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Yeast tRNA Ligase Mutants Are Nonviable and Accumulate tRNA Splicing Intermediates*

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We show here that yeast tRNA ligase protein is essential in the cell and participates in joining together tRNA half-molecules resulting from excision of the intron by the splicing endonuclease. A haploid yeast strain carrying a chromosomal deletion of the ligase gene is viable only if ligase protein can be supplied from a plasmid copy of the gene. When synthesis of the plasmid-borne ligase gene is repressed, cells eventually die and accumulate endonuclease cut but unligated half-molecules and intervening sequences. Half-molecules that accumulate appear to be fully end-processed. Two temperature-sensitive ligase mutant strains have been isolated; these strains accumulate a similar set of unligated half-molecules at the nonpermissive temperature.

Intron-containing tRNAs are ubiquitous in eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, in which splicing is best understood, about one-tenth of the 360 tRNA genes contain intervening sequences (see Culbertson and Winey (1989) for review). Although only a portion of the tRNA genes in this yeast contain introns, tRNA splicing is essential. When introns occur in a tRNA gene in yeast, they occur in all members of the corresponding isoaccepting tRNA gene family. Ten such intron-containing tRNA gene families have been identified (see Ogden *et al.* (1984) and Stucka and Feldmann (1988)); thus, splicing is required to generate functional tRNAs to decode each of the respective codons. In other eukaryotes, fewer intron-containing tRNAs have been discovered; however, since all known human and other eukaryotic $tRNA^{Tyr}$ genes contain introns (van Tol and Beier, 1988), it seems likely that splicing is essential in all eukaryotes. *In vitro* results indicate that tRNA splicing in *S. cerevisiae* requires three enzymes: an endonuclease to excise the intron, a ligase to join together the resultant half-molecules, and a dephosphorylating enzyme to remove the 2′-phosphate left at the splice junction by the ligase protein (Fig. 1).

Splicing is initiated by recognition of the pre-tRNA by endonuclease. The hallmark of any nuclear-derived tRNA intron is its position in space one base 3′ of the anticodon (see Ogden *et al.*, 1984). It is this location of the intron and its lack of perturbation of the structure of the mature domain of the tRNA that dictate the initial step in splicing (Swerdlow and Guthrie, 1984; Lee and Knapp, 1985; Szekely *et al.*, 1988). The splicing endonuclease recognizes some structure common to the mature domain of all the pre-tRNAs (Peebles *et al.*, 1983) and excises the intron by making two precisely measured endonucleolytic cuts in the precursor molecule (Greer *et al.*, 1987; Reyes and Abelson, 1988). The ends formed by endonuclease bear a 5′-OH group and a 2′-3′-cyclic phosphate (Peebles *et al.*, 1983). A similar endonuclease is found in wheat germ (Stange *et al.*, 1988), *Xenopus laevis* (Gandini-Attardi *et al.*, 1985; Mattoccia *et al.*, 1988), and humans (Filipowicz and Shatkin, 1983; Laski *et al.*, 1983). Genetic evidence suggests that this endonuclease is responsible for splicing *in vivo* (Ho *et al.*, 1990).

Two different ligases have been implicated in tRNA splicing in different organisms. In yeast, ligation of the half-molecules has been attributed to tRNA ligase protein (Greer *et al.*, 1983). This 95-kDa protein is 827 amino acids long (Westaway *et al.*, 1988) and has three distinct activities required for the reaction (Greer *et al.*, 1983; Phizicky *et al.*, 1986): a cyclic phosphodiesterase at the C terminus to open the cyclic phosphate to a 2′-phosphate; a kinase, probably located in the middle of the polypeptide, to phosphorylate the 3′-half-molecule; and an adenylylation domain at the N terminus which is used to ligate the half-molecules after first activating the 3′-half-molecule (Xu *et al.*, 1990; Apostol *et al.*, 1991). A similar ligase has been found in wheat germ (Konarska *et al.*, 1981; Pick *et al.*, 1986; Pick and Hurwitz, 1986), *Chlamydomonas* (Tyc *et al.*, 1983), and humans (Zillman *et al.*, 1991). Ligation by the yeast-like tRNA ligase results in a mature sized tRNA molecule bearing a 2′-phosphate at the splice junction; this phosphate can be efficiently removed *in vitro* in yeast extracts (McCraith and Phizicky, 1990, 1991) and in HeLa extracts (Zillman *et al.*, 1991) by an NAD-dependent 2′-phosphate-specific dephosphorylating enzyme. Another ligase, which ligates tRNA half-molecules without formation of a 2′-phosphate has been found in HeLa extracts (Laski *et al.*, 1983; Filipowicz and Shatkin, 1983), and the *Xenopus* version of this HeLa-type RNA ligase has been implicated *in vivo* in tRNA splicing by microinjection experiments (Nishikura and DeRobertis, 1981).

In this report we provide genetic evidence demonstrating that active functional tRNA ligase protein is critical for tRNA splicing in the yeast cell. Ligase is an essential protein in the cell. Consistent with its biochemical activity in splicing pre-tRNA molecules in yeast, lack of ligase protein in the cell leads to accumulation of endonuclease cut but unjoined tRNA half-molecules. These half-molecules are fully processed at both the 5′ and 3′ ends. Surprisingly, excised introns also accumulate in the cell. Temperature-sensitive ligase mutant strains accumulate a similar set of unligated half-molecules under nonpermissive conditions.

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MATERIALS AND METHODS

Strains—Yeast haploid strains SS328 (*MATα his3-Δ200 lys2-801^a ade2-101^o ura3-52 GAL$^+$ suc2*) and SS330 (*MATa his3-Δ200 tyr1 ade2-101^o ura3-52 GAL$^+$ suc2*) were obtained from S. Scherer (Department of Microbiology, University of Minnesota, Minneapolis, MN), and the corresponding diploid is strain EMPY20. Strain T481 is SS328 *HIS3*$^+$ and was made by transformation of SS328 with *HIS3* DNA. Strain EMPY 370-12 is EMPY20 heterozygous for a *Kpn*I chromosomal deletion of the ligase gene. It was constructed by transforming EMPY20 with the *Sac*II-digested integrating plasmid p298-1, purification of the Ura$^+$ prototrophs, selection for Ura$^-$ segregants on 5-fluoroorotic acid (Boeke *et al.*, 1984), and then Southern blotting to find a segregant with the *Kpn*I deletion left in the chromosome. The *Kpn*I-deleted ligase gene has the potential for encoding a protein of 128 amino acids, encompassing the first 123 of the 827 amino acids of the ligase protein, followed by the amino acids Arg-Asp-Pro-Glu-Arg and two translation termination signals (Westaway *et al.*, 1988). Strain EMPY439 (*MATα tyr1 ade2-101^o ura3-52 rlg1-ΔKpn*I GAL$^+$ *suc2* (pBM150-RLGX), which has its one tRNA ligase gene on a plasmid under control of the *GAL10* promoter, is a haploid segregant of EMPY370-12 obtained after transformation with the plasmid and sporulation of the transformant. The starting strain for ligase mutant screening is strain EMPY488 (*MATa his3-Δ200 ura3-52 trp1-Δ901 leu2-3, 112 tyr1 ade2-101^o rlg1-ΔKpn*I (pY11)). This strain was obtained by transforming EMPY370-12 with pY11 and crossing an appropriate haploid segregant EMPY411-2a (*MATα ade2-101^o his3-Δ200 tyr1 rlg1-ΔKpn*I GAL$^+$ (pY11)) with strain SEY6211 obtained from S. Emr (Department of Biology, California Institute of Technology, Pasadena, CA) (*MATa leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101^o suc2-Δ9 mel$^-$ GAL$^+$*). After sporulation, haploids were screened for relevant markers, inability to survive selection with 5-fluoroorotic acid, and healthy growth at 37 °C. Strain EE16 [*MATa ura3-52 tyr1* (*ade2-1* or *ade1*) *rna1*] was obtained from A. Hopper (Department of Biological Chemistry, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA).

Plasmids—Plasmid p298-1 is an integrating plasmid (YIP5) that contains an altered ligase gene which is lacking a 1307-base pair *Kpn*I fragment within the coding region (see Fig. 2). The *Kpn*I fragment was removed from a 6.4-kilobase pair genomic *Xba*I fragment spanning the ligase gene (Westaway *et al.*, 1988), and the resulting shortened *Xba*I fragment was inserted into the *Nhe*I site of YIP5 to create the plasmid. The unique *Sac*II site of p298-1 is located immediately after the end of the ligase coding region, 3′ of the *Kpn*I deletion.

Plasmid pSEYC58 contains *CENIV*, *ARS1*, a *URA3*$^+$ marker, and the pUC8 multi-cloning site and was constructed by S. Emr. pY11 is pSEYC58 containing the *Eco*RI fragment spanning the ligase gene. pY436-RLG1 is a centromere-containing plasmid bearing a *LEU2*$^+$ marker and a *Xho*I-*Xba*I fragment spanning the ligase gene. Plasmid pBM150-RLGX is a derivative of pBM150 (Johnston and Davis, 1984) and contains *CENIV*, *ARS1*, *URA3*$^+$, and the tRNA ligase gene under control of the *GAL10* promoter (Phizicky *et al.*, 1986).

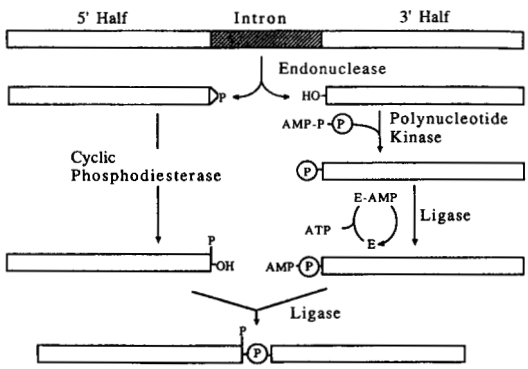

FIG. 1. **The mechanism of tRNA ligation in yeast.** tRNA splicing *in vitro* requires the tRNA ligase protein, which catalyzes three separate steps to join together tRNA half-molecules generated by the splicing endonuclease.

FIG. 2. **A partial restriction map of the region around the ligase gene.** The *arrow* indicates the tRNA ligase coding region, which begins just before the *Hpa*I site and ends just before the *Sac*II site. The distance between *two vertical marks* is 1 kilobase pair.. Restriction sites used in this report are indicated as follows: *X*, *Xba*I; *Xh*, *Xho*I; *R*, *Eco*RI; *H*, *Hpa*I; *K*, *Kpn*I; *S*, *Sac*II. Two other *Eco*RI sites further 5′ to the ligase gene are not indicated.

rlg1^{ts} Strains—Temperature-sensitive ligase mutant strains were obtained by the plasmid shuffle method (Budd and Campbell, 1987). Plasmid pY436-RLG1, after random mutagenesis *in vitro*, was transformed into strain EMPY488, and *LEU2*$^+$ transformants were transferred to plates containing 5-fluoroorotic acid to remove the original resident ligase gene on the *URA3*$^+$ plasmid (Boeke *et al.*, 1984). Ura$^-$ segregants were purified and screened for temperature sensitivity. Candidate temperature-sensitive mutants were further screened for plasmid-borne temperature sensitivity by crossing them with a tester strain carrying the ligase chromosomal deletion and a *GAL10*-controlled ligase gene. The resulting diploids (relevant genotype: *rlg1-ΔKpn*I/*rlg1-ΔKpn*I (py436-RLG1)/(pBM150-RLGX)) were tested for temperature resistance when ligase was expressed from the *GAL10* promoter on the plasmid and for temperature sensitivity after 5-fluoroorotic acid-mediated loss of the *GAL10*-controlled ligase gene and its plasmid. In this way four candidate temperature-sensitive ligase mutant strains were obtained. The ligase DNA from two of these plasmids resulted in a temperature-sensitive phenotype when inserted into the yeast chromosome to replace the wild type gene. This was done by extracting the plasmid bearing the ligase mutation, transferring the mutated *Hpa*I-*Sac*II ligase fragment to plasmid p298-1, digesting the resulting plasmid with *Sac*II and transforming the DNA into strain T481, and screening 5-fluoroorotic acid selected Ura$^-$ segregants for temperature sensitivity. In this way T404 (T481 *rlg1-4*) was obtained from hydroxylamine-mutagenized DNA and T410 (T481 *rlg1-10*) was obtained from formic acid-mutagenized DNA. DNA sequencing indicates that the ligase lesion in T410 is a T → C transition at nucleotide 1059 of the original *Eco*RI fragment of the ligase clone (Westaway *et al.*, 1988), resulting in a Phe → Ser mutation at amino acid 170 of the protein. Similarly, one of the lesions in T404 is a C → T transition at position 1089, corresponding to a Thr → Ile mutation at amino acid 180 of the protein. T404 may contain another lesion further downstream.

Mutagenesis and Transformation—Plasmid DNA was mutagenized either with 1 M hydroxylamine for 45 min at 75 °C (Busby, 1982) or with formic acid for 30 min (Myers *et al.*, 1985), followed by three successive ethanol precipitations with 0.5 M Tris-HCl, pH 8, and two volumes of ethanol. These mutagenic doses were chosen to yield mutations within the ligase coding region at about 1-2 sites/molecule. DNA transformations into yeast were performed as described (Ito *et al.*, 1983).

Labeling Cells—Yeast strains were labeled with $^{32}P_i$ as described (Ogden *et al.*, 1979). Strain EMPY439 was grown overnight in YP medium (1% yeast extract, 2% peptone), containing 2% ethanol, 3% glycerol and 2% glucose-free galactose. Then cells were harvested, washed, resuspended in either the same medium or in YP medium containing 2% glucose, and grown at 30 °C. Prior to labeling, aliquots were harvested, washed, resuspended at 2×10^7 cells/ml in low phosphate medium with the appropriate carbon source, adapted to the low phosphate medium for one generation, and labeled with $^{32}P_i$ for 30 min.

Extraction, Purification, and Analysis of RNA—Labeled RNA was extracted from cells with hot phenol as described (Knapp *et al.*, 1978), and tRNA processing intermediates were displayed on two-dimensional polyacrylamide gels essentially as described by Ogden *et al.*, (1984). The first dimension consisted of electrophoresis through 10% polyacrylamide (30:1), 4 M urea in TBE (90 mM Tris-borate, 1 mM EDTA, pH 8.3) until the bromphenol blue dye front migrated 25 cm. The second dimension consisted of electrophoresis in 20% polyacrylamide, until the xylene cyanol marker was just beginning to elute. Electrophoresis in both dimensions was run so that the plates remained cool to the touch. Samples treated with RNase T1 (5 units at 37 °C for 45 min in 10 mM Tris-HCl, 1 mM EDTA, pH 8) were analyzed on 20% acrylamide (30:1), 7 M urea TBE gels. Calf intestinal phosphatase treatment was with 1 unit for 30 min at 37 °C, followed by addition of 10 μg of carrier RNA, heating at 65 °C for 30 min, phenol extraction, and ethanol precipitation of the samples.

RESULTS

tRNA Ligase Is an Essential Protein in the Cell—To determine if the ligase gene is essential in the cell, we first con-

structed a diploid strain deleted for most, but not all, of one copy of the chromosomal ligase gene. This was done by integration of a plasmid bearing both an internal *Kpn*I deletion of the ligase gene (see Fig. 2) and a *URA3*$^+$ marker at the ligase locus of a diploid, followed by selection against the *URA3*$^+$ marker with 5-fluoroorotic acid (Boeke *et al.*, 1984). Some portion of the Ura$^-$ segregants were expected to leave the deletion in the chromosome. DNA from 14 independent Ura$^-$ segregants was analyzed, and 6 proved to contain one wild type copy of the ligase gene and one deleted copy of the gene (data not shown). One of these diploids heterozygous for the ligase deletion (EMPY370–12, relevant genotype: *RLG1*$^+$/*rlg1*-Δ*Kpn*I *GAL*$^+$/*GAL*$^+$) was studied further.

We proved that the ligase gene and its protein was essential in yeast by sporulation and tetrad analysis of EMPY370–12 and related derivatives of this strain, as follows. First, as expected of a diploid strain bearing a heterozygous deletion of an essential gene, EMPY370–12 segregated 2 live and 2 dead spores in each of 15 tetrads, whereas the control diploid (*RLG1*$^+$/*RLG1*$^+$) segregated 4 live spores in each of a corresponding number of tetrads (data not shown). Second, spores containing the deletion could be recovered if an exogenous source of the ligase gene was present. EMPY370–12 was transformed either with a centromere plasmid (pSEYC58) or with the same plasmid carrying in addition the ligase gene (pY11), and then sporulated, dissected, and analyzed. As expected, diploids bearing the centromere plasmid vector (still of genotype *RLG1*$^+$/*rlg1*-Δ*Kpn*I) continued to segregate 2 live and 2 dead spores in each of 6 tetrads. However, diploids bearing the ligase gene-containing plasmid could segregate more than 2live spores/tetrad. From 17 such tetrads analyzed, 7 contained 3 live spores and 2 contained 4 live spores. Moreover, in each of these tetrads no more than 2 spores could grow on medium containing 5-fluoroorotic acid, which selects against the *URA3*$^+$ plasmid bearing the ligase gene. This is the expected result if spores containing the chromosomal ligase deletion can live only when an exogenous source of the ligase gene is present. Thus the ligase gene is necessary either for spore viability or for vegetative growth.

To prove that presence of functional ligase protein is essential for vegetative growth, the same strain (EMPY370–12) was transformed with a *URA3*$^+$ centromere plasmid containing the ligase gene under control of the *GAL10* promoter (Phizicky *et al.*, 1986), sporulated, and then dissected on medium containing galactose. From 4 tetrads analyzed, we obtained 1 single spore tetrad, 1 two spore tetrad, and 2 three spore tetrads. Each of the 3 spore tetrads contained a spore that died when placed on medium containing glucose but not on medium containing galactose. These two strains therefore contained a chromosomal ligase deletion and the *GAL10*-controlled ligase gene on the plasmid. In these strains, functional ligase could only be obtained when the *GAL10* promoter was active on galactose medium; on glucose-containing medium ligase could not be made and the cells died. Therefore, ligase protein is necessary for vegetative growth.

One of the haploids bearing the ligase deletion and the *GAL10*-controlled ligase gene (EMPY439) was chosen for further study. EMPY439 takes several generations after the shift from galactose-containing medium to glucose-containing medium to manifest the lack of ligase protein by a decrease in growth rate. After an initial lag when transferred to glucose-containing medium, cells resume logarithmic growth for between six and seven generations before absorbance measurements show a slowing of growth. After this point the cultures increase in absorbance values at a slower and slower rate. After 24 h in glucose-containing medium the estimated doubling time (as measured by absorbance) is about 16 h. At this point, all of the cells are abnormal in the microscope; they have either multiple buds or long protrubances.

Cells Repressed for Ligase Accumulate Small RNAs—We have examined in more detail the fate of tRNA processing in cells deprived of ligase protein. If tRNA ligase protein is involved in tRNA splicing *in vivo* as depicted in Fig. 1, then the expected result of such an experiment is an accumulation of endonucleolytically cut but unligated tRNA half-molecules. Such tRNA half-molecules may, however, be rapidly degraded *in vivo*. Alternatively, tRNA ligase protein might be more intimately involved in the tRNA splicing machinery, and lack of the protein might prevent the initiation of splicing by endonuclease; if so, unspliced precursor tRNAs may accumulate.

To examine the consequences of a lack of ligase protein, cells with ligase under *GAL10* control were grown to mid-log phase in galactose, shifted to fresh medium, and labeled at appropriate times with $^{32}P_i$. Then extracted RNAs were displayed on two-dimensional polyacrylamide gels, which separate small molecules due to secondary structure. The results of this experiment are shown in Fig. 3. Inspection of the gels reveals two results. First, a number of small oligonucleotides accumulate during ligase-repression (Fig. 3, *upper panels*). These molecules are first evident four generations after the shift to glucose-containing medium while the cells are still in logarithmic phase, and much more pronounced two generations later, when the cells are beginning to come out of log phase. The oligonucleotides appear in a region of the gel that corresponds to molecules substantially smaller than mature tRNA. One likely explanation of their identity was that they

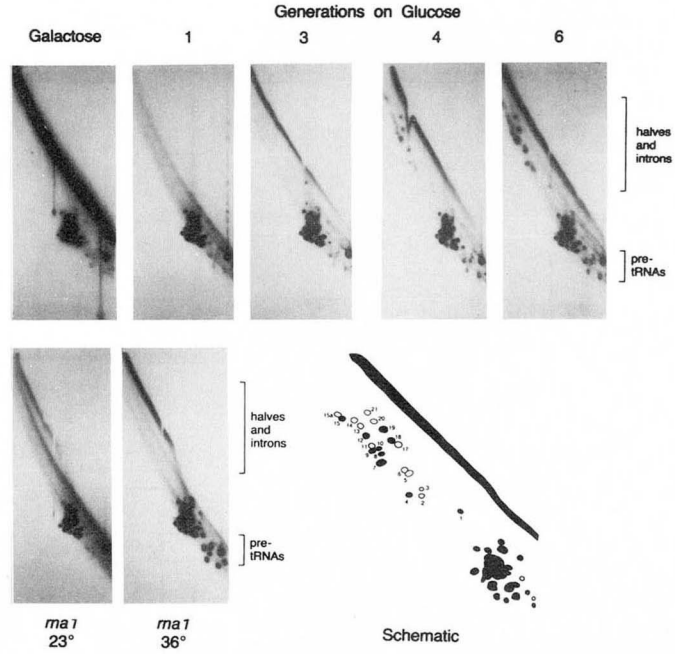

FIG. 3. **Cells lacking tRNA ligase protein accumulate small oligonucleotides.** *Top panels* and *schematic*, strain EMPY439 (relevant genotype: *rlg1*-Δ*Kpn*I pBM150-RLGX) was grown overnight in galactose-containing medium, transferred to fresh medium containing either galactose or glucose, and labeled with $^{32}P_i$ after the number of generations indicated. RNAs were extracted and displayed on two-dimensional polyacrylamide gels as described under "Materials and Methods." First dimension *right* to *left*, second dimension toward the *top*. *Bottom panels*, strain EE16 (relevant genotype: *rna1*) was grown in YPD at 23 °C, labeled with $^{32}P_i$, and grown either at 23 or 37 °C for 30 min to incorporate the label. RNAs were extracted and analyzed on two-dimensional polyacrylamide gels as above.

were unligated half-molecules. This would explain their size and their number. We document below that some of these are indeed unligated half-molecules, and that others are intervening sequences. Second, there is only a marginal increase in the amount of unspliced precursor tRNA (that has not yet reacted with endonuclease) that accumulates during the labeling of ligase-repressed cells (*upper panels*); quantitation of the results shows that compared to the amount of 5 S RNA (which varied about 20%), the amount of unspliced pre-tRNAs increased only about 2-fold during the glucose repression of ligase synthesis. By contrast *rna1* cells accumulate little if any of the small RNAs and substantial amounts of unspliced pre-tRNAs when incubated at high temperature (*lower panels*).

The Small RNAs Are Endonucleolytically Cut but Unligated tRNA Half-molecules and Intervening Sequences—To identify the oligonucleotides that accumulate in ligase-repressed cells, the RNAs were compared with endonuclease-treated precursor tRNAs which accumulate in *rna1* cells at high temperature (Hopper *et al.*, 1978; Ogden *et al.*, 1984). The results of this experiment, from which two conclusions can be drawn, are shown in Fig. 4. First, all of the oligonucleotides are of similar size as fragments produced by endonuclease treatment of tRNA precursors. Second, some of the oligonucleotides (oligonucleotides 1–6) are of the same size as particularly diagnostic fragments. For example, oligonucleotide 1 is the same size as the largest endonuclease product of $tRNA^{Ile}$ cleavage (the intron, of length 60 nucleotides), and oligonucleotide #2 corresponds to the large 3′ half-molecule of $tRNA^{Ser}$. Similarly, oligonucleotides 4–6 correspond either to the 3′-half-molecule of $tRNA^{Leu}_{CAA}$ or to that of $tRNA^{Leu}_{UAG}$.

To confirm these preliminary assignments, the RNase T1 digestion patterns of oligonucleotides 1, 4, 5, and 6 were compared with those generated from *rna1*-derived precursor tRNAs after endonuclease treatment. The results are shown in Fig. 5*A*. The RNase T1 cleavage pattern of oligonucleotide 1 is virtually identical to that of the intron of pre-$tRNA^{Ile}$ (compare *lanes l* and *n*); it is likely therefore that oligonucleotide 1 is the $tRNA^{Ile}$ intron.

Oligonucleotide 4 is likely the 5′-phosphorylated 3′-half-molecule of $tRNA^{Leu}_{UAG}$. Its RNase T1 cleavage pattern is nearly identical to that of the 3′-half-molecule from pre-$tRNA^{Leu}_{UAG}$; one band, however, migrates faster in the oligonucleotide 4 digest (compare *lanes e* and *g*). Since this discrepancy disappears if oligonucleotide 4 is first treated with calf intestinal phosphatase (*lane f*), we conclude that oligonucleotide 4 is a phosphorylated version of the 3′-half-molecule of $tRNA^{Leu}_{UAG}$. This phosphorylation is likely at the 5′-position of the half-molecule since the size of the band that shifts after phosphatase treatment corresponds to a 5-mer (predicted for the 5′ end) rather than to the 9-mer predicted for the 3′ end (see Ogden *et al.* (1984) for sequence). Oligonucleotide 4 also likely has the mature CCA 3′-end of 3′-half-molecules. The unspliced pre-tRNAs that accumulate in *rna1* cells have mature 5′ and 3′ ends containing CCA (Knapp *et al.*, 1978) (see Ogden *et al.* (1984)). The predicted RNase T1 cleavage pattern of the $tRNA^{Leu}_{UAG}$ 3′-half-molecule should have a 10-mer, two 9-mers (one of which corresponds to the 3′ CCA end), and no other bands larger than a 5-mer. This predicted pattern closely resembles the pattern visualized in Fig. 5*A* (*lane g*). The two 9-mers resolve well presumably because the 3′-terminal 9-mer is not phosphorylated at its end. Since the RNase T1 cleavage pattern is identical in this region for oligonucleotide 4 (Fig. 5*A*, *lane f*), we conclude that it also carries CCA. A similar set of arguments indicates that oligonucleotide 5 corresponds to the 3′-half-molecule of $tRNA^{Leu}_{CAA}$ phosphorylated at its 5′ end; the faster mobility of one band in oligonucleotide 5 disappears after pretreatment of the

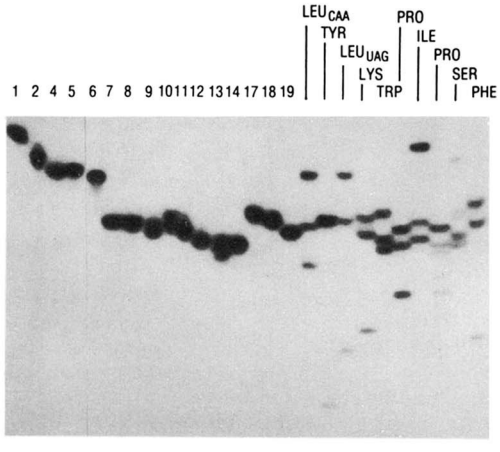

FIG. 4. **Small molecules that accumulate in cells lacking ligase protein are the same size as unligated tRNA half-molecules and introns.** Small molecules, numbered as shown in the *schematic* in Fig. 3, were eluted from two-dimensional gels and displayed on a sequencing length 10% polyacrylamide, 7 M urea gel. For comparison, unspliced pre-tRNAs that accumulate in *rna1* cells incubated at 36 °C were eluted from two-dimensional gels, treated with splicing endonuclease, and displayed on gels, as indicated.

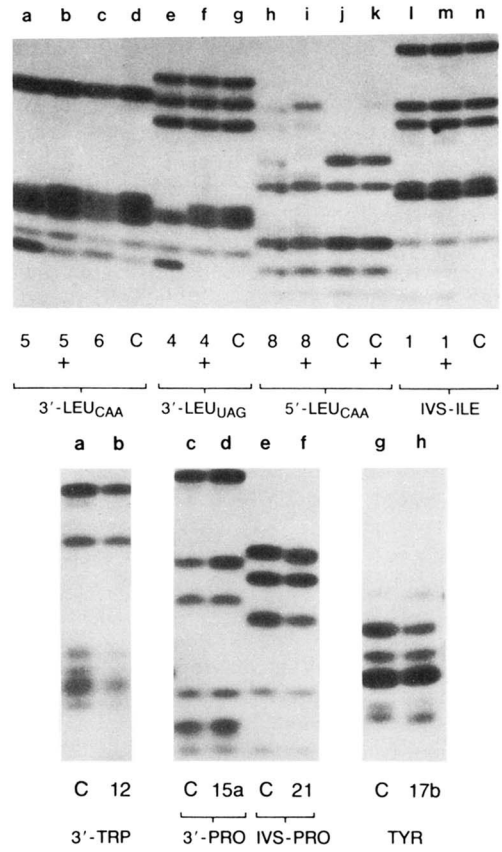

FIG. 5. **RNase T1 digestion patterns of oligonucleotides from cells lacking tRNA ligase are similar to those of tRNA half-molecules and introns.** Oligonucleotides that accumulate in cells lacking ligase protein, as indicated by the *numbers* at *bottom*, were digested with RNase T1 and displayed on a 20% polyacrylamide, 7 M urea gel. Where indicated by a + sign, samples were first treated with calf intestinal phosphatase, and the phosphatase was inactivated before treatment with RNase T1. Samples labeled *C* are the RNase T1 digestion patterns of the corresponding half-molecules or introns derived from *rna1* cells.

oligonucleotide with phosphatase (compare *lanes a* and *b* with *d*).

Oligonucleotide 8 is likely the 5′-half-molecule of $tRNA^{Leu}_{CAA}$, bearing either a terminal 2′- or 3′-phosphate. Its RNase T1 cleavage pattern only differs from that of the corresponding half-molecule derived from *rna1* cells in that the largest band is significantly less intense in the oligonucleotide 8 digest (compare *lanes h* and *j*). The largest band predicted for this half-molecule is a 3′-terminal 7-mer oligonucleotide which, in the case of pre-$tRNA^{Leu}_{CAA}$ treated with endonuclease, bears a 2′-3′-cyclic phosphate (Peebles *et al.*, 1983). Consistent with this (compare *lanes j* and *k* of Fig. 5*A*), the mobility of this largest band does not shift if the sample is pretreated with calf intestinal phosphatase, which does not open cyclic phosphates (Peebles *et al.*, 1983). However, the corresponding and fainter band of oligonucleotide 8 is sensitive to prior treatment with phosphatase and results in a band of reduced mobility after the subsequent RNase T1 treatment. We conclude that oligonucleotide 8 bears either a 2′- or a 3′-phosphate at its end.

The results of some of the other RNase T1 nuclease digestions are shown in the gallery in Fig. 5*B*. It is clear from the comparisons that RNase T1 patterns very similar to those generated from the $tRNA^{Trp}$ 3′-half-molecule, $tRNA^{Pro}$ 3′-half-molecule, $tRNA^{Pro}$ intron, and one of the $tRNA^{Tyr}$ half-molecules are associated with specific oligonucleotides. The oligonucleotides corresponding to the 3′-half-molecules of $tRNA^{Trp}$ and $tRNA^{Pro}$ also bear CCA on their ends, by the same reasoning we used for that of $tRNA^{Leu}_{UAG}$ above. Other experiments indicate that oligonucleotide 7, the most prominent oligonucleotide that accumulates when ligase synthesis is repressed (Fig. 3), is a poorly resolved mixture of the $tRNA^{Lys}$ 3′ half-molecule and the other $tRNA^{Tyr}$ half-molecule (data not shown).

Temperature-sensitive Ligase Mutants Accumulate a Similar Set of Molecules—We have isolated two temperature-sensitive ligase mutant strains, described under "Materials and Methods." These strains have a similar phenotype to that described above for a ligase-repressed strain. First, they display a similar odd cell morphology with long protrubances when incubated at high temperature in liquid. Second, they take several generations to die on plates incubated at high temperature; usually the plates have to be re-replica plated after 2 days of growth at high temperature before they stop growing. Third, they accumulate a similar set of half-molecules within half a generation of growth in liquid at nonpermissive temperature. This is shown in Fig. 6. The parent strain shows no evidence of any half-molecules at this temperature, and neither the temperature-sensitive mutants nor the wild type parent yields significant amounts of half-molecules after growth at permissive temperature (data not shown).

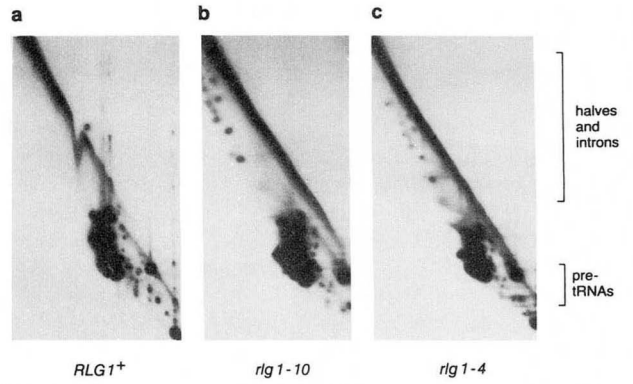

FIG. 6. **Temperature-sensitive ligase mutants accumulate a similar set of oligonucleotides as cells lacking ligase.** Strain EMPY481 (*RLG1*$^+$) and its *rlg1–10* or *rlg1–4* derivatives were grown in YPD medium at 23 °C, labeled with $^{32}P_i$, and shifted to nonpermissive temperature for 30 min. RNAs were extracted and displayed on two-dimensional polyacrylamide gels as described.

DISCUSSION

We have shown that tRNA ligase protein is essential in yeast and presented evidence that this protein is responsible, as expected, for the ligation step of tRNA splicing *in vivo*. Lack of tRNA ligase protein leads to an accumulation of endonucleolytically cut but unligated tRNA half-molecules and intervening sequences which, like those from *rna1* cells, are fully mature at the ultimate 5′ and 3′ ends of the tRNA. The evidence that these are tRNA half-molecules rests both on the similarity in size (Fig. 4) and on the virtual identity of the RNase T1 cleavage patterns (Fig. 5), when compared with those from *rna1*-derived pre-tRNAs cut with endonuclease. The similarity of the RNase T1 cleavage patterns represents more than just placement of the corresponding G residues, since bands of the same predicted size consistently migrate to slightly different positions if they are derived from different precursors. In this way at least one half-molecule or intron from 8 of the 10 pre-tRNAs have been identified in cells repressed for ligase synthesis. These results are consistent with the biochemical role ascribed to tRNA ligase in ligating all of these precursors (Greer *et al.*, 1983; Phizicky *et al.*, 1986). Since mature tRNAs are themselves essential for viability, the likely cause of death in cells lacking ligase protein is the lack of tRNAs. It remains possible, however, that other RNAs, small or otherwise, also accumulate in cells deprived of ligase protein and that the absence of their ligation is the primary cause of cell death. We note in this connection that we do not know the identity of all the oligonucleotides shown in Fig. 3.

It is puzzling that the 3′-half-molecules we have identified are all substantially phosphorylated at their 5′ terminus. One would have thought that the efficient polynucleotide kinase activity of the ligase protein (Greer *et al.*, 1983; Phizicky *et al.*, 1986; Xu *et al.*, 1990; Apostol *et al.*, 1991), was responsible for ligation in the cell. In the case of the 3′-half-molecule of $tRNA^{Leu}_{CAA}$, at least 62% is phosphorylated at its 5′ terminus. This is not likely a consequence of massive preferential degradation of the unphosphorylated 3′-half-molecule since the total number of moles of the 3′-half-molecule recovered in this experiment is 86% of the number of moles of 5′-half-molecules recovered. Perhaps as the ligase protein is diluted out of the cell after shift to ligase-repressing medium, the ligase protein is proteolytically degraded, leaving an active kinase domain, but no ligase domain. Alternatively, there may be another protein in the cell that is capable of phosphorylating the 3′-half-molecules that accumulate. In this latter case the kinase activity of the ligase protein may prove to be dispensable. Similarly, the one 5′-half-molecule we have unequivocally identified, that of $tRNA^{Leu}_{CAA}$, does not contain the 2′-3′-cyclic phosphate at its end; instead it bears a monophosphate at either the 2′ or 3′ positions. This may be a result of hydrolysis during the hot phenol extraction of cellular RNAs, survival of the cyclic phosphodiesterase domain of the ligase protein, or of a compensating activity present in yeast extracts.

It was a surprise to see that intervening sequences accumulate in strains that lack ligase protein, with roughly the same time course as the 5′- and 3′-half-molecules. This is most clearly seen for the $tRNA^{Ile}$ intron (oligonucleotide 1 of

Figs. 3 and 5*A*), although the $tRNA^{Pro}$ intron also accumulates (Fig. 5*B*). Although excised introns have been detected under wild type growth conditions (O'Connor and Peebles, 1991), they do not accumulate to this extent until the cell lacks ligase protein. Presumably, the excised introns are degraded soon after being excised. This result might suggest that there is a saturable degradation system in the cell, which is being swamped by the accumulation of large amounts of half-molecules. Alternatively, it is possible that the endonucleolytically cut tRNA precursor still has the intervening sequence lodged in place and that ligase is required to displace it from the precursor.

The effect of ligase conditional mutants on tRNA splicing is uniquely different from that of all other mutants known to affect tRNA splicing in the cell, in that tRNA half-molecules accumulate under nonpermissive conditions. There is no evidence in either type of ligase mutant strain characterized here of more than a nominal accumulation of unspliced precursor tRNAs. In all other tRNA splicing mutants splicing is blocked at the endonuclease step. The SEN2 protein is likely one of the subunits of the endonuclease protein (Ho *et al.*, 1990), and mutations in this gene, as expected, affect the endonuclease step *in vivo* and *in vitro* (Winey and Culbertson, 1988; Ho *et al.*, 1990). The other 6 genes whose products affect splicing include *LOS1* (Hurt *et al.*, 1987), *PTA1* (O'Connor, 1990; see Culbertson and Winey (1989)), *TPD1* (van Zyl *et al.*, 1989), *SEN1* (Winey and Culbertson, 1988), *STP1* (Wang and Hopper, 1988), and certain alleles of *RNA1* (Hopper *et al.*, 1978; Traglia *et al.*, 1989). Although none of these genes appear to encode an endonuclease subunit, all of them affect the endonuclease step *in vivo*.

Available evidence suggests that ligase is present in a functional splicing complex near the nuclear membrane. Ligase has been localized by indirect immune electron microscopy to a saturable, and therefore probably specific, site immediately adjacent to the inner nuclear membrane (Clark and Abelson, 1987). Since the splicing endonuclease is membrane-associated (Peebles *et al.*, 1983; Rauhut *et al.*, 1990), and since endonucleolytic cleavage and ligation are concerted *in vitro* (Greer, 1986), it seems likely that the two enzymes form a splicing complex in the cell (Greer, 1986). A role for ligase has also been postulated whereby ligase shuttles precursor tRNAs to the nuclear membrane for splicing. This model is based on the fact that ligase protein interacts with pre-tRNAs in the intron (Tanner *et al.*, 1988) and on the fact that a secondary region of tRNA ligase localization occurs in an annulus about 300 nm from the nuclear envelope (Clark and Abelson, 1987). Since both of the conditional ligase mutants described here do not accumulate significant levels of unspliced pre-tRNAs but do accumulate endonucleolytically cut half-molecules, we conclude that there is no mandatory requirement for ligase to shuttle the pre-tRNAs to the membrane.

It has been shown recently that human cells have two ligation pathways capable of fusing tRNA half-molecules (Zillman *et al.*, 1991). The classical HeLa cell ligase catalyzes fusion of tRNA half-molecules without formation of a splice junction 2′-phosphate; this protein represents the vast majority of ligation *in vitro* (Laski *et al.*, 1983; Filipowicz and Shatkin, 1983), and the corresponding *X. laevis* version of the HeLa ligase (Gandini-Attardi *et al.*, 1985) has been implicated in tRNA splicing *in vivo* by microinjection experiments (Nishikura and DeRobertis, 1981). The other human ligase is very similar in properties to the yeast tRNA ligase (Zillman *et al.*, 1991); it generates a mature tRNA bearing a 2′-phosphate, which is subsequently removed *in vitro* in an NAD-dependent reaction. The one known yeast tRNA ligase, which generates a splice junction 2′-phosphate, has been shown here to be responsible for a large portion (if not all) of the tRNA splicing in yeast cells. Whether or not there is a second RNA ligase in yeast cells is unknown.

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