Cytoplasmic and Mitochondrial tRNA Nucleotidyltransferase Activities Are Derived from the Same Gene in the Yeast Saccharomyces cerevisiae*

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ATP (CTP):tRNA-specific tRNA nucleotidyltransferase is an enzyme required for the synthesis of functional tRNAs in eukaryotic cells. Neither the tRNA genes in the nucleus nor in organelles encode the CCA end, so it must be added post-transcriptionally. The gene that codes for the enzyme that adds the CCA end to nuclear coded tRNAs in Saccharomyces cerevisiae has been isolated (Aebi, M., Kirchner, G., Chen, J.-Y., Vijayraghavan, U., Jacobson, A., Martin, N. C., and Abelson, J. (1990) J. Biol. Chem. 265, 16216–16220). We now demonstrate that there is a mitochondrial tRNA nucleotidyltransferase activity in yeast and that it is a matrix enzyme. A comparison of purified mitochondrial enzyme with its cytoplasmic counterpart revealed no differences. These results suggest that proteins responsible for this step in the maturation of tRNAs in the nucleus and mitochondria might be identical and coded by the same nuclear gene. Accumulation of shortened mitochondrial as well as cytoplasmic tRNAs in a strain with a temperature-sensitive tRNA nucleotidyltransferase is consistent with this hypothesis. Alteration of the wild type gene such that amino-terminal truncated proteins are produced leads to a defect in mitochondrial function and a decrease in mitochondrial nucleotidyltransferase activity. This provides a direct demonstration that one gene provides this enzyme activity for the biosynthesis of tRNAs in both the nuclear/cytoplasmic and mitochondrial compartments in yeast.

Precursor RNAs made from transfer RNA genes must be processed in a series of steps to yield mature products. Nuclear and organelar precursor RNAs must be trimmed at their 5′ and 3′ ends. Base modifications as well as the post-transcriptional addition of the CCA end must also occur (reviewed in Ref. 1). In addition, some nuclear tRNA gene transcripts contain intervening sequences that must be removed. The removal of intervening sequences and the end maturation steps occur in the nucleus in Xenopus (2) as do some of the base modifications (3, 4). Precursor tRNAs in yeast that contain intervening sequences and are nuclear restricted have base modifications and CCA ends (5, 6) suggesting that these processing steps and the enzymes that carry them out are nuclear localized in yeast as well. Other base modifications occur in the cytoplasm (3, 7) as does repair of the 3′-terminal A of the CCA end (8). Organellar tRNA precursor processing occurs inside organelles, and both mitochondrial (9–13) and chloroplast (14–16) extracts containing tRNA processing activities have been described. In cases where tRNAs are coded by nuclear genes but imported into mitochondria (17–21), it is not clear whether processing occurs before, during, or after import into the organelle. Regardless of the location, it is clear that tRNA biosynthetic pathways have many similarities and require enzymes with analogous functions in multiple cellular locations. There are two mechanisms whereby enzymes that carry out analogous functions in eukaryotic cells are provided to multiple cellular locations. Many isoenzymes carry out identical biochemical reactions but are structurally distinct and arise from different genes. A smaller class of enzymes, recently named sorting isozymes (22), is made up of proteins which carry out analogous reactions in more than one cellular location but arise from the same gene. In yeast, enzymes that are shared between the mitochondria and cytoplasm and are coded by the same gene include fumarase (23), isopropylmalate synthetase (24), and valyl (25) and histidyl tRNA synthetases (26). N²,N²-Dimethylguanosine-specific tRNA methyltransferase is also a member of this class, but is unique in that it is shared by the mitochondria and the nucleus and does not appear to be present in the cytoplasm (27), whereas isopentenyl pyrophosphatetRNA isopentenyl transferase (28) appears in the nucleus, mitochondria, and cytoplasm.

As described above, ATP (CTP):tRNA-specific nucleotidyltransferase activity is required for tRNA biosynthesis in multiple cellular compartments and for repair of tRNAs in the cytoplasm. A comparison of the enzymes isolated from the mitochondria and the rest of the cell in yeast suggested to us that the proteins responsible for nucleotidyltransferase activity in these compartments might be identical. Analysis of tRNA biosynthesis in a mutant with temperature-sensitive nucleotidyltransferase activity indicated that both mitochondrial and cytoplasmic tRNAs were affected at the restrictive temperature. Site-directed mutagenesis of the previously identified and characterized CCAI gene from yeast (29) was used to make alterations in the gene that result in amino-terminal truncations in the protein produced from this gene. Strains carrying the altered genes are respiratory-deficient as judged by their growth characteristics on non-fermentable carbon sources, and they have decreased mitochondrial but not cytoplasmic nucleotidyltransferase activity. These results dem-

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1 L. Hunter, N. C. Martin, and A. K. Hopper, unpublished observation.
onstrate that the same gene codes for all of the nucleotidyltransferase activity in yeast and thus ATP (CTP):tRNA-specific nucleotidyltransferase joins the sorting isozyme class in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast and Escherichia coli Strains—**MH41-7B (Matα ade2 his1) was used for enzyme isolations and characterizations. 352-1A (Matα cca1-1 ade2-101 his3-200 ura3-52 lys2) (29) was used as a recipient for mutant CCA1 genes. E. coli strains RZ1032 and JM101 were used for mutagenesis and cloning, respectively.

**Enzyme Assays and Isolations—**Mitochondria were prepared as described previously (11) except that, in the experiment reported in Table III, the mitochondria were further purified by sucrose gradient centrifugation (30). For the experiment involving digitonin treatment, the mitochondria were suspended in 10 mM KPO₄, 0.6 M sorbitol, pH 7.2, and mixed with increasing concentrations of digitonin in the same buffer. After 5 min the sample was centrifuged in a microcentrifuge to separate a supernatant and pellet fraction, and the pellets were suspended in the same buffer containing 1% Tween 20. Assays and isolation procedures for nucleotidyltransferase were as described by Chen et al. (31). Cytochrome oxidase was assayed as described in Ref. 32, except that the difference between the absorbance at 550 and 540 nm was used to determine activity. Assays for malate dehydrogenase (33) and glucose-6-phosphate dehydrogenase activity (34) were followed by three 3-min washes in 0.8 M NaCl, 0.08 M sodium citrate at room temperature.

**Site-directed Mutagenesis and Transformations—**Site-directed mutagenesis was carried out according to the procedure of Geisselsoeder et al. (35). The oligonucleotides used to change the ATG at position +1 of the CCA1 gene (accession number 505612) to an ATC were 5′ ATCTTCTGGTTCGCAGCCAG and 5′ GCGCCTGACCTTTTGGCTTC, complementary to cytoplasmic tRNA₂G and mitochondrial tRNA₃G, respectively, were used for Northern analyses. The oligonucleotides were labeled at their 5′ ends with Tₐ and detected by Northern analysis as described by Aebi et al. (36). The oligonucleotide used to change the ATG at position +1 of the CCA1 gene (accession number 505612) to an ATC was followed by three 3-min washes in 0.8 M NaCl, 0.08 M sodium citrate at room temperature.

**RESULTS**

ATP (CTP):tRNA-specific nucleotidyltransferase is clearly required for mitochondrial tRNA biosynthesis because the CCA end is not encoded in tRNA genes. Although the activity is quite easy to assay in mitochondrial extracts, care must be taken to ascertain the activity is inside the mitochondria and not a result of cytoplasmic nucleotidyltransferase contamination of mitochondrial preparations. The results of three experiments collectively demonstrate the enzyme activity we measure in mitochondrial preparations is sequestered and that it behaves as expected for a matrix enzyme. We assayed intact and broken mitochondria for nucleotidyltransferase by increasing concentrations of digitonin parallels the release of the known matrix enzyme malate dehydrogenase (Fig. 1). Therefore, we conclude that the enzyme activity we measure is indeed inside the mitochondria and appears to be a matrix enzyme.

We have highly purified the mitochondrial enzyme (Table II) using a procedure we previously developed for the purification of the nuclear/cytoplasmic enzyme (30). We characterized the organelle enzyme with regard to its Km for ATP and CTP, its pH optimum, and its isoelectric point, and none of these characteristics differed significantly from those of the cytoplasmic enzyme (data not shown). In addition, when the two enzymes were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis they appeared identical (Fig. 2). This apparent identity, and the precedent for the same gene providing enzymes to mitochondria and the rest of the cell, led us to hypothesize that the CCA1 gene known to code for the enzyme in the nuclear/cytoplasmic compartment (29) also coded for the mitochondrial activity.

**TABLE I**

| Step | Protein | Specific activity (%) | Yield % |
|------|---------|-----------------------|---------|
| Cell extract | 4316 | 1.74 | 100 |
| DE52-Septarose | 2079 | 3.39 | 93 |
| Hydroxylapatite | 418 | 9.34 | 52 |
| Affi-Gel Blue | 5.5 | 289 | 21 |
| RNA-Septarose ND⁵ | ND⁵ | ND⁵ | 7 |

⁵ ND, not determined.

One prediction of this hypothesis is that shorter mitochondrial tRNAs should accumulate when strain 352-1A, containing a temperature-sensitive allele of the CCA1 gene, cca1-1 (29), is grown at the restrictive temperature. To determine if cca1-1 affects mitochondrial tRNA biosynthesis we carried out the following experiment. A culture of 352-1A cells was grown at the permissive temperature, then divided, and one
half was continued at the permissive temperature, while the other was shifted to the restrictive temperature. At various time intervals we removed samples, prepared tRNA, and probed for either cytoplasmic or mitochondrial tRNA using oligonucleotide probes specific for a cytoplasmic tRNA\textsuperscript{AAG} and a mitochondrial tRNA\textsuperscript{ATG}. The results presented in Fig. 3 demonstrate that shorter tRNAs do accumulate in both cellular compartments, although the amount of shorter tRNA relative to longer tRNA is greater in the cytoplasm than in the mitochondria. The result of this experiment is consistent with our hypothesis, but it could also be explained if the decrease in cytoplasmic protein synthesis caused by the lack of a CCA end on tRNA led to a decrease in the synthesis of a mitochondrial specific isoform of the enzyme.

Homology to the amino terminus of the E. coli enzyme begins at amino acid 90 of the longest open reading frame predicted by the yeast gene sequence (29). This suggested to us that some of the first 90 amino acids might not be necessary for enzyme activity. The importance of amino-terminal sequences to the import of nuclear coded mitochondrial proteins (37, 38) is well known, and we reasoned that we might remove enough of the amino-terminal amino acids to prevent localization of the protein to mitochondria without abolishing the essential nuclear/cytoplasmic function of this enzyme. The lack of nucleotidyltransferase activity in mitochondria would result in an inability of the cells to carry out mitochondrial protein synthesis so that they would be unable to grow on non-fermentable carbon sources.

We altered the CCA\textsuperscript{1} gene such that we changed the first or the first two ATGs to ATC or ATC and CAG (Fig. 4A). Plasmids carrying no CCA\textsuperscript{1} gene (YCP50), a wild type copy of the CCA\textsuperscript{1} gene (CCA\textsuperscript{1} ATG-ATG), or CCA\textsuperscript{1} genes with either the first ATG (CCA\textsuperscript{1} ATC-ATG) or the first and second ATG missing (CCA\textsuperscript{1} ATC-CAG) were transformed into the cca\textsuperscript{l-1} mutant. To determine whether these altered genes could complement the temperature-sensitive lethal phenotype, we tested their ability to grow at both temperatures demonstrating that sufficient nucleotidyltransferase activity to support life is made by use of glycerol plates. As can be seen in Fig. 4C, cells transformed with the wild type gene grow fine on glycerol at 23 °C. Growth on glycerol at 37 °C is poor for all of the transformants. Nonetheless, it is clear that there is no growth at 37 °C when the vector alone is present and cells transformed with the gene predicted to produce a protein with a 9-amino acid amino-terminal truncation (CCA\textsuperscript{1} ATC-ATG) or with a 17-amino acid amino-terminal truncation (CCA\textsuperscript{1} ATC-CAG) show impaired growth on glycerol compared with those containing the wild type gene (CCA\textsuperscript{1} ATG-ATG). The cells with the 9-amino acid truncation, however, seemed to show better growth on glycerol than the cells producing the 17-amino acid truncation protein. Thus the altered genes cannot compensate for the temperature-sensitive phenotype on glycerol medium, whereas they can do so on glucose medium. This demonstrates that the CCA\textsuperscript{1} gene product is required for mitochondrial function.

If the glycerol-negative growth phenotype of the cells transformed with mutant genes is due to a lack of import of the CCA enzyme, then the enzyme activity in mitochondria from transformants with mutant genes should be decreased. The cca\textsuperscript{l-1} strain transformed with vector alone, with the vector carrying the wild type CCA\textsuperscript{1} gene, or with the vector carrying the CCA\textsuperscript{1} gene without the first ATG or without the first two ATGs were grown for four generations at 23 °C in rich media to assure a good yield of mitochondria from respiratory competent cells. Mitochondria were isolated and the CCA enzyme measured. We have already shown that the enzyme from the temperature-sensitive mutant is much less active than wild type enzyme regardless of temperature of assay (29). This means that, in vitro, the activity measured in the transform-
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**TABLE III**

| Gene construct       | Post-mitochondrial supernatant (CCA) | Mitochondrial extract |
|----------------------|--------------------------------------|-----------------------|
|                      | units/mg                             | units/mg              |
| Plasmid alone        | 0                                    | 20                    |
| ATG-ATG-ATG          | 177                                  | 334                   |
| ATG-ATC-ATG          | 129                                  | 358                   |
| ATG-CAG-ATG          | 142                                  | 316                   |

**FIG. 4.** Growth characteristics of *cca1–1* containing strains transformed with wild type and mutant *CCAI* genes. A, sequences and predicted protein products from wild type and mutant *CCAI* genes with altered translational start site. B, growth of strains transformed with wild type and mutant *CCAI* genes on glucose medium at 23 °C (1) and 37 °C (2). C, growth of strains transformed with wild type and mutant *CCAI* genes on glycerol medium at 23 °C (1) and 37 °C (2).

We have demonstrated that yeast mitochondria contain a nucleotidyltransferase activity. Although this was the expected result since the yeast mitochondrial tRNA genes do not encode the CCA end, detection of the activity had not been reported previously. The fact that it behaves as a matrix enzyme is also expected, since mitochondrial tRNA biosynthesis occurs in this compartment and the tRNAs function there. Our comparison of the activities from mitochondria and the rest of the cell, although not extensive, did suggest that the enzyme in different compartments could be the same.

The results of the site-directed mutagenesis experiments are unambiguous; a mitochondrial phenotype can be caused by altering the open reading frame such that amino-terminal truncated proteins would be formed. Mitochondrial nucleotidyltransferase activity, but not cytoplasmic activity, is markedly affected by these changes.
Nucleotidyltransferase activity has also been detected in wheat mitochondrial extracts (12), but the relationship of this activity to that found in the rest of the cell is not known. One interesting possibility is that mitochondria, chloroplasts, and the nuclear/cytoplasmic compartments all share the same gene product. Rat liver mitochondria are reported to contain 30% of the total nucleotidyltransferase activity, but the activities in the two compartments differ in their properties (39, 40). It is possible that these differences would not persist in purified preparations and the differences observed are more apparent than real. Alternatively, maybe the unusual structures of some mammalian mitochondrial tRNAs relative to cytoplasmic tRNAs would preclude sharing of this particular enzyme between the mitochondria and the rest of the cell. At least one enzyme, fumarase, appears to be coded by a single gene but shared between the mitochondria and cytoplasm in mammals (41, 42). The MOD5 (22), FUM1 (23), LEU4 (24), VAL1 (25), HTS1 (26), and TRMI (43) genes contain two in-frame ATG codons that are both used to produce proteins differing in the presence or absence of an amino-terminal extension. The longer proteins are either exclusively (22–26) or more efficiently (45) imported into mitochondria, while the shorter form of the protein fulfills non-mitochondrial requirements for the activity. There are three in-frame ATGs in the CCA1 gene prior to sequences that are homologous to the E. coli enzyme so different sized proteins could, in theory, be produced from the CCA1 gene. Yet, all of the enzyme appears to be the same size in yeast. The fact that a phenotype is observed when the first ATG is removed demonstrates that it is used as a translational start site in the wild type gene. There are several explanations for our observation that mitochondrial and cytoplasmic activities comigrate on 10% polyacrylamide gels. The first is that translation does start at multiple ATGs, but our gels did not separate the different sized proteins that would result. The second is that all of the enzyme is produced from the first ATG, but only a portion of it is imported into mitochondria. Only a portion of the longer form of the MOD5 gene product is targeted to mitochondria and what remains in the cytoplasm is active (22). The third possibility is that there are two forms of the protein produced by the CCA1 gene, and the longer form, like most proteins imported into the mitochondrial matrix, is processed to remove amino-terminal sequences already have their CCA ends added (5, 6). Thus CCA addition precedes the removal of intervening sequences which is known to occur in the nucleus in yeast (45). Nucleotidyltransferase participates in the repair of the CCA end in yeast (8), and we presume that this occurs in the cytoplasm. Certainly the activity is present in the cytoplasm of rat liver cells (40) and Xenopus oocytes (44). Thus the CCA1 gene product must function in three cellular compartments. Our results demonstrate that amino-terminal sequences do play a role in providing the activity to mitochondria. The enzyme at 62.5 kDa is slightly larger than the 60-kDa size commonly given as the size which would preclude diffusion into nuclei (46), but even much smaller proteins contain nuclear import signals that increase their rate of import into nuclei (47). A scan of the protein sequence does not immediately identify a stretch of basic amino acids similar to other known nuclear targeting sequences (48). Future experiments will be necessary to address the nuclear import of this enzyme and to determine how a portion of it comes to remain in the cytoplasm for tRNA repair.

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