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Mitochondrial poly(A) polymerase is involved in tRNA repair

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ABSTRACT

Transcription of the mitochondrial genome results in polycistronic precursors, which are processed mainly by the release of tRNAs interspersed between rRNAs and mRNAs. In many metazoan mitochondrial genomes some tRNA genes overlap with downstream genes; in the case of human mitochondria the genes for $tRNA^{Tyr}$ and $tRNA^{Cys}$ overlap by one nucleotide. It has previously been shown that processing of the common precursor releases an incomplete $tRNA^{Tyr}$ lacking the 3′-adenosine. The 3′-terminal adenosine has to be added before addition of the CCA end and subsequent aminoacylation. We show that the mitochondrial poly(A) polymerase (mtPAP) is responsible for this A addition. *In vitro*, a $tRNA^{Tyr}$ lacking the discriminator is a substrate for mtPAP. *In vivo*, an altered mtPAP protein level affected $tRNA^{Tyr}$ maturation, as shown by sequencing the 3′ ends of mitochondrial tRNAs. Complete repair could be reconstituted *in vitro* with three enzymes: mtPAP frequently added more than one A to the 3′ end of the truncated tRNA, and either the mitochondrial deadenylase PDE12 or the endonuclease RNase Z trimmed the oligo(A) tail to a single A before CCA addition. An enzyme machinery that evolved primarily for other purposes thus allows to tolerate the frequent evolutionary occurrence of gene overlaps.

INTRODUCTION

Human mitochondria have their own 16.6 kb circular genome (mtDNA) encoding 13 proteins involved in oxidative phosphorylation, as well as 2 rRNAs and 22 tRNAs, which are needed for mitochondrial translation (1,2). The transcription of both mtDNA strands results in polycistronic RNA molecules in which rRNAs and mRNAs are flanked by tRNAs (3–6). Two endonucleases cleave the tRNAs at their 5′ (RNase P) and 3′ ends (RNase Z) and, by doing so, release the other RNA species (7–10). Mitochondrial mRNAs are then substrates for polyadenylation by the mitochondrial poly(A) polymerase (mtPAP, PAPD1) (11,12), while tRNAs undergo CCA addition by the CCA-adding enzyme and subsequently aminoacylation. The enzymes responsible for the different processing steps are all imported into mitochondria, and RNase Z and the CCA-adding enzyme originate from the same gene as their isoform responsible for the processing of nuclear-encoded tRNAs (8,12–15).

Interestingly, in many metazoan mitochondrial genomes, the genes of some tRNAs overlap on the same DNA strand (16–19). This overlap introduces a problem, since only one of the tRNAs can be processed from the common precursor in a complete form. In the case of human mitochondria the genes for $tRNA^{Tyr}$ and $tRNA^{Cys}$ share one nucleotide (1). In principle, the overlapping residue could serve either as the 5′-terminal nucleotide of $tRNA^{Cys}$ or as the 3′-terminal nucleotide of $tRNA^{Tyr}$. It was reported that processing of the precursor releases an incomplete $tRNA^{Tyr}$ molecule lacking the overlapping 3′-terminal nucleotide, but a complete $tRNA^{Cys}$ (20); the enzyme(s) responsible for this non-canonical pathway were not identified. In another study, 3′-end processing of $tRNA^{Tyr}$ was demonstrated to occur at the canonical site, after the overlapping nucleotide (21); this 3′ cleavage was catalyzed by mitochondrial RNase Z and the processing of a dual $tRNA^{Tyr}$-$tRNA^{Cys}$ precursor by mitochondrial extracts or recombinant mitochondrial RNase P and RNase Z resulted in complete and incomplete forms of both tRNAs by alternative canonical processing at either the 3′ end of $tRNA^{Tyr}$ (by RNase Z) or the 5′ end of $tRNA^{Cys}$ (by RNase P), in about equal proportions *in vitro* (Jahn and Rossmanith, unpublished). In both scenarios, a $tRNA^{Tyr}$ lacking the 3′-terminal nucleotide is released, either as a sole product, or in addition to the complete tRNA. The 3′ nucleotide, an adenosine in the case of $tRNA^{Tyr}$, is called discriminator due to its importance for correct aminoacylation of the tRNA (22) and has to be added before the tRNA can finally be matured by the addition of the CCA end and subsequent aminoacylation. In human mitochondrial extracts a discriminator-adding activity was described (20,23), but the enzyme(s) responsible for this repair reaction have not been identified so far.

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Here we show that mtPAP is able to add the discriminator A to $tRNA^{Tyr}$. Because A addition by mtPAP is not limited to a single nucleotide, either the 3′ exonuclease phosphodiesterase 12 (PDE12) or RNase Z are required to trim the oligo(A) tail to a single nucleotide before CCA addition completes the processing of $tRNA^{Tyr}$.

MATERIALS AND METHODS

Cell culture, siRNA treatment

HEK293A cells were grown in DMEM GlutaMax (Invitrogen) supplemented with 10% FBS at 37°C with 5% CO_2 and 90% humidity. For the RNAi experiments, cells were transfected with siRNAs (Supplementary Data, Supplementary Table S1; Eurofins MWG Operon; final concentration 100 nM) by Lipofectamin RNAiMAX (Invitrogen) according to the manufacturer's instructions. Analyses were carried out 72 h after transfection. In all experiments, knock-down efficiencies were checked by qRT-PCR.

RNA analysis

Total RNA was extracted by the TRIZOL method. For qRT-PCR analysis, 2 μg of the total RNA, treated with RNase free DNase I (Roche), were reverse-transcribed with a $(dT)_{12}$ primer and MMLV RT RNaseH Minus (Promega). qRT-PCR was performed using LightCycler®480 SYBR green master I in a LightCycler 480 II (both Roche Diagnostics). Identities of qPCR products were confirmed by sequencing. Steady state mRNA levels for mtPAP and PDE12 were normalized to RPLP0 (primer sequences Supplementary Data, Supplementary Table S3). Relative changes of mRNA amounts were calculated by the $\Delta\Delta C_t$ method (24). For Northern blot analysis, total RNA was separated on a 10% urea polyacrylamide gel and transferred to Hybond-N (GE Healthcare) by semidry electro-blotting. After UV irradiation and hybridization with radioactively labeled synthetic DNA oligonucleotides (Supplementary Data, Supplementary Table S2) in Church buffer at 42°C over night, hybridization signals were detected with a PhosphorImager and analyzed with the software AIDA Image Analyzer (Raytest).

For the analysis of aminoacylated tRNAs, total RNA was isolated under acidic conditions, and the RNA was separated by electrophoresis at 4°C in an acidic 6.5% polyacrylamide gel (pH 5.0) containing 8 M urea as described (25).

tRNA 3′-end analysis

0.5 μg total RNA were ligated to 100 pmol adaptor oligonucleotide (Supplementary Data, Supplementary Table S3, #7) with T4 RNA Ligase 1 (NEB). Reverse transcription was performed using MMLV RT RNase H Minus (Promega) and an oligonucleotide complementary to the adaptor sequence. The cDNA was amplified by standard PCR followed by a one-side nested PCR with Pfu polymerase (Fermentas) and primers as described in the supplement (Supplementary Data, Supplementary Table S3). The PCR products were phosphorylated, ligated into the EcoRV site of pBluescript II SK, transformed into *E. coli* XL1 blue, and individual clones were sequenced with the BigDye®Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and T7 promotor primer on an ABI Prism™310 Genetic Analyzer (Applied Biosystems).

Alternatively, tRNA 3′-ends were analyzed by next-generation sequencing. For this purpose, the reaction products of the *in vitro* processing reactions were extracted with phenol/chloroform and ethanol-precipitated. Adaptor ligation, cDNA synthesis and one-side nested PCR were carried out as described above. These PCR amplicons were used for sequencing library preparation. Blunt end repair, Illumina-adapter ligation, adapter fill-in and amplification were done according to Meyer and Kircher (26). The barcoded libraries were purified and quantified with the Library Quantification Kit - Illumina/Universal (KAPA Biosystems) according to the instructions of the manufacturer. A pool of up to 10 libraries was used for cluster generation at a concentration of 10 nM using an IlluminacBot (Illumina, San Diego, CA, USA). Sequencing of 2 × 100 bp was performed in one lane with an IlluminaHighScan-SQ sequencer at the sequencing core facility of the IZKF Leipzig (Faculty of Medicine, University Leipzig) using version 3 chemistry and flowcell according to the instructions of the manufacturer. Basecalling and demultiplexing of raw reads was done with BCL2FASTQ conversion software (Illumina). At first, we removed Illumina-adapter sequences from raw reads (fastq-files) using Cutadapt software (27). Then all individual read isoforms in the fastq-files of each sample were defined and counted with the Perl script SequenceCounter.pl (28). All isoforms containing the complete first adapter sequence and having a median of at least five counts over all samples were extracted and kept for further analysis. Finally, adapter sequences were trimmed off as well, and the remaining sequences were mapped against the last 44 nucleotides of the reference sequence of mature human mitochondrial $tRNA^{Tyr}$ containing the discriminator residue and the CCA tail. (Due to the set-up of the one-side nested PCR, PCR products contained this part of $tRNA^{Tyr}$ instead of the full tRNA sequence.) No mismatch was allowed within the first 40 bases of a read (stopping at the -1 position of the $tRNA^{Tyr}$), and any number of mismatches was permitted in the following bases, which correspond to the nucleotides added during the base incorporation or reconstitution assay. For the base incorporation assay, the number of nucleotides from position 41 were counted for all reads and grouped into 'A residues' or 'other residues'. Based on the total number of these 3′-end nucleotides, the frequency of A incorporation could then be determined.

RNA substrates

For the production of tRNAs with 5′ OH and homogeneous 3′ ends, constructs were designed containing a T7 promotor, a hammerhead ribozyme (HH) at the 5′ end of the tRNA, the tRNA sequence of interest and the autocatalytic domain of the hepatitis delta virus (HDV) at the 3′ end. These constructs were generated by a PCR-based method (29,30) with the primers indicated (Supplementary Data, Supplementary Table S4) and cloned into the EcoRV site of pBluescript II SK (Stratagene). For *in vitro* transcription the plasmid was linearized with XhoI and NotI. Tran-

scription with T7 RNA polymerase (Agilent) was carried out according to the manufacturer's instructions in the presence of 10 mM $MgCl_2$ and a reaction volume of 50 μl. To allow the ribozyme cassettes of the primary transcript to adopt their active structures, the transcript was repeatedly heated to 60°C for 3 min and cooled to 25°C for 3 min (10 cycles). The desired tRNA products were gel-purified, 3′-dephosphorylated to remove the 2′,3′ cyclic phosphate at their 3′ ends (a consequence of the self-cleavage reaction of the HDV ribozyme) (29) and 5′-end labeled using T4 polynucleotide kinase (NEB).

To produce L3pre RNA and L3pre$(A)_{45}$ RNA, plasmids pSP64-L3pre (31) and pSP-L3pre$(A)_{45}$ (32) were linearized with RsaI or BsbI, respectively, and used for run-off transcription with SP6 RNA polymerase (Roche). Transcription products were gel-purified, dephosphorylated and 5′-end labeled using T4 polynucleotide kinase (NEB).

A 5′-end labeled $tRNA^{Tyr}$ precursor consisting of full length $tRNA^{Tyr}$ and the first 35 nucleotides of $tRNA^{Cys}$ was used in the RNase Z activity assays; the template for this RNA was generated from phY1 (8) through deletion of the leader sequence by *in vitro* mutagenesis (primers listed in Supplementary Data, Supplementary Table S4).

Protein purification

The coding sequence of mtPAP (UniProt accession number Q9NVV4) was amplified from HeLa cell cDNA and cloned into pcDNA5/FRT (Invitrogen) for expression as a fusion protein with C-terminal His8 and FLAG tags. The resulting pcDNA5/FRT-mtPAP.His8.FLAG plasmid was used to generate a stable FLP-In HEK293A (Invitrogen) cell line. The D325A active-site mutant was generated by site-directed mutagenesis of this plasmid. For the pcDNA5/FRT-PDE12.His8.FLAG plasmid, the coding sequence of PDE12 was amplified from a PDE12 cDNA plasmid (IMAGE clone: 4823249) and cloned as above. The E352A active-site mutant was generated by site-directed mutagenesis. For expression of mtPAP and PDE12 as well as their active-site mutants, Flp-In HEK293A cells were cultivated in DMEM medium containing 10% FBS, and cell lines expressing the proteins of interest were selected according to the manufacturer's instructions (Invitrogen). Subcellular localization of the recombinant Flag-tagged proteins was analyzed by subcellular fractionation followed by western blotting and by immunostaining with mouse anti-Flag antibody (Sigma-Aldrich) according to the manufacturer's instructions.

For purification of the proteins, the cells were lysed by sonification in 50 mM Tris (pH 7.5), 200 mM KCL, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM AEBSF, 1 mg/l pepstatin, 1 mg/l leupeptin and 0.2 mM EDTA. The cleared lysate was incubated with M2 anti-FLAG agarose (Sigma-Aldrich; 1 ml matrix per 5 ml packed cell volume). The matrix was washed with lysis buffer and the protein was eluted with lysis buffer containing 5 mM FLAG peptide. All fractions containing recombinant proteins of interest as determined by SDS-PAGE were frozen in liquid nitrogen and stored at −80°C.

His-tagged human CCA-adding enzyme was expressed in *E.coli* BL21(DE3) from the expression plasmid pET-30Ek/LIC-hmtCCA kindly provided by Mario Mörl (University of Leipzig) (14). Freshly transformed cells were grown in LB medium at 37°C to an OD_{600} of 0.8, and expression of the CCA-adding protein was induced with 1 mM IPTG for 3 h. Cells were harvested by centrifugation and lysed by lysozyme treatment and sonification in ice-cold binding buffer (20 mM Tris/HCl (pH 7.9), 0.5 M NaCl, 20 mM imidazole and 0.75 mg/ml lysozyme). The cleared lysate was incubated with Ni-NTA agarose (Qiagen), washed with binding buffer and eluted stepwise with binding buffer containing 10% (v/v) glycerol) and 50, 200 or 500 mM imidazole. Fractions containing the enzyme as determined by SDS-PAGE were pooled, dialyzed against binding buffer II (20 mM Tris/HCl (pH 8.0), 10% (v/v) glycerol, 40 mM KCl, 0.1 mM EDTA) and loaded onto a Mono Q FPLC column (GE Healthcare). Bound protein was eluted with a KCl gradient (40–500 mM in binding buffer II containing 10 mM $MgCl_2$), and fractions containing protein as determined by SDS-PAGE were pooled, frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined either by Bradford (Roti®Quant protein assay, Roth) or by comparison of the band in a Coomassie Blue-stained SDS gel to a BSA standard.

The coding sequence of human *ELAC2* (encoding mitochondrial RNase Z (13), UniProt accession number Q9BQ52) starting from the second, internal ATG (and thereby without its mitochondrial targeting sequence) was PCR amplified (for primers see Supplementary Data, Supplementary Table S4) and cloned into pET-21b(+) (Novagen). The C-terminally His-tagged protein was expressed in *E.coli* Rosetta2(DE3) (Novagen) at 15°C in LB medium containing 1 mM IPTG and 10 μM $ZnCl_2$, and purified on a HisTrap HP column (GE Healthcare) in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 15% glycerol, 0.1 mM DTT, containing 40 mM, 80 mM, and 300 mM imidazole for loading, washing and elution, respectively. The purified protein was concentrated and buffer exchanged to 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 50% glycerol, 5 mM DTT and 0.1% Tween-10, by ultrafiltration. Protein concentration was determined with a Bradford protein assay (BioRad).

Enzyme assays

Base incorporation assays were carried out at 37°C for 30 min in a 10 μl reaction mixture containing 100 nM of 5′-labeled tRNA, up to 100 nM recombinant protein, reaction buffer (50 mM HEPES/KOH (pH 8.0), 20 mM KCl, 7 mM $MgCl_2$, 0.1 mg/ml BSA, 0.02% NP-40 and 1 mM DTT) in the presence of NTPs (0.25 mM each for mtPAP, 0.5 mM ATP and 0.5 mM CTP for CCA-adding enzyme) as indicated. After ethanol precipitation, reaction products were separated by electrophoresis in a 10% polyacrylamide gel containing 8.3 M urea and visualized by autoradiography. The nature of the incorporated nucleotides was determined by 3′-end sequence analysis.

Nuclease assays were carried out at 37°C for 30 min or the times indicated in 10 μl reaction mixtures containing 100 nM 5′-labeled RNA, 1–100 nM recombinant PDE12 or 50 or 100 nM RNase Z and the reaction buffer as described for the base incorporation assay.

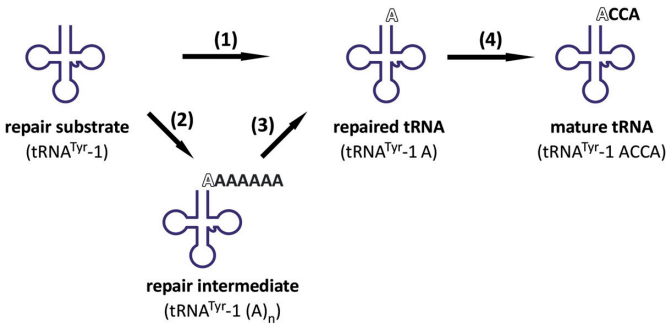


Figure 1. Model of the repair reaction of human mitochondrial $tRNA^{Tyr}$. The model anticipates the results described in this publication. The repair reaction may result from A addition catalyzed by the mitochondrial poly(A) polymerase (mtPAP) (**1**) or a combination of oligoadenylation (by mtPAP) (**2**) and removal of excess A residues either by PDE12 or RNase Z (**3**). The resulting $tRNA^{Tyr}$ (including the discriminator nucleotide) is a substrate for CCA-addition (**4**), and the mature tRNA can finally be aminoacylated. CCA-tail addition and aminoacylation remove $tRNA^{Tyr}$ (including discriminator) from the extension-shortening equilibrium resulting in an overall efficient maturation.

For the reconstitution assays, 100 nM 5′-labeled $tRNA^{Tyr}$ lacking the discriminator ($tRNA^{Tyr}$-1) was incubated in the presence of ATP and CTP (0.5 mM each) with one or a mixture of the following enzymes: 20 nM CCA-adding enzyme; 10 nM mtPAP or the active site mutant D325A; 1–100 nM PDE12 or the active site mutant E351A. Reaction products were separated by electrophoresis in a 10% polyacrylamide gel containing 8.3 M urea and visualized by autoradiography. The nature of the incorporated nucleotides was determined by next-generation sequence analysis.

RESULTS

Since the missing discriminator nucleotide of $tRNA^{Tyr}$ is an adenosine, we hypothesized that mtPAP might be involved in the repair reaction. Three possibilities can be envisioned (Figure 1): first, if mtPAP added just a single A residue to the repair substrate (the truncated tRNA), it would directly generate the required substrate for the CCA-adding enzyme. Second, if mtPAP added more than one A residue to the 3′ end of the repair substrate, a nuclease would be required to generate an appropriate substrate for CCA addition. Third, if mtPAP added several nucleotides, but one A at a time, i.e. in a distributive reaction, the CCA-adding enzyme could remove the monoadenylated intermediate from the reaction equilibrium. The three scenarios are not mutually exclusive, but might operate alternatively or in parallel. The only mitochondrial deadenylase known so far is PDE12 (33,34). Alternatively, endonucleolytic cleavage converting an oligoadenylated intermediate into a tRNA containing just the discriminator might be catalyzed by RNase Z, the enzyme also responsible for 3′ processing of non-overlapping tRNA precursors (7,9).

Oligoadenylated mitochondrial tRNAs exist in human cells

To test whether oligoadenylation of tRNA species takes place in human mitochondria, we performed a BLAST search on human EST data with all human mitochondrial tRNA sequences, taking the last 25 nucleotides of the mature tRNAs as input sequence. EST sequences for 20 of the 22 tRNAs were identified, and sequences containing additional A's at the mature, i.e. CCA-containing 3′ end of the sequence were found for 13 of them (Supplementary Data, Supplementary Table S5). From this we conclude that oligoadenylation of tRNAs indeed occurs in human mitochondria.

Mitochondrial PAP and PDE12 affect $tRNA^{Tyr}$ maturation

We investigated whether altered levels of mtPAP and PDE12 affected the processing of mitochondrial $tRNA^{Tyr}$. HEK293 cells were treated with siRNAs directed against mtPAP or PDE12 (two different siRNAs each) or with a control siRNA. In addition, cell lines stably expressing HisFLAG-tagged versions of the proteins in addition to the endogenous protein were used; the parental cell line served as a control. The levels of mtPAP and PDE12 transcripts were determined by qPCR to verify knockdown or overexpression (Figure 2A). Total RNA was isolated in three to four independent experiments per knockdown or overexpression and used for sequence analysis of the 3′ end of mitochondrial tRNAs for tyrosine, cysteine or phenylalanine, respectively. In this procedure only tRNAs with free 3′ ends are analyzed, as aminoacylated tRNAs are no substrates for adapter ligation. Therefore the amount of correctly processed, mature tRNAs is underestimated (see below).

The resulting sequences for $tRNA^{Tyr}$ were first clustered into two groups: transcripts containing non-encoded adenosine residues at the 3′ end other than the discriminator A and the A within CCA and transcripts without additional As (Figure 2A). In that way we determined the general oligoadenylation status of $tRNA^{Tyr}$. In cells treated with control siRNA or untreated cells (control), the frequency of oligoadenylated $tRNA^{Tyr}$ species was 19% or 9%. This frequency was decreased in cells depleted of mtPAP (0% and 6.5% for the two siRNAs) and increased in cells overexpressing mtPAP (30%). For PDE12, the reverse effect was observed: the frequency of oligoadenylated $tRNA^{Tyr}$ species increased in PDE12-depleted cells (39% and 25%) and decreased after PDE12 overexpression (0%). In addition to non-encoded As, a misincorporation of cytidine residues was detected at a low frequency, e.g. CCCA. Similar misincorporations were observed previously in HeLa cells (23). The incorporation of cytidine residues at the discriminator position might be due to the CCA-adding enzyme. If a $tRNA^{Tyr}$ with cytidine at the discriminator position were functional, which is not known, the CCA-adding enzyme would also be involved in the repair of $tRNA^{Tyr}$, albeit to a very low extent. Incorporations of other nucleotides were not found. From the data we conclude that mitochondrial $tRNA^{Tyr}$ is a substrate for mtPAP and that oligoadenylated products in turn are substrates for PDE12.

The tRNA sequences were then clustered into four groups according to their repair status: sequences representing the repair substrate (lacking the discriminator nucleotide ($tRNA^{Tyr}$-1)), the putative (oligo)adenylated repair intermediate(s) ($tRNA^{Tyr}$-1 $(A)_n$), mature tRNA ($tRNA^{Tyr}$-1 ACCA) and misprocessed species. This last class contains

A

	si-control	si-mtPAP (1)	si-mtPAP (2)	si-PDE12 (1)	si-PDE12 (2)	control	mtPAP	PDE12
steady state mRNA level [fold change]		0.27 ± 0.08	0.23 ± 0.11	0.22 ± 0.07	0.17 ± 0.08		24 ± 5.4	66 ± 19
number of clones analyzed (biological replicates)	83 (5)	20 (3)	62 (4)	49 (4)	36 (3)	44 (4)	37 (4)	35 (3)
tRNA species with 3' non-encoded A residues [%]	19.3	0.0	6.5	38.8	25.0	9.1	29.7	0.0

B

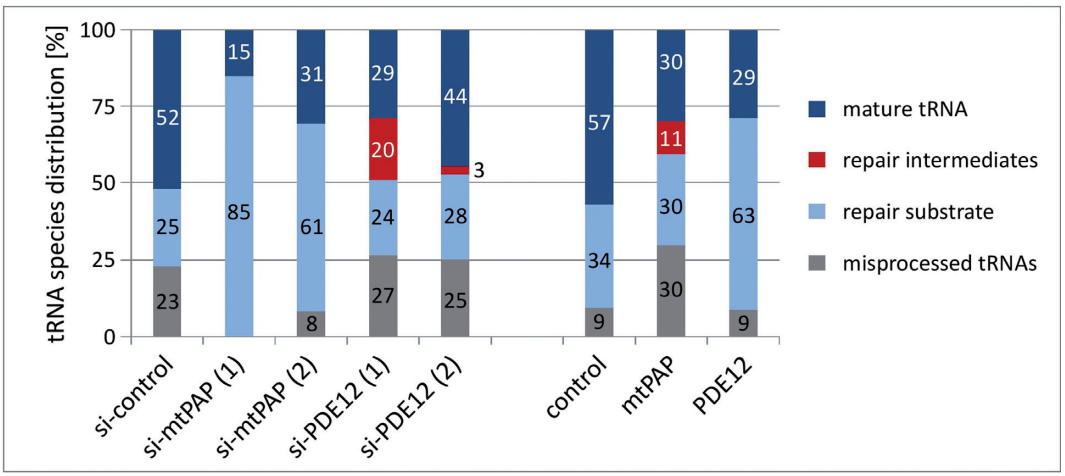


Figure 2. Sequence analysis of the 3′-end of mitochondrial $tRNA^{Tyr}$ upon mtPAP or PDE12 knockdown or overexpression. (**A**) HEK293A cells were treated with control siRNA (si-control) or siRNAs specific for mtPAP or PDE12 (two different siRNAs each) (left part of the table). Alternatively, cells were stably transfected with expression constructs for mtPAP or PDE12 (right part of the table; control, untransfected cells). Total RNA from three to four independent experiments per condition was extracted, a 3′-adapter was added, and, after reverse transcription with an oligonucleotide complementary to the adapter, two rounds of nested PCR were performed with upstream primers specific for the mitochondrial $tRNA^{Tyr}$. The resulting PCR products were cloned and single clones analyzed by sequencing. Steady-state mRNA levels for mtPAP and PDE12 were determined by qPCR and normalized to RPLP0. The frequency of sequences with 3′-non-encoded A residues except the discriminator A and the A residue of the CCA tail is shown. (**B**) 3′-end sequences of $tRNA^{Tyr}$ were clustered as follows: the repair substrate missing the discriminator nucleotide ($tRNA^{Tyr}$-1), the putative processing intermediate carrying an oligo (A)-tail ($tRNA^{Tyr}$-1 $(A)_n$), mature tRNA carrying the correct discriminator and the CCA tail ($tRNA^{Tyr}$-1 ACCA), and misprocessed species (sequences with partial CCA terminus, sequences containing the correct adenosine discriminator but misincorporation in the following nucleotides, and sequences with a misincorporated discriminator nucleotide). Note that aminoacylated tRNA is not a substrate for 3′-adapter addition, therefore the amount of mature tRNA is underestimated as roughly 50% of the tRNA remained aminoacylated during adapter ligation (Supplementary Data, Supplementary Figure S1).

tRNAs with partial CCA tails, sequences with C residues at the discriminator position or more than two C residues in the CCA tail as well as tRNAs carrying the correct discriminator and CCA tail but additional A residues (Figure 2B). In cells treated with control siRNA and parental control cells for the overexpression experiments, the main proportion of sequences corresponds to the mature tRNA (about 55%), whereas a minor proportion corresponded to the repair substrate (about 30%) or the misprocessed species (about 10–20%). These proportions of mature $tRNA^{Tyr}$ and repair substrate missing the discriminator nucleotide resembled the distribution previously observed in HeLa cells (40 and 25%, respectively) (23). Oligoadenylated repair intermediates were not detected.

Upon depletion of mtPAP, fewer sequences representing mature tRNA (15% or 31%, depending on the siRNA used) and correspondingly more sequences representing the repair substrate were observed (85% or 61%). The proportion of misprocessed tRNAs was also lower, because this class contains sequences with the correctly added CCA end but additional A residues at the 3′ end. When mtPAP was overexpressed, the amount of repair substrate was similar to what was observed in control cells (30%), however the percentage of reads for mature $tRNA^{Tyr}$ was decreased to 30%. In the samples of mtPAP overexpression we instead observed the proposed oligoadenylated repair intermediate ($tRNA^{Tyr}$-1 $(A)_n$), 11%). When cells were depleted of the mitochondrial deadenylase PDE12, the read distribution resembled the mtPAP overexpression, with a percentage of repair substrate reads similar to controls (24% and 28%, respectively), detectable amounts of the repair intermediate (20% and 3%) and consequently lower amounts of mature $tRNA^{Tyr}$ (29% and 44%). In contrast, the read distribution in cells overexpressing PDE12 was similar to the distribution in mtPAP-depleted cells. Again, the percentage of mature tRNA was lower than in control cells (29%), and the percentage of repair substrate was higher (63%). The high deadenylase level probably leads to trimming of the cor-

rectly added A residue at the discriminator position, however the possibility of PDE12 negatively regulating mtPAP cannot be ruled out. Misprocessed RNAs were again lower, as these contain reads with oligo(A)-tails following a correctly added CCA end.

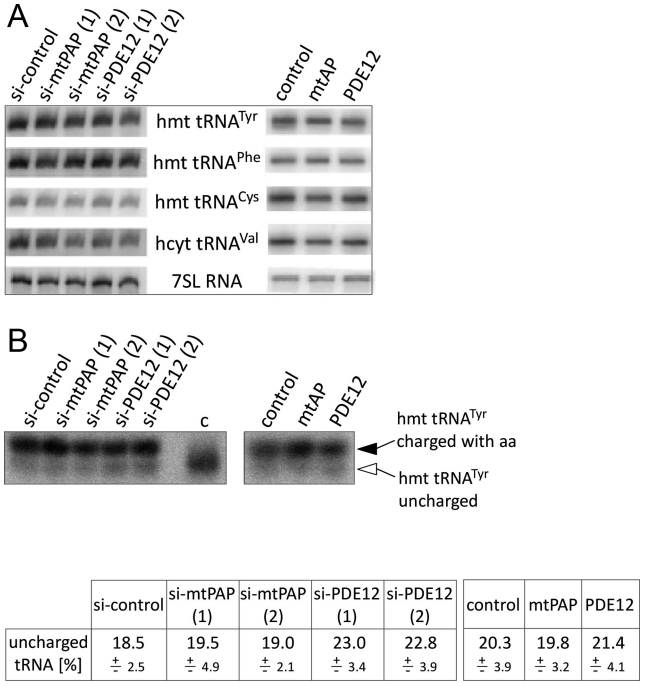
In summary, both overexpression and depletion indicate that mtPAP and PDE12 affect the oligoadenylation and repair of $tRNA^{Tyr}$ in human mitochondria in a manner consistent with the model outlined above. A detailed list with sequences found in our analysis is given in the supplement (Supplementary Data, Supplementary Table S6). For one set of samples, the analysis was extended by RNA deep sequencing (Supplementary Data, Supplementary Table S7). Even though the accumulation of the oligoadenylated repair intermediate after PDE12 knockdown or mtPAP overexpression was not observed in this data set, the changes in the ratio of repair substrate to mature $tRNA^{Tyr}$ were the same as in the analysis above, with higher percentages of repair substrates in mtPAP-depleted cells and cells overexpressing PDE12.

For comparison, the 3′ ends of mitochondrial $tRNA^{Phe}$ and $tRNA^{Cys}$ were also sequenced (Supplementary Data, Supplementary Table S6). Whereas only the mature form of $tRNA^{Cys}$ was found under all conditions, $tRNA^{Phe}$ with additional adenosyl residues at the 3′ end of the CCA tail was sometimes observed in control cells. As for $tRNA^{Tyr}$, the percentage of these reads decreased after depletion of mtPAP or overexpression of PDE12, and an increase was observed for mtPAP overexpression or after depletion of PDE12 (note that in cells depleted of mtPAP or overexpressing PDE12 the amount of reads with an incomplete CCA end, missing the last adenosine residue, was increased). This shows that $tRNA^{Tyr}$ is not the only tRNA substrate for oligoadenylation and deadenylation in human mitochondria, although different tRNAs appear to be modified by oligoadenylation to different extents.

The knockdown of RNase Z (*ELAC2*) was reported to lead to a general accumulation of 3′-unprocessed mitochondrial tRNA precursors and depletion of mature molecules (7,10). Since this general effect on 3′ processing cannot be uncoupled from an effect on the specific repair reaction of $tRNA^{Tyr}$, the effect of altered RNase Z protein levels *in vivo* was not investigated.

The level of total and aminoacylated $tRNA^{Tyr}$ is unchanged in cells depleted of mtPAP

To test whether the levels of mtPAP or PDE12 expression influence the level of total $tRNA^{Tyr}$, RNA samples from knockdown and overexpression cells were analyzed by Northern blotting (Figure 3A). The levels of three mitochondrial tRNAs ($tRNA^{Tyr}$, $tRNA^{Phe}$, $tRNA^{Cys}$), compared to cytoplasmic $tRNA^{Val}$ and 7SL RNA as controls, did not differ appreciably between the samples. Acidic gels were used to separate aminoacylated and free $tRNA^{Tyr}$ (Figure 3B). In all RNA samples about 80% of the mitochondrial $tRNA^{Tyr}$ were aminoacylated. Altered mtPAP or PDE12 protein level (knockdown or overexpression) did not lead to detectable changes in the amount of uncharged tRNA.



	si-control	si-mtPAP (1)	si-mtPAP (2)	si-PDE12 (1)	si-PDE12 (2)	control	mtPAP	PDE12
uncharged tRNA [%]	18.5 ± 2.5	19.5 ± 4.9	19.0 ± 2.1	23.0 ± 3.4	22.8 ± 3.9	20.3 ± 3.9	19.8 ± 3.2	21.4 ± 4.1

Figure 3. Altered levels of mtPAP or PDE12 do not affect the abundance of mature and charged $tRNA^{Tyr}$. (**A**) Total RNA from HEK293A cells treated with control siRNA (si-control) or siRNAs specific for mtPAP or PDE12 (two different siRNAs each) (left part of the figure) or from cells stably expressing mtPAP or PDE12 (right part of the figure) was extracted and analyzed by Northern blotting with probes for mitochondrial $tRNA^{Tyr}$, $tRNA^{Phe}$, $tRNA^{Cys}$, cytosolic $tRNA^{Val}$ and 7SL RNA. (**B**) Total RNA was separated on an acidic urea gel followed by Northern blotting. A representative Northern blot is shown with probing for mitochondrial $tRNA^{Tyr}$. Band intensities corresponding to aminoacylated tRNA (upper band) and uncharged tRNA (lower band) from three experiments were quantified. The amount of uncharged $tRNA^{Tyr}$ is displayed. As a control, the bond between tRNA and amino acid was hydrolyzed by boiling the RNA for 10 min.

The cloning and sequencing experiments described above relied on the 3′ ligation of an adapter, and aminoacylated tRNAs are expected to escape this detection procedure; due to the proofreading activity of aminoacyl-tRNA synthetases, all aminoacylated tRNAs should be mature tRNAs (35,36). In order to determine to which extent this skewed the results of our analyses, we analyzed the stability of aminoacylation under the conditions of the adapter ligation procedure. For this purpose, the aminoacylation status of $tRNA^{Tyr}$ in an RNA preparation subjected to a mock adapter-ligation procedure was compared to untreated RNA and to RNA deacylated by boiling. Again, 80–90% of the tRNA was aminoacylated in the untreated RNA preparation, whereas only about 10% remained after boiling. In the reaction corresponding to an adapter-ligation reaction, about 50% of the tRNA was still aminoacylated (Supplementary Data, Supplementary Figure S1). The level of mature tRNA was thus obviously underestimated in our previous sequencing analyses. However, as this applies to all samples, the significance of the changes in the relative amounts of repair substrate, intermediates and mature tRNA remains largely unaffected.

The repair reaction can be reproduced in vitro

As an additional test whether the combined activity of mtPAP and PDE12 can indeed lead to the production of properly matured $tRNA^{Tyr}$, an attempt was made to reconstitute the reaction *in vitro*. Both proteins were expressed as fusion proteins with C-terminal HisFLAG-tags in HEK293 cells to allow co-purification of additional protein subunits if such should exist. After cell lysis, the proteins were bound to anti-FLAG agarose, washed and eluted with FLAG peptide. As controls, active-site mutants of both proteins were expressed and purified in parallel, and additionally a mock purification of extract from cells not expressing FLAG-tagged protein (parental cell line) was performed. The enzyme preparations were analyzed by mass spectrometry, however no additional subunits could be identified (data not shown). The human CCA-adding enzyme was expressed as His-tag fusion protein in *E. coli* and purified via Ni^{2+} affinity and ion exchange chromatography.

The preparations of all three enzymes were first tested separately with *in vitro* synthesized RNA substrates. All showed the expected activities: The CCA-adding enzyme incorporated two nucleotides into the substrate $tRNA^{Tyr}$ (already carrying the discriminator) in the presence of only CTP and three nucleotides when a mixture of ATP and CTP was present (Figure 4A). For the repair substrate ($tRNA^{Tyr}$-1) a weak incorporation of nucleotides was observed. This was not expected, but likely corresponds to a misincorporation of C residues, because the product pattern was the same in the reactions containing only CTP, or CTP and ATP. When the mature tRNA carrying the discriminator and the CCA tail ($tRNA^{Tyr}$-1 ACCA) was used as substrate, no nucleotide incorporation was observed (data not shown). A putative intermediate carrying an oligo(A) tail ($tRNA^{Tyr}$-1 $(A)_5$) was not a substrate either (Figure 4A).

mtPAP incorporated several nucleotides into the repair substrate ($tRNA^{Tyr}$-1) in the presence of a mixture of all four nucleotides (Figure 4B). Sequencing of the reaction products revealed that 99% of the incorporated nucleotides were adenosyl residues (Supplementary Data, Supplementary Table S8). This is comparable to the analysis of poly(A) tails on mitochondrial mRNAs, where only 12 out of 1875 nucleotides incorporated were not A's (12). Thus, the high fidelity of mtPAP is maintained *in vitro* and with tRNA substrates, even though the acceptor stem of the tRNA is double-stranded, which is an unusual substrate for a poly(A) polymerase. Although the reaction conditions did not permit definitive conclusions regarding the processivity of the enzyme, AMP incorporation did not appear processive. In all cases, the reaction products were heterogeneous in size, the smallest product corresponding to the incorporation of a single nucleotide. The enzyme preparation of the active site mutant of mtPAP (D325A) showed a weak activity with the same characteristics as the wild-type. Since the enzyme was expressed in cells containing endogenous mtPAP, and mtPAP was previously shown to dimerize (37), we assume that this weak activity in the D325A preparation was due to copurification of the endogenous protein.

For PDE12, substrates mimicking an mRNA with or without a 45 nt long poly(A) tail were used (Figure 4C). As shown previously (33), the poly(A)-tail was efficiently removed, but the RNA body was degraded from the 3′ end with much lower efficiency. Interestingly, a reaction product corresponding in size to the RNA body still carrying the first A of the poly(A) tail accumulated at an intermediate enzyme concentration. When the same RNA substrate lacking a poly(A) tail was used, a similar, weak 3′ exonuclease activity was seen. When $tRNA^{Tyr}$ constructs, either containing an oligo(A) tail ($tRNA^{Tyr}$-1 $(A)_5$), or the discriminator and a CCA tail ($tRNA^{Tyr}$-1 ACCA), or missing both ($tRNA^{Tyr}$-1), were used as substrates, degradation of the 3′-terminal adenosine residues or the CCA tail was more efficient than degradation of the tRNA body (Figure 4D). Again, tRNA containing one adenosine residue at the 3′ end, the discriminator nucleotide, accumulated as an intermediate. In the case of tRNA, the relative stability of this degradation intermediate could be due to the structure of the molecule, as the vestigial A would correspond to the last 3′ overhanging nucleotide. In the case of the mRNA substrate, however, this should not be the case. Thus, it is possible that the nucleotide preceding the one to be cleaved off affects the activity of the enzyme. For the mutant PDE12, a weak background activity was observed.

Since the intermediate observed in the PDE12 reactions corresponds to the repair product and would be a substrate for CCA addition, we tested different combinations of mtPAP, PDE12 and CCA-adding enzyme to reconstitute the entire repair/maturation reaction *in vitro* (Figure 5). As controls for this reconstitution experiment, mtPAP and PDE12 as well as their active-site mutants were incubated with the repair substrate $tRNA^{Tyr}$-1. Like in the initial experiments (Figure 4), mtPAP incorporates nucleotides into the repair substrate, while PDE12 degrades $tRNA^{Tyr}$-1 to some extent. Upon incubation of $tRNA^{Tyr}$-1 with CCA-adding enzyme in the presence of ATP and CTP, several nucleotides were incorporated with some accumulation of product corresponding to the addition of four nucleotides, presumably corresponding to the misincorporation of C residues at the discriminator position followed by CCA addition or oligo(C) addition (Figure 5A, lane 10). When mtPAP was included in the reaction, the accumulation at the position '+4' (the expected size of the mature tRNA) was more prominent and the running behavior was slightly different, presumably due to a different sequence of the reaction product (lane 11). In addition, longer reaction products were observed, probably due to the oligoadenylation of the tRNA substrate and/or CCA transferase reaction products. When mutant mtPAP was included instead of the wild-type protein, the reaction products resembled the products of the incubation with CCA-adding enzyme alone (lane 12). When PDE12 instead of mtPAP was included in the reaction the nucleotides incorporated by the CCA-adding enzyme were degraded by PDE12 depending on the amount of PDE12 (lane 13–15). When mutant PDE12 was included instead of the wild-type protein, the reaction products resembled the products of the incubation with CCA-adding enzyme alone (lane 16).

When the tRNA was incubated with CCA-adding enzyme, mtPAP and different amounts of PDE12 (lanes 17–19), again the accumulation of a product at the position of $tRNA^{Tyr}$-1 with four additional nucleotides was observed,

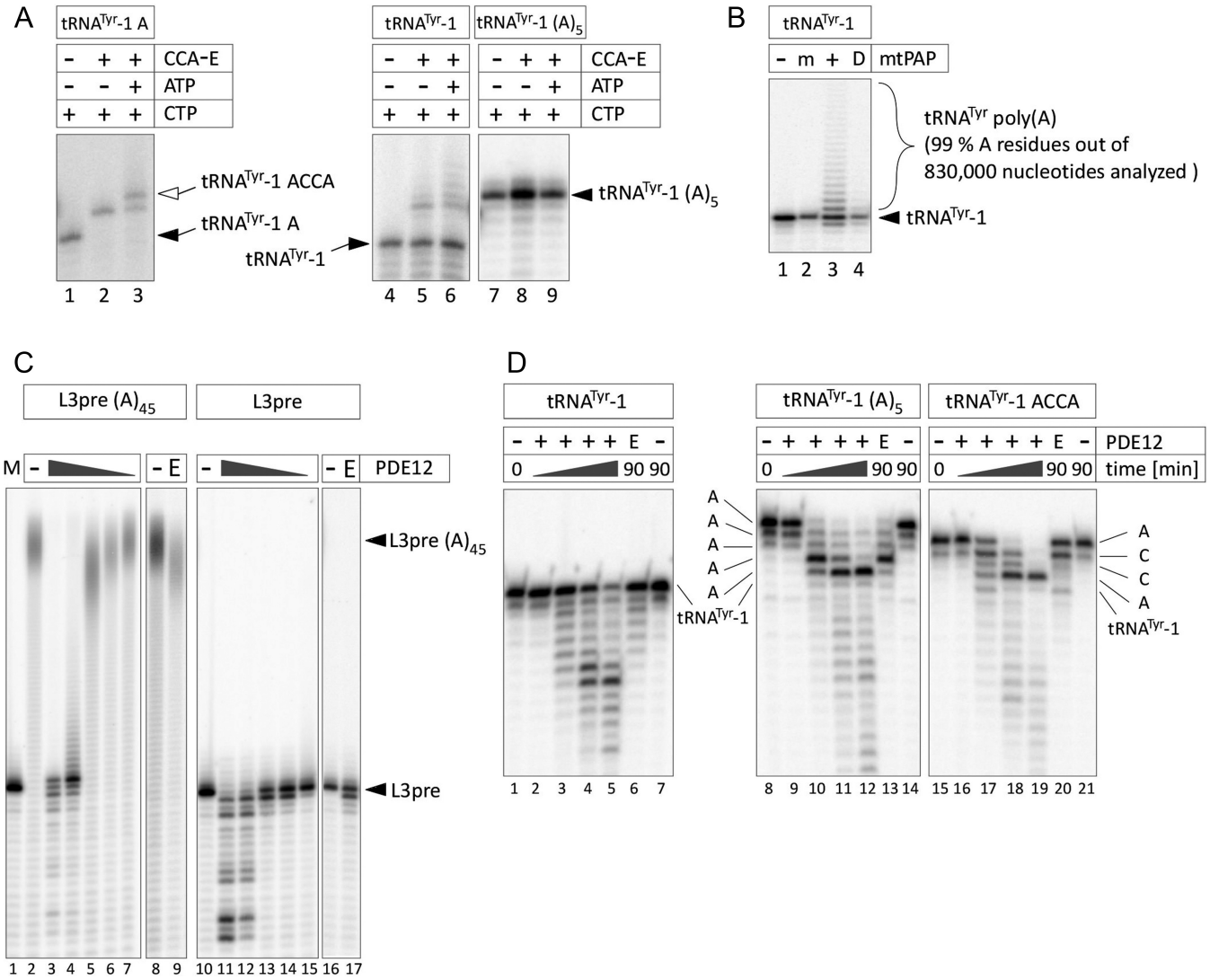


Figure 4. *In vitro* activity of enzymes possibly involved in the repair process. (**A**) 20 nM recombinant CCA-adding enzyme (CCA-E) was incubated with 100 nM of the following variants of *in vitro* synthesized and 5′-end labeled human mitochondrial $tRNA^{Tyr}$ (from left to right): first, a variant carrying the discriminator nucleotide but lacking the CCA end ($tRNA^{Tyr}$-1 A); second, a variant lacking the discriminator (RNA^{Tyr}-1); and third, a variant carrying the discriminator and four additional A residues ($tRNA^{Tyr}$-1 $(A)_5$). CTP and ATP were present at 0.5 mM each as indicated. Reaction products were assigned on the basis of their migration. Black arrows mark the RNA substrates, while the white arrow marks the reaction product corresponding to mature $tRNA^{Tyr}$. (**B**) 10 nM recombinant wild-type mtPAP (+) or the active-site mutant D325A (labeled D) was incubated with 100 nM *in vitro* synthesized and 5′-end radiolabeled human mitochondrial $tRNA^{Tyr}$-1 in the presence of a mixture of all four NTPs (0.25 mM each). As an additional control (m), the FLAG eluate from HEK293 cells not expressing mtPAP was used. Reaction products corresponding to lane 3 were analyzed by 3′-end next-generation sequencing. Lane (-), RNA substrate incubated in the absence of protein (**C**) 1–100 nM recombinant PDE12 or 100 nM active-site mutant E351A (labeled E) was incubated with 100 nM *in vitro* synthesized and 5′-end radiolabeled L3pre RNA with an $(A)_{45}$ tail (L3pre $(A)_{45}$) or lacking the poly(A) tail (L3pre). As controls, both RNA substrates were incubated in the absence of protein. M, *in vitro* synthesized and 5′-end labeled L3pre RNA as a size marker. (**D**) 100 nM recombinant PDE12 (+) or active-site mutant E351A (labeled E) was incubated with 100 nM *in vitro* synthesized, 5′-end labeled $tRNA^{Tyr}$-1, $tRNA^{Tyr}$-1 $(A)_5$, or $tRNA^{Tyr}$-1 ACCA for up to 90 min (0, 5, 30 and 90 min). Lane (-), RNA incubated for the time indicated in the absence of protein. In (**A**)–(**D**), reaction products were separated on 10% polyacrylamide-urea gels and detected by phosphorimaging.

as in the reaction lacking PDE12. However, the longer reaction products visible in the reaction with CCA-adding enzyme and mtPAP were reduced with increasing amounts of PDE12. At high concentrations of PDE12, degradation products of the tRNA became visible.

A high percentage of mature tRNA is generated by in vitro repair

Products from reactions either with the CCA-adding enzyme alone, with a combination of CCA-adding enzyme and mtPAP, or with these two enzymes plus PDE12 at 1 or 10 nM (Figure 5A, lanes 10, 11, 18 and 19) were analyzed by next-generation sequencing (Supplementary Data, Supplementary Table S9). Between 500 000 and 1 200 000 reads were obtained per sample. First, sequences corresponding

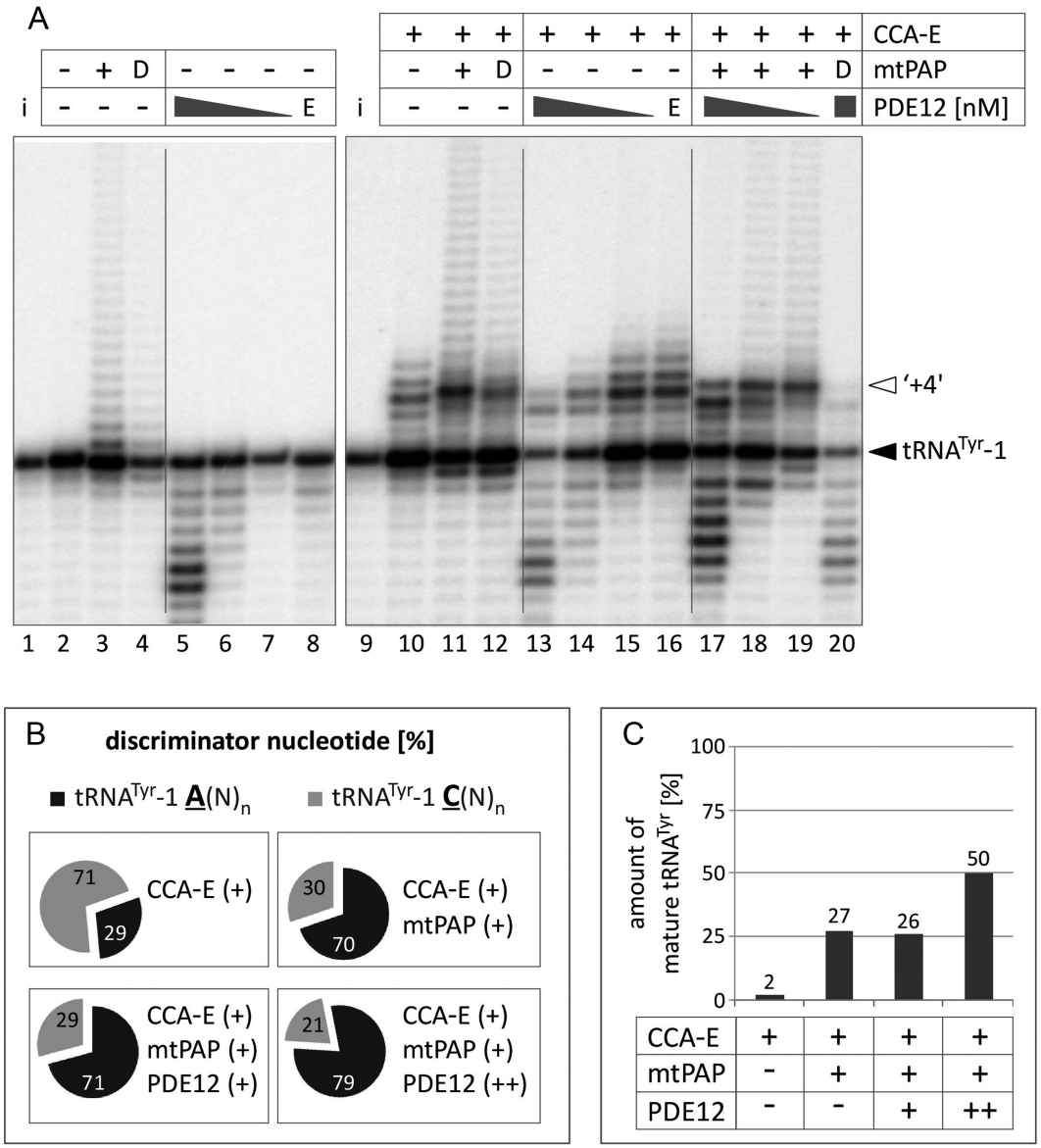


Figure 5. *In vitro* recapitulation of the repair process. (**A**) 100 nM *in vitro* transcribed, 5′-end labeled human mitochondrial $tRNA^{Tyr}$ lacking the discriminator ($tRNA^{Tyr}$-1) was incubated with ATP and CTP (0.5 mM each) in the presence of one or more of the following recombinant enzymes as indicated: 20 nM CCA-adding enzyme (CCA-E); 10 nM mtPAP (mtPAP) or its active-site mutant D325A (D); 1, 10 or 100 nM PDE12 (PDE12) or 100 nM of its active-site mutant E351A (E). Reaction products were separated in a 10% polyacrylamide-urea gel and detected by phosphorimaging. Lanes 1 and 9, RNA incubated in the absence of protein; lane 2, RNA incubated in the presence of a FLAG eluate from HEK293 cells not expressing FLAG-tagged protein. Products were assigned on the basis of their migration; note that a single product band visible in the gel may contain heterogeneous sequences. (**B**) Reaction products of lanes 10, 11, 18 and 19 were analyzed by 3′-end next-generation sequencing. Reads corresponding to tRNA with at least one nucleotide incorporated were clustered as follows: tRNA species with correctly added discriminator ($tRNA^{Tyr}$-1 $\underline{A}(N)_n$); and tRNA species with misincorporation of C at the discriminator position ($tRNA^{Ty}$-1 $\underline{C}(N)_n$). In both clusters we did not distinguish between reads with or without additional nucleotides following the discriminator nor the nature of these additional nucleotides. (**C**) Reaction products of lanes 10, 11, 18 and 19 were analyzed by 3′-end next-generation sequencing, reads with four nucleotides incorporated ('+4') were selected, and the fraction of mature tRNA carrying both the correct discriminator and the CCA tail ($tRNA^{Tyr}$-1 ACCA) within this class was determined.

to the tRNA substrate with at least one nucleotide added were analyzed for the nucleotide at the discriminator position, independently of the presence of additional downstream nucleotides. Less than 0.1% of all sequences contained G or U incorporations, showing the sequencing error rate to be very low. The incorporation of the correct nucleotide A was low with only the CCA-adding enzyme but much higher when mtPAP was included. The reaction with all three enzymes showed a small additional increase in A addition (Figure 5B). Note that Figure 5B only displays the relative proportions of the various elongated products. While 29% of correct A incorporation by CCA transferase alone may seem high, the efficiency of elongation of the $tRNA^{Tyr}$-1 substrate was low (Figure 4A). As the 3′-end sequence of the substrate is …TTTACC, A addition may

reflect the ability of the CCA-adding enzyme to repair a incomplete, pseudo CCA tail (38,39).

We next analyzed all reads with four nucleotides incorporated and counted the reads corresponding to mature $tRNA^{Tyr}$ (Figure 5C). The number of such reads was very low among the reaction products of the CCA-adding enzyme alone, even though a clear band corresponding to the incorporation of four nucleotides was visible in the gel (Figure 5A, lane 10). Despite the fact that the CCA-adding enzyme incorporated sometimes an A residue at the discriminator position (Figure 5B), we often found the incorporation of an oligo(C) tail instead of the CCA tail at positions '+2' to '+4'. In the reaction containing mtPAP, the amount of mature tRNA increased to 27%. In the reaction containing the highest amount of PDE12, the number of mature tRNAs increased even further (50%). The lower proportion of correct products in the absence of PDE12 can be explained by the high amount of sequences containing four A residues in these reaction (33%). Such oligoadenylated tRNAs can serve as substrates for PDE12 and be converted into substrates for CCA addition. Taken together, the sequencing data demonstrate that a combination of mtPAP, PDE12 and CCA transferase can efficiently process $tRNA^{Tyr}$-1 to the mature form.

RNase Z can contribute to the repair of intermediates with longer oligo(A) tails

In 'normal' cases, the 3′ ends of mitochondrial tRNAs are generated by RNase Z followed by CCA addition (7,9). To test whether RNase Z could also remove excess A residues added by mtPAP, we incubated *in vitro* synthesized tRNA substrates corresponding to the oligoadenylated repair intermediate ($tRNA^{Tyr}$-1 $(A)_5$) with RNase Z. Even after prolonged incubation, no cleavage product was observed (Figure 6B). Mature tRNA (with discriminator and CCA end, $tRNA^{Tyr}$-1 ACCA) was not cleaved, in agreement with the reported inhibition of RNase Z activity by the CCA tail (40). In contrast, an *in vitro* transcript comprising full length $tRNA^{Tyr}$ and the first 35 5′-nucleotides of $tRNA^{Cys}$ was readily cleaved to result in the expected cleavage product ($tRNA^{Tyr}$-1 A; Figure 6A). Therefore, longer poly(A) tails were added to the substrate $tRNA^{Tyr}$-1 $(A)_5$ by means of mtPAP. The reaction products were purified and then incubated with RNase Z. A band corresponding to the size of a tRNA carrying the discriminator appeared and increased over time (Figure 6B, lane 12). When the reaction additionally contained the CCA-adding enzyme (the assay was performed in the presence of ATP and CTP), the band corresponding to the correct monoadenylated repair intermediate disappeared and a band corresponding to the size of mature tRNA ($tRNA^{Tyr}$-1 ACCA) became visible (lane 15). RNase Z is thus able to contribute to the overall repair reaction by removing A extensions of more than five nucleotides.

DISCUSSION

In this report we propose a model for the repair of the 3′ end of the human mitochondrial $tRNA^{Tyr}$ precursor (Figure 1). When the tRNA is processed from the primary transcript, a truncated $tRNA^{Tyr}$-1 is released. This repair substrate is adenylated by mtPAP: if only one nucleotide is added, the product directly becomes a substrate for CCA addition; if several adenosyl residues are incorporated into the tRNA, either the deadenylase PDE12 or the endonuclease RNase Z remove the supernumerous nucleotides, producing again a substrate for CCA addition. In both cases CCA addition and subsequent aminoacylation remove the correctly repaired $tRNA^{Tyr}$ from the equilibrium.

This scenario is supported by a set of *in vivo* and *in vitro* experiments: (1) Mitochondrial tRNAs are oligoadenylated *in vivo*, since non-encoded adenosyl residues are observed at the mature CCA terminus of $tRNA^{Tyr}$ and $tRNA^{Phe}$, and these species are also present in EST data for several other mitochondrial tRNAs. In addition, for $tRNA^{Tyr}$, oligoadenylated processing intermediates occur. (2) The amount of $tRNA^{Tyr}$ carrying non-encoded As is affected by the level of the proposed repair enzymes, mtPAP and PDE12, in a way consistent with mtPAP oligoadenylating mitochondrial tRNAs and PDE12 removing such tails. In the same data set, the ratio between mature $tRNA^{Tyr}$ and the repair substrate $tRNA^{Tyr}$-1 is also affected, showing mtPAP and PDE12 to be involved in the repair of $tRNA^{Tyr}$. (3) *In vitro*, a mixture of mtPAP, PDE12 and CCA-adding enzyme can convert the repair substrate $tRNA^{Tyr}$-1 into mature $tRNA^{Tyr}$, carrying the correct discriminator as well as the CCA end. Similarly, the combination of mtPAP, RNase Z and CCA-adding enzyme can repair $tRNA^{Tyr}$-1 resulting in mature $tRNA^{Tyr}$ ($tRNA^{Tyr}$-1 ACCA) *in vitro*.

The first step in the repair reaction is the addition of the discriminator by mtPAP either as a single nucleotide due to the distributive activity of mtPAP or as part of an oligo(A) tail. Polyadenylation of mitochondrial mRNAs is the most obvious function of mtPAP, and at the same time the enzyme also repairs some of the mRNAs by completing their stop codons (41–44). In addition, polyadenylation of a fraction of mitochondrial rRNAs has been observed, even though the function of such tails is not clear (6,45). Oligoadenylation of truncated transcripts of different kinds, including tRNAs, has been reported (46), showing mtPAP to accept a wide variety of RNA substrates *in vivo*. Although the crystal structure of the enzyme has been solved (37), the question of RNA substrate binding and specificity is still open. The RBD-like domain present in mtPAP was shown to be involved in dimerization. Interestingly only the dimer is active in polyadenylation, but RNA binding has not been directly observed for either monomeric or dimeric proteins (37). Other poly(A) polymerases rely on protein cofactors for substrate recognition, and a stimulatory effect of several proteins on polyadenylation by mtPAP was reported (47,48). However, no stable complex of mtPAP and a RNA binding protein has been reported, and in all cases the protein cofactors only enhanced the intrinsic polyadenylation activity of mtPAP.

Previously, oligoadenylation of truncated transcripts of various kinds was observed and a function in RNA degradation similar to the role of polyadenylation in bacteria was suggested (46). However, the RNA decay pathways in human mitochondria are still not well described, and neither is the importance of adenylation for RNA degradation. We also observe oligoadenylation of tRNAs carrying the CCA tail. These tRNAs may be on the way to decay, however we

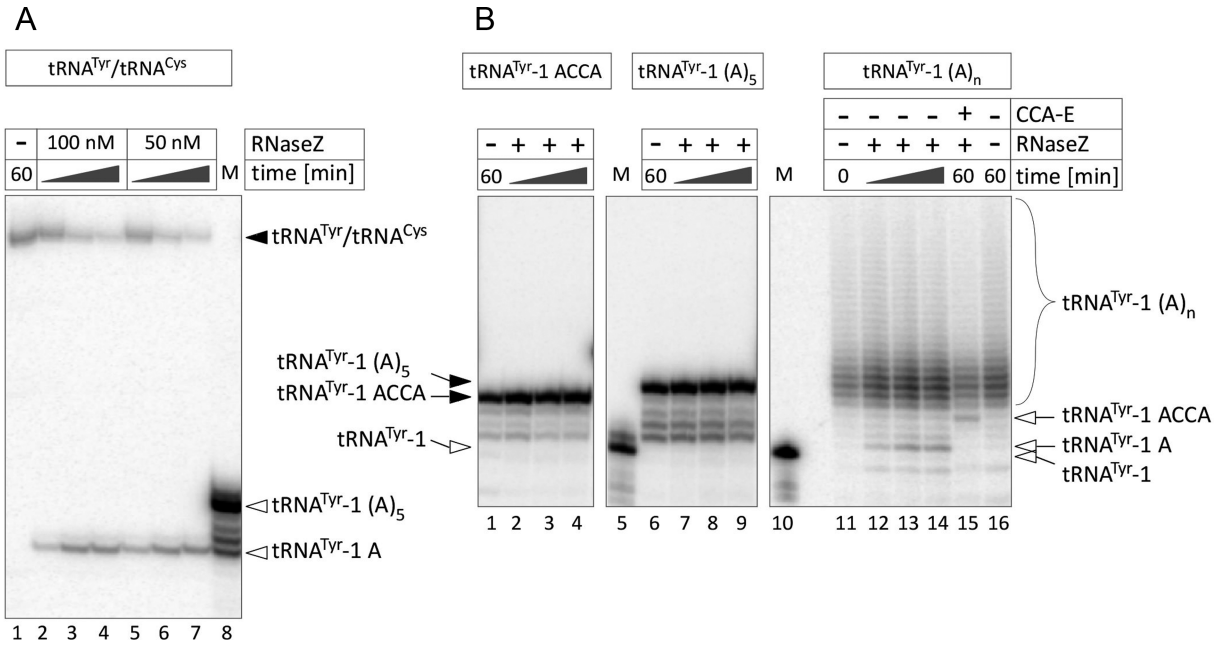


Figure 6. RNase Z and CCA-adding enzyme process polyadenylated $tRNA^{Tyr}$ *in vitro*. (**A**) 100 nM or 50 nM recombinant RNase Z was incubated with 100 nM *in vitro* synthesized and 5′-end labeled human mitochondrial $tRNA^{Tyr}/tRNA^{Cys}$ precursor ($tRNA^{Tyr}/tRNA^{Cys}$) for up to 60 min (5, 30 and 60 min). Reaction products were analyzed on a 10% polyacrylamide-urea gel and detected by phosphorimaging. In a control reaction (-), RNA was incubated in the absence of protein; M, $tRNA^{Tyr}$-1 $(A)_5$ was used as a size marker. (**B**) 100 nM recombinant RNase Z (+) was incubated with 100 nM *in vitro* synthesized and 5′-end labeled $tRNA^{Tyr}$, either carrying the discriminator and the CCA tail ($tRNA^{Tyr}$-1 ACCA), or carrying the discriminator and four additional A residues ($tRNA^{Tyr}$-1 $(A)_5$), or carrying the discriminator and a longer oligo(A) tail ($tRNA^{Tyr}$-1 $(A)_n$) for up to 60 min (5, 30 and 60 min). In the case of the $tRNA^{Tyr}$-1 $(A)_n$ substrate, the reaction also contained recombinant CCA-adding enzyme and ATP and CTP (0.5 mM each) where indicated. Lanes labeled '-' represent incubations in the absence of enzyme. Reaction products were analyzed as in (A). M, $tRNA^{Tyr}$ lacking the discriminator was used as a marker.

did not observe changes in the amount of total or aminoacylated tRNA among the tRNA species analyzed in our knockdown and overexpression experiments. Another explanation for the occurrence of oligo(A) tails on different RNAs could be a generally low substrate specificity of mtPAP, resulting in oligoadenylation of any free RNA 3′ end. In general, mature tRNAs would be protected by aminoacylation, but not truncated tRNAs like the $tRNA^{Tyr}$-1 repair substrate. Oligoadenylation may enhance degradation for certain RNAs, but allows repair in case of $tRNA^{Tyr}$-1.

After oligoadenylation, PDE12 or RNase Z are able to remove supernumerous nucleotides. In addition, PDE12 increases the fidelity of the repair reaction by removing misincorporated nucleotides. In the *in vitro* reactions, addition of PDE12 resulted in a higher percentage of tRNAs with the correct discriminator as well as a higher amount of mature $tRNA^{Tyr}$. Whereas RNase Z was shown to efficiently remove short as well as long trailer sequences and even single nucleotides (49), we observed appreciable cleavage of oligo(A) tails only when they were longer than five nucleotides. This may be a specific feature of oligoadenylated tRNAs, a substrate not previously tested, but we cannot exclude either that the result is due to subtle differences in reaction conditions.

The addition and removal of adenosyl residues leads to a certain amount of tRNA carrying the correct discriminator. These molecules are substrates for CCA addition and the repair product is thereby removed from the reaction equilibrium. In that sense the CCA-adding enzyme acts as a quality control step. In the reconstitution experiments only nucleotidyl transferases and nucleases are present. In contrast the correctly matured $tRNA^{Tyr}$ is a substrate for aminoacylation *in vivo*. The discriminator position is an important identity element for $tRNA^{Tyr}$ (22). Therefore, correctly matured tRNA is protected from degradation by being charged with tyrosine, whereas tRNAs with misincorporations are not, resulting in the high fidelity of $tRNA^{Tyr}$ repair observed *in vivo*.

Our model for the 3′-end repair of human $tRNA^{Tyr}$ involves a set of enzymes with primary functions other than tRNA repair: mtPAP and PDE12 adenylate or deadenylate mitochondrial mRNAs, and RNase Z is responsible for tRNA 3′-end processing. The combination of these enzymes allows the addition of the missing discriminator nucleotide to the truncated $tRNA^{Tyr}$ precursor. Thereby the deleterious effects of the truncated tRNA genes are overcome and microdeletions, resulting in the overlap of tRNA genes, can be tolerated. In the case of $tRNA^{Tyr}$ repair, mtPAP is promiscuous enough to add adenosyl residues not only to mRNAs but also to tRNAs. A truncated $tRNA^{Tyr}$ is not a substrate for CCA-addition and aminoacylation, but the free 3′-end can be adenylated by mtPAP. In the same way, oligoadenylated $tRNA^{Tyr}$ can be deadenylated by PDE12, which is not specific for mRNAs, or RNase Z recognizes the oligo(A) tail as a trailer sequence. In both cases, the result is a restored $tRNA^{Tyr}$.

The repair reaction described above is not specific for $tRNA^{Tyr}$, but could restore missing adenosyl residues at

other tRNA 3′-ends and in any organism containing mtPAP. Interestingly, another overlap of two tRNAs found in vertebrates, between the $tRNA^{Ser}$ and $tRNA^{Leu}$, also consists of one adenosyl residue. In addition, polyadenylation also completes stop codons of several mitochondrial mRNAs. Besides its function in polyadenylation per se, mtPAP therefore seems to act as a repair enzyme for different kinds of mitochondrial RNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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