A General Screen for Mutants of *Saccharomyces cerevisiae* Deficient in tRNA Biosynthesis

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ABSTRACT

We have devised a general screen for isolating conditional lethal mutants defective in synthesis of mature tRNA in *Saccharomyces cerevisiae*. Using this screen, we have identified several new genes in yeast that are required for production of mature tRNA. These genes most likely encode essential functions, since the mutations we isolated are recessive and cause temperature-sensitive growth. One of the mutants, *tpd3*, is defective in *de novo* transcription of 4S RNA at the nonpermissive temperature. A second mutant, *tpd1*, is specifically defective in excision of intervening sequence from a variety of tRNA species. Finally, two other mutants are defective in production of tRNA from a suppressor tRNA locus, as measured by an *in vitro* suppression assay. The specific lesion in these strains, though, is not known. These data confirm that the screen does, in fact, yield a broad spectrum of mutants defective in tRNA maturation.

PRODUCTION of mature eukaryotic tRNAs requires extensive posttranscriptional modification. This includes endonucleolytic trimming of 5′ and 3′ ends, addition of CCA residues to the 3′ end, various modifications of specific nucleotides, and, for about a fifth of the tRNA species, excision of intervening sequences located adjacent to the anticodon. A wealth of information has been accumulated regarding the enzymology of many of these reactions (DEUTSCHER 1984; VENKSTERN 1985). However, many questions regarding the maturation process have not been addressed, due, in part, to the absence of an extensive collection of mutants defective in tRNA maturation. For instance, the functional significance of many of the base modifications in tRNAs is still not clear, an issue most easily resolved by mutants deficient in the particular modification reactions (BJORK *et al.* 1987). The absence of mutants has also restricted efforts on cloning genes involved in tRNA maturation. In turn, this lack of cloned genes has precluded molecular genetic analysis of many of the steps in the maturation process.

Although a number of strategies have been used to identify mutants in yeast defective in pre-tRNA processing, each of these has had only limited success (*cf.* HOPPER 1989). The most effective approach to date has been to screen individual, randomly mutagenized clones using various biochemical assays to identify mutants deficient in a particular step in the maturation pathway. This has yielded mutants deficient in excision of intervening sequences—*rna1*, *sen1*, *sen2* and *ptal*—as well as mutants deficient in particular base modification reactions—*trm1* and *trm2* (HOPPER, BANKS and EVANGELIDIS 1978; WINEY and CULBERTSON 1988; HOPPER *et al.* 1982; PHILLIPS and KJELLIN-STRABY 1967; P. O'CONNOR and C. PEEBLES, personal communication). However, this method is limited by the labor intensive nature of the screens and by the fact that one can identify mutants only in the particular activity being assayed.

A second, extensively explored, procedure for identifying mutants affecting genes required for production of mature, functional tRNA has been the use of screens based on loss of suppressor activity. In these screens, a strain would be constructed that carried both a nonsense suppressor locus and nonsense mutations in several different genes, all of which were suppressed by the selected suppressor locus. The suppressible loci would be chosen so that loss of suppression could be either selected—through acquisition of drug resistance, for example—or readily observed at the colony level—as a change in colony color (RASSE-MESSENGUY and FINK 1973; HOPPER *et al.* 1981). Mutants that no longer produced functional suppressor tRNA were then sought by selecting or identifying those mutant clones that exhibited the loss of suppression phenotype. This approach yielded several genes that affect suppressor tRNA function, such as *mod5* and *los1* (LATEN, GORMAN and BOCK 1978; HOPPER, SCHULTZ and SCHAPIRO 1980). However, none of the genes identified by this approach has proved to be essential for growth. Since many of the genes involved in tRNA maturation are certainly essential for growth,

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the absence of such mutants from these screens indicates that they have missed a large class of tRNA maturation genes.

A reasonable explanation for the failure of loss-of-suppression selection schemes to identify essential genes in tRNA maturation is that such schemes require a mutant to fulfill two mutually exclusive conditions. Since tRNA species are stable, manifestation of an antisuppressor phenotype requires sufficient growth of the mutant strain at the nonpermissive condition to allow the preexisting suppressor tRNA to be eliminated by dilution. On the other hand, a mutant incapable of producing functional tRNA would be incapable of growth, since no new tRNA could be synthesized. To circumvent this dilemma in *Escherichia coli*, SCHEDL and PRIMAKOFF (1973) devised a method to introduce the suppressor gene on a phage only after mutagenized colonies had been shifted to the nonpermissive conditions. By using a *lacZ* nonsense mutation as a metric of suppression, formation of functional suppressor tRNA in the mutant colonies could be monitored by color formation in individual colonies without the need for additional growth at the nonpermissive temperature.

In this report we describe a genetic screen in yeast for essential genes required for production of mature tRNAs. This procedure is an adaptation to yeast of the screen successfully used by SCHEDL and PRIMAKOFF (1973). The screen is designed to be quite general, in that mutations that abolish any step in production of functional, mature tRNA should score as positives in the screen. Thus, genes involved in transcription of tRNAs, specific transport from the nucleus, endo- and exonucleolytic processing of precursor molecules, excision of intervening sequences, or formation of specific base modifications should be identified in this screen. We have documented the feasibility of this procedure by isolating mutants in five previously unidentified genes required for production mature tRNA. Isolation and initial characterization of these mutants are described in this report.

MATERIALS AND METHODS

Strains: Yeast strains used in this study are listed in Table 1. Cultures were grown in either rich medium (YEPD) or appropriate synthetic complete (SC) selective media as described by SHERMAN, FINK and HICKS (1982). Transformation of *Saccharomyces cerevisiae* was performed by the lithium acetate procedure of ITO *et al.* (1983). Gene transplacements were performed as described by ROTHSTEIN (1983) and verified by Southern blot analysis (MANIATIS, FRITSCH and SAMBROOK 1982). Plasmid constructions and amplification were done in the *E. coli* strain MC1066 (Δ(*lacIPOZYA*)*X74 galU galK strA*^{r} *hsdR*$^-$ *trpC9830 leuB6 pyrF74*::Tn*5*) (CASADABAN *et al.* 1983).

To construct an *hm1*::*SUP4*$_o$ clone, a *Bam*H1 fragment from plasmid YC723 (SHAW and OLSON 1984) spanning the *SUP4*$_o$ gene was cloned into the silent mating locus *HMLα*, replacing those sequences between the *Xba*I sites in the **W** and **X** regions of *HMLα*. The construct was used to replace the normal *HMLα* locus of a *sir4-1*^{ts} strain, JR72-6D, and a *sir3-8*^{ts} strain, JR87-6C, by gene transplacement. The *sir4-1* and *sir3-8* alleles were derived by appropriate crosses with strains 62C and JRY188, respectively.

Plasmid YEp62 (BROACH *et al.* 1983) was used as source of the inducible *GAL10-lacZ* fusion protein. The *GAL10* and *lacZ* moieties in the plasmid were placed in frame by deleting the 10-bp *Bam*HI fragment between the two genes. The resulting replicative plasmid was rendered integrative by deleting the *Nsi*I fragment spanning the 2μm ARS region. As an additional selectable marker, the yeast *URA3* gene was introduced on a *Hin*dIII fragment into the unique *Hin*dIII site of plasmid. Ochre mutations in the *lacZ* moiety of this integrative plasmid were isolated by ethyl methane sulfonate (EMS) mutagenesis of *E. coli* strain MC1066 harboring the plasmid, screening for LacZ$^-$ clones. Plasmid DNA was isolated from individual LacZ$^-$ clones, transformed into *E. coli* strain ES514 (*F*$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 argE*$_{am}$ *SuC rif*^{r}) (provided by T. SILHAVY, Princeton University), which contains the tyrosine-inserting ochre-suppressor, SuC, and the resultant transformants scored for LacZ. One plasmid that yielded LacZ$^-$ transformants of strain MC1066 and LacZ$^+$ transformants of ES514 was retained and integrated as a single copy into the genome of the *sir3*^{ts} strain JR260-7A at the *leu2* locus.

A clone containing the *SUC2* gene in *HMR* was constructed by isolating a 3.0-kb *Eco*RI/*Sna*B1 fragment from plasmid pBR58 (CARLSON and BOTSTEIN 1982), spanning the *SUC2* gene, attaching synthetic *Sal*I linkers and cloning the resulting fragment into the unique *Xho*I site of the plasmid pJA44 (ABRAHAM *et al.* 1984). Plasmid pJA44 carries the silent mating locus *HMRa* in which the **X**, **Ya** and **Z1** region have been deleted and replaced with an *Xho*I linker. The resulting *hmr*::*SUC2* construct was used to replace the *HMRa* locus of the *sir4*^{ts} strain JR222-52A by gene transplacement. From crosses between the JR222-52A *hmr*::*SUC2* transformant and a JR260-7D *leu2*::*LEU2-GAL10-lacZ*$_o$ transformant, three strains, EZ5-9a, EZ6-4b and EZ9-6c, with the *hml*::*SUP4*$_o$, *leu2*::*LEU2-GAL10-lacZ*$_o$ and *hmr*::*SUC2* loci were obtained and used for EMS mutagenesis.

For evaluating protein synthesis capacity of the various mutant strains, we replaced the *GAL10*::*lacZ*$_o$ construction in these strains with the parental *GAL10-lacZ*$^+$ construct. This was accomplished by first isolating 5-fluoroorotic acid (FOA)-resistance variants of the desired strain (BOEKE, LA CROUTE and FINK 1984). Most of these arose by deletion of the *GAL10-lacZ*$_o$-*URA3* segment, since it is flanked by directly repeated *leu2* sequences. Selected FOA-resistant clones were then transformed to Ura$^+$ with the *LEU2-GAL10*::*LacZ*$^+$-*URA3* plasmid described above. The expected structures of both the FOA-resistant isolates and the retransformants were confirmed by Southern analysis.

Enzymatic assays: β-Galactosidase activities were determined according to MILLER (1972). Invertase assays were performed according to GOLDSTEIN and LAMPEN (1975). Protein concentrations during enzyme assays were determined according to BRADFORD (1976).

EMS mutagenesis: EMS mutagenesis was performed according to FINK (1970). About 2×10^7 exponentially growing cells of the three strains EZ5-9A, EZ6-4B and EZ9-6C were suspended in 100 μl 0.1 M phosphate buffer, pH 7.0. Three microliters of EMS were added, the tube was briefly vortexed and the cell suspension incubated at room temperature for 60 min. Aliquots of 20 μl from the mutagenesis mixture were suspended in 1.0 ml 5% sodium thiosulfate

TABLE 1

Strains used in this study

Strain	Genotype	Source
201-1-5	*MATα ade2-1 can1-100 his5-2 los1-1 lys1-1 SUP4 trp5-48 ura3-1*	HOPPER, SCHULTZ and SHAPIRO (1980)
EE1b	*MAT***a** *rna1-1* (*ade2-1* or *ade1*) *ura3-52 tyr1*	HOPPER, BANKS and EVANGELIDIS (1978)
62C	*MAT***a** *sir4-1 SUP4-3 cry1 his4-580 trp1 ade2-1 tyr1 lys2*	HARTWELL (1980)
JRY188	*MATα sir3-8 leu2-3,112 trp1 ura3 his4 rme1*	J. RINE
MCY528	*MATα ade2-101 ura3-52 suc2Δ9 gal2*	MARION CARLSON
JR72-6D	*MAT***a** *sir4-1 ade2-1 lys2-1 leu2-3,112 tyr1*	Laboratory stock
JR87-6C	*MAT***a** *sir3-8 ade2-1 his5-2 trp1 trp5-48 lys1-1 leu2-3,112*	Laboratory stock
JR159-6A	*MATα ade2-1 lys1-1 his*(3 or 5) *hm1::SUP4$_o$ leu2::LEU2-GAL10-lacZ* (ochre)	This work
JR222-52A	*MATα sir4-1 ade2-1 ura3-52 leu2-3,112 trp1 his3 hm1::SUP4$_o$ suc2Δ9*	This work
JR260-7A	*MAT***a** *sir3-8 ade2-1 leu2-3,112 ura3-52 lys1-1 his5-2* (*trp1* or *trp5*) *hm1::SUP4$_o$*	This work
EZ5-9A	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 his3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52*	This work
EZ6-4B	*MAT***a** *leu2::leu2-GAL10-lacZ*(ochre)-*URA3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52*	This work
EZ9-6c	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 hm1::SUP4$_o$ hmr::SUC2 sir3-8 sir4-1 ade2-1 ura3-52*	This work
TPD1	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 his3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52 tpd1-1*	This work
TPD2	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 his3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52 tpd2-1*	This work
TPD3	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 his3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52 tpd3-1*	This work
TPD4	*MATα leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 his3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52 tpd4-1*	This work
TPD5	*MAT***a** *leu2::LEU2-GAL10-lacZ*(ochre)-*URA3 hm1::SUP4$_o$ hmr::SUC2 sir4-1 ade2-1 ura3-52 tpd5-1*	This work
ts63-1	*MATα prt1-1 ade2*	HARTWELL (1967), HANIC-JOYCE, SINGER and JOHNSTON (1987)
POC8-36A	*MAT***a** *ade2 leu2Δ1 lys2-801 trpΔ101 ura3-52 pta1-1*	PAT O'CONNOR
T-404	*MATα ura3-52 ade2-101 lys2-801 lig1-4*	ERIC PHICICKY

and appropriate dilutions made in YEPD medium to obtain about 300 to 500 colonies per plate when plated on SC minus uracil selective medium. Plates were incubated at 23° until reasonable sized colonies developed before the colonies were replica plated to SC minus glucose minus uracil plus galactose medium, buffered to pH 7.0 and containing 70 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (ROSE and BOTSTEIN 1983).

Protein synthesis assays: The ability of mutant strains to support protein synthesis at the nonpermissive temperature was first assessed by determining the level of *in vivo* [^{35}S]-methionine incorporation. Cultures were grown in SC minus methionine medium at 23° to 10^7 cells/ml and 5.0-ml aliquots were placed at 23° and 38°, respectively, for 6 hr. Aliquots of 30 μCi [^{35}S]methionine (>800 mCi/mmol) were added to each culture and after a 30-min incubation at the appropriate temperature, the cells were harvested by centrifugation and resuspended in ice-cold 500 μl TNE buffer [25 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA]. About 2/3 volume glass beads were added and the suspensions were vortexed five times for 1-min intervals. The cell debris and beads were removed by centrifugation and washed with 200 μl ice-cold TNE buffer, which was added to bring the supernatants up to 500 μl. After 100 μl of the supernatants were removed for protein determination, 100 μl of 100% trichloroacetic acid (TCA) was added to the remaining 400 μl of supernatant solution and these suspensions were boiled for 10 min. TCA precipitates were collected on millipore filters, washed three times with 5.0-ml aliquots of 10% TCA and twice with 95% ethanol. The filters were transferred to scintillation vials, dried, and counted.

Northern analysis: Cultures were grown in YEPD medium to 10^7 cells/ml and then shifted to 38° in a shaking waterbath. Samples (3.0 ml) were removed at hourly intervals over a period of 6 hr and immediately chilled on ice. Cells were collected by centrifugation and small RNAs extracted with phenol at 65° according to KNAPP *et al.* (1979). RNA species were resolved on 10% polyacrylamide/7 M urea gels at 700 V for 12 hr and then transferred to GeneScreen nylon filters by electroblotting. Transfers were performed for 3 hr at 0.7 A in 25 mM sterile sodium phosphate buffer (pH 6.5). RNAs were UV cross-linked to the nylon filters.

Hybridizations were performed overnight in Church buffer (CHURCH and GILBERT 1984) at room temperature with mixtures of end-labeled single-stranded oligonucleotide probes. Oligonucleotides (19–60 nucleotides in length) complementary to the intronic and/or mature regions of specific tRNAs or to a portion of yeast 5S RNA were obtained from C. HO and J. ABELSON or synthesized on an Applied Biosystem Model 380A DNA Synthesizer. Oligonucleotides were end-labeled with polynucleotidyl kinase in the presence of [γ-^{32}P]ATP (MANIATIS, FRITSCH and SAMBROOK 1982). Hybridization filters were twice washed for 3

min at 50° in 6 × SSC. The wet filters were wrapped in plastic wrap and exposed to Kodak XAR5 film.

***In vivo* polymerase III transcription assay:** Cultures were grown in low phosphate YEPD medium (RUBIN 1975) to 10^7 cells/ml at 23°. Samples (2.5 ml) were then removed and incubated at either 23° or 38° in shaking waterbaths. After 6 hr, 0.5-ml samples were withdrawn from every culture to determine the cell concentration and the remaining 2.0-ml cultures were each labeled for 20 min with 80 μCi [^{32}P]orthophosphoric acid (825 Ci/mg P) according to KNAPP *et al.* (1979). Small RNAs were extracted with phenol at 65° and resolved on 10% polyacrylamide gels with 7 M urea at 700 V for 12 hr. The gel was covered with plastic wrap and overnight exposed to a Kodak XAR5 film. The fractionated RNA was transferred to GeneScreen nylon filters (see *Northern analysis*) and the filters hybridized to oligonucleotide probes against 5S RNA and tRNA^{Tyr} to normalize for the total amount of RNA present in each lane.

***In vitro* suppression assays:** Mutant and wild-type strains were grown in 50-ml YEPD cultures at 23° to 2 × 10^7 cells/ml and then shifted to 37° for an additional 6 hr. Cells were harvested and low molecular weight RNA was isolated as described above. RNA samples (approximately 0.5 mg) were ethanol precipitated and dissolved in 0.5 ml 0.5 M Tris-HCl (pH 8.7) and incubated at 30° for 30 min to deacylate the tRNA. The entire samples were ethanol precipitated, dissolved in 20 mM Tris-HCl (pH 7.6) and applied to a 1-ml DE52 column equilibrated in the same buffer. After washing the columns with the same buffer, tRNA was eluted with 20 mM Tris-HCl plus 1 M sodium chloride. The samples were ethanol precipitated and dissolved in TE buffer to a concentration of 0.5 mg/ml. The actual amount of tRNA in each sample was determined by fractionating a portion of each sample on a 10% polyacrylamide 4 M urea gel, staining the gel with ethidium bromide and then scanning a negative photograph of the stained gel.

In vitro translations were performed essentially as described by GESTELAND *et al.* (1977) using a reticulocyte lysate system purchased from Promega and programmed with 300 μg/ml Qβ RNA. Reactions (15 μl) contained a mixture of amino acid minus methionine to which was added [^{35}S]-methionine from NEN at 1 μCi/reaction (800 Ci/mM). Magnesium acetate was present at 2 mM, a value determined experimentally to be optimum for suppression in the translation system, using SUP4-containing total tRNA isolated from strain FM6 (RASSE-MESSENGUY and FINK 1973). Total tRNA from the test strains were added at approximately 100 μg/ml. The actual amounts added were adjusted on the basis of the total 4S RNA in each sample, as judged by gel fractionation described above, so that each translation reaction contained the same amount of 4S RNA. The level of suppression in the translation system was determined to be linear with increasing tRNA concentration up to 200 μg/ml.

After incubation, the translation reactions were fractionated on a 10% SDS-polyacrylamide gel. The gel was autoradiographed using Kodak XAR film.

RESULTS

A screen for isolating mutants defective in tRNA maturation: We have adapted the screen developed by SCHEDL and PRIMAKOFF (1973) to yeast by developing a method for conditional expression of a suppressor tRNA gene. This allows us to activate transcription of the suppressor locus only after shifting mutagenized colonies to the nonpermissive growth conditions. In this way, preexisting mature suppressor tRNA does not have to be diluted out and production of functional tRNA can be assessed without requiring mutant strains to undergo further growth. By this approach, conditional lethal mutations in genes essential for production of mature tRNA can be recovered.

TABLE 2

Conditional expression of a *SUP4*$_o$ tRNA gene inserted into a silent mating type cassette

Genotype[a]	β-Galactosidase specific activity[b] 23°	35°
sir4-1 hm1::SUP4$_o$	0.08	0.06
sir4-1 leu2::GAL10-lacZ$_o$	0.05	0.01
sir4-1 hm1::SUP4$_o$ *leu2::GAL10-lacZ*$_o$	33	62
sir4-1 leu2::GAL10-lacZ$_o$ [YCp50-*SUP4*$_o$][c]	61	70
SIR4 hm1::SUP4$_o$ *leu2::GAL10-lacZ*$_o$	19	22
sir4-1 hm1::SUP4$_o$ *leu2::GAL10-lacZ*	665	680

[a] All *sir4-1* strains were derived by transformation of strains JR-72-6D with the appropriate plasmid or plasmids described in MATERIALS AND METHODS. The *SIR4* strain is JR159-6A.

[b] Cells were grown in synthetic complete medium at the indicated temperature and harvested at a cell density of 1–2 × 10^7 cells/ml. β-Galactosidase and protein assays were then performed as described in MATERIALS AND METHODS. Specific activity is given as nmoles ONPG hydrolyzed/min/mg protein.

[c] Cells were grown in synthetic complete medium minus uracil in order to select for maintenance of the YCp50 plasmid.

We obtained conditional expression of a suppressor tRNA gene by taking advantage of the observation by SCHNELL and RINE (1986) that a tRNA gene inserted into the silent mating type cassettes of yeast is subject to repression by the *SIR* regulatory proteins. Accordingly, we have inserted a tyrosine-inserting, ochre-suppressing tRNA gene, *SUP4*$_o$, into the silent mating type cassette, *HMLα*, of strains bearing a temperature sensitive mutation in one of the *SIR* genes. To monitor formation of functional suppressor tRNA we incorporated into these strains a *GAL10-lacZ* fusion gene containing an ochre mutation in the *lacZ* coding domain (*cf.* MATERIALS AND METHODS). Consistent with SCHNELL and RINE's observations, we find that when the strain is grown at 23°—permissive temperature for the *sir3* or *sir4* alleles used—little functional β-galactosidase activity is produced (Table 2) and colonies of the strains are white or light blue on medium containing the chromogenic substrate X-Gal (Figure 1). When the strain is grown at 37°—nonpermissive temperature for the *sir* alleles—active β-galactosidase is synthesized, and colonies of the strain are blue on X-Gal plates. In contrast, an isogenic strain carrying the *SUP4*$_o$ gene on a single copy centromeric plasmid, rather than at *HMLα*, produces β-galactosidase at both temperatures. Thus, conditional production of β-galactosidase in the test strain is a result of the condi-

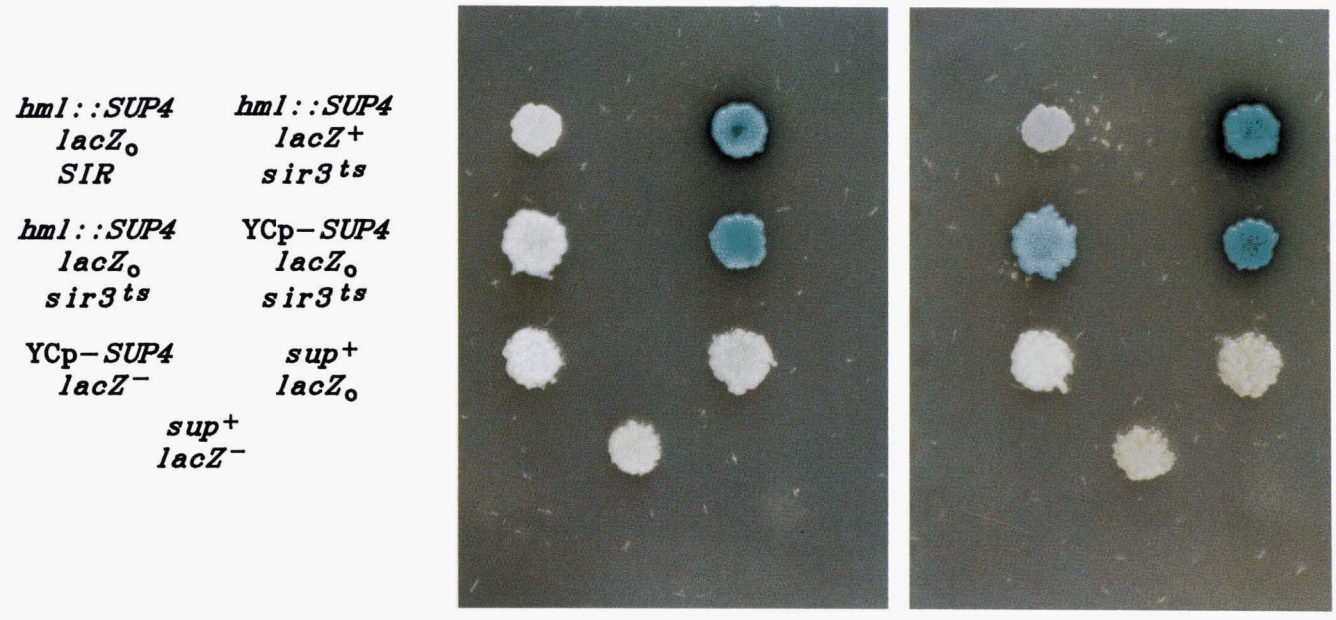

FIGURE 1.—Conditional expression of a $SUP4_o$ tRNA gene inserted in the *HML* silent mating type cassette. Strains with the genotypes indicated on the left were patched onto YEPD plates and grown at 23° overnight. The patches were then replicated to two SC minus uracil plates containing 2% galactose and 70 μg/ml X-Gal. One plate was incubated at 23° for 3 days (left) and the other at 37° for 2 days (right).

tional expression of the $SUP4_o$ gene resident within the silent mating type cassette.

Identification of mutants defective in tRNA production using this system is accomplished as outlined in Figure 2. An *hml*::$SUP4_o$ LEU2::$GAL10$-$lacZ_o$ $sir3^{ts}$ or $sir4^{ts}$ strain is mutagenized, plated at an appropriate dilution on minimal medium containing glucose to yield several hundred colonies per plate, and incubated at 23° for several days until reasonable sized colonies are formed. The plate is then replica plated to a synthetic minimal galactose plate containing X-Gal, incubated at 23° for 2 days, and then shifted to 37° and incubated for an additional 2 days to permit color production. The replica plating is required (1) to preserve a master plate at the permissive temperature and (2) to obviate the low plating efficiency of our strains on the high pH plates that have to be used for X-Gal color development. Most of the clonal isolates are still capable of converting the primary transcript from the derepressed $SUP4_o$ gene into mature suppressor tRNA. Thus, most of the colonies turn blue following incubation on X-Gal. However, clones with a temperature sensitive mutation in a gene required for maturation of the $SUP4_o$ primary transcript would not produce functional suppressor tRNA. Such clones would remain white or light blue. These colonies are then tested for temperature sensitivity. Since our goal is to identify essential genes in tRNA processing, only those clones that do not turn blue and are also temperature sensitive for growth are retained as potential tRNA maturation mutants.

One class of mutants, other than those in tRNA maturation, that would not be eliminated by this screen would be those involved in protein synthesis. To exclude these mutants in a reasonably facile manner, we have further modified the parent strain by integrating the gene for yeast invertase, *SUC2*, into the second silent mating type cassette, *HMR* (*cf.* MATERIALS AND METHODS). In this manner, shifting the strain to 37° yields *de novo* synthesis of invertase as well as $SUP4_o$ tRNA. Mutants deficient in protein synthesis per se would fail to yield either invertase activity or β-galactosidase activity upon a shift to the non-permissive temperature. However, mutants deficient in tRNA maturation would still be capable of producing invertase activity immediately following the shift to the non-permissive temperature, even though they would be incapable of producing β-galactosidase.

Finally, it should be noted that strains with mutations or deletions of *lacZ*, revertants of the sir^{ts} allele, or revertants or deletions of the $SUP4_o$ allele would appear as white or light blue colonies in the screen. However, since these mutations would not cause temperature sensitive growth of the clone, such mutant strains would be eliminated from consideration. A summary of the expected phenotypes of the various classes of mutants is provided in Table 3.

Isolation of putative mutants deficient in tRNA production: We have applied the above screen to three different *hml*::$SUP4_o$ $LEU2$-$GAL10$-$lacZ_o$ *mr*::$SUC2$ sir^{ts} yeast strains, with the results shown in Table 4. These strains were mutagenized with EMS and plated directly onto master plates. Colonies (3.5×10^5) were examined for reduced color production on X-Gal as described above and in MATERIALS AND

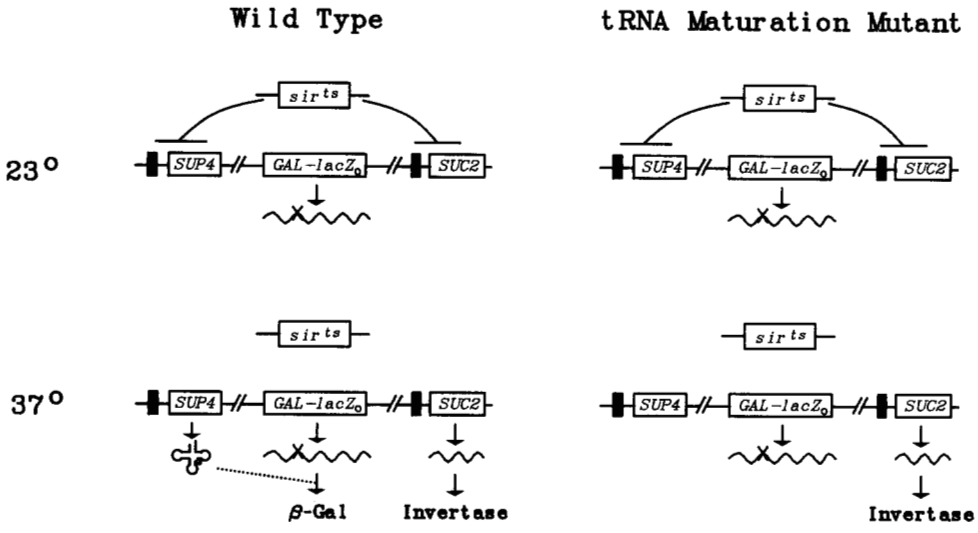

FIGURE 2.—Rationale for a screening procedure to identify mutants defective in tRNA processing. The behavior of a wild type strain and one with a putative tRNA maturation defect, each carrying identical *sir^{ts} hm1::SUP4 hmr::SUC2 leu2::GAL10-lacZ$_o$* alleles, is shown. In both strains at 23°, expression of *SUP4* and *SUC2* is repressed by the *SIR* genes acting on the contiguous chromosomal silencers (black boxes). Accordingly, neither invertase nor β-galactosidase is produced. At 37° in the wild type strain, synthesis of tRNA from *SUP4$_o$* occurs to allow suppression of the nonsense mutation in the *lacZ* gene and production of functional β-galactosidase. At the same time expression of *SUC2* is induced, yielding production of invertase. A mutant defective in tRNA maturation, on the other hand, would fail to produce active suppressor tRNA from the derepressed *SUP4* locus and, accordingly, no β-galactosidase would be synthesized. However, invertase production would be essentially normal. This difference in β-galactosidase production between the wild type and putative mutant strain is the basis of the screening procedure described in the text.

TABLE 3

Effect of various mutations on the phenotypes examined in the screen for tRNA processing deficient mutants

	Phenotype					
	Color		Growth		Invertase activity	
Strain	23°	37°	23°	37°	23°	37°
Wild type	White	Blue	+	+	Low	High
tRNA processing deficient	White	White/light blue	+	−	Low	High
Protein synthesis deficient	White	White/light blue	+	−	Low	Low
GAL10-lacZ deletion/ mutation	White	White	+	+	Low	High
Revertant to Sir$^+$	White	White	+	+	Low	Low

TABLE 4

Summary of mutant isolation

	Strain			
	EZ5-9A	EZ6-4B	EZ9-6C	Total
Colonies screened	112,900	114,200	117,700	344,800
Average killing rate of mutagenesis	55%	60%	61%	
Light blue colonies initially picked	1,818	1,092	837	3,747
Light blue colonies on retesting	352	207	79	638
Light blue and temperature sensitive	76	47	12	135
Retained for further study				46
Light blue, ts, and invertase positive				31
Invertase positive and cosegregates light blue and ts				5

METHODS and 630 white to light blue colonies were obtained. Of these, approximately 20% also exhibited temperature sensitive growth at 37°. We characterized only 46 of these light blue, ts further. Fourteen of the 46 showed reduced invertase activity following a shift to 37°, and were eliminated from further consideration.

Since the desired mutants are those in which the ts phenotype and the reduced color production on X-Gal are the consequence of the same lesion, we tested the linkage of the two phenotypes by genetic segregation analysis. We crossed each of the 31 light-blue, ts strains to a *hm1::SUP4$_o$ LEU2-GAL10-lacZ$_o$ sir^{ts}* strain of the opposite mating type, sporulated the resulting diploid, and dissected a number of tetrads from each. Five of the mutants gave the segregation pattern shown in Figure 3, in which reduced color and temperature sensitivity cosegregated in every te-

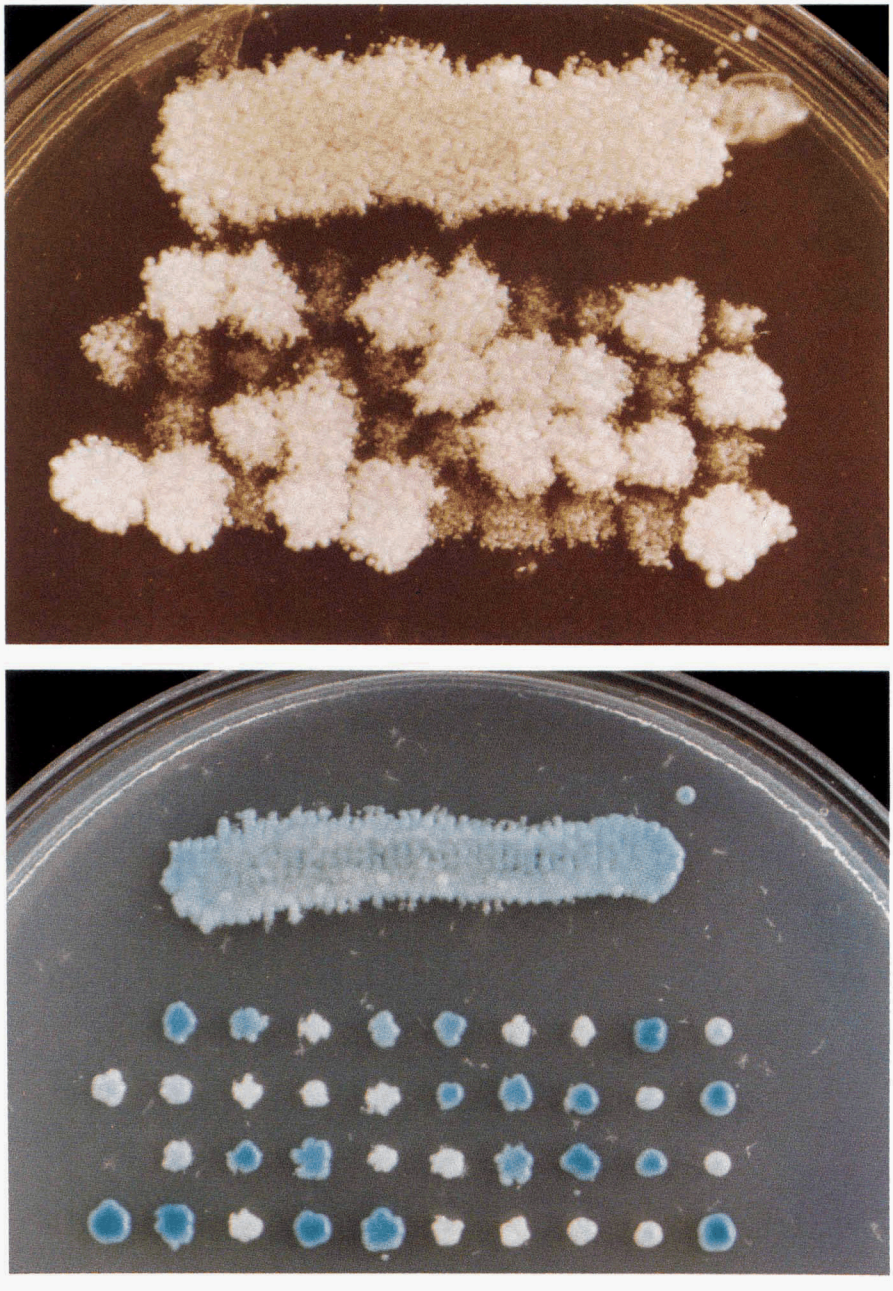

FIGURE 3.—Cosegregation of temperature sensitivity and diminished tRNA maturation from a *tpd1-1* mutant strain. Strains TPD1 and EZ6-4B were crossed, sporulated and dissected onto YEPD plates and the spore clones grown at 23°. The clones were then replicated to a YEPD plate and to a SC plate containing 2% galactose and 70 μg/ml X-Gal. The YEPD plate was incubated at 37° for 1 day and then replicated to a second YEPD plate. The upper portion of the figure shows this second YEPD plate after incubation for one day at 37°. The lower portion of the figure shows the SC + X-Gal plate after 1 day at 23° and 2 days at 37°. The four colonies in each column are the spore clones from a single ascus, representing the meiotic products of one diploid cell.

trad, both phenotypes showing predominantly 2:2 segregation. Thus, in these five mutant strains, the two phenotypes are the result of a single mutation or of two closely linked mutations. Given the number of tetrads examined, if the two phenotypes are the result of two separate mutations, then they lie within 3 centimorgans of each other. The other 26 mutants examined showed either independent segregation of temperature sensitivity and color production or aberrant segregation of one or the other phenotype.

At the end of the screen we retained five strains that met the criteria for those with temperature sensitive mutations in an essential gene required for tRNA maturation. Since we characterized in detail only a third of the possible candidates, our recovery rate from the original mutagenized population was approximately 0.005%.

Characterization of mutants: *Mutants are not deficient in protein synthesis per se*: The initial isolation of conditional lethal mutants in tRNA production included a screen to eliminate those mutants defective in protein synthesis *per se*. To confirm that our mutants were competent for protein synthesis, we measured the rate of protein synthesis of the mutants at the nonpermissive temperature by determining the rate of incorporation of [^{35}S]methionine into acid insoluble counts in intact cells. These results are presented in Table 5. As is evident, the rate of incorporation of label in all five mutant strains is essentially equivalent to that of the parent strain, both at 1 hr and 6 hr after the shift to 37°. On the other hand, a

TABLE 5

tRNA processing defective mutants are protein synthesis competent

Strain	Mutation	Protein synthesis [^{35}S]methionine incorporation[a] 23°	38°[b]
EZ5-9A	*TPD*	6.2	7.2
TPD1	*tpd1-1*	7.7	2.6
TPD2	*tpd2-1*	2.1	3.9
TPD3	*tpd3-1*	1.2	2.0
TPD4	*tpd4-1*	5.4	3.7
TPD5	*tpd5-1*	8.5	6.4
ts63-1	*prt1-1*	2.4	0.05

[a] Aliquots of 30 μCi [^{35}S]methionine (>800 Ci/mM) were added to 5-ml cultures growing in SC minus methionine at the indicated temperature. Incubation was continued for 30 min and the cells then harvested by filtration. After washing cells with cold medium, they were resuspended in 10% TCA, boiled for 1 min and then filtered through a glass fiber filter. Samples were removed for protein determinations prior to labeling. Results are presented as cpm ($\times 10^{-6}$)/mg protein.

[b] Strains were grown at 23° and then shifted to 38° for 6 hr prior to labeling.

mutant strain containing the *prt1-1* allele, a temperature sensitive mutation in a gene required for initiation of protein synthesis (HANIC-JOYCE, SINGER and JOHNSON 1987), shows a rapid decline in the rate of incorporation following a shift to the nonpermissive temperature. Thus, the overall rate of protein synthesis is essentially undiminished in the mutant strains after transfer to the nonpermissive conditions.

Mutants from the screen define novel, independent genes: To determine the number of loci defined by the five mutants recovered from our screen, we performed complementation analysis among all the mutants. From the segregation analysis we recovered **a** and α versions of each mutant. We used these to construct diploids between all pairwise combinations of mutants and between each mutant and the parent strains. We then tested each of the diploids for growth at 37°. All five mutant strains were recessive to wild type for temperature sensitive growth. In addition, each mutant complemented every other mutant for growth at 37°. From this we conclude that the five mutant define five separate loci. We have tentatively designated these five loci *TPD1* through *TPD5*, for tRNA processing deficient.

To date, conditional lethal mutations affecting tRNA maturation have been isolated in only five different genes in yeast: *rna1*, *sen1*, *pta1*, *lig1* and *rpc160* (HOPPER, BANKS and EVANGELIDIS 1978; WINEY and CULBERTSON 1988; PHIZICKY, SCHWARTZ and ABELSON 1986, GUDENUS *et al.* 1988; P. O'CONNOR and C. PEEBLES, personal communication). To determine whether any of the mutations we obtained represent new alleles of any of these previously identified genes, we performed complementation analysis of our mutant strains against strains carrying conditional lethal alleles of three of these genes. (Complementation analysis with an *rpc160* or *sen1* strain was not performed. For reasons described below, we do not believe any of our mutations are in these genes.) We found that each one of our mutants complemented the temperature lethality of each of the three strains. In addition, mutations in three other genes, *los1*, *spd1* and *sen2*, affect tRNA maturation in yeast. However, null mutations of *los1* and *spd1* are not lethal and none of the alleles of *sen2* cause a significant growth defect, so these loci probably do not correspond to the five genes we have identified (HURT *et al.* 1987; WANG and HOPPER 1988; WINEY and CULBERTSON 1988; C. HO and J. ABELSON, personal communication). Thus, all five of our mutations are most likely in genes not previously identified as involved in tRNA maturation.

One of the mutant strains is deficient in tRNA accumulation: As an initial step in identifying the specific biochemical lesions in the various mutant strains, we asked whether any of the mutants are defective at the non-permissive temperature in synthesis of tRNAs. To do so, we pulse labeled mutant and wild-type cells with $^{32}PO_4$ at 23° and at 6 hr after a shift to 38°. We then determined the amount of label incorporated into total tRNA by isolating RNA from the pulse-labeled cells, fractionating it on a polyacrylamide gel, and autoradiographing the gel. To normalize for the amount of total tRNA present in each sample, we transferred the labeled, fractionated material to nitrocellulose and then hybridized it with a labeled oligonucleotide complementary to mature tRNA^{tyr}. Since the specific activity of the oligonucleotide was substantially higher than that of the *in vivo* labeled tRNA, the tRNA^{tyr} specific band could readily be seen above the background of *in vivo* labeled tRNA (Figure 4B). This provided us with an internal control for the total amount of tRNA present in each sample.

The result of this analysis of the parental strain and of one carrying the *tpd3-1* allele is shown in Figure 4. As is evident, the level of incorporation of label into tRNA in strain TPD3 at 38° is significantly reduced compared to that of the parental strain. This contrasts with the situation at 23°, where both strains yield essentially equivalent incorporation of label into tRNA. As is evident from results shown in Figure 4B, the total amount of tRNA^{tyr} in all four samples is essentially equivalent, indicating the same amount of tRNA was loaded for each sample. In addition, this control attests that the reduced level of label in the tRNA fraction in the 38° TPD3 sample is not the consequence of degradation of tRNA during incubation of the strain at the elevated temperature. We conclude that TPD3 is most likely defective specifically in synthesis of tRNA at its non-permissive tem-

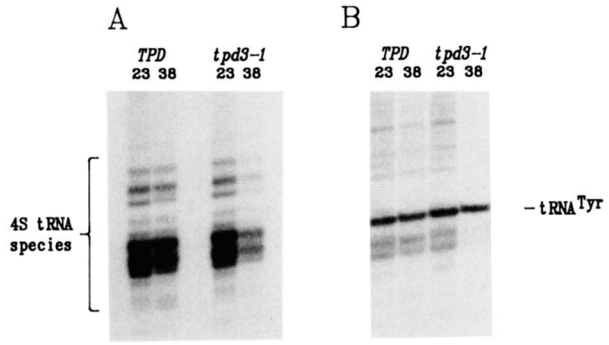

FIGURE 4.—Analysis of tRNA synthesis in *TPD* and *tpd3-1* mutant strains. Cultures of strains EZ5-9B (*TPD*$^+$) and TPD3 (*tpd3-1*) were grown at 23° in low phosphate YEPD medium to 10^7 cells/ml. Samples (2.5 ml) were then incubated at 23° and 38° for 6 hr and labeled with [^{32}P]orthophosphate for 15 min as described in MATERIALS AND METHODS. RNA was extracted from cells and fractionated identically on two 10% polyacrylamide gels. One gel was subjected to autoradiography directly, a portion of the autoradiogram is shown in panel A. The labeled and fractionated RNA in the second gel was transferred to GeneScreen nylon filters and hybridized to end-labeled oligonucleotide probes against 5S RNA and tRNA^{Tyr}. The autoradiogram of the hybridized filter is shown in panel B. The position of migration of tRNA^{Tyr} is indicated.

perature. None of the other mutant strain showed any significant reduction of incorporation of label into tRNA during incubation at 38°.

One of the mutant strains accumulates unspliced tRNA precursors: As a second step in our analysis of the tRNA maturation defect in each of our mutant strains, we looked for tRNA precursor accumulation in the strains by Northern blot analysis. To accomplish this, small molecular weight RNA was isolated from those strains of interest, fractionated on an appropriate acrylamide gel, and transferred by electroblotting to a nitrocellulose membrane. The membrane was then hybridized with various oligonucleotide probes, specific either for particular mature tRNA species or for intron-containing precursors to those tRNA species. The results of one such experiment indicated that RNA from strain *tpd1* grown at the nonpermissive temperature contains a significantly greater amount of unspliced $tRNA^{ser}_{UCG}$ precursor, compared to the level of mature $tRNA^{ser}_{UCG}$, than does either of the parent strains or any of the other four mutant strains (data not shown). On the basis of the size of the accumulated precursor, we conclude that it is fully processed at both the 5′ and 3′ ends. Thus, this mutant strain appears to be blocked specifically in excision of tRNA intervening sequences.

We have examined the time course and extent of accumulation of intron-containing precursor in mutant *tpd1*, following a shift to the nonpermissive condition, and have compared it to that in two other mutant strains—*rna1* and *los1*—that accumulate intron-containing tRNA precursors. The results of this analysis is shown in Figure 5B. The ratio of intron-containing to mature tRNA^{ser} in RNA from the wild type strain remained constant over the 6 hr following a shift to 38°. However, for a strain containing the *tpd1-1* allele, the ratio increased steadily over the course of the experiment, attaining a level at 6 hr of approximately 7 times that at the point of the temperature shift. A similar pattern was obtained with RNA from the *los1-1* strain, although the ratio of precursor to mature did not reach as high a level as that seen with *tpd1*. The strain containing the *rna1-1* allele exhibited a different pattern, with the ratio of precursor to mature reaching a peak within 20 min of the shift to nonpermissive temperature, and then declining to pre-shift levels within three hours of incubation.

tpd1-1 strains are defective for excision of intervening sequences from a variety of tRNA precursors. We probed small RNA from wild type and *tpd1-1* strains with oligonucleotides specific for the introns of tRNA^{tyr} and tRNA^{ile}. In both cases, *tpd1-1* showed an approximately fivefold increase in precursor levels, compared to that in wild-type strains, within 6 hr of a shift to 38° (data not shown). In both cases, the accumulated precursors were the appropriate sizes to be fully matured at both their 5′ and 3′ ends. Thus, we conclude that *tpd1* mutant strains are generally deficient in splicing out intervening sequences of intron-containing tRNA precursors. It is worth noting that accumulation of unspliced tRNA molecules is not a general feature of cessation of growth. We probed small RNA isolated from a temperature sensitive *prt1* strain, at various times following a shift of the strain to 37°. At no point did the ratio of unspliced precursor to mature $tRNA^{ser}_{UCG}$ deviate from that of a wild type strain (Figure 5C).

Some mutants fail to produce mature suppressor tRNA at the nonpermissive temperature: To obtain additional evidence that the mutants are deficient explicitly in tRNA production, we used an *in vitro* suppression assay to measure accumulation of mature *SUP4*$_o$ tRNA in the mutant strains. Total tRNA was purified from a wild-type strain and from each of the mutant strains after a temperature shift to 37° for 6 hr. The level of *SUP4*$_o$ tRNA in each of the total tRNA preparations was determined by measuring the extent of ochre suppression in an *in vitro* protein synthesis system primed with MS2 RNA. Suppression of the ochre stop codon at the end of the Qβ synthetase yields a readthrough product that can be visualized by one dimensional SDS polyacrylamide gel electrophoresis. Thus, within an appropriate range of added tRNA, the amount of readthrough product reflects the amount of suppressor tRNA added to the *in vitro* translation reaction.

The results of this analysis are shown in Figure 6. First, several of the mutant strains exhibit reduced levels of suppressor activity, compared to that for the *TPD* strain. This is most evident for the strain carrying

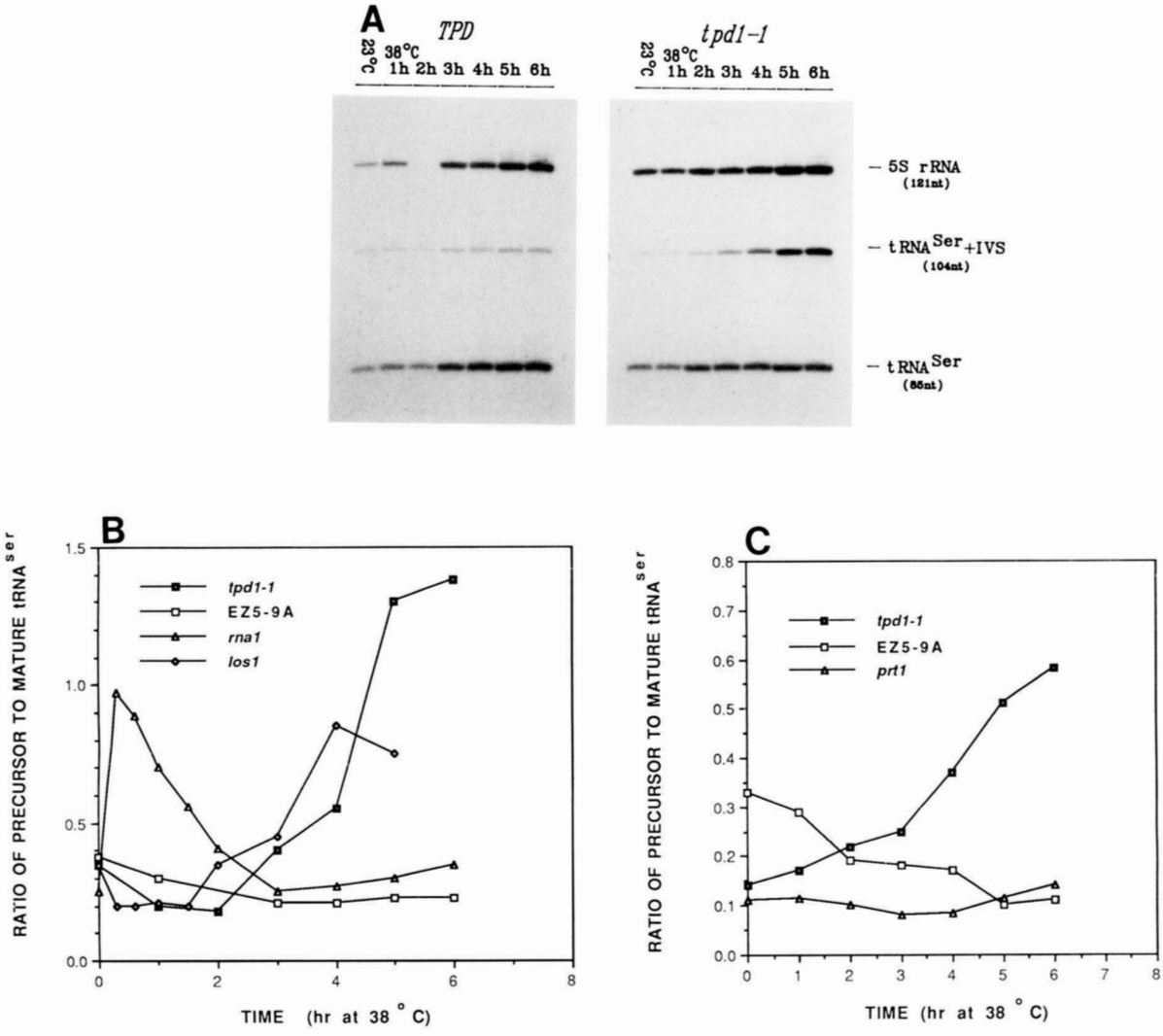

FIGURE 5.—Time course of tRNA precursor accumulation in various wild-type and mutant strains. A, Strains EZ5-9A and TPD1 were grown at 23° in YEPD to 10^7 cells/ml and shifted to 38°. Samples were removed at one hour intervals and RNA was extracted from the cells as described in MATERIALS AND METHODS. RNA samples were fractionated by electrophoresis on a 10% acrylamide/7 M urea gel and the RNA transferred to GeneScreen filters by electroblotting. Filters were hybridized with a mixture of three end-labeled oligonucleotides complementary, respectively, to a portion of 5S RNA, $tRNA^{ser}_{UCG}$ intervening sequence, and a portion of mature $tRNA^{ser}_{UCG}$. The autoradiogram of a 24-hr exposure of the filter is shown. B and C, The ratios of the levels of IVS-containing $tRNA^{ser}_{UCG}$ precursor to mature $tRNA^{ser}_{UCG}$ are plotted as a function of time following a shift of the indicated strain to 38°. Data for strains TPD1 and EZ5-9A in panel B were obtained by densitometer scans of the autoradiogram shown in A. Data for other strains were obtained in an identical fashion. Ratios are given in arbitrary units.

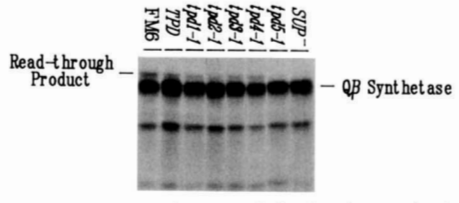

FIGURE 6.—Mutant strains are defective in producing mature $SUP4_o$ tRNA. Total tRNA, prepared from the indicated wild type and mutant strains grown for six hours at 37°, were added to *in vitro* translation reactions programmed with Qβ RNA as described in MATERIALS AND METHODS. The primary translation product is the Qβ synthetase (65 kD). Suppression of the ochre nonsense codon at the end of the gene yields a read-through product 2.7-kD larger.

tpd5-1 but is also clear for a *tpd2-1* strain. Thus, consistent with their *in vivo* phenotype, these strains have reduced levels of specific suppressor tRNA production. For the other strains, a marked reduction in *in vitro* suppressor activity is not as evident, although the suppression activity is somewhat diminished. This is true even for those strains in which a specific defect in tRNA biosynthesis is clearly demonstrable. Thus, the *in vitro* suppression assay is not as sensitive an assay as those for a specific function. This is due in part to the presence of residual suppressor tRNA accumulated prior to the temperature shift. It may also be due to *in vitro* complementation of the mutant phenotypes or to masking *in vitro* of activities that might be limiting *in vivo*. Nonetheless, the fact that

several of the mutant strains show diminished suppressor activity *in vitro* is consistent with their identification as tRNA biosynthesis deficient.

DISCUSSION

We have devised a general screen for isolating conditional lethal mutants defective in synthesis of mature tRNA in yeast. Specifically, we identify mutants of a strain carrying an ochre-specific tRNA suppressor gene that have lost the ability to suppress an ochre mutation in *lacZ*. The method differs from previous loss-of-suppression screens, in that suppressor tRNA synthesis is repressed until the mutant clones are switched to the nonpermissive temperature. Since dilution of preexisting suppressor tRNA molecules by growth of a mutant clone is not required in order to manifest the mutant phenotype, we can in theory isolate mutations in genes essential for growth. Using this screen, we have identified five new genes in yeast that appear to be required for production of mature tRNA. These mutant strains are temperature sensitive for growth and the mutant alleles are recessive. Two of the mutants can be shown to be defective in specific steps in tRNA biosynthesis. In addition, two other mutants are deficient in their ability to yield functional suppressor tRNA from the *SUP4$_o$* gene carried in the strain, although the specific lesion responsible for this defect has not yet been pinpointed. Thus, we conclude that the screen does, in fact, yield mutants defective in tRNA maturation.

The frequency of mutant isolation from our screen was somewhat low. We identified five mutants from approximately 10^5 mutagenized clones. By comparison, screens for cell division cycle mutants have yielded mutants at a frequency of 0.01% to 0.1%, albeit using cells substantially more mutagenized than those we used. Several factors could have contributed to the low frequency of mutant recovery in this screen. First, many potential mutants may have slipped through the screen. This could be due in part to the fact that the available *sir^{ts}* alleles do not fully repress expression of the silent cassettes at the permissive temperature. As a consequence, the color distinction between mutant and wild type cells is not absolute. An additional explanation for the low frequency of recovery is that the mutants may have been represented at a low level in the mutagenized cultures used. This could be due to the relatively light mutagenesis applied in this particular case or to the low mutability to temperature sensitivity of many of the genes involved in tRNA maturation. We are currently repeating the screen with these caveats in mind.

Several different approaches have been used by others to isolate mutants in tRNA maturation, the nature and effectiveness of which have been recently reviewed (HOPPER 1989). These approaches fall into four basic categories: loss-of-suppression screens or selections, biochemical screens of mutagenized clones, reversion of *cis* active mutations in tRNA genes, and reverse genetics. Each of these is inherently limited in its applicability. The first method has not yielded mutations in genes essential for tRNA maturation and, accordingly, for growth. This is most likely due to the requirement for diluting preexisting suppressor tRNA by growth, as suggested in the introduction. The latter three schemes are restricted by the fact that one can recover only mutants defective in the particular process being examined. Both of these problems are circumvented by the screen described in this report. Thus, we anticipate that this current screen, unlike the others previously used, has the potential to yield a broad spectrum of tRNA maturation mutants.

Possible function of mutants isolated in our screen: Five mutant strains were isolated from our screen. We have not yet pinpointed the specific lesion in all of these strains. Because of the generality of the screen, the particular lesion in tRNA production in each of the mutants could be at any one of a wide variety of steps, including transcription of gene, endo- and exonucleolytic processing of the primary transcript, transport from the nucleus to the cycloplasm, and formation of specific essential base modifications. We have examined the mutant strains for their capacity to carry out many of these reactions, with the results summarized in the following.

We have examined the tRNA transcription capacity of the mutant strains. In pulse labeling experiments, one of the mutant strains—that containing *tpd3-1*—showed a tenfold diminished incorporation of label into the 4S fraction following a shift to the nonpermissive temperature. We are currently assessing the ability of this strain to yield extracts that are capable of synthesizing tRNA in an *in vitro* transcription system primed with *SUP4$_o$* DNA. In addition, we have cloned the gene and have determined by restriction analysis that it does not correspond to either of two previously cloned genes—*RPC160* and *RPC40*—encoding subunits of RNA polymerase III (GUDENUS *et al.* 1988; MANN *et al.* 1987). Thus, to date at least one of the mutants behaves as though it might be defective in transcription of tRNA genes.

Our Northern analysis of RNA probed with oligonucleotides specific for various tRNA species, an example of which is presented in Figure 5, failed to yield any indication of a defect in exonucleolytic trimming of pre-tRNA in any of the mutants. In addition, except for strains carrying *tpd1-1*, none of the mutants show an accumulation of intermediates in tRNA splicing. However, two alleles of *sen2* (*sen2-1* and *sen2-2*) [WINEY and CULBERTSON (1988 and personal communication)] yield extracts that are deficient in endonucle-

ase activity *in vitro* but do not show accumulation of splicing intermediates *in vivo*, whereas a third allele (*sen2-3*) (C. Ho and J. Abelson, personal communication) does show such an accumulation. Therefore, the absence of observable intermediates in the mutant strains cannot be taken as evidence that the mutants are not defective in tRNA trimming or splicing.

We have examined the mutant strains to determine whether any of them show evidence of a defect in formation of specific modified bases. We purified $tRNA^{Tyr}$ and $tRNA^{Ser}_{UCA}$ from mutant strains incubated at the nonpermissive temperature and pulse labeling with ^{32}P-sodium phosphate. The pattern of modified bases was then determined by appropriate thin layer chromatography of RNAse T_2 digested material. In no case did we detect an alteration in the presence or amounts of any of the modified bases in tRNAs isolated from the mutant strains *vs.* those isolated from the parent strain. Thus, we do not have evidence that any of our mutant strains are defective in catalyzing specific base modification.

Although we have not identified a specific defect in three of our putative tRNA processing mutants, some of them appear to be diminished in their capacity to synthesize functional tRNA at the nonpermissive temperature. We used an *in vitro* suppression assay to assess whether the mutant strains were able to produce functional suppressor tRNA following induction of transcription from a suppressor tRNA gene. For the most part the effects were small: the mutants exhibited only a two- to threefold lower suppression activity than the parent strain. However, we would not expect a large effect, since the repressed level of suppressor tRNA expression appears to be only two- to threefold lower than the induced level in our strains (*cf.* Table 1). Thus, a mutant unable to produce additional suppressor tRNA following induction would contain only one-half to one-third that of the parent strain. In contrast, a strain carrying *tpd5-1* yielded almost no suppressor activity. This might indicate that the mutant exhibits some deficiency even at the permissive temperature, that the lesion is semidominant, or that the *in vitro* analysis exacerbated the *in vivo* deficiency. In sum, the *in vitro* suppression assays are consistent with a role of the identified genes in tRNA production.

Intron-excision deficient mutant: The mutation we have most thoroughly characterized, *tpd1-1*, reduces the efficiency of excision of intervening sequences from a variety of tRNA species. By Northern analysis of RNA from strains containing this mutation, we do not detect any reduction in the level of mature actin mRNA, whose gene contains an intron. Thus, *tpd1* does not appear to be defective in RNA transport in general, as is the case for *rna1*, or in splicing pre-mRNA species. Rather, the mutation alters tRNA splicing specifically. From the recessive nature of our *tpd1* alleles, we suspect that the defect in endonuclease function results from loss of *TPD1* activity.

Results presented in Figure 5 show that the *tpd1-1* mutant manifests a block in precursor accumulation relatively slowly following a shift to high temperature. This lag in accumulation could be due to the fact that the *tpd1-1* allele renders the gene product temperature sensitive for synthesis rather than function or because precursor accumulation is a secondary effect of the mutation. Nonetheless, the final accumulation of precursor in *tpd1-1* strains is as great or greater than that in other strains, such as those carrying *los1* or *rna1*, that have been classified as tRNA splicing deficient.

Even though *tpd1* strains accumulate intervening sequence-containing tRNA molecules, we do not know whether the gene encodes a component of the yeast tRNA splicing endonuclease or whether it affects splicing only indirectly. We have not rigorously tested whether the mutant strains are deficient in endonuclease activity *in vitro*. However, *TPD1* is not allelic to *SEN1*, mutations in which have been shown to diminish endonuclease activity *in vitro*, since *TPD1* lies on chromosome *VI* and *SEN1* lies on chromosome *XII* (Winey and Culbertson 1988; van Zyl 1989). We have not excluded the possibility that *TPD1* is allelic to *SEN2*, mutations of which are also defective in endonuclease activity *in vitro*. However, the fact that none of the three alleles identified to date exhibit a growth defect suggests that these two genes are not the same (Winey and Culbertson 1922; C. Ho and J. Abelson, personal communication). It is possible that the primary role of the *TPD1* product is in some process that affects tRNA splicing indirectly, such as nuclear export of tRNA or maintenance of the integrity of nuclear structure. Circumstantial evidence suggests that tRNA splicing and nuclear export of tRNA are intimately associated functions. Endonuclease activity behaves in cell fractionation and biochemical extraction procedures as though it were a component of the nuclear membrane (Peebles, Gegenheimer and Abelson 1983) and, by immunoelectron microscopy, Clark and Abelson (1987) have shown that tRNA ligase resides in or near the yeast nuclear membrane. In addition, *rna1* strains, whose primary defect appears to be in RNA export from the nucleus, accumulate unspliced tRNA at the non-permissive temperature (Shiokawa and Pogo 1974; Hopper, Banks and Evangelidis 1978). Thus, *tpd1* strains could accumulate unspliced precursor because the tRNAs are not exported from the nucleus and, therefore, to not see the splicing endonuclease. Alternatively, the mutation could alter the structural framework in which the splicing apparatus/tRNA transport system is embedded. This altered context could result

in reduced efficiency of the splicing apparatus *in vivo*.

Since the primary lesion in *tpd1* strains has not been pinpointed, we do not know if the lethality of the mutants results specifically from the block in tRNA splicing. This issue is highlighted by the fact that *los1* strains yield similar kinetics of accumulation of IVS-containing tRNAs as do *tpd1-1* strains and yet *LOS1* is not an essential gene (HURT *et al.* 1987). Thus, either the splicing deficiency is more severe in *tpd1* strains than in *los1* strains, an hypothesis supported by the data presented in Figure 5, and this difference is critical with respect to cell viability, or lethality and tRNA precursor accumulation are separate consequences of the primary lesion in *tpd1* strains. Further analysis should clarify this point.

TPD1 falls into a class of genes, including *RNA1* and *LOS1*, that are required for efficient tRNA splicing but are not necessarily defective in the endonuclease or ligase specifically. In addition, since at least two separate genes—*SEN1* and *SEN2*—are all required to yield full tRNA endonuclease activity *in vitro*, some of them may be required in the reaction in some other capacity than actual endonucleolytic cleavage. Thus, the genetics of tRNA maturation has begun to define a more complex processing pathway for production of eukaryotic tRNA—and specifically for tRNA splicing—than might have been anticipated from previous studies on prokaryotic tRNA maturation or from the biochemical analysis of tRNA splicing.

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