tRNA synthesis: identification of in vivo precursor tRNAs from parental and mutant yeast strains

Anita K. Hopper^{1,3} and Janet Kurjan²

¹Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033, and ²Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

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ABSTRACT

In vivo yeast precursor tRNAs have been identified using a modification of the Northern-hybridization procedure. Two species of pre-tRNA^{Tyr}, 1 species of pre-tRNA^{Ser}₂ and 2 species of pre-tRNA^{Ser}_{minor} have been found in all yeast strains examined, including parental strains and strains harboring mutations affecting tRNA function. One of the tRNA^{Tyr} precursors and one of the pre-tRNA^{Ser}₂ are the same size as the unspliced pre-tRNAs which accumulate in the yeast mutant rnal. The in vivo tRNA^{Tyr} precursors detected in these studies also appear similar with the RNA species identified when cloned yeast tRNA^{Tyr} is transcribed and processed by *Xenopus* oocytes and/or *Xenopus* extracts. We have also studied the precursor and mature tRNA^{Tyr} species from 22 mutants which contain mutations in the SUP4 tyrosine-inserting suppressor locus. The RNA from 2 mutants mapping at the G52 position showed an aberrantly migrating "mature" tRNA^{Tyr}. Although several of those cloned mutant genes showed transcript products of altered size in in vitro transcription studies (1), we did not detect such altered transcripts in vivo.

INTRODUCTION

Recent studies of tRNA synthesis have indicated that there are many processing steps necessary for the production of the mature tRNAs. In prokaryotes, production of mature tRNA involves processing of the 5' and 3' termini of the primary transcripts, as well as modification of a number of nucleotides (review, 2). In eukaryotes an additional step is often necessary as indicated by the presence of intervening sequences in a number of eukaryotic tRNA genes (3,4).

The conditional lethal yeast mutant rnal, which is putatively defective in the transport of RNA from the nucleus to the cytoplasm (5), accumulates precursor tRNAs which contain the intervening sequence and have the 5' and 3' termini of mature tRNA (6-10). Assuming a mandatory linear sequences of processing events, these data indicate that removal of intervening sequences from precursor tRNAs follows any 5' and 3' nucleolytic steps and that most tRNA processing occurs in the nucleus. Studies employing *Xenopus in vitro* and oocyte injection systems have

shown that the tRNA^{Tyr} gene is transcribed into an RNA species of about 104 nucleotides which contains 5' leader, 3' trailer, and the intervening sequence (11, 1). Consistent with the data from the yeast mutant, the primary precursor synthesized in Xenopus oocytes is processed to an RNA with mature 5' and 3' termini and which contains the intervening sequence (11, 12).

A large number of mutants within the yeast SUP4 tRNA^{Tyr} gene have previously been isolated, cloned into E. coli, sequenced (13, 1) and analyzed in Xenopus cell extracts and oocyte injection systems. In these systems the mutants show a variety of defects including large reductions in transcription, changes in transcription start or stop points and defects in processing (ref.1; DeRobertis, personal communication).

Although transcription and processing of cloned heterologous tRNA genes by Xenopus oocytes or extracts appears to proceed overall with fidelity (11, 14-16), there may be some differences between in vivo homologous and "in vitro" heterologous transcription and processing. For example, for a Bombyx mori tRNA^{Ala}₂ gene, the 5' flanking sequences are not essential for transcription using Xenopus oocyte extracts. However, they were shown to be essential for transcription by the homologous in vitro system (17). It has also been found that Xenopus oocytes add a modification (the Q base) to the yeast tRNA^{Tyr} which is normally not present and fails to add a modification (Gm) which is normally present in the yeast tRNA (18). Therefore, it is desirable to compare results from the Xenopus systems with the in vivo precursors to yeast tRNAs.

We have examined the in vivo RNA from wild type and several mutants using the Northern-hybridization procedure (19, 20) to detect RNAs complementary to clones containing tRNA^{Tyr}, tRNA^{Ser}₂, and tRNA^{Ser}_{minor} sequences. Using this approach we have been able to identify in vivo yeast pre-tRNAs and compare them to the species detected in the Xenopus systems. We report that this is an easy alternative method to heterologous transcription and processing systems to identify tRNA precursors and aberrant tRNAs in wild-type and mutant cells.

MATERIALS AND METHODS

Yeast Stains and Methods: The yeast strains and procedures for growing yeast were as previously described (6, 13, 21, 22).

Recombinant DNA Clones: All tRNA gene-containing recombinant DNA clones were obtained from M.V. Olson (Washington University School of Medicine, St. Louis). PYT-G contains the tRNA^{Tyr} sequence on a 1.2 kb insert of yeast

DNA into plasmid pBR322 (3). The 1.2 kb insert does not contain any tRNA genes other than tRNA^{Tyr} (23). pPB10 contains the tRNA₂^{Ser} sequences on a 0.45 kb fragment of yeast DNA which has been shown not to contain any tRNA sequences other than tRNA₂^{Ser} (24). pPM5 contains the tRNA^{Ser}_{UGG} sequence on a 2.55 kb fragment. pPM16 contains a tRNA^{Ser}_{UCA} sequence on a 1.1 kb fragment. It has not been determined whether pPM5 and pPM16 contain any other tRNA sequences (Olson, Personal Communication). The tRNA^{Ser}_{minor} species, tRNA^{Ser}_{UGG} and tRNA^{Ser}_{UCA}, are extensively homologous and cross hybridize (25,10,29). The tRNA^{Ser}_{minor} and tRNA₂^{Ser} differ by 11 nucleotides, but do not cross hybridize (25). tRNA^{Tyr} does not hybridize to any of the tRNA^{Ser} genes (3, 23, 24, 25).

Resolution of RNA and Electrophoretic Transfer to DBM-paper: RNA was extracted by the Holley procedure (26) and resolved on 3.5-15% linear gradient polyacrylamide gels in 7M urea as previously described (6, 21). 10x20 cm pieces of ABM-paper (Schleicher and Schuell) were diazotized (19). During diazotization the gel was equilibrated in 0.05 M NaAc, pH 5.0. The equilibrated gel was placed directly onto the diazotized paper; a layer of porous polyethylene was placed above and below the paper and gel and approximately 7 cm of 3 MM paper (presoaked in 0.05 M NaAc buffer) was placed atop. RNA was electrophoretically transferred to the paper (27) by using a BioRad 1200 destaining chamber. Transfer proceeded at 200 mA ($\sqrt{9}$ V) for 2 hr. Greater than 50% of the RNA was transferred from the gel.

DNA Isolation, Nick Translation, Hybridization, and Autoradiography: Plasmid DNA was prepared by standard procedures. ³²P DNA probes (2×10^7 cpm/ μ g) were labeled in vitro by the nick translation procedure (28) using α -³²P-dCTP (New England Nuclear) and nick translation reagents from Bethesda Research Laboratories. Prehybridization and hybridization buffers ($\sim 100 \mu$ /cm² Northern paper) (5xNaCl/Cit/5x Denhardt's reagent/10% dextran sulfate) were as previously described (20). Hybridization ($\sim 2.5 \times 10^{-2}$ μ g/ml ³²P DNA) proceeded for 16 or 2 hr. at 35.5°C. Following hybridization the paper was washed extensively with 0.03M sodium citrate, 0.3M NaCl, pH 7.0 at room temperature. Autoradiography using Kodak X-Omat R film and Dupont Cronex Xtra Life intensifying screens proceeded at -70°C for 5-25 hr. For fluorography the paper was sprayed with 6% PPO in ether and then exposed to Kodak X-Omat film at -70°C for 1-3 days.

RESULTS

Precursor tRNAs can be detected in RNA isolated from SUP4 cells: The "Northern" procedure detects, by hybridization, specific fractionated RNA

sequences covalently bound to diazobenzyloxymethyl-paper (19, 20). Using a modification of this procedure (27) which allows the transfer of highly resolved small RNAs from nonporous polyacrylamide gels to "Northern" paper, we have studied the pre-tRNAs synthesized by wild-type and mutant yeast cells.

RNA from cells was resolved on polyacrylamide gels, transferred electrophoretically to diazotized paper, and hybridized to ^{32}P DNA (labeled in vitro by the nick translation procedure (28)) from recombinant genomic clones containing tRNA genes. When the DNA from a clone containing a tRNA^{Tyr} gene sequence (pYT-G; ref. 3) was hybridized to RNA from SUP4 yeast cells by the Northern procedure, 3 bands of hybridization were evident (Fig. 1A, lane c). All three bands could be competed out with excess cold purified tRNA^{Tyr} (data not shown). Since it appears that there is a direct relationship between migration in 7M urea gradient polyacrylamide gels and RNA length (Fig. 2), we can estimate the length of the three bands to be 78, 92, 108 nucleotides. The 78 and 92 nucleotide bands correspond in size with, respectively, mature tRNA^{Tyr} and the intervening sequence-containing 92 nucleotide pre-tRNA^{Tyr} which accumulates in rna1 and los1-1 cells (6, 7, 21) and which is detected by the Xenopus studies (11, 12). The Xenopus studies detected a minor tRNA^{Tyr} intermediate migrating at 97 nucleotides (11, 12); we have not detected this species in in vivo RNA.

Transcription by Xenopus oocytes using cloned DNA from 3 of the 8 tRNA^{Tyr} genes indicated that the primary transcripts of these genes vary from ~102-108 nucleotides in length. In contrast to these results, we do not see heterogeneity of the largest RNA homologous to tRNA^{Tyr} (~108 nucleotides); this band does not appear to be more disperse than either the 92 or 78 nucleotide band (Fig. 1A, lanes c-h). However, each of these bands is somewhat broad. This broadness does not appear to be due to poor electrophoretic resolution nor to diffusion of RNA species during transfer to Northern paper, since in vivo ^3H -ura labeled total RNA which was co-electrophoresed, co-transferred to Northern paper, and exposed to hybridization conditions showed well resolved RNA patterns (Fig. 1A, lanes j,k).

Similar studies have been performed using cloned DNA from the yeast tRNA₂^{Ser} and tRNA_{minor}^{Ser} genes (pPB10 ref. 24 and pPM5, ref. 25). Two bands of hybridization at approximately 85 and 102 nucleotides were detected using the tRNA₂^{Ser} probe (Fig. 1A, lanes k-o; Fig. 2). Presumably, the 102 species is a precursor to mature 85 nucleotide tRNA₂^{Ser}. The tRNA_{minor}^{Ser} species, tRNA_{UGG}^{Ser} and tRNA_{UCA}^{Ser}, are extensively homologous and cross hybridize (10, 25, 29); however, the tRNA_{UGG}^{Ser} gene contains an intervening sequence whereas at least one of the three tRNA_{UCA}^{Ser} genes does not (10, 25). Using either DNA homologous to

Fig. 1: Northern hybridization of RNA from various yeast strains using ^{32}P DNA probes for tRNA^{Tyr}, tRNA^{Ser} and tRNA^{UCG}.

RNA from various yeast strains was extracted, resolved on polyacrylamide gels, transferred to DBM-papers, and hybridized with ^{32}P DNA probes as described in Materials and Methods.

A. ^{32}P DNA probes were as follows: Lanes, a-h, pYT-G (tRNA^{Tyr}); k-o, pPB10 (tRNA^{Ser}); p-q, pPM5 (tRNA^{UCG}). Hybridization proceeded for 16 hours.

RNA samples were as follows: Lane (a) $\sim 190,000$ cpm semi-degraded ^{32}P tRNA from *E. coli* (gift R. Coleman) (b) ≤ 0.5 μg , ^3H -ura labeled RNA from rnal cells, (c) 10^{-6} μg RNA from the SUP4 strain (J15-8d); lanes (d-h) 10^{-6} μg RNA each from loss of suppressor strains: (d) mutation in the intervening sequence, (e) A40, (f) C62, (g) U21, (h) C62 (lanes f and h contain the RNA from 2 independent mutations to C62); lanes (i-j), fluorography of lanes a and b; lane (k), fluorography of ≤ 0.5 μg tRNA from rnal cells; lanes (l and p) 10^{-6} μg RNA from the los2 strain; lanes (m and q) 10^{-6} μg RNA from J15-8d; lanes (n and o) longer exposure of lanes l and m.

B. A similar experiment to A using different preparations of RNA and newly labeled DNA probes. Hybridization proceeded for 2 hr. Lanes (a-d) were hybridized with the tRNA^{Tyr} probe. Lanes (a,b) RNA from the mutant SUP4 strains C62 and A52; lane (c) RNA from the los2 strain; lane (d) RNA from the SUP4 strain. Lanes (e,f) were hybridized using the tRNA^{Ser} probe. Lane (e) RNA from los2; lane (f) RNA from the SUP4 strain.

Fig. 2: Migration of RNAs vs. log number of nucleotides. Data are derived from Fig. 1 and from the straining pattern of 5.8S, 5S and tRNA species remaining in the gel after transfer to Northern paper. The top of the Northern paper was used as the origin for measurement of migration distances. ●, 5.8S (158 nucleotides, ref. 33) and 5S (120 nucleotides, ref. 34). (5.8S and 5.0S RNAs were detected by fluorography and staining). RNAs were detected by hybridization: ▲, tRNA^{Tyr} (78 nucleotides, ref. 35); △, presumptive pre-tRNA^{Ser}₂; ■, tRNA^{Ser}₂ (85 nucleotides, ref. 36); ◻, presumptive pre-tRNA^{Ser}₂; ◻, tRNA^{Ser}_{UGG} (85 nucleotides, ref. 10, 25); ◻, presumptive pre-tRNA^{Ser}_{UGG}.

tRNA^{Ser}_{UGG} or to tRNA^{Ser}_{UCA} three bands of hybridization at approximately 85, 104 and 120 nucleotides were detected (Fig. 1A, lanes p,q; Fig. 1B, lanes e,f; Fig. 2; data not shown for tRNA^{Ser}_{UCA}). It is not known whether any other tRNA sequences are contained on the tRNA^{Ser}_{UGG} and tRNA^{Ser}_{UCA} recombinant DNA clones; however, since both of these clones (which are derived from genes on different yeast chromosomes, ref. 25) showed the same hybridization pattern, it is likely that all 3 species detected by hybridization represent tRNA^{Ser}_{minor} sequences. The small species corresponds with the expected migration for tRNA^{Ser}_{minor} and the ~104 species with the tRNA^{Ser}_{UGG} precursor containing an intervening sequence which accumulates in the rna1 mutant (10). It is not known if the 120 species is a precursor to the tRNA^{Ser}_{UGG} or to tRNA^{Ser}_{UCA}.

tRNA species synthesized by mutant yeast cells: There are two kinds of

mutations which can affect tRNA synthesis: (1) mutations with lesions in a structural or surrounding region of a tRNA gene and (2) mutations in genes whose function is necessary for proper tRNA synthesis or function. In yeast the tRNA genes are redundant; for example, there are eight genes encoding tRNA^{Tyr} (30, 27). The Northern hybridization results must reflect the transcription and processing of products from all eight of these loci. Assuming equal transcriptional outputs for all eight loci, a mutant with a lesion in only one of these eight genes should affect the synthesis of only 1/8 of the gene products and one would not detect alterations in the hybridization profile if the mutant in question resulted in a loss of transcription or a relatively small decrease in the rate of processing one of the intermediates. However, if a mutation caused a disproportionate accumulation of the 108 or 92 nucleotide species, the accumulation of pre-tRNA not detected in wild-type cells, misprocessing of a tRNA precursor, or aberrant transcription resulting in a transcript of altered size, this might be detectable in our studies.

Twenty-nine of the cloned SUP4 tRNA^{Tyr} mutant genes were transcribed in vitro by Xenopus oocytes; mutations at U15, U21, -A36A37, A51, A52, G56, U56, C62, -C(63-67), and a mutation at the 4th nucleotide of the intervening sequence all resulted in transcripts of faster mobility on polyacrylamide gels (1). In at least two cases (-A36A37 and the intervening sequence mutation) the altered mobility is due to premature termination at TA tracts resulting from the mutation (1). Northern hybridizations were performed on the RNA isolated from SUP4 mutants with lesions U3, G3, U6, U10, G14, U15, U21, G27, U29, A32, G32, G37, A40, A45, A52, G60, C62, -C(63-67) U67, C68, G73, and the mutation in the intervening sequence. In all but two cases the results of the hybridization experiments, using the tRNA^{Tyr} probe, showed the same 3 bands for SUP4 and mutant RNAs (Fig. 1, data shown for positions 21 (Fig. 1A, lane g), 40 (Fig. 1A, lane e), 52 (Fig. 1B, lane b), and the intervening sequence mutation (Fig. 1A, lane d). In no cases, even with long exposures of the hybridized Northern paper to film (not shown), were transcripts of altered mobility observed. The RNA from two mutants with lesions in the SUP4 locus at the G62 position (both C62; ref. 13) reproducibly produced a 4th band migrating slightly faster than mature tRNA^{Tyr} (Fig. 1A, lanes f,h; Fig. 1B, lane a). A mutation at the C52 (A52) position, which normally base pairs with G62, did not result in similarly altered migration (Fig. 1B, lane b).

Mutants in the second category, (that is, mutations which affect tRNAs, but do not map at a tRNA locus) have also been described. The los1 and rnal mutations cause the accumulation of pre-tRNAs which contain intervening

sequences and the los2 mutation causes an altered migration of a few bands of RNA in the mature tRNA region in a polyacrylamide gel (21, 22). Since this type of mutant contains alterations in gene products necessary for tRNA synthesis or expression they cannot be studied in heterologous in vitro systems. One might expect that the los1 mutation would cause an increased ratio of the intervening sequence-containing precursors to mature tRNA species and that this altered ratio could be detected by hybridization. However, the ratios of the precursors to mature tRNA in SUP4 cells has been somewhat variable (Fig. 1) making a quantitative analysis of differences difficult. Since the los2 mutation simultaneously causes the alteration of migration of a few tRNAs and a loss of suppression of a tyrosine-inserting suppressor, one might expect the migration of tRNA^{Tyr} to be altered in polyacrylamide gels. However, no reproducible difference was detected in the migration of tRNA^{Tyr} (Fig. 1B, lanes c,d), tRNA^{Ser}₂ (Fig. 1A, lanes l-o), or tRNA^{Ser}_{minor} (Fig. 1A, lane p,q; Fig. 1B lanes e,f) for RNA extracted from los2 and SUP4 was detected.

DISCUSSION

The ability to identify pre-tRNAs of tRNA^{Tyr}, tRNA^{Ser}₂ and tRNA^{Ser}_{minor} even in wild-type cells indicates that Northern hybridizations will be useful for the identification of other precursor tRNAs in yeast and in other organisms. It was surprising that pre-tRNAs could be so easily detected. The ratio of precursor species to mature tRNA species seemed peculiarly high in consideration of the fact that these pre-tRNA are not detected in pulse-chase labeling studies of yeast RNA (6). This is not due to preferential transfer of larger RNA species to diazotized paper, since our results show that very small polynucleotides are slightly more efficiently transferred than are larger polynucleotides (data not shown). It is still possible, however, that the ratios obtained by the Northern procedure do not reflect the in vivo quantities of RNA. For example, it has been observed that nonhomologous RNA migrating in the region of homologous RNA can inhibit binding of the DNA probe (32); perhaps the presence of other tRNAs in the tRNA region of the gels inhibits the binding of DNA probe to specific tRNAs.

The Northern procedure should potentially allow investigations of the effect of mutant genes upon the synthesis of individual species of tRNA. Our identification of a mutant (C62) with an altered tRNA^{Tyr} species demonstrates this point. Sequencing studies should determine whether the aberrant migration of this mutant tRNA^{Tyr} results from altered sequence, modification, or transcription. However the presence of pre-tRNAs in wild-type cells complicates the study of

mutants. Only products from genes containing a mutation which results in a size or migration change in the products or a large excess of product will be distinguishable from wild-type. Our failure to detect the in vivo shortened transcripts synthesized in vitro (1) from mutant tRNA^{Tyr} genes may be due to a difference between heterologous and homologous transcription and processing or may result from degradation of abnormal transcription products in vivo. Resolution of this will probably await establishment of a homologous yeast in vitro transcription system.

The major limitation of using the Northern hybridizations to study tRNA intermediates is that there is no obvious easy means to determine the sequence of the identified precursors. This is due to the very small quantities of these species; we estimate that there is 0.002-0.02 µg of the pre-tRNA^{Tyr} species per gel slot.

The in vivo species of RNA homologous to tRNA^{Tyr} identified by these studies coincide in estimated size to the pre-tRNAs which accumulate in the rnal and los1 yeast mutants and to the three major bands detected by Xenopus studies (11, 1). Although not proven, it seems likely that they are identical in nucleotide sequence. This again points to the conclusion that Xenopus transcription and processing enzymes can synthesize yeast tRNAs with fidelity. In addition, we have identified two tRNA^{Ser} precursors which had not been previously reported -- the 102 nucleotide precursor tRNA^{Ser}₂ and 120 nucleotide precursor to either tRNA^{Ser}_{UCC} or tRNA^{Ser}_{UCA}. Thus, our results confirm and complement many of the results from the Xenopus systems and indicate that although it has certain limitations, the Northern procedure will be a useful tool in the further studies of tRNA processing.

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³To whom correspondence should be addressed.

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