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In Vivo Pre-tRNA Processing in *Saccharomyces cerevisiae*

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We have surveyed intron-containing RNAs of the yeast *Saccharomyces cerevisiae* by filter hybridization with pre-tRNA intron-specific oligonucleotide probes. We have classified various RNAs as pre-tRNAs, splicing intermediates, or excised intron products according to apparent size and structure. Linear, excised intron products were detected, and one example was isolated and sequenced directly. Additional probes designed to detect other precursor sequences were used to verify the identification of several intermediates. Pre-tRNA species with both 5′ leader and 3′ extension, with 3′ extension only, and with mature ends were distinguished. From these results, we conclude that the processing reactions used to remove the 5′ leader and 3′ extension from the transcript are ordered 5′ end trimming before 3′ end trimming. Splicing intermediates containing the 5′ exon plus the intron were detected. The splice site cleavage reactions are probably ordered 3′ splice site cleavage before 5′ splice site cleavage. Surprisingly, we also detected a splicing intermediate with the 5′ leader and a spliced product with both 5′ leader and 3′ extension. Evidently, splicing and end trimming are not ordered relative to each other, splicing occurring either before or after end trimming.

The biosynthesis of eucaryotic tRNAs requires several RNA processing reactions. These include trimming of both the 5′ leader and 3′ extension sequences of the primary transcript, addition of the -CCA_{OH} tail to form the mature acceptor end, nucleoside modifications, RNA splicing in some instances, and transport from the nucleus to the cytoplasm. Most of these RNA processing reactions have been studied to various levels of detail in vitro (reviewed in references 7 and 24). Much less is known of these reactions in vivo. Consequently, we have undertaken an examination of the in vivo pre-tRNA processing pathway in the yeast *Saccharomyces cerevisiae*.

At least 10 of the estimated 46 nuclear tRNA gene families in *S. cerevisiae* contain intervening sequences (21, 46, 58; for a recent compilation of *S. cerevisiae* tRNA sequences, see reference 44b). The intervening sequences have remarkable size and sequence similarity within a tRNA gene family yet have little similarity among the 10 different tRNA gene families (59). The intervening sequences range in size from 14 to 60 bp and are all located 1 bp 3′ of the anticodon sequence. The intervening sequences are transcribed into the primary transcript and removed by an RNA splicing mechanism different from group I, group II, or pre-mRNA splicing (11, 35, 50). We chose to study the in vivo processing pathway of these tRNA genes since their processing pathways would also include splicing events.

On the basis of *Xenopus* oocyte microinjection studies of cloned *S. cerevisiae* tRNA genes (8, 9, 42, 43) and in vitro experiments with *S. cerevisiae* and *Xenopus* partially purified and purified enzymatic activities (12, 29, 32, 45), a generally accepted pathway for pre-tRNA processing has been deduced. *S. cerevisiae* nuclear tRNA genes are transcribed by RNA polymerase III, yielding primary transcripts with 5′ leaders, 3′ extensions, and in appropriate instances, introns (reviewed in reference 17). The primary transcripts lack -CCA_{OH} tails and nucleoside modifications. Except for some nucleoside modifications, all of the processing reactions occur in the nucleus (43, 44). The first RNA processing reaction appears to be the endonucleolytic removal of the 5′ leader by RNase P (12, 37). The 3′ extension is subsequently removed by a different nuclease activity(s) (3, 12, 15). However, it is unclear whether the 3′ end trimming activity is an endonuclease, exonuclease, or combination. Pre-tRNAs with both 5′ leaders and 3′ extensions do not appear to be substrates for the *Xenopus* 3′ end trimming activity (3). Nucleotidyltransferase polymerizes the -CCA_{OH} tail onto end-trimmed pre-tRNAs, producing an end-mature form (ptRNA) (10). The end-mature ptRNA is thought to be the splicing substrate, and indeed the majority of in vitro pre-tRNA splicing studies have used end-trimmed or end-mature ptRNAs as substrates. These studies have demonstrated that *S. cerevisiae* ptRNA splicing occurs in three separable steps. The first step is the ATP-independent, endonucleolytic excision of the intron by pre-tRNA-splicing endonuclease (48, 49). In vitro, the ptRNA is cut first at the 3′ splice site and second at the 5′ splice site (53). The reaction generates 2′,3′ cyclic phosphates and 5′ hydroxyl groups. The *S. cerevisiae* pre-tRNA-splicing endonuclease behaves as an integral membrane protein and has recently been purified (48, 52a). In the second step, tRNA ligase forms a new phosphodiester bond between the two tRNA exons in an ATP-dependent reaction that also leaves a 2′ phosphate at the splice junction (19, 49). The 2′ phosphate is subsequently removed by what appears to be an NAD^+-dependent phosphatase (41, 41a). The end-mature, spliced tRNA is transported from the nucleus to cytoplasm either during or immediately after splicing (42, 43). This deduced order of processing reactions indicated that pre-tRNA processing occurred vectorially in that the product of one reaction was the sole substrate for the next reaction.

tRNA nucleoside modifications are added in a definite order by *Xenopus* oocytes (44). However, analysis of *S. cerevisiae* mutations that cause reduced modification of specific tRNA nucleotides has demonstrated that the order of nucleoside modification additions is not obligatory in vivo (26, 36, 39).

Comparatively little is known of these RNA processing reactions in vivo. Feldmann and co-workers observed at

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least 27 different RNA species from briefly radiolabeled *S. cerevisiae* cells; these RNAs contained RNase T_1 oligonucleotides characteristic of tRNAs (2, 14). These RNA species were of the appropriate size to be tRNA precursors and could be converted to tRNA-size molecules with *S. cerevisiae* extracts. More recently, filter hybridization analysis of *S. cerevisiae* total RNA detected two pre-tRNA SerCGA species of 104 and 120 bases and two pre-tRNA TyrGUA species of 92 and 108 bases (27). The 92- and 104-base species represent the end-mature, intron-containing ptRNAs, while the larger species were presumed to be primary transcripts.

Analysis of *S. cerevisiae* pre-tRNA processing mutants has so far given little insight into the in vivo pre-tRNA processing pathway. Exclusive of nucleoside modification mutations, 12 *S. cerevisiae* mutations or genes have been identified that affect the pre-tRNA processing pathway (*rna1-1* [25, 33], *los1* [28], *RLG1* [51], *sen1-1* and *sen2* [65], *STP1* [64], *tpd1-tpd5* [63], and *pta1-1* [44c]). For example, the *rna1-1* mutation results in the accumulation of end-mature but unspliced ptRNAs under restrictive conditions (25, 33). Of these mutations, only *sen2-3* causes an appreciable accumulation of an intron-containing species other than end mature ptRNA (23). The *SEN2* gene apparently encodes one of the pre-tRNA-splicing endonuclease subunits (22a). The *sen2-3* allele results in defective pre-tRNA-splicing endonuclease activity that causes inefficient cleavage of the 5′ splice site in vivo and in vitro. The so-called 2/3 intermediate that accumulates contains the 5′ exon and the intron. tRNA ligase is encoded by the *RLG1* gene; conditional loss of this activity results in the in vivo accumulation of unspliced exons (50a). Several other mutations result in the accumulation of end-mature but unspliced ptRNA yet display no obvious defect in the known processing activities (*rna1-1*, *los1*, and *pta1-1*), suggesting that this may be an important control point in the processing pathway.

We have surveyed the pre-tRNA intron-containing RNAs of *S. cerevisiae* by filter hybridization analysis of total RNA extracted from log-phase cells. By using differential oligonucleotide hybridization, size estimation, and primer extension analysis, structures were assigned to several of the pre-tRNA intron-containing species. Our results are consistent with an in vivo pre-tRNA processing pathway in which end processing is ordered 5′ end trimming before 3′ end trimming. The 3′ splice site appears to be preferentially cut first during splicing, and the intron is excised as a linear molecule. Unexpectedly, we have found that splicing may occur either before or after end trimming, at least for certain pre-tRNAs.

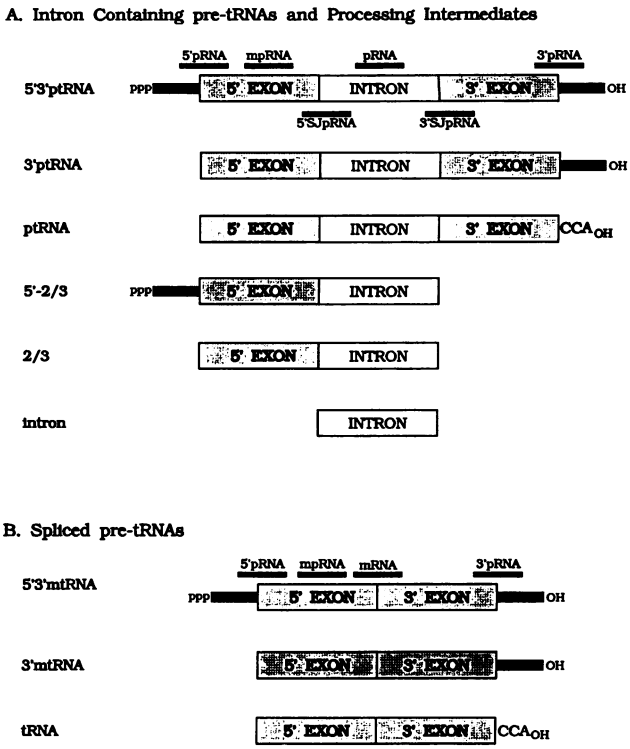

FIG. 1. Schematic representation of hybridization probes and pre-tRNA processing intermediates and products. Shown are diagrams for pre-tRNA intron-containing species (A) and spliced tRNA species (B). Oligonucleotide hybridization probes are represented by thin lines drawn above the pre-tRNA or tRNA regions to which each is complementary. The shorthand notation for each kind of probe is indicated; for different tRNA genes, the RNA is replaced with the amino acid (and anticodon where necessary) designation for that tRNA gene.

MATERIALS AND METHODS

Strains. *S. cerevisiae* A364A (*MATa ade1 ade2 gal1 his7 lys2 tyr1 ura1*), X2180-1A (*MATa gal2 mal mel CUP1 SUC2*), M25 (relevant genotype +/+), and M304 (relevant genotype *rna1-1/rna1-1*) were used for these experiments. Cultures were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Growth was monitored by optical density readings at 660 nm.

Pre-tRNA, tRNA, and probe nomenclature. Pre-tRNAs, tRNAs, and tRNA gene families are designated by their cognate amino acid and the tRNA anticodon sequence without base modifications, for instance, ptRNA SerCGA and tRNA SerGCU. Specific pre-tRNA species, splicing intermediates, and products are designated as shown in Fig. 1. Probes are designated also as shown in Fig. 1 except that "RNA" is substituted with the cognate amino acid three-letter code for the target tRNA in uppercase letters and with the anticodon sequence in lowercase letters where necessary. For example, the intron-specific probe for ptRNA ProUGG is designated pPRO, and the intron-specific probe for ptRNA SerCGA is designated pSERcga.

Nucleic acid preparation. Total RNA was isolated from *S. cerevisiae* cells grown to mid-log phase in YPD (optical density at 660 nm of 0.5 to 1.0) by phenol extraction. Cells were harvested by pouring the culture onto crushed ice (70 g of ice per 200 ml of culture), collected by centrifugation (2,000 × *g* for 5 to 10 min at 4°C), and then washed in ice-cold water. The cell pellet was resuspended on ice in 5 ml of RNA extraction buffer (0.1 M sodium acetate [pH 5.2], 1% sodium dodecyl sulfate [SDS], 20 mM EDTA, and 1% 2-mercaptoethanol added just before use) per 200 ml of original culture volume. The cell suspension was transferred on ice to another tube containing an equal volume of phenol (with 0.1% 8-hydroxyquinolone and equilibrated with 0.1 M sodium acetate [pH 5.2] and 1% 2-mercaptoethanol) and 1 g of acid-washed glass beads per ml of phenol. This cell suspension was vortexed vigorously, incubated at 65°C with intermittent vortexing for 10 min, and then placed on ice for 10 min. The aqueous and phenolic phases were separated by centrifugation (2,000 to 3,000 × *g* for 10 min at 4°C). The aqueous phase was carefully removed without disturbing the interface into another tube kept on ice. The aqueous phase

was then phenol extracted as described above except that no glass beads were used. The aqueous phase from the second phenol extraction was then extracted with chloroform (24 parts chloroform to 1 part isoamyl alcohol) as described above except that no glass beads were used. The aqueous phase from the last extraction was ethanol precipitated, and the RNA pellet was resuspended in 0.5 to 1.0 ml of diethyl pyrocarbonate-treated water. The RNA concentration and purity were determined spectrophotometrically. RNA preparations typically had A_{260}/A_{280} ratios between 1.9 and 2.0. RNA preparations with A_{260}/A_{280} ratios less than 1.8 were reextracted with phenol and chloroform until the A_{260}/A_{280} ratio was greater than 1.8.

S. cerevisiae genomic DNA was isolated as described by Cryer et al. (6). DNA was isolated from strain X2180-1A grown to saturation in YPD medium. DNA concentration was estimated by agarose gel electrophoresis and ethidium bromide staining intensity relative to known standards.

Oligonucleotide synthesis, purification, and labeling. Oligonucleotides were synthesized manually by using the phosphotriester chemistry (1) or made with an Applied Biosystems 380A automated DNA synthesizer. All oligonucleotides were purified by preparative polyacrylamide gel electrophoresis. Oligonucleotides were radiolabeled with T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, Ohio) and [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear, Boston, Mass.), using standard protocols (40). Unincorporated ^{32}P was removed by applying the reaction mix to a small DE52 column, with subsequent differential elution of nucleotides from oligonucleotide or by G25 spin column chromatography.

Polyacrylamide gel electrophoresis. Denaturing gels were either 10% polyacrylamide (29 parts acrylamide to 1 part bisacrylamide) and 8 M urea or 20% polyacrylamide (19 parts acrylamide to 1 part bisacrylamide) with 7 M urea. Partially denaturing gels were 7% polyacrylamide (29 parts acrylamide to 1 part bisacrylamide) and 4 M urea. Two-dimensional gels were partially denaturing 7% polyacrylamide gels in the first dimension and 20% polyacrylamide (29 parts acrylamide to 1 part bisacrylamide) with 4 M urea in the second dimension. All gels were run at room temperature in a Hoefer SE600 or SE620 gel apparatus, using 1× TBE (40) as the running buffer. Running conditions and amounts of RNA used are indicated in the figure legends.

Filter hybridization analysis. For electrophoretic transfers, polyacrylamide gels and Gene Screen were equilibrated in 25 mM sodium phosphate (pH 6.5) for 20 to 30 min. Transfers were done in a Hoefer TE42 Transphor unit. The polyacrylamide gel-Gene Screen sandwich was made as instructed by the manufacturer. Transfer of the RNA from the gel onto the Gene Screen was done in 25 mM sodium phosphate buffer (pH 6.5) with 0.25 to 0.5 A for 8 to 12 h at 20°C. RNA was covalently attached to the Gene Screen by UV cross-linking (4). The blot was then prehybridized in OHyB (1% SDS, 6× SSPE [40], 100 μg of calf liver RNA per ml) for at least 4 h at 37°C.

Blots were hybridized overnight at 37°C in fresh OHyB (50 μl/cm^2) unless otherwise stated. At least 10 pmol of ^{32}P-end-labeled oligonucleotide per 10 μg of total RNA was used as the hybridization probe. Blots were washed three times in 6× SSPE with 0.1% SDS at 25 to 37°C. Kodak XAR5 film was used for autoradiography. Exposures were made at room temperature without intensifying screens.

Blots to be hybridized with another probe were stripped in TE7/SDS (10 mM Tris chloride [pH 7.0], 1 mM EDTA, 1% SDS). A blot (15 by 15 cm) was placed in 1 liter of TE7/SDS and heated to 95°C for 20 to 30 min. The TE7/SDS was discarded, and the procedure was repeated. The blot was then prehybridized and hybridized as described before.

TABLE 1. Oligodeoxynucleotide hybridization probes

Probe	Sequence
Intron	
pILE	5′-CCT GTT TGA AAG GTC TTT GGC ACA-3′
pILE-17mer	5′-CGG AAA CCG AAT GTT GC-3′
pLEUcaa	5′-CCC ACA GTT AAC TGC GGT C-3′
pLEUuag	5′-AAT TTA GAG GTT AAA TCC A-3′
pLYS	5′-ATC CTT GCT TAA GCA AAT GCG CT-3′
pPHE	5′-AAC TTG ACC GAA GTA TTT-3′
pPRO	5′-TGC TTT GTC TTC CTG TTT-3′
pSERcga	5′-AGC GAA CTT TTT TAT TCC A-3′
pSERgcu	5′-AAT TGC TTT TCT GAG GAA A-3′
pTRP	5′-CCG TGG AAT TTC CAA GAT TT-3′
pTYR	5′-AAG ATT TCG TAG TG$^{A}_{G}$ TAA ATT ACA-3′
pTYR-14mer	5′-TTC GTA GTG $^{A}_{C}$TA AA-3′
End	
5′pILE	5′-CTA CAC GAG CAT TTT CGA AAG A-3′
3′pILE	5′-AAA AAG AG$^{C}_{A}$ AAG TGC TCG AGG-3′
5′pLEUcaa	5′-CCA AAC AAC CAC TTA TTT GTT GA-3′
3′pLEUcaa	5′-AAA AAA TTA TTG GTT GCT AA-3′
5′pSERcga	5′-GTG CCA TTT CGA TTT GAA A-3′
3′pSERcga	5′-AAA AAT TAA ACG ACA CC-3′
Other	
mpILE	5′-GCA CGA AGC TCT AAC CAC TGA G-3′
mILE	5′-CCG TCG CGT TAT AAG CAC G-3′
5′SJpILE	5′-CGA ATG TTG CTA TAA GCA CG-3′
3′SJpILE	5′-ACG GTC GCG TTG CTT TTA AA-3′

Dot blots were made by using a Schleicher & Schuell Mini-Fold apparatus and Gene Screen. The Gene Screen filter was equilibrated in 25 mM sodium phosphate buffer (pH 6.5) before sample application. DNA applied to the filter was denatured by boiling in 0.2 N NaOH. Total RNA was applied in 25 mM sodium phosphate buffer (pH 6.5). Hydrolyzed RNA was made by boiling in 0.2 N NaOH for 10 min and was then applied to the filter. Nucleic acids were fixed onto the filter with UV light. Dot blots were prehybridized and hybridized as described above.

Hybridization probes. Sequences for the oligonucleotide hybridization probes used in these experiments are shown in Table 1. Probe nomenclature and the position at which each probe hybridizes to its target tRNA are described above and indicated in Fig. 1. Probes for tRNA IleUAU are based on the two known tRNA IleUAU genes (46). The 5′pLEUcaa and 3′pLEUcaa probes were designed from the tRNA LeuCAA gene present in plasmid pJB-2k (31). The 5′pSERcga and 3′pSERcga probes were designed from the tRNA SerCGA gene present in plasmid pPM-5 (47). The pSERgcu probe is based on the tRNA SerGCU gene present on plasmid pYH82 (58). Other probes were designed from the ptRNA sequences summarized by Ogden et al. (46).

5.8S rRNA size markers. Size markers were made from *S. cerevisiae* 5.8S rRNA purified by preparative polyacrylamide gel electrophoresis of total RNA. The 5.8S rRNA was labeled at its 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP in an exchange reaction (40). Partial RNase T_1 and U_2 reaction conditions were empirically determined. Fragment sizes were determined from the 5.8S rRNA sequence (54, 55).

Primer extension analysis. *S. cerevisiae* total RNA (1 to 2 mg) from strain A364A was separated on a 10% denaturing polyacrylamide gel (0.3 by 15 by 30 cm). The gel was cut into

1- or 0.5-cm strips, and the RNA from each strip was crush eluted. Each strip fraction was assayed for individual intron-containing RNA species by filter hybridization with an oligonucleotide probe. Intron-containing pre-tRNA IleUAU species were partially sequenced by primer extension, using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), dideoxynucleotides (Pharmacia), oligonucleotide primers, and [α-^{32}P]dATP (3,000 Ci/mmol; New England Nuclear). Primer extension reactions were analyzed on denaturing 20% sequencing gels, with subsequent autoradiography.

RESULTS

Survey of RNAs containing pre-tRNA introns. We have used ^{32}P-labeled oligonucleotide probes to identify intron-containing RNA species by filter hybridization analysis. The pRNA probes are complementary to the intron sequences of the 10 known pre-tRNA families (Table 1). It has previously been shown that the hybridization conditions used are highly specific (44a). Only hybridization to intron-containing RNA sequences occurs. Total RNA isolated from two wild-type strains, A364A and X2180-1A, was analyzed. These two strains represent the most common yet diverse laboratory *S. cerevisiae* genetic backgrounds. By analyzing these RNA preparations in parallel, we hoped to identify both the variation inherent in the extraction of RNA and any variation due to genetic differences. Size markers and *S. cerevisiae* total RNA were separated by denaturing polyacrylamide gel electrophoresis and then electrophoretically transferred onto Gene Screen for hybridization analysis. Filters were cut into lane-specific pieces, and each lane except marker lanes was hybridized with a different pRNA probe. The filter hybridization pattern of the higher-molecular-weight intron-containing RNA species of A364A can be seen in Fig. 2A; that for lower-molecular-weight species is shown in Fig. 2B. The panels represent composites from several autoradiographic exposures. The exposures were chosen to show the major hybridizing species, and consequently many minor species are not visible. Each pRNA probe detected a characteristic set of pre-tRNAs and processing intermediates distinct for each tRNA gene family. Different intron probes for the same pre-tRNA family detected identical patterns of bands (data not shown). Results obtained with X2180-1A total RNA were very similar (Table 2 and data not shown).

Intron-containing RNA sizes were estimated relative to size markers derived from partial RNase T_1 and U_2 digestion of 5.8S rRNA (Fig. 2, lanes M1 and M2). Linear regression was used to calculate a best line for each blot on the basis of predicted RNase T_1 and U_2 product sizes and the distances migrated (Fig. 3 and data not shown). Intron-containing RNA sizes were estimated from the best line and are summarized in Table 2. We conclude that the size markers reliably predict the sizes of the various intron-containing RNAs with errors of no more than 1 or 2 bases. A more complete discussion of these size estimates has been presented elsewhere (44b).

The precision of our RNA size estimates is limited by the resolution of the electrophoresis system chosen, as demonstrated by comparing the autoradiograph shown in Fig. 2 with that shown in Fig. 4. The 118-base 3′ ptRNA TrpCCA species appeared as a single band in our initial survey (Fig. 2A, lane 9). When the RNA was separated in a gel twice as long, at least three bands were resolved in that region (Fig. 4C). These RNAs appeared to differ by single-nucleotide steps over a range of 3 bases, the same range of sizes indicated in Table 2 for this 3′ ptRNA species. Resolution improvements can be seen for other RNAs as well. Some of the size variation may be due to distinct initiation and termination sites used by different genes of the same family, or even heterogeneity of initiation and termination sites on the same gene. Because of these limitations, accurate prediction of transcription initiation and termination sites would be difficult without additional, more direct transcript-mapping experiments.

We clearly saw numerous intron-containing RNAs in growing cells for each tRNA gene family. While this finding was not unexpected, we were surprised by the complexity of some of the patterns. Previously, *S. cerevisiae* pre-tRNAs had been detected in vivo by radioactive labeling (2, 14, 25, 33, 34). Most of the well-characterized examples are end-mature, intron-containing ptRNAs. Larger species have been detected by Northern (RNA) analysis and identified as probable primary transcripts (27). It is curious that in vivo labeling experiments failed to detect the more abundant pre-tRNAs, such as the 128-base 5′3′ ptRNA TrpCCA species.

Identification of the end-mature ptRNAs. Each pRNA probe detected a major hybridizing species of the appropriate size to be the end-mature ptRNA (Table 2). These species had the same electrophoretic mobility as purified end-mature ptRNAs (44a). Two ptRNA ProUGG species were detected of 100 and 103 bases. This heterogeneity is probably due to intron size differences in this tRNA gene family (46). On the basis of size estimates, 3 of the 10 ptRNAs appeared to lack the -CCA_{OH} tail. They are ptRNA IleUAU, ptRNA Pro-UGG, and ptRNA TrpCCA. This observation suggests that -CCA_{OH} tail addition may not be necessary for pre-tRNA splicing.

We wanted to confirm that the species we had identified as ptRNAs accumulated in *rna1-1* mutant cells following a temperature shift to 37°C. The RNA samples for this experiment were prepared from either a wild-type diploid or an *rna1-1* homozygous diploid. Both strains were grown in rich medium at 23°C and then shifted to 37°C before RNA isolation. The pattern of intron-containing RNA species was identical between the wild-type and *rna1-1* strains from the unshifted cultures (Fig. 4 and data not shown). The pattern of intron-containing RNAs remained similar between the two cultures after the temperature shift except that the *rna1-1* strain had more ptRNA, as expected (25, 33). In fact, the ptRNAs were resolved in the unshifted cultures as a mixture of species differing by a single base in mobility. We suspect that this mixture of bands represents -CCA_{OH} tail polymerization intermediates. After the temperature shift, both the wild-type and *rna1-1* strains had less -CCA_{OH} tail polymerization intermediates and more end-mature ptRNA. The temperature shift might temporarily slow splicing in wild-type cells, thereby causing an accumulation of ptRNAs with mature acceptor ends. Curiously, the accumulation of ptRNAs seen in *rna1-1* cells might be interpreted simply as an exaggeration of the effect normally seen in wild-type cells.

Identification of excised introns. Nine of the ten pRNA probes also detected smaller RNA species of the appropriate size to be excised introns (Fig. 2 and Table 2). From DNA sequence analysis, the intervening sequence for both known tRNA IleUAU genes is 60 bp (46). The fastest-migrating species detected with the pILE probe was 60 bases (Fig. 1, lane 1). We confirmed that this RNA was indeed the pre-tRNA IleUAU intron by primer extension. *S. cerevisiae*

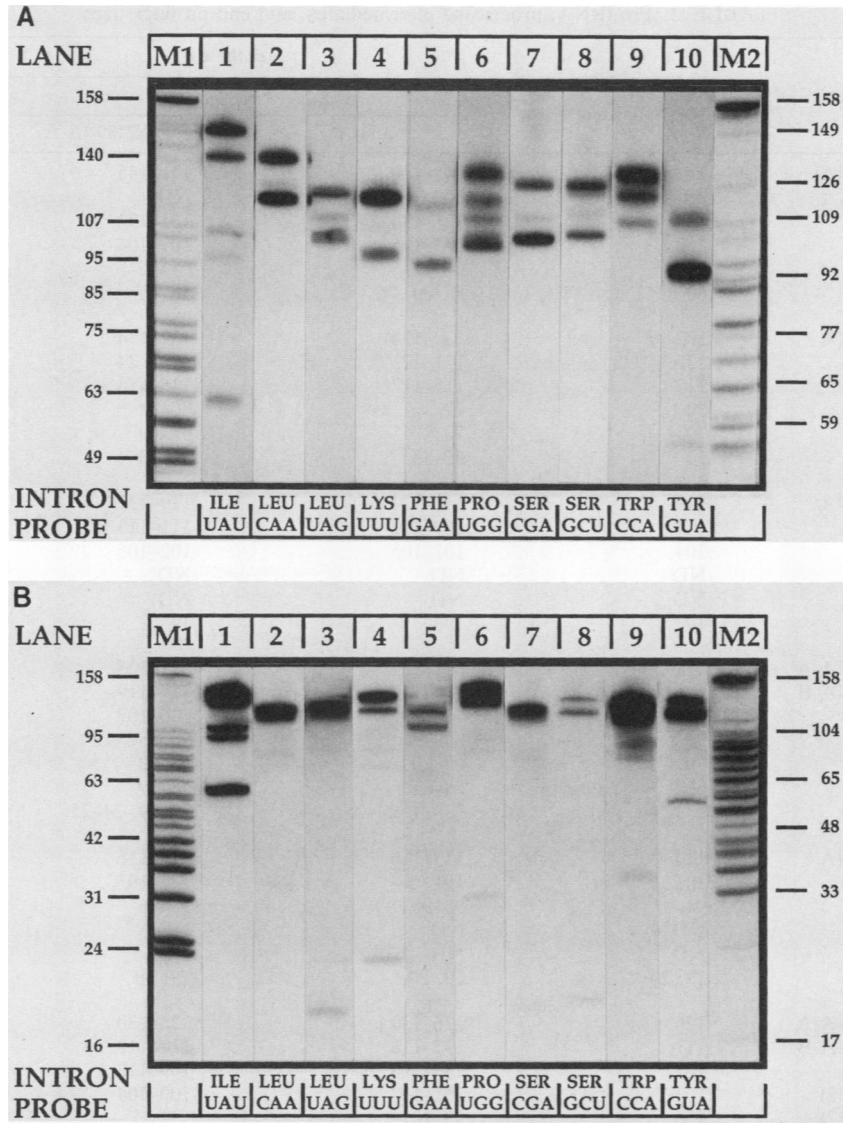

FIG. 2. Survey of transcripts and processing intermediates detected with probes for intron-containing pre-tRNA genes. Total RNA was isolated from *S. cerevisiae* A364A grown in YPD medium at 30°C. The RNA was separated by electrophoresis on a denaturing 10% polyacrylamide gel (14 cm long) for 4 h at 400 V (A) or on a denaturing 20% polyacrylamide gel (14 cm long) for 7 hours at 200 V (B). Lanes 1 to 10 were loaded with 10 μg of RNA. ^{32}P-labeled size markers applied to lanes M1 and M2 were RNase T_1 and U_2 partial digestion products of 5.8S rRNA, respectively. Selected size standards are indicated by their number of bases. After electrophoresis, the RNA was transferred onto Gene Screen. Lanes cut from the filter were hybridized with different ^{32}P-labeled probes. Lanes were probed with pre-tRNA intron probes as follows: 1, IleUAU (pILE); 2, LeuCAA (pLEUcaa); 3, LeuUAG (pLEUuag); 4, LysUUU (pLYS); 5, PheGAA (pPHE); 6, ProUGG (pPRO); 7, SerCGA (pSERcga); 8, SerGCU (pSERgcu); 9, TrpCCA (pTRP); 10, TyrGUA (pTYR). Lanes M1 and M2 were not hybridized to any probe. Composites of several autoradiographic exposures are shown.

total RNA was fractionated by polyacrylamide gel electrophoresis. Each fraction was assayed for the various pre-tRNA IleUAU species by Northern analysis with a pILE probe. Sequencing was then performed on the fraction containing the 60-base species, using the pILE probe as the primer (Fig. 5). The primer was extended 24 bases to the expected 5′ end of the excised intron, and the expected complementary sequence was also evident. Appropriate primer extension termination and no aberrant polyacrylamide gel mobility indicated that this RNA molecule has a linear structure. We conclude that the 60-base species detected with the pILE probe is the excised, linear pre-tRNA IleUAU intron. By analogy, we infer that the RNA species of the appropriate sizes detected with the other pRNA probes are also excised, linear introns. The amounts of these RNAs were reduced in *rna1-1* cells shifted to a nonpermissive temperature (data not shown), consistent with their assignment as excised introns. No species of the appropriate size was detected for the excised pre-tRNA Tyr-GUA intron. Since the pre-tRNA TyrGUA intron is only 14 bases, this is probably due to the technical limitations of identifying such a small RNA molecule by filter hybridization.

More than a single band near the excised intron size was detected by several of the pRNA probes. Two intron-size species of 19 and 20 bases were detected with the pPHE probe. Two different pre-tRNA PheGAA introns are known;

TABLE 2. Pre-tRNA, processing intermediates, and end product sizes

tRNA	Species	Size (bases)			
		Estimated	Range		Predicted
			A364A	X2180-1A	
IleUAU	5′3′ptRNA	145	143–146	141–145	?
	3′ptRNA	ND[a]	ND	ND	?
	ptRNA	133	131–134	131–133	133–136[b]
	5′-2/3	103	103–105	103–104	103[c]
	2/3	97	94–97	95–96	97
	Intron	60	59–60	58–61	60
LeuCAA	5′3′ptRNA	132	132–134	132–134	?
	3′ptRNA	122	121–123	122–124	?
	ptRNA	117	115–117	116–118	114 (115)-117 (118)[d]
	5′-2/3	80	77	80–81	79–83[e]
	2/3	70	70	68–69	70 (71)
	Intron	32	32–33	32–33	32 (33)
LeuUAG	5′3′ptRNA	119	118–121	119–121	?
	3′ptRNA	111	109–111	111–113	?
	ptRNA	104	101–105	102–105	101–104
	5′-2/3	ND	ND	ND	64–69
	2/3	ND	ND	ND	57
	Intron	19	19	19	19
LysUUU	5′3′ptRNA-A[f]	121	121–124	121–125	?
	5′3′ptRNA-B	116	115–120	117–119	?
	3′ptRNA	106	106–107	106–107	?
	ptRNA	99	96–99	96–98	96–99
	5′-2/3	70	68–70	69–70	67–72
	2/3	60	58–61	58–59	60
	Intron	23, 24	23–24, 24–25	23–24, 24–25	23
PheGAA	5′3′ptRNA	114	113–116	114–116	?
	3′ptRNA	101	101	101–103	?
	ptRNA	94	93–95	93–96	91 (92)-93 (94)
	5′-2/3	ND	ND	ND	67 (68)-70 (71)
	2/3	ND	ND	ND	55 (56)
	Intron	19, 20	19, 20	19, 20	18 (19)
ProUGG	5′3′ptRNA-A	126	126–129	126–130	?
	5′3′ptRNA-B	117	117–118	117–119	?
	3′ptRNA	109	109–110	109–112	?
	ptRNA(31)	103	101–104	100–104	103–106
	ptRNA(27)	100	99–101	—[g]	100–103
	5′-2/3	80	81	78–80	74 (71)-87 (84)
	2/3	67	65–67	64–67	67 (64)
	Intron	25, 26, 27, 29, 31	25, 26, 27, 29, 30–31	25, 26, 27, 29, 30	27, 31[h]
SerCGA	5′3′ptRNA	123	122–126	123–126	?
	3′ptRNA	111	110–112	111–113	?
	ptRNA	104	103–104	105–106	101–104
	5′-2/3	68	68	68–69	66–72
	2/3	56	53–54	55	56
	Intron	19, 20	19, 20	19–20, 20–21	19
SerGCU	5′3′ptRNA-A[i]	123	121–123	122–125	?
	5′3′ptRNA-B	120	118–120	121	?
	3′ptRNA	113	111–113	113–114	?
	ptRNA	104	104–105	103–106	101–104
	5′-2/3	67	66–67	66–68	64–71
	2/3	57	56–57	56–58	57
	Intron	20, 21	20, 21	20, 21	20
TrpCCA	5′3′ptRNA	128	125–128	126–130	?
	3′ptRNA	118	117–119	118–121	?
	ptRNA	109	109–110	108–109	109–112
	5′-2/3	80	83–86	79–80	76–81
	2/3	70	67–69	68–69	70
	Intron	34, 35	33–34, 34–35	34, 35	34

Continued on following page

TABLE 2—*Continued*

tRNA	Species	Size (bases)			
		Estimated	Range		Predicted
			A364A	X2180-1A	
TyrGUA	5'3'ptRNA	109	108–111	109–113	?
	3'ptRNA	ND	ND	ND	?
	ptRNA	92	91–93	92–94	89–92
	5'-2/3	ND	ND	ND	?
	2/3	53	52–53	52–53	53
	Intron	ND	ND	ND	14

[a] ND, Not detected.
[b] ptRNA sizes vary depending on addition of -CCA_{OH} tail.
[c] Known to have a 6-base 5' flank by primer extension.
[d] Sizes enclosed in parentheses indicate variation due to intron length.
[e] Size range of 5'-2/3 determined from size ranges of 5'3'ptRNA and 3'ptRNA.
[f] The A form of 5'3'ptRNA LysUUU is less abundant than the B form.
[g] The two ptRNA ProUGG species were not clearly distinguished on this blot.
[h] The ptRNA ProUGG intron has at least five sequence variants with two different sizes.
[i] The A form of 5'3'ptRNA SerGCU is more abundant than the B form.

one is 18 bases, and the other is 19 bases. Two intron-size RNA molecules were also detected with the pLYS, pSERcga, pSERgcu, and pTRP probes. For pre-tRNA SerCGA, this intron size heterogeneity cannot be due to intervening sequence differences, since tRNA SerCGA is transcribed from a single-copy gene (13, 47). We believe that these discrepancies result from the presence of both a 5' and a 3' phosphate on the 5.8S rRNA size markers (causing increased mobility of the smaller size markers) and possible loss of the 3' phosphate from the excised intron. Five intron-size RNA species were detected with the pPRO probe. Five different end-mature pre-tRNA ProUGG species have been detected in *rna1-1* cells, and five different intervening sequence variants of two different sizes (28 and 31 bp) have been reported for the tRNA ProUGG family (46). In this case, some of the intron size heterogeneity may be due to gene variants not yet known.

Identification of end-extended pre-tRNAs. Each pRNA probe detected one or more intron-containing RNA species larger than its cognate ptRNA species. The pILE and pTYR probes detected one larger species, the pLYS, pPRO and pSERgcu probes detected three larger species, and the remaining five pRNA probes detected two larger species (as judged from the resolution of this gel system; Fig. 2). From in vitro transcription and oocyte injection studies, we presumed these larger, intron-containing RNA species to be primary transcripts (5'3'ptRNA) and end-processing intermediates (3'ptRNA). We confirmed the structure of the larger RNA species from three tRNA gene families by differential hybridization. Oligonucleotide probes were designed that could hybridize only to pre-tRNAs with a 5' leader (5'pRNA probes) or a 3' extension (3'pRNA probes) under appropriate hybridization and wash conditions. Probe sequences were based on one gene sequence for each tRNA gene family. Consequently, hybridization signals were not as great for the 5'pRNA and 3'pRNA probes as for other oligonucleotide probes. The pILE probe detected two major pre-tRNA IleUAU species, the 133-base ptRNA and a 145-base species. The 145-base pre-tRNA IleUAU hybridized to both 5'pILE and 3'pILE probes (Fig. 6, lanes 1 and 2). The ptRNA IleUAU species did not hybridize to either probe. Primer extension analysis demonstrated that the 133-base ptRNA IleUAU had the expected 5' end of mature tRNA IleUAU, while the 145-base pre-tRNA IleUAU spe-

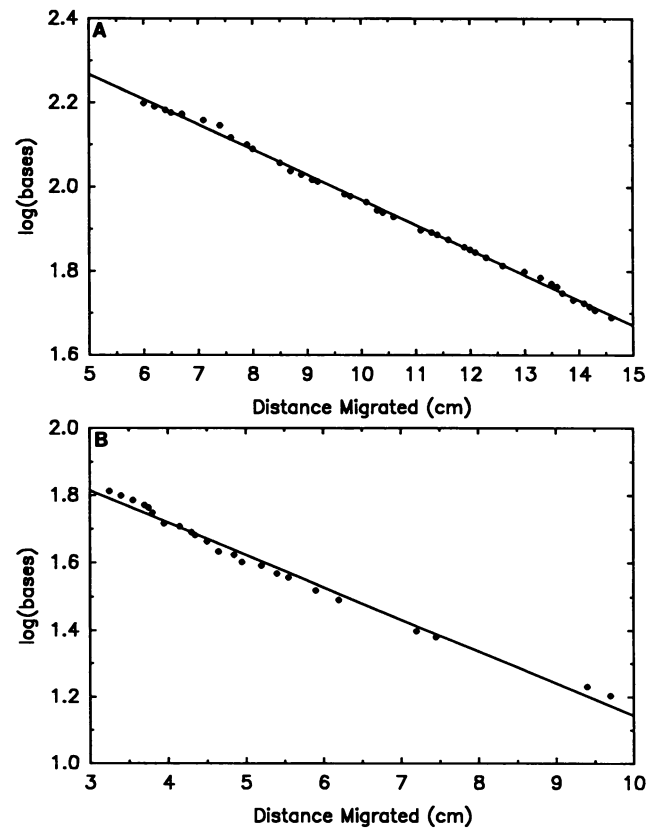

FIG. 3. Calibration curves for molecular weight determination of intron-containing RNAs. Sizes of fragments generated by partial T_1 or U_2 digestion of 5.8S rRNA were determined from the published sequence (54). The distance that each fragment migrated during electrophoresis was measured from autoradiographs of the filters onto which the fragments had been transferred. The logarithm of the number of bases in each fragment was then plotted against the distance that it had migrated. Linear regression was used to generate the best line shown. (A) Calibration curve made from the size markers shown in Fig. 2A; (B) calibration curve made from the size markers shown in Fig. 2B. Size estimates of various pre-tRNA intron-containing RNA species were made from these calibration curves (Table 2).

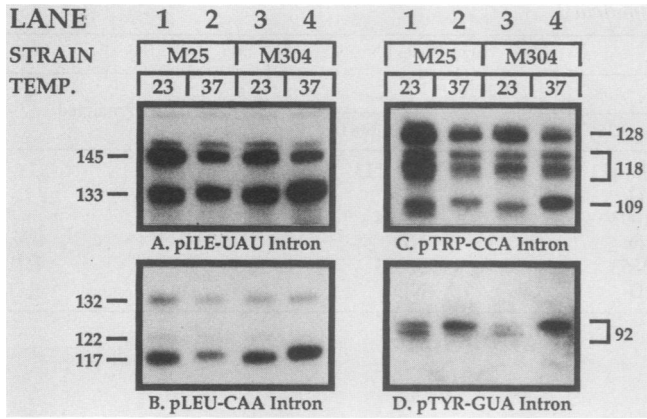

FIG. 4. Induction by heat shock of accumulation of fully end mature ptRNA in both wild-type and *rnal-1* cells. Total RNA was extracted from M25 (wild-type) and M304 (*rnal-1*) cells grown in YPD at 23°C or in YPD at 23°C and then shifted to 37°C for 30 min. Samples (10 μg) of each RNA preparation were separated on a 10% denaturing polyacrylamide gel (30 cm long) and transferred onto Gene Screen. Four sets of RNA samples were used in parallel, each set consisting of four lanes: 1, M25 RNA, 23°C culture; 2, M25 RNA, heat-shocked culture; 3, M304 RNA, 23°C culture; 4, M304 RNA, heat-shocked culture. Each set was hybridized with a different ^{32}P-labeled pre-tRNA intron probe: (A) pILE-17mer; (B) pLEUcaa; (C) pTRP; (D) pTYR-14mer. For pTyr-14mer probe, hybridization was done at 30°C before washing at room temperature.

cies had a 6-base 5′ leader (data not shown). These results indicate that the 145-base pre-tRNA IleUAU species is the 5′3′ptRNA IleUAU with a 5′ leader and 6-base 3′ extension. Two intron-containing RNA species larger than the 104-base ptRNA SerCGA were detected with the pSERcga probe. The 123-base species hybridized to both a 5′pSERcga and a 3′pSERcga probe (Fig. 6, lanes 4 and 5). The 111-base species hybridized to only the 3′pSERcga probe. The 104-base ptRNA SerCGA hybridized to neither probe. This finding indicates that the 123-base species is the 5′3′ptRNA SerCGA and that the 111-base 3′ ptRNA SerCGA species retains its 3′ extension but lacks a 5′ leader. A similar experiment done with 5′pLEUcaa and 3′pLEUcaa probes gave the same pattern of results (Fig. 6, lanes 7 to 9). In this example, the 132-base pre-tRNA LeuCAA species is the 5′3′ptRNA and the 122-base pre-tRNA LeuCAA is the 3′ptRNA. By analogy, we have designated the other intron-containing RNA species larger than their cognate ptRNAs as either 5′3′ptRNAs or 3′ptRNAs. No evidence was found to indicate that a pre-tRNA with a 5′ leader and lacking a 3′ extension exists in vivo for these three tRNA gene families.

Identification of splicing intermediates. The pILE probe detected two intron-containing RNA species (103 and 97 bases) that had sizes between those of ptRNA IleUAU and its excised intron. The 97-base species is of the appropriate size to be a splicing intermediate consisting of the 5′ exon and intron or 3′ exon and intron. Oligonucleotide probes designed to hybridize specifically to intact splice sites (5′SJpILE and 3′SJpILE) were used to distinguish between

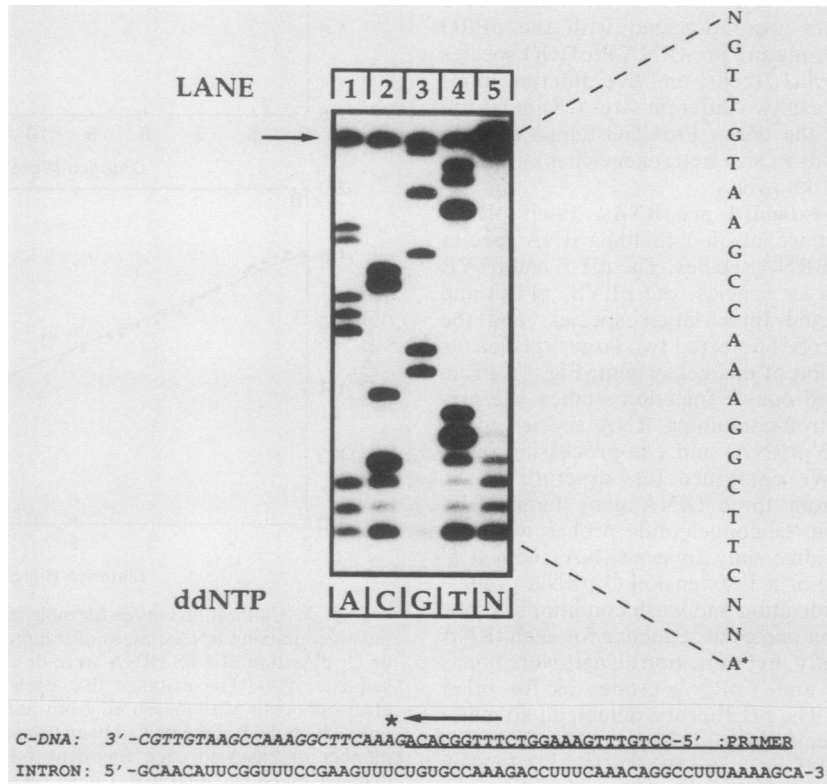

FIG. 5. Primer extension on pretRNA IleUAU excised intron. Total RNA prepared from strain A364A was fractionated by polyacrylamide gel electrophoresis. The fraction containing a 60-base RNA that hybridized to the pretRNA IleUAU intron probe (pILE probe) was annealed to an oligonucleotide complementary to nucleotides 61 to 85 of the tRNA IleUAU gene and extended with reverse transcriptase. These reactions contained four deoxynucleoside triphosphates plus ddATP in lane 1, ddCTP in lane 2, ddGTP in lane 3, ddTTP in lane 4, or no dideoxynucleotide (ddNTP) in lane 5.

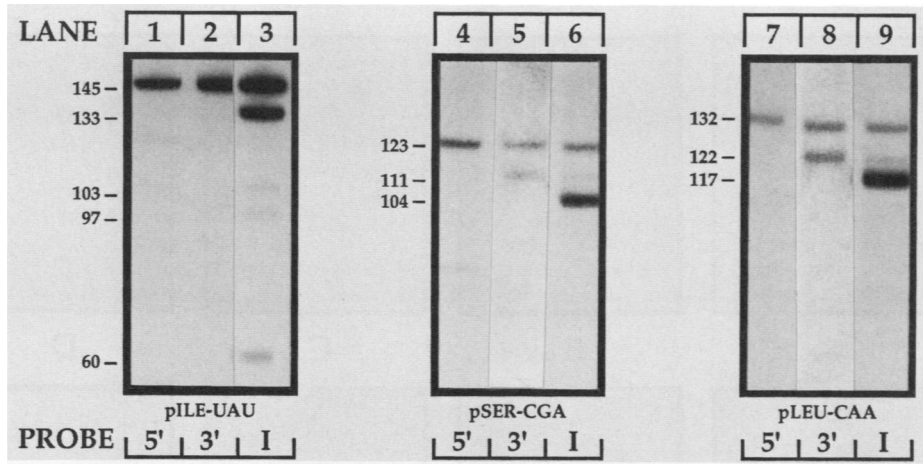

FIG. 6. Demonstration that pre-tRNA processing to remove the 5′ leader and 3′ extension occurs in a definite order. Samples (10 μg) of total RNA from *S. cerevisiae* A364A were separated by electrophoresis in a partially denaturing 7% polyacrylamide gel for 2.5 h at 200 V, and the RNA was transferred onto Gene Screen. Lane-specific pieces of the filter were hybridized with different ^{32}P-labeled probes in the lanes as follows: 1, 5′pILE; 2, 3′pILE; 3, pILE; 4, 5′pSERcga; 5, 3′pSERcga; 6, pSERcga; 7, 5′pLEUcaa; 8, 3′pLEUcaa; 9, pLEUcaa. Size is indicated by the number of bases.

these two possibilities. Total RNA isolated from strain A364A was used in this experiment. RNA was isolated from cultures grown at 23°C and grown at 23°C with a temperature shift to 37°C for 30 min. The 5′SJpILE probe detected the 97-base species (Fig. 7, lanes 5 and 6). The 3′SJpILE probe detected only the 5′3′ptRNA and ptRNA IleUAU species (lanes 3 and 4). This result indicates that the 97-base species is a splicing intermediate consisting of the 5′ exon and intron (2/3 molecule). Curiously, the 5′SJpILE probe also hybridized to the 103-base species. Since the 3′SJpILE failed to detect the 103-base species, we assumed that this species was extended at its 5′ end. The 5′pILE probe also hybridized to the 103-base species (lanes 7 and 8). Primer extension analysis confirmed that the 97-base 2/3 molecule had the same 5′ end as the ptRNA IleUAU and that the 103-base species had the same 5′ end as the 5′3′ptRNA IleUAU (data not shown). We believe the 103-base species is another splicing intermediate consisting of the 5′ leader, 5′ exon, and intron (5′-2/3 molecule). Pre-tRNA IleUAU splicing intermediate abundance varied with growth conditions (Fig. 7; compare lanes 1 and 2; 44b). Wild-type cells grown at 23°C had more 5′-2/3 molecule than 2/3 molecule, whereas cells that had been temperature shifted to 37°C had more 2/3 molecule than 5′-2/3 molecule. This result again suggests that splicing is slowed in wild-type cells that have been temperature shifted.

Intron-containing RNA species of appropriate sizes to be 2/3 molecules were identified with pRNA probes from seven of the remaining nine tRNA gene families (Fig. 2 and Table 2). In six of the remaining nine tRNA gene families, RNA species of the size appropriate for 5′-2/3 molecules were also detected (Fig. 2 and Table 2). Though we did not detect a splicing intermediate consisting of the intron and 3′ exon, we cannot rule out the possibility that such an intermediate exists at a low level for pre-tRNA IleUAU or even as the major intermediate for other tRNA gene families.

Splicing can precede end processing. The 5′-2/3 molecule is unexpected if pre-tRNA processing occurs in a strictly vectorial manner. Identification of the pre-tRNA IleUAU 5′-2/3 molecule suggests that pre-tRNA splicing can precede end processing. If the pre-tRNA IleUAU 5′-2/3 molecule is a true splicing intermediate, then a spliced but end-extended pre-tRNA (5′3′mtRNA) should also be present. Filter hybridization analysis of total RNA fractionated on two-dimensional polyacrylamide gels was used to identify the 5′3′mtRNA IleUAU species. Four two-dimensional blots were made in parallel. Each blot was hybridized with a

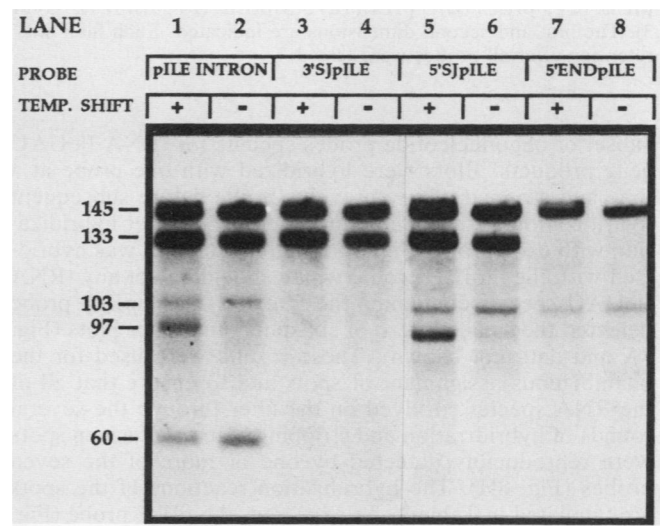

FIG. 7. Identification of a novel pre-tRNA splicing intermediate. Total RNA was isolated from *S. cerevisiae* A364A grown in YPD at 23°C or at 23°C and shifted to 37°C for 30 min prior to RNA extraction. Samples (10 μg) of total RNA from each preparation were applied to a partially denaturing 7% polyacrylamide gel and separated by electrophoresis for 2.5 h at 200 V, and the RNA was transferred onto Gene Screen. Even-numbered lanes contained RNA from the 23°C culture; odd-numbered lanes contained RNA from the temperature-shifted culture. Pieces of the filter containing one lane of RNA from each preparation were hybridized with different ^{32}P-labeled probes as follows: 1 and 2, pILE; 3 and 4, 3′SJpILE; 5 and 6, 5′SJpILE; 7 and 8, 5′pILE. Size is indicated by the number of bases.

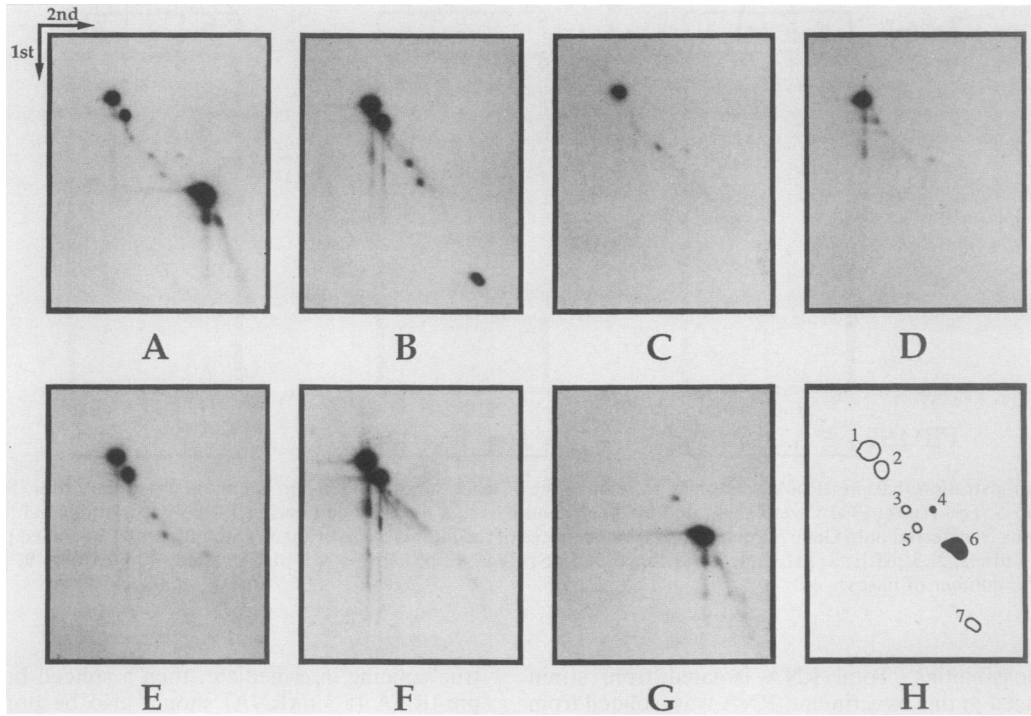

FIG. 8. Demonstration that pre-tRNA splicing and end processing are not ordered. Total RNA was isolated from *S. cerevisiae* X2180-1A grown in YPD at 23°C and separated by two-dimensional polyacrylamide gel electrophoresis. For the first dimension, samples (10 μg) were applied to a partially denaturing 7% polyacrylamide gel and separated by electrophoresis for 2.5 h at 200 V. For the second dimension, four lane-specific pieces from the first-dimension gel were removed, and short 7% gels were cast around each. Partially denaturing 20% polyacrylamide running gels were then cast on top of the first-dimension gel pieces. RNA was separated in the second dimension at 200 V for 18 h. All four gels were run in parallel, and the RNA was transferred onto Gene Screen. The filters were hybridized with different ^{32}P-labeled probes, autoradiographed, stripped free of radioactivity, and hybridized again until all seven probes were used. (A) mpILE; (B) pILE; (C) 5'pILE; (D) 3'pILE; (E) 5'SJpILE; (F) 3'SJpILE; (G) mILE; (H) schematic summary of all species identified, spots 1 to 7 (Table 3). The first and second dimensions are indicated. Each filter was hybridized to the mpILE probe to ensure accurate alignment and RNA retention after all probes were tested.

subset of oligonucleotide probes specific for tRNA IleUAU gene products. Blots were hybridized with one probe at a time and stripped of any previous probe before subsequent hybridizations. Autoradiographs were made after hybridization with each probe (Fig. 8). Finally, each blot was hybridized with the mpILE probe, which should detect any tRNA IleUAU species containing the 5' exon. The mpILE probe detected the same pattern of six spots on all four blots (Fig. 8A and data not shown). These results were used for the unambiguous assignment of spots and to ensure that all of the RNA species survived on the filter through the several rounds of hybridization and stripping. A total of seven spots were reproducibly detected by one or more of the seven probes (Fig. 8H). The hybridization reactions of the spots are tabulated in Table 3. As expected, the pILE probe (Fig. 8B) detected the 5'3'ptRNA (spot 1), the ptRNA (spot 2), the 5'-2/3 molecule (spot 3), the 2/3 molecule (spot 5), and excised intron (spot 7). All of the intron-containing RNAs are displayed along a single diagonal. The mpILE probe hybridized to six species (Fig. 8A): 5'3'ptRNA, ptRNA, 5'-2/3, 2/3, an abundant species migrating at the correct size for mature tRNA IleUAU (spot 6), and spot 4. As expected, the mpILE probe failed to hybridize to the excised intron. Spot 4 migrated near the 5'-2/3 molecule in the first dimension but migrated somewhat faster than the 5'-2/3 molecule in the second dimension. Spot 4 was not detected with the intron probe. Neither spot 4 nor mature tRNA Ile migrated on the same diagonal as the intron-containing RNAs, but both would fall on a line parallel to that diagonal. This finding suggests a similar secondary structure. As expected, the 5'pILE probe hybridized to both the 5'3'ptRNA and the 5'-2/3 molecule (Fig. 8C). The 5'pILE probe also hybridized to spot 4 (Fig. 8C), indicating that spot 4 contains the 5' leader. The 3'pILE probe hybridized to the 5'3'ptRNA and also to spot 4 (Fig. 8D). This result shows that spot 4 contains a 3' extension like the 5'3'ptRNA. The 5'SJpILE probe hybridized to 5'3'ptRNA, to ptRNA, and to 5'-2/3 and 2/3 molecules but did not hybridize to spot 4, mature tRNA

TABLE 3. Differential probe hybridization results for tRNA IleUAU

Species	Hybridization with given oligodeoxynucleotide probes							Description
	pIle	mpIle	5'pIle	3'pIle	5'SJpIle	3'SJpIle	mIle	
Spot 1	+	+	+	+	+	+	−	5'3'ptRNA
Spot 2	+	+	−	−	+	+	−	ptRNA
Spot 3	+	+	+	−	+	−	−	5'-2/3
Spot 4	−	+	+	+	−	−	+	5'3'mtRNA
Spot 5	+	+	−	−	+	−	−	2/3
Spot 6	−	+	−	−	−	−	+	mtRNA
Spot 7	+	−	−	−	−	−	−	Intron

IleUAU, or the excised intron (Fig. 8E). A small amount of radioactivity was detected in the mature tRNA IleUAU position with the 5'SJpILE probe. This represents a small amount of 5'SJpILE probe binding to mature tRNA IleUAU. The 3'SJpILE probe hybridized only to the 5'3'ptRNA and ptRNA (Fig. 8F). Finally, a tRNA IleUAU-specific probe (mILE) that spans the splice junction hybridized to both mature tRNA IleUAU and spot 4 (Fig. 8G). Spot 4 contains sequences from the pre-tRNA IleUAU D-stem and D-loop region, the 5' leader, the 3' extension, and the anticodon stem and loop region. Spot 4 does not have sequences complementary to the pre-tRNA IleUAU intron and lacks sequences wholly complementary to the 5' or 3' splice site. We conclude that spot 4 represents the 5'3'mtRNA, a spliced pre-tRNA IleUAU that retains both the 5' leader and the 3' extension.

Another possibility exists for the source of spot 4 RNA. This RNA could be the transcript from a related tRNA IleUAU gene that does not contain an intervening sequence. Different members of tRNA gene families often retain considerable sequence similarity, even extending into the flanking sequences (44b). Therefore, it is possible that probes designed from sequences of tRNA IleUAU genes with intervening sequences might hybridize to transcripts of related tRNA Ile genes. Only one *S. cerevisiae* tRNA Ile gene without an intervening sequence has been sequenced to date, tRNA IleAAU (22, 52). The sequences of the tRNA IleAAU and the tRNA IleUAU genes are sufficiently different that we would not expect any of the probes to cross-hybridize. We also tested this possibility empirically. *S. cerevisiae* genomic DNA and control samples were applied to Gene Screen and then hybridized with four different probes (Fig. 9). With the mpILE probe, a positive signal was detected for genomic DNA, plasmid DNA that contains a tRNA IleUAU gene with an intervening sequence, and *S. cerevisiae* total RNA; no signal was detected for the hydrolyzed RNA, as expected (Fig. 9, column A). The same results were obtained with the pILE probe (column B). No signal was detected with mILE probe for genomic DNA, the plasmid DNA, or the hydrolyzed RNA (column C). This result indicates that there is no sequence in the *S. cerevisiae* genome complementary to the mILE probe. The mILE probe did hybridize to total RNA, indicating that sequences complementary to this probe do arise after RNA processing. As a further control, we assayed for the tRNA SerCGA gene, which is known to be single copy (13, 47). The pSERcga probe gave a positive signal for the genomic DNA and total RNA but not for the plasmid DNA or hydrolyzed RNA (column D). We interpret this control experiment as showing that there is less than a single copy of any tRNA gene that could produce spot 4 RNA directly by transcription.

Relative abundances of pre-tRNAs, splicing intermediates, and products. We have estimated relative pre-tRNA abundance on the basis of hybridization signal intensity from the series of autoradiographic exposures used to make Fig. 2 (data not shown). This assessment assumes that the hybridization efficiency for each pRNA probe is about the same, that X-ray film density is proportional to radioactivity and exposure time, and that efficiencies of pre-tRNA binding to Gene Screen are nearly equal. All of the pRNA probes were labeled to the same specific radioactivity, and an excess of probe over filter-bound RNA was used for hybridization. Comparing between tRNA gene families, the order of decreasing abundance is (i) TrpCCA, (ii) IleUAU, (iii) ProUGG, (iv) TyrGUA, (v) LeuCAA, (vi) LysUUU, (vii) SerGCU, (viii) SerCGA, (ix) LeuUAG, and (x) PheGAA. The apparent range of abundance may span as much as 50-fold from the most to least abundant pre-tRNA set.

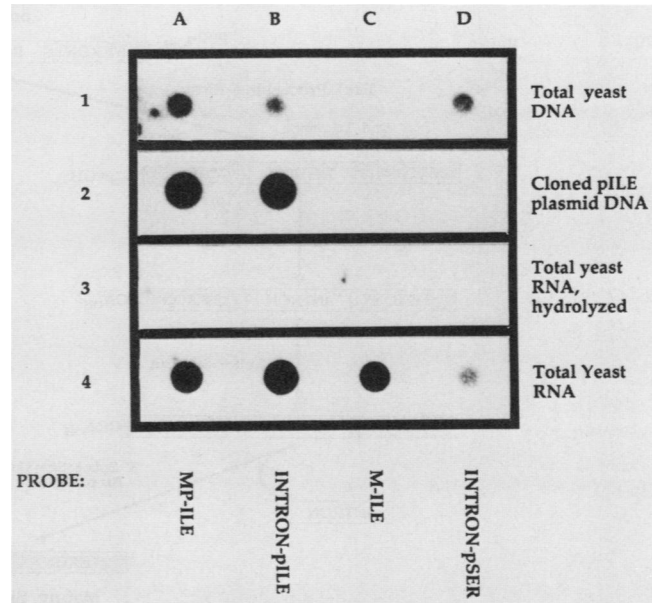

FIG. 9. Demonstration that all *S. cerevisiae* tRNA IleUAU genes contain an intron. Samples of several different nucleic acid preparations were applied to Gene Screen in defined areas and hybridized with four different ^{32}P-labeled probes. Samples (1 μg) of genomic DNA from strain X2180-1A were applied to each dot of row 1; samples (10 pg) of pGKN-7 plasmid DNA (containing a cloned tRNA IleUAU gene) were applied to each dot of row 2; samples (1 μg) of alkaline-hydrolyzed total RNA from strain X2180-1A were applied to each dot of row 3; samples (50 pg) of total RNA from strain X2180-1A were applied to each dot of row 4. Column A was hybridized with the mpILE probe; column B was hybridized with the pILE probe; column C was hybridized with the mILE probe; column D was hybridized with the pSERcga probe.

Within any ptRNA set, the relative amounts of 5'3'ptRNA, 3'ptRNA, and ptRNA can be estimated (Fig. 2). There is relatively more 5'3'ptRNA than ptRNA for Ile UAU, LysUUU, and TrpCCA. The amounts of 5'3'ptRNA and ptRNA are approximately equal for LeuCAA, LeuUAG, ProUGG, and SerGCU. There is relatively less 5'3'ptRNA than ptRNA for PheGAA, SerCGA, and Tyr GUA. For all pre-tRNA families, the relative amount of 5'3'ptRNA was greater than that of 3'ptRNA.

The relative abundances of the ptRNA splicing intermediates and products were also estimated. The relative order of excised introns was, in decreasing abundance, (i) IleUAU, (ii) LysUUU, (iii) TrpCCA, (iv) ProUGG, (v) SerGCU, (vi) SerCGA, (vii) LeuCAA, (viii) LeuUAG, and (ix) PheGAA. It is possible that the smaller intron amounts are significantly underestimated by this technique. The 2/3 splicing intermediate was most abundant for pre-tRNA IleUAU, followed by TyrGUA and then TrpCCA. No 5'-2/3 splicing intermediate was detected for pre-tRNA TyrGUA. The 5'-2/3 molecule of pre-tRNA IleUAU was the most abundant, followed by TrpCCA and LysUUU. Curiously, those tRNA gene families with the most 5'-2/3 molecule also have more 5'3'ptRNA than ptRNA. Since the relative amounts of the various species represent steady-state levels, estimation of processing kinetics is not possible.

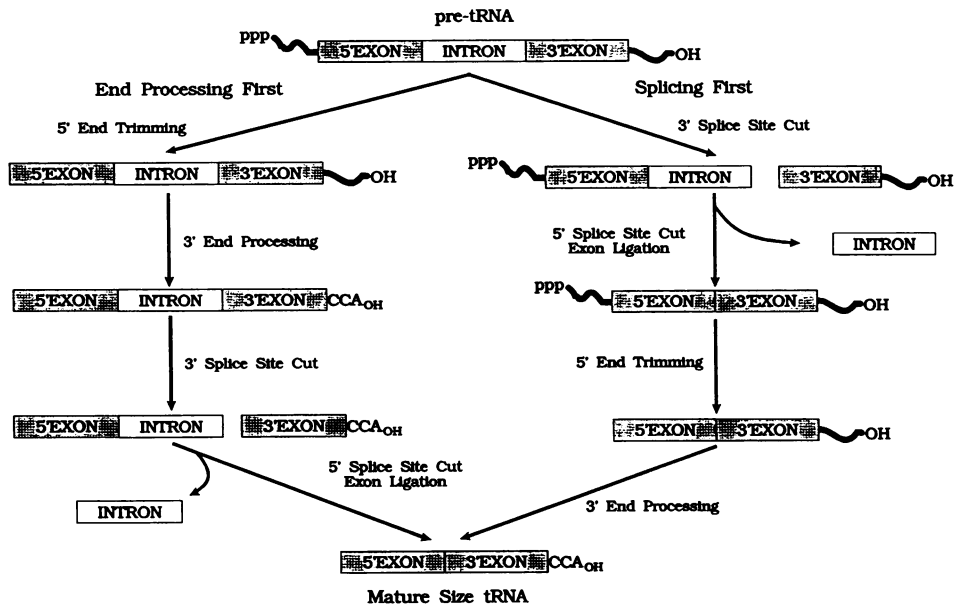

FIG. 10. Schematic representation of the two nucleolytic processing pathways used by pre-tRNA IleUAU.

DISCUSSION

The *S. cerevisiae* pre-tRNA processing pathway. One interpretation of our results is summarized in Fig. 10, a schematic diagram of two alternative pre-tRNA processing pathways operating in vivo for *S. cerevisiae*. The two pathways are designated end processing first and splicing first. The steps in the end-processing-first pathway are (i) production of the transcript by RNA polymerase III, (ii) 5′ end trimming by RNase P, (iii) 3′ end trimming, (iv) addition of the -CCA_{OH} tail by nucleotidyltransferase, (v) cleavage at the 3′ splice site by the pre-tRNA-splicing endonuclease, (vi) cleavage at the 5′ splice site by the pre-tRNA-splicing endonuclease, (vii) ligation of the two exons, (viii) removal of the 2′ phosphate, and (ix) transport from nucleus to cytoplasm. The steps in the splicing-first pathway are (i) production of the transcript, (ii) cleavage at the 3′ splice site, (iii) cleavage at the 5′ splice site, (iv) ligation of the two exons, (v) removal of the 2′ phosphate, (vi) 5′ end trimming by RNase P, (vii) 3′ end trimming, (viii) addition of the -CCA_{OH} tail, and (ix) transport from nucleus to cytoplasm. While removal of the 2′ phosphate must occur after splicing, the exact stage of 2′ phosphate removal is not known, and none of our results address that issue. We also do not know whether addition of the -CCA_{OH} tail is necessary prior to splicing or nuclear export. None of our results address the order of nucleoside modification additions.

Another interpretation of our results is that splicing and end processing may occur concurrently in vivo. The experimental approach that we used to determine the in vivo pathway cannot distinguish this possibility from the end-processing-first or splicing-first pathway. One presumed splicing substrate in a concurrent pathway would be 5′-end-trimmed, 3′-end-extended ptRNA (3′ptRNA). The splicing intermediates and products of this substrate would be identical to those already identified in the end-processing-first and splicing-first pathways. In a similar vein, splicing intermediates could also be substrates for end processing activities without generating distinctive intermediates. Consequently, the concurrent pathway remains a distinct possibility.

We conclude that the events of processing to remove the 5′ leader and 3′ extension are ordered in vivo for *S. cerevisiae*. Our data clearly indicate that processing to remove the 5′ leader may occur without prior removal of the 3′ extension. Furthermore, we do not detect any RNA containing the 5′ leader but lacking the 3′ extension. We suggest that 5′3′ptRNA is the preferred substrate for 5′ end processing by RNase P. The 3′ptRNA is the substrate for an uncharacterized activity that removes the 3′ extension. Finally, the mature-CCA_{OH} tail is added by nucleotidyltransferase. This order is consistent with in vitro transcription and processing results obtained by using soluble yeast extracts and with results of oocyte extract or microinjection experiments (12, 29, 32, 42, 43, 45). Since we are analyzing steady-state RNA for the presence of possible intermediates, one could always argue that the steps of still other alternative pathways are so rapid that the intermediates are undetectably rare. Therefore, we cannot rule out the possibility that 3′ end trimming might sometimes occur prior to 5′ end trimming. Nevertheless, we suggest that there is one definite order of processing to remove the terminal extensions from pre-tRNAs containing introns: 5′ end trimming followed by 3′ end trimming.

The processing activities responsible for end trimming appear to be endonucleolytic. We did not detect a ladder of faint bands between 5′3′ptRNAs and 3′ptRNAs or between 3′ptRNAs and ptRNAs. We could, however, detect presumed nucleotidyltransferase polymerization intermediates for some ptRNA families (Fig. 4). Since pre-tRNA 5′ leaders are trimmed by the known endonuclease RNase P, we do not expect any intermediates between 5′3′ptRNA and 3′ptRNA. However, both endonucleolytic and exonucleolytic activities have been characterized from a variety of sources that are potentially responsible for processing pre-tRNA 3′ extensions (3, 12, 15). It is not yet clear that these purified activities are genuine pre-tRNA maturation nucleases for

removal of the 3′ extension. Our results indicate that in *S. cerevisiae* this processing activity is endonucleolytic, though failure to detect exonucleolytic intermediates does not prove that an endonucleolytic activity is involved. One further observation is that 5′3′ptRNAs are always more abundant than their cognate 3′ptRNA species. This suggests that 3′ end trimming is closely coordinated with RNase P cleavage of the 5′ leader. This could explain why we have not detected 3′ptRNA and 3′mtRNA IleUAU species and can only conditionally detect a 3′ptRNA TyrGUA species (44b).

The existence of a ptRNA IleUAU 5′-2/3 molecule implies that 5′3′ptRNA IleUAU is a substrate for splicing. Identification of the 5′3′mtRNA IleUAU indicates that the 5′-2/3 molecule is a genuine splicing intermediate. In contrast with previously described pathways (9, 43), these results demonstrate that pre-tRNA IleUAU splicing can occur before or after end trimming. RNA species of the appropriate size to be 2/3 or 5′-2/3 molecules were identified in several pre-tRNA families. This finding suggests that the alternate processing pathway is operative for other pre-tRNA families. Our interpretation is supported by previous observations in that HeLa cell extracts were shown to splice some end-extended, intron-containing pre-tRNAs (16, 56, 62). Since end processing need not precede splicing of intron-containing pre-tRNAs, these events are not related in a dependent order.

The proposed pathway indicates that the 3′ splice site is cut first, generating either 5′-2/3 or 2/3 molecules. For the case of pre-tRNA IleUAU, we have shown that there is little or no RNA that could represent splicing intermediates for an alternative order of cleavage. We have not directly addressed whether other pre-tRNAs are spliced by cleavage first at the 5′ splice site. Mutations in *Schizosaccharomyces pombe* tRNA Ser genes have been analyzed for effects on splicing in *S. cerevisiae* extracts (20). Certain mutations interfered with cleavage at either the 5′ or 3′ splice site. This finding provides evidence that the two cleavage events are independent in vitro. It is possible that some pre-tRNA families have splicing intermediates composed of the intron and 3′ exon. However, the splicing intermediates that we detect appear at sizes that are most simply accommodated by splicing consistently proceeding by cleavage first at the 3′ splice site.

As diagrammed in Fig. 10, the initial cleavage event of splicing liberates a free 3′ exon, with or without a 3′ extension. The second cleavage event liberates the free 5′ exon, with or without a 5′ leader. Although we have not yet attempted to detect such products, we expect that end-extended exons exist and are most abundant for families like pre-tRNA IleUAU and under conditions that favor production of the 5′-2/3 molecule. Separate exons have been detected in mutant cells deficient in tRNA ligase activity (50a).

Apparent preference for the splicing-first or end-processing-first pathway varies among ptRNA species and according to growth conditions. The pre-tRNA TyrGUA 2/3 molecule appears to be the second most abundant, yet no 5′-2/3 molecule was detected for TyrGUA (Fig. 2 and Table 2). For IleUAU, TrpCCA, and LysUUU, both 5′-2/3 and 2/3 molecules are clearly evident. These observations suggest that each pre-tRNA may be preferentially processed along one or the other pathway. We have observed that the pre-tRNA IleUAU 5′-2/3 and 2/3 molecules vary in relative abundance, depending on growth temperature (Fig. 7; 44b). This observation suggests that the preferred processing pathway for pre-tRNA is influenced by growth conditions.

We have also noticed that increased levels of 5′3′ptRNA relative to cognate ptRNAs correlate with increased abundance of 5′-2/3 for IleUAU, LysUUU, and TrpCCA (Fig. 2). We believe that this finding indicates that pre-tRNAs IleUAU, LysUUU, and TrpCCA are the three precursors most likely to follow the splicing-first processing pathway. Once spliced, precursors to these tRNAs would no longer be detected with an intron probe. Thus, the relative abundances of the 5′3′ptRNAs for IleUAU, LysUUU, and TrpCCA do not indicate how well these pre-tRNAs are processed by the end trimming activities but indicate the pathway by which they are preferentially processed.

The splicing-first pathway may also represent a dead-end pathway that produces nonfunctional 5′3′mtRNAs. We believe that this possibility is unlikely. tRNA genes from which the intervening sequences have been removed are functional in vivo (30, 57, 67). Conversely, functional tRNA was produced from a tRNA gene in which an intervening sequence was inserted (66). These studies indicate that the intron has little or no effect on end processing of pre-tRNAs in vivo. RNase P cleavage of mutant tRNA LeuCAA gene transcripts has also been studied in vitro (38). Crude RNase P preparations but not highly purified RNase P preparations efficiently end trimmed pre-tRNA LeuCAA transcripts from a gene without an intervening sequence. The structure of a pre-tRNA transcribed from a tRNA gene without an intervening sequence is identical to that of 5′3′mtRNA, a splicing product of 5′3′ptRNA. Therefore, 5′3′mtRNAs must be substrates for the end processing activities. The 5′3′mtRNA produced from the splicing-first pathway would have to be sequestered from nuclear end processing activities for it to be a dead-end product. Sequestration could be accomplished by nuclear-cytoplasmic transport. Implicit in this argument is that the tRNA transport machinery cannot always differentiate between mature and end-extended tRNAs. We believe that this is unlikely, especially since the *Xenopus* oocyte tRNA transport machinery efficiently discriminates against tRNAs with altered structures (61, 68).

Organization of the processing activities. Here we propose a model in which *S. cerevisiae* pre-tRNA processing and transport occur at a nuclear membrane complex. In this model, primary transcripts are bound by tRNA ligase and directed to a pre-tRNA processing complex located on the nuclear membrane. The ligase-pre-tRNA assembly binds to the complex, the pre-tRNA is nucleolytically processed as depicted in Fig. 10, and the tRNA product is transported to the cytoplasm. This model is supported by several observations. First, *S. cerevisiae* pre-tRNA splicing activities apparently work as a complex. The tRNA ligase and pre-tRNA-splicing endonuclease work cooperatively in vitro (18). tRNA ligase has been immunolocalized to a diffuse sphere just on the inside of the nuclear envelope and to distinct spots on the nuclear envelope (5). The pre-tRNA-splicing endonuclease behaves as an integral membrane protein complex (48). The model would explain that the tRNA ligase found in the diffuse sphere binds the nascent pre-tRNA transcripts and translocates them to the pre-tRNA-splicing endonuclease. This accounts for the tRNA ligase located on the nuclear membrane as forming part of the processing complex. Second, pre-tRNA splicing and nuclear-cytoplasmic transport are thought to be coupled processes in *Xenopus* oocytes. Spliced tRNAs from microinjected genes have not been found in the nucleus (42, 43). This result is consistent with our model of a processing-transport complex. Finally, the pre-tRNA end processing activities are presumed to be nuclear enzymes in *S. cerevisiae*, since these

activities are localized to the nucleus of *Xenopus* oocytes (43). Identification of two alternate pre-tRNA processing pathways is consistent with this model. A concurrent end processing and splicing pathway would also be consistent with this model. Our model differs from previously described pre-tRNA splicing complex models (5, 18, 60) in that splicing as well as all other nucleolytic processing events occur in a single complex.

We propose that pre-tRNAs without introns would also be processed by this same complex. Ligation, but not endonucleolytic cleavage of intron-containing pre-tRNAs, is competitively inhibited by mature tRNA in vitro (49). This result is consistent with the ptRNA processing complex model in that tRNA ligase could bind all nascent tRNA gene transcripts and direct them to the processing complex. One prediction of this processing complex model is that pre-tRNA splicing endonuclease and RNase P are part of all processing complexes.

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