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Characterization of Gtf1p, the Connector Subunit of Yeast Mitochondrial tRNA-dependent Amidotransferase*[S]

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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 F_1-F_0 ATP synthase that is degraded, thereby preventing assembly of the F_0 sector. Allotopic expression of recoded *ATP8* and *COX2* did not significantly improve growth of *gtf1* mutants on respiratory substrates. However, ts *gft1* 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

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials and Methods, Tables S1 and S2, and Figs. S1 and S2.

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[2] The abbreviations used are: AdT, amidotransferase; mtDNA, mitochondrial DNA; ts, temperature-sensitive; ρ^o mutant, respiratory deficient mutant lacking mitochondrial DNA; ρ^- mutant, respiratory deficient mutant with a partially deleted mitochondrial genome.

TABLE 1
Genotypes and sources of *Saccharomyces cerevisiae* strains

Strain	Genotype	mtDNA	Source
W303-1A	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1*	ρ^+	R. Rothstein[a]
W303-1B	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+	R. Rothstein
aW303/I^0	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	Intronless	Ref. 15
W303/ρ^0	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^0	Ref. 15
MR6	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+	Ref. 16
MR6ΔATP8	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	*atp8::ARG8*^{m}	This study
aMR6ΔATP8/GTF1ts	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 gtf1::HIS3 arg8::HIS3* + *pG172/ST25*	*atp8::ARG8*^{m}	This study
aW303 /GTF1ts/nATP8	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3*+ *pG172/ST25* + *pAT8/ST4*	ρ^+	This study
aW303 /GTF1ts/nCOX2	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3*+ *pG172/ST25* + *pCOX2-W168*	ρ^+	This study
aW303 /GTF1ts/nATP8/nCOX2	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3*+ *pG172/ST25* + *pAT8/ST4*+ *pCOX2-W168*	ρ^+	This study
aW303ΔPET111	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 pet111::HIS3*	ρ^+	Ref. 17
aW303ΔCOX20	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox20::URA3*	ρ^+	Ref. 18
W303ΔIMP1	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 imp1::HIS3*	ρ^+	Ref. 17
W303ΔIMP2	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 imp2::URA3*	ρ^+	Ref. 17
W303ΔOXA1	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 oxa1::URA3*	ρ^+	This study
W303ΔGTF1	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3*	ρ^+	This study
aW303ΔGTF1	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3–1 gtf1::HIS3*	ρ^+	This study
a/αW303ΔGTF1	**MAT**a/α *ade2-1/ade2-1 his3-1,15/his3-1,15 leu2-3,112/leu2–3,112 trp1-1/trp1-1 ura3-1/ura3-1 gtf1::HIS3*	Intronless	W303/ρ^0 × aW303/I^0
aW303ΔHER2ts9	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 her2::HIS3* + pG27/TS9	ρ^+	This study
B66	**MAT**a *ade1 her2–1*	ρ^+	Ref. 19
W303/GTF1ts1	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3* + pG172/ST25	Intronless	This study
W303/GTF1ts2	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 gtf1::HIS3* + pG172/ST26	Intronless	This study
W303ΔCOX20/GTF1ts1	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1cox20::URA3 gtf1::HIS3* + pG172/ST25	ρ^+	W303/GTF1ts × aW303ΔCOX20
aW303ΔPET111/GTFts1	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 PET111::HIS3 gtf1::HIS3* + pG172/ST25	ρ^+	W303/GTF1ts × aW303ΔPET111
W303ΔGTF1/ST2	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 gtf1::HIS3 leu2::pG172/ST2*	ρ^+	This study
aW303ΔGTF1/ST21	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1ura3-1 gtf1::HIS3* + pG172/ST21	ρ^+	This study
aW303ΔGTF1/ST22	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 gtf1::HIS3 ura3::pG172/ST22*	ρ^+	This study

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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 $MnCl_2$, 1.5 mM $MgCl_2$, 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′-acgaaccaagcTttccttgg (the capital T was inserted in the place of a "G" in order to create a HindIII site) and 5′-ggcaagctttcaagcgtagtctgggacgtcgtatgggtacttcctgtttttgagtagtccctct (which introduces a HindIII site and HA tag at the 3′-end of *GTF1*). *HER2 was* amplified from plasmid pG27/ST3, consisting of the shuttle vector YEp351 with a 3-kb SmaI-PstI fragment containing *HER2*. The primers used for the amplification were 5′-ggcgagctccctggttcaacgatg (SacI site) and 5′-gggtaacgccagggttttccc (this primer is complementary to YEp351). 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

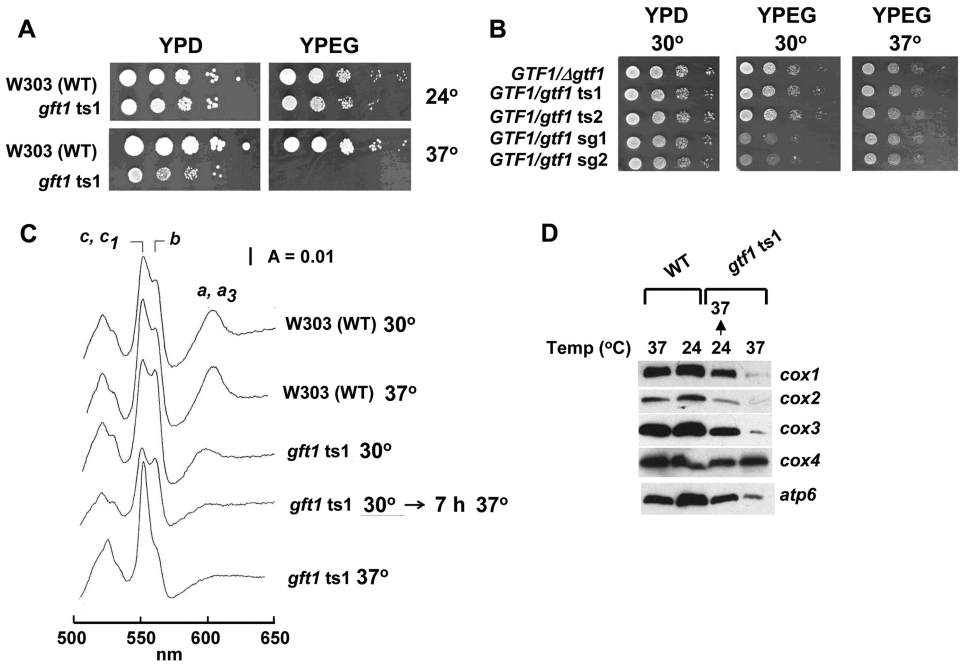

FIGURE 1. **Growth properties, mitochondrial spectra, and steady-state concentrations of mitochondrial products in the *gtf1* ts mutant.** *A*, the wild type strain W303-1A and the *gtf1* null mutant harboring a ts allele of the gene on a CEN plasmid (*gtf1* ts1) were grown at 24 °C in liquid YPD (rich glucose medium) and were serially diluted and spotted on solid rich glucose (YPD) and rich glycerol plus ethanol (YPEG) media. The plates were incubated at 24 and 37 °C for 2.5 days. *B*, the heterologous diploid mutant a/αW303ΔGTF1 (*GTF1/Δgtf1*), the heterozygous mutant transformed with either the ts1 or ts2 alleles (*GTF1/gtf1* ts1 and *GTF1/gtf1* ts2), 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 ρ^-/ρ^0 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 ρ^-/ρ^0 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).

the heterozygous diploid strains a/αW303ΔGTF1 and a/αW303ΔHER2. The heterozygous *gtf1/GTF1* strain was obtained by crossing the *gtf1* null mutant aW303ΔGTF1 to W303/I°. 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- and tryptophan-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 (YEPG). Two conditional *gtf1* mutants (W303/GTF1ts and W303/GTF1ts2) were used to purify the plasmids pG172/ST25 and pG172/ST26 with the ts1 and ts2 allele, respectively. Similarly, one *her2* ts mutant (W303/HER2ts9) was used to purify the plasmid pG27/TS9.

Miscellaneous Methods—Standard methods were used for plasmid manipulations (23) and transformation of yeast (24). Protein concentrations were determined by the method of Lowry *et al.* (25). ATPase and NADH cytochrome *c* reductase activity were measured as described previously (26). The buffer system of Laemmli (27) was used for SDS-PAGE with the modifications indicated in the figure legends. A yeast genomic library constructed from a partial Sau3A digest of nuclear DNA cloned in the shuttle vector YEp24 (28) was kindly provided by Dr. Marian Carlson (Department of Genetics and Development, Columbia University).

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 ρ^- and ρ^0 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_1-F_0 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_1-F_0 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. 1*A*). 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

TABLE 2

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 described (30). The values in experiment 1 are reported as averages of duplicates with the ranges indicated. Oligo, oligomycin.

Strain	Temperature	ρ^-, ρ^0	ATPase		Inhibition
			Without Oligo	With Oligo	
	°C	%	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

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. 1*B*). 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. 1*B*). 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^-/ρ^0 mutants after growth at 30 °C. The percentage of secondary ρ^-/ρ^0 mutants was increased to 12% after growth at 37 °C for 7 h. When grown continuously at 37 °C, the population of ρ^-/ρ^0 mutants increased to 40–50%.

Mitochondrial spectra indicated an abnormally low ratio of cytochromes *a* and a_3 relative to cytochrome *b* in the ts mutant (Fig. 1*C*). 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 F_0 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. 1*D*) 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. 1*D*). The decrease in these mitochondrial gene products was even more pronounced when the mutant was grown continuously at 37 °C.

Analysis of Mitochondrial Gene Products Translated in Vivo in a gtf1 ts Mutant—The explanation for the cytochrome oxidase and F_1-F_0 ATP synthase deficits in the ts mutant grown at the restrictive temperature emerged from an analysis of the mitochondrial gene products synthesized *in vivo* by cells that 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 Atp8p labeling in wild type but a 20-fold decrease in the mutant (Fig. 2, *A* and *B*).

TABLE 3

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

NADH cytochrome *c* reductase activity was measured in the absence and presence of 2 μM antimycin A. The values in experiment 1 are reported as averages of duplicates with the ranges indicated.

Strain	Temperature	ρ^-, ρ^0	Cytochrome *c* reduced
	°C	%	μmol/min/mg protein
W303-1A	30	<1	0.795 ± 0.035
W303-1A	37	<1	0.758 ± 0.055
W303/GTF1ts1	30	2	0.350 ± 0.024
W303/GTF1ts1	37	12	0.035 ± 0.007

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. 2*A*). 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 *imp1* 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. 2*C*, *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. 2*C*). The middle band migrated identically to the unprocessed precursor (pCox2p) of the *imp1* and *oxa1* mutant (32, 33). The third novel protein (Cox2p*), migrating slightly slowed than the precursor, was detected only in the *gtf1* ts mutant labeled at 37 °C (Fig. 2*C*, *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. 2*D*) 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-

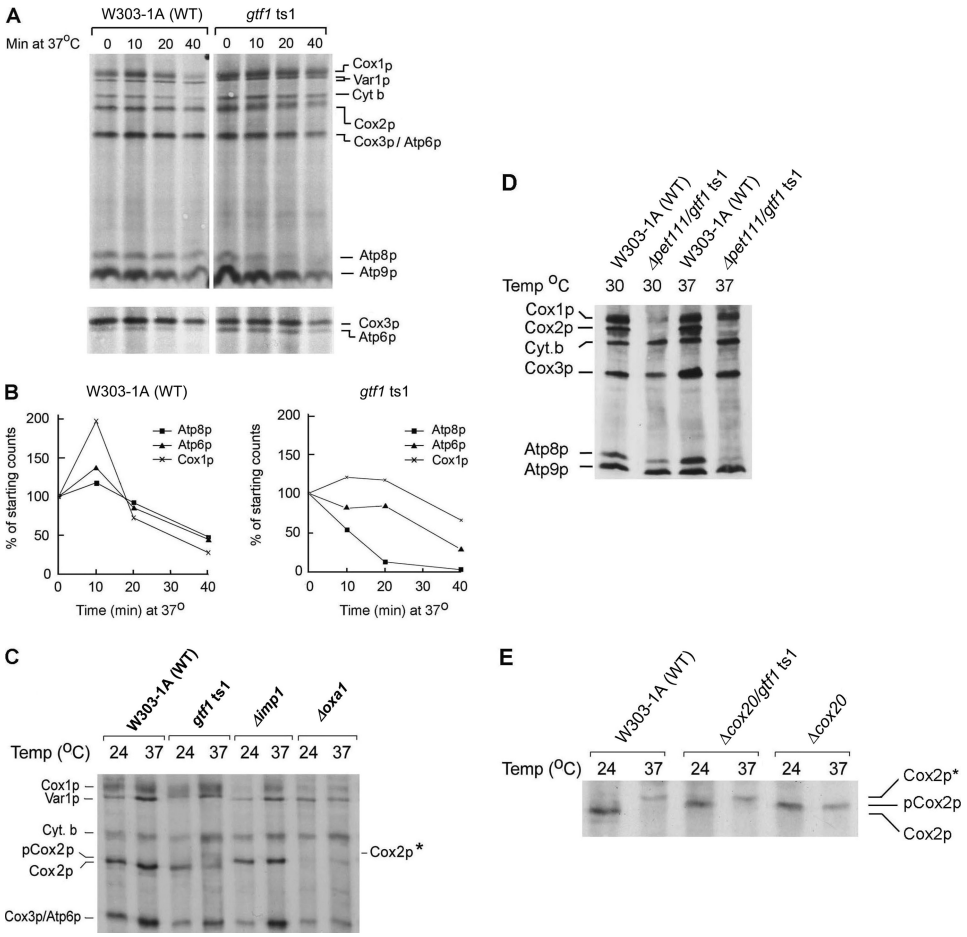

FIGURE 2. ***In vivo* translation of mitochondrial gene products in wild type and the *gtf1* ts mutant.** *A*, W303-1A and the *gtf1* ts1 mutant were grown at 24 °C, and incorporation of [^{35}S]methionine into the mitochondrial translation products was assayed *in vivo* as described previously (18, 31). Cells were incubated at 37 °C for 0, 10, 20, and 40 min prior to labeling at 37 °C for 20 min. Total proteins were separated by SDS-PAGE on a 17% polyacrylamide gel with a 30:0.8 ratio of acrylamide to bisacrylamide (*top*) and on a 12% acrylamide gel in the presence of 6 M urea (*bottom*) to separate Cox3p and Atp6p. Western blots were processed as in Fig. 1. In the 17% polyacrylamide gel, cytochrome *b* migrates slower than Cox2p. *B*, the radiolabeled products in *A* were quantified with a PhosphorImager and are expressed as a percentage of the values measured in cells that had not been incubated at 37 °C. *C*, W303-1A, the *gtf1* ts1 mutant, and the *imp1* (Δ*imp1*) and *oxa1* (Δ*oxa1*) null mutants were first incubated for 20 min at either 24 or 37 °C for 20 min and labeled with [^{35}S]methionine for 20 min at the same temperatures. Proteins were separated by SDS-PAGE on a 17% polyacrylamide gel with the pH of the running buffer adjusted to 8.3. *D*, W303-1A and the *gtf1* ts mutant with a *pet111* null allele (Δ*pet111*/*gtf1* ts1) were grown at 30 °C and preincubated for 20 min at 30 or 37 °C prior to initiating translation at 24 °C for 20 min. Total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide. *E*, the wild type W303-1A, the single *cox20* (Δ*cox20*) and ts *gtf1* (*gtf1* ts1) mutants, and the double *cox20* and *gtf1* ts1 mutant (Δ*cox2*, *gtf1* ts1) were labeled, and mitochondrial products were separated as in *C*. The radiolabeled bands corresponding to mature Cox2p, the precursor pCox2p, the novel Cox2p* polypeptide, subunit 1 (*Cox1p*) and subunit 3 (*Cox3p*) of COX, cytochrome *b* (*Cyt. b*), subunit 6 (*Atp6p*), subunit 8 (*Atp8p*), and subunit 9 (*Atp9p*) of the ATPase are marked in the *margins*.

types are not interdependent was confirmed by the significant reduction of i*n vivo* translation of Atp8p in the double mutant grown at 30 °C and shifted to 37 °C for 20 min (Fig. 2*D*). 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. 2*E*). 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. 2*E*). The dominance of the ts phenotype in the double mutant constitutes strong evidence that Cox2p* has an unprocessed N terminus.

Translation of Cox2p and Atp8p in Wild Type and gtf1 Mutant Mitochondria—The unusual Cox2p phenotype of the *gtf1* ts mutant, disclosed by *in vivo* translation, was corroborated by analyzing the products translated *in organello* by mitochondria of a respiratory competent strain, of two *gtf1* ts

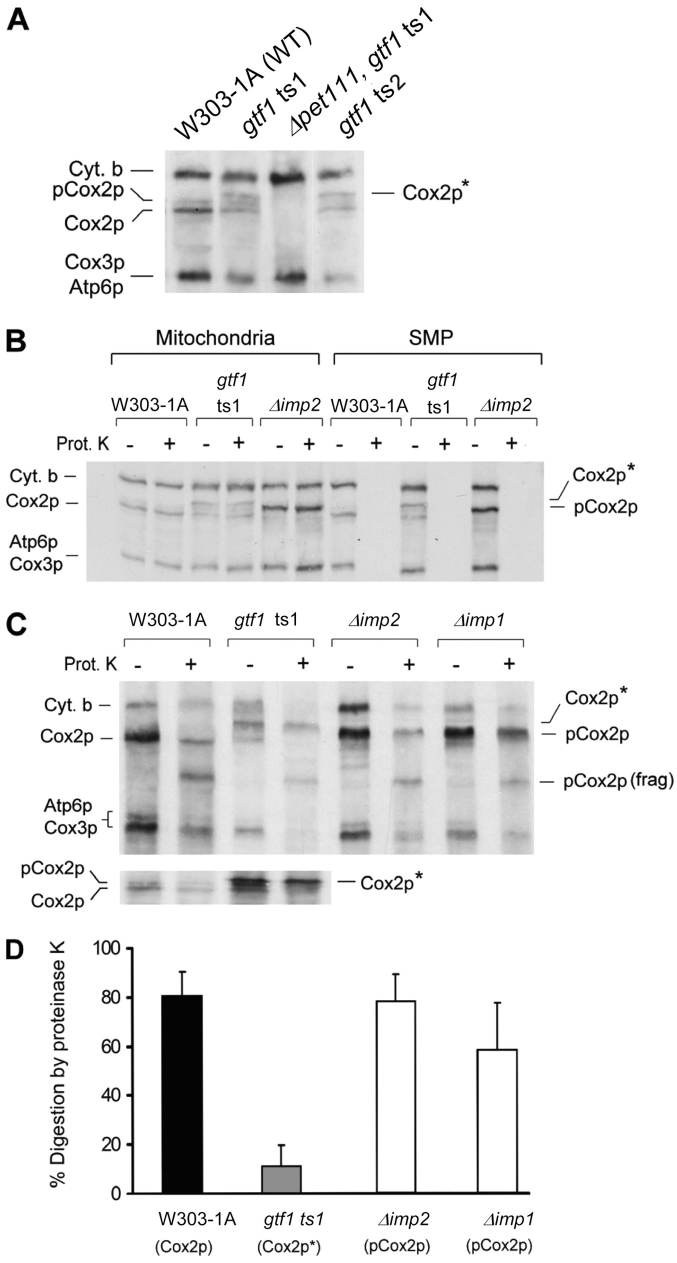

FIGURE 3. ***In organello* translation and processing of the Cox2p precursor in wild type and mutants.** *A*, W303-1A, the *gtf1* ts mutants W303/GTF1ts1 and ts2 (*gtf1* ts1 and *gtf1* ts2) and the double *pet111* and *gtf1* ts1 mutant (Δ*pet111*, *gtf1* ts1) were grown in liquid YPGal medium, and mitochondria prepared by the method of Herrmann *et al.* (39) were preincubated at 37 °C for 20 min and labeled at the same temperature for 20 min in a volume of 320 μl as described previously (18). The labeled mitochondria were separated by SDS-PAGE and visualized as in Fig. 2*C*. *B*, mitochondria were prepared from W303-1A, from the ts mutant W303/GTF1ts1 (*gtf1* ts1), and from the *imp2* null mutant (Δ*imp2*) blocked in processing of the Cox2p precursor. Mitochondria were incubated and labeled at 37 °C as in *A*. One-half of the labeled mitochondria were converted to submitochondrial particles (*SMP*) by sonication. One-half of the remaining mitochondria and of the SMPs were treated at 4 °C for 45 min with proteinase K at a final concentration of 0.1 mg/ml. The proteinase K-treated and -untreated samples were precipitated with TCA, dissolved in Laemmli sample buffer (27), and separated by SDS-PAGE as in *A*. *C*, mitochondria, labeled as in *A*, were diluted with seven volumes of cold 20 mM Hepes, pH 7.5, and one-half was treated with proteinase K as in *B*. The mitoplasts formed under the hypotonic conditions were precipitated with TCA and separated by SDS-PAGE as in *A*. The Cox2p fragment (*frag*) produced by the proteinase K treatment is indicated on the *right*. A clearer separation of the three *COX2*-related bands of the *gtf1* ts1 mutant is shown at the *bottom*. *D*, the radioactivity associated with mature Cox2p of wild type mitochondria, the precursor pCox2p of the two different *imp* mutants, and the novel Cox2p* band in the *gtf1* ts1 mutant is presented as the percentage of radioactivity remaining after proteinase K treatment. *Error bars*, S.D.

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 [^{35}S]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. 3*A*). 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. 3*A*). 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. 3*C*). 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. 3*B*).

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 Cox3p (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 mutant (Fig. 3, *C* and *D*). Similar results were obtained with the pCox2p accumulated in *imp1* and *imp2* mutants, blocked in processing of the precursor (38). Digestion of Cox2p* in the *gtf1* ts mutant, however, was significantly less. The 5–20% loss of this protein measured in four different experiments may represent the fraction of mitoplasts that had become leaky to proteinase K during the hypotonic treatment.

Northern Analysis of Mitochondrial Transcripts—The absence of Atp8p among the products labeled *in vivo* in *gtf1* tsl mutants at the restrictive temperature (Fig. 2*A*) 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 polycistronic transcript removes the *COX1* sequence and produces a 5.2-kb transcript containing *ATP8* and *ATP6* (and *ENS2*, if present in the genome). The 5.2-kb transcript is further processed at a site 600 nucleotides from the 5′-end to yield a

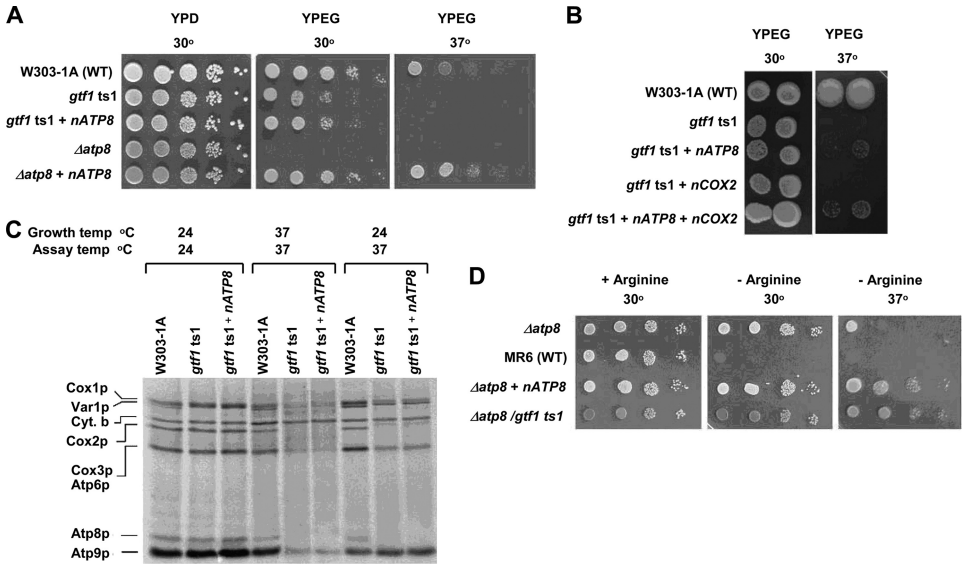

FIGURE 4. **Rescue of an *atp8* but not *gtf1* mutant by *nATP8* and combined expression of *nATP8* and *nCOX2*.** *A*, serial dilutions of W303-1A, the ts mutant W303/GTF1ts1 (*gtf1* ts1), the ts mutant harboring recoded *nATP8* under the control of the *ADH1* promoter (*gtf1* ts1 + *nATP8*), the *atp8* null mutant (Δ*atp8*), and the *atp8* null mutant transformed with the recoded *nATP8* (Δ*atp8* + *nATP8*) were spotted on YPD and YPEG and incubated at 30 °C or 37 °C for 2 days. *B*, strains shown in *A* plus the *gtf1* ts mutant harboring the recoded $nCOX2^{W56R}$ (*gtf1* ts1 + *nCOX2*) and both *nATP8* and $nCOX2^{W56R}$ (*gtf1* ts1 + *nATP8* + *nCOX2*) were tested for growth on YPEG at 30 °C for 2 days and at 37 °C for 7 days. *C*, the wild type strain W303-1A, the ts *gtf1* mutant (*gtf1* ts1), and the ts mutant transformed with recoded *nATP8* in the high copy plasmid YEp351 under the control of the *ADH1* promoter (*gtf1* ts1 + *nATP8*) were grown at 24 °C and labeled with [^{35}S]methionine in the presence of cycloheximide at either 24 or 37 °C as in Fig. 2. The same strains were also grown and labeled at 37 °C. The radioactive bands corresponding to mature Cox2p, the novel Cox2p* protein, Atp8p, and other mitochondrial gene products are marked in the *margins*. *D*, MR6 is a respiratory competent haploid strain with an *arg8* null mutation. MR6ΔATP8 (Δ*atp8*) has the *arg8* null mutation and mtDNA in which the mitochondrial *ATP8* gene has been replaced with $ARG8^{m}$. MR6ΔATP8 was also transformed with pATP8/ST4, a high copy plasmid with *nATP8* under the control of the *ADH1* promoter (Δ*atp8* + *nATP8*). aMR6ΔATP8/GTF1ts1 (Δ*atp8/gtf1* ts1) has the *gtf1* ts1 and the mitochondrial *ATP8* gene replaced by $ARG8^{m}$. The strains were grown in liquid minimal glucose medium supplemented with all of the auxotrophic requirements of MR6, and serial dilutions were spotted on solid minimal glucose medium with and without arginine. The plates were incubated at 30 or 37 °C for 2 days. The poor growth of the Δ*atp8* null mutant at 37 °C in the absence of arginine is due to extensive conversion of this strain to $\rho^{-/o}$ mutants that are unable to express $ARG8^{m}$. The red coloration of aMR6ΔATP8/GTF1ts1 (Δ*atp8/gtf1* ts1) tends to obscure the robust growth of this strain under all three conditions in the photograph.

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 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 gft1 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 [^{35}S]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/ST3) and in the related plasmid pGML3 (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. 4*A*). 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. 4*C*). Some growth of the *gtf1* ts2 was detected but only after 7 days of incubation at 37 °C (Fig. 4*B*).

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 $nCOX2^{W56R}$ in the *gtf1* ts1 mutant did not improve its growth on respiratory substrates at the non- permissive temperature. Co-expression of *nATP8* and $nCOX2^{W56R}$ achieved some improvement in growth at 37 °C but only after a prolonged incubation (7 days; Fig. 5*B*). This indicated that the respiratory defective phenotype of the *gtf1* ts1 mutant was not significantly altered by the presence of *nATP8* and *nCOX2*.

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/GTF1ts1 + *nATP8* are transformants harboring recoded *nATP8* in the high expression vector pGML3 (pATP8/ST4). Oligo, oligomycin.

Strain	Temperature	ρ^-, ρ^0	ATPase		Inhibition
			Without Oligo	With Oligo	
	°C	%	μmol/mg/min		%
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/GTF1ts1	37	46	1.98	1.97	1
W303/GTF1ts1 + *nATP8*	37	52	1.54	1.52	1

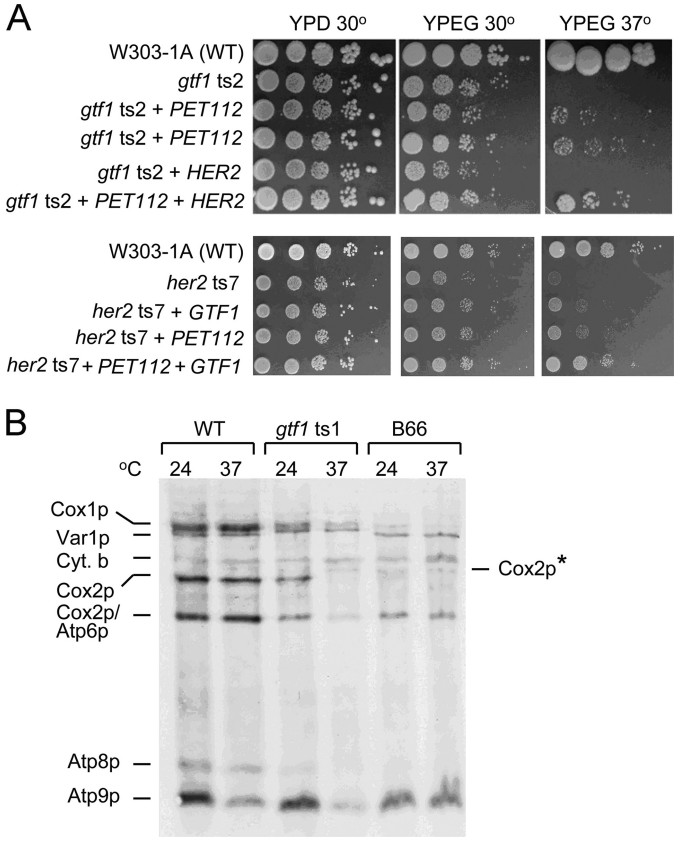

FIGURE 5. ***PET112* suppress ts *gtf1* and *her2* mutants.** *A*, growth of W303-1A, of the *gtf1* ts mutant (*gtf1* ts2), and the *gtf1* ts harboring multicopy plasmids expressing *PET112* (*gtf1* ts2 + *PET112*), *HER2* (*gtf1* ts2 + *HER2*), and both *PET112* and *HER2* (*gtf1* ts2 + *PET112*, + *HER2*), as well the *her2* ts mutant (*her2* ts) and the *her2* ts harboring multicopy plasmids expressing *PET112* (*her2* ts + *PET112*), *GTF1* (*her2* ts + *GTF1*), and both *PET112* and (*her2* ts + *PET112* + *GTF1*). The different strains were serially diluted, spotted on YPD and YEPG, and incubated for 2 days at 30 °C and 7 days at 37 °C. *B*, W303-1A, the *gtf1* ts1 mutant (*gtf1* ts1), and the *her2* point mutant B66 were grown at 24 °C and labeled with [^{35}S]methionine in the presence of cycloheximide at either 24 or 37 °C as in Fig. 2. The same strains were also grown and labeled at 37 °C. The radioactive bands corresponding to mature Cox2p, the novel Cox2p* protein, Atp8p, and the other mitochondrial gene products are marked in the *margins*.

The *gtf1* ts mutation was also tested on expression of mitochondrial *ARG8^m* at the *ATP8* locus of mtDNA. *ARG8^m* 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^m* (Fig. 4*D*). 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^0 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^m* (Fig. 4*D*). Surprisingly, expression of *ARG8^m* at the *ATP8* locus was not affected by the *gtf1* ts allele at either the permissive or restrictive temperature (see Δ*atp8*/*gtf1* ts1 in Fig. 4*D*).

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. 6*A*). 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. Overexpression of both *GTF1* and *PET112*, if anything, decreased the rescue (Fig. 5*A*).

The *her2* point mutant B66 displays the same phenotype as *gtf1* ts mutants; B66 is a *her2* point mutant assigned to complementation group G27 of our *pet* mutant collection (19). *In vivo* labeling of mitochondrial gene products in B66 with [^{35}S]methionine revealed the absence of Atp8p and presence of Cox2p* in this strain (Fig. 5*B*). The similarity of the phenotypes of the *her2* point and *gtf1* ts mutant suggests that the products of these genes have related functions in mitochondrial translation.

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

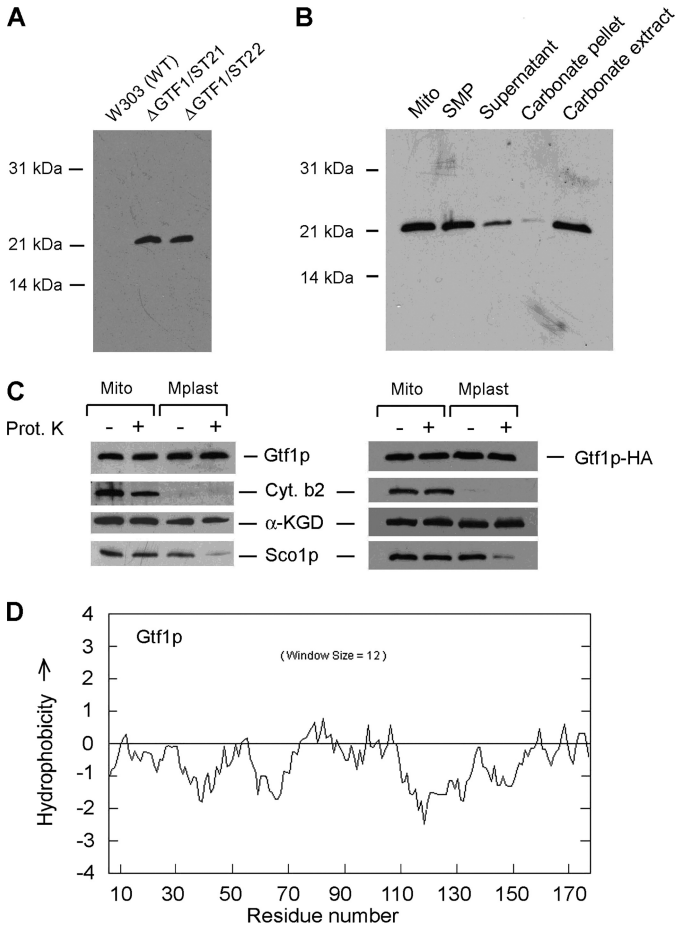

FIGURE 6. **Gtf1p is a peripheral mitochondrial inner membrane protein facing the matrix compartment.** *A*, mitochondria were prepared from W303-1B and from W303ΔGTF1/ST21 (ΔGTF1/ST21) and W303ΔGTF1/ST22 (ΔGTF1/ST22) expressing Gtf1p-HA, respectively, from a high copy plasmid and as a single copy integrated 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. 1*D*. *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 $\times$ g_{av} 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 separated from the membranes (carbonate pellet) by centrifugation at 90,000 $\times$ g_{av} for 20 min. The samples, adjusted to the starting volume of mitochondria, were separated by SDS-PAGE. Gtf1p-HA was visualized as in *A*. *C*, mitochondria were prepared by the method of Glick and Pon (51) from the respiratory competent strain W303-1A and from W303ΔGTF1/ST22. Mitochondria (*Mito*) and hypotonically lysed mitoplasts (*Mplast*) were incubated on ice for 1 h in the absence or presence of 0.1 mg/ml proteinase K. Mitochondria and mitoplasts were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a rabbit polyclonal antibody against Gtf1p (*left*) and a mouse monoclonal antibody against the HA epitope (*right*). The Western blot was also reacted with rabbit polyclonal antibodies against Sco1p, cytochrome b_2 (*Cyt. b2*), and α-ketoglutarate dehydrogenase (α-*KGD*). *D*, hydropathy profile of Gtf1p.

tag was inserted after the 27th residue, assuming that the primary translation product is synthesized with an N-terminal sequence cleaved after the phenylalanine at position 24. The choice of the cleavage site was based on the alignment of the *S. cerevisiae* sequence with other fungal homologues and the Mitoprot (50) prediction of the signal peptide. This gene complemented the *Gtf1* null mutant when present either on a multicopy plasmid or in single copy, integrated at the chromosomal *URA3* locus. A monoclonal antibody against the HA tag detected a protein of 23 kDa (Fig. 6*A*), which is some 4 kDa larger than expected based on the sequence and the assumed cleavage site. It is not clear if this discrepancy is due to anomalous migration of the protein or if the prediction of where the protein is cleaved is incorrect. A similar size protein was detected with the Gtf1p antibody (not shown).

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. 6*B*). 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.

Gtf1p is located in the inner membrane and is protected against proteinase K digestion in intact mitochondria and in mitoplasts (Fig. 6*C*). The resistance of Gtf1p to the protease in mitoplasts indicates that it faces the matrix side of the inner membrane. Lysis of the outer membrane after exposure of mitochondria to hypotonic conditions was verified by the loss of soluble cytochrome b_2 located in the intermembrane space and the sensitivity of Sco1p, an inner membrane protein facing the intermembrane space, to proteinase K in mitoplasts. The presence of α-ketoglutarate dehydrogenase in mitoplasts confirmed the intactness of the inner membrane.

DISCUSSION

A previous study reporting the presence of a nuclear encoded $tRNA^{Gln}$ in yeast mitochondria was interpreted to indicate that translation of mitochondrial gene products does not require AdT to generate glutaminyl-$tRNA^{Gln}$ from glutamyl-$tRNA^{Glu}$ (14). More recently, however, yeast mitochondrial glutaminyl-$tRNA^{Gln}$ 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 F_0 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 *ATP8/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 $ARG8^m$ at the *ATP8* locus. Mutations leading to a loss of AdT activity should block transamidation of glutamyl-$tRNA^{Gln}$ to glutaminyl-$tRNA^{Gln}$ with a consequent incorporation of glu-

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 a_3 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 *gft1* mutation on these subunits.

The *gtf1* ts mutant containing a nuclear *arg8* null allele and a recoded copy of $ARG8^{m}$ (MR6ΔATP8/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 leakiness 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 $tRNA^{Gln}$ 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-$tRNA^{Asn}$ (59). Assuming that at the restrictive temperature *gtf1* ts mutants have predominantly mischarged glutamyl-$tRNA^{Gln}$, 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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