Mechanisms Leading to and the Consequences of Altering the Normal Distribution of ATP(CTP):tRNA Nucleotidyltransferase in Yeast*

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CCA1 codes for mitochondrial, cytosolic, and nuclear ATP(CTP):tRNA nucleotidyltransferase. Studies reported here examine the mechanisms leading to and the consequences of altering the distribution of this important tRNA processing enzyme. We show that the majority of Cca1p-I, translated from the first in-frame ATG, is in mitochondria but surprisingly, there is a small contribution to nuclear and cytosolic tRNA processing by this form as well. The majority of Cca1p-I and Cca1p-III, translated from ATG2 and ATG3, respectively, is in the cytosol but both are also located in the nucleus for processing precursors. Altering the cytosolic/nuclear distribution of Cca1p by fusing the SV40 nuclear localization signal to the 5′ end of CCA1 causes a growth defect and results in the accumulation of end-shortened tRNAs in cells resuming exponential growth are more severely affected than those continuing exponential growth.

Transfer RNA biosynthesis is compartmentalized in eukaryotes as both nuclear and mitochondrial DNA generally contain tRNA genes. In the instances in which nuclear transcribed tRNAs are imported into mitochondria, it is not clear in which cellular compartment maturation occurs but it is clear that all steps in the biosynthesis of tRNAs coded by mitochondrial DNA occurs in the organelle (for review, see Ref. 1). Mitochondrial pre-tRNAs are processed to their mature form by steps in which sequences are trimmed from their 5′ and 3′ ends, bases are modified, and the oligonucleotide CCA is added to the 3′ terminus. The order of mitochondrial pre-tRNA processing can vary. Removal of 5′ leader sequences from pre-tRNAs need not precede the removal of the 3′ trailer sequence and the addition of the end terminal CCA (1, 2).

In contrast, cytosolic tRNA biosynthesis begins in the nucleus and is completed in the cytosol. The processing of nuclear precursor tRNA to form mature tRNA also requires the trimming of 5′ leader and 3′ trailer sequences from the primary transcript, the post-transcriptional addition of oligonucleotides CCA to the 3′ terminus, base modifications, and in some instances, splicing to remove intervening sequences (for review, see Ref. 2). Two lines of evidence demonstrate that end maturation, intron splicing, and certain base modifications take place in the nucleus. First, yeast pre-tRNAs microinjected into Xenopus oocyte nuclei are processed (3–5). Second, nuclear restricted pre-tRNAs, those tRNAs with intervening sequences, have base modifications and CCA ends (6, 7, 8). Following maturation in the nucleus, which includes CCA addition, tRNAs are exported to the cytosol where additional base modifications (4, 9) are added. 3′ terminal CCA ends turn over in the cytosol (10).

Multiple locations of tRNA biosynthesis means that tRNA maturation enzymes with analogous functions are required in multiple cellular locations. Many of these isozymes are structurally distinct and are coded by separate genes. However, three tRNA maturation enzymes have now been identified as sorting isozymes, enzymes that function in more than one cellular compartment. These include N2,N2-dimethylguanosine-specific tRNA methyltransferase, Trm1p (11–13); D2-isopentenyl-pyrophosphate: tRNA isopentenyltransferase, Mod5p (14); and ATP(CTP): tRNA-specific nucleotidyltransferase, Cca1p (15). Sorting isozymes have heterogeneous amino-terminal ends which are the result of translation initiation from more than one in-frame AUG. Selection of the AUG to be used for translation initiation can depend on whether transcription initiates upstream or downstream of ATG1 of the ORF1 (16). Heterogeneous amino termini can also result when the translation initiation machinery bypasses an AUG in a “poor” context in favor of a downstream AUG (17–19). Translation from the first ATG provides a mitochondrial targeting signal to promote efficient import into the organelle while translation from a downstream ATG eliminates this information and provides enzyme for nuclear and/or cytoplasmic located tRNA processing.

CCA1 codes for mitochondrial, cytosolic, and nuclear Cca1p which catalyzes the addition of CCA to the 3′ termini of tRNA (15, 20, 21). In yeast, this enzyme is essential because the CCA end is not encoded by either nuclear or mitochondrial tRNA genes (for review, see Ref. 2). The CCA1 gene has 3 in-frame ATGs at the 5′ end of its ORF. An examination of mRNA transcripts from CCA1 demonstrated 5′ ends which fall upstream of ATG1 and between ATG1 and ATG2, but not between ATG2 and ATG3. Cca1p synthesis initiates from all three ATGs. This means that translation initiation from ATG3 requires that ribosomes bypass ATG2 to initiate at the downstream ATG3. Thus, an interplay of transcription initiation

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The abbreviations used are: ORF, open reading frame; NLS, nuclear localization signal; kb, kilobase(s); DAPI, 4′,6-diamidino-2-phenylindole.
and translational selection of ATGs leads to the biosynthesis of three isoforms of Cca1p (15, 19). Site-directed mutagenesis experiments which altered ATG1 demonstrated that protein translated from the first in-frame ATG is necessary for mitochondrial protein synthesis. Mutant genes retaining either both ATG2 and ATG3 provide nuclear/cytosolic enzyme activities (15, 19).

The fact that there are three isoforms and three compartments raises the question as to whether each isoform is compartment specific. To address this issue, we investigated the ability of each isozyme to catalyze the addition of CCA to nuclear restricted pre-tRNA by Northern blot analysis. We also examined the cellular distribution of Cca1p by indirect immunofluorescence. Our studies indicate that both Cca1p-II and Cca1p-III are in the nucleus and the cytosol. Cca1p-I is primarily in mitochondria but minor activity from Cca1p-I can also be detected in the nucleus.

We also determined the consequences of altering the cytosolic/nuclear distribution of nucleotidyltransferase activity. By providing Cca1p with a surrogate nuclear localization signal (SV40-NLS-Cca1p) we have substantially reduced the cytosolic pool of Cca1p and concomitantly increased the nuclear pool of this enzyme. At the nonpermissive temperature SV40-NLS-Cca1p is unable to complement a ts cca1-1 allele. This result directly demonstrates an important role for Cca1p in the cytosol of eukaryotes.

**EXPERIMENTAL PROCEDURES**

Strains and Media—Saccharomyces cerevisiae strain W3031B (MATa ade2-1 his3-11, 15 eu2-2, 112 ura3-1 can1-100 trpl-1) was used for immunofluorescence experiments. Strain NT33-5 was used for growth studies and isolation of RNA for Northern blot analysis. NT33-5, obtained from backcrossing of strain 352-1A (MATa ade2-1 his3-11, 15 eu2-2, 112 ura3-1 can1-100 trpl-1) to W3031B, has the ts cca1-1 allele and requires uracl and leucine for growth. The preparation of media and growth of yeast cells were performed by standard procedures described by Sherman et al. (22). Yeast strains were transformed with wild-type or mutant CCA1 genes on yeast shuttle vectors: Ycp50 (23), pRS426 (24), and pBluescript KS (25) by the one-step lithium acetate method described by Chen et al. (26).

Construction, Expression, and Purification of TrpE-Cca1p—A 656-base pair PstI/XbaI fragment from cca1-M2 (19) was inserted into the PstI/XbaI site in pATH 23 (27) creating a fusion gene with nucleotides coding for amino acids 11–230 of CCA1 fused to the 3’ end of the TRPE ORF. Expression of TrpE-Cca1p was expressed on Escherichia coli strain JM101 cells by tryptophan depletion. Fusion of the purification protein for injection into rabbits was performed as described by Koerner et al. (27).

Isolation of Antibody—Preimmune serum was drawn prior to injection of the antigen. The rabbit was then subcutaneously injected with 200 μg of TrpE-Cca1p fusion protein in 1 ml of Ribi adjuvant (Ribi Immunochim Research Inc.) prepared according to the manufacturer’s instructions. At monthly intervals the rabbit was injected subcutaneously with 100 μg of TrpE-Cca1p in 500 μl of incomplete Freund’s adjuvant (Sigma). Ten to fifteen ml of blood was collected 7–14 days after each boost.

Affinity Purification of Antibody—Anti-Cca1p antibody was affinity purified from nitrocellulose blots using the method described by Pringle et al. (28) except that anti-TrpE antibodies were first removed by preabsorption of crude sera to a blot of TrpE before affinity purification of anti-Cca1p antibodies with a blot of TrpE-Cca1p.

Construction of Mutant CCA1 Genes—cca1-M1,M2, cca1-M1,M3, and cca1-M2,M3 were constructed as described previously (15, 19). SV40-NLS-CCA1 and SV40-nls-CCA1 were synthesized in a thermocycling reaction with CCA1 as a template. The gag nucleotide 5’-GAAACTGCCCTTCATGGG-3’ which is a PstI site (underlined) at its 5’ end followed by an AUG (italic) for translation initiation sequence that codes for the SV40 large T antigen nuclear localization signal (bold), and sequence complementary to nucleotides 50–72 of the CCA1 ORF was used as the upstream primer. An oligonucleotide with a sequence complementary to nucleotides 1454–1478 of the CCA1 ORF, 5’-GGTTTGGATCTGACAATTATTTA-3’, was used as the down-stream primer. The amplified product was ligated into the TA cloning vector (Invitrogen). The vector was digested with PstI and NdeI and a 1.2-kb fragment was isolated. The fragment now having the SV40 NLS coding sequence was used to replace a similar PstI/NdeI fragment in cca1-M1,M2 cloned in pBluescript KS+ (Stratagene). The 4.4-kb BamHI/Sall fragment now containing SV40-NLS-CCA1 was transferred into the BamHI/Sall site of pRS426 and Ycp50. This fusion in detail including the amino acid sequence was cloned in pBluescript KS+ to produce SV40-nls-CCA1. The 4.4-kb BamHI/Sall fragment containing SV40-nls-CCA1 was then transferred to pRS426 and Ycp50.

Indirect Immunofluorescence—Indirect immunofluorescence was carried out as described by Rose et al. (30) with W303-1B cells carrying multiple copies of CCA1. Affinity purified anti-Cca1p antibody was the primary antibody.

**RESULTS**

To examine exponential growth at the nonpermissive temperature, overnight cultures were diluted in synthetic complete medium to a cell density of about 0.05 OD 600 nm and allowed to resume log phase growth at room temperature. The cells were then shifted to 37°C and cell density was monitored. To examine the transition between nonexponential and exponential growth at 37°C, cells were grown as described above except that after dilution of cells to 0.05 OD 600 nm, growth was immediately monitored at 37°C.

Isolation of Small RNAs for Northern Blot Analysis—Overnight cultures were diluted in 50 ml of synthetic complete medium to an OD 600 nm of 0.1 and allowed to resume log phase growth. The cells were then shifted to 37°C and harvested after either 4 or 18 h growth by centrifugation at 10,000 × g for 8 min. The cell pellet was washed before thawing in 2 ml of water per 0.5 g of cells in a 15-ml disposable test tube (Sarstedt). Fifty μl of 0.5 M magnesium acetate, 0.25 M β-mercaptoethanol, pH 6.0, was added followed by 1.2 ml of phenol saturated in the same buffer described above. The tube was laid on its side and gently agitated for 1 h on a platform shaker. The layers were separated by centrifugation in a tabletop centrifuge. The aqueous layer, containing total cell small RNAs, was transferred to another tube. Sodium dodecyl sulfate was added to 0.5% to inhibit RNases before storage at –20°C. Prior to an experiment, an ethanol precipitation was removed from each E. coli cell. J. M. Salton

**Northern Blot Analysis**—Total RNA (15 μg) was electrophoretically separated on a 6% acrylamide, 8 M urea sequencing gel and the RNAs transferred to Zetaprobe (Bio-Rad) in a transblot apparatus (Bio-Rad) in the presence of 40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0. Prewashing, prehybridization, and hybridization steps were carried out as described by Morris et al. (31). Probes were labeled with [32P]ATP at the 5’ end using polynucleotide kinase. The following probes complementary to trnA Phe , trnA 20S , trnA 30S , trnA 50S , trnA 50S , trnA 50S , trnA 50S , trnA 50S , trnA 50S , trnA 50S , and trnA 50S were used.

**RESULTS**

The Majority of Cca1p Is Cytosolic in S. cerevisiae—Although it is clear from an analysis of trnA processing and repair that ATP(CTP):trnA nucleotidyltransferase activity is located in both nucleolus and cytosol, the distribution of the and the necessity for the enzyme in both compartments in yeast was not known. Cca1p is a low abundance protein (19) and antibodies raised against Cca1p do not detect endogenous Cca1p by immunoblot analysis of cell extracts. The immunofluorescence signal is weak at best in wild-type cells. Also, CCA1 is an essential gene; therefore, we cannot determine by deletion of the gene if the fluorescence is due to Cca1p-specific immunoreactive complexes. The results of epitope tagging Cca1p also indicate that Cca1p is a low abundance protein. We fused the 12CA5 epitope
from influenza virus hemagglutinin (32) to the 3' end of CCA1 and confirmed the fusion was in-frame by DNA sequencing. This fusion protein complements the ts cca1-1 strain. However, no protein was detected by immunoblot analysis of cell extract nor by immunofluorescence microscopy with the 12CA5 monoclonal antibody (BABCO).

We are able to detect by immunoblot analysis Cca1p in extracts of cells containing multiple copies of CCA1. The immunofluorescence experiments reported here were done with extracts of cells carrying CCA1 on multicopy vectors, pRS426 (10–20 copies/cell) and pJDB207 (100–200 copies/cell). Fig. 1 (A, C, and E) shows the fluorescent isothiocyanate detection of Cca1p immunoreactive complexes. Fig. 1 (B, D, and F) shows the 4',6-diamidino-2-phenylindole (DAPI) staining of nuclear and mitochondrial DNA. A very weak cytosolic signal is detected in cells carrying vector alone and expressing endogenous levels of Cca1p (Fig. 1A). In cells carrying 10–20 copies of CCA1, most Cca1p is in the cytosol with only a little present in nuclei (Fig. 1, C and D). Fig. 1, E and F, shows the subcellular distribution of Cca1p in cells carrying 100–200 copies of CCA1. Again Cca1p is primarily localized to the cytosol. However, we now observe an enrichment of Cca1p in the region surrounding the nucleus.

Distribution of Protein Expressed from Each AUG by Indirect Immunofluorescence—To determine the distribution of each Cca1p isoform by immunofluorescence microscopy, we transformed wild-type cells with mutant CCA1 genes missing ATG1 and ATG3; ATG1 and ATG2; or ATG2 and ATG3 (cca1-M1,M3, cca1-M1,M2, or cca1-M2,M3) on pRS426. Indirect immunofluorescence of cells carrying cca1-M1,M3 shows that Cca1p-II initiating from AUG2 is localized primarily to the cytosol with little detected in the nucleus and mitochondria (Fig. 2, A and B). Cca1p-III, initiating from AUG3, is also confined to the cytosol with little detected in the nucleus or mitochondria (Fig. 2, C and D). Close examination of the micrographs actually reveals nonreactive areas corresponding to mitochondria in these cells. However, Cca1p-I, originating from AUG1 in cells carrying cca1-M2,M3, localizes almost exclusively to mitochondria (Fig. 2, E and F). These results are consistent with previous experiments which show that protein initiating from the first ATG in the CCA1 open reading frame is required for respiration on non-fermentable carbon sources but alone is not able to support life. It is clear from these studies that ATG1 provides mitochondrial enzyme and does not make a significant contribution to enzyme found in the cytosolic/nuclear compartments (Fig. 2, E and F).

Cca1p-II and Cca1p-III Expressed from AUG2 and AUG3, Respectively—Provide Nuclear Nucleotidyltransferase Activity—We have demonstrated that Cca1p-I is targeted to mitochondria and Cca1p-II and Cca1p-III localize in the cytosol. To determine which isoform of Cca1p provides nuclear enzymatic function, we examined, by Northern blot analysis, tRNA isolated from cca1-1 cells having only Cca1p-I, Cca1p-II, or Cca1p-III. Since tRNA that lack the 3'CCA end migrate faster on polyacrylamide gels than tRNA with a 3'CCA end, nucleotidyltransferase activity can be assessed in vivo under varying growth conditions. To specifically examine nuclear Cca1p activity we used a probe complementary to the intron region of nuclear restricted pre-tRNA\textsubscript{Ser}\textsuperscript{CCA}. At the nonpermissive temperature, cca1-1 cells carrying vector alone accumulate pre-tRNA\textsubscript{Ser}\textsuperscript{CCA}, that lack CCA at its 3' terminus (Fig. 3, lane 1). Pre-tRNA\textsubscript{Ser}\textsuperscript{CCA} isolated from cells containing only Cca1p-I, the longest form of Cca1p, is primarily without a CCA end, however, some 3' end matured pre-tRNA\textsubscript{Ser}\textsuperscript{CCA} is present (Fig. 3, lane 2). A larger fraction of pre-tRNA\textsubscript{Ser}\textsuperscript{CCA} having CCA extended termini accumulates in cells carrying either Cca1p-II or Cca1p-III (Fig. 3, lanes 3 and 4). Consequently, all three isoforms of Cca1p provide nuclear Cca1p activity but the shorter isoforms provide the majority of nuclear activity.

The SV40 Nuclear Localization Signal Targets Cca1p to the Nucleus—Immunofluorescence studies clearly show that the majority of Cca1p is in the cytosol. To examine the importance of cytosolic Cca1p, we designed a protocol predicted to reduce the cytosolic level of the enzyme. We replaced the 5' end of the CCA1 ORF (nucleotides +1 to +79, ATG3 starts at +52) with...
the SV40 NLS to produce SV40-NLS-Cca1p. We also made the companion construct containing a mutant SV40 NLS (SV40-nls-Cca1p). Indirect immunofluorescence shows that the SV40 NLS targets Cca1p to the nucleus and substantially reduces the cytosolic pool of Cca1p (Fig. A and B). Cells carrying SV40-nls-Cca1p do not show this radical change in distribution (Fig. C and D). SV40-nls-Cca1p does, however, appear to have a slightly altered distribution relative to Cca1p-III alone in that there appears to be slightly more SV40-nls-Cca1p in the nucleus (compare Fig. 2C and Fig. 4C).

Altering the Nuclear/Cytosolic Distribution of Cca1p Causes a Growth Defect—To determine if altering the distribution of Cca1p between the nucleus and cytosol has phenotypic consequences, we examined the ability of nuclear localized SV40-NLS-Cca1p to complement the defective allele in cca1-1 cells (Fig. 5). cca1-1 cells carrying vector alone, CCA1, SV40-NLS-CCA1, or mutant SV40-nls-CCA1 in single copy were spotted onto agar plates containing glucose as a carbon source. All of the cells grow at the permissive temperature because chromosomally expressed Cca1p is active (Fig. 5A). However, at the nonpermissive temperature, functional Cca1p must be provided by the plasmid. Therefore, cells transformed with vector alone cannot grow at 37 °C (Fig. 5B, lane 1). Cells carrying CCA1 (Fig. 5B, lane 2) grow slightly better than cells carrying mutant SV40-nls-CCA1 (Fig. 5B, lane 4). However, cells carrying SV40-NLS-CCA1 grow poorly at 37 °C (Fig. 5B, lane 3). cca1-1 cells carrying SV40-NLS-CCA1 also grew slower in liquid culture. Early log phase cca1-1 cells incubated in synthetic complete minus uracil media were shifted to the nonpermissive temperature. cca1-1 cells carrying vector alone do not grow (Fig. 6A). cca1-1 cells producing SV40-nls-Cca1p have the same doubling time as cells producing wild-type Cca1p. In comparison, the doubling time for cells producing SV40-NLS-Cca1p increases 30%. The inability of SV40-NLS-CCA1 to complement cca1-1 becomes more pronounced when overnight cultures that are no longer in log phase are diluted into synthetic complete minus uracil media and grown at the nonpermissive temperature. Initially, the growth rate for cca1-1 cells carrying Cca1p, SV40-nls-Cca1p, or SV40-NLS-Cca1p is similar to that of the same cells inoculated from log phase cells (Fig. 6, compare A and B). However, cells producing SV40-NLS-Cca1p begin to lag much earlier than cells carrying Cca1p or SV40-nls-Cca1p. It is clear that SV40-NLS-Cca1p is not able to fully complement the temperature-sensitive phenotype of cca1-1 cells, in particular the transition between nonexponential and exponential growth.

There are several possible explanations for this. One is that fusion of the SV40 NLS to the amino-terminal end of Cca1p inhibits its enzyme activity. This seems unlikely since SV40-nls-Cca1p, which differs in only one amino acid from SV40-NLS-Cca1p, provides sufficient nucleotidyltransferase activity for wild-type growth. Another is that an increase in nuclear nucleotidyltransferase activity is inhibitory to some other nuclear function essential for normal cell growth. This is unlikely as cells expressing SV40-NLS-Cca1p at the permissive temperature grow fine. The third explanation is that the reduction of the cytosolic pool of Cca1p by nuclear mislocalization is responsible for the growth defect. To address this issue, we examined tRNA biosynthesis in cells carrying each of these constructs on single copy vectors as described below.

SV40-NLS-Cca1p Is Active—To determine if SV40-NLS-Cca1p is active in the nucleus, we examined the 3′ termini of pre-tRNA isolated from temperature-sensitive cca1-1 cells carrying mutant and wild-type CCA1 genes. Early log phase cells carrying CCA1, SV40-NLS-CCA1, or SV40-nls-CCA1 on a single copy vector were shifted to the nonpermissive temperature and RNA was isolated after 3.5 h of additional growth. An oligonucleotide complementary to the intron of pre-tRNA$_{Cca1}$ was used to detect nuclear confined pre-tRNA$_{Cca1}$ intron. At the nonpermissive temperature, cca1-1 cells carrying vector...
alone accumulate pre-tRNAs lacking the CCA modification because the chromosomally expressed temperature-sensitive Cca1p is inactive (Fig. 7, lane 1). Cells carrying CCA1 accumulate 3' end mature pre-tRNA\textsuperscript{Ser}\textsubscript{CGA} (Fig. 7, lane 2). Pre-tRNA\textsuperscript{Ser}\textsubscript{CGA} with CCA ends also accumulate in cells carrying SV40-NLS-CCA1 (Fig. 7, lane 3). Surprisingly, cells expressing SV40-nls-Cca1p, which differs by only one amino acid from SV40-NLS-Cca1p and is not enriched in the nucleus, accumulate more pre-tRNA\textsuperscript{Ser}\textsubscript{CGA} with complete CCA ends than do cells carrying wild-type Cca1p or Cca1p-III (compare Fig. 7, lane 4 with lane 2, and Fig. 3, lane 4). Thus Cca1p that has either a functional or nonfunctional surrogate NLS is active in the nucleus. The difference in accumulation of 3' end-mature pre-tRNA\textsuperscript{Ser}\textsubscript{CGA} between cells expressing SV40-NLS-Cca1p and SV40-nls-Cca1p may be a secondary affect resulting from the reduction of cytosolic protein synthesis in cells carrying SV40-NLS-CCA1 rather than any real difference in the activities of the two different fusion proteins.

The Rate of 3' End Shortening Varies among tRNAs—At the permissive temperature cca1-1 cells accumulate very little 3' end-shortened cytosolic tRNA\textsuperscript{Trp}\textsubscript{CCA}, tRNA\textsuperscript{Cys} and tRNA\textsuperscript{His}\textsubscript{GTG} (Fig. 8). However, at the nonpermissive temperature these tRNAs that lack the CCA end accumulate in the cytosol in cca1-1 cells carrying plasmid alone (Fig. 9, lanes 1 and 5). Moreover, the susceptibility of cytosolic tRNA to 3' end shortening varies among tRNA families. A larger proportion of tRNA\textsuperscript{Cys}\textsubscript{CGA} is end-shortened compared to tRNA\textsuperscript{Trp}\textsubscript{CCA} and tRNA\textsuperscript{His}\textsubscript{GTG} (Fig. 9, lanes 1 and 5).

Very little end-shortened tRNA\textsuperscript{Trp}\textsubscript{CCA}, tRNA\textsuperscript{Cys}\textsubscript{CGA} and tRNA\textsuperscript{His}\textsubscript{GTG} accumulates in cca1-1 cells carrying wild-type CCA1 at the nonpermissive temperature (Fig. 9, lane 2). A trace of tRNA\textsuperscript{Trp}\textsubscript{CCA} and a small fraction of tRNA\textsuperscript{Cys}\textsubscript{CGA} and tRNA\textsuperscript{His}\textsubscript{GTG} have shortened 3' termini in log phase cca1-1 cells carrying SV40-NLS-Cca1p which produces enzyme largely confined to the nucleus (Fig. 9, lane 3). In contrast, end-shortened tRNA\textsuperscript{Trp}\textsubscript{CCA}, tRNA\textsuperscript{Cys}\textsubscript{CGA}, and tRNA\textsuperscript{His} do not accumulate in cca1-1 cells carrying SV40-nls-Cca1p which contains the majority of Cca1p in the cytosol (Fig. 9, lane 4). These results further demonstrate that Cca1p is required for the repair of cytosolic tRNAs. Although the ratio of
end-shortened to mature tRNA is not as dramatic when nuclear Cca1p is present (SV40-NLS-CCA1) (Compare Fig. 9, lanes 1 and 3), the slight accumulation of cytosolic tRNAs, particularly rare tRNAs, lacking 3'CCA termini could affect cell growth as seen in Figs. 5 and 6.

The Requirement for Cytosolic Cca1p Is Greater in Non-Log Phase Cells Than in Log Phase Cells—At the permissive temperature very little end-shortened tRNA^{Trp}, tRNA^{His}, and tRNA^{Cys} accumulates in either log phase or non-log phase cca1-1 cells (Fig. 8). In cells with reduced cytosolic Cca1p (SV40-NLS-CCA1), more tRNA^{Trp} and tRNA^{His} accumulate in exponentially growing cells (Fig. 9, lane 7), than in exponentially growing cells (Fig. 9, lane 3). This accumulation is similar to that observed in cells lacking nucleotidyltransferase activity altogether (Fig. 9, lanes 1 and 5). Very little end-shortened tRNA^{CCG} is observed in either log phase or non-log phase cells carrying CCA1 or SV40-nls-CCA1 (Fig. 9, lanes 2 and 4 and lanes 6 and 8).

In the absence of nucleotidyltransferase activity, the majority of tRNA^{CCG} lacks CCA ends (Fig. 9, lanes 1 and 5). Although end-shortened tRNA^{CCG} is not detected in exponentially growing cells producing wild-type Cca1p (Fig. 9, lane 2), a small amount is detected in nonexponentially growing cells (Fig. 9, lane 6). In cells lacking cytosolic Cca1p, the level of end-shortened tRNA^{CCG} increases dramatically during nonexponential growth (Fig. 9, lane 7) compared to exponential growth (Fig. 9, lane 3). Cells carrying SV40-nls-Cca1p do not accumulate 3' end-shortened tRNA^{CCG} (Fig. 9, lanes 4 and 8).

End-shortened tRNA^{His}_{SV40} does not accumulate in either log phase or non-log phase cells carrying SV40-nls-Cca1p (Fig. 9, lanes 4 and 8). Although there is very little end-shortened tRNA^{His}_{SV40} in exponentially growing cells carrying wild-type Cca1p (Fig. 9, lane 2) that amount increases in nonexponentially growing cells (Fig. 9, lane 6). The level of end-shortened tRNA^{His}_{SV40} is greatest in cells lacking cytosolic Cca1p and it is increased during nonexponential growth (compare Fig. 9, lanes 3 and 7).

DISCUSSION

ATP(CTP):tRNA nucleotidyltransferase is a sorting isozyme that is localized to the nucleus, mitochondria, and cytosol. Although previous studies demonstrated that mitochondrial enzyme was provided by protein translated from the first in-frame ATG in the CCA1 open reading frame, (19) the source of nuclear and cytosolic activity was not known. Through indirect immunofluorescence studies described here, we have demonstrated that Cca1p-I is highly enriched in mitochondria and that the majority of Cca1p produced from the second and third ATGs in the open reading frame is cytosolic. An examination of pre-tRNAs by Northern blot analysis showed that Cca1p-II and Cca1p-III produced from these ATGs also provide nuclear activity sufficient for normal cell growth and that even Cca1p-I is capable of reaching the nucleus, albeit in amounts too small to support the needs of the cell. Altering the distribution of Cca1p-III such that the majority is located in the nucleus leads to the accumulation of 3' end-shortened tRNAs and causes a growth defect.

Subcellular distribution of nucleotidyltransferase varies among organisms. In rat liver cells, one-third of the nucleotidyltransferase activity is mitochondrial and the remainder is primarily cytosolic (33). Very little nucleotidyltransferase is found in the nucleus. In Xenopus, however, 30% of nucleotidyltransferase is nuclear (34). Most of yeast Cca1p is clearly localized to the cytosol. We do not know if the nuclear/cytosolic distribution is a result of strong cytosolic interactions (35), inefficient nuclear targeting, or a strong nuclear export signal (36–38). Since Cca1p does not contain any of the nuclear localization signals described to date (for review, see Ref. 39 and 40), we have not examined the strength of its NLS nor have we tried to prevent its delivery to the nucleus. By incorporating the strong SV40 large T antigen NLS into the amino terminus of Cca1p we were able to shift the distribution of Cca1p such that it accumulates in the nucleus. This suggests that a "weak" NLS is the determining factor for low levels of nuclear Cca1p.

Cca1p-I is localized primarily to mitochondria. While the absence of Cca1p-II and Cca1p-III might change the distribution of Cca1p-I and lead to this accumulation, it is also possible that in wild-type cells, a small amount of Cca1p-I is normally
located to nuclei and the cytosol. There are two routes that could lead to the nuclear/cytosolic activity provided by the longest form of Cca1p. First of all, unlike the mitochondrial form of the enzyme which is processed upon import (19), Cca1p-I could retain its mitochondrial targeting signal but not engage with the mitochondrial targeting machinery. Alternatively, all of the Cca1p-I produced in the cell could begin the process of import into mitochondria but a portion of it be released before import is complete as occurs with the enzyme fumarase. Fumarase is translated from a single ATG and all of the protein initiates import into mitochondria and is processed by the mitochondrial matrix processing enzyme but only a portion continues to be fully translocated while the majority is released to the cytosol (41). Both models for Cca1p are consistent with the observation that the amino terminus of Cca1p-I does not have the relatively high hydrophobic moment and the α-helical amphipathic structure characteristic of mitochondrial targeting signals. Indeed, Mod5p-I, another sorting isozyme with a less than optimal mitochondrial targeting signal is about evenly divided between mitochondria and the cytosol (14). Unfortunately, Mod5p-I is not processed making it difficult to determine whether all of the protein begins import but only a portion completes it. In the former scenario, cytosolic accumulation of Mod5p-I and Cca1p-I could occur if interaction with a mitochondrial import receptor were compromised. In the latter scenario, a weak interaction with mitochondrial Hsp70 might lead to retrograde movement of a portion of the protein (42). Since Cca1p-I is processed, we would be able to differentiate between these two models if we could separate the processed from the unprocessed form and if there were sufficient Cca1p-I in the cell to detect. Unfortunately, Cca1p does not fulfill either of these criteria.

Our studies have also allowed us to address the functional role of the cytosolic pool of nucleotidyltransferase. Cca1p-II and Cca1p-III provide the bulk of nuclear/cytosolic enzyme activity with the largest fraction of Cca1p-II and Cca1p-III remaining in the cytosol. Unlike nuclear and mitochondrial located Cca1p, cytosolic Cca1p does not play a role in tRNA biosynthesis. Instead, cytosolic Cca1p catalyzes the repair of 3′ end-shortened tRNA.

Yeast cells that lack cytosolic nucleotidyltransferase exhibit a growth phenotype. This phenotype is relatively mild in early log phase cells with only a 30% increase in doubling time. However, non-log phase cells are unable to properly regain log phase growth when diluted into synthetic complete minus ura medium (Fig. 6). An increase in the cytosolic pool of 3′ end-shortened tRNA accompanies this growth defect in both phases of cell growth, suggesting that this accumulation of end-shortened tRNA is responsible for the growth phenotype.

Cytosolic Cca1p is functionally analogous to E. coli nucleotidyltransferase; for the only known function of E. coli nucleotidyltransferase is in repair, since E. coli tRNAs are transcribed with CCA ends (43). In E. coli, aminoacylation or charging protects the 3′ termini of tRNA from ribonucleases (43). However, the efficiency of charging varies among tRNAs. Consequently in E. coli cells that lack nucleotidyltransferase activity, 10% of tRNAs have 90% of the end-shortened termini (43). In yeast, as in E. coli, tRNAs differ in their susceptibility to ribonuclease removal of their 3′ CCA end. In cells lacking nucleotidyltransferase activity, almost all tRNAs have lost its 3′ terminal CCA sequence; whereas much of tRNAs retains its CCA end. Although a large fraction of tRNA<sup>His</sup> also contains a CCA end, a sizeable portion of tRNA<sup>His</sup> does not (Fig. 9).

More end-shortened tRNAs accumulate in non-log phase cells than in log phase cells, at least for the tRNAs we examined (see Fig. 9, lanes 3 and 7). One explanation for the difference in accumulation of end-shortened tRNA is that more tRNA is synthesized in log phase cells allowing them to maintain a pool of mature cytosolic tRNA which serves to lessen the growth phenotype. This idea is supported by the observation that in cells that lack a nucleotidyltransferase activity, a much greater proportion of the tRNAs examined is end-shortened (Fig. 9, lanes 1 and 5). Another explanation is that, as in E. coli (43), there is an increase in ribonuclease catalyzed removal of the 3′ CCA end during non-log phase growth. Thus, it is possible that changes in de novo synthesis of tRNA and ribonuclease activity both contribute to the differences observed in the cytosolic pool of end-shortened tRNAs in log phase and non-log phase cells.

The accumulation of a pool of end-shortened uncharged tRNA causes a growth defect in yeast. One possible model to explain this is that uncharged tRNA competes with charged tRNA for the A site on the ribosome. This competition might result in ribosomal stalling and induction of a “stringent response” similar to that observed in E. coli. The stringent response is characterized by a decrease in tRNA synthesis, tRNA synthesis, rRNA transcription, protein synthesis, and an increase in amino acid biosynthesis (44). A second model is that accumulation of uncharged tRNA induces a response similar to that induced by amino acid starvation. In this case, uncharged tRNA levels leads to the induction of the GCN2 catalyzed phosphorylation of translation initiation factor, eIF-2α. Phosphorylation of eIF-2α, leads to down-regulation of protein synthesis and cell division as well as up-regulation of transcription factor GCN4 which activates expression of amino acid biosynthetic genes (45, 46).

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