Human Mitochondrial mRNAs Are Stabilized with Polyadenylation Regulated by Mitochondria-specific Poly(A) Polymerase and Polynucleotide Phosphorylase*

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Mammalian mitochondrial (mt) mRNAs have short poly(A) tails at their 3′ termini that are post-transcriptionally synthesized by mt poly(A) polymerase (PAP). The polyadenylation of mt mRNAs is known to be a key process needed to create UAA stop codons that are not encoded in mtDNA. In some cases, polyadenylation is required for the tRNA maturation by editing of its 3′ terminus. However, little is known about the functional roles the poly(A) tail of mt mRNAs plays in mt translation and RNA turnover. Here we show human mt PAP (hmtPAP) and human polynucleotide phosphorylase (hPNPase) control poly(A) synthesis in human mitochondria. Partial inactivation of hmtPAP by RNA interference using small interfering RNA in HeLa cells resulted in shortened poly(A) tails and decreased steady state levels of some mt mRNAs as well as their translational products. Moreover, knocking down hmtPAP generated markedly defective mt membrane potentials and reduced oxygen consumption. In contrast, knocking down hPNPase showed significantly extended poly(A) tails of mt mRNAs. These results demonstrate that the poly(A) length of human mt mRNAs is controlled by polyadenylation by hmtPAP and deadenylation by hPNPase, and polyadenylation is required for the stability of mt mRNAs.

The polyadenylation of mRNAs plays a pivotal role in gene expression. In eukaryotes, polyadenylation is known to confer mRNA stability, promote translation initiation, and play a role in the transport of processed mRNAs from the nucleus to the cytoplasm, which are mediated by poly(A)-binding proteins (1–4). In prokaryotes, in contrast, polyadenylation is involved in the normal turnover of the mRNAs. In Escherichia coli, polyadenylation of mRNAs allows the degradosome, a large degradation machine containing polynucleotide phosphorylase (PNPase), which is a phosphate-dependent exonuclease, to facilitate mRNA degradation (5–8). In chloroplasts, polyadenylation of mRNAs also constitutes the signal for RNA degradation as in bacteria (9). Therefore, control of poly(A) tail synthesis is a key regulatory step in gene expression in both eukaryotes and prokaryotes.

Polyadenylation in mitochondria is very varied among organisms. In plant mitochondria, polyadenylation promotes mRNA degradation as in bacteria and chloroplasts (10). In yeast mitochondria, mRNAs lack the poly(A) tail, and mt gene expression seems to be regulated by the mtDNA-encoded conserved dodecamer sequence at its 3′ terminus (11). It is known that the stability of yeast mitochondrial (mt) mRNA is controlled by mRNA-binding proteins (12–14). In trypanosome mitochondria, the length of the poly(A) tail is dependent on the edited status of the mRNA (15–17). The role of long poly(A) is unknown, whereas short poly(A) seems to stimulate mRNA degradation (18).

In animal mitochondria, polyadenylation is a key process in the expression of mtDNA-encoded genes at the post-transcriptional level. It has been suggested that in some mt mRNAs, the polyadenylation creates the UAA stop codons during the maturation of mRNAs, because some of the mRNAs encoded by mtDNA lack stop codons. Thus, polyadenylation functions as an mRNA processing factor that can be translated in mitochondria (19). It has also been suggested that polyadenylation is involved in editing the 3′-acceptor region of some mt tRNAs (20, 21). However, little is known about the functional roles the poly(A) tails play in mt translation and RNA turnover of animal mt mRNAs.

Recently, Lightowlers and co-workers (22) show that the decreased steady state level of human mt ATP6 mRNA harbors a microdeletion (Δ9205) at its 3′ end, which is associated with human mt cytopathies. This microdeletion results in shortening the poly(A) tail of mt ATP6 mRNA. Normally, precursor ATP6 mRNA is processed so that its 3′-terminal nucleotide is a uridine. This terminal uridine serves as the 1st letter of the UAA codon, which is subsequently completed by the polyadenylation of the mRNA. The Δ9205 microdeletion removes this terminal uridine and thus results in aberrant ATP6 mRNA that lacks a stop codon. This non-stop mRNA is degraded rapidly in a deadenylation manner, suggesting that the poly(A) tail is required for human mt mRNA stability as in eukaryotic cytoplasm (22).

Conversely, Stepień and co-workers (23) reported that long poly(A) is not required for mt mRNA stability. Several mt mRNAs were shown to be relatively stable if they were significantly deadenylated by knocking down human mt poly(A) polymerase (hmtPAP). This suggests that the long poly(A) tails do not play any specific role in the stability of mammalian mt mRNAs. This could be associated with the presence of high levels of poly(A)-binding proteins (12–14). However, this possibility has not been determined experimentally.

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‡ The abbreviations used are: PNPase, polynucleotide phosphorylase; PAP, poly(A) polymerase; hmt, human mitochondria; mt, mitochondrial; siRNA, small interfering RNA; np, nucleotide position; CO, cytochrome c oxidase; ATP6, ATP synthase F$_{1}$ subunit 6; ND, nicotinamide adenine dinucleotide dehydrogenase; HSP60, heat shock 60-kDa protein; DMEH, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; EGFP, enhanced GFP; RT, reverse transcription; PAT, poly(A) tail-length assay.
not act directly as the determinants of mt mRNA stability (23). They argue against the direct role of long poly(A) tails in the stabilization of human mt mRNAs.

In this study, we demonstrate the regulatory mechanisms and functional role of polyadenylation in human mt mRNAs.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]ATP and [ε-32P]dCTP were obtained from Amersham Biosciences. T4 RNA ligase was purchased from New England Biolabs. E. coli PAP and E. coli pNPase were obtained from Takara and Sigma, respectively.

**Expression Vectors**—The full-length coding region of the candidate gene for human mt PAP (hmtPAP) cDNA was amplified from human poly(A)+ mRNA using primers designed to bind outside of the coding region. The sense and antisense primers were 5'-taagctggagctccacctgatgacctgtg-3' and 5'-gccagatttcagcatgacctgtg-3', respectively. A second round of PCR was performed by using an upstream primer with an NdeI site (5'-aagagggctgtcgttaagggagcagcagtctcgc-3') and a downstream primer with a Sall site (5'-ctttggtgtagcagtactgttcttcactgtttggatatgas-3'). The first codon of the predicted mature enzyme (GAC) in the upstream primer was changed to ATG to create an initiation codon (underlined). The PCR products were ligated into the corresponding sites of the pET-28b(+) vector (Novagen) to obtain the expression vector pET-hmtPAP, which produces C-terminal His tag-fused hmtPAP.

To analyze cellular localization, a nested PCR product was generated by using an upstream primer with a SacI site (5'-aaagagggctgtcgttaagggagcagcagtctcgc-3') and an downstream primer with a Sall site (5'-ctttggtgtagcagtactgttcttcactgtttggatatgas-3') and inserted into pEGFP-N1 (Clontech) to generate pEGFP-hmtPAP.

Construction of expression vector containing hPNPase was performed as described above. The sense and antisense primers used for amplification of hPNPase cDNA from human poly(A)+ mRNA were 5’-gagctggagctccacctgatgacctgtg-3’ and 5’-ccacagtaaatgtaactgtcagactgtctcttcccactggtttttgaaattttct-3’. The PCR product was cloned into the corresponding sites of the pET-28b(+) vector (Novagen) to obtain the expression vector pET-hPNPase, which produces N-terminal His tag-fused hPNPase.

**Cell Culture and RNA Interference**—HeLa cells were cultured in a humidified atmosphere with 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 4 mML-glutamine, and 1% penicillin and streptomycin. The sequences of the inner three strands of each siRNA are as follows: 5’-cucucgauccucaug-3’ for hmtPAP, which targets nucleotide position (np) 189–207 of hmtPAP, and 5’-gaccgcgtgc-3’ for hPNPase, which targets np 1900–1918 of hPNPase. The DNA templates were obtained from invitrodiagnostik (Bremen, Germany) and inserted into pEGFP-N1 (Clontech) to generate pEGFP-hmtPAP.

**Northern Blotting**—5 μg of total RNA from each transfectant were electrophoresed on a 1% agarose-formaldehyde gel, transferred onto a Hybond-C membrane (Amersham Biosciences), and then cross-linked to the membrane. The probe was labeled with [α-32P]dCTP by using a Random Priming Labeling Kit (Takara) and hybridized at 42 °C (24). The membranes were then dried and analyzed by an imaging analyzer BAS5000 (Fuji Film). The DNA templates were obtained from invitrodiagnostik (Bremen, Germany) and inserted into pEGFP-N1 (Clontech) to generate pEGFP-hmtPAP.

**Oxygen Consumption Rate**—The O2 consumption rate by cells 72 h after siRNA transfection was measured at 37 °C using a Clark-type oxygen electrode (YSI 5300A Biological Oxygen Monitor System) as described previously (26, 27).

**In Vivo Transcription of mt mRNA**—The long-length coding region of bovine mt ND6 DNA was amplified from bovine mtDNA using a sense primer with Apal site (5’-gagcgaccctgatcataagtcattttc-3’) and a gene-specific forward primer (upper primer). A second round of PCR was then carried out using a forward primer internal to the first PCR product (lower primer) and the anti-linker. A five-cycle PCR was performed as above with the first PCR product using 30 μl of both the anti-linker and 5’-32P-labeled lower primer. The amplified products were separated by electrophoresis in a 10% polyacrylamide deaturing gel containing 7 μM urea and visualized by an imaging analyzer (LAS-5000, Fuji Film). In Vivo Transcription of mt mRNA was performed as described (25). Total protein was extracted from each siRNA-treated cell, and the protein concentration was determined by a protein assay kit (Bio-Rad). 50 μg of total protein were separated on an SDS-polyacrylamide gel and transferred onto a Hybond-ECL membrane (Amersham Biosciences). Subsequently, the proteins were detected specifically using antibodies against CO1, CO2, and CO4 (Molecular Probes), heat shock 60-kDa protein (HSP60), and β-actin (Sigma).

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sites of the pGEM-TZ is (+) Vector (Promega). The cloned plasmid linearized with HindIII was used as a template for T7 transcription, and the transcripts obtained were purified by RNasy Midi Kit (Qiagen).

In Vivo PAP Assay—The assays were carried out at 37 °C for 30 min in reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2.5 mM MnCl₂, 40 mM KCl, 0.1 mM ATP, 0.033 µM [α-³²P]ATP, 1 mM diithiothreitol, 0.01 A₅₅₀ units of oligo(A), or 0.05 A₅₅₀ units of bovine mt ND6 mRNA, and 300 ng of hmtPAP or 500 ng of hPNPase. After the reaction, the RNA substrate was subjected to electrophoresis on a denaturing polyacrylamide gel containing 7 M urea, and the gel was exposed to an imaging plate. The radioactivity was analyzed by an imaging analyzer BAS5000 (Fuji Film).

RESULTS

Retrieval of a Candidate Gene Encoding Human mt PAP in Silico—Previously, while we were purifying the mt CCA-add- ing enzyme from bovine liver mitochondria, we detected mt PAP activity for 5 S rRNA as a substrate along with mt CCA- adding activity for tRNAs (28). However, we were not able to purify PAP from bovine mitochondria because its purification through several column chromatographic steps caused the en- zyme to lose its activity. This induced us to turn to a reverse genetics approach to identify mt PAP.

Many mt proteins show high homology to bacterial counter- parts. In particular, it is known that mt CCA-adding enzyme (28), which, like PAP, belongs to the nucleotidyltransferase superfamily, is an apparent homolog of its bacterial counter- parts. Consequently, we speculated that mt PAP may show high sequence similarity to bacterial type PAP, which belongs to the class II proteins of the nucleotidyltransferase superfamily. However, we were not able to detect bacterial PAP-like sequences in the human and mouse EST data base except for one encoding an mt CCA-adding enzyme (28). However, when we searched for a gene having homology to the family of regul- atory cytoplasmic PAPs that was recently identified in Caec- norhabditis elegans (GLD-2 (29)) and Schizosaccharomyces pombe (Cid1 (30) and Cid13 (31)), we identified an identical human regulatory cytoplasmic PAP homolog bearing an mt targeting pre-sequence. We have designated this protein hmt- PAP. While our study on hmtPAP was in progress, Stapen and co-workers (23) also identified and reported the same gene.

Orthologs of hmtPAP were found in mice, rats, chickens, flies, mosquitoes, and ascidians. All had the mt transit peptide at their N terminus (data not shown). Shorter forms bearing the mt transit peptide were also readily identified in Trypanosoma brucei and Leishmania major (data not shown). The hmtPAP or- thologs diverge from the canonical bacterial class II or eukaryotic class I PAPs and lack the apparent RNA-recognition motif region thought to be critical for substrate RNA binding (29). All or- thologs bear a conserved putative DAD motif essential for the coordination of the metal ions during the nucleotidyltransfer reaction. Therefore, we speculated that all these gene products may function as PAPs in the mitochondria of the organisms listed above. We could not find regulatory cytoplasmic PAP homologs with an mt targeting signal peptide in yeast, which is consistent with the report that yeast mt RNAs lack a poly(A) tail (1) (data not shown).

Subcellular Localization of hmtPAP in HeLa Cells—To de- termine whether hmtPAP is in fact targeted to mitochondria, HeLa cells were transfected with pEGFP-hmtPAP, which tran- siently expresses hmtPAP with GFP fused to its C-terminal end. Confocal microscopy of the cells showed that GFP-hmtPAP (Fig. 1, left panel) co-localized with the mitochondrial marker MitoTracker CMXRos (Fig. 1, right panel), indicating that hmtPAP mainly localizes to mitochondria, as expected.

hmtPAP Is Responsible for Polyadenylating mt mRNAs—Next, we examined whether hmtPAP in mitochondria is in- volved in the polyadenylation of mt mRNAs. Thus, we used siRNA to knockdown hmtPAP in HeLa cells and measured the poly(A) tails of the mt mRNAs by using the PAT assay (22). We also knocked down hPNPase because PNPase is reported to have polyadenylation activity in bacteria (32-34) and spinach chloroplasts (35). PNPase is a reversible enzyme that can de- grade RNA by using inorganic phosphate or synthesize RNA by using any nucleotide diphosphate as substrate. Nucleotide triphosphate can also be used as a substrate for polymerization activity (32, 35). Because hPNPase is localized in mitochondria (36), it may be possible that hPNPase can also polyadenylate human mt mRNAs. To estimate the efficacy of the siRNA- mediated knockdown, RT-PCR for the target mRNAs was car- ried out, and the PCR products were detected by ethidium bromide staining. This showed that both the hmtPAP and hPNPase genes were specifically targeted by their respective siRNAs because hmtPAP mRNA was barely detectable even after 25 cycles of RT-PCR, whereas hPNPase mRNA could not be detected after 20 cycles (data not shown). The GAPDH mRNA levels were not affected by either siRNAs. Thus, these siRNAs were employed in the following experiments.

We employed PAT assay (22) to examine the poly(A) length of the mt mRNAs CO1, CO2, CO3, ATP6, and ND3 in the cell, which was transfected by siRNA targeting for hmtPAP. As a control, the poly(A) tail of cytoplasmic β-actin mRNA was ana- lyzed at the same time. The wild type cells and the siRNA- scramble-treated cells show two populations of poly(A)™ mt mRNAs (Fig. 2, lanes 1 and 2), namely mt mRNAs with longer tails consisting of 40~50 adenes, and mt mRNAs with shorter tails consisting of 0~15 adenes (22). In the siRNA-hmtPAP- treated cells, the lengths of the longer poly(A) tails were slightly shortened, and the population of shorter poly(A) tails of mt mRNAs had increased (Fig. 2, lanes 3). This pattern was similar to that observed when the ATP6 gene had pathogenic micro-deletions that caused severe deadenylation (22). The knockdown of hmtPAP did not affect the length of the poly(A) tail of cytoplasmic β-actin mRNA. These data, together with those shown in Fig. 1, showed that hmtPAP is involved in the polyadenylation of mt mRNAs but not cytoplasmic mRNAs. In contrast, in the siRNA-hPNPase-treated cells, the population of mRNAs with the longer poly(A) tails apparently extended about 20 adenes, on average, than those in the wild type cells (Fig. 2, lanes 4). The poly(A) tail of the cytoplasmic β-actin mRNA was not affected by the knockdown of hPNPase. This result indicates hPNPase functions primarily in vivo in human mitochondria as an exonuclease rather than as a polymerase.

Steady State mt mRNA and Translated Product Levels Are Significantly Decreased When hmtPAP Is Partially Inactivat- ed—We examined the role polyadenylation plays in the stabi- lization and translation of mt mRNAs by Northern and West- ern blotting analyses of the siRNA-transfected HeLa cells, respectively. A marked decrease in the steady state levels of the mt mRNAs for CO1, CO2, CO3, and ATP6 was observed when hmtPAP but not hPNPase was knocked down, whereas ND3 mRNA was not affected (Fig. 3A, see “Discussion”). As a control, mt 16 S rRNA and cytosolic β-actin mRNA levels were unaffected by the siRNA-hmtPAP transfection (Fig. 3A). These results indicate that the function of hmtPAP differs from that of bacterial PAP, whose polyadenylation of mRNAs is involved in
their degradation (7, 8). Western blotting also showed that knockdown of hmtPAP markedly reduced the steady state levels of mt encoded proteins (CO1 and CO2) but had no effect on the levels of nuclear encoded mt proteins (CO4 and HSP60) or the cytosolic β-actin protein (Fig. 3B, lane 3). This is consistent with the effect of hmtPAP knockdown on mt mRNA levels shown in Fig. 2A. Knockdown of hPNPase had no effect on protein levels (Fig. 3B, lane 4); a similar effect has been reported for Arabidopsis chloroplast PNPase (37).

Knocking Down of hmtPAP Results in Mitochondrial Dysfunction—The morphology and membrane potential (ΔΨ) of the mitochondria in the siRNA-transfected HeLa cells were stained with both MitoTracker CMXRos and GreenFM and analyzed by confocal fluorescent microscopy (Fig. 3C). Both agents are fluorescent indicators of mitochondria, and MitoTracker CMXRos is also an indicator of ΔΨ. The mitochondria of the control siRNA-scramble- or siRNA-hPNPase-transfected cells display good staining with both dyes and a well developed filamentous meshwork. In contrast, the hmtPAP-knockdown cells had granular-shape mitochondria because of fragmentation of the meshwork and stained poorly with MitoTracker CMXRos. Judging from the overlay images obtained using the two dyes (Fig. 3C, middle panels), the ΔΨ of the hmtPAP-knockdown cells was severely decreased.

We also analyzed the rate of oxygen consumption of the hmtPAP-knockdown cells, which revealed a 50% decrease in oxygen consumption by these cells compared with the siRNA-scramble- or siRNA-hPNPase-treated cells (Fig. 3D). The hPNPase-knockdown cells did not show decreased oxygen consumption compared with the siRNA-scramble-treated cells (Fig. 3D). These results suggest that mitochondrial activity is reduced by the knocking down of hmtPAP, which accords well with the fact that the steady state levels of mtDNA-encoded mRNAs and their protein products are reduced in siRNA-hmtPAP-treated cells (Fig. 3).

Recombinant hmtPAP Has Polyadenylation Activity In Vitro—So far, our data show that hmtPAP is required for the polyadenylation of mt mRNAs. To confirm that hmtPAP polyadenylates mt mRNAs, hmtPAP cDNA lacking the predicted mt targeting sequence was cloned into an expression vector, and recombinant hmtPAP bearing a His tag was overexpressed as a soluble form in E. coli and purified by cobalt affinity purification (Fig. 4A). We also prepared recombinant
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hPnPase without the predicted mt transit peptide (Fig. 4B) to examine whether it has polyadenylation activity in vitro. *E. coli* or chloroplast PNPase can both synthesize RNA by using nucleotide diphosphate or triphosphate as substrate and exonucleolytically degrade RNA in the presence of inorganic phosphate (33, 35). The polymerization activity of hPNPase has not been reported, whereas the phosphate-dependent exonuclease activity of hPNPase was confirmed (38). The ability of the purified hmtPAP or hPnPase protein to polyadenylate in vitro was examined using oligo(A) or bovine mt ND6 mRNA, and [α-^32^P]ATP as substrates. Denaturing PAGE clearly revealed elongated poly(A) tails, which indicates that recombinant hmtPAP and hPnPase induces the incorporation of [α-^32^P]AMP into oligo(A) as well as bovine mt ND6 mRNA (Fig. 4, A and B, lanes 2 and 5). To rule out the possibility that the polyadenylation is because of contamination of host proteins, mock-purified materials from an *E. coli* strain harboring the empty pET plasmid was used (lanes 3 and 6). Lane 1 shows [α-^32^P]-labeled oligo(A)₅₃ before polyadenylation.

**FIG. 4.** *In vitro* PAP activity of recombinant hmtPAP and hPnPase. A, *in vitro* PAP activity of recombinant hmtPAP stained with Coomassie Brilliant Blue (right lane). The molecular marker protein sizes are indicated. Middle and right panels, PAP activity of recombinant hmtPAP. oligo(A)₃₅ middle panel) or bovine mt ND6 mRNA (right panel was incubated with recombinant hmtPAP (lanes 2 and 5) or *E. coli* PAP (lanes 4 and 7) under the conditions described under “Experimental Procedures.” As a negative control, mock-purified material from cells harboring the empty pET plasmid was used (lanes 3 and 6). Lane 1 indicates the position of the substrate RNA ([α-^32^P]-labeled oligo(A)₅₃). Reactions were analyzed by denaturing PAGE containing 7 M urea. The positions of RNA markers are indicated. The expected size of mt ND6 mRNA is 544 bases. B, *in vitro* PAP activity of recombinant hPnPase. Left panel, SDS-PAGE analysis of the His-tagged recombinant hPnPase stained with Coomassie Brilliant Blue (right lane). The molecular marker protein sizes are shown. Middle and right panels, PAP activity of recombinant hPnPase. oligo(A)₃₅ middle panel) or bovine mt ND6 mRNA (right panel) was incubated with recombinant hPnPase (lanes 2 and 5) or *E. coli* PNP (lanes 4 and 7) under conditions described under “Experimental Procedures.” Mock-purified material from cells harboring the empty pET plasmid was used (lanes 3 and 6). Lane 1 shows [α-^32^P]-labeled oligo(A)₅₃ before polyadenylation.

**DISCUSSION**

We showed in this paper that mt polyadenylation is regulated by the mitochondria-specific enzymes hmtPAP and hPnPase and that the polyadenylation is involved in mRNA stabilization, as well as generating stop codons in some mt mRNAs. Recently, Lightowler and co-workers (22) reported that the decreased stability of human mt *ATP6* mRNA with a pathogenic microdeletion (μ9205) at its 3’ end correlates with its shortened poly(A) tail. This deletion results in the loss of terminal uridine, which serves as the 1st letter of the UAA codon that is generated by the polyadenylation of the mRNA. This non-stop mRNA is degraded rapidly in a deadenylation manner, suggesting that the poly(A) tail is required for mRNA stability (22). However, it remains possible that an unnatural RNA decay pathway such as nonsense-mediated decay exists in mitochondria and that this is degrading the non-stop transcripts in this system (39). Here we describe the data showing that poly(A) is indeed involved in normal mt mRNA stability (Fig. 2 and Fig. 3A). When hmtPAP was knocked down by siRNA, steady state level of several mt mRNAs, including *ATP6* mRNA, decreased significantly. Thus, the polyadenylation was found to be needed for mRNA stabilization in human mitochondria. This result is rather surprising because polyadenylation promotes mRNA degradation in bacteria (7, 8), plant chloroplasts (9), and mitochondria (10). It has been suggested by Gagliardi et al. (40) that, similar to the human cytoplasmic situation, human mitochondria may have evolved a system where the longer poly(A) in human mt mRNAs recruits mt poly(A)-binding proteins that promote mt mRNA stability (40).

However, ND3 mRNAs was not degraded if severe deadenylation occurs by the knocking down of hmtPAP (Figs. 2 and 3). Although Stepiew and co-workers (23) reported that several mt mRNAs were relatively stable when they were deadenylated by knocking down of hmtPAP and they suggested long poly(A) is not required for the mt mRNA stability, we clearly showed that mt mRNAs for *CO1*, *CO2*, *CO3*, and *ATP6* were severely degraded when hmtPAP was knocked down, indicating that polyadenylation has a critical role in stabilization of several (but not all) mt mRNAs. In yeast mitochondria, all mRNAs lack the poly(A) tail, and mt mRNAs are stabilized by mRNA-specific factors. For example, ATP8/6 mRNA requires four nuclear genes, *NCA2*, *NCA3*, *NAM1*, and *AEF3*, for its stabilization (12, 41–44). Although no human homolog of these yeast genes has been identified, it can be speculated that the mRNA-specific protein factor(s) with similar function might be involved in the stabilization of human ND3 mRNA.

Conversely, it is also possible that poly(A)-dependent degradation of RNA also occurs in human mitochondria. Polyadenylation has been suggested to be required for the editing of some mt tRNAs during maturation (20, 21). For example, in human mitochondria, tRNA^Tyr^ lacks its discriminator base (adenine) after cleavage from its precursor RNA and thus may require polyadenylation by hmtPAP to obtain this critical nucleotide. After polyadenylation, the extra poly(A) tail must be removed by an exonuclease followed by CCA addition. In this case, the poly(A) does not seem to be long enough for RNA stabilization and seems rather to function as a toehold for an exonuclease such as hPnPase. There are also some reports (45, 46) suggesting that RNA degradation via polyadenylation occurs in eukaryotes and is associated with RNA quality control.

We also showed the steady state levels of mtDNA-encoded proteins are significantly reduced in hmtPAP-knockdown cells (Fig. 3B). When we analyzed *de novo* mt protein synthesis in wild type and the hmtPAP-knockdown cells by [^35^S]methionine pulse-labeling experiments, only a small reduction in the rate
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The evolution of polyadenylation is quite interesting. We did not find an hmtPAP homolog in bacteria, which suggests that hmtPAP may not have originated from bacteria. Rather, hmtPAP may have come from the nucleus as the mt genome became more complex in evolution and polyadenylation was needed for creating stop codons or for the RNA editing that leads to tRNA maturation. The yeast Saccharomyces cerevisiae lacks an hmtPAP homolog, probably because yeast mitochondria do not require polyadenylation for mt gene expression and thus have not had to acquire a PAP from the nucleus. Plants also apparently lack an hmtPAP homolog; however, plants instead have a bacterial class II PAP homolog bearing a mt transit peptide that may function as a PAP in plant mitochondria (50). It is possible that plant mitochondria inherited class II PAP from eubacteria during its endosymbiosis, whereas the animal and yeast mitochondria have evolved otherwise as described above.

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Fig. 5. Schematic depiction of postulated poly(A) metabolism in human mitochondria. A poly(A) tail is added by hmtPAP to produce the UAA stop codon in many mt mRNAs. hmtPAP lacks an RNA-binding domain and its efficient polyadenylating activity may require partnership with an RNA-binding protein. PAP-mediated polyadenylation may also be needed to stabilize mt mRNA to protect it from exonuclease activity (for example, hPNPase). Deadenylation by hPNPase regulates the poly(A) length.

of de novo mt protein synthesis was observed in the hmtPAP-knockdown cells (data not shown). Thus, it seems that mt translation proceeds even when hmtPAP is knocked down. Because siRNA-mediated gene silencing leads to partial inactivation of the target gene, most UAA stop codons seem to be undamaged if severe deadenylation occurs. Furthermore, Lightowlers and co-workers (47) reported that functional polypeptides can still be synthesized from pathogenic ATP6 mRNA lacking the UAA stop codon. However, the slight decrease in the translation rate due to hmtPAP knockdown may cumulatively result in the severely decreased steady state levels of mtDNA-encoded proteins (Fig. 3B). Furthermore, as shown in Fig. 4, C and D, hmtPAP knockdown cells showed deficient mitochondrial activities such as oxygen consumption rate and ΔΨ, which is consistent with the decreased steady state levels of mtDNA-encoded proteins. In contrast, knockdown of hmtPAP did not affect the steady state levels of mtDNA-encoded proteins (Fig. 3B) or the activities of the mitochondria (Fig. 3, C and D). A previous study of a PNPase-deletion mutant from E. coli has shown that the lack of PNPase function can be compensated by the remaining exonuclease activity in the cell (48). Similarly, other mt localized exonuclease(s) may explain the lack of effect of hPNPase knockdown on mitochondrial function.

Fig. 5 shows a model of poly(A) metabolism in human mitochondria. Mitochondrial mRNAs are polyadenylated by hmtPAP, which lacks a RNA-binding motif and may need to be stimulated by a partner RNA-binding protein and/or other components. Poly(A) addition is needed not only for creating the UAA stop codon of many mt mRNAs but also for stabilizing mt mRNAs; the latter function may be facilitated by a poly(A)-binding protein. Therefore, hmtPAP is a key enzyme regulating both translation termination and mRNA metabolism in human mitochondria. In contrast, hPNPase acts degradatively, similar to E. coli or Arabidopsis organellar PNPases (6, 37, 49), and thus controls the extent of mitochondrial polyadenylation. However, it is also shown that hPNPase has in vitro PAP activity (Fig. 4B). Thus, it may be possible that hPNPase, like E. coli PNPase (33), acts as the polyadenylating enzyme toward mt mRNAs.
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