Mutations Affecting the tRNA-Splicing Endonuclease Activity of *Saccharomyces cerevisiae*

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ABSTRACT

Two unlinked mutations that alter the enzyme activity of tRNA-splicing endonuclease have been identified in yeast. The *sen1-1* mutation, which maps on chromosome *12*, causes temperature-sensitive growth, reduced *in vitro* endonuclease activity, and *in vivo* accumulation of unspliced pre-tRNAs. The *sen2-1* mutation does not confer a detectable growth defect, but causes a temperature-dependent reduction of *in vitro* endonuclease activity. Pre-tRNAs do not accumulate in *sen2-1* strains. The *in vitro* enzyme activities of *sen1-1* and *sen2-1* complement in extracts from a heterozygous diploid, but fail to complement in mixed extracts from separate *sen1-1* and *sen2-1* haploid strains. These results suggest a direct role for *SEN* gene products in the enzymatic removal of introns from tRNA that is distinct from the role of other products known to affect tRNA splicing.

SPLICING of precursor tRNAs in the yeast *Saccharomyces cerevisiae* requires two separate enzymatic functions, a membrane-associated endonuclease and a soluble ligase (PEEBLES *et al.* 1979). The endonuclease cleaves the pre-tRNA, releasing the intron as a linear molecule (PEEBLES, GEGENHEIMER and ABELSON 1983). The ligase joins the half tRNAs through an ATP-dependent, three-step mechanism involving cyclic phosphodiesterase, polynucleotide kinase, and RNA ligase activities (GREER *et al.* 1983). There is evidence for the formation of a splicing complex *in vitro*, suggesting the formation of such a complex *in vivo* (GREER 1986).

In previous studies, mutations in two genes, *RNA1* and *LOS1*, were found to affect tRNA splicing *in vivo* (HOPPER, BANKS and EVANGELIDIS 1978; HOPPER, SCHULTZ and SHAPIRO 1980). At the non-permissive temperature, strains carrying a temperature-sensitive allele of the *RNA1* gene accumulate pre-tRNAs, unprocessed rRNAs, and are defective for mRNA metabolism (ATKINSON, DUNST and HOPPER 1985). Given this pleiotropic phenotype, it seems unlikely that the *RNA1* product participates directly in the enzymology of tRNA splicing. Indeed, *in vitro* extracts from *rna1* strains have normal tRNA splicing activities (ATKINSON, DUNST and HOPPER 1985; this study). ATKINSON, DUNST and HOPPER (1985) suggest that *RNA1* may be involved in the maintenance of nuclear structure. Hence, mutant alleles of this gene probably affect tRNA splicing indirectly.

Mutations in the *LOS1* gene confer loss of nonsense suppression in strains carrying *SUP4-o*, an intron-containing, tyrosine-inserting ochre suppressor tRNA gene (HOPPER, SCHULTZ and SHAPIRO 1980). These mutations result in pre-tRNA accumulation. However, like *rna1* strains, *in vitro* tRNA splicing activities are normal in extracts from *los1* strains (HURT *et al.* 1986; this study).

The gene encoding the tRNA-splicing ligase (*LIG1*) was identified by reverse genetic techniques (PHIZICKY, SCHWARTZ and ABELSON 1986). The soluble ligase protein is a single polypeptide with an M_r of 90 kD that co-purifies with all three activities required for ligation of tRNA half molecules (cyclic phosphodiesterase, polynucleotide kinase, and RNA ligase). In contrast, attempts to identify mutations in the structural gene(s) for the tRNA-splicing endonuclease as well as attempts to purify the enzyme to homogeneity have thus far been unsuccessful. The difficulties with these approaches can be attributed largely to the lack of specificity in mutant selections based on loss or gain of suppression, and to problems inherent in stabilizing the membrane-associated endonuclease during purification. For these reasons, knowledge of the genetics and biochemistry of endonucleolytic cleavage of tRNA introns is severely limited.

In this communication we report the results of a direct screen for mutations affecting tRNA-splicing endonuclease activity. We have identified two new genes, *SEN1* and *SEN2*, whose products are likely to participate in the enzymatic reaction catalyzing removal of introns from tRNA precursors. We expect that further study of these genes and their products will provide a more complete description of the tRNA splicing pathway.

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TABLE 1

Yeast strains

Strain	Genotype	Source
A354a	**a** *ade1 ade2 ura1 his7 lys2 tyr1 gal1*	R. K. MORTIMER
S288C	α *gal2*	R. K. MORTIMER
R14	**a** *leu2-3*	R. F. GABER
R15	α *leu2-3*	R. F. GABER
M10	**a** *leu2-3 leu2-112 ura3-52 trp1-7*	W. R. BOORSTEIN
M11	α *leu2-3 leu2-112 ura3-52 trp1-7*	W. R. BOORSTEIN
tsx17-6b	**a** *sen2-1 leu2-3 leu2-112 ura3-52 pep4-3*	This study
tsx17-13b	α *sen2-1 leu2-3 leu2-112 ura3-52 pep4-3*	This study
tsx24-5b	**a** *sen1-1 leu2-3 leu2-112 ura3-52 pep4-3*	This study
201-1-5	α *los1-1 SUP4-o his5-2 lys1-1 can1-100 trp5-48 ade2-1 ura3-1*	A. K. HOPPER
SS4-17C	**a** *rna1-1 met6*	S. SILVERMAN
BJ926	α/**a** *prc1-126/prc1-126 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1 gal2/gal2 HIS1/his1 trp1/TRP1*	E. JONES

MATERIALS AND METHODS

Yeast strains and genetic techniques: The collection of temperature-sensitive yeast strains (HARTWELL 1967) used to screen for tRNA-splicing endonuclease activity was obtained from C. MCLAUGHLIN. A strain containing the *los1-1* mutation was provided by A. HOPPER. Plasmid pSEYC58 containing the yeast *LIG1* gene was provided by E. PHIZICKY, and the plasmids YEpRNA1 and YEpLOS1 were provided by A. HOPPER. Yeast media and genetic techniques have been described (GABER and CULBERTSON 1982). The original isolates of the *sen1* and *sen2* mutant strains were successively out-crossed to strain S288C and its derivatives, R14, R15, M10 and M11 (Table 1). *sen1-1* and *sen2-1*-containing strains derived from the final outcrosses (Table 1) were used in the experiments reported in this communication.

Labeling of RNA: *In vivo* tRNA synthesis was assayed by pulse-labeling phosphate-starved yeast for 30 min with ^{32}P-orthophosphate. KNAPP *et al.* (1978) describe the yeast labeling, RNA extraction and purification, and gel fractionation techniques. Steady-state levels of small RNAs were assayed in a similar manner except that labeling periods were extended to 4 to 6 hr. Steady-state levels of pre-tRNAs and tRNAs were also assayed by *in vitro* ^{32}P-3′-end-labeling using yeast tRNA nucleotidyl transferase. Small RNAs were isolated from log phase cultures as described above and end-labeled as described by LEE and KNAPP (1985). Yeast nucleotidyl transferase was prepared by a modified method of STERNBACH *et al.* (1971).

***In vitro* techniques:** End-matured proline pre-tRNA$_{UGG}$ transcripts of the *suf8* wild-type gene (CUMMINS, CULBERTSON and KNAPP 1985) were synthesized *in vitro* using a yeast RNA polymerase III transcription system (ENGELKE, GEGENHEIMER and ABELSON 1985). The pre-tRNA was uniformly labeled with ^{32}P-UTP (Amersham, 400 Ci/mmol). Labeled pre-tRNAs were eluted from polyacrylamide gels, ethanol-precipitated (MAXAM and GILBERT 1977), and used as substrate for tRNA-splicing endonuclease (referred to throughout as the endonuclease).

The method of PEEBLES, GEGENHEIMER and ABELSON (1983) was used for large scale endonuclease partial purification. The endonuclease fraction used in experiments described in this communication is equivalent to the Triton X-100 extract of yeast membranes designated as fraction IV in PEEBLES, GEGENHEIMER and ABELSON (1983). Total protein in this fraction was determined as in LOWRY *et al.* (1951). Endonuclease assays were performed as previously described in WINEY *et al.* (1986). The reactions were terminated as in LEE, MILLIGAN and KNAPP (1988). The reaction products were fractionated on 10% (19:1) polyacylamide gels containing 4 M urea. To obtain quantitative data from these reactions, bands were excised from the gels and analyzed by Cerenkov counting.

Screening procedure: A rapid procedure for preparing extracts with endonuclease activity was developed to screen the temperature-sensitive mutant collection. The extract is a small-scale version of the yeast nuclear extract used for *in vitro* RNA polymerase III transcription (ENGELKE, GEGENHEIMER and ABELSON 1985). Individual extracts (designated throughout as mini-extracts) from each of 300 temperature sensitive strains were assayed for tRNA splicing endonuclease activity as described below.

Yeast cells were grown to early log phase (OD_{600} = 0.3–1.2). The equivalent of 1.0 ml of cells at OD_{600} = 0.9 were collected in 1.5-ml microfuge tubes. The cell pellet was suspended in 35 μl of an isotonic buffer [1.0 M sorbitol, 50 m*M* Tris (pH 7.9), 10 mM $MgCl_2$, 10 mM dithiothreitol] and left at room temperature for 15 min. The cells were collected and resuspended in another 35 μl of the isotonic buffer with 0.1 mM dithiothreitol and 0.15 mg/ml Zymolyase 60,000 (Kirin), followed by incubation for 1 hr with gentle agitation at room temperature to allow formation of spheroplasts. The spheroplasts were collected by centrifugation at 4°, and all subsequent steps were performed at 4°. Ice-cold hypotonic buffer [100 μl, 15 mM KCl, 10 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 3 mM dithiothreitol] was added to the spheroplast pellet. The pellet was resuspended and the spheroplasts lysed by grinding with a pestle (Kontes, Chicago). The lysate was spun in a microfuge for 10 sec. The supernatant was discarded and the nuclei-containing pellet was resuspended in 20 μl of endonuclease buffer [25 mM Tris (pH 8.0), 0.5 mM EDTA, 100 mM $(NH_4)_2SO_4$, 10 mM spermidine, 1% Triton X-100, 5.0 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol] (PEEBLES, GEGENHEIMER and ABELSON 1983). This final fraction was used for endonuclease assays. A 35-μl reaction containing 7 μl of extract and 28 μl of a cocktail (final reaction conditions described in WINEY *et al.* 1986) was assembled on ice. Aliquots (10 μl) of this reaction mix were removed and incubated at 25°, 30° and 34° for 15 min. The reactions were terminated, and fractionated on gels as described above, and analyzed by autoradiography.

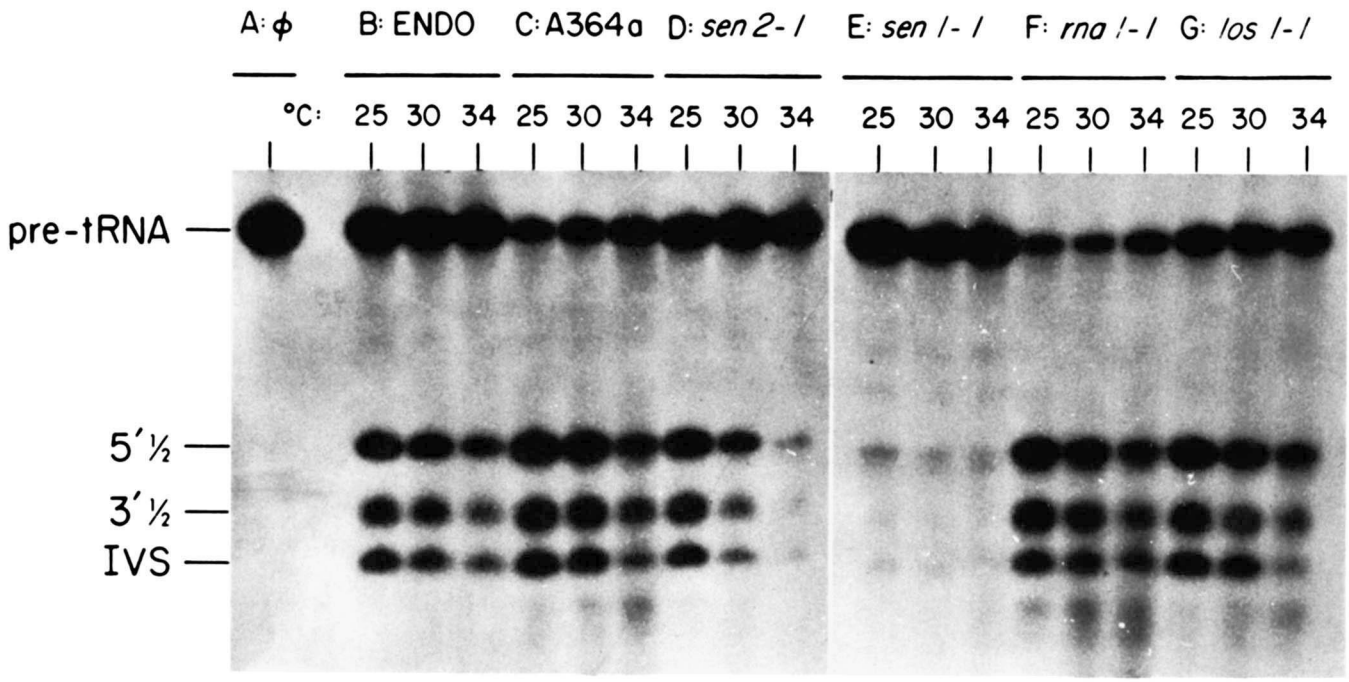

FIGURE 1.—*In vitro* assays of tRNA splicing endonuclease activity. Assays were performed at the indicated temperatures using reaction conditions described in Materials and Methods. Reaction products were fractionated on 10% polyacrylamide gels containing 4M urea. (A) shows the uncleaved proline pre-tRNA$_{UGG}$ substrate in the absence of enzyme. (B) shows the products of cleavage with partially purified endonuclease (PEEBLES, GENGENHEIMER and ABELSON 1983). The identity of products was determined previously (WINEY *et al.* 1986). The remaining assays show endonuclease activity in mini-extracts (see MATERIALS AND METHODS) as follows: (C), wild-type (strain A364a, Table 1); (D), *sen2-1* (original isolate); (E), *sen1-1* (original isolate); (F), *rna1-1* (strain SS14-17C, Table 1); (G) *los1-1* (strain 201-1-5, Table 1).

RESULTS

Identification of mutations affecting tRNA-splicing endonuclease. The endonuclease activity was assayed in mini-extracts from 300 temperature-sensitive strains grown at permissive temperature. Endonuclease activity is detected by the cleavage of pre-tRNA to half tRNAs and introns (Figure 1). *In vitro* assays were carried out at three different temperatures. These include the permissive temperature (25°), the optimal temperature for *in vitro* endonuclease activity (30°), and the maximum nonpermissive temperature giving detectable wild-type endonuclease activity (34°). At 34°, wild-type endonuclease activity is present at comparable albeit reduced levels in both partially-purified endonuclease (fraction IV) (PEEBLES, GEGENHEIMER and ABELSON 1983) (Figure 1B) and in mini-extracts (Figure 1C). Since the mini-extracts were comparable in activity to partially purified enzyme at all temperatures, they were regarded as suitable for use in screening for mutants. Altered endonuclease activity was detected in mini-extracts from two out of the 300 strains screened. Data provided below show that these strains contain mutations in two unlinked genes, designated *SEN1* and *SEN2* (*s*plicing *en*donuclease).

In mini-extracts the *sen1-1* mutation causes reduced endonuclease activity at all assay temperatures (Figure 1E). The *sen2-1* mutation confers temperature-sensitive endonuclease activity with near normal activity at 25°, reduced activity at 30°, and little to no activity at 34° (Figure 1D). For comparison, we assayed strains containing two previously identified mutations, *rna1-1* (Figure 1F) and *los1-1* (Figure 1G), that cause *in vivo* accumulation of intron-containing pre-tRNA (HOPPER, BANKS and EVANGELIDIS 1978; HOPPER, SCHULTZ and SHAPIRO 1980). The endonuclease activity in these strains was similar to wild-type activity, as shown previously (ATKINSON, DUNST and HOPPER 1985; HURT *et al.* 1986), and did not resemble the altered activities associated with the *sen1-1* and *sen2-1* mutations. The reduced endonuclease activities conferred by the *sen1-1* and *sen2-1* mutations are not due to the presence of an inhibitor in the *in vitro* system since mixtures of wild-type and mutant extracts resulted in wild-type endonuclease activity.

Co-purification of wild-type and mutant endonuclease activities. To verify whether results obtained from mini-extracts are reliable, the endonuclease was partially purified by the method of PEEBLES, GEGENHEIMER and ABELSON (1983) from wild-type, *sen1-1*, and *sen2-1* strains. Fraction IV (PEEBLES, GEGENHEIMER and ABELSON 1983) was used to assay endonuclease activity. This fraction is the detergent extract of a membrane fraction that is devoid of soluble proteins including tRNA-splicing ligase (see MATERIALS AND METHODS).

The rates of pre-tRNA cleavage catalyzed by frac-

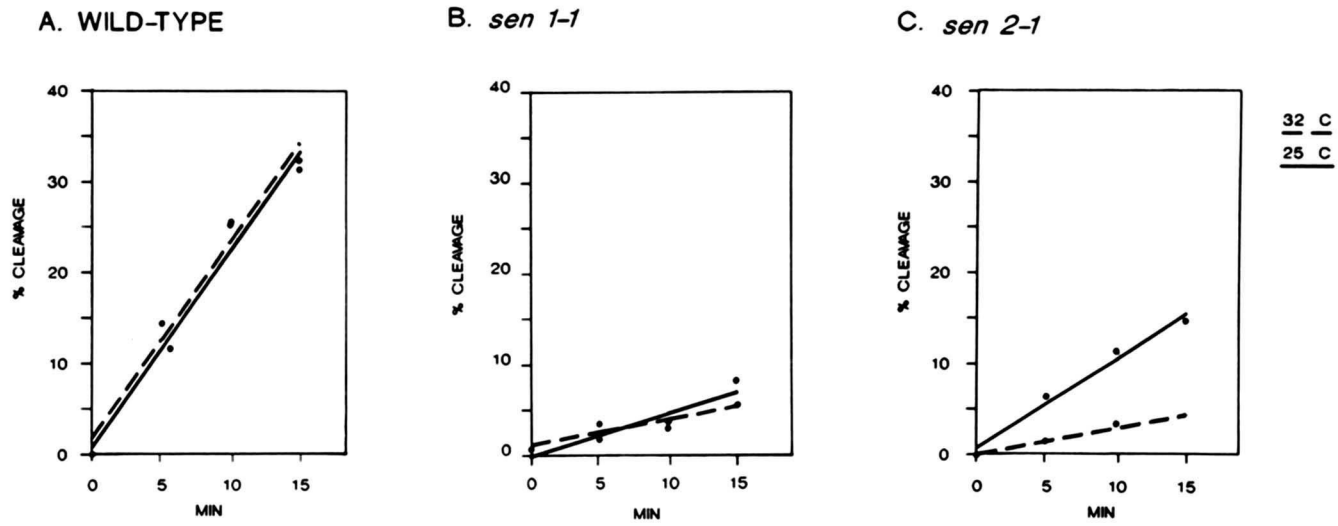

FIGURE 2.—*In vitro* activities of partially purified endonuclease were determined for (A) wild type (strain BJ926, Table 1); (B) *sen1-1* (strain tsx24-5b, Table 1); and (C) *sen2-1* (strain tsx17-6b, Table 1). Reactions were carried out at 25° and 32° as described in MATERIALS AND METHODS. Instead of using equal units of enzyme per reaction, equal quantities of protein were used per reaction (3.8 μg protein/10 μl reaction). Percent cleavage was determined by the method of GREER, SÖLL and WILLIS (1987). The maximum standard deviation for any point was $s^2 = 2.5\%$.

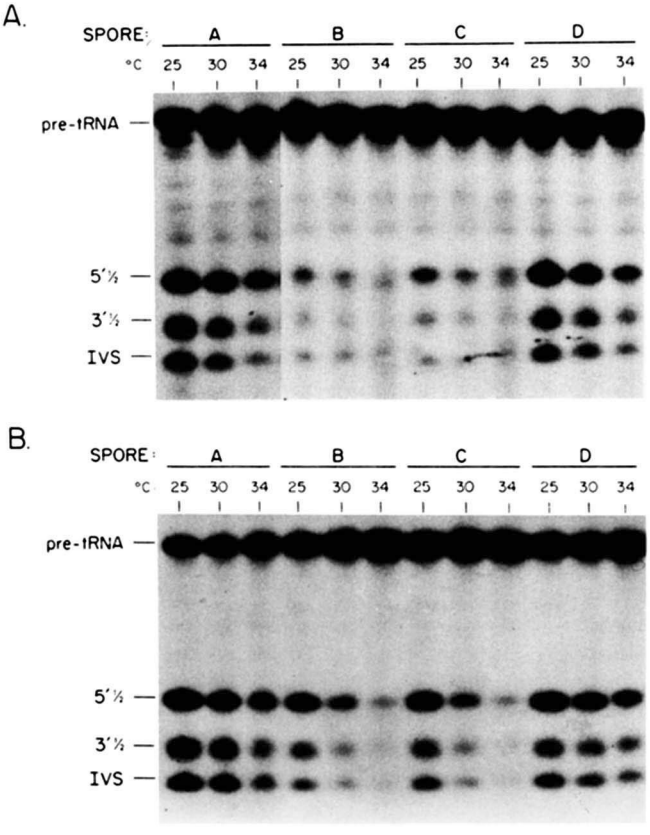

FIGURE 3.—*In vitro* endonuclease activity was assayed as in Figure 1 in mini-extracts from the four spore clones of two representative tetrads, one from the cross *sen1-1* × *SEN1* (A) and one from the cross *sen2-1* × *SEN2* (B). In both tetrads the mutant phenotype segregates 2:2 (spore A, +; B, −; C, −; and D, +).

tion IV endonuclease from the wild-type and mutant strains were determined by measuring the percentage of precursor cleavage as a function of reaction time at 25° and 32° (Figure 2). The latter assay temperature was chosen for these experiments because 32° is the highest temperature yielding an endonuclease activity equivalent to that found at 25° in a wild-type strain (Figure 2A). Higher temperatures result in inhibition of the wild-type endonuclease activity. Using these reaction conditions, the endonuclease from a *sen1-1* strain has reduced activity at both temperatures (Figure 2B), similar to that found in mini-extracts (Figure 1E). The endonuclease activity from a *sen2-1* strain decreases with increasing temperature (Figure 2C), also in a manner similar to that found in mini-extracts (Figure 1D).

We have also performed mixing experiments with partially purified fractions and shown that *sen1-1* and *sen2-1* strains do not contain inhibitors of wild-type endonuclease activity. We conclude from these experiments that the endonuclease from wild-type and mutant strains shows similar behavior in both mini-extracts and a partially purified endonuclease fraction.

Genetic segregation analysis: The altered *in vitro* endonuclease activity found in strains containing the *sen1-1* mutation was analyzed in tetrads from *sen1-1*/*SEN1* diploids. Figure 3A shows a 2:2 Mendelian segregation for altered *vs* normal endonuclease activity in mini-extracts from spore segregants of a representative tetrad. A similar 2:2 segregation was observed in 34 tetrads derived from eight successive outcrosses of the original *sen1-1* isolate. In the initial outcrosses, several temperature-sensitive mutations were found to segregate in tetrads. By successively crossing spore segregants containing altered endonuclease activity as determined by assaying mini-extracts, we derived a strain in which a single temperature-sensitive mutation was shown to co-segregate

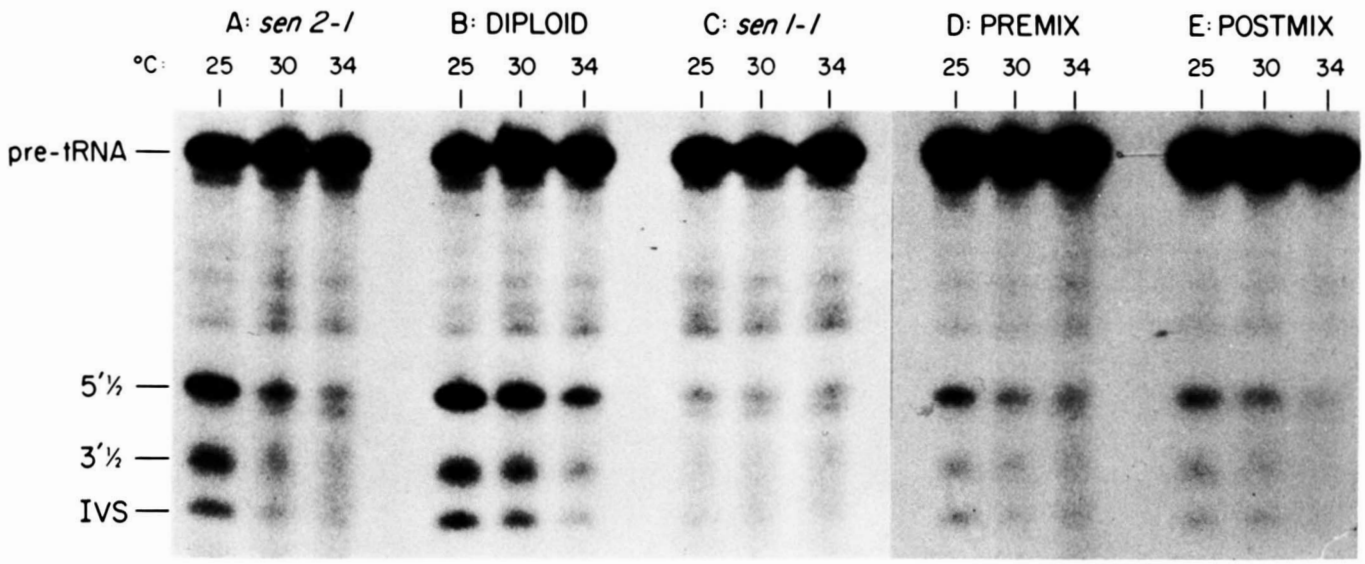

FIGURE 4.—Complementation of *sen1-1* and *sen2-1*. Endonuclease activity was assayed as in Figure 1 in mini-extracts from strains carrying (A) *sen2-1* (strain tsx17-13b, Table 1), (C) *sen1-1* (strain tsx24-5b, Table 1), and (B) a diploid strain derived from mating these haploids. (D) Extracts from the *sen2-1* and *sen1-1* haploid strains (A and C) were also mixed and assayed for their ability to complement *in vitro*. (E) As a control, the extracts from (A) and (C) were incubated with reaction components separately. The reactions were then terminated, mixed, and fractionated on the gel. The results show that the mutants fail to complement *in vitro*.

with the altered *in vitro* phenotype. Both the *in vitro* and *in vivo* phenotypes of *sen1-1* are recessive to wild type.

In order to confirm that temperature-sensitive growth and altered *in vitro* endonuclease activity are related phenotypes resulting from the *sen1-1* mutation, we assayed endonuclease activity in mini-extracts from seven temperature-independent revertants. Assays of the mini-extracts showed that all seven revertants had regained wild-type endonuclease activity, indicating phenotypic co-reversion of temperature sensitivity and reduced *in vitro* enzyme activity. It is therefore likely that both phenotypes are related and are the result of a single mutation. These assays were not sensitive enough to discriminate between partial versus complete reversion.

The *SEN1* gene maps on chromosome 12 as determined by the method of GABER *et al.* (1983). Using the temperature-sensitive phenotype to follow the segregation of *sen1-1*, linkage was detected between the *sen1-1* and *ura4* loci (11.3 cM; 41 parental ditype asci, 0 nonparental ditype asci, 12 tetratype asci).

The altered endonuclease activity in *sen2-1* strains is recessive to wild-type and also shows a 2:2 Mendelian segregation in mini-extracts from spore segregants of a representative tetrad derived from a *sen2-1/SEN2* heterozygous diploid (Figure 3B). A similar 2:2 segregation was observed in 31 tetrads from six successive outcrosses. The *sen2-1* mutation did not co-segregate with any of several temperature-sensitive mutations in the original isolate. We conclude that, unlike *sen1-1*, the *sen2-1* mutation does not confer detectable growth inhibition.

Further segregation tests demonstrate that the *sen1-1* and *sen2-1* mutations are separable by recombination and are distinct loci in the yeast genome. We followed the segregation of both mutations by *in vitro* assays using mini-extracts from tetrads derived from a *sen1-1/SEN1, sen2-1/SEN2* heterozygous diploid. Among ten tetrads, seven were recombinant (3 parental ditype; 2 nonparental ditype; 5 tetratype), indicating nonlinkage of the two genes. Furthermore, double mutant haploid strains derived from these crosses were phenotypically similar to the *sen1-1* single mutant (temperature-sensitive-for-growth; reduced *in vitro* endonuclease activity at all assay temperatures), indicating that *sen1-1* is epistatic to *sen2-1*.

Complementation analysis: As expected for mutations in unlinked genes, *sen1-1* and *sen2-1* complement each other in a heterozyogus diploid, since a mini-extract from the diploid has wild-type endonuclease activity (Figure 4, A–C). The heterozygous genotype of the diploid was confirmed by showing the segregation of the *sen1-1* and *sen2-1 in vitro* phenotypes among spore progeny.

We also determined whether complementation occurs upon mixing either mini-extracts or partially purified fraction IV endonuclease extracts prepared from two haploid strains, one containing *sen1-1* and the other containing *sen2-1* (pre-mixed extracts, Figure 4D). As a control, the additive activity from the separate extracts was assayed by mixing the products following reaction termination (post-mixed extracts, Figure 4E). The endonuclease activities of the pre-mixed extracts were qualitatively and quantitatively

indistinguishable from the activities of the post-mixed extracts. Since mixing extracts from these two mutants did not enhance endonuclease activity above that found in the extracts separately, we conclude that these extracts fail to complement *in vitro*. Additionally, crude homogenates (fraction I) (PEEBLES, GEGENHEIMER and ABELSON 1983) from the mutant strains also failed to complement *in vitro*. This suggests that no factors are lost in the preparation of mini-extracts or partially purified endonuclease extracts that are required for complementation to occur.

Since tRNA-splicing endonuclease and ligase physically interact *in vitro* (GREER 1986), the altered endonuclease activities in *sen1-1* and *sen2-1* strains might be caused indirectly by a defective ligase. To examine this possibility, strains containing *sen1-1* and *sen2-1* were tested for complementation with a wild-type *LIG1* gene (tRNA-splicing ligase) (PHIZICKY, SCHWARTZ and ABELSON 1986), which was introduced into the strains on a centromere-containing plasmid (see MATERIALS AND METHODS) by transformation (ITO *et al.* 1983). The mutant *in vitro* phenotypes in mini-extracts of both strains were unaltered by the presence of the *LIG1* plasmid. This result shows that *LIG1* fails to complement *sen1-1* or *sen2-1*, and indicates that these genes probably do not encode tRNA-splicing ligase.

Since the *sen2-1* mutation has not yet been mapped due to the lack of a convient *in vivo* phenotype, we tested *sen2-1* allelism by complementation with the wild-type *RNA1* and *LOS1* genes. The *sen2-1* strain tsx17-6b (Table 1) was transformed with the 2-micron-derived plasmids YEp-RNA1 (ATKINSON, DUNST and HOPPER 1985) or YEp-LOS1 (HURT *et al.* 1986). The endonuclease activities of transformants were assayed *in vitro* using the mini-extract procedure and found to be unaltered by the presence of the *RNA1* gene or the *LOS1* gene (data not shown). These results indicate that *sen2-1* is not functionally complemented by *RNA1* or *LOS1* and therefore, is not likely to be an allele of either of these genes.

***In vivo* accumulation of intron-containing tRNA precursors:** Mutations that reduce endonuclease activity might be expected to result in substrate accumulation *in vivo*. To test this possibility, wild-type, *sen1-1* and *sen2-1* strains were grown at 25°C, labeled with ^{32}P-orthophosphate at 25° or 37°, and small RNAs were analyzed (see MATERIALS AND METHODS). To examine small RNA synthesis, cells of pulse-labeled wild-type and mutant strains were phenol-extracted, and the resulting small RNAs were fractionated on polyacrylamide gels (Figure 5). Compared to a wild-type strain, the *sen1-1* strain contains increased amounts of pre-tRNAs at both permissive and nonpermissive temperatures. Furthermore, the pre-tRNAs that accumulate in *sen1-1* strains appear to be identical to those that accumulate in *rna1-1*

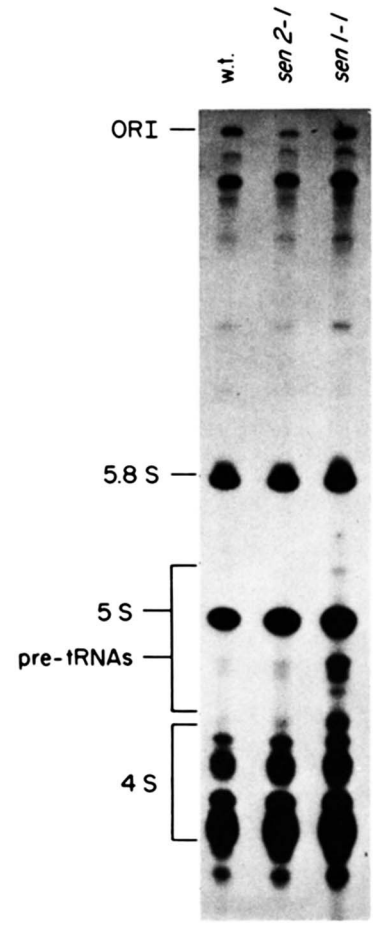

FIGURE 5.—Pre-tRNA accumulation in *sen* strains. Wild-type, *sen2-1*, and *sen1-1* strains (S288C, tsx-17-6b, and tsx24-5b, respectively, Table 1) were pulse-labeled for 30 minutes at 25°. The small RNAs were extracted and fractionated on a 10% polyacrylamide, 8 M urea gel (see MATERIALS AND METHODS). Equal counts-per-minute of labeled RNA were loaded per lane. The results show that pre-tRNAs accumulate only in the *sen1-1* strain. Similar results were obtained when these strains were labeled at 37° for 30 min (see text).

strains (data not shown, see below). Strains containing *sen2-1* resembled wild-type and did not appear to accumulate pre-tRNAs at 25° (Figure 5) or 37° (data not shown). These results suggest that the rate of precursor tRNA utilization in *sen1-1* strains is significantly reduced *in vivo*. In *sen2-1* strains the rates of precursor utilization are not reduced to a level sufficient to cause precursor accumulation.

The steady-state levels of pre-tRNAs were examined in the mutants by two methods. Unlabeled small RNAs were extracted from logarithmic phase cultures grown at 25°, labeled *in vitro* (see MATERIALS AND METHODS), and analyzed on polyacrylamide gels (Figure 6). Secondly, cultures of the mutant strains were steady-state labeled *in vivo*, and tRNAs were extracted and analyzed on polyacrylamide gels (see MATERIALS AND METHODS, data not shown). Both methods showed that strains containing *sen1-1* had increased steady-

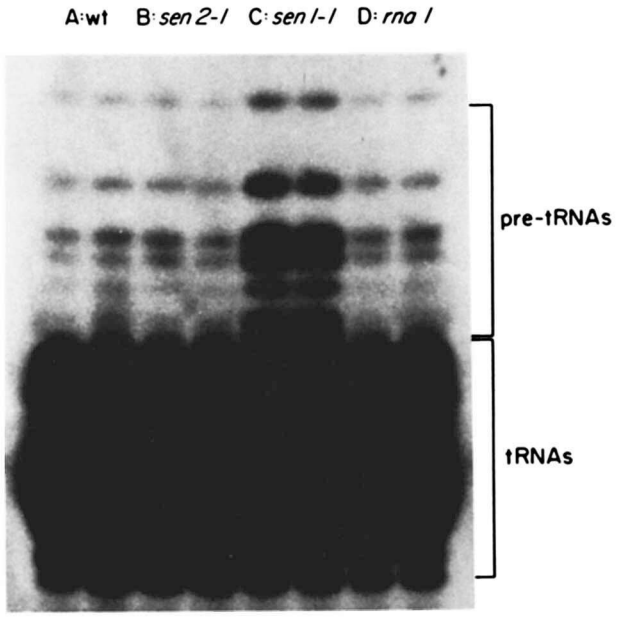

FIGURE 6.—Steady-state levels of pre-tRNAs in (A) a wild-type strain (S288C, Table 1), (B) a *sen2-1* strain (tsx17-6b, Table 1), (C) a *sen1-1* strain (tsx24-5b, Table 1), (D) an *rna1-1* strain (SS4-17C, Table 1). RNA was prepared from log-phase cultures and 3′ end-labeled as described in MATERIALS AND METHODS. Duplicate reactions are shown for each RNA sample and the autoradiogram was purposely over-exposed so the relative level of pre-tRNAs could be visualized in all strains.

state levels of pre-tRNAs at 25°, whereas *rna1-1* and *sen2-1* showed no significant increase in steady-state pre-tRNA levels at this growth temperature compared to wild type (Figure 6). Figure 6 also shows that the pre-tRNAs accumulated in *sen1-1* strains are likely to have mature 5′ and 3′ ends because they comigrate with the pre-tRNAs observed in *rna1* strains (KNAPP *et al.* 1978). These results phenotypically distinguish *sen1-1* from the other mutants since the *sen1-1* mutation appears to impose a block in the splicing pathway that results in an increase in the steady-state levels of pre-tRNA.

DISCUSSION

Mutations in the *SEN1* and *SEN2* genes: An *in vitro* screen for altered tRNA-splicing endonuclease activity resulted in the identification of recessive mutations in two unlinked genes, *SEN1* and *SEN2*. This approach was attempted in light of previous success of other workers looking for mutations in other enzymatic functions (DINARDO, VOELKEL and STERNGLANZ 1984; THRASH *et al.* 1984). Table 2 summarizes the properties of these mutations compared with mutations in two other single copy genes that affect *in vivo* tRNA splicing, *RNA1* and *LOS1* (ATKINSON, DUNST and HOPPER 1985; HURT *et al.* 1986). It is noteworthy that, unlike the mutations reported in this study, mutations in *RNA1* and *LOS1* do not affect the activity

TABLE 2

Properties of yeast tRNA-splicing mutants

Gene	*In vitro* endonuclease activity	Effect on growth	Map location	Pre-tRNA accumulation	
				Pulse	Steady state
sen1-1	Reduced (all temperatures)	ts[a]	12R	+	+
sen2-1	tl[b]	No effect	?	–	–
rna1-1[c]	wt[d]	ts[a]	13R	+	–
los1-1[e]	wt[d]	No effect	11L	+	–

[a] ts = temperature-sensitive-for-growth.
[b] tl = thermoliabile enzyme activity.
[c] HOPPER, BANKS and EVANGELIDIS (1978); ATKINSON, DUNST and HOPPER (1985).
[d] wt = activity resembles wild-type enzyme activity.
[e] HOPPER, SCHULTZ and SHAPIRO (1980); HURT *et al.* (1986).

of tRNA-splicing endonuclease *in vitro* and probably do not play a direct role in the enzymology of tRNA intron removal. Genes of this type might affect tRNA splicing indirectly by, for example, affecting compartmentalization of splicing enzymes or the integrity of the substrates.

The results of genetic mapping show that *SEN1* is a previously unknown gene whose function is required for normal *in vitro* endonuclease activity and *in vivo* tRNA splicing. Furthermore, we have shown by complementation tests that *SEN1* does not encode tRNA-splicing ligase. The unique phenotype of the *sen1-1* mutation suggests that this gene plays a direct role in cleavage of tRNA introns. In addition to altered *in vitro* endonuclease activity, the *sen1-1* mutation causes temperature-sensitive growth and an increase in the steady-state level of pre-tRNA at both permissive and restrictive temperatures. Based on these results it appears likely that *SEN1* is an essential gene in which loss of function confers lethality due to a defect in tRNA splicing. However, we cannot at present rule out the formal possibility that the wild-type *SEN1* gene product is not essential for viability and that the *sen1-1* mutant gene product causes lethality only by acting as a poison subunit. This question can be resolved in future experiments by assaying the phenotype of a null allele constructed through gene cloning techniques.

The *sen2-1* mutation confers temperature sensitive endonuclease activity *in vitro*, but has no detectable growth or pre-tRNA accumulation phenotype *in vivo*. The phenotype of a null allele of the *SEN2* gene conferring complete loss of function has not yet been determined. Given that the *sen2-1* allele may retain substantial residual endonuclease activity *in vivo*, the possibility remains that this gene encodes an essential tRNA splicing function. We have, however, ruled out the possibility that *sen2-1* is an allele of the *LIG1*, *RNA1* or *LOS1* genes.

Relationship between *SEN1* and *SEN2* gene products: The components of tRNA splicing may physically associate in a complex consisting of tRNA splicing ligase, endonuclease, and perhaps other factors (GREER 1986). Since the endonuclease is extractable only in the presence of a detergent, it has been suggested that the putative splicing complex may be associated with the nuclear membrane (GREER 1986; CLARK and ABELSON 1987).

The results of complementation tests between *sen1-1* and *sen2-1* bear on the possibility of such a membrane-associated complex. Whereas complementation between the mutations was observed in extracts from a heterozygous diploid, similar complementation was not observed when mini-extracts or partially purified endonuclease extracts from separate *sen1-1* and *sen2-1* strains were mixed and assayed. A lack of complementation was also observed when crude unfractionated extracts from the mutant strains were mixed. It is therefore unlikely that a factor required for complementation was lost during fractionation of extracts. The failure of extracts from these mutants to complement *in vitro* when mixed suggests that the two genes encode products that are functionally related.

We envision two alternative generic models for the function of the *SEN1* and *SEN2* gene products (**SEN1P** and **SEN2P**, respectively; Figure 7). In these models tRNA-splicing endonuclease activity is directly attributed to the function of the *SEN1* and/or *SEN2* gene product(s). Since the temperature-sensitive mutant collection was screened by assaying *in vitro* endonuclease activity at different temperatures in extracts from cells grown only at permissive temperature, the mutants most likely affect gene product function rather than synthesis (transcription or translation). The models do not encompass functions that are unnecessary for normal *in vitro* endonuclease activity such as the *RNA1* and *LOS1* gene products.

In the first model, **SEN1P** and **SEN2P** are subunits of the endonuclease (Figure 7A), such that changes in either product directly affect enzyme activity. The term subunit is used in the broad sense, and could represent a polypeptide containing an active site for the enzyme, sites for binding of pre-tRNA, a membrane-docking protein, a scaffolding protein, or any other structural component that might be required in a splicing complex. The failure of extracts from mutant strains to complement *in vitro* is explained in this model by the failure of subunits to freely dissociate and reassociate, possibly because the enzyme is already assembled in a membrane-bound complex.

The second model differs from the first in that **SEN1P** is a structural component of the endonuclease, whereas **SEN2P** is not such a component (Figure 7B). **SEN1P** was chosen for this role because the *sen1-1* mutation is epistatic to *sen2-1*, an assumption that could be misleading if the *sen2-1* phenotype differs substantially from that of a *sen2* null allele. The **SEN2P** function is imagined to be required for the wild-type *in vitro* activity of **SEN1P**. In the absence of any direct interaction with pre-tRNA or the splicing complex, **SEN2P** could be required to convert **SEN1P** from an thermolabile form to a temperature-resistant form. This could be accomplished by events such as proteolytic cleavage or protein modification. Thus, both mutants would be defective for the same enzymatic activity but for different reasons. According to this model, the lack of *in vitro* complementation arises from the inability of **SEN2P** to function in the *in vitro* system. This might occur for two different reasons. **SEN1P** might be inaccessible to **SEN2P** action or reaction conditions in the *in vitro* system may not favor **SEN2P** function.

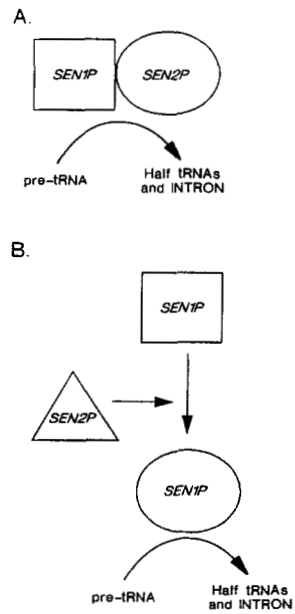

FIGURE 7.—Function and interaction of *SEN1* and *SEN2* gene products. In (A) the products are imagined to function as subunits of tRNA-splicing endonuclease. In (B) **SEN2P** is imagined to be required for the activity of **SEN1P**, where **SEN1P** is a component of the enzyme.

By analyzing the structures of *SEN* genes and their products and by examining the phenotypes of additional mutant alleles, we hope to resolve these models by providing information on the structure, function, and localization of tRNA splicing components. The most immediate question to be answered centers on which products are structural components of the endonclease. Once this central question is resolved it should be possible to discover how and where tRNA splicing occurs in intact cells.

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