Separation and Characterization of 5'- and 3'-tRNA Processing Nucleases from Rat Liver Mitochondria*

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The 5'- and 3'-tRNA processing nucleases have been isolated from rat liver mitochondria. The two activities co-purified through heparin-agarose and phenyl-Sepharose columns and then efficiently separated on a DEAE-cellulose column. The 5' processing nuclease was found in the flow-through fraction, and the 3' processing activity eluted with 0.5 M KCl. Both enzymes were greater than 500-fold purified over the high speed supernatant of a mitoplast extract. The 159-base pre-tRNA*Tyv used as a substrate in this study was synthesized in vitro and contained the Escherichia coli suppressor III tRNA*Tyv plus a 49-base leader sequence and a 25-base trailing sequence. The 5' processing nuclease converted the pre-tRNA*Tyv into two discrete RNA species, identified as the 5'-processed intermediate and the 5' flanking fragment, by endonucleolytic cleavage at the 5' end of the mature tRNA*Tyv sequence. The 3' processing nuclease was inactive with the intact pre-tRNA*Tyv as substrate but efficiently converted the 5'-processed intermediate to the mature tRNA*Tyv, indicating an obligatory order of processing in which 5' maturation was necessary before cleavage by the 3' processing nuclease could occur. The mitochondrial enzymes exhibited optimal activity in the presence of about 2 mM Mg²⁺, but both enzymes were nearly fully active without addition of exogenous Mg²⁺ to the reaction mixtures. In contrast, a partially purified 5' processing endonuclease present in the postmitochondrial cytosolic fraction required higher [Mg²⁺] for activity, thus providing a means for differentiating between these similar enzyme activities obtained from the cytosolic and mitochondrial fractions.

The current concept of mammalian mitochondrial RNA synthesis is that transcription of both the heavy and light strands of mtDNA is initiated in the noncoding region near the displacement loop, resulting in the production of polycistrionic RNA species (for a review see Clayton, 1984). This model was strongly supported by the identification of the major heavy and light strand promoters that have been localized proximal to each other in the region near the displacement loop (Chang and Clayton, 1984; Bogenhagen et al., 1984; Chang et al., 1985, 1986). The spatial arrangement of the genes in mammalian mtDNA is extremely economical in that there are few, if any, noncoding nucleotides separating the gene sequences (Anderson et al., 1981, 1982; Bibb et al., 1981). A common feature among these genomes is that the genes for rRNAs and most of the protein-encoding genes are flanked by contiguous or nearly contiguous tRNA genes. This observation led to the prediction that most of the required nucleolytic processing of primary mitochondrial transcripts is likely to be carried out by one or several endonucleases that recognize tRNA structures and cleave precisely at the 5' and 3' ends of the tRNA sequences. Cytoplasmic 5'- and 3'-tRNA processing nucleases with these properties have been identified in eukaryotic cells (Frendewey et al., 1985; Castaño et al., 1986). Characterization of these enzymes in in vitro assays suggests that nuclear pre-tRNA transcripts are trimmed by precise 5'- and 3'-tRNA endonucleases that function in temporal sequence with 5' processing preceding cleavage at the 3' processing site.

In prokaryotes, 5' processing of tRNA precursors is carried out by the well studied RNase P (Kole and Altman, 1982), a precise endonuclease containing a protein moiety and a catalytically active RNA subunit (Guerrier-Takada et al., 1983). Maturation at the 3' end of prokaryotic pre-tRNAs is less well characterized, but both endo- and exonucleases appear to be involved (Kole and Altman, 1982).

Yeast mitochondria contain an RNase P-like enzyme composed of a nuclear-encoded protein moiety and an essential mitochondrial 9 S RNA known to be the product of the mitochondrial tRNA synthesis locus. This enzyme accurately removed the 5' leader sequence from a homologous precursor tRNA by endonucleolytic scission (Hollingsworth and Martin, 1986). The first described mammalian mitochondrial tRNA processing nuclease was an RNase P-like enzyme isolated from HeLa cells (Doersen et al., 1985). This enzyme exhibited the same specificity as Escherichia coli RNase P using the precursor to E. coli suppressor tRNA*Tyv as substrate. The HeLa cell mitochondrial enzyme was sensitive to pretreatment with micrococcal nuclease and Pronase and was differentiated from a similar activity obtained from a cytosolic fraction primarily on the basis of chromatic differences. Hereafter, mitochondrial enzymes involved in the nucleolytic maturation at the 3' end of tRNA precursors have not been described.

In this report, we describe the purification and separation of two rat liver mitochondrial enzymes that sequentially process the 5' leader and 3' trailer sequences from an in vitro synthesized precursor transcript containing the E. coli suppressor tRNA*Tyv. The optimal Mg²⁺ and KCl concentrations for the mitochondrial 5'- and 3'-tRNA processing nucleases are similar, but differ substantially from the optimal conditions required by a partially purified 5'-tRNA processing activity obtained from a postmitochondrial cytosolic fraction.

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Portions of these results were presented previously in abstract form (Manam and Van Tuyle, 1986).

**EXPERIMENTAL PROCEDURES AND RESULTS**

Partial Purification and Separation of the 5'- and 3'-tRNA Processing Nucleases from Rat Liver Mitochondria—Mitochondria were isolated from rat liver tissue by differential centrifugation and treated with digitonin to remove the outer mitochondrial membrane to reduce nuclear and cytosolic contamination of the mitochondrial enzyme preparations. The resulting inner membrane-matrix preparations (mitoplasts) were lysed with 0.5% Triton X-100 in the presence of 0.35 M KCl and clarified by centrifugation. An in vitro synthesized 159-base transcript (pre-tRNATyr) (Fig. 1) containing the mature suppressor tRNA5'. The supernatant fraction was then applied to a column of heparin-agarose, washed with 0.1 M KCl, and eluted stepwise with 0.25 and 0.5 M KCl. Assay of the 0.25 M KCl fraction produced two products of 110 and 85 bases (Fig. 2). Subsequent analyses of the 110- and 85-base products confirmed their identity as the 5'-processed intermediate and the mature tRNA\(^\text{Tyr}\), respectively (see the Miniprint). To further purify the processing nucleases, the 0.25 M KCl fraction from heparin-agarose was applied directly to a phenyl-Sepharose column. The 5'- and 3'-tRNA processing activities co-eluted at the 50% (v/v) ethylene glycol step (Fig. 3). The final step in the purification made use of a DEAE-cellulose column to which the active fractions from the phenyl-Sepharose column (50% ethylene glycol step) were applied. The material that was not retained by the DEAE-cellulose (flow-through fraction) contained an activity that converted the pre-tRNA\(^\text{Tyr}\) primarily to the mature tRNATyr. Portions of this results were presented previously in abstract form (Manam and Van Tuyle, 1986).

**Fig. 1.** Nucleotide sequence and probable secondary structure of the precursor transcript used as a substrate in this study. The transcript was produced *in vitro* by SP6 polymerase from pGEM 1 transcription vector containing the gene for the *E. coli* suppressor III tRNA\(^\text{Tyr}\) as described under "Experimental Procedures." The complete sequence has been numbered sequentially from the 5' to the 3' end, and every 10th ribonucleotide is identified by a bold dot. The arrows delineate the 5' and 3' ends of the mature tRNA\(^\text{Tyr}\).
used in the DEAE-cellulose column fractionation and the details of the assay are described under “Experimental Procedures.”}

The 5'- and 3'-tRNA processing nucleases have been isolated and separated from extracts of rat liver mitoplasts. The 5'-processing enzyme was shown to be an endonuclease in that it cleaved the pre-tRNA\textsuperscript{Pry} at the 5'-end of the mature tRNA\textsuperscript{Pry} sequence yielding the leader sequence and a 110-base 5'-processed intermediate. Endonucleolytic cleavage at the 5'-end of pre-tRNA\textsuperscript{Pry} sequences appears to be a common feature in tRNA processing in both prokaryotic (Kole and Altman, 1976; Garber and Altman, 1979; Kline et al., 1981; Akaboshi et al., 1985) and mitochondrial (Hollingsworth and Martin, 1986; Doersen et al., 1985) systems. The mitochondrial 3'-processing nuclease exhibited no detectable activity with the intact pre-tRNA\textsuperscript{Pry} transcript, but converted the 5'-processed intermediate to the mature tRNA\textsuperscript{Pry}. It appears that the 3'-processing nuclease is restricted from cleaving at the 5'-processing site of substrates containing a 5'-leader sequence due to either steric hindrance by the leader sequence or due to a requirement for the presence of the mature 5'-end that is likely to be adjacent to the 3'-cleavage site in the folded tRNA structure. This apparent requisite order in the sequence of the processing reactions in vitro is consistent with the 5'- and 3'-tRNA processing enzymes from Xenopus laevis (Carlini et al., 1985), Bombyx mori (Garber and Altman, 1979), KB cells (Zasloff et al., 1982), and Drosophila (Frendewey et al., 1985).

The major product produced by the 3'-processing nuclease was the mature tRNA\textsuperscript{Pry} possessing the E. coli gene-encoded terminal trinucleotide CCA. However, secondary products that were several nucleotides shorter on the 3'-end were also identified. This apparent staggered cleavage by the 3' nuclease may have been exacerbated by a termination CCA sequence present in the precursor transcript. None of the known mammalian mitochondrial tRNA genes encode for the terminal CCA of the acceptor stem (Clayton, 1984), thus requiring addition of these three nucleotides subsequent to 3'-end processing of the encoded mitochondrial tRNA sequence. Thus, in vivo the probable role of the 3'-processing nuclease would be to cleave the 5'-processed intermediate precisely at the 3'-end of the encoded tRNA sequence, i.e. one nucleotide beyond the mature 5'-end on the duplex acceptor stem, in preparation for addition of the CCA extension by tRNA nucleotidyltransferase (Mukerji and Deutscher, 1972). It is also logical that the 3'-processing nuclease must recognize terminally added CCAs as requisite extensions of mature tRNAs in vivo and thus leave them intact. We, therefore, suggest that the staggered cleavage observed with the 3'-processing nuclease probably reflects complications introduced by the E. coli pre-tRNA\textsuperscript{Pry} substrate and that the products obtained represent (a) a dominant cleavage at the 3'-end of the mature tRNA\textsuperscript{Pry} due to recognition of the terminal CCA, and (b) cleavage within the CCA sequence at a distance one or two nucleotides from the processed 5'-terminus closely juxtaposed on the duplex acceptor stem, in keeping with the predicted cleavage site in mitochondrial systems. An alternative explanation for the observed staggered cleavage by the 3'-processing nuclease is that this enzyme might be an exonuclease and that the staggered ends may be the result of incomplete exonucleolytic processing. We believe this is unlikely since products intermediate in size between the mature tRNA\textsuperscript{Pry} and the 110-base precursor were not observed during assay of the more highly purified preparations of the enzyme, even under conditions of high substrate-to-enzyme ratios employed during measurement of activity units. However, confirmation of an endonucleolytic mode of action for this enzyme will require demonstration of removal of an intact trailer sequence.

For direct comparison, a cytosolic 5'-processing nuclease was partially purified from the postmitochondrial supernatant of rat liver extracts. The total units of activity of this enzyme present in the cytosolic fraction were only about four times that of the comparable enzyme obtained from the mitochondrial extract. This enzyme also produced an endonucleolytic scission at the 5'-end of the mature tRNA\textsuperscript{Pry} sequence of the pre-tRNA\textsuperscript{Pry} substrate. The optimal concentrations of Mg\textsuperscript{2+} and K\textsuperscript{+} required by the cytosolic enzyme were higher than those required by either the mitochondrial 5' or 3' processing nucleases. In fact, the mitochondrial enzymes remained highly active in the absence of added Mg\textsuperscript{2+} to the reaction mixtures. Similar differences in the requirements for Mg\textsuperscript{2+} were also observed between the cytosolic and mitochondrial RNase P enzymes isolated from HeLa cells (Doersen et al., 1985).

In studies of mitochondrial enzymes that are known to have cytosolic or nuclear counterparts, care must be taken to ensure that the mitochondrial fraction is free of the corresponding
extramitochondrial components. The importance of carefully separating the subcellular fractions was emphasized by the study of the RNase P-like enzymes from HeLa cells (Doersen et al., 1985) in which there was a tremendous excess of total RNase P activity in the cytosolic fraction, as compared to that found in mitochondria. Although this concern appears to be of lesser magnitude with our rat liver system, in that the ratio of total 5’ processing nuclease activity found in the cytosol versus the mitochondria was only about 4:1, we have nonetheless taken the special precaution of removing the outer mitochondrial membrane and washing the resultant mitoplasts prior to preparation of the mitochondrial enzyme extracts. With these identical procedures, mitoplast extracts were found previously (Ledwith et al., 1982; Sekiya et al., 1980; Pepe et al., 1981) in which there was a tremendous excess of total tRNA processing nucleases with precursor transcripts containing various rat mitochondrial tRNA sequences. To facilitate the enzyme assays during our purification of the mitochondrial 5’ and 3’ processing nucleases, we chose to use the E. coli pre-tRNA 5’ as the substrate because of its high potential for forming normal tRNA secondary structure. In contrast, mammalian mitochondrial tRNA sequences exhibit many unusual features (Anderson et al., 1981, 1982; Böbb et al., 1981; Gortz and Feldmann, 1982; Roe et al., 1982; Brown and Simpson, 1982; Sekiya et al., 1980; Wolstenholme et al., 1982; Pepe et al., 1982; Grosskopf and Feldmann, 1981a, 1981b; Kobayashi et al., 1980, 1981; Koike et al., 1982; Saccone et al., 1981). For example, they are less G+C-rich than their cytosolic or prokaryotic counterparts, and many lack the “constant” bases known to be involved in the stabilization of tertiary folding. It would seem, therefore, that mitochondrial tRNAs have considerably less potential for stabilizing higher-order structures. Furthermore, the length of the TWC-loop varies among mitochondrial tRNAs; the universal sequence GTYCRAC is variable; and in the case of tRNA Ser the dihydrouridine arm is completely missing. Thus, these potential substrates for the mitochondrial 5’ and 3’ processing nucleases are highly variable and present a challenging array of recognition features. It will now be of considerable interest to analyze the breadth of specificity of our purified processing nucleases with precursor transcripts containing various rat mitochondrial tRNA sequences.

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Rat Mitochondrial 5'-\text{LTR} and 3'-\text{TRNA} Processing Nucleases

**Separation and Partial Purification of the 5'-3' and 3'-5' Processing Nucleases from Rat Liver Mitochondria**

Suzie Manne and Glenn C. Van Tuyle

**Experimental Procedure and Results**

**Materials**

- Hepatitis virus, restriction endonucleases, plasmid pH222, E. coli DNA polymerase I
- Large fragment DNA, RNA, poly(A), and RNA from research laboratories.
- RNA polymerase and [\text{32P}]ribonucleotides

**Methods**

**Separation and Partial Purification of the 5'-3' and 3'-5' Processing Nucleases** from Rat Liver Mitochondria

- Purification of the 5'-3' and 3'-5' processing nucleases from rat liver mitochondria was achieved by isoelectric focusing. To analyse contamination of the mitochondrial enzyme preparations by cytoplasmic and nuclear factors, the mitochondria were treated with digitonin to remove the non-mitochondrial membrane as previously described (Manne and Van Tuyle, 1975a).

**Results**

**DNA Synthesis**

- DNA synthesis was initiated with 5'-3' and 3'-5' processing nucleases in the presence of 5'-O-(3' 5')-diphosphate and dNTPs. The 5'-O-(3' 5')-diphosphate was used to prevent premature termination of the RNA polymerase reaction.

**RNA Synthesis**

- RNA synthesis was initiated with 5'-3' and 3'-5' processing nucleases in the presence of 5'-O-(3' 5')-diphosphate and dNTPs. The 5'-O-(3' 5')-diphosphate was used to prevent premature termination of the RNA polymerase reaction.

**Chemical Assays and Protein Determinations**

- Chemical assays and protein determinations were performed as described in a previous study (Manne and Van Tuyle, 1975a).
Partial purification of a cytosolic (extra-mitochondrial) 5' 3'-processing nuclease from rat liver. For purposes of direct comparison with the mitochondrial 5' processing nuclease, the corresponding cytosolic nuclease was partially purified from a post-mitochondrial supernatant fraction. This fraction was subjected to centrifugation at 100,000 g and the high-speed supernatant (S-100) was chromatographed on a heparin-agarose column. The retained material was eluted stepwise with 0.1M, 0.25M, and 0.5M KCl. The cytosolic 5' tRNA processing nuclease, eluting in the 0.25M KCl step, converted the pre-tRNA^{III} into the 110-base, 5'-processed intermediate (Fig. 5B). Even at this level of purity of the enzyme preparation, the 49-base, 5'-flanking fragment appeared to remain intact as well. However, no evidence of a 3' -processing activity was seen with this cytosolic fraction using either the complete pre-tRNA^{III} or the 5'-processed intermediate as substrate. At this stage of purification, the active fraction contained a total of 4.44 x 10^3 units of activity and a specific activity of 2.36 x 10^4 units/mg. The degree of purification of this cytosolic enzyme achieved by heparin-agarose chromatography was 37-fold over the S-100.

![Fig. 5. Preparative polyacrylamide-urea gel electrophoresis of the products produced by the mitochondrial and cytosolic 5' processing nucleases.](image)

![Fig. 6. T1 oligonucleotide fingerprints of the mitochondrial processing nuclease products.](image)
The RNase T1 fingerprint of the [γ-32P]GTP-labeled, 110-bare products contained the oligonucleotides expected from a 5'-processed intermediate (Fig. 6B), except for the internal 32-base species which was visible after electrophoresis of the RNase T1 digestion products on a polyacrylamide-urea gel (Fig. 8). The fingerprint of the [γ-32P]GTP-labeled, 110-base species (data not shown) revealed in addition an aberrantly migrating species (see the 5'-end fragment) that we deduced from fingerprint analysis to be the 3-nt terminal of the unprocessed pre-tRNA(THP). To identify the 5'-end residue of the 110-base intermediates, the [γ-32P]GTP-labeled species were completely digested with RNase A and T2, and the resulting mononucleotides were separated by PEI-TLC on 1M sodium formate (Fig. 9, lanes A.1.a. and C.1.a.). Under these conditions, the nucleoside 5'-diphosphate pGp was virtually the only diphosphorylated species seen. Pre-treatment of the 110-base samples with alkaline phosphatase prior to digestion to mononucleotides with RNases A and T2 and the resulting mononucleotides were separated by PEI-TLC in 1M sodium formate (Fig. 9, lane B.1.a.).

Our interpretation of the fingerprint analysis unless the 5'-end of the mature tRNA(TH') is known to migrate aberrantly under these conditions. Thus to identify the 5'-end nucleotide of the 110-base species, labeled [γ-32P]GTP (lanes 1A), and only a background level of pGp was evident using [γ-32P]GTP-labeled precursor (Fig. 9, lane B.2.a.). Therefore, using the same logic as described for the determination of the 5'-end nucleotide of the 110-base, 5'-processed intermediates, we conclude that the 5'-end of the mature tRNA(TH') was not efficient in converting to the 85-base product by the 3'-processing activity. The fingerprint of the 85-base product revealed the presence of all the expected internal fragments (Fig. 6C), but the correct 5'-end product pGp was not identifiable from fingerprints since both species were known to migrate aberrantly under these conditions. Thus to identify the 5'-end nucleotide of the mature tRNA(TH'), we deduced that the 5'-end nucleotide was pGp. Furthermore, because the fingerprints of the 85-base leader sequence contained the 8-mer CRUCBECGQ and lacked the 19G (Fig. 8A) whereas those of the 110-base intermediate contained a 19G (Fig. 5F), we deduced that cleavage must have occurred at the 5'-end of the 85-base product at position 50 (Fig. 8A). The inability to label the pGp with [γ-32P]GTP (Fig. 8, lanes A.1.a. and C.1.a.) ruled out the G residue at position 51 and confirmed that the pGp at position 50 corresponded to the 5'-end of the mature tRNA(TH'). The 5'-diphosphate pGp was efficiently converted to the 85-base product by the 3'-processing activity. To identify the 3'-end of the 85-base RNA species, the RNase T1 digestion products labeled with [γ-32P]GTP, were analyzed in a polyacrylamide-urea gel using alkaline phosphatase-treated RNA fragments as markers (Fig. 10). The longest and most prominent RNase T1 product obtained (Fig. 10, lane E) migrated exactly with the 19-base, 5'-diphosphorylated marker (Fig. 9, lane B.1.a.) indicating the dominant 3'-cleavage site corresponded to the 3'-end of the mature tRNA(TH'). The 3'-end of the mature tRNA(TH') was identified by the [γ-32P]GTP-labeled, 19-base product given Ca++, Mg++, and ATP (data not shown). Since the mature tRNA sequence was followed by a U residue in the precursor transcript, the absence of a labeled Ap in the secondary digestion products of the 19-base fragment confirmed the absence of a 3'-phosphate on the 85-base product. However, a second prominent RNase T1 fragment of about 17 nucleotides as well as a less prominent 16-mer (Fig. 10, lane E) were also seen indicating that the 3'-processing activity exhibited some degree of imprecision in the choice of cleavage sites with the substrate used in these studies.
Optimal Reaction Conditions for the Mitochondrial 5' and 3' Processing Nucleases and the Cytosolic 5' Processing Nuclease - Table III presents a summary of optimal reaction conditions for the three processing nucleases. The optimal concentrations of K+ and Mg++ required by both of the arachnoid nucleases were lower than those of the cytosolic 5' activity. These differences have provided a means for differentiating between the mitochondrial and cytosolic 5' processing nucleases. Using the optimal reaction conditions for the cytosolic activity, the arachnoid 5' nuclease was completely inactive, regardless of the stage of purification of the enzyme preparation. Based upon these results, we believe that the two 5' processing nucleases we isolated were different enzymes and that the 5' processing nuclease isolated from arachnoid was probably the arachnoid enzyme, rather than an extramitochondrial contaminant. The observed low Mg++ requirement for both of the mitochondrial nucleases is noteworthy. In fact, both of these nucleases exhibited nearly full activity in the absence of exogenous Mg++ in the assay mixtures. This observation is consistent with results obtained for a RNase P-like activity isolated from HeLa cell mitochondria (Shaw et al., 1980). However, addition of NH4EDTA to our reaction mixture completely inhibited the mitochondrial nucleases resulting in no detectable processing or degradation of the precursor transcripts. It appears, therefore, that efficient processing by these nucleases does require a divalent cation that is probably present in our incubations by virtue of its association with either the precursor transcript or the enzyme preparation.

### Table III

**Optimal Reaction Conditions**

| Conditions | Mitochondrial a | Cytosolic b |
|------------|-----------------|------------|
| pH         | 8.0             | 7.2        |
| KCl        | 10.25           | 10-11      |
| MgCl2 (mM)| 2-5             | 5-2        |

a Assayed after DEAE-cellulose chromatography

b Assayed after heparin-agarose chromatography