Characterization of Gtf1p, the Connector Subunit of Yeast Mitochondrial tRNA-dependent Amidotransferase

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The bacterial GatCAB operon for tRNA-dependent amidotransferase (AdT) catalyzes the transamidation of mischarged glutamyl-tRNA^Gln to glutaminyl-tRNA^Gln. Here we describe the phenotype of temperature-sensitive (ts) mutants of GTF1, a gene proposed to code for subunit F of mitochondrial AdT in Saccharomyces cerevisiae. The ts gtf1 mutants accumulate an electrophoretic variant of the mitochondrially encoded Cox2p subunit of cytochrome oxidase and an unstable form of the Atp8p subunit of the F1–F0 ATP synthase that is degraded, thereby preventing assembly of the F0 sector. Allotopic expression of rescued ATP8 and COX2 did not significantly improve growth of gtf1 mutants on respiratory substrates. However, ts gtf1 mutants are partially rescued by overexpression of PET112 and HER2 that code for the yeast homologues of the catalytic subunits of bacterial AdT. Additionally, B66, a her2 point mutant has a phenotype similar to that of gtf1 mutants. These results provide genetic support for the essentiality, in vivo, of the GatF subunit of the heterotrimeric AdT that catalyzes formation of glutaminyl-tRNA^Gln (Frechin, M., Senger, B., Brayé, M., Kern, D., Martin, R. P., and Becker, H. D. (2009) Genes Dev. 23, 1119–1130).

Aminoacyl-tRNA synthetases catalyze acylation of tRNAs with their cognate amino acids (1). Aminoacyl-tRNA synthetases are highly specific enzymes, but in some cases, non-discriminating attachment can occur (2, 3). Synthesis of glutaminyl-tRNA in some organisms involves a non-discriminating glutamyl-tRNA synthetase that charges tRNA^Gln with glutamic acid. The resultant glutamyl-tRNA^Gln is then processed by a tRNA-dependent amidotransferase (AdT)^2 complex to glutaminyl-tRNA^Gln (4). The transamidation of glutamyl-tRNA^Gln is known to occur in prokaryotes and more recently has also been shown in eukaryotic organelles (3, 5, 6). In bacteria, the AdT reactions are catalyzed by the heterotrimeric enzyme encoded by the GatCAB operon (7, 8). Transamidation starts with phosphorylation by the GatB subunit of the attached glutamyl γ-carboxyl group (9), followed by amidation of the activated phosphorylated intermediate by the GatA subunit (10). GatC codes for a stable constituent of the complex that has been proposed to be required for linking and proper folding of the catalytic subunits (7, 11).

The HER2 and PET112 gene products of Saccharomyces cerevisiae share significant sequence similarity with the GatA and GatB products, respectively. Pet112p was shown to affect mitochondrial translation (12). The respiratory defect of pet112 mutants is rescued by expression of Bacillus subtilis GatB fused to a mitochondrial targeting sequence (13). More recently, Pet112p was shown to exist in a complex with Her2p and Ygr102p, which catalyzes transamidation of a mischarged glutamyl-tRNA^Gln in vitro (3). These results indicate the existence in yeast mitochondria of a heterotrimeric AdT consisting of the product of YGR102c, Pet112p, and Her2p, the latter two being orthologues of the bacterial GatB and GatA subunits, respectively. These findings contradicted an earlier report of the presence in mitochondria of import from the cytoplasm of glutaminyl-tRNA synthetase and tRNA^Gln, which function in mitochondrial translation (14).

Because the product of YGR102c did not share sequence similarity with any bacterial GatC subunit, it remained questionable if this protein was also needed for transamidation of the mischarged tRNA^Gln. In the present study, the YGR102c reading frame, here named GTF1 (glutaminyl transamidase subunit F), was tested for in vivo expression of mitochondrial gene products.

The high instability of mitochondrial DNA in gtf1 null mutants precluded their use for functional studies. This problem was circumvented by examining mitochondrial translation in temperature-sensitive (ts) mutants. Our evidence indicates that at the restrictive temperature, gtf1 ts mutants translate aberrant forms of the mitochondrially encoded Cox2p and Atp8p subunits of cytochrome oxidase and ATP synthase, respectively, and that this phenotype is suppressed by overexpression of PET112 or HER2. Assays of mitochondrial respiratory activities in the ts gtf1 mutants also indicate that they translate non-functional cytochrome b. These results are consistent with a role of Gtf1p in transamidation of mitochondrial glutamyl-tRNA^Gln.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—The genotypes and sources of the yeast strains used in this study are listed in

SEPTEMBER 23, 2011 • VOLUME 286 • NUMBER 38

32937 JOURNAL OF BIOLOGICAL CHEMISTRY

The abbreviations used are: AdT, amidotransferase; mtDNA, mitochondrial DNA; ts, temperature-sensitive; ρo mutant, respiratory deficient mutant lacking mitochondrial DNA; ρ− mutant, respiratory deficient mutant with a partially deleted mitochondrial genome.
### Table 1

| Strain         | Genotype                  | mtDNA       | Source     |
|---------------|---------------------------|-------------|------------|
| W303-1A       | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303-1B       | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/s⁰       | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/s¹       | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| MR6           | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| MR6ΔATP8      | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| aMR6ΔATP8/GTF1ts | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/GTF1ts/nATP8 | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/GTF1ts/nCOX2 | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/GTF1ts/nATP8/nCOX2 | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔPET111   | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| aW303ΔCOX20    | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔIMP1      | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔIMP2      | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔOXA1      | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/GTF1      | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| a/aW303/GTF1    | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔHER2ts9   | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| B66            | MATa ade1 her2-1           | p⁺         | Ref. 15    |
| W303/GTF1ts1   | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303/GTF1ts2   | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔCOX20/GTF1ts | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| aW303ΔPET111/GTF1ts | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| W303ΔGTF1/ST2  | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |
| aW303ΔGTF1/ST22 | MATa ade2-1 his3-1,15 leu2-3,112 trpl-1 ura3-1 | p⁺         | Ref. 15    |

Table 1. The compositions of YPD, YPEG, and minimal glucose medium (supplemented with the appropriate auxotrophic requirements) have been described (20).

**Isolation of Mitochondrial RNA** — Yeast was grown either at 30 or 37 °C in YPGal (2% peptone, 1% yeast extract, and 2% galactose) and mitochondria were prepared by the method of Faye et al. (21) except that zymolyase instead of glusulase was used to convert cells to spheroplasts. RNA was extracted from mitochondria by the addition of an equal volume of 1% SDS, 0.5 mM EDTA, and 100 mM NaCl. The solubilized mitochondria were mixed with an equal volume of water-saturated phenol. The mixture was centrifuged for 2 min in a microcentrifuge, and 0.6 ml of the upper phase was transferred to a fresh tube. Following three washes with ether, nucleic acids were precipitated by the addition of 0.05 volumes of 5 M NaCl and 3 volumes of ethanol, rinsed with 80% ethanol, and dried.

**Construction of ts Mutants** — Temperature-sensitive gtf1 and her2 alleles were obtained by PCR amplification of the genes in four separate reactions containing 0.25 mM MnCl₂, 1.5 mM MgCl₂, 0.2 mM concentrations of three deoxynucleotides, and a 0.04 mM concentration of the fourth deoxynucleotide (17). The primers for amplification of a 1362-bp fragment containing GTF1 fused to a sequence coding for the HA tag were 5’-acgaacaagATTctctgg (the capital ‘T’ was inserted in the place of a “G” in order to create a HindIII site) and 5’-ggaattccttagtaacagagctag (Sac site) and 5’-ggaatatcgaagttttttct (this primer is complementary to YeEp351). The products from each set of reactions were pooled, digested with either HindIII (GTF1 fragment) or with Pst1 and SacI (HER2), and cloned in the centromeric plasmid YCplac22 with the TRP1 marker (22). The plasmid libraries were used to transform
the heterozygous diploid strains a/aW303ΔGTF1 and a/aW303ΔHER2. The heterozygous gtf1/GTF1 strain was obtained by crossing the gtf1 null mutant aW303ΔGTF1 to W303/1A. Similarly, the her2 null mutant aW303ΔHER2 was crossed to W303-1B to isolate the heterozygous her2/HER2 strain. The pooled tryptophan-independent transformants were sporulated on solid potassium acetate medium. Uracil-independent meiotic progeny were selected at 30 °C and further checked for growth at 37 °C. This screen yielded several mutants that displayed a clear ts phenotype for growth on non-fermentable substrates (YPG). Two conditional gtf1 mutants (W303/GTF1ts and W303/GTF1ts2), respectively, or the dominant sg alleles (GTF1/gtf1 sg1 and GTF1/gtf1 sg2) were spotted on YPD and YPEG and incubated at 30 °C for 2 days. C, the wild type W303-1A and the gtf1 ts1 mutant, grown to early stationary phase in YPGal at 30 °C, were transferred to fresh YPGal medium and incubated at either 30 or 37 °C for an additional 7 h. Mutant cells were also grown continuously at 37 °C. Mitochondria at 5 mg of protein/ml were extracted with deoxycholate (26), and difference spectra of the ferricyanide-oxidized versus dithionite-reduced extracts were recorded at room temperature. The percentages of p<sup>−</sup>/p<sup>+</sup> mutants in the cultures of the ts mutant were 2% for cells grown at 30 °C and 12% for cells grown at 37 °C. Less than 1% of p<sup>−</sup>/p<sup>+</sup> mutants were present in the wild type culture grown at either temperature. The α-absorption bands of mitochondrial cytochromes are identified. D, mitochondria were prepared from the W303-1A grown at 37 °C and from the gtf1 ts1 mutant grown under three conditions described in A. Total mitochondrial proteins were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with monoclonal antibodies against Cox3p and with polyclonal antibodies against Cox1p, Cox2p, Cox4p, and Atp6p. The blots were then treated either with peroxidase-conjugated anti-mouse or anti-rabbit IgG and were visualized with the Super Signal chemiluminescent substrate kit (Pierce).

RESULTS

Phenotype of GTF1 Null and Temperature-sensitive Mutants—The YGR102C null mutant obtained from the Yeast Deletion Strain collection and the W303ΔGTF1 mutant convert quantitatively to secondary p<sup>−</sup> and p<sup>+</sup> clones with deletions and complete loss, respectively, of mtDNA. As a result, they display a pleiotropic phenotype with deficiencies in respiratory chain complexes and the F<sub>1</sub>-F<sub>0</sub> ATP synthase (data not shown). Mitochondrial DNA instability is generally, but not exclusively, a property of mutants defective in mitochondrial protein synthesis (20) or in the F<sub>1</sub>-F<sub>0</sub> ATP synthase complex (29). Because of the similarity in their phenotype, protein synthesis and ATP synthase mutants are often difficult to distinguish from one another.

To analyze the biochemical lesion responsible for the pleiotropic phenotype in the gtf1 null mutants, we isolated ts alleles of the gene. A plasmid library, constructed by mutagenic PCR amplification of GTF1, was used to isolate temperature-sensitive gtf1 mutants, two of which (W303/GTF1ts1 and W303/GTF1ts2) were analyzed in more detail. Both mutants showed a clear temperature-sensitive growth defect on non-fermentable carbon sources at 37 °C and a partial growth defect at 24 and 30 °C (shown for ts1 in Fig. 1A). The GTF1 gene in W303/GTF1ts1 was found to have two mutations, resulting in amino acid substitutions L46S and A71G in the protein. Only the L46S...
change is at a conserved position (supplemental Fig. S1). The gene in W303/GTF1ts2 sustained a deletion of a nucleotide in a run of five As ending at position 392 of the gene. The frameshift introduced by the deletion causes a replacement of the terminal 15 amino acid residues by a penultimate methionine and a C-terminal alanine. When introduced into a heterozygous GTF1/gtf1 mutant, the ts1 and ts2 alleles behaved as recessive mutations (Fig. 1B). Two other mutants obtained from the screen had a dominant phenotype when the mutations (gtf1 sg1 and gtf1 sg2) were present in a CEN plasmid. These mutations suppressed growth of the GTF1/gtf1 diploid strain on glycerol/ethanol at 30 °C but not at 37 °C (Fig. 1B). The dominant phenotype at 30 °C suggests that the mutant protein had a competitive advantage over the normal protein produced from wild type GTF1, perhaps by binding to some other component(s).

The loss of dominance at 37 °C can be explained by an alteration in the tertiary structure of the mutant protein that either causes it to be degraded or prevents it from interacting with other proteins.

Unlike the null mutation, the ts alleles had a much less destabilizing effect on mtDNA. Only 2% of the ts mutants converted ρ−/ρ0 mutants after growth at 30 °C. The percentage of secondary ρ−/ρ0 mutants was increased to 12% after growth at 37 °C for 7 h. When grown continuously at 37 °C, the population of ρ−/ρ0 mutants increased to 40–50%.

Mitochondrial spectra indicated an abnormally low ratio of cytochromes a and a3 relative to cytochrome b in the ts mutant (Fig. 1C). This was especially evident after the mutant was shifted to the restrictive temperature for 7 h. Growth at 37 °C caused a 4-fold reduction of oligomycin-sensitive ATPase activity (Table 2), a hallmark of a lesion in the F0 sector of the ATP synthase complex. Although the cytochrome b spectrum of mitochondria from cells grown for 7 h at the non-permissive temperature was not significantly affected, their NADH cytochrome c reductase activity was only 5% of wild type and 10% of the mutant grown at the permissive temperature (Table 3). Western analysis of cytochrome c oxidase and ATPase subunits (Fig. 1D) revealed a decrease in the steady-state concentrations of Cox1p, Cox2p, Cox3p, and Atp6p after the mutant grown at 24 °C was transferred to 37 °C for a period of 7 h (Fig. 1D). The decrease in these mitochondrial gene products was even more pronounced when the mutant was grown continuously at 37 °C.

**TABLE 2**
Mitochondrial ATPase activities in wild type and the gtf1 ts mutant

| Strain         | Temperature | ρ−, ρ0 | ATPase Without Oligo | ATPase With Oligo | Inhibition |
|----------------|-------------|--------|----------------------|-------------------|-----------|
|                | °C          | %      | μmol/mg/min           | μmol/mg/min       | %         |
| W303-1A        | 30          | <1     | 6.15 ± 0.15           | 1.50 ± 0.10       | 76        |
| W303-1A        | 37          | <1     | 6.30 ± 0.10           | 1.35 ± 0.05       | 79        |
| W303/GTF1ts1   | 30          | 2      | 5.35 ± 0.15           | 1.95 ± 0.15       | 67        |
| W303/GTF1ts1   | 37          | 12     | 3.55 ± 0.15           | 3.00 ± 0.10       | 18        |

**TABLE 3**
Antimycin-sensitive NADH cytochrome c reductase activities in wild type and gtf1 ts mutants

| Strain         | Temperature | ρ−, ρ0 | Cytochrome c reduced | ρ−, ρ0 | Cytochrome c reduced |
|----------------|-------------|--------|----------------------|--------|----------------------|
|                | °C          | %      | μmol/min/mg protein  | %      | μmol/min/mg protein  |
| W303-1A        | 30          | <1     | 0.795 ± 0.035        | <1     | 0.758 ± 0.055        |
| W303-1A        | 37          | <1     | 0.350 ± 0.024        | <1     | 0.035 ± 0.007        |
| W303/GTF1ts1   | 30          | 2      | 0.10                 | 3.1     | 0.05                 |
| W303/GTF1ts1   | 37          | 12     | 0.035 ± 0.007        | 0.10   | 0.007                |

had been grown at 24 °C and incubated and labeled at 37 °C. The incubation at the higher temperature resulted in a time-dependent loss of translation efficiency, as witnessed by the decreased labeling of proteins in both wild type and the gtf1 ts mutant. However, whereas all of the translation products in the wild type strain decrease proportionally, this was not true in the mutant. Incubation at 37 °C for 20 min resulted in less than 10% reduction of Atp6p labeling in wild type but a 20-fold decrease in the mutant (Fig. 2, A and B).

In vivo translation of mitochondrial gene products at 37 °C in the gtf1 ts mutant led to the appearance of a progressively slower migration form of radiolabeled Cox2p when the translation products were separated on a 17% polyacrylamide gel run in the Laemmli buffer system (27) (Fig. 2A). In contrast, at 37 °C newly translated Cox2p in the wild type strain migrated as the mature protein at all time points of the experiment. To obtain a better resolution of the Cox2p region, the length of the gel was increased from 10 cm to 15 cm, and the products were separated by SDS-PAGE using a 17.5% polyacrylamide gel with the pH of the running buffer adjusted to 8.3. Wild type, the gtf1 ts mutant, and impl and oxa1 mutants (both of which accumulate the unprocessed precursor form of Cox2p) were labeled in vivo at 30 and 37 °C. Under these conditions, Cox2p separated into three distinct bands in the gtf1 ts mutants labeled at 37 °C (Fig. 2C, lane 4). The faster migrating band corresponded to mature Cox2p, which was also evident in the wild type and in the gtf1 ts mutant labeled at 24 °C (Fig. 2C). The middle band migrated identically to the unprocessed precursor (pCox2p) of the impl and oxa1 mutant (32, 33). The third novel protein (Cox2ap0), migrating slightly slowed than the precursor, was detected only in the gtf1 ts mutant labeled at 37 °C (Fig. 2C, lane 4; also see Fig. 3, A and B).

Pet111p is required for Cox2p translation initiation (34). The absence of the novel Cox2p* band in a pet111 and gtf1 double mutant (Fig. 2D) defective in translation of the COX2 mRNA (34, 35), confirmed that this translation product is related to Cox2p. That the novel Cox2p* and the Atp8p-deficient pheno-
types are not interdependent was confirmed by the significant reduction of in vivo translation of Atp8p in the double mutant grown at 30 °C and shifted to 37 °C for 20 min (Fig. 2D). Similarly, other studies have shown Cox2p to be normally translated in an atp8 null mutant (36). This indicates that formation of Cox2p* is not dependent on the absence of Atp8p.

Does the Novel Cox2p Polypeptide Contain the N-terminal Presequence?—The migration of some proteins can be altered by a single amino acid change (37). The electrophoretic properties of hydrophobic mitochondrial proteins are also affected by the degree of cross-linking of the polyacrylamide matrix. For example, the migration of cytochrome b relative to Cox2p can be reversed by increasing the cross-linkage of the polyacrylamide gel. The slower migration of Cox2p* in SDS-PAGE, therefore, does not necessarily indicate that it has a larger mass than the precursor.

The Cox20p chaperone functions in conjunction with the Imp protease complex (18, 38) to promote cleavage of the presequence in pCox2p (18). To ascertain if the N-terminal presequence of the Cox2p precursor (pCox2p) is present in the novel polypeptide, the cox20 single mutants and a cox20/ gtf1 ts double mutant were labeled in vivo at the permissive and non-permissive temperature. As expected, the cox20 mutant accumulated pCox2p at both assay temperatures (Fig. 2E). The precursor was also the only detectable product in the cox20/ gtf1 ts double mutant grown and labeled at 24 °C. In contrast, the major product in the double mutant labeled at 37 °C corresponded to the Cox2p* polypeptide seen in the single gtf1 ts mutant at 37 °C (Fig. 2E). The dominance of the ts phenotype in the double mutant constitutes strong evidence that Cox2p* has an unprocessed N terminus.
mutants, and of a pet111, gtf1 ts double mutant. The latter was used to identify the products derived from COX2. Mitochondria were isolated from the different strains grown at 24 °C and after incubation at 37 °C for 20 min were labeled with [35S]methionine 37 °C. In organello translation assays revealed the presence in the gtf1 ts mutants of the mature and precursor forms of Cox2p and of the Cox2p* polypeptide noted in the in vivo assays (Fig. 3A). The most prominent band in mitochondria of the gtf1 mutants labeled at 24 °C corresponded to mature Cox2p (not shown). The major product detected in wild type mitochondria was mature Cox2p, although some precursor was also present. The products identified as Cox2p, pCox2p, and Cox2p* were absent in the pet111/gtf1 ts double mutant, confirming that all three were derived from COX2 (Fig. 3A). The abundance of Cox2p* relative to mature Cox2p and to the pCox2p precursor was somewhat variable. In most experiments, Cox2p* was the more strongly labeled of the three bands at the restrictive temperature (Fig. 3C). The novel protein was inaccessible to proteinase K in intact mitochondria but, like other mitochondrial translation products, was quantitatively digested by proteinase K in submitochondrial particles obtained by sonic disruption of mitochondria (Fig. 3B).

Approximately 60% of Cox2p consists of a C-terminal hydrophilic domain that protrudes into the intermembrane compartment of mitochondria (40). When newly translated Cox2p assumes its correct topology, this domain, which houses the CuA binding site of the protein, is accessible to externally added protease in mitoplasts but not in mitochondria (41, 42). The proteolytic product detected after digestion of mitoplasts with proteinase K migrates as a discrete band between Cox2p and pCox2p (18, 42). The accessibility of Cox2p*, pCox2p, and Cox2p* to externally added proteinase K was compared after conversion of labeled mitochondria to mitoplasts. Approximately 70–90% of mature Cox2p was proteolyzed by proteinase K in mitoplasts from wild type and the gtf1 ts mutants labeled at 24 °C, whereas some precursor was also present. The products identified as Cox2p, pCox2p, and Cox2p* were absent in the pet111/gtf1 ts double mutant, confirming that all three were derived from COX2 (Fig. 3A). The abundance of Cox2p* relative to mature Cox2p and to the pCox2p precursor was somewhat variable. In most experiments, Cox2p* was the more strongly labeled of the three bands at the restrictive temperature (Fig. 3C). The novel protein was inaccessible to proteinase K in intact mitochondria but, like other mitochondrial translation products, was quantitatively digested by proteinase K in submitochondrial particles obtained by sonic disruption of mitochondria (Fig. 3B).

Northern Analysis of Mitochondrial Transcripts—The absence of Atp8p among the products labeled in vivo in gtf1 ts1 mutants at the restrictive temperature (Fig. 2A) could indicate a defect in transcription or processing of the ATP8 mRNA. ATP8 is co-transcribed with COX1 and ATP6 (43). In some strains of yeast, the primary transcript also includes ENS1 that codes for an endonuclease (44). Endonucleolytic cleavage of the polycistrionic transcript removes the COX1 sequence and produces a 5.2-kb transcript containing ATP8 and ATP6 (ENS2, present in the genome). The 5.2-kb transcript is further processed at a site 600 nucleotides from the 5′-end to yield a
smaller transcript of 4.6 kb. In most but not all strains, the 4.6- and 5.2-kb mRNAs are present in equimolar amounts (45, 46).

Northern analyses of mitochondrial RNA from wild type and the mutant confirmed the presence of the two ATP8/ATP6 mRNAs, although the abundance of the 4.6-kb relative to the 5.2-kb transcript was decreased in the ts mutant, particularly when incubated or grown at 37 °C (supplemental Fig. S2). The gtf1 ts mutation did not significantly affect the COX2 mRNA of cells grown at either temperature. This was also true of a gtf1 ts mutant that had been shifted to 37 °C for 7 h after growth at 30 °C (supplemental Fig. S2). The presence of the ATP6/ATP8 transcripts strongly argues against a direct role of Gtf1p in processing of the polycistronic COX1/ATP8/ATP6 transcript. This was also supported by the presence of the mature COX1 mRNA in the ts mutant grown under the three different conditions. Interestingly, growth of the mutant at the higher temperature led to markedly increased COX1 mRNA. This was especially evident in cells that had been grown continuously at 37 °C (supplemental Fig. S2). We have no explanation for the apparent greater stability of the COX1 mRNA in the gtf1 ts mutant at the non-permissive temperature.

**Allotopic Expression of ATP8 and COX2 in the gtf1 ts Mutant**—
The presence of ATP8/ATP6 mRNA in the gtf1 ts mutant grown at the restrictive temperature suggested that the nearly complete absence of radiolabeled Atp8p following in vivo incorporation of [35S]methionine is a consequence of either a translational defect or instability of the newly translated protein.

We reasoned that if the gtf1 mutant is impaired in translation of Atp8p, its ATPase defect should be corrected by expression of Atp8p from a nuclear gene (nATP8), recoded for translation on cytoplasmic ribosomes. A modified ATP8 gene was fused to a sequence coding for the Neurospora crassa import signal of ATP9 and was cloned in the high copy episomal plasmid YEp351 (pATP8/ST4) and in the related plasmid pGM13 (pATP8/ST4) containing the ADH1 promoter (see supplemental material regarding construction of these plasmids). This gene had previously been shown to complement an atp8 mutant (47). This was confirmed in the present study by the ability of nATP8 to restore oligomycin-sensitive ATPase (Table 3) and growth of the atp8 null mutant on glycerol/ethanol (Fig. 4A). Neither the ATPase nor COX defect of the gtf1 ts mutant, however, was rescued by nATP8 at the non-permissive temperature (Table 4 and Fig. 4C). Some growth of the gtf1 ts2 was detected but only after 7 days of incubation at 37 °C (Fig. 4B).

Recently, allotopic expression was achieved with recoded COX2 containing a single mutation (W56R) in the first transmembrane domain of Cox2p (48). The expression of nuclear encoded nCOX2W56R in the gtf1 ts1 mutant did not improve its growth on respiratory substrates at the non-permissive temperature. Co-expression of nATP8 and nCOX2W56R achieved some improvement in growth at 37 °C but only after a prolonged incubation (7 days; Fig. 5B). This indicated that the respiratory defective phenotype of the gtf1 ts1 mutant was not significantly altered by the presence of nATP8 and nCOX2.
**Gtp1p Subunit of Yeast AdT**

**TABLE 4**

Mitochondrial ATPase activities in wild type and the gtf1 ts mutant

ATPase activity was measured at 37 °C in the absence and presence of 10 μg/ml oligomycin as in Table 2. MR6ΔATP8 + nATP8 and W303/GTF1s1 + nATP8 are transformants harboring recoded nATP8 in the high expression vector pGML3 (pATP8/ST4). Oligo, oligomycin.

| Strain                  | Temperature | ρ\(^{+}\)/ρ\(^{-}\) | Without Oligo | With Oligo | Inhibition |
|-------------------------|-------------|----------------------|---------------|------------|------------|
| W303-1B                 | 37          | 22                   | 5.14          | 1.90       | 63         |
| MR6ΔATP8                | 37          | 98                   | 1.54          | 1.49       | 3          |
| MR6ΔATP8 + nATP8        | 37          | 20                   | 2.79          | 1.54       | 45         |
| W303/GTF1s1             | 37          | 46                   | 1.98          | 1.97       | 1          |
| W303/GTF1s1 + nATP8     | 37          | 52                   | 1.54          | 1.52       | 1          |

In contrast to the results obtained in the *in vivo* assays, some Atp8p was detected in mitochondria of the gtf1 ts mutant labeled *in organello* at 37 °C (not shown). Detection of radiolabeled Atp8p in this assay together with the other two lines of evidence also supports synthesis of Atp8p in the gtf1 ts mutants at the non-permissive temperature and points to turnover as the more likely explanation for the observed absence of Atp8p under *in vivo* conditions.

**Temperature-sensitive Mutants of GTF1 and HER2 Are Suppressed by Overexpression of PET112—Temperature-sensitive gtf1 ts2 mutants were transformed with a yeast genomic library in order to isolate multicopy suppressors. Partial respiratory growth at the non-permissive temperature was restored in some transformants. These were ascertained to contain plasmid with PET112 as one of the genes in the nuclear DNA insert. PET112 was previously proposed to be required for mitochondrial translation (17) and was subsequently shown to be associated with Her2p and Gft1p (3). Subclones of the nuclear DNA insert confirmed that PET112 is a multicopy suppressor of the gtf1 ts2 mutant. Although HER2 alone did not have any effect on growth of the gtf1 ts2 mutant, co-expression of PET112 and HER2 in multicopy plasmids produced a significant improvement in the mutant’s ability to grow on respiratory substrates (Fig. 6A). A her2 ts mutant was partially suppressed by PET112. This mutant was found to have two mutations, E330K and S399F, of which the first is in a conserved region of the protein.

**Localization of Gtf1p**—The intramitochondrial localization of Gtf1p was studied with antibodies against the native protein and against the HA epitope, the latter in a strain expressing Gtf1p with an N-terminal HA tag. The sequence coding for the

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The gtf1 ts mutation was also tested on expression of mitochondrial ARG8\(^{mn}\) at the ATP8 locus of mtDNA. ARG8\(^{mn}\) is a version of nuclear ARG8 but modified to accommodate for the difference in the genetic code of yeast mitochondria (49). Growth of an arg8 mutant was arginine-dependent in a wild type mtDNA background but not in mutant in which mitochondrial ATP8 was replaced with ARG8\(^{mn}\) (Fig. 4D). The compromised growth of the atp8 null mutant at 37 °C in the absence of arginine is due to the large fraction of ρ\(^{-}\)/ρ\(^{+}\) cells produced under these conditions. The instability of mtDNA in atp8 null mutant is suppressed by expression of Atp8p from the nuclear gene resulting in normal complementation of the arginine auxotrophy by ARG8\(^{mn}\) (Fig. 4D). Surprisingly, expression of ARG8\(^{mn}\) at the ATP8 locus was not affected by the gtf1 ts allele at either the permissive or restrictive temperature (see Δatp8/gtf1 ts1 in Fig. 4D).

![FIGURE 5. PET112 suppress ts gtf1 and her2 mutants.](image-url)
Gtf1p is a peripheral mitochondrial inner membrane protein facing the matrix compartment. A, mitochondria were prepared from W303-1B and from W303Δgtf1/ST21 (Δgtf1/ST21) and W303Δgtf1/ST22 (Δgtf1/ST22) expressing Gtf1p-HA, respectively, from a high copy plasmid and as a single copy integrated at the chromosomal URA3 locus. Mitochondria (50 μg of protein) were separated by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose, and the Western blot was treated as in Fig. 1D, B. W303Δgtf1/ST22 mitochondria at a protein concentration of 5 mg/ml in 0.6 M sorbitol, 20 mM HEPES, pH 7.4, were sonically irradiated for 5 s with a Branson microprobe at half-maximal output. The soluble protein fraction (supernatant) was separated from the submitochondrial membrane vesicles (SMP) by centrifugation at 90,000 × gsw for 20 min. The soluble and peripheral membrane proteins were extracted from mitochondria by the addition of an equal volume of 0.2 M sodium carbonate. After incubation on ice for 30 min, the soluble protein fraction (carbonate extract) was solubilized when mitochondria were treated with sodium carbonate, indicating that it is a peripheral membrane protein. The sequence of Gtf1p has only one region of marginal hydrophobicity located in the middle of the sequence. Sonic disruption of mitochondria resulted in only partial recovery of the protein in the soluble fraction (Fig. 6B). This may have been due to the presence of some small membrane fragments that failed to sediment under the conditions used to separate the submitochondrial membrane vesicles. All of Gtf1p, however, was solubilized when mitochondria were treated with sodium carbonate, indicating that it is a peripheral membrane protein.

**DISCUSSION**

A previous study reporting the presence of a nuclear encoded tRNA\(^{\text{Gln}}\) in yeast mitochondria was interpreted to indicate that translation of mitochondrial gene products does not require AdT to generate glutaminyl-tRNA\(^{\text{Gln}}\) from glutamyl-tRNA\(^{\text{Glu}}\) (14). More recently, however, yeast mitochondrial glutaminyl-tRNA\(^{\text{Gln}}\) was shown to be formed by a transamidation pathway (3) similar to that operating in human mitochondria (6), chloroplasts (5), and bacteria (7, 8). The results of our assays of mitochondrial translation and of respiratory and ATPase activities in gtf1 and her2 mutants, summarized below, lend further support to the proposal that the Gtf1p, Her2p, and Pet112p are part of mitochondrial AdT required for translation of functional yeast mitochondrial gene products (3).

The temperature-sensitive gtf1 mutants reported here are defective in assembly of the F0 sector of the mitochondrial ATP synthase. Growth of the ts mutants at the non-permissive temperature leads to a large decrease in mitochondrial oligomycin-sensitive ATPase activity. Analysis of the mitochondrial gene products synthesized in vivo at the non-permissive temperature indicates a deficit of Atp8p but not Atp6p or Atp9p. We ascribe this to a rapid turnover of newly translated Atp8p. The partial decrease of the Atp8p/ATP6 mRNAs at 37 °C was not sufficient to explain the almost complete absence of Atp8p. Translation of Atp8p by the gtf1 mutant at the non-permissive temperature was also supported the mutant’s ability to express ARG8\(^{\text{m}}\) at the Atp8 locus. Mutations leading to a loss of AdT activity should block transamidation of glutamyl-tRNA\(^{\text{Glu}}\) to glutaminyl-tRNA\(^{\text{Gln}}\) with a consequent incorporation of glu-
Gtp1p Subunit of Yeast AdT

tamic acid instead of glutamine residues at CAA and CAG codons. Atp8p has the highest percentage of glutamine residues among mitochondrial gene products (6.3%; supplemental Table S2). This may explain the rapid turnover of newly synthesized Atp8p compared with the other mitochondrial gene products in gtf1 and her2 mutants.

The gtf1 ts and her2 point mutant display a novel COX2 translation product not seen in any of the other known mutants affected either in Cox2p translocation or maturation. The abundance of this novel protein relative to mature and precursor forms of Cox2p varies somewhat, perhaps because of its lability. Proteinase K accessibility has been a useful tool to study the topology of newly translated Cox2p in various mutants. For example, Cox2p in mitoplasts of oxa1 mutants is protected against proteinase K because of its failure to translocate across the inner membrane (33, 52). The novel COX2 product translated at the non-permissive temperature in mitochondria of the gtf1 ts mutant was also almost completely resistant to proteinase K in mitoplasts. These results indicate that the C-terminal hydrophilic domain of the novel Cox2p protein, like that of the precursor in oxa1 mutants, is not transferred to the intermembrane space. The failure of proteinase K to alter the electrophoretic migration of the novel Cox2p polypeptide in mitoplasts indicates that its N terminus too is not transported to the intermembrane space. This is not surprising in view of the presence of a glutamine in the presequence of the Cox2p precursor.

The introduction of a negatively charged glutamic acid in this hydrophobic domain probably inhibits its transfer across the lipid bilayer. The gtf1 mutant phenotype is dominant to the cox20 processing defect, as evidenced by the presence of the novel polypeptide at the non-permissive temperature in the double mutant, a finding consistent with its instability and inaccessibility to proteinase K in mitoplasts. These results suggest that the loss of AdT function in the gtf1 and her2 mutants prevents membrane insertion and processing of the Cox2p precursor, which contains the second highest percentage of glutamines (3.6%). Other newly translated mitochondrial gene products were stable in the mutant, and their electrophoretic properties were similar to those of the wild type.

When grown at the restrictive temperature, the gtf1 ts mutant was deficient in the spectral absorption bands of cytochromes a and a3 and had reduced amounts of Cox1p and Cox3p, the other two mitochondrially encoded subunits of cytochrome oxidase. Because depletion of ATP synthase is known to repress translation of Cox1p and stability of Cox3p (16, 53), it is not possible to distinguish between a direct and indirect effect of the gtf1 mutation on these subunits.

The gtf1 ts mutant containing a nuclear arg8 null allele and a recoded copy of ARG8m (MR6∆ATP8/GTP1ts1) in mtDNA grows in the absence of arginine at the non-permissive temperature, indicating that it is able to express functional Arg8p (acetylornithine aminotransferase). Similarly, the presence of functional Var1p in the gtf1 ts and the her2 point mutants is evident from their ability to translate mitochondrial gene products. Var1p, a component of the small subunit of mitochondrial ribosomes, is essential for ribosome assembly (54). The low glutamine content (1%) of Var1p combined with some leakage of the gtf1 ts and her2 point alleles may help to explain the retention of mitochondrial translation in these strains. A combination of factors may contribute to the presence in the mutants of functional Arg8p, which has a relatively high glutamine content (3%). These include the leakiness of the mutations, tolerance for glutamic acid at some glutamine positions without loss of catalytic activity, and expression from the mitochondrial locus of more Arg8p than is needed for arginine prototrophy.

It is known that Ef-Tu, including that of organelles is able to discriminate against mischarged tRNAs in vivo (55, 56). This has been shown to stem from the low binding affinity of mischarged tRNAs to the elongation factor (57). Nonetheless, several studies indicate that this line of defense against amino acid misincorporation is not fool-proof and can be overcome when the mischarged tRNA is present in excess. Overexpression of non-discriminating glutamyl-tRNA synthetase in E. coli, which lacks GatCAB, has been interpreted to be toxic because of incorporation of glutamic acid from mischarged tRNA\(^{\text{Gln}}\) at glutamine codons (58). Evidence for mischarging is also supported by missense suppression of an E. coli mutant that incorporates aspartic acid at an asparagine codon. The suppression in this case depended on overexpression of a non-discriminating aspartyl-tRNA synthetase to produce aspartyl-tRNA\(^{\text{Asn}}\) (59). Assuming that at the restrictive temperature gtf1 ts mutants have predominantly mischarged glutamyl-tRNA\(^{\text{Gln}}\), this would be analogous to the situation in the above mentioned studies in which the misacylated aminoacyl-tRNAs were present in excess.

Gtf1p is a hydrophilic protein bound to the internal side of the mitochondrial inner membrane. It is an extrinsic membrane protein extractable at alkaline pH with sodium carbonate. The existence of gtf1 dominant mutant alleles is consistent with the presence of Gtf1p in a complex with one or more proteins and is in agreement with the proposed role of Gtf1p as a linker protein for the Pet112p and Her2p components of the mitochondrial heterotrimeric AdT complex (3). In addition to the similarity of the phenotype of gtf1 and her2 mutants, this is also supported by our findings that PET112 is a multicopy suppressor of gtf1 and her2 ts mutants and that co-expression of PET112 and HER2 improved the respiratory growth of gtf1 ts mutant at the non-permissive temperature.

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