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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 18, Issue of May 5, pp. 13291–13296, 2000

Printed in U.S.A.

Expression of *Arabidopsis thaliana* Mitochondrial Alanyl-tRNA Synthetase Is Not Sufficient to Trigger Mitochondrial Import of $tRNA^{Ala}$ in Yeast*

Received for publication, December 9, 1999, and in revised form, February 8, 2000

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It has often been suggested that precursors to mitochondrial aminoacyl-tRNA synthetases are likely carriers for mitochondrial import of tRNAs in those organisms where this process occurs. In plants, it has been shown that mutation of U^{70} to C^{70} in *Arabidopsis thaliana* $tRNA^{Ala}$(UGC) blocks aminoacylation and also prevents import of the tRNA into mitochondria. This suggests that interaction of $tRNA^{Ala}$ with alanyl-tRNA synthetase (AlaRS) is necessary for import to occur. To test whether this interaction is sufficient to drive import, we co-expressed *A. thaliana* $tRNA^{Ala}$(UGC) and the precursor to the *A. thaliana* mitochondrial AlaRS in *Saccharomyces cerevisiae*. The *A. thaliana* enzyme and its cognate tRNA were correctly expressed in yeast *in vivo*. However, although the plant AlaRS was efficiently imported into mitochondria in the transformed strains, we found no evidence for import of the *A. thaliana* $tRNA^{Ala}$ nor of the endogenous cytosolic $tRNA^{Ala}$ isoacceptors. We conclude that at least one other factor besides the mitochondrial AlaRS precursor must be involved in mitochondrial import of $tRNA^{Ala}$ in plants.

Import of cytosolic tRNAs into mitochondria has been described in a wide range of organisms, including yeast, trypanosomatids, and plants (1, 2). The number of imported tRNA species varies widely between these organisms: only one in *Saccharomyces cerevisiae* (3), seven to ten in higher plants (4–6), and all mitochondrial tRNAs in trypanosomatids (7, 8). These differences may reflect different import mechanisms. According to *in vitro* studies, tRNAs and proteins seem to be imported by distinct mechanisms into the mitochondria of *Trypanosoma brucei* (9), and results obtained with *Leishmania tropica* indicate that tRNAs may bind directly to a mitochondrial outer membrane receptor (10, 11). Aminoacyl-tRNA synthetases (aaRSs)[1] are clearly not involved in the process in trypanosomatids (12). In contrast, mitochondrial targeting of $tRNA^{Lys}$(CUU) in *S. cerevisiae* requires a functional mitochondrial protein import system (13) and participation of both the cytosolic and the mitochondrial lysyl-tRNA synthetase (LysRS) (14). The cytosolic LysRS is necessary for aminoacylation of the tRNA, which is a prerequisite for mitochondrial import of the natural $tRNA^{Lys}$(CUU). Subsequently, the precursor to the mitochondrial LysRS and other unknown protein factor(s) are needed for the actual import step (14, 15).

In higher plants, nucleus-encoded $tRNA^{Ala}$ isoacceptors partition between the cytosol and the mitochondria in all species investigated (4–6). The implication of the aminoacyl-tRNA synthetases in the tRNA import process has been suggested in the case of *Arabidopsis thaliana* $tRNA^{Ala}$, because a mutant tRNA that is not aminoacylatable by the alanyl-tRNA synthetase (AlaRS) (16) is also not imported into plant mitochondria *in vivo* (17). We previously cloned the *A. thaliana* nuclear gene encoding mitochondrial AlaRS and showed that it also encodes the cytosolic AlaRS by the alternative use of two transcription initiation sites and two translation initiation codons, leading to the synthesis of a long form of the protein with a N-terminal mitochondrial targeting peptide and a short one remaining in the cytosol (18). To clarify the involvement of *A. thaliana* AlaRS in mitochondrial import of $tRNA^{Ala}$, we addressed the question of whether this enzyme is sufficient to direct its cognate tRNA into the mitochondria of a eukaryote that does not naturally import $tRNA^{Ala}$. To do this, we expressed the *A. thaliana* mitochondrial AlaRS precursor and $tRNA^{Ala}$ in *S. cerevisiae* and analyzed the presence of the enzyme and the tRNA in the mitochondria of the transformed cells.

MATERIALS AND METHODS

Strains, Media, and Transformation Procedures—The strains used in this study are listed in Table I. Culture conditions used the classical yeast medium SD (minimal medium) and YPD (rich medium) according to Ref. 19. Respiratory growth was assessed on YPEG medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 3% (v/v) ethanol, 3% (v/v) glycerol). *S. cerevisiae* nuclear transformation was carried out using the one step transformation described by Gietz *et al.* (20).

Construction of Plasmids—All the constructs that were transferred to *S. cerevisiae* were made in a modified version of the pFL61 yeast expression vector (21), in which the *URA3* marker was replaced by the *LEU2* marker recovered from pFL36 (22) following digestion with *Bgl*II. This modified pFL61 was named p61L. In all constructs, the AlaRS coding sequence was inserted as a *Not*I fragment between the phosphoglycerate kinase promoter and terminator of p61L. The successive steps were as follows. The *A. thaliana* AlaRS cDNA corresponding to the cytosolic form of the enzyme (from the ATG to the TGA codon, excluding 5′ and 3′ untranslated sequences) was amplified by PCR from the plasmid 2WT (18) using the oligonucleotides 5′-CTGCAGAACCAGTGTGCTGGAAA**ATG**CCGGGTTCCGAA-3′ and 5′-CTGCAGAACCAGT-

* This work was supported by funds from the Institut National de la Recherche Agronomique, the Center National de la Recherche Scientifique, and the Université Louis Pasteur, a grant from the Groupement de Recherche et d'Etude des Génomes, and National Institutes of Health Grant GM29362. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; AlaRS, alanyl-tRNA synthetase; DIFP, diisopropylfluorophosphate; LysRS, lysyl-tRNA synthetase; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction.

TABLE I
S. cerevisiae strains used in this study

Mitochondrial genotypes are in brackets. Genes not in brackets are nuclear.

Strain	Genotype	Source or reference
W303–1B	*MAT*a *ade2–1 leu2–3,112 his3–11,15 trp1-1 ura3–1 can1–100[rho$^+$]*	F. Lacroute[a]
MCC123r^0	*MAT*a *ade2 ura3 kar1-1 [rho^0]*	Thorsness and Fox, 1993 (37)
HMD3	*MAT*a *lys2 [rho$^+$ cox2–103]*	Dunstan *et al.*, 1997 (38)
HMD13	*MAT*a *ade2 ura3–52 kar 1-1 [rho$^+$ cox2–103]*	H. Dunstan, unpublished
NB40–16B	*MAT*a *leu2–3, 112 lys2 ura3–52 arg8::hisG [rho$^+$ cox2–62]*	N. Bonnefoy[a], unpublished
HM4	*MAT*a *leu2–3, 112 lys2 ura3–52 arg8::hisG [rho$^+$ cox2-amb]*	This study
YPH102	*MAT*a *ade2–101(ochre) leu2-delta1 lys2–801 (amber) his3 delta200 ura3–52 [rho$^+$]*	Sikorski and Hieter, 1989 (39)

[a] Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France.

GTGCTGG**TCA**GTTGAGCTTCAT-3′ (introduced *Bst*XI sites are underlined; the ATG initiation codon and the codon complementary to the TGA stop codon are indicated in bold type). Only the 5′ and 3′ parts of the 3-kilobase amplification product obtained were used for cloning into pFL61 to minimize the incorporation of PCR-amplified DNA. A *Bst*XI-*Sst*I DNA fragment from the PCR product, an *Sst*I-*Spe*I fragment directly isolated from the 2WT plasmid, and an *Spe*I-*Bst*XI fragment from the PCR product were ligated together and inserted into *Bst*XI-digested pFL61. Cloning of the mitochondrial *A. thaliana* AlaRS cDNA was done following the same strategy, except that the PCR used the plasmid 1WT2m (18) as a template and a 5′ primer 5′-CTGCAGAACCAGTGT-GCTGGAAA**ATG**AGATTAGTGAAG-3′ (the *Bst*XI site is underlined, and the ATG initiation codon is indicated in bold type) designed such that the PCR product included the mitochondrial targeting sequence. In both cases, the AlaRS coding sequence flanked with *Bst*XI sites was recovered by *Not*I digestion and cloned into p61L, giving the plasmids p61L-2WT and p61L-1WT2m, respectively (not shown). The *A. thaliana* tRNA^{Ala}(UGC) gene was recovered from a previous construct in pBluescript SK(+) (16) by an *Xba*I-*Ssp*I double digestion and recloned into the *Xba*I and *Sma*I sites of p61L, giving the p61L-tRNA^{Ala} construct (see Fig. 1). The same *Xba*I-*Ssp*I fragment was also inserted between the *Xba*I and *Sma*I sites in pBluescript KS(+) (Stratagene, La Jolla, CA), giving the plasmid KS$^+$/tRNA^{Ala}. An *Eco*RI-*Hin*dIII fragment of pFL61 containing the pUC19 polylinker was cloned into the same sites of KS$^+$/tRNA^{Ala} to allow the *A. thaliana* tRNA^{Ala} gene to be flanked on both sides by a *Bam*HI restriction site. The tRNA^{Ala} gene was then isolated from a *Bam*HI digest and inserted into the *Bam*HI site of the p61L-2WT and p61L-1WT2m plasmids. These constructs were named p61L-2WT/tR and p61L-1WT2m/tR (see Fig. 1). The *A. thaliana* amber suppressor tRNA^{Ala}(CUA) gene was cut out with a *Kpn*I-*Bam*HI double digestion from a plasmid previously constructed for transient expression in tobacco protoplasts (16) and recloned into pFL61 at the same sites.

Preparation of S. cerevisiae Total Proteins and RNA—To prepare total *S. cerevisiae* protein samples for Western blot analysis, cells from log phase cultures ($A_{600\ nm}$ = 1.5–2.0) were collected by centrifugation, washed twice with water, once with mitochondrial breakage buffer (see below), and directly extracted in 200 μl of denaturing sample buffer (23) including 1 mM PMSF, 1 mM DIFP, Complete™ protease inhibitor mixture (according to the instructions of the manufacturer, Roche Molecular Biochemicals) and a droplet of antifoam (Sigma). Following cell disruption for 1 min on a vibrating homogenizer (mini-beadbeater, Biospec Products, Bartlesville, OK) in the presence of 1 volume of 0.5-mm glass beads, the suspension was incubated for 5 min at 100 °C, transferred into a new tube, and cleared by centrifugation for 5 min at 15,000 × *g*.

Total *S. cerevisiae* RNA was extracted from a 500-μl aliquot of the initial cell homogenate when isolating mitochondria (see below). The suspension was adjusted to 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1% (w/v) SDS, and extracted twice with water-saturated phenol (24). Large RNAs were eliminated by selective precipitation in the presence of 1 M NaCl. The tRNA fraction was finally ethanol-precipitated and resuspended in water.

S. cerevisiae Enzyme Extracts and Aminoacylation Assays—*S. cerevisiae* enzyme extracts for aminoacylation assays were prepared from 800 ml of log phase cultures ($A_{600\ nm}$ = 1.5–2.0). All steps were carried out at 4 °C. Cells were collected by centrifugation, washed twice with water, and resuspended in chilled two times concentrated enzyme buffer (100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 20% (v/v) glycerol, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20 μg/ml α_2-macroglobulin, 20 μg/ml leupeptin, 1 mM PMSF, 1 mM DIFP, and Complete™ protease inhibitor mixture). The suspension (about 1 ml) was completed with one volume of chilled acid-washed 0.5-mm glass beads and cells were disrupted by shaking for 6 min in a vibrating homogenizer (Vibrogen Zellmühle, E. Bühler, Tübingen, Germany). Protein extracts were subsequently enriched by centrifugation followed by DEAE-cellulose and Sephadex G-75 chromatography as described previously (24). Protein concentrations (usually 2–15 mg/ml) were estimated according to Bradford (25). Mitochondrial enzyme extracts were obtained by the same procedures following disruption of the organelles (the equivalent of 5–10 mg proteins as estimated according to Ref. 25, see below for the isolation protocol) by sonication for twice 30 s in 500–800 μl of the above enzyme buffer.

Aminoacylation assays were conducted at 37 °C in the presence of limiting amounts of *S. cerevisiae* total or mitochondrial enzyme extracts or of aliquots from hydroxyapatite chromatography fractions (see below). The aminoacylation reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 10 mM ATP, 15 mM $MgCl_2$, 0.4 mM glutathione, 0.1 mg/ml bovine serum albumin, 1–100 μM L-[^{3}H]amino acid (NEN Life Science Products) at 1–74 Ci/mmol, 3 mg/ml total yeast tRNA, and appropriate amounts of proteins.

Preparation of Mitochondrial Proteins and RNA—*S. cerevisiae* mitochondria were isolated from 1 l of end log phase cultures ($A_{600\ nm}$ = 12–15) grown in the presence of 3% (v/v) glycerol, 0.5% (w/v) glucose, and 2% (v/v) ethanol, essentially following procedures described previously (13, 26). Cells were washed twice with water and once with breakage buffer (10 mM Hepes-KOH, pH 6.8, 0.6 M mannitol, 1 mM EDTA, 0.3% (w/v) bovine serum albumin), resuspended in 10 ml of breakage buffer and disrupted by shaking for 5 min in a vibrating homogenizer (Vibrogen Zellmühle). A 500-μl aliquot was withdrawn from the suspension for total RNA extraction (see above), and mitochondria were isolated through two cycles of low and high speed centrifugation followed by a 0.6 M/1.85 M stepwise sucrose gradient. Upon washing and resuspension in a small volume of breakage buffer, two-thirds of the mitochondria were incubated for 10 min at room temperature in the presence of 0.1 μg/μl RNase A and 2 units/μl RNase T1 and recovered by centrifugation. Mitochondrial RNA was obtained from RNase-treated mitochondria by resuspension in extraction buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1% (w/v) SDS), phenol extraction (twice) and ethanol precipitation. The remaining third of the mitochondria was treated for 15 min at 4 °C with 0.1 μg/μl proteinase K and recovered by centrifugation upon addition of 1 mM PMSF, 1 mM DIFP, and Complete™ protease inhibitor mixture. Mitochondrial protein samples for Western blotting were extracted by resuspending proteinase K-treated mitochondria in 70 μl of denaturing sample buffer (23) containing 1 mM PMSF.

Western Blot Analysis—10–50 μg of total or mitochondrial proteins were run on SDS-polyacrylamide gels (23) and analyzed by Western blotting following classical protocols (6, 19). The antisera against *S. cerevisiae* or *A. thaliana* AlaRS were used at a 1:10000 dilution. The antiserum against the yeast enzyme was a gift from A. Tzagoloff (Columbia University, New York, NY). The antiserum against the *A. thaliana* enzyme was prepared previously (18). Binding of the primary antibody was revealed by chemoluminescence using a peroxidase-conjugated secondary antiserum and ECL reagents (Amersham Pharmacia Biotech).

Hydroxyapatite Chromatography of S. cerevisiae Mitochondrial Enzyme Extracts—*S. cerevisiae* mitochondrial enzyme extracts were fractionated by medium pressure chromatography on a 0.8-ml Bio-Scale Ceramic hydroxyapatite Type I (CHT-I) column driven by a BioLogic integrated system (Bio-Rad). The samples (2 mg of proteins in 1 ml) were loaded at 0.5 ml/min on the CHT-I column equilibrated with a 75 mM potassium phosphate buffer, pH 7.5, containing 1 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) 1,2-propanediol, 5 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.5 mM DIFP. After washing with the same buffer (3 ml), elution

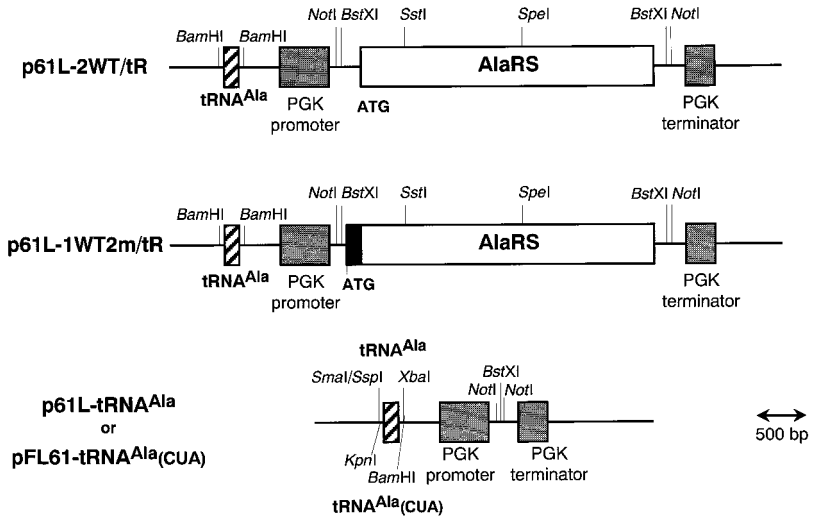

FIG. 1. **Constructs used for heterologous expression of *A. thaliana* AlaRS and tRNA^{Ala}.** All constructs were made in p61L, except for the amber suppressor tRNA^{Ala}(CUA), which was inserted into pFL61. The *A. thaliana* AlaRS coding sequence is indicated by an *open box*, the sequence encoding the mitochondrial targeting peptide by a *black box*, the *A. thaliana* tRNA^{Ala} gene by a *striped box*, and the *S. cerevisiae* phosphoglycerate kinase (*PGK*) promoter and terminator by *shaded boxes*. The restriction sites used during the construction of the plasmids are also indicated.

was carried out at 0.75 ml/min with a linear potassium phosphate gradient (8 ml, 75–350 mM). Fractions of 0.35 ml were collected, and aliquots were submitted to aminoacylation assays and Western blot analyses.

Northern Blot Analysis—Total and mitochondrial RNA samples were run on 15% (w/v) polyacrylamide gels under denaturing conditions (27) and then transferred to Hybond N membranes (Amersham Pharmacia Biotech). Northern analysis was essentially as described in Ref. 28. The ^{32}P-labeled probe for *A. thaliana* cytosolic tRNA^{Ala}(UGC) was obtained by *in vitro* antisense transcription of the corresponding gene with T7 RNA polymerase using a previously described construct in pBluescript SK(+) (17). The following oligonucleotides, ^{32}P-labeled with T4 polynucleotide kinase, were used as probes for *S. cerevisiae* tRNAs: 5′-GGTGGACG(C/A)(G/A)(A/T)CCGGAATCG-3′ (degenerated oligonucleotide complementary to both *S. cerevisiae* tRNA^{Ala} isoacceptors), 5′-CCAAGCATGGGTTGCTTAAAAGAC-3′ (complementary to *S. cerevisiae* mitochondrially encoded tRNA^{Lys}), and 5′-GGTGAAACGGACAGGAA-3′ (complementary to *S. cerevisiae* cytosol-specific tRNA^{Trp}).

Creation of the cox2 Amber Mitochondrial Mutant of S. cerevisiae—An amber codon was introduced in place of an alanine codon at position 114 of the *S. cerevisiae cox2* gene by oligonucleotide-directed mutagenesis of pJM2 (29) using the Mutagene kit (Bio-Rad) and the oligonucleotide 5′-GCTTTAATAGTTAT**CTA**TGGTGAAATAACTTC-3′ (the codon complementary to the amber mutation is indicated in bold type). The *S. cerevisiae* HM4 strain containing this mutant *cox2* allele was constructed in a two-step scheme. First, the plasmid bearing the *cox2* amber allele was transformed into the mitochondria of the MCC123ρ^0 strain by high velocity microprojectile bombardment as described previously (30). Transformant strains harboring the mutant plasmid were identified by screening for marker rescue of the *cox2* amber allele after mating to the strain HMD3. In the second step, the amber mutation was integrated into the mitochondrial genome by cytoduction of the ρ^- mitochondria into the NB40–16B strain. Recombinant ρ^+ progeny were identified by their ability to marker rescue the *cox2-103* mutation after crossing to the HMD13 strain. The presence of the amber mutation was verified by sequencing the relevant region of the *cox2* gene of the ρ^+ mitochondrial DNA. The genotypes of the strains used are given in Table I.

RESULTS

The Mitochondrial A. thaliana AlaRS Can Be Expressed in S. cerevisiae—*A. thaliana* AlaRS cDNAs with or without the region encoding the mitochondrial targeting sequence of the enzyme were cloned, together with an *A. thaliana* tRNA^{Ala}(UGC) gene, into a derivative of the yeast expression vector pFL61 (21) (Fig. 1). The resulting constructs (p61L-1WT2m/tR and p61L-2WT/tR in Fig. 1) were transformed into *S. cerevisiae*, and their expression was tested by Western blot analysis using anti-*A. thaliana* AlaRS antibodies (18) to probe total proteins (data not shown). The strain carrying the complete cDNA including the region encoding the targeting sequence produced significant amounts of the *A. thaliana* AlaRS, which migrated as expected on denaturing gels (apparent molecular mass of about 105 kDa). On the contrary, we saw no sign of significant expression of the *A. thaliana* AlaRS upon transformation of yeast with a cDNA encoding the cytosolic form of the enzyme, despite the abundant accumulation of the corresponding mRNA in transformed cells (data not shown).

To validate these results and confirm that the mitochondrial *A. thaliana* AlaRS expressed in *S. cerevisiae* was functional, *i.e.* still capable of aminoacylating tRNA^{Ala}, total enzyme extracts were prepared from the strains transformed with the AlaRS gene (p61L-1WT2m/tR and p61L-2WT/tR in Fig. 1) and from a control strain transformed with a construct lacking the AlaRS gene (p61L-tRNA^{Ala} in Fig. 1). The extracts were tested for aminoacylation activity in the presence of [^{3}H]alanine and *S. cerevisiae* total tRNA, which is a good substrate for both plant and yeast AlaRS. In agreement with the Western blot results, no increase in AlaRS activity was measured with the strain expressing the cytosolic form of plant AlaRS (Fig. 2*A*), further suggesting the instability of this form of the plant enzyme in yeast. Conversely, an approximately 2-fold increase in AlaRS activity, as compared with the control, was observed in the case of the *S. cerevisiae* strain expressing the plant mitochondrial AlaRS (Fig. 2*A*). Leucyl-tRNA synthetase activity was unchanged between the control strain and the strains transformed with the plant AlaRS gene (Fig. 2*B*), showing that the observed increase in aminoacylation activity was specific to AlaRS and did not result from differences in the overall efficiency of the extracts. These data imply that functional *A. thaliana* mitochondrial AlaRS is produced in *S. cerevisiae*.

The A. thaliana Mitochondrial AlaRS Precursor Is Efficiently Imported into S. cerevisiae Mitochondria in Vivo—Mitochondria were purified from untransformed *S. cerevisiae* and from *S. cerevisiae* strains transformed with construct p61L-1WT2m/tR, carrying the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS or with construct p61L-tRNA^{Ala}, carrying only the *A. thaliana* tRNA^{Ala}(UGC) gene. Western blot experiments were carried out to probe the proteins extracted from these samples with the anti-*A. thaliana* AlaRS antibody. A strong, specific signal corresponding in size to the *A. thaliana* AlaRS was detected only with the mitochondrial protein extracts from the *S. cerevisiae* strain expressing the plant mitochondrial AlaRS (Fig. 3). This implies that the plant AlaRS presequence is functional in *S. cerevisiae* and promotes efficient import of the enzyme into yeast mitochondria, despite the probable instability of the cytosolic form of the protein in this organism. That no signal was observed when the plant AlaRS gene was not present indicated that the antibodies against the *A. thaliana* AlaRS did not significantly cross-react with the

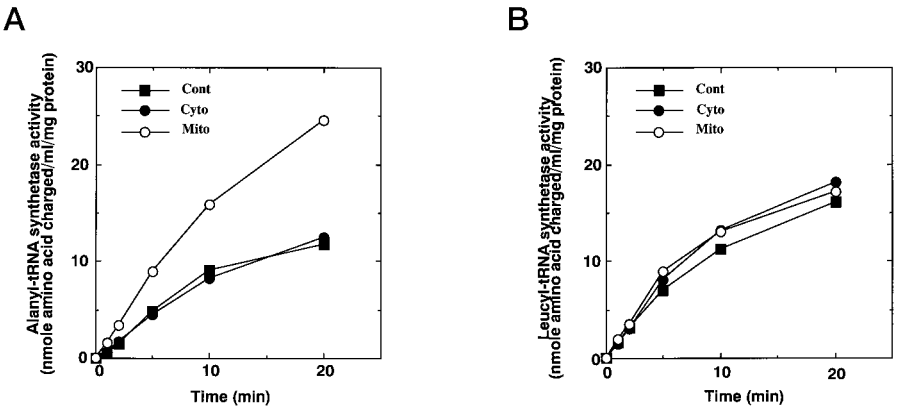

FIG. 2. **Aminoacylation kinetics with *S. cerevisiae* total enzyme extracts.** Aminoacylation of total *S. cerevisiae* tRNA (3 mg/ml) was run with [^{3}H]alanine (*A*) or [^{3}H]leucine (*B*) in the presence of limiting amounts of total enzyme extracts from *S. cerevisiae* strains transformed with a plasmid containing either the *A. thaliana* tRNA^{Ala}(UGC) gene but no AlaRS gene (*Cont*), the *A. thaliana* genes for tRNA^{Ala}(UGC) and cytosolic AlaRS (*Cyto*), or the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS (*Mito*). Protein concentrations were 0.3 mg/ml for all assays.

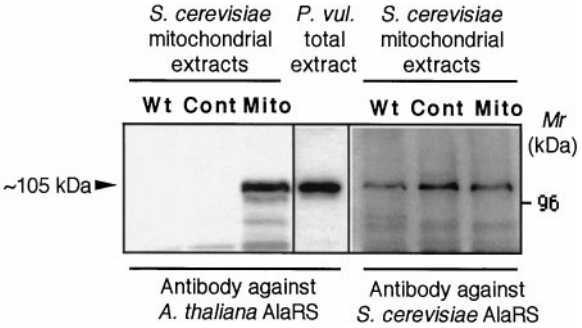

FIG. 3. **Western blot analysis of mitochondrial proteins using anti-*A. thaliana* AlaRS or anti-*S. cerevisiae* AlaRS antibodies.** Mitochondrial proteins were extracted from untransformed *S. cerevisiae* (*Wt*) and from *S. cerevisiae* strains transformed with a plasmid containing either the *A. thaliana* tRNA^{Ala}(UGC) gene but no AlaRS gene (*Cont*) or the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS (*Mito*). Only the latter strain contained a polypeptide of the expected size (~105 kDa) detected by the anti-*A. thaliana* AlaRS antibody. A plant total protein extract from bean (*Phaseolus vulgaris*) hypocotyls (24) was run in parallel with the mitochondrial samples (*P. vul.*). The chemoluminescence reaction was developed for a much longer time with the antibody against the *S. cerevisiae* AlaRS to obtain signal intensities comparable to those with the antibody against *A. thaliana* AlaRS.

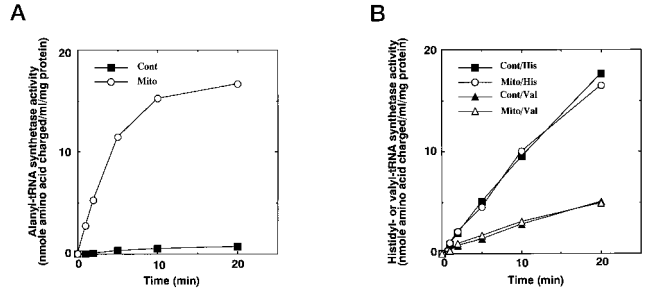

FIG. 4. **Aminoacylation kinetics with *S. cerevisiae* mitochondrial enzyme extracts.** Aminoacylation of total *S. cerevisiae* tRNA (3 mg/ml) was run with [^{3}H]alanine (*A*), [^{3}H]histidine (*B*, *His*), or [^{3}H]valine (*B*, *Val*) in the presence of limiting amounts of mitochondrial enzyme extracts from *S. cerevisiae* strains transformed with a plasmid containing either the *A. thaliana* tRNA^{Ala}(UGC) gene but no AlaRS gene (*Cont*) or the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS (*Mito*). Protein concentrations were 0.5 mg/ml for all assays.

endogenous yeast mitochondrial AlaRS. Conversely, there was no evidence for cross-recognition of the plant enzyme when probing similar Western blots with antibodies against the *S. cerevisiae* mitochondrial AlaRS, which enabled us to show that the level of the endogenous enzyme was not significantly affected by the presence of the plant AlaRS (Fig. 3).

The A. thaliana AlaRS Present in the Mitochondria of Transformed Yeast Is Functional—To test whether the *A. thaliana* AlaRS imported into *S. cerevisiae* mitochondria was functional, mitochondrial enzyme extracts were prepared from the strains transformed with the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS or with the *A. thaliana* tRNA^{Ala}(UGC) gene alone. These extracts were tested for aminoacylation with [^{3}H]alanine, using *S. cerevisiae* total tRNA as substrate. In yeast as in plants, the mitochondrial and cytosolic AlaRSs are likely to be encoded by the same gene (31),[2] so yeast cytosolic tRNAs^{Ala} are appropriate substrates for both the plant and yeast mitochondrial enzymes. The AlaRS activity was roughly 20-fold higher in mitochondrial extracts of the strain expressing the plant mitochondrial AlaRS, as compared with extracts from the control strain transformed only with the plant tRNA^{Ala} gene (Fig. 4*A*). Mitochondrial histidyl- and valyl-tRNA synthetase activities were unchanged between the control strain and the strain transformed with the plant AlaRS gene (Fig. 4*B*), showing that the observed increase in aminoacylation activity was specific to AlaRS and did not result from differences in the overall efficiency of the extracts. For histidyl- and valyl-tRNA synthetase, as for AlaRS, the same gene encodes both the cytosolic and the mitochondrial form of the enzymes in yeast (32, 33), which again enabled the use of total *S. cerevisiae* tRNA for testing mitochondrial extracts.

To confirm that the observed increase in AlaRS activity was due to the presence of the plant enzyme, and not to a stimulation of the endogenous enzyme, the mitochondrial extracts were fractionated by chromatography on a hydroxyapatite column, and the individual fractions were tested for aminoacylation with alanine. The plant and the yeast AlaRS appeared to elute from the column as two distinct peaks, with an activity ratio similar to that obtained when testing the unfractionated mitochondrial extracts (Fig. 5*A*). The identity of each peak was confirmed by Western blot analysis of the individual fractions with the AlaRS antiserum (Fig. 5, *B* and *C*). Considering all the data, it appears that functional *A. thaliana* mitochondrial AlaRS accumulates at a high level in mitochondria of the *S. cerevisiae* strain transformed with the corresponding gene.

Import of pre-AlaRS Does Not Lead to Mitochondrial Import of Cytosolic tRNA^{Ala}—Putative mitochondrial import of tRNA^{Ala} was analyzed in the *S. cerevisiae* strains transformed with the *A. thaliana* tRNA^{Ala}(UGC) gene alone, combined with the gene for the *A. thaliana* cytosolic AlaRS, or combined with the gene for the *A. thaliana* mitochondrial AlaRS. Total RNA and mitochondrial RNA were extracted from the *S. cerevisiae* transformants and analyzed by Northern blotting. The absence of any hybridization signal with a probe specific for the cytosolic tRNA^{Trp} proved the absence of significant cytosolic contamination of the mitochondrial tRNA samples (Fig. 6, *panel*

[2] S. Li and A. Tzagoloff, unpublished results.

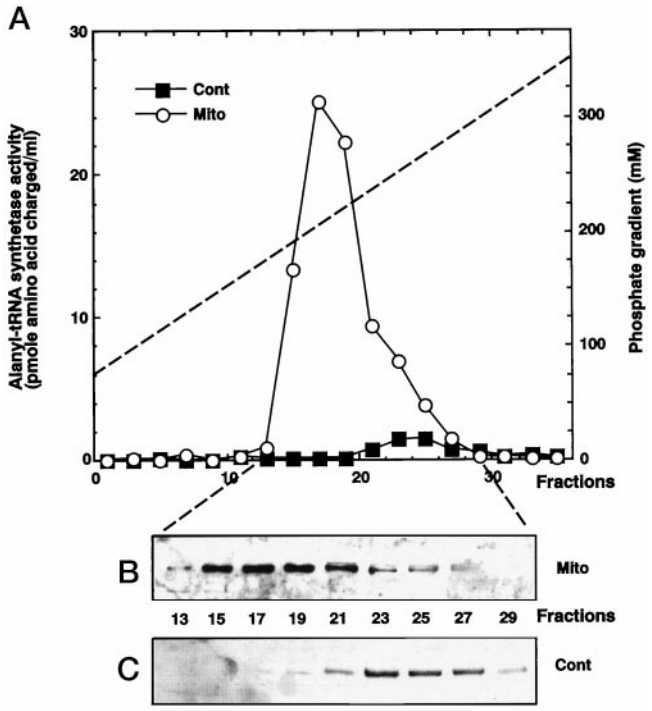

FIG. 5. **Chromatographic characterization of *A. thaliana* AlaRS and *S. cerevisiae* AlaRS.** Mitochondrial enzyme extracts from *S. cerevisiae* strains transformed with a plasmid containing either the *A. thaliana* tRNA^{Ala}(UGC) gene but no AlaRS gene (*Cont*), or the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS (*Mito*) were fractionated in identical run conditions on a CHT-I column. Aliquots of the collected fractions were tested for AlaRS activity in the presence of [^{3}H]alanine (*A*) and for recognition by the antibodies against *A. thaliana* (*B*) or *S. cerevisiae* (*C*) AlaRS. The chemoluminescence reaction in *C* was developed for a much longer time to obtain signal intensities comparable to those in *B*.

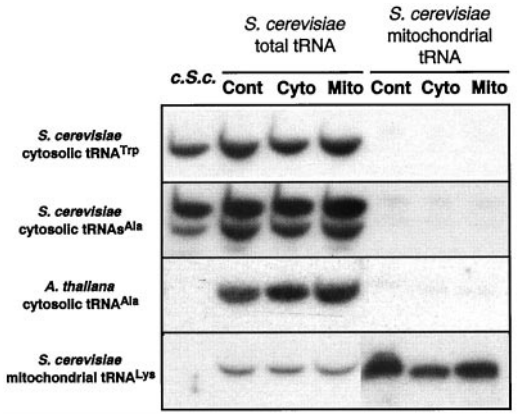

FIG. 6. **Northern blots of total and mitochondrial tRNA.** Total and mitochondrial tRNA from *S. cerevisiae* strains transformed with a plasmid containing either the *A. thaliana* tRNA^{Ala}(UGC) gene but no AlaRS gene (*Cont*), the *A. thaliana* genes for tRNA^{Ala}(UGC) and cytosolic AlaRS (*Cyto*), or the *A. thaliana* genes for tRNA^{Ala}(UGC) and mitochondrial AlaRS (*Mito*) were run on polyacrylamide gels, transferred to nylon membranes, and probed for the tRNAs indicated on the *left* of each panel. *c.S.c.* corresponds to commercial *S. cerevisiae* total tRNA used as a negative control.

a). Hybridization with a probe recognizing the two endogenous *S. cerevisiae* cytosolic tRNA^{Ala} isoacceptors also gave signals only with total RNA samples, which proved the absence of significant contamination of the mitochondrial RNA preparations with cytosolic tRNA^{Ala} (Fig. 6, *panel b*). Moreover, this result showed that expression of the *A. thaliana* AlaRS had no effect on the expression level of the endogenous cytosolic tRNA-

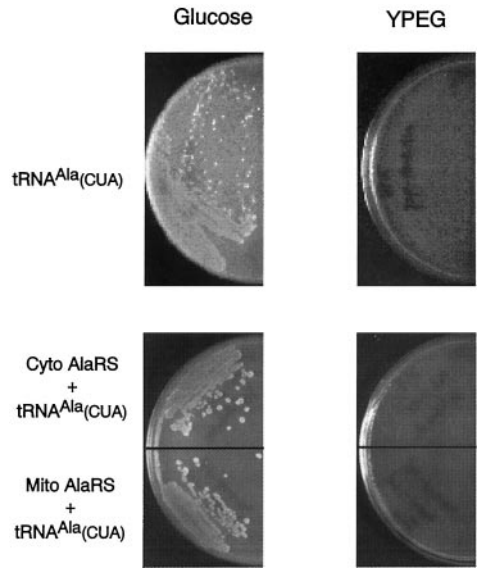

FIG. 7. **Expression of the amber suppressor tRNA^{Ala}(CUA) in an amber *cox2* yeast mutant.** The *S. cerevisiae* HM4 strain, containing an amber mutation in the mitochondrial *cox2* gene, was transformed with a plasmid encoding the *A. thaliana* amber suppressor tRNA^{Ala}(CUA) alone or with constructs encoding the suppressor tRNA and either the cytosolic (*Cyto*) or the mitochondrial (*Mito*) *A. thaliana* AlaRS, as indicated. Transformants were selected on minimal medium containing glucose and then printed to YPEG plates containing nonfermentable carbon sources.

s^{Ala} and, more pertinently, did not lead to import of these tRNAs into mitochondria. Similarly, we could only detect a specific hybridization signal corresponding to the *A. thaliana* tRNA^{Ala} from the *S. cerevisiae* total tRNA samples (Fig. 6, *panel c*). Considering the hybridization signal observed, compared with that of the endogenous tRNAs^{Ala} and tRNA^{Trp}, it seems unlikely that the expression level of the *A. thaliana* tRNA^{Ala} in transformed *S. cerevisiae* was a limiting factor for putative mitochondrial import of this tRNA. The identity and integrity of the mitochondrial tRNAs was verified by using a probe for the *S. cerevisiae* mitochondrially encoded tRNA^{Lys} (Fig. 6, *panel d*).

A functional test for the import of the *A. thaliana* tRNA^{Ala} into *S. cerevisiae* mitochondria was pursued by creating a yeast mitochondrial mutant in which an alanine codon of the *cox2* gene was mutated to an amber codon (strain named HM4, see "Materials and Methods"). The presence of the premature stop codon prevents the mutant strain from synthesizing a full-length COX2 protein and thus prevents respiration (Fig. 7). The amber mutation could potentially be suppressed by mitochondrial import of an amber suppressor tRNA^{Ala}(CUA) from the cytosol. Such a suppressor tRNA, derived from the *A. thaliana* tRNA^{Ala}(UGC) (16), was subcloned into pFL61, and its expression and function in *S. cerevisiae* were tested by the ability to suppress the amber mutation of the *lys2* nuclear gene present in the YPH102 strain (data not shown). The HM4 strain was then transformed with a plasmid encoding the *A. thaliana* amber suppressor tRNA^{Ala}(CUA) alone or with a plasmid encoding both the tRNA^{Ala}(CUA) and either the cytosolic or the mitochondrial *A. thaliana* AlaRS. The mitochondrial import of the *A. thaliana* tRNA^{Ala}(CUA) was tested by transferring the different transformants to plates containing a nonfermentable carbon source. None of the strains was able to grow under such conditions (Fig. 7). This inability of the different transformants to respire implies that little or no *A. thaliana* tRNA^{Ala}(CUA) was entering mitochondria in each strain. This test should be very sensitive because the presence of very low levels of COX2 protein are known to support de-

tectable growth in this assay (29). Taken together, the results show that the *A. thaliana* tRNA^{Ala} is correctly expressed in *S. cerevisiae* but is not imported into the mitochondria of this organism, even when the *A. thaliana* AlaRS is targeted to mitochondria in the same transformants.

DISCUSSION

Following the initial observations of tRNA import into *Tetrahymena* mitochondria (34, 35), it was suggested that import might occur as co-import of a tRNA/mitochondrial aaRS precursor complex. Since these early experiments, some evidence has been accumulated that aaRSs are indeed involved in mitochondrial tRNA import, at least in plants and yeast (14, 17), although ironically this does not seem to be the case in *Tetrahymena* (36) nor in *Leishmania* (12). However, the exact role that aaRSs might play in the plant tRNA import mechanism has not been elucidated, and at least three hypotheses are quite plausible: (i) The true import factor(s) require(s) aminoacylated tRNA, hence the requirement for the corresponding aaRS. The cytosolic LysRS clearly plays this role in *S. cerevisiae*, because it is no longer necessary for mitochondrial import of tRNA^{Lys}(CUU) *in vitro* if charged tRNA^{Lys} is used for the assay (14). (ii) tRNAs are imported through the mitochondrial protein import channel in a complex with the cognate aaRS. Evidence for the involvement of a complex between tRNA^{Lys}(CUU) and the precursor to the *S. cerevisiae* mitochondrial LysRS (pre-MSK) prior to import of the tRNA and for the importance of a functioning protein import channel is quite strong in yeast (13, 14). (iii) The aaRS acts as a specificity factor, singling out specific tRNAs for import but does not actually participate in the transfer of the tRNA through the mitochondrial membrane, which occurs separately (perhaps through a still-to-be-identified RNA import channel).

In eukaryotes, all the mitochondrial aaRS precursors are imported from the cytosol, and yet only in a few cases does this import correlate with tRNA import. In these special cases, it has been unclear until now whether the aaRS precursor has some unusual intrinsic ability to promote mitochondrial tRNA import or whether other factors are involved. The experiments described here address the fundamental point of whether a mitochondrial aaRS precursor implicated in tRNA import is still capable of directing its cognate tRNA to mitochondria when expressed in a different host. To be a fair test, the experimental system should meet several criteria. First, one should use a homologous aaRS/tRNA pair known to be imported in their natural host. Second, the enzyme and the tRNA must be expressed correctly and in sufficient amounts in the transformed strains. Third, the enzyme produced from the transgene must be shown to be active and imported efficiently into mitochondria. Finally, the mitochondria from the host organism should be competent for tRNA import, *i.e.* they should be known to import tRNAs other than the one being tested. We believe that the experiments described here meet these criteria.

It is clear from our results that the *A. thaliana* mitochondrial AlaRS precursor is not sufficient to direct tRNA^{Ala} to yeast mitochondria. Plant cells must therefore contain at least one other factor missing from yeast cells that is necessary for mitochondrial import of tRNA^{Ala}. Following the hypotheses put forward above, the possibilities include a factor that requires aminoacylated tRNA^{Ala} (hence the necessity for aminoacylation shown previously (17)), a factor that acts to stabilize a putative pre-AlaRS/tRNA^{Ala} complex, or an RNA import channel lacking from yeast mitochondria. A great deal of further experimentation will be required to identify which of these possibilities is nearest to being correct.

Acknowledgments—We thank Ivan Tarassov for many fruitful discussions and for help in isolating *S. cerevisiae* mitochondria, Heather Dunstan and Nathalie Bonnefoy for supplying strains and plasmids, Alexander Tzagoloff for providing us with an antiserum against *S. cerevisiae* AlaRS and with unpublished experimental information about this enzyme, and Gérard Keith for the gift of specific oligonucleotides.

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