A Mutation in a Methionine tRNA Gene Suppresses the *prp2-1* Ts Mutation and Causes a Pre-mRNA Splicing Defect in *Saccharomyces cerevisiae*

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ABSTRACT

The *PRP2* gene in *Saccharomyces cerevisiae* encodes an RNA-dependent ATPase that activates spliceosomes for the first transesterification reaction in pre-mRNA splicing. We have identified a mutation in the elongation methionine tRNA gene *EMT1* as a dominant, allele-specific suppressor of the temperature-sensitive *prp2-1* mutation. The *EMT1-201* mutant suppressed *prp2-1* by relieving the splicing block at high temperature. Furthermore, *EMT1-201* single mutant cells displayed pre-mRNA splicing and cold-sensitive growth defects at 18°. The mutation in *EMT1-201* is located in the anticodon, changing CAT to CAG, which presumably allowed *EMT1-201* suppressor tRNA to recognize CUG leucine codons instead of AUG methionine codons. Interestingly, the *prp2-1* allele contains a point mutation that changes glycine to aspartate, indicating that *EMT1-201* does not act by classical missense suppression. Extra copies of the $tRNA^{Leu}(UAG)$ gene rescued the cold sensitivity and *in vitro* splicing defect of *EMT1-201*. This study provides the first example in which a mutation in a tRNA gene confers a pre-mRNA processing (*prp*) phenotype.

PRE-mRNA splicing is a multistep process in which introns are removed from pre-mRNAs and exons are joined. Reactions occur in a dynamic RNA-protein complex called the spliceosome, which is composed of U1, U2, U4/U6, and U5 small ribonucleoprotein (snRNP) particles, non-snRNP proteins, and the pre-mRNA (reviewed in Sharp 1994). Two classes of proteins involved in pre-mRNA splicing have been identified (Guthrie 1991; Krämer 1996; Will and Lührmann 1997): integral proteins, which are the snRNP proteins that bind to one or more snRNAs, and extrinsic factors, which are non-snRNP proteins that are not tightly associated with snRNAs and may interact only transiently with the spliceosome. Following spliceosome assembly splicing proceeds via two sequential transesterification reactions. ATP is not required for the transesterification reactions *per se*, since catalysis occurs by two phosphoryl transfer reactions (reviewed in Moore *et al.* 1993; Staley and Guthrie 1998). However, ATP hydrolysis is required for pre-mRNA splicing, perhaps for rearrangements of RNAs that occur during the assembly and maturation of the spliceosome (Ares and Weiser 1995; Nilsen 1998). Several RNA-dependent ATPases that are essential for pre-mRNA splicing and that may play a role in these rearrangements have been identified (reviewed in Beggs 1993; Staley and Guthrie 1998). The RNA-dependent ATPases identified in the yeast *Saccharomyces cerevisiae* include Prp2 (Kim *et al.* 1992), Prp5 (O'Day *et al.* 1996), Prp16 (Schwer and Guthrie 1991), Prp22 (Company *et al.* 1991), Prp28 (Strauss and Guthrie 1991), Prp43 (Arenas and Abelson 1997), and Rss1 (Lin and Rossi 1996) [also known as Brr2 (Noble and Guthrie 1996), Slt22 (Xu *et al.* 1996), and Snu246 (Lauber *et al.* 1996)]. All contain conserved RNA helicase motifs, including the signature DEAD or DEAH sequence and some have RNA helicase activity *in vitro* (Laggerbauer *et al.* 1998; Schwer and Gross 1998; Wagner *et al.* 1998; Wang *et al.* 1998).

The Prp2 protein is an extrinsic factor that interacts transiently with spliceosomes prior to the first transesterification reaction in pre-mRNA splicing (King and Beggs 1990; Kim and Lin 1993; Plumpton *et al.* 1994). Prp2 binds to the pre-mRNA-containing spliceosome in the absence of ATP *in vitro* and is released from the spliceosome following ATP hydrolysis (Kim and Lin 1993; Plumpton *et al.* 1994); as a result, the spliceosome is rearranged and activated for the first transesterification reaction (Kim and Lin 1996). Prp2 has not been shown to have RNA helicase activity *in vitro*; however, Prp2 may require additional protein factor(s) for this activity (Kim *et al.* 1992). Thus far, one factor, Spp2, has been identified that interacts directly with Prp2 (Roy *et al.* 1995). *SPP2* was identified as a high-copy suppressor of temperature-sensitive *prp2* mutant strains (Last *et al.* 1987). Biochemical analysis demonstrated that Spp2 is an essential splicing factor required for Prp2 binding to the spliceosome prior to the first transesterification reaction (Roy *et al.* 1995).

Genetic approaches to the identification of factors

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involved in yeast pre-mRNA splicing have been fruitful. Thus far, at least 40 mutants have been identified that have defects in RNA processing (*prp*), as measured by the accumulation of pre-mRNA or splicing intermediates (reviewed in Ruby and Abelson 1991; Rymond and Rosbash 1992). Successful strategies for isolation of mutants in pre-mRNA splicing have included screens of heat-sensitive (Hartwell 1967; Vijayraghavan *et al.* 1989; Blanton *et al.* 1992; Maddock *et al.* 1996) or cold-sensitive (Strauss and Guthrie 1991; Noble and Guthrie 1996) banks of mutants. Identification of extragenic suppressors or of mutants that show a synthetic lethal phenotype with mutations in known splicing factors has also been informative (Couto *et al.* 1987; Chapon and Legrain 1992; Frank *et al.* 1992; Liao *et al.* 1993; Wells *et al.* 1996; Xu *et al.* 1998).

Extragenic suppressors and synthetic lethal mutations are often in genes whose products physically interact with the original mutant gene product (Huffaker *et al.* 1987). To identify factors that interact with Prp2, we isolated an extragenic suppressor of *prp2-1*. The suppressor is an anticodon mutation in *EMT1*, one of the genes encoding elongation $tRNA^{Met}$, which changes CAT to CAG. This anticodon mutation in *EMT1-201* presumably allowed *EMT1-201* suppressor tRNA to recognize CUG leucine codons instead of AUG methionine codons. We identified the *prp2-1* mutation as a change from glycine to aspartate at amino acid 360, indicating that *EMT-201* does not act by missense suppression of *prp2-1*. In addition, cells carrying *EMT1-201* have a Cs^- phenotype and a splicing defect at 18°, which could be rescued by extra copies of a $tRNA^{Leu}$ gene. This study provides the first example in which a mutation in a tRNA gene confers a pre-mRNA processing (*prp*) phenotype.

MATERIALS AND METHODS

Yeast strains: Yeast strains used in this study are listed in Table 1. All genetic manipulations, tetrad dissections, and yeast growth conditions were performed as described in Rose *et al.* (1990). Yeast were transformed with DNA using the lithium acetate method as described in Rose *et al.* (1990). The *prp2-1::URA3* marked strain was constructed as follows to distinguish intragenic from extragenic suppressors by genetic crosses. A *Hin*dIII/*Bam*HI fragment of *PRP2* (Chen and Lin 1990) was cloned into the *Hin*dIII and *Bam*HI site of the plasmid YIp5 and named YIp5-H/B. The plasmid YIp5-H/B was linearized with *Xba*I, which cut within the *Hin*dIII/*Bam*HI fragment, before transformation into the *prp2-1* strain P30-6D, which has a point mutation between the *Hin*dIII and *Xba*I sites of *PRP2*. Homologous recombination results in one copy of the complete *prp2-1* allele and a 5′-truncated *PRP2*, with the integrated *URA3* marker lying between them. All Ura^+ transformants had Ts^- phenotypes. Transformants were crossed with a wild-type strain and the resulting tetrads were analyzed, showing two Ts^-/Ura^+ and two Ts^+/Ura^- spores (data not shown). Ts^-/Ura^+ strains with two different mating types were obtained as the *prp2-1::URA3* marked strain A (*MAT***a**) and B (*MAT*α).

Isolation of suppressors: The *prp2-1* strain 3.2A10C was mutagenized using ethyl methanesulfonate (EMS) as described in Vijayragnathan *et al.* (1989). The treatment resulted in 10–30% survival. Spontaneous Ts^+ revertants of the *prp2-1* strain SS304 were isolated from cells plated on YPD plates (about 1×10^8 cells per plate), and incubated for 4 days at 35°. Ts^+ colonies were selected and tested for cold sensitivity on YPD plates incubated at 18°.

RNA isolation and analysis: RNA was extracted from yeast by the glass bead method described by Lin *et al.* (1996). A 10-ml yeast culture grown in YPD, OD_{600} of 1.5, was shifted to different temperatures of 34°, 26°, or 18° for 2 or 4 hr. Cells were harvested by centrifugation and cell pellets were frozen at −80° prior to RNA extraction. A total of 20 μg of total cellular RNA per lane was separated on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. Northern blot analysis was performed according to Rose *et al.* (1990). The blots were probed with a 2.2-kb *Hin*dIII fragment of the *CRY1* gene and an *Eco*RI fragment of the *ACT1* gene, which were uniformly labeled with ^{32}P by random priming.

Preparation of extracts and *in vitro* splicing reactions: Preparation of splicing extracts and actin pre-mRNA substrate was done as described (Lin *et al.* 1987; Kim and Lin 1993, 1996). Heat-inactivated *prp2* extract (*prp2*Δ) was made by incubating the *prp2* splicing extract at 37° for 30 min. Glass bead extracts were used as a source of the Prp2 protein and were prepared as follows: cells from 5 ml of yeast culture ($OD_{600} \approx 3$) were harvested, washed with water, and frozen at −80° until use. Frozen cells were thawed and washed with prechilled buffer A (10 mm HEPES, pH 7.8, 1.5 mm $MgCl_2$, 10 mm KCl, 0.5 mm DTT) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mm), benzamidic acid (1 mm), leupeptin (1 μm), and pepstatin A (1 μm), and finally resuspended in 300 μl of the same solution. An equal volume of glass beads (0.45 mm, Sigma, St. Louis) was added and the cells were broken by vortexing for 1 min, followed by chilling on ice for 1 min. The vortexing procedure was repeated three times. After a 1-min spin at 4°, supernatants were collected and mixed with 1/10 volume of 2 m KCl. The mix was centrifuged at 14,000 rpm at 4° for 15 min, and the resulting supernatant was collected and used as glass bead extract.

Spliceosome assembly assay: Nondenaturing composite gel electrophoresis was performed essentially as described (Seraphin and Rosbash 1989). The polyacrylamide-agarose mixture was cooled to 36° before *N,N,N′,N′*-tetramethylenediamine was added. The gel was polymerized between two glass plates at room temperature for 1 hr and stored at 4°. *In vitro* splicing reactions (10 μl) were stopped by the addition of 10 μl ice-cold buffer R (2 mm $(MgOAc)_2$, 50 mm HEPES, pH 7.5), and 1 μl yeast total RNA (10 mg/ml). The mixture was incubated at 0° for 10 min and then 5 μl loading buffer was added and 5 μl of the final mixture was loaded onto the gel.

Library construction and cloning: Yeast genomic DNA from strain P30-4C (*prp2-1*, *EMT1-201*) was prepared from 500 ml of an overnight culture using glass beads and polyethylene glycol precipitation as described in Fujimura and Sakuma (1993). Purified DNA (200 mg) was divided into five aliquots, and each was digested in a 100-μl reaction with 10 units of restriction enzyme *Dpn*II (New England Biolabs, Beverly, MA) at 37°. The extent of digestion was monitored by gel electrophoresis of a small aliquot, while the remaining samples were frozen in liquid nitrogen and stored at −80°. Digestion with *Dpn*II was continued until the average length of DNA fragments was ~10 kb. DNA fragments around the 10-kb marker were purified from agarose gels by phenol extraction. Purified DNA fragments were ligated overnight at 18° with an equal amount of YCp50 DNA, which had been digested with *Bam*HI and treated with calf intestine alkaline phosphatase. The ligated DNA was separated from unligated DNA by electropho-

TABLE 1

Yeast strains used in this study

Strain	Relevant genotype	Source
SS304	*MAT*α *prp2-1 ade2-101 his3-Δ200 tyr1-901 ura3-52*	Vijayraghavan (1989)
SS330	*MAT***a** *ade2-101 his3-Δ200 tyr1-901 ura3-52*	Vijayraghavan (1989)
SS328	*MAT*α *ade2-101 his3-Δ200 lys2-801 ura3-52*	Vijayraghavan (1989)
P30-4C	*MAT*α *prp2-1 EMT1-201 ade2-101 his3-Δ200 lys2-801 ura3-52*	This study
P30-6D	*MAT***a** *prp2-1 his3-Δ200 ura3-52*	This study
P25-7A	*MAT***a** *ade2-101 his3-Δ200 tyr1-901 ura3-52*	This study
P25-7C	*MAT*α *EMT1-201 ade2-101 his3-Δ200 lys2-80 ura3-52*	This study
P30-13	*MAT***a** *EMT1-201 ade2-101 his3-Δ200 leu2-3 lys2-801*	This study
prp2-1:URA3$_A$	*MAT***a** *prp2-1*^{URA3} *ade2-101 his3-Δ200 lys2-801*	This study
prp2-1:URA3$_B$	*MAT*α *prp2-1*^{URA3} *ade2-101 his3-Δ200 tyr1-901*	This study
3.2A10B	*MAT*α *prp2-1 ade2-101 his3-Δ200 lys2-801 ura3-52*	This study
3.2A10C	*MAT*α *prp2-1 ade2-101 his3-Δ200 tyr1-901 ura3-52*	This study
2-5.1D1D	*MAT***a** *prp2-5 ade2-101 his3-Δ200 lys2-801 ura3-52*	This study
2-8.3A1D	*MAT***a** *prp2-8 ade2-101 his3-Δ200 lys2-801 ura3-52*	This study

resis on agarose gels and purified by phenol extraction. A total of 5 μg of the ligated, unamplified genomic library from P30-4C (*prp2-1, EMT1-201*) was used to transform strain P30-6D (*prp2-1*) by the lithium acetate method. After transformation, plates were incubated at 26° for 12 hr and then shifted to 35° for 3 to 4 days.

A wild-type library in YCp50 (a gift of Dr. Clarence Chan, University of Texas, Austin) was used to transform strain P30-13 (*EMT1-201*) to select for Cs$^+$ clones and isolate the gene encoding *tRNA*^{Leu} *(UAG).* After transformation, plates were incubated directly at 18°.

For mapping the *prp2-1* mutation, *PRP2* clones with sequential deletions from the N terminus were used in a marker rescue experiment. The YCp50-EB plasmid was constructed by inserting the 3.2-kb *Eco*RI/*Bam*HI fragment containing the *PRP2* gene into YCp50 (Figure 5b). YCp50-HB contains the *Hin*dIII/*Bam*HI fragment of *PRP2*, which removes the N-terminal amino acids 1–205, YCp50-XB contains the *Xba*I/*Bam*HI fragment of *PRP2*, removing amino acids 1–464, and YCp50-PB contains the *Pvu*II/*Bam*HI fragment of *PRP2*, removing amino acids 1–668. After transformation of each plasmid into the *prp2-1* strain P30-6D, Ura$^+$ colonies were selected. One milliliter of culture ($OD_{600} \approx 3$) was washed with sterile water, spread on Ura$^-$ plates, and incubated at 37°. Ts$^+$ revertants were counted after 4 days.

The plasmid YCp51 was derived from YCp50 by removal of the *Eco*RI and *Hin*dIII sites. A *Bam*HI/*Bam*HI fragment of *PRP2* was inserted into YCp51 and the resulting plasmid was used to clone the *prp2-1* mutation by the gap repair method (Orr-Weaver *et al.* 1981). A gap was created by digestion of YCp51-*PRP2* with *Hin*dIII and *Xba*I since the mutation is located between those restriction sites. The plasmid with the gap was gel purified and used to transform the *prp2-1* strain P30-6D. Plasmids from Ura$^+$/Ts$^-$ transformants were recovered through *Escherichia coli* transformation as described in Rose *et al.* (1990).

PCR: Primers EMT1-1 (5′ ATGAGAATTCAGGATAATG TATTG 3′) and EMT1-2 (5′ ATTTGAATTCTCAAATAAAT GAGC 3′) were designed to PCR amplify the entire *EMT1* gene from wild-type and suppressor strains. The PCR product was gel purified and ligated to the *Eco*RI site of the CEN vector pRS316 (Sikorski and Hieter 1989) or the 2μ vector pRS426 (Christianson *et al.* 1992). Primers Leu-1 (5′ TTCTTGAATT CATTTAAAGATATT 3′) and Leu-2 (5′ TTCAGGTACCATTT GCCATTTTGC 3′) were used to amplify the *tRNA* ^{Leu} *(UAG)* gene. The PCR product was gel purified and ligated to the *Eco*RI and *Kpn*I sites of the CEN vector pRS316. Primers Leu-3 (5′ GGGAGTTTGGCCGAGTGG 3′) and Leu-4 (5′ TGA GAGCTAAGGGATTCG 3′) were used to test DNA fragments for the presence of the *tRNA*^{Leu}*(UAG).* Sequencing of *EMT1-1* was performed using Sequenase (Stratagene, La Jolla, CA) and the primer 5′ TCATATGTGTTCTAT 3′.

RESULTS

Isolation and characterization of extragenic suppressors: To understand the nature of the binding of Prp2 to the spliceosome, we used a genetic suppression approach to identify Prp2-interacting factors. Spontaneous or EMS-induced Ts$^+$ (at 35°) revertants of temperature-sensitive *prp2-1* strains SS304 and 3.2A10C were selected (see Table 1 for a complete description of the strains used in this study). There were 89 Ts$^+$ revertants from 1×10^9 EMS-treated cells and 31 spontaneous Ts$^+$ revertants from 2×10^9 untreated SS304 cells. To eliminate intragenic revertants as well as to facilitate genetic analysis of the suppressors, the cold sensitivity of single Ts$^+$ colonies was tested on YPD plates at 18°. Seven Ts$^+$Cs$^-$ suppressors were isolated from the two *prp2-1* strains. Revertants were twice backcrossed to *prp2-1* strain 3.2A10B to obtain clean Ts$^+$Cs$^-$ cosegregation. Analysis of at least 12 tetrads from each of the seven revertants showed two Ts$^+$Cs$^-$, two Ts$^-$Cs$^+$ segregation patterns, indicating that suppression and Cs$^-$ were linked and could be caused by a single mutation. One of the Ts$^+$Cs$^-$ suppressors, *SPP201-1* (suppressor of *prp2-1*), was characterized further; the remaining six suppressors later were found to be identical to *SPP201-1* (see below).

The putative suppressor-carrying strain was crossed to the *prp2-1* strain 3.2A10B and to the wild-type strain SS330 to determine the dominance of the Ts suppression and Cs growth traits. Diploid cells with the genotype

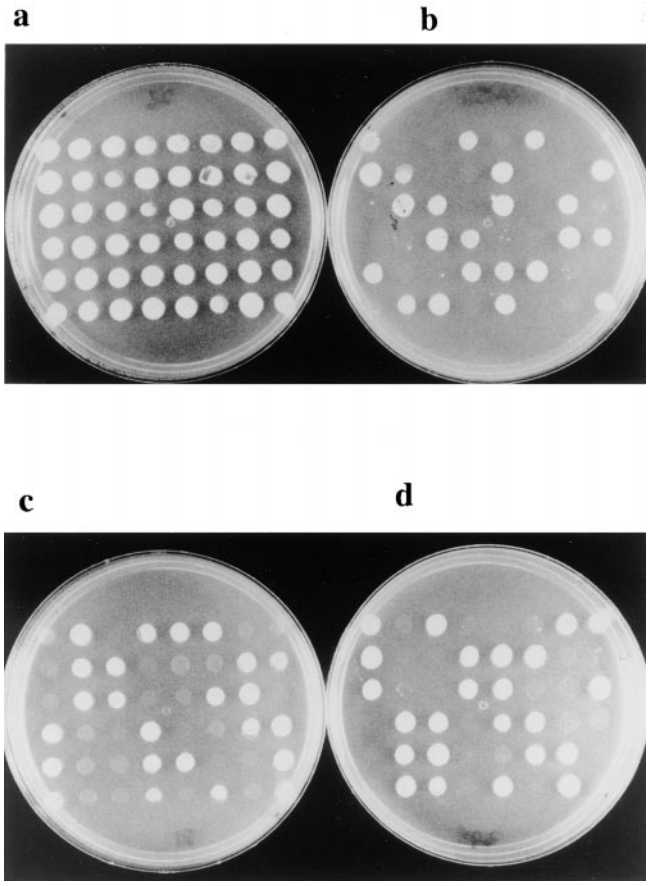


Figure 1.—*SPP201-1* is not linked to *PRP2*. The *prp2-1, SPP201-1* strain 5-23 was crossed with a *prp2-1* marked strain (*prp2-1::URA3*), sporulated, and the tetrads dissected. Progeny cells were suspended in water and aliquots spotted on YPD or −Ura plates. Each row contains 2 tetrads, and 12 complete tetrads were tested on (a) YPD at 26°, (b) −Ura at 26°, (c) YPD at 34°, and (d) YPD at 18°.

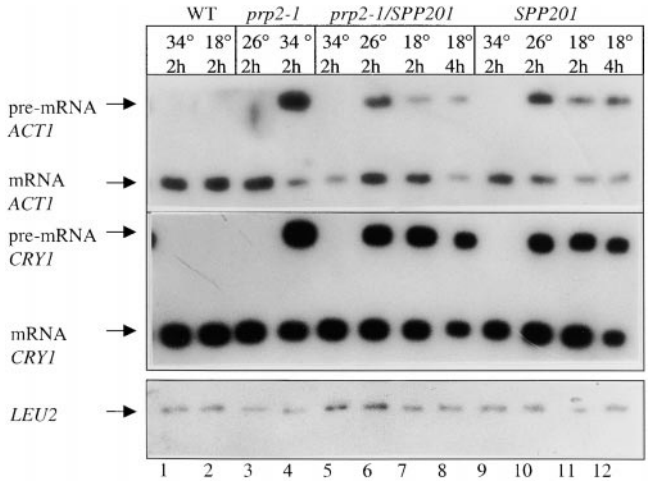


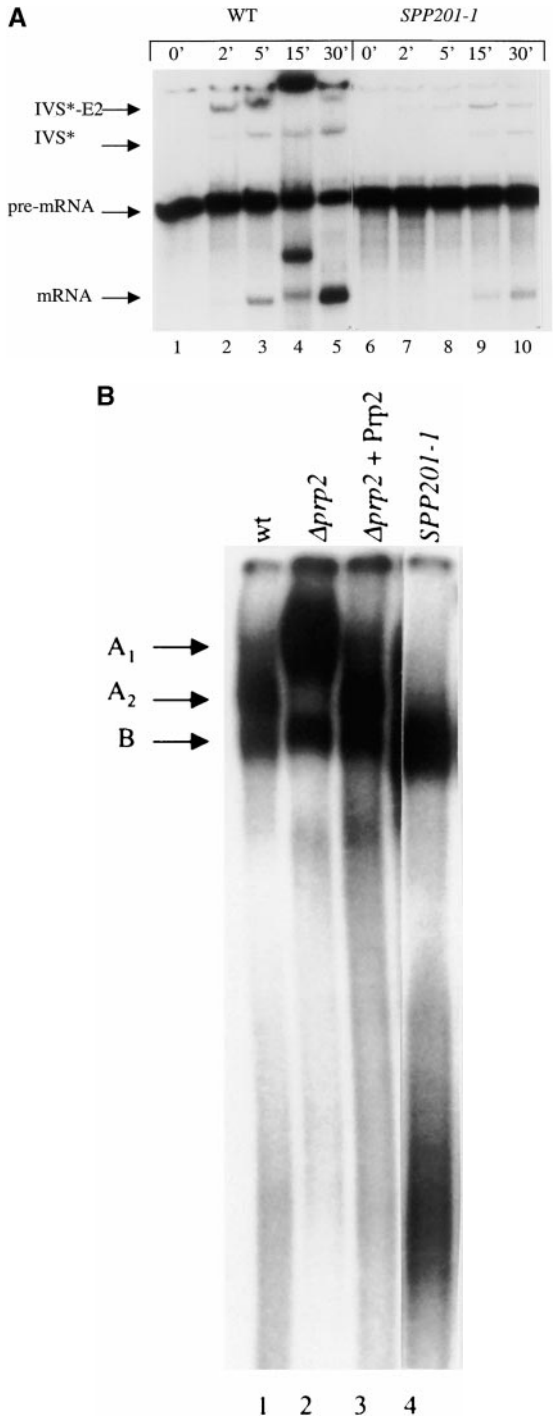
Figure 2.—*SPP201-1* suppresses the splicing defect of *prp2-1* at 34° and has a splicing defect at low temperatures *in vivo*. Cells from the indicated haploid strains were grown at 26° in YPD to OD_{600} of 1.5 and then were shifted to 34° for 2 hr, 18° for 2 or 4 hr, or maintained at 26° for 2 hr. RNA was extracted from each culture, and 20 μg of total RNA per lane was separated on a 1.2% denaturing agarose gel and blotted. Blots were hybridized with ^{32}P-labeled *CRY1*, *ACT1*, or *LEU2* DNA probes, as indicated. The positions of the unspliced pre-mRNAs and spliced mRNAs are indicated. Lanes 1 and 2, RNA from wild-type strain SS328; lanes 3 and 4, RNA from *prp2-1* strain P30-6D; lanes 5–8, RNA from *prp2-1, SPP201-1* strain P30-4C; lanes 9–12, RNA from *SPP201-1* strain P30-13.

prp2-1/*prp2-1*, *spp201*$^+$/*SPP201-1* were Ts$^+$ at 35°, and diploid cells with *PRP2*/*prp2-1*, *spp201*$^+$/*SPP201-1* were Cs$^-$ at 18°, indicating that suppression of *prp2-1* and cold-sensitive growth were both dominant traits (data not shown). Interestingly, further analysis of *SPP201-1* revealed that while it can grow at 35° it is Ts$^-$ at 37°, suggesting that *SPP201-1* not only caused the Cs$^-$ phenotype but also is partially Ts$^-$. In fact, *SPP201-1* strains grew more slowly than wild-type strains at 26°. To confirm that the Cs$^-$ phenotype cosegregated with suppression and the mutation was not linked to the *PRP2* locus, the *prp2-1, SPP201-1* strain was crossed to a strain carrying a *prp2-1::URA3* marked allele. Upon sporulation, tetrads were dissected and the spore colonies were tested for temperature sensitivity, cold sensitivity, and uracil auxotrophy. Cold sensitivity and resistance to high temperature always cosegregated in 96 tetrads but did not cosegregate with uracil prototrophy, indicating that suppression and cold sensitivity were tightly linked but were not linked to the original *prp2-1* allele (Figure 1).

We also tested the specificity of suppression by *SPP201-1*. A *prp2-5* strain (2-5.1D1D) and a *prp2-8* strain (2-8.3A1D) were crossed individually with a *prp2-1::URA3, SPP201-1* strain and the resulting tetrads were analyzed. Consistent with *SPP201-1* being an allele-specific suppressor of *prp2-1*, only spore colonies that were cold sensitive and uracil prototrophs could also grow at 35° (data not shown).

***SPP201-1* relieves the splicing defect of *prp2-1* and has a *prp* phenotype at low temperature:** If *SPP201-1* suppressed the splicing defect of *prp2-1*, spliced messages should be detected in *prp2-1, SPP201-1* double mutant cells at high temperature. Analysis of RNA from *prp2* mutant strains isolated after a temperature shift to the nonpermissive temperature shows accumulation of unspliced pre-mRNA. To examine the effects of *SPP201-1* on the *prp2-1* splicing defect, total RNA was prepared from four different haploid strains: wild type (SS328); *prp2-1* (P30-6D); *prp2-1, SPP201-1* (P30-4C); and *SPP201-1* (P30-13), with or without temperature shifts. Northern blot analysis was used to observe the steady-state levels of two intron-containing pre-mRNAs: *CRY1*, which encodes a ribosomal protein, and *ACT1*, which encodes actin. Intron-less *LEU2* mRNA was used as a loading control (Figure 2, bottom). As expected, the wild-type strain showed only fully processed *ACT1* or *CRY1* mRNAs at both high and low temperatures (Figure 2, lanes 1 and 2). The *prp2-1* strain did not accumulate pre-mRNA at the permissive temperature (lane 3) but

accumulated both *ACT1* and *CRY1* pre-mRNAs after a temperature shift to 34° for 2 hr (lane 4). The *prp* phenotype was relieved in the *prp2-1, SPP201-1* strain, as seen by the lack of pre-mRNA accumulation after a temperature shift to 34° for 2 hr (lane 5). This result is in accord with the Ts^+ phenotype of the *prp2-1, SPP201-1* strain at 34°, and suggests that *SPP201-1* suppressed the Ts growth defect of the *prp2-1* mutant by rescuing the splicing defect at 34°. Interestingly, Northern blots of RNA prepared from cells maintained at 26° show accumulation of *ACT1* and *CRY1* pre-mRNAs in both the *prp2-1*, *SPP201-1* and *SPP201-1* strains, suggesting that *SPP201-1* has a splicing defect at 26°, the permissive temperature for *prp2-1* (lanes 6 and 10). Pre-mRNA also accumulated in *SPP201-1* strains after a shift to 18° for 2 or 4 hr (lanes 7, 8, 11, 12). Therefore, *SPP201-1* rescues the temperature sensitivity and splicing defect of *prp2-1*, but confers a *prp* phenotype at low temperatures.



***In vitro* splicing activity of *SPP201-1*:** Data from the Northern analysis indicated that *SPP201-1* is involved in pre-mRNA splicing *in vivo*. Splicing extracts from *SPP201-1* prepared at low temperatures might therefore be defective for splicing *in vitro*. Splicing extracts were prepared in parallel from a $spp201^+$ wild-type (P25-7A) and a *SPP201-1* mutant (P25-7C) strain and tested for their abilities to process ^{32}P-labeled *ACT1* pre-mRNA. Splicing reactions were incubated at 23° for the indicated times, and the RNA was extracted and analyzed on a denaturing acrylamide gel (Figure 3A). The *SPP201-1* extract showed a low level of splicing activity after 15 min (Figure 3A, lane 9), whereas the wild-type extract showed a noticeable amount of pre-mRNA processing by 2 min of incubation (Figure 3A, lane 2). At 30 min, much more mRNA was produced in the wild-type extract than in the *SPP201-1* extract (Figure 3A; compare lanes 5 and 10). To show that the low splicing activity of the *SPP201-1* extract was not due to poor extract preparation, splicing complexes were formed with ^{32}P-labeled actin pre-mRNA and analyzed on nondenaturing gels (Figure 3B). Complexes corresponding to A_1, A_2, and B complex were formed after incubation with wild-type extract (Figure 3B, lane 1; Cheng and Abelson 1987). Heat-treated *prp2-1* extract (Δ*prp2*) accumulates A_1 complex, which is the complex formed prior to active spliceosome A_2 (Figure 3B, lane 2). Addition of purified Prp2 protein to Δ*prp2* permits formation of the A_2 complex (Figure 3B, lane 3). B complex and a low level of A complexes formed in the *SPP201-1* extract (Figure

Figure 3.—Extract from the *SPP201-1* mutant strain is not efficient in *in vitro* splicing. (A) Splicing extracts were prepared from the indicated strains, which were derived from a single tetrad. Extracts were assayed separately for splicing activity by incubating each with ATP and ^{32}P-labeled actin pre-mRNA as described in Kim *et al.* (1992) for either 0, 2, 5, 15, or 30 min, as indicated. After incubation, RNA was extracted and separated on a denaturing acrylamide gel. The positions of the pre-mRNA, mRNA, released intron lariat (IVS*), and intron lariat-exon 2 (IVS*–E2) intermediates are indicated. Lanes 1–5, extract derived from the wild-type strain P25-7A; lanes 6–10, extract derived from the *SPP201-1* strain P25-7C. (B) Spliceosome complex assembly. Splicing extracts prepared as above were incubated with ATP and ^{32}P-labeled actin pre-mRNA under splicing conditions and assayed for spliceosome complex assembly on a nondenaturing polyacrylamide-agarose gel. Complexes are designated as described in Cheng and Abelson (1987). Lane 1, wild-type extract; lane 2, heat-treated *prp2-1* extract; lane 3, heat-treated *prp2-1* extract with Prp2 protein added; lane 4, *SPP201-1* extract.

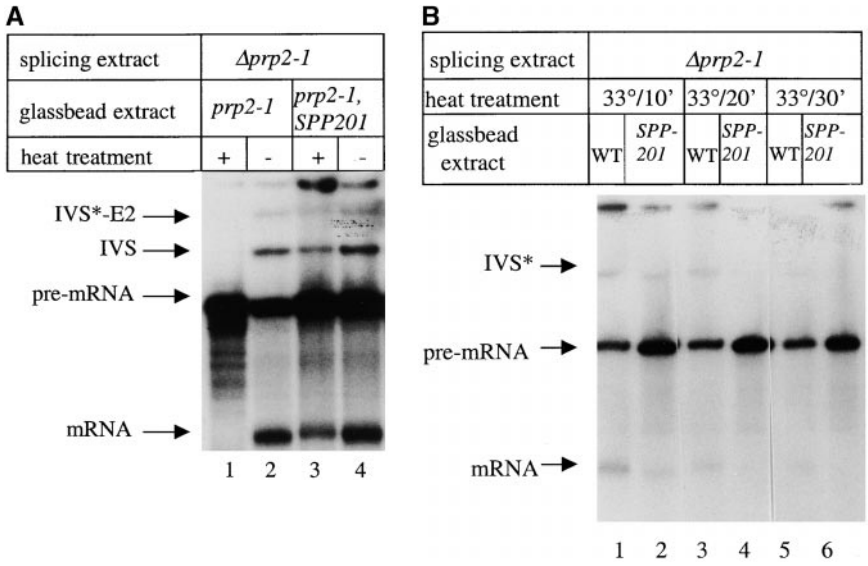


Figure 4.—Heat sensitivity of Prp2 activity in extracts. (A) The Prp2 protein from a *prp2-1*, *SPP201-1* strain is active in splicing after heat treatment. The heat sensitivities of Prp2 proteins derived from strains P30-6D (*prp2-1*) and P30-4C (*prp2-1*, *SPP201-1*) were compared for their abilities to complement heat-inactivated *prp2-1* extract. Glass bead extracts derived from the *prp2-1* strain P30-6D (lanes 1 and 2) and the *prp2-1*, *SPP201-1* strain P30-4C (lanes 3 and 4) were either incubated at 33° for 10 min (+ heat treatment, lanes 1 and 3) or left on ice (− heat treatment, lanes 2 and 4). One microliter of glass bead extract was added to 4 μl of heat-inactivated *prp2-1* splicing extract (Δ*prp2-1*) in a 10-μl splicing reaction with ATP and ^{32}P-labeled actin transcript. After incubation for 30 min at 23°, RNA was recovered by phenol extraction and ethanol precipitation and separated on a 7.5% acrylamide/7 m urea gel. The positions of the pre-mRNA, mRNA, released intron lariat (IVS*), and intron lariat-exon 2 (IVS*–E2) are indicated. (B) The Prp2 protein from a *SPP201-1* strain is less stable than Prp2 from a wild-type strain. Glass bead extracts were prepared from the wild-type strain P25-7A (lanes 1, 3, and 5) and the *SPP201-1* strain P25-7C (lanes 2, 4, and 6). Both express the Prp2 protein from the chromosomal wild-type gene. Aliquots of each glass bead extract were incubated at 33° for 10 min (lanes 1 and 2), 20 min (lanes 3 and 4), or 30 min (lanes 5 and 6) prior to use. One microliter glass bead extract was added to 4 μl heat-inactivated *prp2-1* extract (Δ*prp2-1*) in a 10-μl splicing reaction containing ATP and ^{32}P-labeled actin pre-mRNA, and processed as described in Figure 4A.

3B, lane 4), and the IVS*–E2 splicing intermediate was seen in an aliquot of the reaction separated on a denaturing polyacrylamide gel (data not shown). The *SPP201-1* extract was quite active in forming the B complex, indicating that the extract preparation was fine. The partial block to the transition from B to A_1 in the *SPP201-1* extract suggested that some splicing factors involved in the loading of U4/U6.U5 tri-snRNP might be affected by the mutation. Thus, both the *in vivo* and *in vitro* assays indicated that the gene product of *SPP201* may be involved in pre-mRNA splicing and possibly interacts with Prp2.

To examine the mechanism by which *SPP201-1* suppressed the Ts^- growth of *prp2-1*, the thermal stability Prp2 activity was tested in *SPP201-1* and *spp201*$^+$ extracts. Glass bead extracts, which by themselves cannot carry out a complete splicing reaction, were prepared from a *prp2-1* strain (P30-6D) and a *prp2-1*, *SPP201-1* strain (P30-4C) and compared for their ability to complement heat-inactivated *prp2-1* splicing extracts (*prp2*Δ; Figure 4A). The mutant Prp2 protein encoded by *prp2-1* can be inactivated after heat treatment of the *prp2-1* extract (Lustig *et al.* 1986); thus, the only active Prp2 must be supplied by the complementing extract (Lin *et al.* 1987). Both glass bead extracts could complement the *prp2*Δ extract, indicating that Prp2 from either complementing extract was active (Figure 4A, lanes 2 and 4). Extract from the *prp2-1* strain could no longer complement *prp2*Δ after heat treatment as expected (Figure 4A, lane 1), whereas the *prp2-1, SPP201-1* extract remained active after the same treatment (Figure 4A, lane 3). These results suggest that the Prp2 protein in the *prp2-1*, *SPP201-1* extract is more heat resistant than the Prp2 protein in the *prp2-1* extract. Therefore the suppression of *prp2-1* by *SPP201-1* may be due to the synthesis of a more heat-stable protein in the suppressor-containing strain.

It is possible that *SPP201-1* has a global effect on protein stability at high temperature, such as by inactivating a protease. If that were true, the wild-type Prp2 protein should be more heat stable in the presence of *SPP201-1*. To test this, glass bead extracts were prepared from a wild-type (P25-7A) and *SPP201-1* mutant strain (P25-7C), heat treated by incubation at 33° for different times, and compared for their ability to complement heat-treated *prp2-1* splicing extracts (*prp2*Δ) in an *in vitro* splicing assay (Figure 4B). There was an increase in pre-mRNA accumulation and a decrease in processing intermediates in the *SPP201-1* extract relative to wild type after 10 min (Figure 4B, lanes 1 and 2), 20 min (Figure 4B, lanes 3 and 4), or 30 min (Figure 4B, lanes 5 and 6) of heat treatment. Thus, the *SPP201-1* extract was more heat sensitive than the wild-type extracts, indicating that *SPP201-1* may decrease the stability of the wild-type Prp2 protein at high temperatures. Moreover, these data may also suggest that the growth defect of *SPP201-1* strains at 37° may be due to increased heat sensitivity of the Prp2 protein.

***SPP201-1* is an allele of the *EMT1* gene:** Because *SPP201-1* was a dominant suppressor, cloning of the *SPP201*-1 gene required a library made from a suppressor strain. We made a yeast genomic library from the

prp2-1, SPP201-1 strain P30-4C and transformed the *prp2-1* strain P30-6D with it. Seventeen thousand transformants of P30-6D were screened for their ability to grow at 35° since the *SPP201-1* allele is dominant for reversion of the *prp2-1* Ts growth phenotype. Eleven isolates were identified that met the screening criterion. Strains of *prp2-1* transformed with these plasmids grew at 35° but not at 18°, indicating that the clones could contain the *SPP201-1* gene since both suppression and cold sensitivity were dominant. Further restriction enzyme analysis showed that all clones had a common 1.7-kb *Bst*BI fragment (data not shown). A 2.4-kb *Hin*dIII/*Hin*dIII fragment containing the common 1.7-kb fragment was cloned into the vector pRS316 and the DNA sequence was determined. The sequence information was used to search the yeast genomic sequence database and a unique segment of the yeast genome was identified. The fragment included part of a Ty1 element and the *EMT1* gene, one of the five genes encoding elongator methionine tRNA in *Saccharomyces cerevisiae* (Åström *et al.* 1993). Since the tRNA gene was the only complete gene on the 2.4-kb segment, it was likely that it was *SPP201*. The *EMT1* region on the plasmid was amplified by PCR and inserted into pRS316. Complete sequence determination of the *EMT1* gene on the recombinant plasmid pRS316-EMT1 demonstrated that the anticodon sequence of *EMT1* in the complementing clone had a mutation from CAT to CAG (data not shown), which presumably would cause the tRNA to recognize a leucine codon instead of a methionine codon. To confirm that the mutant *EMT1* encoded *SPP201-1*, primers from the *EMT1* gene were used to amplify the *EMT1* gene from wild type and six additional *SPP201-1* strains by PCR. Sequencing of the PCR products showed that the *emt1*$^+$ gene from the wild-type strain had CAT at the anticodon, whereas *EMT1* from all seven *SPP201-1* strains had CAG at the anticodon.

The relevance of the anticodon mutation in *EMT1* to the suppression of *prp2-1* was demonstrated by transformation of the *prp2-1* strain P30-6D with *EMT1* from either the wild-type (P30-6D) or *SPP201-1* (P30-4C) strain. Transformation of *prp2-1* by pRS316-EMT1 from *SPP201-1* converted the phenotype from Ts$^-$Cs$^+$ to Ts$^+$Cs$^-$, whereas the gene from the wild-type strain did not (data not shown). Transformation of a wild-type strain with *EMT1* from the suppressor strain caused the cell to be Cs$^-$ at 18° and Ts$^-$ at 37° (data not shown). The transformation data indicate that the suppressor is an allele of *EMT1*, and therefore it was renamed *EMT1-201*.

We sequenced the mutation in *prp2-1* to determine if suppression by *EMT1-201* was due to missense suppression. The location of the *prp2-1* mutation was determined by marker rescue using sequentially deleted *PRP2* clones. The mutated region in the *prp2-1* gene was cloned by gap repair and sequenced (Orr-Weaver *et al.* 1981; see materials and methods). The mutation in *prp2-1* is a G to A, changing a glycine to aspartate at amino acid 360 (data not shown). Because *EMT1-201* codes for a mutated tRNA^{Met}, which could recognize a leucine codon, it is unlikely that *EMT1-201* suppresses *prp2-1* by missense suppression.

If *EMT1-201* misincorporates methionine into leucine codons during translation, cells with a high-copy number of *EMT1-201* could be unable to grow due to a decrease in the fidelity of protein synthesis. The *EMT1-201* mutation creates a perfect anticodon:codon recognition for the CUG leucine codon. *S. cerevisiae* does not have a tRNA^{Leu} with a CAG anticodon, but instead requires a G:U wobble pairing with tRNA^{Leu} (UAG; Percudani *et al.* 1997). Therefore, the mutant tRNA in *EMT1-201* expressed at high levels may have a higher affinity than the endogenous tRNA^{Leu}(UAG) for recognition of the CUG leucine codon in the mRNA. To test this idea, plasmids carrying the *EMT1-201* gene were constructed in pRS316, a single copy *CEN* vector, and in pRS426, a multicopy 2μ vector. The *prp2-1* strain P30-6D was transformed with three different *EMT1-201* constructs and the vector alone (Figure 5). Cells transformed with pRS316/*EMT1-201*, a single copy plasmid with one copy of *EMT1-201*, grew somewhat slower than the control at 26° (Figure 5b). Cells transformed with pRS426/*EMT1-201*, a multicopy plasmid with one copy of *EMT1-201*, grew more slowly than both the pRS316/*EMT1-201* and control transformants, forming only tiny colonies (Figure 5c). No transformants were observed when cells were transformed with a multicopy plasmid with three copies of *EMT1-201*, indicating that overexpression of *EMT1-201* is deleterious to wild-type yeast cells (Figure 5d).

The cold-sensitive growth of *EMT1-201* could be caused by misincorporation of methionine into leucine codons during translation. If that is so, additional copies of the leucine tRNA may rescue the Cs$^-$ and splicing defects conferred by *EMT1-201* by competing with the *EMT1-201* mutant tRNA. We addressed this possibility by screening a wild-type yeast genomic library on the CEN vector YCp50 for complementation of Cs growth. The library was used to transform an *EMT1-201* mutant strain (P30-13) and select for the ability to grow at 18°. Three cold-resistant clones were isolated among 7200 Ura$^+$ transformants. Sequence analysis of a 0.9-kb complementing fragment identified a 104-bp region with complete sequence identity to the *tRNA^{Leu}(UAG)* gene. The gene was amplified by PCR and subcloned into the single-copy centromere vector pRS316. The resulting plasmid, pRS316-*tRNA^{leu}(UAG)*, suppressed the Cs$^-$ phenotype of *EMT1-201* strains *in vivo* (data not shown).

Suppression of the *in vitro* splicing defect was tested by preparing extracts from *EMT1-201* strains that either did or did not carry the pRS316-*tRNA^{Leu} (UAG)* plasmid. Splicing of actin pre-mRNA was detected in wild-type extract (Figure 6, lane 1) but not in the *EMT1-201* extract (Figure 6, lane 2). The *EMT1-201* extract could

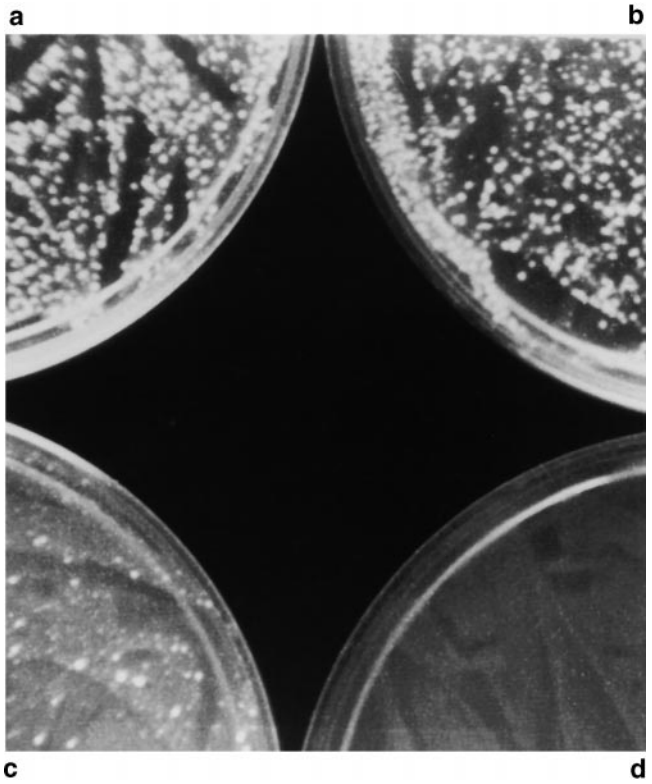


Figure 5.—An elevated copy number of *EMT1-201* is lethal to the cell. The *prp2-1* strain P30-6D was transformed with 2 μg of the indicated plasmid DNA and then incubated at 26° for 3–4 days. (a) pRS426 vector alone; (b) pRS316/*EMT1-201*, which has a single copy of *EMT1-201* on a single copy vector; (c) pRS426/*EMT1-201*, which has a single copy of *EMT1-201* on a multicopy vector; (d) pRS426/3 copies of *EMT1-201*.

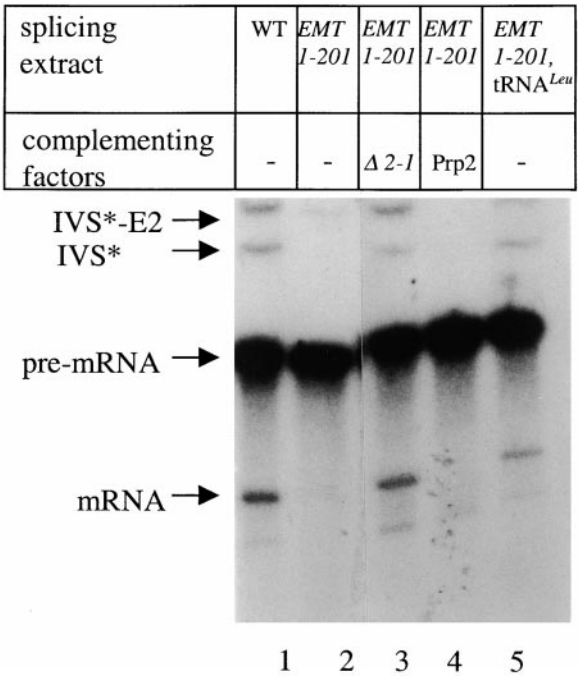


Figure 6.—A *tRNA^{Leu}* gene rescues the splicing defect of *EMT1-201*. Splicing extracts were prepared from the wild-type strain P25-7A, the *EMT1-201* strain P25-7C, and strain P25-7C carrying the plasmid pRS316/*tRNA^{Leu}(UAG)*. Splicing reactions were done in 10 μl, containing 4 μl splicing extract, ATP, and ^{32}P-labeled actin pre-mRNA, with or without 1 μl of the complementing factor. After incubation at 23° for 30 min, RNA was extracted and separated on a 7.5% acrylamide/7 m urea gel. Lane 1, wild-type extract (P25-7A); lane 2, *EMT1-201* extract (P25-7C); lane 3, *EMT1-201* extract plus 1 μl heat-treated *prp2-1* splicing extract (Δ *2-1*); lane 4, *EMT1-201* extract plus 1 μl purified Prp2 protein; lane 5, extract from the *EMT1-201* strain carrying the plasmid pRS316/*tRNA^{Leu}(UAG)*. The positions of the actin pre-mRNA, mRNA, released intron-lariat (IVS*), and intron lariat-exon 2 (IVS*–E2) are indicated.

be complemented by a splicing extract missing only functional Prp2 (Figure 6, *prp2*Δ, lane 3), but not by purified Prp2 protein (Figure 6, lane 4), supporting the *in vivo* evidence that *EMT1-201* confers a splicing defect at low temperatures that is independent of its suppression of the *prp2-1* splicing defect. Splicing activity was restored in the *EMT1-201*/*tRNA^{Leu} (UAG)* strain (Figure 6, lane 5), indicating that the leucine tRNA was able to suppress the Cs$^-$ and splicing defects conferred by *EMT1-201* both *in vivo* and *in vitro*. These suppression data support the idea that the *EMT1-201* phenotype is caused by misincorporation of methionine into leucine codons during translation.

DISCUSSION

Prp2 is an RNA-dependent ATPase required before the first transesterification reaction in pre-mRNA splicing. Temperature-sensitive *prp2-1* mutants are defective in pre-mRNA splicing *in vivo* and *in vitro*. To identify factors that interact genetically with *PRP2*, dominant Cs$^-$ suppressors of *prp2-1* were isolated. The Cs mutation suppressed the *prp2-1* splicing defect *in vivo*. Interestingly, the Cs suppressor by itself has a splicing defect *in vivo* and *in vitro*. We identified the suppressor as one of the genes encoding elongator methionine tRNA, which had a mutation allowing it to read a leucine codon. We identified the *prp2-1* mutation as a glycine to aspartate substitution, indicating that the mutant tRNA does not act by classical missense suppression. Our work is the first example of a mutation in a tRNA gene showing a *prp* phenotype.

Genetic suppression studies have been used successfully to identify new splicing factors. For example, *SPP2* was identified as a high-copy suppressor of *prp2* mutants (Last *et al.* 1987). The genetic interaction was confirmed biochemically; *SPP2* is an essential gene whose product interacts with Prp2 protein and is required prior to the first step in splicing (Roy *et al.* 1995). Genetic suppression experiments have also identified *PRP16* as a suppressor of a branchpoint mutation (Couto *et al.* 1987). *SPP91-1* was initially identified as a suppressor of *PRP9* (Chapon and Legrain 1992) and

is an allele of *PRP21*, a gene whose product is associated with the U2 snRNP (Arenas and Abelson 1993).

Extragenic suppressors of mutations in splicing factors that affect splicing only indirectly have also been identified. *SPP41*, an allele of *SRN1*, was isolated in a screen for suppressors of *prp4* (Maddock *et al.* 1994). The gene product of *SRN1* is a negative regulator of glucose-repressed genes (Tung *et al.* 1992) and is allelic to *REG1*, which encodes a regulatory subunit of protein phosphatase 1 (Tu and Carlson 1995). It was thought that *SRN1*/*REG1*/*SPP41* suppresses the *prp4* mutant by derepressing its expression (Maddock *et al.* 1994). Interestingly, mutations in *SRN1* can suppress a *prp2*, *prp6* double mutant (Pearson *et al.* 1982). Another example is *spp81*, an allele of the *DED1* gene, which encodes a putative RNA helicase (Jamieson *et al.* 1991). *Spp81* was identified as a suppressor of *prp8* (Jamieson *et al.* 1991), whose gene product is a U5-associated protein. Recent evidence, however, indicates that Ded1 is a cytoplasmic protein required for translation, and no role for Ded1 in pre-mRNA splicing has been found (Chuang *et al.* 1997).

In our study, the suppressors of *prp2-1* were mapped to the *EMT1* locus, which is one of five genes encoding elongator methionine tRNAs (Åström *et al.* 1993). All suppressors have a T to G mutation in the anticodon, changing the anticodon from CAT, which reads the AUG methionine codon, to CAG, which reads the CUG leucine codon. The *EMT1-201* mutation suppressed the *in vivo* splicing defect of *prp2-1* cells (Figure 2). However, unlike the indirect suppressors of splicing defects described above, *EMT1-201* has a splicing defect even in a wild-type background (Figures 2, 3, and 6). The *EMT1-201* mutation could be suppressed by overexpression of *tRNA^{Leu} (UAG)*, which reads the CUG leucine codon (Figure 6).

We identified the mutation in *prp2-1* as Gly360 to Asp360, which is in the ATPase domain between the DEAH box (motif II) and the SAT motif (motif III). Since *EMT1-201* has a mutation in a tRNA for methionine, *EMT1-201* probably does not act by missense suppression of *prp2-1*. Although tRNA plays a role in many cellular functions in addition to translation, the mutant tRNA most likely works as an intragenic suppressor of *prp2-1* by insertion of methionine at some leucine codons of *prp2-1* so that the protein is active at high temperature. This model is supported by several observations. First, Senger *et al.* (1992) reported that in *S. cerevisiae*, a methionine tRNA with a CAG anticodon could be charged with methionine by the methionyl tRNA synthetase, although with reduced efficiency relative to wild type. Second, there are six CUG leucine codons in *PRP2*, and two are near codon 360. Third, the Prp2 protein isolated from a *prp2-1*, *EMT1-201* double mutant strain is more heat resistant than Prp2 from a *prp2-1* single mutant strain (Figure 4A), but not as heat resistant as the wild-type Prp2 protein (Figure 4B). These data suggest that the Prp2 protein is altered in the presence of *EMT1-201*. *EMT1-201* suppressed the *prp2-1* allele but not *prp2-5* or *prp2-8*, the two other *prp2* alleles tested (data not shown). The *prp2-5* mutation is not known. The *prp2-8* mutation is in codon 701, a G to A mutation that causes a predicted change of aspartate to asparagine (data not shown). Presumably the substitution of methionine for leucine in the Prp2 proteins in these strains does not make them heat stable as it does in *prp2-1*.

Expression of one additional copy of the *tRNA^{Leu} (UAG)* gene rescues the *EMT1-201* mutant (Figure 6). The tRNA^{Leu}(UAG) that recognizes the CUG leucine does so with a G-U wobble interaction, whereas the *EMT1-201* mutant is perfectly matched with CUG, giving the mutant a competitive advantage over the endogenous tRNA^{Leu} (Percudani *et al.* 1997). Therefore, the additional tRNA^{Leu}(UAG) is required to compete with the *EMT1-201* tRNA for incorporation of leucine at CUG codons during translation. This result suggests that *EMT1-201* could suppress *prp2-1* by insertion of methionine at one or more leucine codons in the prp2-1 protein, thereby restoring its ability to function in splicing. It is also possible that misincorporation of methionine in place of leucine occurs in proteins that affect the activity or stability of the Prp2 protein.

Overexpression of *EMT1-201* is lethal to wild-type yeast cells (Figure 5). Although the *EMT1-201* tRNA can be charged with methionine, it is 55-fold less efficient than charging of the wild-type tRNA^{Met} (Senger *et al.* 1992); therefore, overexpression of *EMT1-201* may result in the presence of uncharged tRNA in the cell. Excess uncharged tRNA in the cell acts as a starvation signal and can induce the stringent response in yeast (Warner and Gorenstein 1978). Changes in the cell during the stringent response include decreased rRNA and protein synthesis, and induction of genes specific for amino acid biosynthesis (reviewed in Hinnebusch 1988). The decrease in splicing activity in the *EMT1-201* mutant, therefore, would be a nonspecific effect of the decrease in protein synthesis and increase in transcription of genes whose products are required directly for amino acid biosynthesis. In addition, uncharged tRNA is not exported efficiently from the nucleus (Lund and Dahlberg 1998). The lethality caused by *EMT1-201* overexpression may be due both to decreased fidelity of translation and the presence of excess uncharged *EMT1-201* tRNA in the cell.

The *EMT1-201* mutation confers a defect in pre-mRNA processing. Happel and Winston (1992) identified a frameshift suppressor glycine tRNA as an extragenic suppressor of *spt3*, a gene whose product is involved in initiation of transcription from δ sequences. The glycine frameshift suppressor tRNA did not act by missense suppression of *spt3*, was not allele specific, and showed a transcriptional defect in an *SPT* background. It was not clear whether the effect of the mutant tRNA

on transcription was direct or indirect, but the authors suggest that the frameshift tRNA caused defects in translation that in turn affected transcription (Happel and Winston 1992).

Cells carrying the *EMT1-201* allele are Cs^- and defective in pre-mRNA splicing *in vivo* and *in vitro*. Why does *EMT1-201* have a *prp* phenotype? The rescue of the *EMT1-201* mutant by overexpression of $tRNA^{Leu}$ *(UAG)* suggests that the mutant tRNA may insert methionine into leucine codons of some splicing factors, causing them to be inactive. Guthrie and Abelson (1982) reported that CUG codon usage is rare since none of 10 proteins examined used that codon. We searched for CUG codon usage among randomly selected yeast sequences including some pre-mRNA splicing factors and ribosomal proteins (data not shown). There was a greater distribution of the CUG codon in splicing factors when compared with ribosomal proteins; however, the CUG codon was also found in proteins that play a role in the cell cycle, DNA replication, and transcription. Incorporation of methionine into leucine codons in some of the splicing factors may confer the *prp* phenotype in *EMT1-201*; however, it is unlikely that CUG can be used specifically for regulating the expression of splicing factors. Nevertheless, this study provides the first example describing a mutation in a tRNA gene that leads to a splicing defect *in vivo*.

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