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Sequence and expression of four mutant aspartic acid tRNA genes from the mitochondria of *Saccharomyces cerevisiae*

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ABSTRACT

Expression of the mitochondrial $tRNA^{Asp}$ gene of *Saccharomyces cerevisiae* has been examined in five *syn*$^-$ mutants known to affect $tRNA^{Asp}$ function, and in a *rho*$^-$ mutant which accumulates precursor tRNAs. By comparison of wild-type versus mutant DNA sequence, the lesion in each *syn*$^-$ mutant has been identified as a single base change within the mitochondrial $tRNA^{Asp}$ structural gene. The mutant $tRNA^{Asp}$ genes are transcribed, and the transcripts can be processed to mature 4S-size $tRNA^{Asp}$. The steady-state level of each mutant $tRNA^{Asp}$ is lower than that of wild-type $tRNA^{Asp}$. The RNA from two of the *syn*$^-$ mutants contained a second, slow-migrating form of mitochondrial $tRNA^{Asp}$ which is correctly processed at the 5' end. We conclude that the lesions in the *syn*$^-$ mitochondrial $tRNA^{Asp}$ genes block neither transcription of these genes, nor 5'-end processing of the transcripts. The effect of each point mutation must be manifested at the level of 3'-end processing, or at a functional level.

INTRODUCTION

The mitochondrial genome of *Saccharomyces cerevisiae* encodes a complete set of transfer RNAs essential for intra-mitochondrial protein synthesis. Of the 24 tRNA genes, 16 are clustered in a 9 kilobase region between the genes encoding the 21S rRNA and cytochrome oxidase subunit 2. The remainder are scattered throughout the mitochondrial genome among genes which encode two ribosomal RNAs and a number of proteins. Mutations in any gene, nuclear or mitochondrial, encoding a component of the mitochondrial translational apparatus can cause defects in mitochondrial protein synthesis. If such a mutation occurs in a nuclear gene, the mutants are designated *pet*$^-$; if in a mitochondrial gene, *syn*$^-$. Both classes have a respiratory deficient phenotype because the translational machinery containing the mutant component is unable to translate some or all mitochondrial mRNAs encoding proteins of the respiratory chain. Absence of mitochondrial protein synthesis has been demonstrated directly for the *syn*$^-$ mutant FF1210-170 whose lesion is in the $tRNA^{Asp}$ gene (1) and indirectly for the *syn*$^-$ mutant G116-40 whose

lesion is in the $tRNA^{Tyr}$ gene (2). Characterization of a *syn*$^-$ mutant mapping to $tRNA^{Ser}_{UCN}$ revealed that mitochondria from this strain did synthesize several proteins including *var*1, cytochrome oxidase subunits 2 and 3, and cytochrome b, but did not make cytochrome oxidase subunit I (3). Of these mitochondrial proteins, only cytochrome oxidase subunit I uses the codon UCC for serine. This observation leads us to suggest that a U-C match in the wobble position of the codon-anticodon interaction was not allowed by the mutant tRNA whereas an A-U or U-U was. In any case, it is clear that mutations in mitochondrial tRNA genes can cause at least two types of mitochondrial protein synthetic deficiencies.

Evidence accumulated by a number of investigators shows that yeast mitochondrial tRNAs are generally transcribed as multigenic precursors that may also contain other tRNAs, rRNAs or mRNAs (4-10). These precursor tRNAs are endonucleolytically processed at the 5' and 3' ends to form mature 4S-size tRNAs. Other processing steps include the addition of CCA at the 3' end and modification of nucleotides. The precise temporal sequence in which these steps occurs is undetermined. Our interest in the biosynthesis of mitochondrial tRNA led us to examine the possibility that expression of tRNAs is altered in known *syn* mutants. It has been shown that mutations in tRNA genes from many organisms alter the transcription and RNA processing of their tRNA products. We reasoned that a lesion in a *syn* gene which disrupted any of the steps in tRNA biosynthesis would affect tRNA function and thereby impair or abolish mitochondrial protein synthesis. *Syn*$^-$ mutants mapping to $tRNA^{Cys}$, $tRNA^{His}$, and $tRNA^{Val}$ have been isolated, but only one mutant per locus has been identified (11-14). In contrast, five $tRNA^{Asp}$ mutants have been reported and we chose to examine this series of mutant strains.

One of these $tRNA^{Asp}$ mutants, FF1210-170, has been examined in some detail by Miller *et al*. (1) who reported a single base change within the structural $tRNA^{Asp}$ gene. No mitochondrial protein synthesis was detected in FF1210-170 mutants although a mature-sized $tRNA^{Asp}$ was observed in Northern blot experiments. We have expanded this study to include four other mitochondrial $tRNA^{Asp}$ mutants (Table I). Each mutant has been previously mapped genetically to the region of the mitochondrial genome known to contain the $tRNA^{Asp}$ gene (11,13,14). Each is defective in accepting aspartic acid in vitro. Our findings regarding the identification of the lesion within each gene as well as analyses of transcription from these genes and processing of these transcripts are reported here.

MATERIALS AND METHODS

Yeast Strains and Media

The *syn*$^-$ strains and parental wild-type strains used in this work are described in Table I. The yeast cells were grown in the following media: YPD, 1% yeast extract, 1% bactopeptone, 2% or 10% glucose (as indicated); YPG, 1% yeast extract, 1% bactopeptone, 0.2% glucose, 2% galactose.

Preparation of Nucleic Acids

Mitochondrial DNA was prepared from yeast cells grown to mid-log phase in YPG. The cells were harvested by centrifugation, washed with water and resuspended at 1 ml/g wet weight in 0.6M mannitol, 20mM tris-chloride (pH7.4), 5mM EDTA (MTE buffer). Cells were broken in a glass bead mill (Bronwill) by shaking for 2 min. with CO_2 as coolant. The broken cell suspension was centrifuged at 1200 X g for 10 min. Mitochondria were harvested from the supernatant by centrifugation at 27,000 X g for 10 min. The mitochondria were resuspended in MTE buffer and the centrifugations were repeated. DNA was prepared from the mitochondria by density gradient centrifugation in $CsCl_2$ in the presence of the DNA-binding dye bisbenzimide (Hoechst 33258) (16).

Mitochondrial RNA was isolated from yeast cells grown and harvested as above. Cells were washed in 4 ml/g wet weight of 1.35M sorbitol, 0.1mM EDTA, 10mM tris-chloride (pH7.5). Zymolyase 60,000 (Miles Scientific) was added to a concentration of 0.5 mg/g original wet weight. This mixture was agitated gently at 28° for 1-2 hours. Spheroplasts were pelleted by centrifugation and resuspended in 0.7M sorbitol, 0.1mM EDTA, 0.1% bovine serum albumin, 50mM tris-chloride (pH7.5) at 6 ml/g original wet weight. Spheroplasts were broken by homogenization for 30 sec. in a Waring Blendor. Mitochondria were harvested by differential centrifugation as described above for DNA preparation. RNA was prepared by a variation of the phenol extraction method of Locker (17). Mitochondria were resuspended in 10mM tris-chloride (pH7.4), 2mM EDTA, 0.1% macaloid, 0.2% SDS and extracted with an equal volume of phenol. The aqueous phase was recovered and stored at -70°.

Whole-cell small RNAs were obtained by a modification of the phenol extraction method of Holley (18). Yeast cells, harvested and washed as above, were resuspended at 2 ml/g wet weight in 10mM tris-chloride (pH7.5). An equal volume of equilibrated phenol was added and the mixture was agitated overnight. The aqueous phase was recovered and stored at -70°.

Purified DNA was treated with commercially-available restriction

endonucleases according to the manufacturer's recommendations (New England Biolabs, Bethesda Research Labs). Recombinant pBR322 molecules containing a copy of each mutant $tRNA^{Asp}$ gene were constructed by ligating mitochondrial HpaII fragments into the plasmid pBR322 at the ClaI site according to standard procedures (19). Appropriate clones were identified by *in situ* hybridization (20) using the wild-type $tRNA^{Asp}$ gene as probe (21).

Oligonucleotide probes were radiolabelled with [γ-^{32}P]ATP using polynucleotide kinase (New England Biolabs, 5 units) in a 20µl solution containing 20 pmoles oligonucleotide, 50mM tris-chloride (pH9.5), 10mM $MgCl_2$, 0.1mM spermidine, 5mM dithiothreitol. After incubation for 30 min. at 37°, the reaction was stopped by heating at 65° for 10 min. Removal of unincorporated radionucleotides was achieved by cetylpyridinium bromide precipitation (22).

DNA Sequence Analysis

The DNA sequences were determined by chemical cleavage of radiolabelled DNA fragments (23). Fragment isolation, labelling and gel electrophoresis were described previously (24).

Gel Electrophoresis and Filter Hybridization

RNA samples were analyzed by electrophoresis on 40 cm discontinuous buffer acrylamide-urea gels. The 34 cm separation gel contained 4M urea, 10% acrylamide:bisacrylamide (25:1) in 89mM tris, 89mM borate, 25mM EDTA, pH8.3 (TBE buffer). The 6.5 cm stacking gel contained 4M urea, 5.4% acrylamide:bisacrylamide (19:1) in 0.8mM tris-chloride (pH6.7). Samples were precipitated by ethanol and resuspended in 30µl of sample buffer (50mM Na-acetate pH4.5, 8M urea, 30% sucrose, 0.1% xylene cyanol). Gels were run for 20-24 hrs. at 400V, 65mA in TBE buffer. Gels were stained and urea was removed simultaneously by soaking the gel in a solution of 1µg/ml ethidium bromide, 50mM Na-phosphate (pH5.5) for 30 min. at 4°. RNA was transferred electrophoretically to DBM paper (25) prepared by activation of ABM paper (BioRad) or to ZetaProbe Membrane (BioRad) according to manufacturer's recommendation.

Filters were prehybridized in 6 X SSC [3M NaCl, 0.3M Na-citrate], 5 X Denhardt's solution (26), 0.5% SDS, 100µg/ml herring sperm DNA, at 42° for 4 hrs. Hybridization to the oligonucleotide $tRNA^{Asp}$ probe was performed in the same buffer at 42° overnight.

Primer Extension Analysis

Four pmoles of ^{32}P radiolabelled synthetic oligonucleotide (5'-GCACTGACATCCTCC-3') complementary to the 3' half of wild-type mitochondrial $tRNA^{Asp}$ were annealed to 60µg of mitochondrial or whole-cell

RNA by incubation at 70° for 5 min. in 5µl of 400mM NaCl, 80mM tris-chloride (pH8.3) buffer. After a cooling period, the mixture was adjusted to 6mM $MgCl_2$, 20mM dithiothreitol and 5mM each dATP, dGTP, dCTP, and dTTP in a final volume of 20µl. For unextended samples, a 4µl aliquot of this mixture was adjusted to 170mM NaOH and incubated at 65° for 10 min. For RNase A treated samples, a second 4µl aliquot was brought to 80µg/ml RNase A and incubated at room temperature for 30 min. To this and to the remaining 12 µl of the RNA/oligonucleotide/dNTP solution was added 2 units and 5 units of AMV reverse transcriptase (Life Sciences) respectively. Both mixtures were incubated at 42° for 30 min., then adjusted to 170mM NaOH and incubated at 65° for 10 min. An aliquot of each sample underwent electrophoresis next to a sequence ladder generated by the Sanger method using $tRNA^{Asp}$ oligonucleotide as primer, a cloned copy of the wild-type $tRNA^{Asp}$ as template, a commercially-available sequencing kit (Amersham), and [α-^{35}S] dATP (New England Nuclear Corp.). Samples were run on 8% gels which were fixed and dried (27) prior to autoradiography.

RNase P Assays

Each assay was performed with 30µg mitochondrial RNA in a 50µl solution containing 50mM tris-chloride (pH8), 50mM ammonium chloride, 6mM $MgCl_2$, and yeast mitochondrial RNase P (generous gift of Dr. M. Hollingsworth) or *E. coli* RNase P (generous gift of Dr. S. Altman). The reaction mixtures were incubated at 37° for 30 min., then extracted with an equal volume of phenol:$CHCl_3$(1:1), and again with $CHCl_3$. The RNA was concentrated by precipitation with ethanol. Products of the reactions were examined by gel electrophoresis and Northern blot analysis or by primer extension.

RESULTS

Characterization of Lesions in Mutant Mitochondrial $tRNA^{Asp}$ Genes

Five mitochondrial $tRNA^{Asp}$ mutants were analyzed. Table I lists each *syn*$^-$ strain and relevant genetic information. For each mutant, a 700 base-pair HpaII restriction fragment carrying the $tRNA^{Asp}$ gene was isolated from mitochondrial DNA and inserted into the unique ClaI site of the plasmid pBR322. Sequence analysis of these genes as well as approximately 150 bases of both 5' and 3' flanking regions revealed a single base change within the structural gene itself when compared to the wild-type mitochondrial $tRNA^{Asp}$ gene sequence (21). The deduced cloverleaf structure for the wild-type $tRNA^{Asp}$ gene with the change identified for each *syn*$^-$ mutant, including that previously published for FF1210-170, is indicated in Figure I. In only one

TABLE I
Sources of Strains

Syn⁻ Strain	Genotype	Wild-Type Parent	Reference
FF1210-170	α ura *rho*⁺	FF1210-6C	11
aM551-50	α ade *rho*⁺ ω⁺ *syn*⁻	D273-10B	13
aM516-45	α ade *rho*⁺ ω⁺ *syn*⁻	D273-10B	13
M737	α *rho*⁺ ω⁺ *syn*⁻	D273-10B/A1	14
ts832	α ura *rho*⁺ *syn*⁻	FF1210-6C	11

case, that of 516-45, does the change affect a conserved position in the tRNA.

Based on the DNA sequence information, the mitochondrial DNA from the strains 551-50 and M737 should contain an ScaI restriction enzyme site (5'-AGTACT-3') within the $tRNA^{Asp}$ gene that is not present in parental DNA. The presence of this restriction site was established by restriction digestion of mitochondrial DNA isolated from the *syn*⁻ strains in question, and the pBR322 plasmids containing a copy of each mutant gene. The digested DNAs were fractionated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized to an oligonucleotide probe complementary to the 3' half

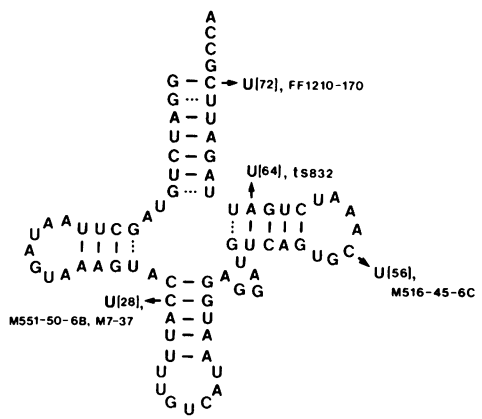

Figure 1. *Deduced cloverleaf structure of mitochondrial* $tRNA^{Asp}$. The structure shown is deduced from the DNA sequence of the wild-type $tRNA^{Asp}$ (21). Arrows indicate changes determined in this study for each *syn*⁻ mutant, plus that previously published for FF1210-170 (1). Numbering is according to the system proposed by Gauss and Sprinzl (28).

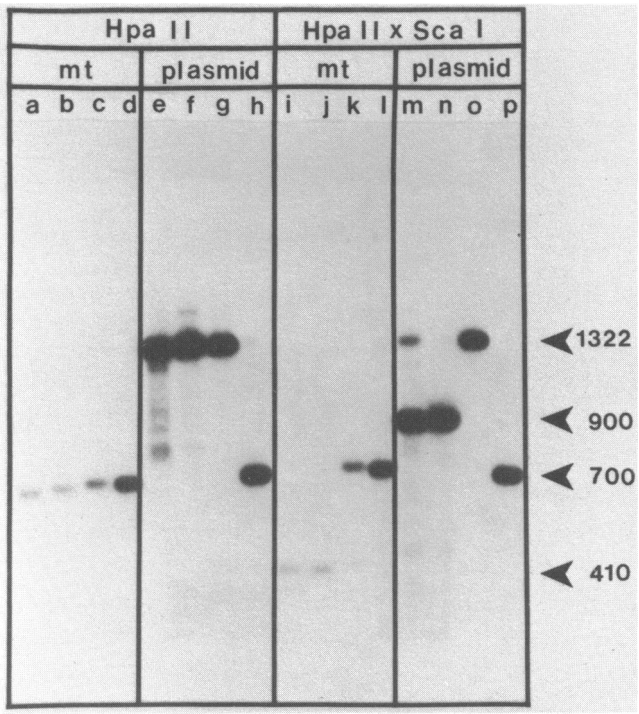

Figure 2. *Southern blot analysis of the tRNA^{Asp} gene in mitochondrial and plasmid DNAs.* Mitochondrial DNA was isolated from strains 551-50 (lanes a and i), M737 (lanes b and j), 516-45 (lanes c and k), and FF1210-6C (lanes d and l). Plasmid DNAs are pBR322 recombinants containing a single copy of the tRNA^{Asp} gene within either (1) a 700 base-pair HpaII fragment of mitochondrial DNA isolated from strain 551-50 (lanes e and m), M737 (lanes f and n) or ts832 (lanes g and o); or (2) a 2.5 kilobase-pair fragment of mitochondrial DNA isolated from wild-type strain D273-10B (lanes h and p). DNAs were treated with the indicated restriction enzyme(s), separated on a 1.5% agarose gel and transferred to nitrocellulose. A ^{32}P radiolabelled oligonucleotide complementary to tRNA^{Asp} was hybridized to the filter-bound DNA. Autoradiographic results are shown above. Fragment sizes are indicated at right.

of tRNA^{Asp}. In all strains tested, the probe hybridized to a 700 base-pair HpaII fragment of mitochondrial DNA (Fig. 2, lanes a-d), and a 1322 or 700 base-pair fragment of a recombinant DNA containing mitochondrial DNA cloned into pBR322 DNA (Fig. 2, lanes e-h). The 1322 base-pair HpaII fragment is a hybrid pBR322-mitochondrial DNA fragment that results from inserting a mitochondrial HpaII fragment into the ClaI site of the plasmid. In such a junction, the HpaII recognition site is not reconstructed. A 700 base-pair fragment is observed in the wild-type sample (lane h) because the insert

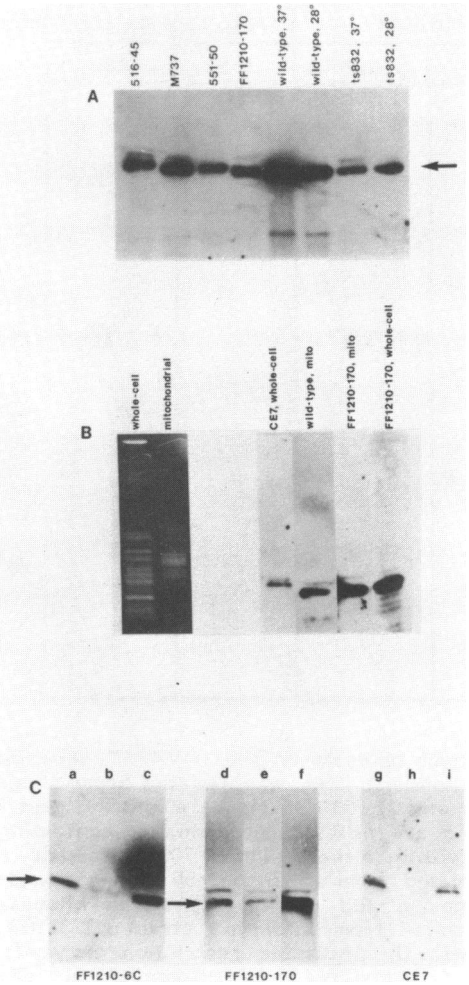

Figure 3. *Northern blot analysis of tRNA^{Asp} gene expression.* A) Whole-cell small RNAs were isolated from the indicated *syn*$^-$ or wild-type strain grown in YPG at 28° unless otherwise indicated. Samples representing RNAs obtained from equivalent quantities of cells were fractionated on discontinuous buffer acrylamide-urea gels and transferred to DBM paper. The filter-bound RNAs were hybridized to a ^{32}P radiolabelled tRNA^{Asp} oligonucleotide. Results of autoradiography are shown. The position of mature tRNA^{Asp} migration is indicated (←). B. Whole-cell small RNAs obtained from strains CE7 or FF1210-170 (grown in YPG) and mitochondrial RNA extracted from isolated FF1210-170 or wild-type mitochondria were analyzed as in A. At left is shown the ethidium bromide staining pattern typical of RNAs run in this gel system. C. Whole-cell small RNAs were extracted from the indicated wild-type, *syn*$^-$ or *rho*$^-$ strains grown in YPD-2% glucose (lanes a,d, and g), YPD-10% glucose (lanes b, e, and h) or YPG (lanes c, f, and i) and analyzed as in A.

carried in this recombinant is a large mitochondrial fragment in which the 700 base-pair HpaII fragment is contained. Digestion of this recombinant DNA with HpaII releases a 700 base-pair fragment containing only mitochondrial DNA. When the same DNAs were treated with both HpaII and ScaI restriction enzymes, hybridization to the 700 base-pair fragment was still observed for the mitochondrial DNA isolated from the wild-type parental strain and the *syn*$^-$ strain 516-45 which carries a lesion that does not generate an ScaI site (Fig. 2, lanes k and l); and to a 1322 or 700 base-pair fragment of the respective plasmids (Fig. 2, lanes o and p). However, hybridization to the 700 base-pair fragment was not observed for mitochondrial DNAs isolated from the strains 551-50 or M737, nor to the 1322 base-pair plasmid fragment. Instead, hybridization to a smaller fragment of 410 base-pairs for mitochondrial DNA (Fig. 2, lanes i and j) or 900 base-pairs for plasmid DNA was observed (Fig. 2, lanes m and n) confirming the presence of the predicted ScaI site within the 700 base-pair HpaII fragment containing the tRNA^{Asp} gene of these two strains . Thus, although the five *syn*$^-$ strains were isolated independently, only four different lesions could be identified because M737 and 551-50 yielded identical results.

In a previous study, respiratory competent recombinants arose with low but significant frequency when FF1210-170, M737, and ts832 were crossed with one another, indicating that although the lesions in these strains are closely linked, they are not identical (11). Direct crosses between ts832 and 516-45 could not be performed since these mutants were derived from strains of the same mating type. Instead, we took advantage of the technique of Kar cytoduction (29). The *kar1-1* mutation blocks nuclear fusion in mating cells. Progeny of a cross between a *kar1-1* mutant and a *KAR* strain are haploid heteroplasmons containing a mixed mitochondrial population. The *syn*$^-$ strain α ts832 and a *rho*^{0} (containing no mitochondrial DNA) *kar1-1* mutant of the a mating type were mated. By appropriate selections, progeny of this cross which retained ts832 mitochondria in the *kar1-1* nuclear background were identified. Upon mating a *kar1-1*/ts832 with α 516-45, respiratory competent progeny were observed at low but significant frequency (data not shown) indicating that these two mutations also are not identical. These genetic data corroborate our sequence information.

Transcription of Mutant tRNA^{Asp} Genes

To determine whether the altered tRNA^{Asp} genes are transcribed *in vivo*, whole-cell small RNAs were isolated from *syn*$^-$ or wild-type cells grown to mid-log phase in 2% galactose media at 28° or 37°. These RNAs were

fractionated on discontinuous buffer acrylamide-urea gels and transferred to DBM paper. Each sample represented total whole-cell small RNAs isolated from equivalent quantities (by wet weight) of cells. Autoradiography was performed on the filter after hybridization to a radiolabeled oligonucleotide complementary to the mitochondrial $tRNA^{Asp}$ (Fig. 3A). All the strains examined transcribed the mutant $tRNA^{Asp}$ genes. The steady-state levels of $tRNA^{Asp}$ transcripts were lower in the mutant cells than in wild-type cells regardless of media or temperature of incubation. In every case, signals corresponding to mature 4S-size transcripts were observed indicating that the mutant transcripts could be processed efficiently. In the cases of FF1210-170 and ts832 grown at 37° an additional, weaker signal was observed at a position above mature $tRNA^{Asp}$.

In a similar experiment, whole-cell small RNAs were obtained from the *rho*$^-$ strain CE7 or the *syn*$^-$ strain FF1210-170 and subjected to electrophoresis on a discontinuous buffer acrylamide-urea gel alongside RNA obtained from purified wild-type or FF1210-170 mitochondria. These RNAs were transferred to DBM paper and hybridized to the same radiolabelled oligonucleotide as used above. The results of autoradiography of this filter are shown in Figure 3B. Wild-type and *syn*$^-$ strains both contain mature 4S-size $tRNA^{Asp}$. Furthermore, the weak, slow-migrating signal seen in blots of whole-cell FF1210-170 small RNA (Fig. 3A) is also present in the blot of RNA from isolated FF1210-170 mitochondria. The lane containing wild-type RNA was exposed for a time period that was 10x shorter than that used for the other lanes. The minor signal observed migrating more slowly than mature $tRNA^{Asp}$ is an artifact of this exposure since it extends beyond the limits of the lane and was not observed in other experiments. Mature 4S-size $tRNA^{Asp}$ is not seen in the lane containing RNA isolated from the *rho*$^-$ strain CE7. Instead, an RNA which appears slightly longer than $tRNA^{Asp}$ is seen. This RNA co-migrates with the slow-migrating RNA of FF1210-170. The *rho*$^-$ strain CE7 retains the cluster of 16 tRNA genes, including a wild-type $tRNA^{Asp}$ gene, but lacks the mitochondrial tRNA synthesis locus. This locus encodes the RNA subunit of mitochondrial RNase P (30). RNase P is required for maturation of the 5' end of mitochondrial tRNAs (31). Strains such as CE7 do not process any tRNA transcripts to mature, 4S-sized tRNAs because they lack RNase P activity. Instead, tRNAs which are extended at the 5' ends accumulate in such strains, and when these RNAs are separated by gel electrophoresis, they migrate more slowly than fully matured tRNAs.

To determine whether growth conditions affect the steady-state levels of

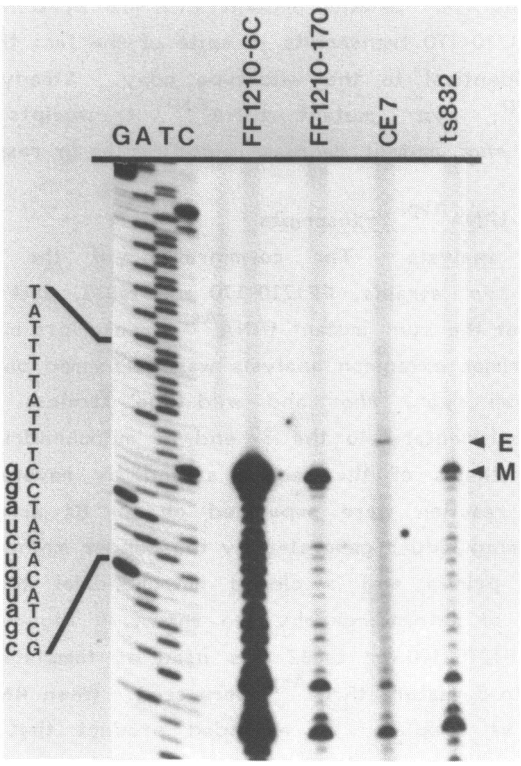

Figure 4. *Primer extension analysis of tRNA^{Asp} transcripts.* Whole-cell small RNAs were obtained from strains FF1210-6C, FF1210-170, CE7 and ts832. These RNAs were annealed to a ^{32}P radiolabelled oligonucleotide complementary to tRNA^{Asp}. The oligonucleotide was extended with AMV reverse transcriptase and the extension products were fractionated by electrophoresis on an 8% acrylamide-urea gel alongside a sequence ladder. The gel was fixed and dried prior to autoradiography. Sequence presented in upper case is that of the coding strand read directly from the gel. Sequence presented in lower case is that of tRNA^{Asp}. Arrows indicate products corresponding to mature tRNA^{Asp} (M) or to 5'-extended tRNA^{Asp} (E).

mutant tRNA^{Asp} gene transcripts, whole-cell small RNAs were extracted from cells grown to log phase in YPD-2% glucose, YPD-10% glucose, or YPG. RNAs were separated on discontinuous buffer acrylamide-urea gels, transferred to DBM paper and probed by hybridization to radiolabelled tRNA^{Asp} oligonucleotide. Results of autoradiography are shown in Figure 3C. For the RNA size range we examined, *syn*$^{-}$ mutant tRNA^{Asp} transcripts are present to a lesser extent than wild-type tRNA^{Asp} transcripts from a wild-type mitochondrial genome regardless of growth conditions. The tRNA^{Asp}

transcripts from the *rho*⁻ deletion mutant CE7 are present in even lesser quantities than FF1210-170 transcripts in spite of the fact that the $tRNA^{Asp}$ gene of CE7 is identical to the wild-type copy. Steady-state levels of wild-type $tRNA^{Asp}$, *syn*⁻ mutant $tRNA^{Asp}$ transcripts and $tRNA^{Asp}$ transcripts from a *rho*⁻ mutant decrease significantly in response to glucose repression.

Analysis of Mutant $tRNA^{Asp}$ Transcripts

Primer extension analysis. The co-migration of the minor $tRNA^{Asp}$ transcripts of the *syn*⁻ strains, FF1210-170 and ts832, and the *rho*⁻ strain, CE7, suggested that the *syn*⁻ mutant $tRNA^{Asp}$ transcripts might be extended at the 5' end. Primer extension analysis was performed on whole-cell small RNA prepared from *syn*⁻, *rho*⁻ and wild-type strains. A radiolabelled oligonucleotide complementary to the 3' end of mitochondrial $tRNA^{Asp}$ was used to prime synthesis of the second strand by reverse transcriptase. Products of this reaction were separated on an 8% acrylamide-urea gel alongside a sequencing ladder generated by the Sanger method using the same oligonucleotide as primer and a cloned mitochondrial $tRNA^{Asp}$ gene as template. Results of autoradiography are shown in Figure 4. When RNA from wild-type, FF1210-170 or ts832 was used as template, only products which correspond to 5'-mature $tRNA^{Asp}$ were seen. When RNA from the *rho*⁻ strain was used as template, no extended product that corresponds to 5'-mature $tRNA^{Asp}$ was observed. Instead, a product was visualized which is longer by 3 nucleotides at the 5' end. In all lanes, products which migrated more rapidly than those corresponding to fully 5'-mature tRNAs are due to incomplete extensions and are probably a consequence of the high degree of secondary structure of tRNA molecules. If the RNA templates were treated with RNase A prior to primer extension, or if no reverse transcriptase was added to catalyze the extensions, then no products larger than the primer itself were visualized. (Data not shown.) We conclude that the results observed are due to extension of $tRNA^{Asp}$ transcripts and not to extraneous $tRNA^{Asp}$ DNA sequences. The slow-migrating *syn*⁻ $tRNA^{Asp}$ transcripts of FF1210-170 and ts832 observed by Northern blot analysis, are correctly matured at the 5' end, whereas the $tRNA^{Asp}$ transcripts of CE7, even though they co-migrate with the slow-migrating *syn*⁻ transcripts, are not correctly processed at the 5' end.

Some 5'-extended $tRNA^{Asp}$ Transcripts from CE7 are Substrates for RNase P. Since the mitochondrial genome of CE7 lacks the tRNA synthesis locus, the $tRNA^{Asp}$ gene transcripts are not fully processed to 4S-size $tRNA^{Asp}$ *in vivo*. We have demonstrated that the smallest RNAs containing $tRNA^{Asp}$

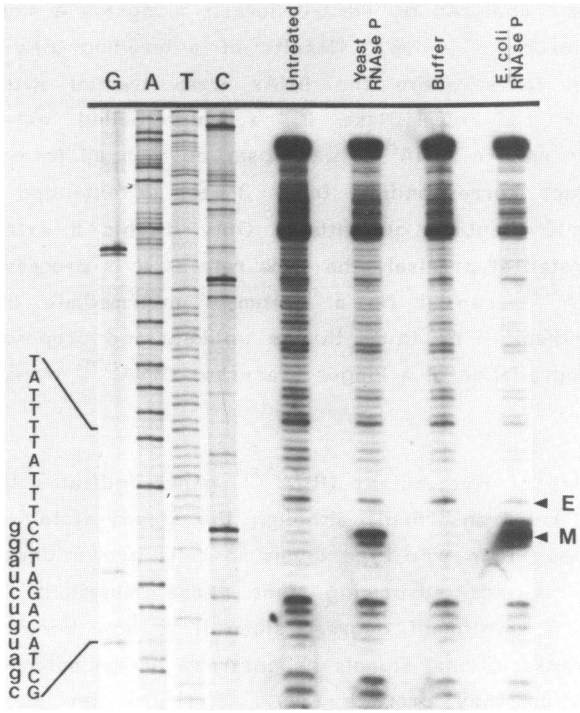

Figure 5. *Analysis of ability of tRNA^{Asp} transcripts in CE7 to be processed by RNase P.* RNA extracted from isolated mitochondria obtained from strain CE7 was treated with yeast mitochondrial or *E. coli* RNase P. Sequence presented in upper case is that obtained from sequence ladder. Sequence presented in lower case is that of tRNA^{Asp}. Arrows indicate position of mature tRNA^{Asp} (M) or 3-base extended tRNA^{Asp} (E).

sequences in this strain are extended by 3 nucleotides at the 5' end. If these RNAs are legitimate intermediates in the maturation of wild-type tRNA^{Asp} then they should be substrates for mitochondrial RNase P. To address this question, total RNA prepared from isolated CE7 mitochondria were treated with partially purified mitochondrial RNase P or purified *E. coli* RNase P. A portion of the reaction products was run on a discontinuous buffer acrylamide-urea gel and stained with ethidium bromide. It is apparent that RNase P treatment drastically alters the size of large CE7 mitochondrial RNAs and 4S sized molecules appear (data not shown). Therefore, there are transcripts present in RNA from the strain CE7 which can be processed by RNase P. A second portion of the RNase P reaction products was extended by reverse transcriptase using as primer a radiolabelled oligonucleotide complementary to the 3' half of mitochondrial tRNA^{Asp}. The products from

this reaction were analyzed by electrophoresis alongside a sequencing ladder generated as described above. Results of autoradiography are shown in Figure 5B. In lanes where the RNAs were treated with either yeast mitochondrial or *E. coli* RNase P, a radiolabelled extension product corresponding to mature $tRNA^{Asp}$ was observed. In all lanes, however, the extension product corresponding to a 3-base 5'-extended $tRNA^{Asp}$ was present in nearly identical quantities. Only if this 5'-extended $tRNA^{Asp}$ species is generated at precisely the same rate as it is processed by RNase P to mature $tRNA^{Asp}$ can it be a legitimate intermediate in mitochondrial $tRNA^{Asp}$ biosynthesis. We think this is unlikely and propose that it arises from nuclease degradation of a longer precursor $tRNA^{Asp}$ transcript.

DISCUSSION

Our analyses of five mutant $tRNA^{Asp}$ genes indicates that the mutant $tRNA^{Asp}$ genes are transcribed, although the steady-state levels of mutant $tRNA^{Asp}$ are lower than wild-type levels in cells grown under a variety of conditions. It is not surprising that these mutations do not affect transcription to a significant degree, since it is now known that in yeast mitochondria, transcriptional signals lie upstream of the mitochondrial gene(s) whose transcription they promote (32). The promoter element has been defined as a nonanucleotide consensus sequence and in many cases the precise site of transcriptional initiation *in vivo* has been confirmed experimentally (6). The transcriptional initiation site(s) for the 16 tRNA genes including $tRNA^{Asp}$ lying between the genes for 21S rRNA and cytochrome oxidase subunit 2 have not been determined although it is known that transcripts from this region are polygenic (4,9). Rapid processing of these multigenic transcripts in wild-type cells makes analysis of primary transcripts difficult. Recently, however, a consensus promoter sequence has been identified upstream of the $tRNA^{Leu}$ gene and it has been suggested that this initiates a long transcript containing the tRNAs for leu, glu, lys, arg, gly, asp, ser_2 and arg_2 (33). It is likely that a single base change in the $tRNA^{Asp}$ gene would have no effect on a promotion event occurring more than 2 kilobases upstream.

Upon inspection of the mutant $tRNA^{Asp}$ genes, the lesions in strains FF1210-170, 551-50, M737 and ts832 do not cause a change in a conserved position on the resultant tRNA molecules, but might be expected to alter stem structures. In FF1210-170, the G1-C72 base pair becomes a G1·U72 non-Watson-Crick pair at the terminal position of the acceptor stem. This may destabilize the double-stranded helix particularly since the adjacent pair is G2·U71 as well. Strains 551-50 and M737 carry the same lesion and encode

$tRNA^{Asp}$ molecules with a C28→U28 change in the anticodon stem. Generally, a G·U non-Watson-Crick pair is stereologically acceptable and does not cause gross helix distortions. However, some accommodation must be made in the stem and this could ultimately affect the position of the anticodon with respect to the remainder of the molecule. The A→T lesion in the $tRNA^{Asp}$ gene of strain ts832 results in a tRNA molecule with a U50 U64 mispair, adjacent to the G49·U65 non-Watson-Crick pair. These two pairs occur at the end of the T pseudouridine (TF) stem leaving only 3 bases to form that structure. Although 3-base TF stems containing two adjacent U U mismatches occur rarely (in the mitochondrial $tRNA^{Met}$ of higher eukaryotes), the mispairings are flanked on both sides by G-C pairs. As with the FF1210-170 mutation, the ts832 mutation may allow more flexibility in a double-stranded helix region. The C→T change identified in the $tRNA^{Asp}$ gene of 516-45 is the only mutation reported here which alters an invariant nucleotide in the resultant tRNA. Position 56, normally a C, is involved in a base-pair interaction with G19 of the D loop. Usually the D loop-TF loop interaction also involves G18-F55 base-pairing. However, wild-type $tRNA^{Asp}$ has an A at position 18 and G at position 55. Thus, there is only one critical D loop-TF loop base-pair in the wild-type $tRNA^{Asp}$ (G19-C56) and it is lost in the $tRNA^{Asp}$ of the *syn*$^-$ mutant 516-45. Although the mutant molecule retains other tertiary interactions involved in maintenance of tRNA structure, the loss of the D loop-TF loop interaction probably has a significant effect on tertiary structure. Thirteen other tRNAs, all mitochondrial, have the sequence TGC in place of TTC at positions 54-56. Of these, eleven can form the G19-C56 pair; the two exceptions have severely shortened D and/or TF loops. In addition, the remainder of yeast mitochondrial tRNAs retain the conserved G18/G19 and T54/T55/C56 bases in the D and TF loops, respectively.

During tRNA biosynthesis, enzymes such as RNase P, nucleotidyl transferase, or enzymes that modify nucleotides must interact with numerous tRNAs and probably recognize the general three-dimensional structure common to all tRNAs. We expect these enzymes to have little trouble recognizing the mutant $tRNA^{Asp}$ molecules as substrates since the common tRNA structures are probably retained by the mutant tRNAs. The structural changes in $tRNA^{Asp}$ wrought by the lesions of the *syn*$^-$ strains are probably more subtle than a complete disruption of the common tRNA structure. We have shown that at least one of these enzymes, RNase P, can process transcripts from the mutant genes. The results presented here also demonstrate that the majority

of mutant $tRNA^{Asp}$ molecules co-migrate with wild-type $tRNA^{Asp}$ indicating that they are the same size molecules. This strongly implies that they have been properly processed at both the 5' and 3' ends. We also observed a minor component of $tRNA^{Asp}$ in RNA isolated from strains FF1210-170 and ts832. Although this RNA migrates more slowly than mature $tRNA^{Asp}$ on discontinuous buffer acrylamide-urea gels, we showed by primer extension analysis that it contains a mature 5' end. The possibility that an extension at the 3' end exists has not been excluded. A similar form of $tRNA^{Asp}$ was observed in RNA isolated from the *rho*⁻ mutant CE7. This $tRNA^{Asp}$ is extended by 3 bases at the 5' end. Because this strain lacks the tRNA synthesis locus, tRNA precursors with 5' extensions are expected. However, such a short leader is unusual. Frontali and her colleagues (9) have reported that other precursor tRNAs from this cluster have long 5' leaders that extend to the 3' end of the nearest upstream tRNA on the transcript. In this study, precursor $tRNA^{Asp}$ molecules with much longer leaders can be observed in RNA extracted from purified CE7 mitochondria (Fig. 5B). We believe that the 3-base 5' extended $tRNA^{Asp}$ molecules are not substrates for RNase P, and are probably degradation products. A $tRNA^{Thr}$ molecule which is 2-3 bases shorter at the 5' end than mature $tRNA^{Thr}$ has been identified in an analysis of $tRNA^{Thr}$ transcripts in another *rho*⁻ mutant lacking the tRNA synthesis locus (2). We propose that these are likewise degradation products. Numerous examples of point mutations in tRNA genes which affect tRNA processing have been reported (34-38). However, the anomalous migration we observed for some FF1210-170 and ts832 $tRNA^{Asp}$ molecules may be due simply to the ability of the mutant $tRNA^{Asp}$ to more easily assume an alternate conformation which then migrates differently from the first. Such electrophoretically separable conformers of tRNAs have been reported previously (39,40).

Perhaps more interesting even than the processing of the mutant tRNAs is the observation that these mutant strains do not make a mitochondrial tRNA which can be charged *in vitro* with radiolabelled aspartate (11,13) despite the synthesis of mature size $tRNA^{Asp}$ molecules. Three regions on a tRNA surface have been implicated as discriminators in tRNA-aminoacyl synthetase reactions: the aminoacyl acceptor stem, the D stem and the anticodon (for review, see 41). The mutations in FF1210-170, 551-50 and M737 probably fall into this category. The mutation in ts832 $tRNA^{Asp}$ may place a strain on the D-TF loop interaction, but not completely disrupt it except at a higher temperature. If this is so, then at the restrictive temperature the ts832 mutation would have the same net result as the 516-45 mutation: disruption

of the G19-C56 tertiary base pair. Since neither of these tRNAs can be charged with aspartate, it is likely that this tertiary structural feature is important in this case for recognition by synthetase. It is noteworthy that it is these two mutants which contain the minor slow-migrating forms of $tRNA^{Asp}$.

The wild-type mitochondrial $tRNA^{Asp}$ molecule has some interesting features. Besides the unusual TF loop, three of the four stems end with a G·U pair. It has been suggested that a G·U pair at a helix terminal provides the flexibility needed to form the "sharp corners" in a properly folded tRNA (42). It is perhaps this flexibility, along with the dependence of the D loop-TF loop interaction on formation of a single base pair, that explains why in screens for *syn*$^-$ mutants, lesions mapping to $tRNA^{Asp}$ were found most often. In other words, it is possible that the $tRNA^{Asp}$ is particularly fragile. Alternatively, the flexibility of $tRNA^{Asp}$ may enable it to tolerate certain alterations that still leave it with residual activity. Mitochondrial protein synthesis is required for maintenance of a wild-type mitochondrial genome in *Saccharomyces cerevisiae* (15). In the absence of protein synthesis, the mitochondrial genome suffers deletions, giving rise to a class of mitochondrial mutants called *rho*$^-$ or petite deletion mutants. *Syn*$^-$ mutations that do not yield leaky or conditional strains should be unstable and degenerate into *rho*$^-$ mutants. Such *syn*$^-$ mutants would never be recovered by the genetic screens used to date.

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REFERENCES

1. Miller,D.L., Najarian,D.R., Folse,J.R. and Martin,N.C. (1981) *J. Biol. Chem.* 256, 9774-9777.
2. Nobrega,M.P. and Nobrega,F.G. (1986) *J. Biol. Chem.* 261, 3054-3059.
3. Miller,D.L., Folse,J.R., Benson,P.J. and Martin,N.C. (1983) Biochemistry 22,1709-1714.
4. Locker,J. and Rabinowitz,M. (1981) *Plasmid* 6, 302-314.
5. Christianson,T., Edwards,J.C., Mueller,D.M. and Rabinowitz,M. (1983) *Proc. Natl. Acad. Scl. USA* 80, 5564-5568.
6. Christianson,T. and Rabinowitz,M. (1983) *J. Biol. Chem.* 258, 14025-14033.
7. Thalenfeld,B.E., Bonitz,S.G., Nobrega,F.G., Macino,G. and Tzagoloff,A. (1983) *J. Biol. Chem.* 258, 14065-14068.
8. Thalenfeld,B.E., Hill,J. and Tzagoloff,A. (1983) *J. Biol. Chem.* 258, 610-615.
9. Palleschi,C., Francisci,S., Zennaro,E. and Frontali,L. (1984) *EMBO J.* 3, 1389-1395.
10. Zassenhaus,H.P., Martin,N.C. and Butow,R.A. (1984) *J. Biol. Chem.* 259, 6019-6027.
11. Faye,G., Bolotin-Fukuhara,M. and Fukuhara,H. (1976) In Th. Bucher *et al.* (eds), *Genetics and Biogenesis of Chloroplasts and Mitochondria*, Elsevier/North Holland Biomedical Press, Amsterdam, pp.547-555.

12. Berlani,R.E., Pentella,C., Macino,G. and Tzagoloff,A. (1980) *J. Bact.* 141, 1086-1097.
13. Bonitz,S.G. and Tzagoloff,A. (1980) *J. Biol. Chem.* 255, 9075-9081.
14. Trembath,M.K., Macino,G. and Tzagoloff,A. (1977) *Mol. Gen. Genet.* 158, 35-45.
15. Myers,A.M., Pape,L.K. and Tzagoloff,A. (1985) *EMBO J.* 4, 2087-2092.
16. Hudspeth,M.E.S., Shumard,D.S., Tatti,K.M. and Grossman,L.I. (1980) *Biochim. Biophys. Acta* 610, 221-228.
17. Locker,J. (1979) *Anal. Biochem.* 98, 358-367.
18. Holley,R.W., Apgar,J., Doctor,B.P., Farrow,J., Marini,M.A. and Merrill,S.H. (1961) *J. Biol. Chem.* 236, 200-202.
19. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor.
20. Grunstein,M. and Hogness,D. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3961-3965.
21. Miller,D.L. and Martin,N.C. (1981) *Curr. Gen.* 4, 135-143.
22. Geck,P. and Nasz,I. (1983) *Anal. Biochem.* 135, 264-268.
23. Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.* 65, 449-580.
24. Miller,D.L., Martin,N.C., Pham,H.D. and Donelson,J.E. (1979) *J. Biol. Chem.* 254, 11735-11740.
25. Stellwag,E.J. and Dahlberg,A.E. (1980) *Nucleic Acids Res.* 8, 299-317.
26. Denhardt,D.T. (1966) *Biochem. Biophys. Res. Comm.* 23, 641-646.
27. Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3963-3965.
28. Gauss,D.H. and Sprinzl,M. (1981) *Nucleic Acids Res.* 9, r1-r24.
29. Conde,J. and Fink,G.R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3651-3655.
30. Hollingsworth,M.J. and Martin,N.C. (1986) *Mol. Cell Biol.* 6, 1058-1064.
31. Martin,N.C., Miller,D.L., Underbrink,K. and Ming,X. (1985) *J. Biol. Chem.* 260, 1497-1483.
32. Osinga,K.A. and Tabak,H.F. (1982) *Nucleic Acids Res.* 10, 3617-3626.
33. Backer and Getz (1986) *J. Biol. Chem.*, in press.
34. Celis,J.E. (1979) *Nucleic Acids Res.* 6, r21-r27.
35. Koski,R.A., Clarkson,S.G., Kurjan,J., Hall,B.D. and Smith,M. (1980) *Cell* 22, 415-425.
36. Kurjan,J., Hall,B.D., Gillam,S. and Smith,M. (1980) *Cell* 20, 701-709.
37. Nishikura,K., Kurjan,J., Hall,B.D. and DeRobertis,E.M. (1982) *EMBO J.* 1, 263-268.
38. Tobian,J.A., Drinkard,L. and Zasloff,M. (1985) *Cell* 43, 415-422.
39. Dubin,D.T., HsuChen,C.-C. and Tillotson,L.E. (1986) *Curr. Gen.*, in press.
40. Pieczenik,G. (1980) *J. Mol. Biol.* 138, 879-884.
41. Schimmel,P.R. (1979) In Schimmel,P.R., Soll,D and Abelson,J.N. (eds) *Transfer RNA: Structure, Properties and Recognition*, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 297-310.
42. Clark,B.F.C. (1978) In Altman,S. (ed) *Transfer RNA*, MIT Press, Cambridge, pp. 14-47.