Processing and Editing of Overlapping tRNAs in Human Mitochondria*

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Overlapping tRNA genes in mitochondria of many metazoans introduce a problem for the processing of such polycistronic primary transcripts. Using runoff transcripts and an S100 extract from HeLa cell mitochondria, the processing of the human mitochondrial tRNA^Tyr/tRNA^Cys^ precursor (carrying an overlap of one base) was investigated: tRNA^Cys^ is released in its complete form carrying the overlapping residue at the first position, whereas tRNA^Tyr^ lacks that nucleotide at the discriminator position.

Partial deletion of tRNA^Cys^ or complete replacement by a non-tRNA-like sequence does not alter the processing reaction and indicates that the upstream tRNA^Tyr^ alone is recognized by a 3'-endonuclease activity. The truncated 3'-end of this tRNA^Tyr^ is then completed in an editing reaction that incorporates the missing residue.

The processing of this tRNA overlap seems to be species-specific, because an overlapping tRNA precursor (tRNA^Ser(AGY)/tRNA^Leu(CUN)) from opossum mitochondria is not recognized by the human extract. Because processing activities for overlapping and nonoverlapping tRNA precursors could not be separated, it seems that one general activity is responsible for the 3'-end processing of mitochondrial tRNAs and that this activity coevolved with the particular overlap between tRNA^Tyr^ and tRNA^Cys^ in human mitochondria, being unable to recognize overlaps between other tRNAs.

In the mitochondrial genomes of many animal species, some tRNA genes overlap by one to six nucleotides with downstream genes on the same strand (1–5). An example is the human gene for tRNA^Tyr^, which shares an A residue with the downstream gene for tRNA^Cys^ such that this nucleotide potentially represents not only the first base of the tRNA^Cys^ but also the discriminator nucleotide of tRNA^Tyr^. These overlaps introduce a problem for the processing of polycistronic precursors in mitochondria during which the tRNAs are released by cleavage reactions at their 5'- and 3'-ends (6): from an individual RNA molecule, only one functional tRNA can apparently be created, whereas the other one lacks the shared nucleotide(s). An alternative processing reaction, in which one of the tRNAs is correctly processed in some transcripts and the other in other transcripts, could potentially produce both complete transcripts.

To approach the problem of how the cleavage of the precursor occurs, a processing system using a human mitochondrial extract and the tRNA^Tyr^/tRNA^Cys^ precursor from human mitochondria was established. This system leads to the release of a complete downstream tRNA and a 3'-truncated upstream tRNA. Furthermore, tRNA deletion and replacement experiments indicate that the cleavage reaction represents the activity of a 3'-endonuclease recognizing solely the upstream tRNA^Tyr^ and exclude the participation of RNase P. The released tRNA^Tyr^ must then be completed posttranscriptionally by an editing mechanism. Using an in vitro transcript that corresponds to this 3'-truncated tRNA, the editing reaction could be observed in vitro.

In addition, the opossum mitochondrial tRNA^Ser(AGY)/tRNA^Leu(CUN) precursor, which also overlaps by one A residue, was not recognized by the human extract. Therefore, it seems that the described mitochondrial processing activity is highly specific in that it does not accept overlapping mitochondrial tRNA precursors found in other species.

**EXPERIMENTAL PROCEDURES**

PCR1 and Primers

Wild type and mutated templates for in vitro transcription were prepared by PCR amplification (standard conditions) of the corresponding mitochondrial genes using the following primers synthesized on an ABI DNA/RNA synthesizer. T7 (humTyr): 5'-CTAATACGACTCATACTAGTATGAAATTCGAGGCT-3'; downstream (humTyr+): 5'-TGGTAAAAAGGCGTAA-3' (*: diagnostic mutation); downstream (humTyr-1): 5'-GTGAAAAAGGAGCTAA-3'; downstream1 (humTyr/Cys): 5'-AGCCCGCCGAGGTAGTTGACG-3'; downstream2 (humTyr/Cys): 5'-TGCAATCTATATGAAAACT-3'; upstream (humCys): 5'-AAGCAGCTCTCAAAACTCTG-3'; T7 (humCys): 5'-CTAATACGACTCATACTAGTATGAAATTCGAGGCT-3'; bridge (humTyr/Cys): 5'-CACCTCGAGAGTTGTTAAAAAGGAGGCGC-3'; downstream (humTyr/Cys): 5'-TTGTAAGACTTCATGCTGTGTTAAATTTTGGTAGT-3'; insA (hum Tyr/Cys): 5'-TTGTAAGACCTCTTGCCTGGCTATGAAATTTTGAGGATTGTTA-3'; downstream (humCys): 5'-CTCAGCTTCTGCTACAGGTTGTTAAATTTTGGTAGT-3'; T7 (humSer): 5'-ACAAGCTTCTACAATGAAATTTTGAGGATTGTTA-3'; downstream (humCys): 5'-TACCAGCTTCTACAATGAAATTTTGAGGATTGTTA-3'; downstream1 (humTyr/Cys): 5'-TTGTAAGACCTCTTGCCTGGCTATGAAATTTTGAGGATTGTTA-3'; insA (hum Tyr/Cys): 5'-CTCAGCTTCTGCTACAGGTTGTTAAATTTTGGTAGT-3'; insA (hum Ser/Leu): 5'-CATCAGCTTCTACAATGAAATTTTGAGGATTGTTA-3'; pUC 18 reverse (–24): 5'-AACGCTTCTACAATGAAATTTTGAGGATTGTTA-3'; pUC sequencing (–20): 5'-GTAAAAAGGAGGCGC-3'.

In Vitro Mutagenesis

The site-directed mutagenesis was carried out using the PCR technique as described (7).

In Vitro Transcription and RNA Purification

Transcription was carried out in a volume of 30 μl according to the manufacturer (New England Biolabs).

Radioactively labeled transcripts or reaction products were purified by denaturing PAA gel electrophoresis. The bands were cut out with a sterile blade, and the RNA was eluted by incubation in 500 mM ammonium acetate, pH 5.7, 0.1 mM EDTA, 0.1% SDS at 4 °C overnight (8) and concentrated by ethanol precipitation.

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1 The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol.
Processing and Editing of Overlapping tRNA Precursors

Dephosphorylation and 5'-End Labeling of RNA

Dephosphorylation using calf intestinal alkaline phosphatase and 5'-end labeling using polynucleotide kinase and 10 μCi of [γ-32P]ATP (ICN) or [γ-32P]ATP (ICN) were carried out in volumes of 20 μl according to the manufacturer (New England Biolabs).

Preparation of Human and Yeast Mitochondrial S100 Extract

Mitochondria were prepared from frozen HeLa cells (Computer Cell Culture Center, Muns, Belgium) or cultivated yeast cells by differential centrifugation and subsequent sucrose step gradient ultracentrifugation (9, 10). For extraction, the mitochondria were incubated on ice for 5 min in 6 mM HEPES (pH 7.9), 30 mM KCl, 0.5 mM DTT, 0.2% Nonidet P-40. The lysate was centrifuged at 20,000 × g at 4 °C for 20 min, followed by a centrifugation of the supernatant at 100,000 × g at 4 °C for 1 h. The supernatant (S100) was dialyzed twice for 8 h against 2 liters of 20 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA, 20% glycerol, and Complete protease inhibitor mixture (Boehringer Mannheim; applied according to the manufacturer’s instructions). The dialyzed extract was stored at −80 °C until use.

Fractionation of the S100 extract was carried out on an Amersham Pharmacia Biotech fast protein liquid chromatography system with a high S cation exchange column (Bio-Rad) using 20 mM HEPES (pH 7.9), 1 mM DTT as buffer. A linear NaCl gradient (0–0.5 M) in 10% polyacrylamide gel containing 8M urea (11) was used in the elution procedure. The obtained fractions were desalted using Millipore Ultrafree 5000 microconcentrators; washed twice with 1 volume of 20 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA; adjusted to 40% glycerol; and stored at −80 °C.

The purity of the mitochondrial preparation was assayed by Northern blot analyses for nuclear (U6) and cytoplasmic (tRNAAsp) marker RNAs and showed no detectable hybridization signal in the mitochondrial fraction, whereas nuclear and cytoplasmic fractions showed strong signals. Additionally, a marker enzyme test for cytoplasm (lactate dehydrogenase) was carried out and revealed 5.6% specific activity in the mitochondrial fraction in comparison to the cytoplasm, indicating a purity of the mitochondrial fraction in comparison to the cytoplasm, indicating a high purity of the mitochondria.

In Vitro Assays

Processing—1 pmol of 32P-labeled tRNA precursor transcript was incubated with 0.5–2 μg of protein of the mitochondrial S100 extract from HeLa cells or with an aliquot of the individual fast protein liquid chromatography fractions in the presence of 30 mM HEPES-KOH (pH 7.6), 6 mM MgCl2, 30 mM KCl, 2 mM DTT, and 30 units of RNase inhibitor (Boehringer Mannheim) in a total volume of 20 μl for 20 min at 25 °C.

Editing—5–10 pmol of 32P-labeled tRNA5′-1 were incubated with 0.5 μg of protein of the mitochondrial S100 extract from HeLa or yeast cells in the presence of 1 mM NTPs (Amersham Pharmacia Biotech), 30 mM HEPES-KOH (pH 7.6), 6 mM MgCl2, 30 mM KCl, 2 mM DTT, and 30 units of RNase inhibitor (Boehringer Mannheim) in a total volume of 20 μl for 6 h at 30 °C.

The reactions were terminated by phenol-chloroform extraction. The products were ethanol-precipitated, separated by electrophoresis on 10% polyacrylamide gels, and visualized by autoradiography (Fig. 1D). The processing product appears as a single band with an apparent size of 65 nucleotides, corresponding to the length of a truncated tRNA77. This result excludes an alternative processing, which would lead to two products differing by one nucleotide in length.

In control experiments, 2% SDS was added to the assay, and the S100 extract was pretreated with 20 μg of proteinase K for 30 min at 37 °C, or heated to 94 °C for 5 min prior to the incubation with the RNA substrate. Each individual treatment led to a complete inactivation of the extract, indicating a proteinaceous activity (data not shown).

Processing of a Heterologous Overlapping Bicistronic tRNA Precursor—In order to test whether the human mitochondrial processing machinery is able to recognize other (heterologous) overlapping tRNA molecules in a precursor transcript, a 5′-end-labeled in vitro transcript of the opossum mitochondrial tRNA genes for serine and leucine was offered. Similar to the human tRNA5′/tRNA5′−precursor, these tRNAs overlap by one residue in the primary transcript (Fig. 1E). The result of the incubation of this transcript in the presence of the human mitochondrial S100 protein extract is shown in Fig. 1E: besides background degradation of the substrate RNA, no reaction product is observed (lane 1). One possible explanation for this is that a precursor containing tRNAs for serine and leucine is generally not processed by the mitochondrial S100 extract. To address this question, the experiment was repeated with the human mitochondrial tRNA5′/tRNA5′−(AGY)/tRNA5′−(CUN) precursor. This transcript is highly similar to the opossum RNA in primary and secondary structure (Fig. 1C) but contains no overlapping region between the two tRNAs (both tRNAs have their own A residue at their 3′- and 5′-ends, respectively). A 5′-end-labeled version of this precursor was again tested as a substrate for the human S100 protein extract. Although the processing extract does not recognize the wild type opossum overlapping tRNA5′/tRNA5′−precursor (lane 1), it is able to cleave the human nonoverlapping transcript and leads to a release of the labeled tRNA5′ (Fig. 1E, lane 2). Therefore, the extract is able to process tRNA5′/tRNA5′−precursors.

Furthermore, an opossum transcript carrying an insertion of one additional A residue at the border of the tRNA5′ and tRNA5′−sequences (which removes the overlap, Fig. 1B, (2)) was constructed. Interestingly, this transcript carrying the A insertion, and therefore mimicking the situation in the human
transcript, is cleaved to release the tRNA\textsubscript{Ser} (Fig. 1E, lane 2). The different migration positions of the released tRNA\textsubscript{Ser} versions in lanes 2 and 3 represent the length difference between the opossum tRNA\textsubscript{Ser} (58 bases, lane 2) and the human tRNA\textsubscript{Ser} (59 bases, lane 3). Whether these cleavage reactions represent a recognition of the downstream located tRNA\textsubscript{Leu} and therefore an RNase P activity or a recognition of the tRNA\textsubscript{Ser} (upstream) by a 3'-9'-endonuclease is not clear at this point (both reactions would lead to the same processing products).

To analyze whether both the overlapping tRNA\textsubscript{Ty}r/tRNA\textsubscript{Cys} precursor and the nonoverlapping tRNA\textsubscript{Ser}/tRNA\textsubscript{Leu} transcripts are cleaved by the same activity, the S100 protein extract was fractionated on a cation exchange chromatography using a High S column and a linear NaCl gradient ranging from 0 to 0.80 M. The resulting 20 fractions were tested for the processing activity using the human overlapping tRNA\textsubscript{Ty}r/tRNA\textsubscript{Cys} transcript and the human nonoverlapping tRNA\textsubscript{Ser}/tRNA\textsubscript{Leu} precursor (Fig. 2). Fractions eluting between 0.24 and 0.60 M NaCl were able to process both overlapping and nonoverlapping precursor transcripts. Furthermore, the fraction eluting at 0.48 M NaCl (showing the highest processing activity) was used for competition studies with the overlapping (tRNA\textsubscript{Ty}r/tRNA\textsubscript{Cys}) and nonoverlapping (tRNA\textsubscript{Ser}/tRNA\textsubscript{Leu}) precursors. Both transcripts showed a similar efficiency in competing with a radioactively labeled overlapping tRNA\textsubscript{Ty}r/tRNA\textsubscript{Cys} precursor (data not shown).

Is RNase P the Responsible Nuclease?—The cleavage between the two tRNA structures in the precursor molecule can in principle be achieved either by recognition of the upstream tRNA\textsubscript{Ty}r, the downstream tRNA\textsubscript{Cys} sequence (RNase P), or both tRNA structures. To be cleaved by RNase P, a tRNA precursor needs to have at least a part of the T loop and the 3'-9'-half of the acceptor stem that serves as an external guide sequence (16, 17), as has been shown for \textit{E. coli}, human, and \textit{Xenopus laevis} RNase P (16–20).

In order to test whether in our transcript the downstream tRNA\textsubscript{Cys} determines the cleavage (representing a 5'-cut by RNase P), an \textit{in vitro} transcript was designed that contained a complete tRNA\textsubscript{Ty}r and a 3'-truncated form of the tRNA\textsubscript{Cys} (consisting of 31 bases and terminating immediately 3' of the anticodon). This transcript was incubated as a 5'-end-labeled molecule in the presence of the S100 protein extract (Fig. 3A). As a control, the complete precursor tRNA\textsubscript{Ty}r/tRNA\textsubscript{Cys} was also tested. Interestingly, both incubations led to products migrating at identical positions in the polyacrylamide gel, indicating identical processing reactions. Therefore, the enzymatic activity probably recognizes the upstream tRNA and possibly ele-
ments in the 5'-half of the downstream tRNA. To distinguish between these two possibilities, the truncated tRNA<sup>Tyr</sup> was completely replaced by the first 30 bases of the mitochondrial mRNA for coxII. This sequence has no similarities to a tRNA structure and carries presumably no recognition elements for a tRNA processing enzyme. The construct was tested as a 5'-end-labeled transcript in an overlapping (the A residue of the AUG start codon in coxII represented also the discriminator base of the tRNATyr) as well as in a nonoverlapping situation (Fig. 3B). The released tRNA molecules migrate at a position identical to that of the processing product of the tRNATyr/tRNACys precursor. These results indicate that the recognition elements for the processing activity are restricted to the upstream tRNATyr and that the downstream sequences do not influence the processing reaction.

**Determination of the Cleavage Position**—In order to get information about the cleavage position on nucleotide level in the human tRNATyr/tRNACys precursor, an RNA molecule carrying a single 32P-labeled 5'-phosphate group at the overlapping nucleotide position was constructed using a bridging technique (13) and verified by reverse transcription PCR and sequencing (data not shown).

The processing reaction was performed using this precursor transcript, and the resulting products were separated on a denaturing 10% polyacrylamide gel (Fig. 4). Because both tRNATyr and tRNACys have lengths of 66 nucleotides (including the overlapping nucleotide), it was not possible to distinguish between the two molecules simply by their migration position. Therefore, the product was isolated and subjected to a nearest neighbor analysis by RNase T2 digestion (14) (Fig. 5). This
enzyme leads to the release of nucleotide 3′-monophosphates (Fig. A, Np) and thus allows the 5′-neighboring base of the labeled nucleotide to be identified. Fig. A shows the products expected for the possible processing products. If the tRNA\textsuperscript{Tyr} is released as a complete molecule, it should carry the overlapping labeled base at its 3′-end. The T2 digest should therefore lead to a labeled cytosine 3′-monophosphate (an identical result is expected for the digestion of the precursor RNA), whereas a complete tRNA\textsuperscript{Cys} should carry the overlapping base at its 5′-end and lead to a 5′-labeled adenine 3′,5′-diphosphate (as would the RNase T2 digestion of the 5′-labeled tRNA\textsuperscript{Cys} used in the bridging experiment). The resulting mononucleotides were separated by thin layer chromatography, and the labeled bases were identified by autoradiography. Fig. B shows that the labeled nucleotide migrates to a position identical to the digestion product of the 5′-labeled tRNA\textsuperscript{Cys}. It therefore indicates that the band in Fig. 4 corresponds to a complete tRNA\textsuperscript{Cys} and that the upstream located tRNA\textsuperscript{Tyr} is released in a truncated form lacking the discriminator base.

In order to further analyze the 5′-end of the processed tRNA\textsuperscript{Cys} obtained in the \textit{in vitro} reaction as well as isolated from HeLa cell mitochondria, the 5′-end (carrying a phosphate group) and the 3′-end (carrying a 3′-OH group) of the tRNA were ligated by T4 RNA ligase, resulting in a circularized tRNA molecule (2). The acceptor stem of the tRNA was reverse transcribed, amplified by PCR, and cloned, and individual clones (representing the 5′- and 3′-termini of individual tRNA molecules) were analyzed by chain termination sequencing. All sequences showed that the tRNA\textsuperscript{Cys} carries an A residue at the first position, indicating that this tRNA is found as a complete molecule not only under the described \textit{in vitro} conditions (Fig. 6) but also in \textit{vivo} (data not shown).

The Truncated tRNA\textsuperscript{Tyr} Is Completed by an Editing Reaction—Because the released tRNA\textsuperscript{Tyr} is truncated at its 3′-end, the acceptor stem has to be completed in order to render the tRNA molecule functional. \textit{In vitro} analysis revealed that this is indeed the case (data not shown), as shown also for overlapping tRNA transcripts in other organisms (2–5): an A residue is incorporated at the discriminator position, and the CCA terminus is added. To investigate whether this incorporation activity is also present in the mitochondrial S100 protein extract, an \textit{in vitro} transcription corresponding to a tRNA\textsuperscript{Tyr} lacking the discriminator base (Fig. 7A; the 3′-end was verified by ligation of the transcript to DNA, cloning, and sequencing according to Ref. 12; data not shown) was incubated as a 32P-5′-end-labeled version with the extract in the absence or presence of NTPs in
FIG. 7. Base incorporation and completion of the 3′-end of the truncated tRNA^Tyr^-. A, ^32^P-5′-end-labeled *in vitro* transcript representing tRNA^Tyr^-1 (lacking the discriminator base) (A) was incubated in the presence of mitochondrial S100 extract and NTPs (B). The nucleotide marked by the asterisk in A represents an introduced diagnostic mutation that allows us to distinguish the substrate tRNA from the endogenous tRNA^Tyr^ found in the extract. The mutation did not affect the processing or base incorporation reactions (data not shown). B, although the tRNA is partially degraded in the presence of the extract, an incorporation of nucleotides can be observed that completes not only the tRNA^Tyr^-1+A, but also the 3′-truncated version (tRNA^Tyr^-1) to a full-length molecule carrying a discriminator base and the CCA end. The control experiment using the complete tRNA^Tyr^ (tRNA(Tyr)+A, ending with the discriminator position) shows a high terminal nucleotidyl transferase activity in the protein extract. C, comparison of nucleotide incorporation by the human and the yeast mitochondrial S100 extracts. Whereas the yeast extract adds only three bases (corresponding to the CCA terminus) to the 3′-end of the tRNA^Tyr^-1 (lacking the discriminator base) (A), the human S100 incorporates an additional nucleotide. This indicates that only the human extract has the ability to incorporate a base at the discriminator position in addition to the CCA terminus.

equimolar amounts (Fig. 7B). This truncated tRNA^Tyr^-1 carried a diagnostic mutation at position 52 (G52A), indicated in Fig. 7A by the asterisk (this mutation did not interfere with the analyzed reactions and allowed us to distinguish between substrate and endogenous tRNA^Tyr^ found in the protein extract (data not shown)).

Fig. 7B shows that in the presence of NTPs, the 3′-end is elongated by incorporation of four bases, leading to a full-length product migrating at a position identical to that of the complete tRNA^Tyr^ (tRNA(Tyr)+A) carrying a CCA end. In order to determine the nature of the incorporated nucleotides, the shifted bands of tRNA^Tyr^-1 were isolated, ligated to double-stranded DNA (12), amplified by reverse transcription PCR, and cloned, and individual clones were sequenced. This analysis revealed that CTP (7 of 14 clones, 50%) and ATP (4 of 14 clones, 29%) are readily accepted by the extract and incorporated at the discriminator position, whereas UTP (2 of 14 clones, 14%) and GTP (1 of 14 clones, 7%) are incorporated at a lower efficiency. An explanation for the misincorporation of a C residue at the discriminator position might be the high activity of the terminal nucleotidyl transferase in the extract, which adds the CCA terminus to the 3′-ends of tRNAs: in a control assay, a transcript representing the complete tRNA^Tyr^ (carrying the discriminator base at the 3′-end) was used (tRNA(Tyr)+A). The observed base incorporation represents the addition of the CCA terminus and indicates that the terminal nucleotidyl transferase is highly active in the S100 extract.

In contrast to the addition of four bases by the human S100 extract, a yeast mitochondrial S100 protein extract incorporated only three residues at the 3′-end of the truncated tRNA^Tyr^ (as it did with the complete tRNA^Tyr^-1+A), corresponding probably to the CCA end (Fig. 7C). This indicates that the yeast extract is not able to incorporate the discriminator base in addition to the CCA terminus, a result that correlates with the fact that in yeast mitochondria, overlapping tRNA genes were not identified.

**DISCUSSION**

In animal mitochondria, many tRNA genes are transcribed as multigristronic precursors consisting of individual tRNA molecules arranged in tandem with no or only a few bases in between (6). In some of these primary transcripts, one tRNA not only abuts the next tRNA or protein coding sequence but overlaps by one to several bases (1, 2). While nonoverlapping tRNA precursors are expected to be processed either by 5′- or 3′-cleavage (or both where spacers exist) (9, 21), overlapping precursors introduce a problem. In principle, the release of complete versions of two overlapping tRNA molecules can be achieved in two ways. One possibility is an alternative processing reaction that leads to the formation of only one functional tRNA per primary transcript. Alternatively, it is conceivable that the processing takes place at one position in all transcripts, leading to the release of only one complete tRNA, whereas the other one is truncated. In the latter case, it is not clear how the primary transcript is recognized by the processing enzyme(s). It is imaginable that only one (either the upstream or the downstream) tRNA sequence in the precursor molecule serves as a recognition element. Another possibility is that both tRNAs are needed for a proper processing.

The established *in vitro* system described in this work served to investigate the processing reaction in more detail. In the analyzed reactions, the release of a truncated upstream and a complete downstream tRNA was detected. Because these findings are compatible with the *in vivo* results (data not shown), the *in vitro* system seems to contain the enzymatic activity that is responsible for the processing reaction. The fact that an overlapping precursor consisting of tRNA^Tyr^ and only 31 bases of the downstream overlapping tRNA^Cys^ is processed in the same way as the full construct (two complete tRNAs) indicates that the cleavage reaction is catalyzed by an enzyme that recognizes primarily the upstream tRNA. Furthermore, replacing the complete downstream tRNA^Cys^ by a non-tRNA-like sequence (the 5′-part of coxII mRNA) did not interfere with the processing reaction and did not lead to a shift of the cleavage position, demonstrating that the upstream tRNA, exclusively, is recognized by the processing activity.

A 3′-truncated version of the tRNA^Tyr^/tRNA^Cys^ precursor (carrying 36 nucleotides of the tRNA^Cys^) very similar to the one used in Fig. 3A was tested in an *in vitro* system by Rossmanith *et al.* (9). In their system, the tRNA^Tyr^ seemed to be liberated as a complete tRNA carrying the overlapping A residue at the
3'-end. A reason for this discrepancy might be the different methods of analysis of the tRNA 3'-ends. Whereas in the described experiments, the analysis was carried out by direct characterization of the 3'-terminal base, Rossmanith et al. (9) compared the migration position of the released 3'-part of the tRNA<sup>CYS</sup><sub>1</sub> to a partially hydrolyzed RNA (alkaline RNA ladder) and to a tRNA precursor cleaved by nuclease T1. Because alkaline hydrolysis and T1 cleavage both produce 2',3'-cyclic phosphate and 5'-hydroxyl groups, the migration properties of the resulting RNA fragments differ from those of the processing products, which have 5'-phosphate and 3'-hydroxyl groups. Furthermore, interpretation of migration properties, particularly of short RNA molecules, can be difficult because partial base pairing, as well as the base composition, may influence the electrophoretic mobility. Another explanation for the different results might be that the construct used by Rossmanith et al. (9) carried five additional bases at the 3'-end and that the incubation conditions were slightly different.

The in vivo and in vitro analyses presented show that the downstream tRNA is released as a full version carrying the overlapping part at its 5'-end (2, 3, 5), whereas all transcripts of the upstream tRNA analyzed to date are either missing the overlap (a likely intermediate) or have been completed post-transcriptionally by an enzymatic activity that is not yet characterized. In the described in vitro system, additionally to the CCA end, a single A residue is incorporated at the truncated 3'-end of tRNA<sup>Tyr</sup>, a reaction that delivers the discriminator base to the tRNA molecule. The efficiency of this fill-in editing mechanism is rather low, which corresponds to the in vivo situation (only 44% of the isolated and analyzed tRNA<sup>Tyr</sup> carried a completed 3'-terminus, whereas 56% were truncated; data not shown). Whereas in vivo, only A is found at the discriminator position of the tRNA<sup>Tyr</sup>, in the in vitro system, not only ATP but also CTP is readily incorporated. Because the used S100 extracts contain a highly active terminal nucleotidyl transferase, as shown in Fig. 7, B and C, it is conceivable that this activity in the human extract also acts on a tRNA substrate lacking the discriminator position at the 3'-terminus (tRNA<sup>Tyr</sup>-1), a reaction that was observed using the yeast mitochondrial extract (Fig. 7C): the gel shows that the human mitochondrial extract incorporates four nucleotides, whereas the yeast extract adds only three bases to the 3'-end of the tRNA<sup>Tyr</sup>-1 and is therefore obviously not able to incorporate an additional base besides the CCA end. An explanation for this difference might be the fact that the yeast mitochondrial genome does not contain overlapping tRNA genes. Therefore, a discriminator nucleotide incorporating activity is not needed.

The apparent gap in the bands shifted by the human extract indicates that the base incorporation at the discriminator position is the rate-limiting step in the completion reaction of the 3'-end of the tRNA. Because GTP and UTP are also incorporated at this position (although at a low efficiency), it is also possible that the fill in activity has a high error rate in the in vitro system. However, a misincorporation of CTP (or G and U residues) at the discriminator position was not observed in vivo, which might be due to either a higher fidelity of the system in the whole cell or to a excision reaction that removes the misincorporated C residue. A scenario is possible in which tRNA<sup>Tyr</sup> molecules possessing an A residue as a discriminator followed by the CCA terminus become protected against exonucleolytic activity by aminoacylation, whereas those variants carrying a misincorporated base at this position are not aminoacylated and undergo the excision reaction to get a new chance for the addition of the correct discriminator nucleotide.

A similar but probably unrelated base incorporation system has been described for mitochondrial tRNAs in the slime mold <i>Acanthamoeba castellanii</i>, in which mismatches in the acceptor stem of tRNA molecules are corrected by nucleotide conversions at the 5'-end (22). Whereas in the latter case, the 3'-part of the acceptor stem could function as a template, in the system described in this work, an unpaired nucleotide has to be incorporated at the 3'-end. Therefore, the activity cannot be guided by a simple template intrinsic to the tRNA. A possible candidate for such a base-incorporating activity is the poly(A) polymerase, which in a template-independent way adds adenosine residues to the 3'-end of mitochondrial RNAs (6). In chicken mitochondria, the tRNA<sup>Tyr</sup> (which overlaps by one nucleotide with the sequence for tRNA<sup>CYS</sup>) was found to carry up to 13 extra A residues at the 3'-end as a possible reaction intermediate. On the basis of these observations, it has been suggested that the poly(A) polymerase might be involved in the addition of the poly(A) tail to this tRNA (4), although the addition of more than one A residue was never observed in the described in vitro system. A recent publication describes another enzyme that is possibly involved (23): in the nucleus and cytoplasm of human cells, several small RNAs, including U2, U6, 7SL, and 5S, carry a single not encoded A residue at the 3'-end. Sinha et al. (23) developed an in vitro system that is capable of adenylation of some of the transcripts. Whereas the function of this adenylation is not known, the fill-in reaction described here obviously restores the functionality of the truncated mitochondrial tRNA. A further indication for the involvement of one of these enzymes might be the fact that only tRNAs carrying adenosine as a discriminator base seem to overlap in their sequence with downstream genes (4).

A third candidate for a template independent base incorporating activity is the terminal nucleotidyl transferase that adds the CCA end to the 3'-end of tRNAs. It is known that this enzyme is able to repair partially degraded CCA ends at tRNA molecules (24). Because the acceptor stem of the released human tRNA<sup>Tyr</sup> carries two C residues (CC) at the 3'-end, it is conceivable that this structure mimics an incomplete CCA end, which could be recognized and repaired by the terminal nucleotidyl transferase. Because the tRNA would then carry a discriminator base, it would represent a conventional substrate for addition of an unpaired CCA terminus by the CCA-adding enzyme. Therefore, this scenario would imply the repair of one and the addition of another CCA end to an individual tRNA molecule. Whether this corresponds to the actual situation remains to be clarified. However, because only the tRNA<sup>Tyr</sup> carries this additional CC(A) sequence at the 3' terminus, whereas other overlapping tRNAs like tRNA<sup>Ser</sup> have a different base composition, this scenario would represent a special case.

In opossum mitochondria, the genes for the tRNA<sup>Ser</sup>(AGY) and tRNA<sup>Leu</sup>CUN(CUN) overlap by one adenosine residue (25). Because a general processing machinery is believed to recognize all of the individual mitochondrial tRNA precursors, one would expect that a system that recognizes two overlapping tRNAs in a homologous assay (human mt proteins and human mt tRNA precursor) would also be able to handle a foreign tRNA precursor consisting of two different tRNAs having a similar overlapping nucleotide. However, the nuclease responsible for processing the human tRNA<sup>Tyr</sup>/tRNA<sup>CYS</sup> does not recognize the opossum transcript, although nonoverlapping tRNA<sup>Ser</sup>/tRNA<sup>Leu</sup> precursors (artificial and natural) are recognized and processed. Because both overlapping and nonoverlapping RNA substrates are cleaved by the same protein fractions obtained from the cation exchange chromatography and showed no different efficiencies in competition experiments, it seems that one individual activity exists in human mitochondria that is able to process conventional tRNA precursors lack-
ing overlapping bases and seems to have coevolved with the overlapping situation found in the tRNA\textsubscript{Tyr}/tRNA\textsubscript{Cys} precursor; this activity is therefore specific for this individual overlap. A necessity for such a coevolution might be that the two overlapping tRNA acceptor stems in the precursor molecule acquire a three-dimensional structure that hinders RNase P in getting access to the cleavage position and renders such a precursor dependent on a 3'−endonuclease. Furthermore, a coevolution of this activity might explain why the overlapping opossum mt tRNA\textsubscript{Dor}tRNA\textsubscript{Leu} precursor is not cleaved by the human mt tRNA\textsubscript{Tyr} in vitro. 3 Although the human mt tRNA\textsubscript{Tyr} has no abnormal number of base pairs in these stems, it is possible that individual bases or base pairs can influence the cleavage site. It remains to be clarified which features of tRNA\textsubscript{Tyr} are responsible for guiding the endonuclease to the observed cleavage position.

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