Interplay of Heterogeneous Transcriptional Start Sites and Translational Selection of AUGs Dictate the Production of Mitochondrial and Cytosolic/Nuclear tRNA Nucleotidyltransferase from the Same Gene in Yeast*

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ATP (CTP):tRNA nucleotidyltransferase catalyzes the addition of the CCA end to tRNAs. In yeast, nucleotidyltransferase is encoded by the CCA1 gene and is localized to three cellular compartments: mitochondria, nucleus, and cytosol. There are three in-frame ATGs near the 5′ end of the CCA1 open reading frame. Primer extension experiments show multiple transcription initiation sites upstream of ATG1 and between ATG1 and ATG2. Fractionation of cells carrying a CCA1-COXIV fusion gene demonstrates that all three in-frame AUGs are used as sites of initiation of translation. Therefore, both transcription of CCA1 mRNA with heterogeneous 5′ ends and translation from downstream AUGs in CCA1 mRNAs play a role in the synthesis of three nucleotidyltransferase isozymes. Protein initiating from AUG1 is required for mitochondrial protein synthesis and, like many other proteins targeted to mitochondria, it is processed at the amino terminus upon import into the organelle. The shorter proteins arising from AUG2 and AUG3 provide nuclear/cytosol activity.

Sorting isozymes carry out analogous functions in more than one cellular location yet are encoded by the same gene (1). They have heterogeneous amino-terminal ends. Most commonly, selection of the AUG to be used for translation initiation depends on whether transcripts initiate upstream or downstream of the first ATG (ATG1) coded by the ORF1 (for review, see Ref. 2). Translational bypass of an AUG that is in a “poor” context for translation from downstream AUGs in CCA1 mRNAs is a common mechanism allowing the initiation of protein synthesis from more than one AUG in a single transcript (2). For sorting isozymes, the site of translation initiation determines the efficiency with which the protein products are localized to different compartments and/or exported from the cell.

Sorting isozymes shared between mitochondria and elsewhere in the cell can be grouped into four classes. The class composed of isozymes partitioned between mitochondria and cytosol includes fumarase (Fumlp), isopropylmalate synthase (Leu4p), and valyl and histidyl tRNA synthetases (Val1p and Hta1p) (3–6). Cytosolic protein is expressed from transcripts initiating between ATG1 and ATG2. Additional amino-terminal sequences necessary for mitochondrial targeting are provided by initiation at ATG1 (3–6).

Serine:pyruvate aminotransferase, encoded by the rat SPT gene, belongs to a class of sorting isozymes partitioned between mitochondria and peroxisomes. Two mRNAs are synthesized from SPT (7). The longer transcript codes for an amino-terminal extension that localizes protein to mitochondria. Peroxisomal protein is synthesized from the shorter transcript and lacks the amino-terminal extension (7).

N2,N6-dimethyladenosine-specific tRNA methyltransferase and human uracil-DNA glycosylase, products of the TRM1 and UNG genes, respectively, are partitioned between the nucleus and mitochondria (8–11) and define a third class. TRM1 transcripts, which initiate upstream of the first ATG, provide protein to the mitochondria. Transcripts that initiate between the first and second ATG provide protein to both mitochondria and nuclei (8–10, 12, 13). A well defined nuclear localization signal effectively targets Trmlp to the nucleus where it remains. The mechanism resulting in two forms of Ungp is not known; the form having a 77-residue amino-terminal extension is mitochondrial, but the shorter isozyme is nuclear (11).

Δ2-Isopentenyl-1-pyrophosphate:tRNA isopentenyltransferase and ATP(CTP):tRNA nucleotidyltransferase, products of MOD5 and CCA1 yeast genes, respectively, belong to a fourth class partitioned between the mitochondria, nucleus, and cytosol (14, 15). MOD5 transcripts include both ATGs (16), but the poor context for translational initiation surrounding AUG1 as well as short 5′-untranslated leaders allow read-through to AUG2 (17). The long form of Mod5p is enriched in the mitochondria, but, unlike Trmlp, a pool of it remains in the cytosol (1). Mod5p initiated from AUG2 is in both the nucleus and the cytoplasm (14).

ATP(CTP):tRNA nucleotidyltransferase catalyzes the addition of CMP and AMP to the 3′ end of tRNA (19). It is required because the CCA end is not coded in the tRNA genes of either nuclei or mitochondria (15, 19). In yeast, tRNA precursors in the nucleus have CCA ends (20, 21). Approximately 30% of nucleotidyltransferase activity has been shown to be in the nucleus in Xenopus oocytes (22). Nucleotidyltransferase activity is present in the cytosol of rat liver cells (23) and Xenopus oocytes (22), where it repairs tRNA that has lost part or all of its 3′-terminal CCA end. Repair of mature tRNA CCA ends, which presumably occurs in the cytosol, has also been demonstrated in yeast (24).

The CCA1 gene is an essential gene and contains multiple in-frame ATGs at the 5′ end of the ORF (25). The definitive experiment that demonstrated the importance of CCA1 to mitochondrial function altered these upstream ATGs. CCA1 mutants temperature-sensitive for growth on all carbon sources...
were transformed with wild-type and mutant genes missing either ATG1 or ATG1 and ATG2. The cells containing a CCA1 gene missing ATG1 have decreased mitochondrial nucleotidyl-transferase activity and decreased respiratory growth. Cytosolic/nuclear enzyme activity was not affected (15). Cells containing a CCA1 gene in which both ATG1 and ATG2 were altered were respiratory-deficient (15). These experiments demonstrated that translation from the upstream ATGs was important for complementation of the temperature-sensitive enzyme in mitochondria and established Cca1p as a sorting isozyme shared among nuclei, mitochondria, and the cytosol.

We have extended our studies of CCA1 to address ATG use and selection in wild-type cells. We report here that all three upstream in-frame ATGs play a role in initiation of translation of Cca1p. Cell fractionation demonstrates that Cca1p synthesized from AUG1 is enriched in mitochondria. The shorter Cca1ps from downstream AUGs provide cytosolic/nuclear activity. AUG selection in CCA1 results from a combined role of transcription initiation and translational selection. Sites of transcription initiation occur upstream of ATG1 and ATG2 so initiation at the first AUG in these CCA1 mRNAs will result in synthesis of the longest and intermediate size Cca1ps. As there are no transcriptional start sites between ATG2 and ATG3, Cca1p arising from the AUG3 is made from mRNAs containing upstream AUGs.

EXPERIMENTAL PROCEDURES

Yeast Strains—Saccharomyces cerevisiae strain MH41-7B (MATa ade2 his1 [CPB10]) (26) and strain D72S-10B (MATa) were used to obtain RNA for Northern blots. Total RNA was also isolated from yeast cells (34) that had been back-crossed with W303-lA. 

To construct the double mutant ccal-M2,M3, the oligonucleotide AG- CACGATCATCTCGAGTAG was used to alter the ATG at position 28 of the yeast shuttle vector pRS426. Strain W303-lA was also used as the recipient for CCA1-COXV fusion genes carried on vector pRS426. Mutant CCA1 genes were transformed into 352-1A (MATa ccal-1 ade2-101 his-200 ura3-52 lys2) that had been back-crossed with W303-lA.

Construction of Mutant CCA1 Genes—DNA for site-directed mutagenesis was prepared using Sequenase (U. S. Biochemical Corp.) as the DNA polymerase (31). Mutant CCA1 genes were subcloned into the multicopy shuttle vector pRS426. Poly(A) RNA was isolated from total cellular RNA with yeast shuttle vector YCp50 (32).

Primer Extension—Total RNA was isolated from yeast cells as described in the manufacturer’s instructions. The 5' end of CCA1 and CCA1-COXV fusion mRNAs were mapped as described by Triezenberg (35). The oligonucleotide CG- TGACGAACTGACAGGTGTTCC that is complementary to CCA1 mRNA from position 109 to 131 of the CCA1 ORF was labeled with γ-32P-ATP and used as a primer for extension. The primer was annealed to the labeled cDNA then extended with avian myeloblastosis virus reverse transcriptase (Life Sciences) at 50°C for 45 min. To provide a sizing ladder, the same oligonucleotide was used for sequencing CCA1 and CCA1-COXV genes.

Northern Blot Analysis—Total RNA was electrophoretically separated on a formaldehyde-agarose gel (31) and transferred to Zetaprobe (Life Sciences) at 50°C for 45 min and extended with avian myeloblastosis virus reverse transcriptase (Life Sciences) at 50°C for 45 min. To provide a sizing ladder, the same oligonucleotide was used for sequencing CCA1 and CCA1-COXV genes.

Northern Blot—Total RNA was electrophoretically separated on a formaldehyde-agarose gel (31) and transferred to Zetaprobe (Bio-Rad) according to the manufacturer’s instructions. The prehybridization and hybridization steps were carried out as described by Morales et al. (36). Probes complementary to a 1.2-kb HindIII fragment carrying URA3 and to the 1-kb EcoRV/PvuII CCA1 DNA fragment were prepared by nick translation using a nick translation kit (Promega).

Cellular Fractionation—Strain W1031A transformed with vector pRS426 carrying wild-type or mutant CCA1-COXV fusion genes was grown in 3 liters of synthetic complete medium (37) lacking uracil with galactose as the carbon source. The cells were harvested in mid log phase by washing and pelleting. The RNA was isolated as described previously (28) except that 2.5 μg of yeast lytic enzyme (ICN)/g of cell pellet was substituted for zymolase. A portion of the spheroplasts was frozen for subsequent Western blot analysis. Remaining spheroplasts were washed and suspended in 2 ml of 0.6 M mannitol, 0.5 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA and collected in a micro-centrifuge and stored frozen as pellets at -70°C.

Western Blot Analysis—Sodium dodecyl sulfate-polyacrylamide electrophoresis was as described previously (38). 100 μg of spheroplast, 100 μg of cell lysate, 100 μg of postmitochondrial supernatant, and 20 μg of mitochondrial extract as determined with the Bradford protein reagent were prepared for Western blot analysis. Mitochondria were suspended in disruption buffer and centrifuged for 15 min at 27,000 x g. The mitochondria were washed with disruption buffer followed by a 5-min centrifugation (1,000 x g) to remove debris. Mitochondria were suspended in disruption buffer and pelleted two more times by centrifugation for 15 min at 27,000 x g. Mitochondria were then suspended in 0.6 M mannitol, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA and collected in a micro-centrifuge and stored frozen as pellets at -70°C.

RESULTS

CCA1 Transcription Copy Number Is Low in S. cerevisiae—To detect CCA1 mRNA, Northern blot analysis was performed using RNA from wild-type cells and cells transformed with the CCA1 gene on a multicopy plasmid. CCA1-specific and URA3-specific radiolabeled probes were used. Two transcripts are detected in total RNA isolated from wild-type cells (Fig. 1, lane 1). A comparison of RNA from cells carrying multiple copies of CCA1 and URA3 (lanes 2) or URA3 alone (lanes 3) identifies the larger RNA as the CCA1 mRNA and the smaller RNA as the 1,000-base URA3 mRNA. The CCA1 mRNA migrates with the...
small ribosomal RNA and thus must be about 1,600 bases. In wild-type cells (lane 1), the amount of CCA1 message is low and appears comparable to the low abundance URA3 mRNA (39).

Transcripts from the CCA1 Gene Initiate Upstream of ATG1 and between ATG1 and ATG2—Primer extensions were performed to locate the 5' ends of CCA1 transcripts. Because of the low abundance of CCA1 transcripts, cells containing a multicopy plasmid carrying CCA1 were used. Total cellular RNA and poly(A) RNA gave the same result in primer extension studies. As the large number of apparent transcript ends were always seen, they are not likely a result of degradation but may arise from a secondary structure that interferes with extension. Regardless, no 5' mRNA ends fall between ATG2 and ATG3 of the CCA1 ORF, although a small proportion of the total transcripts was found to end just 3' to ATG3 (Fig. 2). Even if these result from premature termination and their real 5' ends fall between ATG2 and ATG3, their contribution to the total population of mRNA would be quite small. As translation initiation in yeast usually occurs at the first AUG in a mRNA, this result suggested that AUG3 might not be used in wild-type cells. However, we have shown previously that AUG3 can serve as a site of translation initiation in CCA1 genes without ATG1 and ATG2 (15). As described below, two separate approaches were used to examine further AUG usage in CCA1 transcripts.

Protein Sequence Analysis of the Amino-terminal End of Cca1p—As one approach to understanding AUG usage in the CCA1 gene, we isolated (28) and sequenced Cca1p (29) from whole cell extract. The sequence obtained, TNSNFVLAN, begins 1 amino acid following the methionine coded by AUG3. One interpretation is that synthesis starts at AUG3, and the initiating methionine is removed. Another is that translation begins at all of the AUGs, and physiological processing or proteolysis during purification produces a unique end. As shown below, in addition to AUG3, synthesis initiates from AUG1 and AUG2, but the resulting proteins are not processed between the methionine coded by AUG3 and the following threonine. Since only a small amount of protein (~16 pmol) was available for sequencing, we do not know whether the other termini are not detected because they are blocked or whether we did not begin with enough protein to see them.

Altering ATG Codons Differentially Alters the Ability of CCA1 to Complement Growth on Fermentable and Nonfermentable Carbon Sources—Wild-type and mutant CCA1 genes (Table I) with one or more altered methionine codons were cloned into a single copy vector. The effects of the mutations were tested by transforming them into a temperature-sensitive tRNA nucleotidyltransferase mutant. All strains grow at the permissive temperature because the chromosomal CCA1 gene provides functional Cca1p (Fig. 3). At the nonpermissive temperature, functional Cca1p must be provided by a plasmid.
Lane transformed with wild-type and mutant ATG3 or a combination thereof from the ORF as described in Table I. (panel 37C (panel gone; lane amounts of cells were spotted on glucose medium at 25 °C (panel A) and 6, YCp50 alone.

ATG3 and thus cannot directly measure the level of each protein in the wild-type gene. We have been unable to find electro-

made, type and mutant CCAl-COXN fusion genes on multicopy plasm-

genes missing ATGl same ORF, we sought to learn whether all three ATGs are used

expression genes with the

CoxIVp. Mitochondrial import of the fusion protein could also

carried from processing of a larger protein rather than from

translation initiation from AUGB ac-

CoxIV fusion protein originating from AUGl in immunoblots.

Although we know that Cca1p initiating from AUGl is re-

to AUGl is retained in the protein.

Cca1p initiating from AUGl is re-

COXW similar to the largest protein observed in cells carrying

CCA1-COXW and

although we know that Cca1p initiating from AUGl is re-

CCA1-COXN allelic (Table I) revealed two proteins in cells transcribed with CCA1-COXN (Fig. 5, lane 1) and two similar sized proteins in cells transcribed with cca1-coxIV-M1 (compare Fig. 5, lanes 1 and 4). To determine if the shortest protein arises from processing of a larger protein rather than from translation initiation at different AUGs, a gene retaining only the second ATG (cca1-coxIV-M1, M3) was constructed. Only one protein, that arising from AUG2, is observed. Its mobility is similar to the largest protein observed in cells carrying CCA1-COXIV or cca1-coxIV-M1 (Fig. 5, compare lane 3 with lanes 1 and 4). The mobility of the smallest protein corresponds to a protein originating from AUG3 in cca1-coxIV-M1, M2 extracts (compare Fig. 5, lane 3 with lanes 1 and 4). Therefore, unless changing the methionine encoded by ATG3 abolishes the putative processing event, translation initiation from AUG3 accounts for synthesis of the smallest protein produced from CCA1-COXIV and cca1-coxIV-M1.

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teins are found in cells carrying cca1-coxIV-M2, M3 (Fig. 5, lane 2). The shorter migrates slightly faster than the protein originating from AUG2 in cells carrying cca1-coxIV-M1, M3 (Fig. 5, compare lanes 2 and 3). Although there is much less of the larger protein, it exhibits the mobility expected from a protein

yeast Nucleotidyltransferase expression

table I

translational start sites of wild-type and mutant CCA1 and CCA1-COXN genes

| Gene construct | Translational start sites |
|----------------|--------------------------|
| CCA1           | +1 ATG CTA CGG TCT ACT ATA TCT CTA CTG ATG AAT AGT GCT GCT CAG |
| cca1-M1        | +1 ATG-ATG-ATG           |
| cca1-M1, M2    | ATG-ATG-ATG              |
| cca1-M1, M3    | ATG-ATG-AGA              |
| cca1-M2, M3    | ATG-CAG-AGA              |

Fig. 3. Growth characteristics of a cca-1-containing strain transformed with wild-type and mutant CA1 alleles. Equal amounts of cells were spotted on glucose medium (panel A) and glycerol medium (panel B) at 25 °C (panel C) and 37 °C (panel D). The mutant CCA1 genes were missing either ATG1, ATG2, or ATG3 or a combination thereof from the ORF as described in Table I. Lane 1, wild-type CCA1; lane 2, ATG1 gone; lane 3, ATG2 and ATG3 gone; lane 4, ATG1 and ATG3 gone; lane 5, ATG1 and ATG2 gone; lane 6, YCp50 alone.

Fig. 4. Growth characteristics of a COXIV-deficient strain transformed with wild-type and mutant CCA1-COXIV alleles. Equal amounts of cells were spotted on glucose medium (panel A) and glycerol medium (panel B) at 30 °C. The bottom row of cells is a 1:10 dilution of the top row. The mutant CCA1 genes were missing either ATG1, ATG2, or ATG3 or a combination thereof from the ORF as described in Table I. Lane 1, wild-type CCA1-COXIV; lane 2, ATG1 gone; lane 3, ATG2 and ATG3 gone; lane 4, ATG1 and ATG3 gone; lane 5, ATG1 and ATG2 gone; lane 6, YCp50 alone.

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same ORF, we sought to learn whether all three ATGs are used in the wild-type gene. We have been unable to find electro-

phoretic conditions that will separate translation products made, in vitro, from genes containing only ATG1, ATG2, or ATG3 and thus cannot directly measure the level of each protein in cellular extracts. As an alternate strategy, we have constructed CCA1-COXIV fusion genes that express Cca1-

CoxIV fusion proteins from the CCA1 promoter. The smaller size of the Cca1-CoxIV protein allows proteins translated from each AUG to be separated and detected with an antibody to CoxIVp. Mitochondrial import of the fusion protein could also be assessed in vivo by complementation of a COXIV mutant strain.

CCA1-COXIV Fusion Proteins Complement a COXIV Mutant when Expressed from a Multicopy Plasmid—CCA1-COXIV fusion genes with the CCA1 promoter were unable to complement the COXIV mutant when present on a single copy vector. This is consistent with the low level of CCA1 mRNA observed by Northern analysis. Therefore, all studies were done with wild-
type and mutant CCA1-COXIV fusion genes on multicopy plasmids (Table I). A COXIV mutant transformed with the fusion genes missing ATG1 (cca1-coxIV-M1) or ATG1 and ATG2 (cca1-
coxIV-M1, M2) each grow slower on glycerol medium than cells carrying the wild-type fusion gene (CCA1-COXIV) (Fig. 4B, compare lane 1 with lanes 2 and 5). As with the wild-type gene, the amino-terminal amino acids of the CCA1 ORF are important to the mitochondrial pool of the protein. Lack of these sequences, however, does not completely abolish the complementation. The fusion gene cca1-coxIV-M1 is able to complement the coxIV growth defect better than cca1-coxIV-M1, M3, which shows a dramatic decrease in growth on glycerol (Fig. 4B, lane 4). This comparison suggests that protein is initiating from both AUG2 and AUG3 of cca1-coxIV-M1.

CCA1-COXIV Fusion Proteins Are Produced from the First, Second, and Third ATGs of the CCA1 ORF—Western blot analysis of extracts from cells carrying either wild-type or mutant
Mitochondria-Western blot analysis was performed on cell gone. and ATG3 gone; initiating from AUG1.

More of the first three ATGs of the transformed with wild-type and mutant CCA1-COXN alleles. Procedure. Total extract from cells transformed with ccal-coxN- extract, postmitochondrial supernatant, and mitochondria have the same 5' ends—Primer extension to compare the Mrp2p, were followed to monitor the effectiveness of the process (Fig. 6). Total extract from cells transformed with ccal-coxN-lanes 6, 9, 12) from strain WD1 carrying the CCA1-COXIV fusion gene or CCA1-COXIV in which one or more of the first three ATGs of the ORF have been altered as described in Table 1. Lanes 1-3, wild-type CCA1-COXIV allele; lanes 4-6, ATG2 and ATG3 gone; lanes 7-9, ATG1 gone; lanes 10-12, ATG1 and ATG2 gone. Panel A, probed with antibody to CoxIVp; panel B, probed with antibody to Act1p; panel C, probed with antibody to Mrp2p.

Initiating from AUG1, Protein Expressed from AUG1 Is Processed and Enriched in Mitochondria—Western blot analysis was performed on cell extract, postmitochondrial supernatant, and mitochondria from a COXIV-deficient strain transformed with wild-type and mutant CCA1-COXIV alleles. Total cell extract (lanes 1, 4, 7, and 10), postmitochondrial supernatant (lanes 2, 5, 8, 11), and mitochondria (lanes 3, 6, 9, 12) from strain WD1 carrying the CCA1-COXIV fusion gene or CCA1-COXIV in which one or more of the first three ATGs of the ORF have been altered as described in Table 1. Fractionation of the known cytosolic protein Act1p, and the known mitochondrial protein Mrp2p, were followed to monitor the effectiveness of the procedure. Total extract from cells transformed with cca1-coxIV-M2.M3 has two proteins (Fig. 6, lane 4). These proteins are almost nondetectable in the postmitochondrial supernatant (Fig. 6, lane 5), but they are enriched in the mitochondrial fraction (Fig. 6, lane 6). Thus, the fusion protein initiating from AUG1 is processed as indicated by two protein forms.

A substantial amount of protein initiating from AUG2 and AUG3 remains in the postmitochondrial supernatant (Fig. 6, compare lanes 7 and 8). Neither is enriched in mitochondria (Fig. 6, lane 9). Total extract from cells carrying CCA1-COXIV contains more protein initiating from AUG3 than from AUG1 and AUG2 combined (Fig. 6, lane 1).

mRNA Transcripts from CCA1 and CCA1-COXIV Genes Have the Same 5' Ends—Primer extension to compare the 5' ends of mRNA from cells transformed with the CCA1 gene and the CCA1-COXIV genes were done. Primer extension experiments show that, as for the CCA1 gene, the 5' ends of the CCA1-COXIV transcripts map upstream of ATG1 and between ATG1 and ATG2 (Fig. 2).

Discussion

The CCA1 gene codes for a housekeeping enzyme required for the biosynthesis of all cellular tRNAs. Northern analysis of CCA1 transcripts indicates a low level of expression of this gene, and primer extension shows that not all transcripts extend to the upstream most ATG in the ORF. Experiments that change the three 5' ATGs in the longest ORF in both the wild-type and fusion gene constructs demonstrate the importance of the translation initiation site to cellular location of the enzyme. In S. cerevisiae, synthesis of isozymes from the same ORF is accomplished either because transcriptional initiation sites occur upstream and downstream of the first ATG or because the environment surrounding the first AUG in the mRNA has a primary or secondary structure unfavorable for translation initiation (for review, see Ref. 2). Both play a role in the synthesis of yeast Cca1p isozymes. Primer extension experiments (Fig. 2) show that CCA1 transcripts are heterogeneous. Transcripts initiate 5' to the first ATG and between the first and second ATGs. Immunoblot of Cca1-CoxIV fusion proteins show that protein synthesis originates from each of the three AUGs (Fig. 6). Consequently, Cca1p arising from AUG1 is synthesized from the longest messages. Protein originating from AUG2 is probably made from the shorter messages, but it is also possible that the long messages contribute to translation from AUG2. Translation from AUG3 must occur from mRNAs having upstream AUGs.

Of the 131 yeast genes analyzed by Cigan and Donahue (41), 95% adhere to the scanning mechanism, with initiation occurring at the first AUG encountered by the translational machinery (40). Comparative analysis of yeast genes indicates the presence of the consensus sequence, 5'-AYAA/UAUUUCU-3' surrounding the initiating methionine codon (41, 42). In higher eukaryotes translation initiation is influenced by nucleotide sequence surrounding the AUG codon, by secondary structure upstream or downstream of the AUG, and by the distance between the 5' end of the transcript and the AUG (for review, see Ref. 2). Although severely affected by mRNAs secondary structure (42, 43), translation initiation in yeast is considerably less sensitive than higher eukaryotes to poor sequence context and mRNA leader length (2, 41, 42, 44, 45). However, Slusher et al. (17) demonstrated that the translation initiation region surrounding AUG1 of MOD5 is very sensitive to nucleotide context. Upon altering the nucleotides surrounding AUG1 to match the yeast consensus sequence they observed a 5-10-fold increase in expression from AUG1 as well as a concomitant decrease in expression from AUG2 (17). Creation of a longer mRNA leader also increases expression of the long form of Mod5p (17).

Western blot analysis of Cca11-CoxIV fusion protein shows protein synthesis from AUG3, yet, all messages start upstream of ATG2. Judging from the yeast consensus sequence, AUG1 and AUG2 of the Cca1p ORF are very weak translation initiation sites, neither site possessing the highly conserved purine in position -3. Only the nucleotide sequence surrounding ATG3 has the highly conserved adenine at position -3. Western blot analysis also shows much more protein is synthesized from AUG3 than from AUG2. As a result of these observations we propose that leaky ribosome scanning of AUG2 and possibly AUG1 results in protein initiation at AUG3.

Mitochondrial proteins encoded in the nucleus are generally synthesized with an amino-terminal extension of 20-80 amino acids that carry information necessary for targeting protein to mitochondria. Although there is no known amino acid sequence for mitochondrial targeting, the amino termini of mitochondrial targeted proteins are enriched in basic and hydroxylated amino acids with arginine being favored over lysine (46, 47). Statistical (47) and experimental data (48, 49) show that amphiphilic secondary structure is important for mitochondrial targeting. Often, targeting signals are composed of two domains; the amino-terminal domain is usually an α-helical amphipathic structure with a relatively high hydrophobic moment, and the carboxyl-terminal domain is also amphiphilic but not necessarily α-helical (50).

The amino terminus of Cca1p does not share these characteristics. Although rich in hydroxylated amino acids, it is not particularly rich in basic residues. Protein sequence analysis...
programs (51, 52) do indicate several short α-helical structures from residues 1 to 30. However, the hydrophobic moment of 18-amino acid windows at the amino terminus is lower than for most reported mitochondrial targeting sequences (47). Like Mod5p, a functional pool of Cca1p initiating from AUG1 appears to be present in the cytosol/nucleus, as cca1Δ cells transformed with cca1-M2,M3 do grow at 37 °C, albeit very poorly, on fermentable carbon sources. Thus, not all of the long form is sequestered in mitochondria.

The targeting signal of many mitochondrial bound proteins is cleaved by matrix proteases (53). Some sorting isozymes are on fermentable carbon sources. However, we infer that Ccalp is also processed, although it is unusual in that their mitochondrial targeting signals are not getting signals cleaved by matrix proteases (53). This motif is observed (53). If, as immunoblots indicate, the cleavage site for Ccalp is just downstream of AUG2, then it may occur at the -10 arginine which is one of the few arginines at the amino terminus.

In summary, ATP (CTP):tRNA nucleotidyltransferase is a sorting isozyme that shares some features with other such proteins but that differs as well. Like Mod5p, it is found not only in two compartments but in three: the mitochondria, nucleus, and cytosol. Like other genes coding for sorting isozymes, CCA1 contains more than one in-frame ATG, but it is unusual in having three. There are multiple transcription start sites in the CCA1 gene which fall upstream of all ATGs as well as between ATG1 and ATG2 so that, in part, translation start sites are dictated by the 5′ ends of the mRNAs. However, to produce protein from AUG3, upstream AUGs in CCA1 mRNAs must be bypassed. Like other genes coding sorting isozymes where one destination is mitochondria, protein initiated from AUG1 of the ORF is targeted to mitochondria more efficiently than the other products of the CCA1 gene. Some enzyme synthesized from AUG1 remains in the cytosol/nucleus providing a small amount of activity. The cytosolic/nuclear pool of the longest form of the protein is presumably not processed as it is the majority which is imported into the organelle. Although we have a good understanding of the mechanisms that provide nucleotidyltransferase to mitochondria many questions about the partitioning of CCA1 gene products between nuclei and cytosol remain.

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