Saccharomyces cerevisiae imports the cytosolic pathway for Gln-tRNA synthesis into the mitochondrion

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Aminoacyl-tRNA (aa-tRNA) formation, an essential process in protein biosynthesis, is generally achieved by direct attachment of an amino acid to tRNA by the aa-tRNA synthetases. An exception is Gln-tRNA synthesis, which in eukaryotes is catalyzed by glutaminyl-tRNA synthetase (GlnRS), while most bacteria, archaea, and chloroplasts employ the transamidation pathway, in which a tRNA-dependent glutamate modification generates Gln-tRNA. Mitochondrial protein synthesis is carried out normally by mitochondrial enzymes and organelle-encoded tRNAs that are different from their cytoplasmic counterparts. Early work suggested that mitochondria use the transamidation pathway for Gln-tRNA formation. We found no biochemical support for this in Saccharomyces cerevisiae mitochondria, but demonstrated the presence of the cytoplasmic GlnRS in the organelle and its involvement in mitochondrial Gln-tRNA synthesis. In addition, we showed in vivo localization of cytoplasmic tRNAGln in mitochondria and demonstrated its role in mitochondrial translation. We furthermore reconstituted in vitro cytoplasmic tRNA Gln import into mitochondria by a novel mechanism. This tRNA import mechanism expands our knowledge of RNA trafficking in the eukaryotic cell. These findings change our view of the evolution of organellar protein synthesis.

Keywords: Saccharomyces cerevisiae; aminoacyl-tRNA; glutaminyl-tRNA synthetase; mitochondrion; protein synthesis; tRNA import

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Gln-tRNA formation is the least conserved route of aminoacyl-tRNA (aa-tRNA) synthesis found in nature, and is generated by kingdom-specific pathways that guarantee proper glutamine incorporation during protein synthesis [Ibba and Söll 2004]. In the eukaryotic cytoplasm and in some bacteria, glutaminyl-tRNA synthetase [GlnRS] attaches glutamine directly to tRNA [Fig. 1]. A different pathway, found in most bacteria and all archaea, employs a pretranslational modification to generate Gln-tRNA Gln [Fig. 1]. tRNA Gln is first misaminoacylated with glutamate by a nondiscriminating glutamyl-tRNA synthetase [GluRS], which is able to synthesize both Glu-tRNA Gln and Glu-tRNA Glu [Lapointe et al. 1986; Sekine et al. 2001]. The resulting mischarged tRNA is then converted by glutamyl-tRNA Gln amido-transferase into Gln-tRNA Gln [Feng et al. 2004]. Mitochondria and chloroplasts, in line with their bacterial ancestry, are thought to share the same routes of aa-tRNA synthesis with bacteria and archaea [Yang et al. 1985; Schön et al. 1988; Woese et al. 2000]. This is underscored by the fact that most chloroplast and mitochondrial genomes encode a complete set of tRNAs necessary for organellar protein synthesis [Barrell et al. 1980; Bonitz et al. 1980; Heckman et al. 1980]. In line with this expectation, Gln-tRNA formation in barley chloroplasts was shown to occur by transamidation, and this led to the suggestion that the same route of Gln-tRNA synthesis is also prevalent in mitochondria [Schön et al. 1988].

Proteins orthologous to bacterial amidotransferases are frequently found in the nuclear genomes of most eukaryotes. Bacterial amidotransferases are heterotrimeric enzymes consisting of A, B, and C subunits. Orthologs to the A and B subunits have been annotated in the Saccharomyces cerevisiae genome [PET112 and YMR293c] and are essential to mitochondrial function [Mulero et al. 1994; Hughes et al. 2000]. Most eukaryotes also contain mitochondrial GluRSs that are distinct from their cytoplasmic counterparts and could potentially participate in the transamidation pathway. Despite these observations, a mitochondrial amidotransferase has not been characterized and the ability of a mitochondrial GluRS to form mischarged Glu-tRNA Gln has not been rigorously established.

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To elucidate the mechanism of Gln-tRNA formation in mitochondria we have focused our investigations on \textit{S. cerevisiae}. Our results support a new general pathway for mitochondrial Gln-tRNA formation and highlight a common feature relevant to mitochondrial protein synthesis.

**Results**

**Direct formation of mitochondrial Gln-tRNA by the cytoplasmic GlnRS**

Analysis of the \textit{S. cerevisiae} genome reveals three open reading frames possibly involved in formation of Glu-tRNA and Gln-tRNA: GluRS, mGluRS, and GlnRS. The mGluRS and the GlnRS from \textit{S. cerevisiae} are essential for mitochondrial protein synthesis and cytosolic Glu-tRNA formation, respectively; however, their relevance to mitochondrial Gln-tRNA formation has not been examined \cite{Ludmerer1987, Tzagoloff1995}. We therefore investigated the specificity of the \textit{S. cerevisiae} mGluRS and cytoplasmic GlnRS. We purified both recombinant enzymes [expressed in yeast or \textit{Escherichia coli}] and examined their enzymatic activities with purified, mature mitochondrial tRNAs [tRNA\textsubscript{mGln}] from \textit{S. cerevisiae} [Fig. 2A]. As expected, the mGluRS formed Glu-tRNA\textsubscript{mGln}, but surprisingly, it could not generate Glu-tRNA\textsubscript{Gln}, the required intermediate for the transamidation pathway. This finding was consistent with our previous unsuccessful attempts at identifying a mitochondrial amidotransferase activity, suggesting that the transamidation pathway may not be functioning in yeast. Similar data were obtained with purified native tRNA\textsubscript{mGln} and are consistent with previous reports of yeast GlnRS activity \cite{Ludmerer1993}. The nature of the [\textsuperscript{14}C]-labeled amino acid attached to the different tRNA species was confirmed by thin-layer chromatography [TLC] of in vitro generated aa-tRNA [Fig. 2B]. Cytoplasmic GlnRS attaches glutamine to both mitochondrial tRNA\textsubscript{mGln} and cytoplasmic tRNA\textsubscript{Gln}. Fundamentally, these two types of tRNA are separated by the location of their genes in the nuclear and mitochondrial genomes. However, because the GlnRS can utilize both tRNAs as substrates, we examined the possibility that the mitochondrion may contain tRNA\textsubscript{Gln} outside of its own genomic context. To this end, we designed specific \textsuperscript{32}P-labeled oligonucleotide probes for tRNA\textsubscript{Gln} and tRNA\textsubscript{Gln} [Fig. 2C]. The positive identification of each tRNA was aided by the fact that they have distinct electrophoretic mobilities [Fig. 2C]. Utilizing these probes and acid urea gel electrophoresis, we consistently observed a second set of aminoacylated tRNA\textsubscript{Gln} in total mitochondrial tRNA that hybridized to probe specific for tRNA\textsubscript{Gln} [Fig. 2D, lane 1]. Acid urea gel electrophoresis allowed us to further confirm the identity of this tRNA species by comparing the aa-tRNAs extracted in vivo with the products of in vitro aminoacylation with GlnRS \cite{Varshney1991}. The signals for aa-tRNAs, which are distinct from the tRNA\textsubscript{mGln} species encoded in the mitochondrial genome, are consistent with the Gln-tRNA\textsubscript{Gln} in the in vivo aminoacylated tRNA fraction extracted from mitochondria [Fig. 2D, lanes 1, 3]. Furthermore, neither tRNA\textsubscript{mGln} in the total mitochondrial fraction is a substrate for the \textit{S. cerevisiae} mGluRS [data not shown]. Taken together, these results indicate that mitochondrial Gln-tRNA\textsubscript{Gln} is formed by direct charging of a tRNA\textsubscript{Gln} species not encoded in the mitochondrial genome.
Mitochondrial tRNA\textsubscript{Gln} is of nuclear origin

The presence of aminoacylated tRNA\textsubscript{Gln} in the mitochondrion raised the question as to how these nucleus-encoded tRNAs became localized in the organelle. To investigate this further, we first probed total RNA from purified, micrococcal nuclease-treated mitochondria by Northern hybridization using radioactive oligonucleotides specific for either organellar- or nucleus-encoded tRNA\textsubscript{Gln}. The data [Fig. 3A] show the presence of tRNA\textsubscript{Gln}, implying import of a fraction of cytosolic tRNA\textsubscript{Gln} into mitochondria. Probing of the same mitochondrial RNA fraction with the nuclear/cytosolic marker U6 snRNA showed this fraction to be devoid of any significant nuclear or cytosolic contamination.

RT–PCR with oligonucleotides that allow amplification of all four isoacceptors permitted determination of the population of tRNA\textsubscript{Gln} isoacceptors present in mitochondrial and cytoplasmic fractions [Fig. 3B]. The S. cerevisiae nuclear genome encodes a single tRNA\textsubscript{Gln} \textsubscript{CUG} and three tRNA\textsubscript{Gln} \textsubscript{UUG} genes differing at two or three nucleotide positions apart from the anticodon [Fig. 4]. There was only tRNA\textsubscript{Gln} \textsubscript{UUG1} and tRNA\textsubscript{Gln} \textsubscript{CUC} in the mitochondrial fraction, 23 out of 55 clones examined were tRNA\textsubscript{Gln} \textsubscript{CUG} and 32 clones corresponded to tRNA\textsubscript{Gln} \textsubscript{UUG1} with a G at position 42 [Fig. 3C]. We detected one tRNA\textsubscript{Gln} \textsubscript{UUG2} containing A42 in the cytoplasm, whereas this tRNA was not observed in the 55 clones examined from the mitochondrial fraction. This result further establishes that tRNA\textsubscript{Gln} \textsubscript{UUG} and tRNA\textsubscript{Gln} \textsubscript{CUC} are imported into the mitochondria from the cytoplasm and suggests a difference in the distribution of tRNA species between the two compartments.

In vitro import of cytoplasmic tRNA\textsubscript{Gln} into mitochondria

In light of the above observations, we tested the in vitro import of radioactive tRNA\textsubscript{Gln} purified from total cytosolic RNA fractions [isolated as above]. Incubation of this tRNA with isolated yeast mitochondria led to 4% import of the labeled tRNA in vitro [Fig. 5A, lane 1]. No import was observed in the absence of exogenous ATP, indicating that tRNA\textsubscript{Gln} import is ATP dependent [Fig. 5A, lane 4]. Import of tRNA\textsubscript{Lys}, previously the only imported tRNA known in S. cerevisiae, requires the addition of both the cytosolic and mitochondrial aa-tRNA synthetases during import [Tarassov et al. 1995; Kolessnikova et al. 2000]. The mode of tRNA\textsubscript{Gln} import into yeast mitochondria is more similar to the mechanism of tRNA import described previously in trypanosomatids and plants [Rubio et al. 2000; Delage et al. 2003]. This was demonstrated in a head-to-head comparison in which the tRNA\textsubscript{Lys} was not imported in vitro [Fig. 5B]. The specificity of the tRNA import was further demonstrated by including the Leishmania spliced-leader RNA as a nonimported RNA control [Fig. 5B]. Thus, the system described here is certainly different, as no exogenous cytosolic factors are required for in vitro import of tRNA\textsubscript{Gln}. Therefore, S. cerevisiae has more than one mechanism for the import of nucleus-encoded tRNAs into mitochondria. The direct demonstration of tRNA\textsubscript{Gln} import into isolated mitochondria also corroborates the cytosolic origin of the tRNA substrates used for the Gln-tRNA synthesis in S. cerevisiae mitochondria.

The S. cerevisiae GlnRS is also localized to the mitochondrion

The cytoplasmic S. cerevisiae GlnRS is an essential enzyme required for cytoplasmic Gln-tRNA synthesis [Ludmerer and Schimmel 1987]. We examined the intracellular localization of the GlnRS protein to determine whether it also could play a role in mitochondrial Gln-tRNA formation. The gene for an N-terminal GlnRS-GFP fusion protein was transformed into S. cerevisiae; this protein became localized in the cytoplasm and the mitochondrion, as demonstrated by coincident GFP fluo-
rescence and DAPI staining (Fig. 6A). A second experiment utilizing a C-terminally V5-tagged GlnRS was carried out in an *S. cerevisiae* GlnRS deletion strain. Plasmid exchange experiments with the GlnRS-V5 and GlnRS-GFP showed that both fusion proteins could rescue growth of a GlnRS deletion strain, suggesting that both proteins were fully functional (data not shown). Co-expression of the GlnRS-V5 and of a COXIV-GFP fusion protein targeted separately to mitochondria showed colocalization of both proteins in mitochondrial structures with the GlnRS-V5 showing additional staining of the cytoplasm (Fig. 6B). These results suggest that the native GlnRS enzyme may also be localized in the mitochondrion in vivo.

To investigate this further we examined GlnRS localization by Western blot analysis using pure mitochondria from wild-type *S. cerevisiae*. A clear GlnRS signal was evident in total mitochondrial and mitoplast fractions (Fig. 6C). All mitochondrial fractions were negative for the cytoplasmic marker protein adenine phosphoribosyltransferase (APRT), indicating that the signal observed for GlnRS is not due to cytosolic contamination of the mitochondrial fraction. Additionally, mitoplasts were prepared under conditions rigorous enough to release some of the mitochondrial matrix into the supernatant fraction. Even under these conditions, we observed colocalization of GlnRS and the mitochondrial marker mtHsp70, indicating that GlnRS is localized in the mitochondrial matrix of wild-type cells. However, the mitochondrial localization signal is not obvious. The first N-terminal 127 amino acids of the GlnRS are dispensable and do not affect the respiratory efficiency of wild-type cells and the remainder of the open reading frame contains no predictable targeting sequence (Ludmerer and Schimmel 1987). Furthermore, the fact that both the GFP and V5-tagged GlnRS proteins are localized in the mitochondrion suggests that the GlnRS does not contain a traditional cleavable targeting signal at its N or C terminus.

A single nuclear tRNA<sup>Gln</sup> supports translation in both the cytoplasm and the mitochondrion

The collective in vitro evidence supports a new model of yeast mitochondrial Gln-tRNA function, in which tRNA<sub>Gln</sub> is partitioned between the cytoplasm and mitochondrion to participate in translation in both compartments. To demonstrate this in vivo we introduced a point mutation in a plasmid-borne copy of a yeast tRNA<sub>Gln</sub> gene to create an amber suppressor tRNA<sub>Gln</sub><sup>CUG</sup>. We utilized the tRNA<sub>Gln</sub><sup>CUG</sup> to suppress in frame amber stop codons in both a cytoplasmic and mitochondrial tRNA<sub>Gln</sub> into isolated yeast mitochondria. [A] Nuclease protection of native tRNA<sub>Gln</sub> incubated with isolated yeast mitochondria. Native tRNA<sub>Gln</sub> radioactively labeled at the 5’ end with γ<sup>32</sup>P-ATP (1 × 10<sup>5</sup> cpm) was incubated with isolated yeast mitochondria in the presence (lane 1) and absence (lane 4) of ATP and subsequently digested with micrococcal nuclease (MN). The arrow depicts the migration of the full-length RNA protected from MN digestion. [Lane 2] As a control, input RNA was digested with MN in the absence of mitochondrial DNA. Lane 3 represents one-tenth of the input RNA, without MN digestion and in the absence of mitochondria, used for quantification. [B] In vitro import of native *S. cerevisiae* tRNA<sub>Gln</sub> compared to *S. cerevisiae* tRNA<sub>Lys</sub> and the *Leishmania* spliced leader RNA (slRNA) into isolated yeast mitochondria. Import experiments are presented as in A with the third lane in each panel representing an RNA-only control (one-tenth of the input RNA) used for quantification.
mitochondrial gene [Fig. 7]. A yeast strain containing a mutant trp1-1\textsuperscript{UAG} allele could grow in the absence of tryptophan only when transformed with the tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} [Fig. 7A]. This showed that the tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} was a substrate for translation in the cytoplasm. The yeast strain HM4, which contains a cox2-114\textsuperscript{UAG} mutation, was used to demonstrate the import of tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} and its subsequent role in mitochondrial translation [Fig. 7B]. The HM4 lacked any detectable Cox2p whereas there was a clear signal when the same strain contained the plasmid-borne tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} gene. The HM4 strain is respiration-deficient and tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} expression did not rescue this phenotype [data not shown]. In contrast to the Trp1p, the Cox2p may not be functional with a glutamine inserted at the site of the amber stop codon. BLAST searches revealed a conserved alanine at position 114 in most yeast and plant Cox2. Although the protein is not functional, tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} -mediated suppression clearly produces immunologically detectable Cox2p. Taken together, these data clearly show that yeast partitions a functional tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} between the cytoplasm and mitochondria and uses this imported tRNA in mitochondrial translation.

Discussion

Here we describe a newly discovered strategy used by \textit{S. cerevisiae} mitochondria to activate glutamine for protein synthesis. Following import into mitochondria, the cytosolic GlnRS directly attaches glutamine to tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA}. In addition, a fraction of the tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} present in mitochondria is a consequence of import from the cytoplasm. This pathway contrasts with the mechanism operative in chloroplasts, which, in line with their bacterial ancestry, rely on the transamidation of mischarged Glu-tRNAGln [Schön et al. 1988]. This indirect biosynthesis pathway requires two enzyme activities, a GluRS forming Glu-tRNAGln and an amidotransferase able to convert this mischarged tRNA species to the correctly charged Gln-tRNAGln [Curnow et al. 1997]. While paralogs of the amidotransferase genes are found in the yeast genome, the lack of a mitochondrial activity capable of synthesizing Glu-tRNAGln, the required amidotransferase precursor, may explain why the transamidation route does not function in mitochondria. As switching of tRNA specificity between discriminating and nondiscriminating synthetases has been shown to be quite feasible [Charron et al. 2003; Feng et al. 2003], one may hypothesize that during mitochondrial evolution the organelar GluRS lost its tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} specificity and with it the ability to form Glu-tRNAGln. This event may have prompted the organism to import the total pathway of Gln-tRNA formation from the cytoplasm. The tRNA import may have been advantageous, since the cytoplasmic tRNAs had been evolutionarily adapted to the cytoplasmic GlnRS, and are probably better substrates than the mitochondrial tRNA\textsubscript{n\textsuperscript{Cl}}\textsuperscript{CUA} still present. A similar mechanism, by which amidotransferase is replaced, is established for the introduction of GlnRS, by lateral gene transfer.
transfer, into some bacterial lineages, especially γ-proteobacteria (Fig. 8; Lamour et al. 1994; Brown and Doolittle 1999; Handy and Doolittle 1999). However, mitochondria only needed a horizontal gene “product” transfer, and perhaps this feature increased the chances of the simultaneous acquisition of tRNA\(^\text{Gln}\) and GlnRS from the cytoplasm.

**Mitochondrial translation requires two tRNA\(^\text{Gln}\) isoacceptors**

The observed import of a set of glutamine tRNAs explains how Gln-tRNA is synthesized in yeast mitochondria, but raises new and important questions. What roles are played by the imported tRNA\(^\text{Gln}_{\text{CUC}}\) and the tRNA\(^\text{Gln}_{\text{UUC34}}\)? The yeast mitochondrial genome encodes a tRNA\(^\text{m\_Gln}_{\text{CUU}}\) with anticodon UUG. Two mitochondrial tRNA species, tRNA\(^\text{m\_Gln}_{\text{UUC}}\) and tRNA\(^\text{m\_Gln}_{\text{UUG}}\), were shown to contain 5-carboxymethylaminomethyl-2-thiouridine [mcm\(^5\)s\(^2\)U] in position 34, the first anticodon nucleotide [Nakai et al. 2004]. Wobble base pairing necessitate the import of a tRNA\(^\text{Gln}_{\text{CUC}}\) isoacceptor to decode the CAG codons in mitochondrial mRNA. Similarly, mcm\(^5\)s\(^2\)U34 in tRNA\(^\text{m\_Gln}_{\text{UUG}}\) would only decode CAA codons [for review, see Yokoyama and Nishimura 1995]. This should explain how the yeast mitochondrial genome encodes three different tRNA\(^\text{m\_Gln}_{\text{UUG}}\) species [Fig. 4], and the redundancy in this tRNA population may have some important and as of yet unknown function common to both the cytoplasm and the mitochondria. The selectivity of tRNA\(^\text{Gln}\) import and what features of the import machinery control intracellular tRNA distribution will be the subject of future investigations.

**The S. cerevisiae mitochondrion has two tRNA import mechanisms**

Prior to this report, yeast mitochondria were believed to encode and synthesize all of the necessary tRNAs for protein synthesis. Thus, the notion of mitochondria-imported tRNAs was not seriously considered for yeast and the import of a single functionally redundant tRNA\(^\text{Gln}\) was an interesting exception [Kolesnikova et al. 2000]. The ability to import uncharged tRNA\(^\text{Gln}\) in vitro in the absence of added cytosolic factors differs radically from the mechanism of tRNA\(^\text{Gln}\) import. In the latter mechanism, an aminoacylated tRNA\(^\text{Gln}\) and a precursor mitochondrial lysyl-tRNA synthetase are both required for import. The function of the imported tRNA\(^\text{Gln}\) is unclear given that the mitochondrial lysyl-tRNA synthetase does not aminoacylate it [Tarassov et al. 1995]. We observe localization of both cytoplasmic tRNA\(^\text{Gln}\) and GlnRS to the mitochondrion where they are most likely required for protein synthesis. Future studies will focus on the essential nature of each mitochondrial tRNA\(^\text{Gln}\), of both nuclear and mitochondrial origin, in protein synthesis.

Is mitochondrial tRNA import, at least of tRNA\(^\text{Gln}\), more general and also occurring in higher eukaryotes? The ability to import both the cytoplasmic synthetase together with its cognate tRNA might not be unique to yeast mitochondria. This idea arises from the observation that a tRNA\(^\text{Gln}\) with anticodon CUG is not encoded in any of the >500 mitochondrial genomes sequenced to date [NCBI Organelle Genomes, http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html]. The only exception is the *Ascomobolus immersus* tRNA\(^\text{Gln}_{\text{CUC}}\) “pseudogene” [with an unusual DNA sequence insertion] that is probably not functional [Goyon et al. 1996]. Recent studies showed the presence of a nucleus-encoded GlnRS and the direct synthesis of Gln-tRNA in the mitochondria of kinetoplastid protozoans (*Leishmania tarentolae* and *Trypanosoma brucei*) [Nabholz et al. 1997; Rinehart et al. 2004]. The concept of import of both GlnRS and tRNA\(^\text{Gln}\) is more obvious in these organisms because their mitochondrial genomes do not contain tRNA genes. Indeed tRNA import was first described in these and other organisms, but largely unexplored in higher eukaryotes [for reviews, see Schneider and Marechal-Drouard 2000; Hopper and Phizicky 2003]. If our prediction of the essentiality of cytoplasmic tRNA\(^\text{Gln}\) import into mitochondria were true, then GlnRS-catalyzed Gln-tRNA synthesis should be widespread in the mitochondria of most organisms.

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**Materials and methods**

**Yeast strains**

The wild-type *S. cerevisiae* strain W303 mat a ade2-1 his3 leu2-3, 112 trp1-1 ura3-52 was used for tRNA preparation and protein expression. The mutant trp1-1 allele in W303 is the result of an amber nonsense codon; therefore, this strain was also utilized in suppression experiments. The strain HM4 mat a leu2-3, 112 lys2 ura3-52 arg8 - : hisG [p + cox2-1124\^\text{meta}] [Kolesnikova et al. 2000] was used in mitochondrial suppression studies. INVSc1 mat a/a his3Δ1/1 his3Δ1 leu2/leu2 trp1-189/189 ura3-52 ura3-52 [Invitrogen] was used for immunofluorescence localization. The heterozygous GlnRS deletion strain, Y22424 BY4743 mat a/a his3Δ1/1 his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 MET15/...
met15Δ0 ura3Δ0 ura3Δ0 YOR168w::kanMX4/YOR168w (Research Genetics), was used to generate strains for immunofluorescence localization, as well as complementation via plasmid exchange [5-FOA selection]. The GlnRS was subcloned (described below) into a p416GPD [CEN/ARS, URA+ ] vector and used to create a viable GlnRS chromosomal deletion strain [exchange strain]. Tetrad dissection and other genetic manipulations were carried out as before [Rosas-Sandoval et al. 2002]. All media were prepared as described [Guthrie et al. 1991].

Isolation of mitochondria and Western blot

Yeast W303 was grown in rich media containing 2% galactose, and mitochondria were isolated as described [Daum et al. 1982; Celis 1994; Glick and Pon 1995]. Spheroplasts were prepared by Zymolyase-20T [ICN] treatment and broken by three passes through a EmulsiFlex-C5 [AVESTIN] in 0.6 M sorbitol [Fluka], 10 mM Tris-HCL (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride. Mitochondria were purified by centrifugation and extensive washing in the same buffer as described [Daum et al. 1982]. The cytosolic fraction obtained during this procedure was clarified by ultracentrifugation at 100,000 g for 60 min. The supernatant was TCA precipitated for Western blot analysis.

RNA localization with Northern blot and RT–PCR sequencing

RNA was isolated from total, microccocal nuclease-treated mitochondrial fractions and/or cytosolic yeast fractions by the guanidinium thiocyanate/phenol/chloroform extraction method [Chomczynski and Sacchi 1987]. RNA fractions were separated on denaturing 8% polyacrylamide gels with 8 M urea and electroblotted to a Zeta-probe [Bio-Rad] membrane. Membranes were probed with [32P] 5’ end-labeled oligonucleotides complementary to tRNA<sup>gln</sup> [TACCTATTAGTCTACGACTCA] tRNA<sup>gln</sup> [ATACCTACTACATTAGGACC] and U6 snRNA [CGAAGGGTATCCGCGGAC] and hybridized, washed, and stripped of the probes were carried out according to the manufacturer’s directions [Bio-Rad]. Images were taken with a Storm PhosphorImager and analyzed with ImageQuant [ Molecular Dynamics].

Cloning and purification of GlnRS and mGluRS

Oligonucleotides were synthesized and DNAs were sequenced by the Keck Foundation Biotechnology Resource Laboratory at Yale University. PCR primers were designed for the <i>S. cerevisiae</i> GlnRS (GLN4/YOR168W) and mGluRS [MSE1/YOL033W] according to the sequences published in the Saccharomyces Genome Database [http://www.yeastgenome.org]. The genes were cloned by PCR from a yeast genomic DNA library [ATCC, #77164] using the Expand High Fidelity PCR System [Roche]. Primers GCATATGATCTGTCTGAAATGAAATT GACTCAG and CCGCTCGAGTCACTTTGAAGTTCCGTCT CTTCAAGCTAAC were used to clone the GlnRS in the HIS3 vector p413GPD between the SpeI and XhoI restriction sites, which allowed the overexpression of the native protein in yeast [Mumberg et al. 1995]. Wild-type yeast W303 was transformed with the GlnRS-p413GPD construct and cells were cultured in semi-synthetic, 2% glucose media lacking histidine. Native GlnRS expression and purification was performed essentially as described [Ludmerer et al. 1993]. Ion-exchange chromatography was carried out, in succession, over 5-Sepharose, Hepa- rin HP, and Mono S HR [Amersham] columns in 50 mM Tris-HCL (pH 7.0–7.5), 5% glycerol, 5 mM β-mercaptoethanol [β-ME], protease inhibitor cocktail for fungal and yeast cells [Sigma], and the appropriate counter-ion. Purity was judged by Coomassie-stained SDS–polyacrylamide gel electrophoresis and protein concentrations were determined by the QuantiGold [Diversified Biotech] colloidal gold protein assay [Stoscheck 1987].

tRNA<sup>gln</sup> import into yeast mitochondria

by ion-exchange chromatography on Mono S HR 5/5 [Amersham]. Primers GACTGTATGTCTCTGTAGAAAGATT GACTCAG and CCGCTCGAGTCACTTTGAAGTTCCGTCT CTTCAAGCTAAC were used to clone the GlnRS in the HIS3 vector p413GPD between the SpeI and XhoI restriction sites, which allowed the overexpression of the native protein in yeast [Mumberg et al. 1995]. Wild-type yeast W303 was transformed with the GlnRS-p413GPD construct and cells were cultured in semi-synthetic, 2% glucose media lacking histidine. Native GlnRS expression and purification was performed essentially as described [Ludmerer et al. 1993]. Ion-exchange chromatography was carried out, in succession, over 5-Sepharose, Heparin HP, and Mono S HR [Amersham] columns in 50 mM Tris-HCL (pH 7.0–7.5), 5% glycerol, 5 mM β-mercaptoethanol [β-ME], protease inhibitor cocktail for fungal and yeast cells [Sigma], and the appropriate counter-ion. Purity was judged by Coomassie-stained SDS–polyacrylamide gel electrophoresis and protein concentrations were determined by the QuantiGold [Diversified Biotech] colloidal gold protein assay [Stoscheck 1987].

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tRNA purification

Wild-type yeast [W303] was cultured in rich media supplemented with 2% galactose to increase the mitochondrial content of the cells. Unfractionated tRNA and total cytoplasmic tRNA was prepared with a QIAGEN-tip 100 column [QIAGEN] as described [Rinehart et al. 2004]. Following established procedures, individual tRNAs were purified from unfractionated mitochondria.
tionated tRNA with 5′ biotinylated DNA oligonucleotides, complementary to the target tRNA immobilized on streptavidin agarose beads [Pierce] (Kaneko et al. 2003). Biotinylated oligo-DNAs (150 µg), XTGGTGAAATCTCATTAGTCTTGAGCCTCA for tRNA\textsubscript{Gln}\textsuperscript{m}, (X = 5′ biotin), XTGATTGCTGAACTCCATTTAGACGATAAGGTC for tRNA\textsubscript{Gln}\textsuperscript{m}, and XCGAAGAGCATTACACTACACTAGAAGC for tRNA\textsubscript{Glu}, were bound to the streptavidin beads in 10 mM Tris-HCl [pH 7.5]. The oligo-beads were equilibrated in 6× NTE solution [20× NTE solution is 4 M NaCl, 0.1 M Tris-HCl at pH 7.5, 50 mM EDTA, 5 mM 2-ME] and 3 mg of unfraccionated yeast tRNA (10 mg/mL in 6× NTE) was then added and incubated at 65°C for 30 min. After incubation, the temperature of the mixture was decreased by natural heat dissipation until it reached 30°C and washed, in succession, with 5× NTE three times, 1× NTE twice, and a final 0.1× NTE wash until the absorbance at 260 nm of the was zero. tRNAs retained on the beads were eluted with 0.1× NTE at 65°C and ethanol precipitated. True tRNAs were examined with Northern blot by hybridization with a 5′ 32P-labeled oligodeoxyribonucleotide probe specific to each tRNA [described above].

**Aminoacylation assays:** filter binding, thin-layer chromatography, and acid uria gel electrophoresis

Filter binding assays were performed essentially as described [Ahel et al. 2002]. Aminoacylation reactions were performed at 30°C in 100 mM HEPES-Na [pH 7.2], 30 mM KCl, 12 mM MgCl\textsubscript{2}, 10 mM ATP, 10 mM DTT, 1 µM pure tRNA, and 50 µM [14C]Gln [50 µCi/µL, 242 mCi/mmol; Amersham] or 50 µM [14C]Glu [50 µCi/µL, 254 mCi/mmol; Amersham] using 0.1 μl GlmRS or mGluRS. The identity of the [14C] amino acid was confirmed by thin layer chromatography. In vitro aminoacylation reactions were stopped after 30 min with 1 volume of 0.6 M sodium acetate [pH 4.5] and the aa-tRNA was extracted with acidic-phenol. Samples were ethanol precipitated, and the aa-tRNA (~1 pmol) was treated with 0.5 M borate [pH 9.0] to remove the [14C] amino acid, dried in a speed vac, spotted in water on 20 × 20 cellulose plates (Sigma), and resolved in isopropanol/formic acid/water (80/20/4, vol/vol/vol). TLC plates were dried and visualized with the Storm phosphorimaging system described above. Acid uria gel electrophoresis of tRNA and aa-tRNA was carried out essentially as described [Varshney et al. 1991]. Total aa-tRNAs from pure mitochondria were extracted under acidic conditions (equilibrated with 0.3 M sodium acetate at pH 4.5 and 10 mM EDTA), ethanol precipitated, and resuspended in 10 mM sodium acetate [pH 4.5] and 1 mM EDTA. A fraction of the total aa-tRNA was stored at ~80°C to be used for in vivo aa-tRNA controls. The remaining tRNA was deacylated by incubation in 100 mM Tris-OAc [pH 8.5] at 37°C for 30 min, ethanol precipitated, and used for in vitro aminoacylation assays. In vitro aminoacylation reactions were carried out as described above except with unlabeled amino acids and 10 µg of total deacylated tRNA from mitochondria. The reactions were stopped at 30 min with 1 volume of 0.6 M sodium acetate [pH 4.5]. Parallel aminoacylation reactions with radiolabeled amino acids were performed under the same conditions to monitor aminoacylation. In vitro aminoacylation reactions were analyzed on acid uria gels essentially as described [Polycarpo et al. 2003]. After acidic-phenol extraction and ethanol precipitation, the aa-tRNAs were dissolved in 1.5 µL of 10 mM sodium acetate [pH 4.5] and 1 mM EDTA. Once resuspended, 1.5 µL of loading buffer [7 M urea, 0.1 M sodium acetate at pH 4.5, 0.1% bromophenol blue, 0.1% xylene cyanol] was added to each sample. Samples were loaded on a 9.5% polyacrylamide gel (50 × 20 cm, 0.4 mm thick) containing 7 M urea, 0.1 M sodium acetate [pH 5.0]. The gel was run at 4°C, at 600 V in 0.1 M sodium acetate [pH 5.0] for 38 h. The 0.1 M sodium acetate [pH 5.0] was changed after 24 h. tRNAs were subsequently electroblotted onto a Hybond N+ nylon membrane [Amersham Biosciences] and visualized via Northern blot with the appropriate 5′ 32P-labeled probe [described above].

**Immunofluorescence analysis**

V5-tagged GlnRS was cloned into a URA-2 µm pYES-V5 TOPO vector [Invitrogen] utilizing primers similar to those described above except lacking a stop codon and Xhol restriction site in the reverse primer. The resulting constructs allowed expression of each V5 fusion protein in the yeast strain INVSc1 as verified by Western blot with an HRP-conjugated anti-V5 antibody [Invitrogen]. The GlnRS-V5 plasmid construct was transformed into the yeast strain Y22424, which was then used to generate a GlnRS deletion strain [as described above]. Each strain was cotransformed with a HIS-CEN plasmid that carries an ADH1-COX4-GFP and directs GFP to the mitochondrion [Sasakci and Jensen 1999]. Cells were cultured to early log phase in semi-synthetic, 2% galactose media lacking histidine and uracil, fixed with 4% paraformaldehyde, permeabilized with zymolyase, and prepared with organic solvents for immunostaining as described [Harlow and Lane 1999]. The cells were incubated with mouse anti-V5 [Invitrogen] primary antibody [1:60] followed by Cy3-conjugated goat anti-mouse [Jackson Immuno Research] secondary antibody [1:200] and mounted with ProLong [Molecular Probes] mounting media. Cox2p immunofluorescence was carried out in a similar fashion with mouse anti-Cox2p [Molecular Probes] primary antibody [1:200] and Cy3-conjugated anti-mouse [Jackson Immuno Research] secondary antibody [1:500] followed by staining with 20 nM of MitoTracker Green FM [Molecular Probes] according to the manufacturer’s instructions. An N-terminal GlnRS-GFP was constructed by inserting GFP into the GlnRS-p413GPD. Utilizing PCR primers GACTAGTATGAGTAAAGGAGAACACTTGCTG and GACTAGTTTTGTATGAGTTCATCCATG, the stop codon was removed and 5′ and 3′ Spel restriction sites were added to the GFP gene. The gene was then inserted at the Spel site in GlnRS-p413GPD and the proper orientation of the GlnRS-GFP fusion gene was checked by MscI/XhoI restriction analysis. W303 yeast expressing GlnRS-GFP was cultured to early log phase in semi-synthetic, 2% galactose media lacking histidine, and stained with DAPI [Molecular Probes]. Yeast strains expressing both GFP- and V5-tagged proteins were examined on an Axio- scope [Carl Zeiss Microimaging] fitted with an Axiocam, a ChromaGFP filter set [model C2909], DAPI and TR filters [Chroma Technology], and a Uniblitz Shutter assembly. Images were processed with OpenLab version 3.1.5.

**In vivo suppression**

Primers GGACTAGTCCGGTTAAATAGGGACCATTCATTTC and GGACTAGTCCGGTTAAATAGGGACCATTCATTTC with flanking Spel sites were used to clone the native yeast tRNA\textsubscript{Gln}CUA gene into the low copy yeast vector p316 [URA3]. The tRNA\textsuperscript{Gln}CUA gene was then generated from this clone via PCR using the primers TTGTTCGGATGAAAAAGAAGAATTCGACTTCGGTTAATCCGGATGATTGTATCTGT with flanking Spel sites were used to clone the native yeast tRNA\textsubscript{Gln}CUA gene into the low copy yeast vector p316 [URA3]. The tRNA\textsuperscript{Gln}CUA gene was then generated from this clone via PCR using the primers TTGTTCGGATGAAAAAGAAGAATTCGACTTCGGTTAATCCGGATGATTGTATCTGT with flanking Spel sites were used to clone the native yeast tRNA\textsubscript{Gln}CUA gene into the low copy yeast vector p316 [URA3]. The tRNA\textsuperscript{Gln}CUA gene was then generated from this clone via PCR using the primers TTGTTCGGATGAAAAAGAAGAATTCGACTTCGGTTAATCCGGATGATTGTATCTGT with flanking Spel sites were used to clone the native yeast tRNA\textsubscript{Gln}CUA gene into the low copy yeast vector p316 [URA3].
Cells were cultured to early log phase and prepared for immunofluorescence as described above.

**In vitro tRNA import assays**

Native tRNA^{32P} | purified as above | were first treated with calf intestinal Alkaline Phosphatase (Invitrogen) followed by phenol extraction and ethanol precipitation. The sequence for the imported yeast tRNA^{32P} was as described (Kolesnikova et al. 2000). In vitro transcript of tRNA, tRNA by T7 transcription as described (Rubio et al. 2000; Ahel et al. 2002). The tRNA was then 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (GIBCO-BRL) followed by gel purification on a 7 M urea/8% polyacrylamide gel. In vitro RNA import assays were performed in a 20 µL reaction volume containing 100,000 cpm of 5'-end-labeled tRNA, 1 µg of mitochondria, 0.6 M sorbitol, 20 mM Tris-HCl (pH 8.0), 5 mM ATP, 2 mM DTT, 20 mM MgCl₂, and 2 mM EDTA. After incubation at 27°C for 10 min, 100 U of micrococcal nuclease (MN) (Roche) and 5 mM CaCl₂ were added and the reaction was incubated an additional 30 min to digest the tRNAs that were not imported into the mitochondria. MN was then inhibited by the addition of 10 mM EGTA (pH 8.0). To isolate protected tRNAs, the mitochondria were washed with 0.6 M sorbitol/20 mM Tris-HCl (pH 8.0), pelleted, resuspended in 90 µL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS, and extracted with 100 µL of 1 M water-saturated phenol (pH 4.5) followed by ethanol precipitation. The radioactively labeled tRNAs were separated by electrophoresis through a 7 M urea/6% acrylamide gel. After electrophoresis, the gels were dried onto Whatman 3MM chromatography paper and visualized with the Storm PhosphorImager imaging system as described above.

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