGTGTGACATCCTGGTGGCCACT 1080
 H S L E E I S F S E G C D I L V P A T 317
 CCAGGACGTTTAATTGACTCTTTAGAAAATCACTTACTGTGATGAACAGGTGGAGACA 1140
 P G R L I D S L E N H L L V M K Q V E T 337
 RsaI
 TTAGTACTAGTAGAAGCTGACAAAATGATTGATTAGGTTTGGAGCAAGTAACAAC 1200
 L V L D E A D K M I D L G F E D Q V T N 357
 ATTTTAACTAAAGTCGATATAATGCTGACTCTGCTGAATAGACAAACCTGATGTT 1260
 I L T K V D I N A D S A V N R Q T L M F 377
 ACCGCTACATGACACCCGTTATAGAAAAATTCAGCAGGATACATGCAAAAGCCTGTT 1320
 T A T M T P V I E K I A G Y M Q K P V 397
 TATGCAACCATTTGGGGTGGAGCGGGTCTGAACCTTTGATTCAACAGGTTGTGGAATAT 1380
 Y A T I G V E T G S E P L I Q Q V V E Y 417
 GCAGATAATGACGAAGCAAAATCAAAAAGTTGAAGCAATTTGCTGATAAATATGATCCA 1440
 A D N D E D K F K L K P I V A K Y D P 437
 CCAATAATAATTTTATCAATACAAACAACTGCTGATTGGTTAGCAGAAAAGTTTCAA 1500
 P I I I F I N Y K Q T A D W L A E K F Q 457
 AAGGAAACCAATGAAAGGTGACGATTTTACATGGTTTAAATCGCAAGAGCAAGGGAA 1560
 K E T N M K V T I L H G S K S Q E Q R E 477
 CATTTCTTGAATATTTTGCACCAACAAAGTCCAAATCATGATCGCAACCAAGCTTGCC 1620
 H S L Q L F R T N K V Q I M I A T N V A 497
 EcoRV
 GCAAGAGGATTGGATATCCCTAATGTTTCCCTGGTAGTCAATTTCCAGATTTCACAAAA 1680
 A R L D I P N V S L V N F Q I S K K 517
 ATGGATGACTACATCCATAGAATTGGTAGAAGCGTGCAGCAAAATGAGGCACTGCT 1740
 M D D Y I H R I G R T G R A A N E G T A 537
 GTCTCCTTTGTTAGTGCCTGAGATGAATCATTGATACGTGAACCTTCAAAATACGTC 1800
 V S F V S A A E D E S L I R E L Y K Y V 557
 AGGAACATGATCCCTTGAATAGCAATATCTTTTCCGAAGCGTAAAGAACAAATATAAC 1860
 R K H D P L N S I F S E A V K N K Y N 577
 GTCGGCAACAGCTCAGTAATGAGATTATATTAAGATTATATTTCTTTCTTTT 1920
 V G K Q L S N E I I Y * 588

BglII
 TTTGATGGACCAAGATCTCAACTTTCAAGTTATTGTCTATATTTTGTCTAAAAAGCCAAG 1980
 ACGCAATTTACAACTTCATTGTCTGAAAGTCGGGCTAACTACTTGTAGGCTATTG 2040
 GAAACCCATCTATCTCACCAGATTGTATTAATCATTCCAATATGTTTGTATCTTTAT 2100
 CTTCTGGATTGAGCGCAGAGTGGTATCCATTAGCCCATCAATTTGGCAATCGACACTAC 2160
 CTACAGGTATAGATAATGATGGGAAATTTAAACGTTGAATAGTAAATGTATCGTAAAG 2220
 TTGCTTTAGCATGTCCAGGCTAAATGGCATCAGCGTTGTAGGAGATATGATAACAT 2280
 CAGGCTTTTCTGATTCGGGAAAGAAAGGTAATCTCTCATTAAATTTGTGTCATTAT 2340
 AAACCTTTTGGTCAATCCCACTTATTAACAGTATATGGGGGCTTCCGTTTTCCTCA 2400
 AAACCATTTGGTTTAAATATCTAGGAGTGGTTCTCCGGAATTTCCGAATTC 2452

Figure 4. Nucleotide and predicted protein sequence of 2.5-kb complementing fragment. The *prp28-1* mutation is shown above nucleotide 1019 (*A*), and the predicted amino acid change is shown below amino acid 297 (*E*). Restriction sites referred to in the text are indicated.

mants were viable at the nonpermissive temperature. *PRP28* therefore defines a new complementation group.

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 μ 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 *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. *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 *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 β -strand and an α -helix (de Vos et al. 1988; Drevsick et al. 1988; Pai et al. 1989). This alternating structure, first proposed by Rossmann et al. (1974) as a core component of a canonical "dinucleotide fold" appears to bind the β/γ phosphates of the NTP molecule.

PRP28 contains all of the signature sequences of the ATP-dependent RNA helicase gene family, as seen in Figure 5. With the exception of eIF4A, 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, *MSS116* and *PRP5* (Seraphin et al. 1989; Dalbadie-McFarland and Abelson 1990). Two other members, *SRMB* and *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); (PRP5) Dalbadie-McFarland and Abelson (1990); (MSS116) Seraphin et al. (1989); (Vasa) Hay et al. (1988); (p68) R. Iggo (pers. comm.); see also Hloch et al. (1990); (SrmB) Nishi et al. (1988, 1989); (PL10) Leroy et al. (1989); (SPB4) Sachs and Davis (1990); (DED1) 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

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.

Table 1. Growth phenotypes and tetrad dissection data

A. Growth phenotypes of strains				
Genotype	Growth phenotype (°C)			
	18	25	37	
<i>prp28-1</i>	–	+	+	
<i>prp24-1</i>	+	+	–	
<i>prp5-1</i>	+	+	–	
<i>spp281-1</i>	+	+	–	
<i>prp8-1</i>	+	+	–	
<i>prp28-1/PRP28</i>	+	+	+	
<i>prp24-1/PRP24</i>	+	+	+	
<i>spp281-1/SPP281</i>	+	+	+	
<i>prp8-1/PRP8</i>	+	+	+	
<i>prp24-1, prp28-1</i>	NA	–	NA	
<i>prp5-1, prp24-1</i>	+	+	–	
<i>prp5-1, prp28-1</i>	–	+	–	
<i>prp8-1, prp28-1</i>	–	+	–	
<i>prp28-1/prp28-1, spp281-1/SPP281</i>	+ / –	+	+	
B. Tetrad dissection data				
	Genetic crosses (no. of spores)			
	cs ⁺ , ts ⁺	cs, ts ⁺	cs ⁺ , ts	cs, ts
<i>prp28-1</i> × <i>prp2-1</i>	5	8	7	7
<i>prp28-1</i> × <i>prp3-1</i>	5	13	9	8
<i>prp28-1</i> × <i>prp4-1</i>	6	7	10	7
<i>prp28-1</i> × <i>prp5-1</i>	6	4	9	3
<i>prp28-1</i> × <i>prp6-1</i>	13	11	9	14
<i>prp28-1</i> × <i>prp7-1</i>	9	7	6	15
<i>prp28-1</i> × <i>prp8-1</i>	28	27	24	21
<i>prp28-1</i> × <i>prp11-1</i>	2	7	10	6
<i>prp28-1</i> × <i>prp24-1</i>	29	29	31	1 (sick)
<i>prp28-1spp281-1</i> × <i>prp28-1SPP281</i>	0	19	21	0
	<i>PRP5, PRP24</i> (ts ⁺)	<i>prp5-1, PRP24</i> ts ^a	<i>PRP5, prp24-1</i> ts ^a	<i>prp5-1, prp24-1</i> ts ^a
<i>prp5-1</i> × <i>prp24-1</i>	7	9	13	13

(NA) Not applicable.

^aThe genotypes of temperature-sensitive (ts) spores from this cross were determined by carrying out complementation tests with *prp5-1* and *prp24-1* strains of appropriate mating types. Spores complementable by *prp5-1* were scored as *PRP5, prp24-1*; those complementable by *prp24-1* were scored as *prp5-1, PRP24*; and those not complementable by either were scored as *prp5-1, prp24-1*.

90 viable spores (for summary of tetrad dissection data, see Table 1B). Frequencies for the other classes of spores were as expected: Twenty-nine were completely wild-type (*PRP24, PRP28*), 29 were cold-sensitive (*PRP24, prp28-1*), and 31 were temperature-sensitive (*prp24-1, PRP28*). We conclude that cells cannot tolerate both mutations simultaneously. This phenomenon has been termed synthetic lethality (Dobzhansky 1946). (The double mutant recovered presumably contains a third mutation, which suppresses the lethal phenotype.) It is un-

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.

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* were grown to saturation at 30°C, and 10⁷ cells were plated on YPD. These plates were incubated at 18°C, and eight colonies from each plate were purified. The revertants were then screened for growth at 37°C. We obtained five independent suppressors of *prp28-1* (Table 1B). The *spp281-1* proved to be particularly interesting because suppression of the temperature-sensitive phenotype of *prp28-1* by a single gene: In a backcross between the *prp28-1* and *spp281-1* and spored tetrads were obtained. In all 40 spores, 21 were temperature-sensitive, and 19 were cold-insensitive (Table 1B). *spp281-1* suppresses temperature sensitivity in a wild-type *PRP28* background. 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–prp10*, *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

the vector alone. Transformants containing *PRP8* were able to grow at 37°C, indicating that *spp281-1* 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 VLDEAD 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 de-

tailed 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*, *spp281-1*, which has a temperature-sensitive phenotype on its own. Intriguingly, the temperature-sensitive growth phenotype conferred by *spp281-1* fails to complement that of *prp8-1*. The wild-type *PRP8* gene (supplied on a low-copy plasmid) alleviates the temperature sensitivity in a *spp281-1* strain. Therefore, *spp281-1* 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 diagrammed 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 *spp281-1* 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.

Figure 7. Model for PRP28 activity. PRP28 is proposed to unwind the U4/U6 helix. PRP8 would stabilize the base-paired form of U4/U6 snRNP, and PRP24 would stabilize the unwound form. Decreased activity of PRP8 would favor the right-hand side of the equation, and decreased activity of PRP24 would favor the left-hand side.

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. DEAD) (Burgess et al. 1990; Chen and Lin 1990; Company et al. 1991).

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-

way; it is inviting to speculate that each of the putative ATPases uses ATP hydrolysis to drive conformational changes that sequentially modify RNA/protein interactions in the assembling and disassembling spliceosome.

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 ^{32}P was obtained from Amersham, and [^{32}P]ATP was obtained from ICN. Oligonucleotides were synthesized by the Biomolecular Resource Center at University of California, San Francisco.

Oligonucleotides

The following oligonucleotides were employed in this work:

CHY2 exon 2	5'-TGCTTACCGATACGACCTTTA-3'
RP73 exon 2	5'-TTTGGTTGATAGATTCTTGGG-3'
MATa1 exon 3	5'-GAATTTATTTAGATCTCATACGTTT-3'
cs1-5' end	5'-TCTTGTTTTATACCAGCAATCAGC-3'
cs1-seq	5'-GTCAATTAAACGTCCTGGAGT-3'
Sac-mer	5'-GAGAGAGATTGGAGGATACTA-3'
RV-mer	5'-GGGAACATTCTTTGCAATTATTT-3'
Rsa-mer	5'-GTGACATCCTGGTGGCCAC-3'
2150A	5'-GGCAATCGACACTACCTACA-3'
2150C	5'-CGATTGCCAAATTGATGGGC-3'
900A	5'-AGCGTTAATTTTAGCTCCAACA-3'
900T	5'-CGCTTTGGGGCCATCAATAAT-3'
370	5'-TTCAATGAAACAAATGATAAGAAGA-3'
1360	5'-CGGGTTCTGAACCTTTGATTC-3'
T3 primer	5'-ATTAACCCCTCACTAAAG-3'
T7 primer	5'-AATACGACTCACTATAG-3'

Strains

The following strains were employed in this work:

YJC13, 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*
 YBP67: MATa *prp2 ura3-52 leu2 his ade tyr1*
 RL92: MATa *prp2 ura3-52 leu2-3 leu2-112*
 rna3-1-3A: MATa *prp3 leu ade*
 SPJ3.33: MATa *prp3 ura3-52 leu2 lys2 his3*
 YBP69: MATa *prp4 ura3-52 leu2 lys2 his7 ade*
 YBP70: MATa *prp4 ura3-52 leu2 lys2 ade*
 rna4-1: MATa *prp4 ura1 lys2 his7 ade1 ade2 tyr1 gal1*
 YBP71: MATa *prp5 ura3-52 leu2 his7*
 YBP72: MATa *prp6 ura3-52 lys2 his3*
 rna6-1: MATa *prp6 ura1 lys2 his7 ade1 ade2 tyr1 gal1*
 YBP73: MATa *prp7 leu2 lys2 his*
 rna8 (AH): MATa *prp8 ura1 ade arg trp*
 rna8-1: MATa *prp8 ura1 lys2 his7 ade1 ade2 tyr1*
 YBP80: MATa *prp8 lys2 his3 ade2 trp1*
 SPJ10.2: MATa *prp11 ura leu lys his ade tyr*
 YBP74: MATa *prp11 ura3-52 leu2 his4-512*
 YDL15: MATa *ura3-52 prp16 leu2 lys2 his3 ade2 trp1*
prp17-prp27 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*.

Strauss and Guthrie

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 2 μ 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 *PstI* fragment of pBJ3-2 cloned into the *PstI* site of Bluescript.
- pBJ5: 800-bp *EcoRV* fragment of pBJ3-2 cloned into the *EcoRV* site of Bluescript.
- pBJ6: 950-bp *EcoRV*–*SacI* fragment of pBJ3-2 cloned into the *EcoRV/SacI* sites of Bluescript.
- pBJ8: 450-bp *RsaI*–*SacI* fragment of pBJ3-2 cloned into the *EcoRV/SacI* sites of Bluescript.
- pBJ9: 300-bp *EcoRV*–*BglII* fragment of pBJ3-2 cloned into the *EcoRV* site of Bluescript.
- pBJ10: 500-bp *EcoRV*–*BglII* fragment of pBJ3-2 cloned into the *EcoRV* site of Bluescript.

Plasmids used for gene replacement are as follows:

- pPH336: pBR322 containing *TRP1*.
- pBJ13-11: 2.5-kb *EcoRI* fragment of pBJ3-2 cloned into *EcoRI* site of pUC19.
- pBJ16-5: *TRP1* *BglII* fragment of pPH336 cloned into 3.4-kb *BglII* fragment of pBJ13-11.

Other plasmids

pSE360 was constructed and provided by Steve Elledge (Elledge and Davis 1988). It contains the yeast *ARS1*, *CEN4*, *Amp^r*, *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 2 μ vector; original clone from high copy (2 μ) bank (Carlson and Botstein 1982).

- pHD3: 12-kb complementing fragment in URA3 2 μ vector; original clone from high copy (2 μ) bank (Carlson and Botstein 1982).
- pHD4: 7.5-kb *BamHI*–*SmaI* complementing fragment of pHD1 cloned into *SmaI* site of pSE360.
- pHD14: 5.5-kb *NaeI*–*SnaBI* complementing fragment of pHD4 cloned into *SmaI* site of pSE360.
- pHD20: 2.5-kb fragment containing *PRP28* cloned into *EcoRI* site of pSE360.
- Sac7: One of the original gap-repaired plasmids obtained by digesting pHD20 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 μ g 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 pPH336 (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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