

Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins

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ABSTRACT

Human mitochondrial long noncoding RNAs (lncRNAs) have not been described to date. By analysis of deep-sequencing data we have identified three lncRNAs generated from the mitochondrial genome and confirmed their expression by Northern blotting and strand-specific qRT-PCR. We show that the abundance of these lncRNAs is comparable to their complementary mRNAs and that nuclear-encoded mitochondrial proteins involved in RNA processing regulate their expression. We also identify the 5' and 3' transcript ends of the three lncRNAs and show that mitochondrial RNase P protein 1 (MRPP1) is important for the processing of these transcripts. Finally, we show that mitochondrial lncRNAs form intermolecular duplexes and that their abundance is cell- and tissue-specific, suggesting a functional role in the regulation of mitochondrial gene expression.

Keywords: noncoding RNA; mitochondrial RNA; RNA processing

INTRODUCTION

Eukaryotic transcriptomes have been shown to be extremely complex. The discovery of noncoding RNAs (Okazaki et al. 2002) has expanded the regulatory repertoire of gene expression and our view beyond the central dogma where RNA couples gene expression to protein synthesis (Mattick 2003). An extraordinary range of noncoding RNAs are generated from sense or antisense interspersed intronic or genomic regions overlapping with coding and noncoding sequences, and their expression and function are tightly linked to developmental processes (Carninci et al. 2005; Amaral and Mattick 2008; Mercer et al. 2009). Long ncRNAs (lncRNAs) are diverse in sequence and structure, and genome-wide technologies allow the classification and analysis of their functional relevance (Carninci et al. 2005; Kapranov et al. 2005, 2007). The function of lncRNAs includes the epigenetic control of chromatin structure (Mattick et al. 2009) as well as transcriptional and post-transcriptional gene regulation (Mercer et al. 2009). There are also many types of small regulatory RNAs found in the nucleus and cytoplasm of animal cells (Mattick and Makunin 2005; Taft

et al. 2009, 2010). Interestingly, to date only six small ncRNAs have been identified in mitochondria (Lung et al. 2006) as well as short inverted repeats that form extensions of the mitochondrial 16S rRNA (Villegas et al. 2007). However, lncRNAs generated from the mammalian mitochondrial genome have not been identified.

The mitochondrial genome, unlike the complex nuclear genome, is a compact, circular, double-stranded DNA encoding only 13 proteins, which are all subunits of the electron transport chain, as well as two rRNAs and 22 tRNAs required for their translation (Smeitink et al. 2001). Mitochondrial genes for proteins and tRNAs are located on both the heavy and light strands of the genome, which are transcribed as large polycistronic transcripts covering almost the entire length of each strand (Aloni and Attardi 1971; Murphy et al. 1975; Montoya et al. 1981; Mercer et al. 2011). A third transcript covering the start of the heavy strand and the two rRNA genes is also produced (Christianson and Clayton 1988). These long precursor mitochondrial transcripts undergo processing to form functional RNAs (Ojala et al. 1981). In nearly all cases, coding genes are interspersed by one or more tRNAs, which act as "punctuation" marks for processing by RNase P at the 5' end of tRNAs (Holzmann et al. 2008) and by the mitochondrial RNase Z, *elaC* homology 2 (ELAC2), at the 3' end of tRNAs (Takaku et al. 2003; Brzezniak et al. 2011; Lopez Sanchez et al. 2011). A CCA triplet is added to the 3' ends of tRNAs and specific bases

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within both tRNAs and rRNAs are often modified, while mRNAs are generally polyadenylated at their 3' ends. Recently, using a deep-sequencing approach to characterize the 5' and 3' ends of all 22 mitochondrial tRNAs, we have found that the regulation of the processing of mitochondrial tRNAs has profound effects on mitochondrial gene expression (Lopez Sanchez et al. 2011). We found that knockdown of the four nuclear-encoded mitochondrial proteins ELAC2, mitochondrial RNase P proteins 1 and 3 (MRPP1 and MRPP3), and pentatricopeptide repeat domain protein 1 (PTCD1) affects the levels of mitochondrial RNAs and their final processing sites (Lopez Sanchez et al. 2011).

Here we have used deep-sequencing data to discover RNAs generated from noncoding sequences of the mitochondrial genome. We have identified three abundant mitochondrial lncRNAs and have found that their expression is regulated by nuclear-encoded mitochondrial processing proteins, in particular, those that comprise the mitochondrial RNase P complex. We show that all three lncRNAs form intermolecular duplexes and their abundance varies in different cell lines and tissues, suggesting that mitochondrial lncRNAs may have functional significance that contributes to the regulation of mitochondrial gene expression.

RESULTS/DISCUSSION

The mitochondrial genome generates three stable lncRNAs

The mitochondrial polycistronic transcript encoding the heavy-strand genes has little noncoding sequence. In contrast, the light-strand polycistronic transcript only encodes seven tRNAs and the *ND6* mRNA that are separated by long stretches of noncoding sequences. It is not entirely clear whether the noncoding sequences are degraded or whether any of them are abundant and functionally significant. We used data sets from strand-specific deep sequencing to analyze the presence of lncRNAs in the transcriptome of HeLa mitochondria. We observed that a significant proportion (15.02%, excluding rRNA and tRNA) of reads that uniquely aligned to the mitochondrial genome correspond to noncoding DNA (Fig. 1A). The regions of the mitochondrial genome complementary to the genes that encode *ND5*, *ND6*, and *Cyt b* mRNAs were found to have high levels of lncRNAs (Fig. 1B). The region on the heavy strand that is complementary to the *ND6* mRNA is known to be retained as the 3' untranslated region (UTR) of the mature *ND5* mRNA; however, we also found that it is a lncRNA in its own right (see below). The three mitochondrial lncRNAs are punctuated by numerous stop codons and there are no significant open reading frames present (Supplemental Fig. 1). The longest opening reading frame is only 67 amino acids, located in the middle of lnc*ND6* RNA (Supplemental Fig. 1). Therefore, we identified three mitochondrial lncRNAs, lnc*ND5*, lnc*ND6*, and lnc*Cyt b* RNA within the mitochon-

drial transcriptome (Fig. 1; Supplemental Fig. 1). In addition, the parallel analyses of RNA ends (PARE) and poly(A) analyses of RNA isolated from HeLa cells revealed the canonical 5' and 3' ends of mitochondrial coding transcripts (Ojala et al. 1981) as well as potential transcript ends of the lncRNAs (Fig. 1B), which we further investigated here.

We used strand-specific qRT-PCR (ss qRT-PCR) to measure the abundance of these lncRNAs and compared them with their complementary transcripts, which encode functional mRNAs. We found that these three lncRNAs encoded by the light strand are 58%, 34%, and 14% as abundant as their complementary coding *ND5*, *ND6*, and *Cyt b* mRNAs, respectively (Fig. 1C). We observed that there was a 1867-fold higher abundance of sense to antisense RNA from the 12S rRNA region, indicating that the ssqRT-PCR provides at least three orders of magnitude higher sensitivity for the target strand of interest over its complementary sequence (Fig. 1C).

Nuclear-encoded proteins regulate the levels of mitochondrial lncRNAs

Recently, we have shown that ELAC2, MRPP1, MRPP3, and PTCD1 affect the levels of mature tRNA, rRNA and mRNA expression, and the accumulation of precursor transcripts at the RNA processing level (Lopez Sanchez et al. 2011). Pentatricopeptide repeat (PPR) domain protein 2 (PTCD2) belongs to the family of PPR RNA-binding proteins, which includes PTCD1 and MRPP3, and are involved in the regulation of mammalian mitochondrial gene expression (Rackham and Filipovska 2011), and has been found to affect the processing of the *Cyt b* mRNA (Xu et al. 2008). We performed ss qRT-PCR on RNA isolated from purified mitochondria of HeLa cells where ELAC2, MRPP1, MRPP3, PTCD1, or PTCD2 were knocked down after 6 d to $3.5\% \pm 0.4$, $1.6\% \pm 0.5$, $1.9\% \pm 0.6$, $5.3\% \pm 0.8$, and $2.2\% \pm 0.5$, respectively, by siRNAs and used nontargeting (NT) siRNAs as a control to determine the contribution of these proteins to the regulation and abundance of lncRNA compared with coding mRNA expression (Fig. 2A). In addition, by using antibodies against ELAC2, MRPP1, MRPP3, PTCD1, or PTCD2, we show that their protein abundance is significantly decreased (Supplemental Fig. 2A). We show knockdown of MRPP1 caused the most dramatic decrease in the abundance of the three lncRNAs (Fig. 2A). Knockdown of MRPP3 decreased the levels of the lncRNAs, although the effect was less dramatic compared with MRPP1. This may be a result of the stability of the MRPP3 protein compared with MRPP1, since their respective mRNAs were knocked down to similar levels. Decrease in ELAC2 levels caused a more subtle decrease in all lncRNAs, whereas PTCD1 knockdown caused a specific decrease in the *ND5* lncRNA (Fig. 2A). PTCD2 has been shown to affect *Cyt b* mRNA processing in the heart (Xu et al. 2008). Therefore, we investigated the effect of PTCD2 knockdown

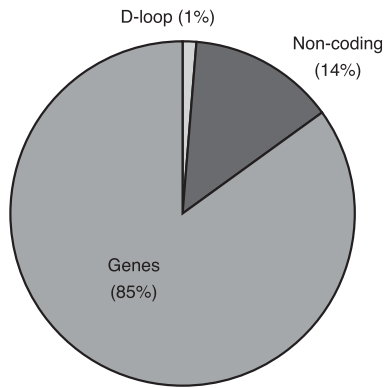
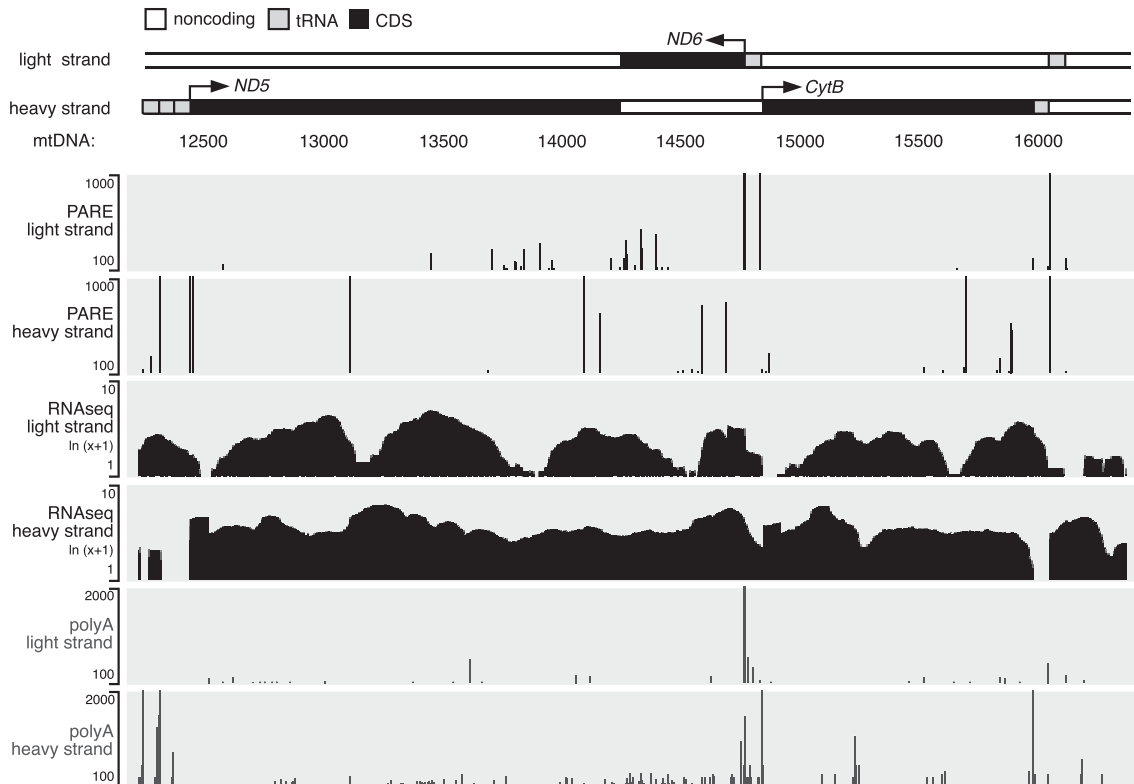
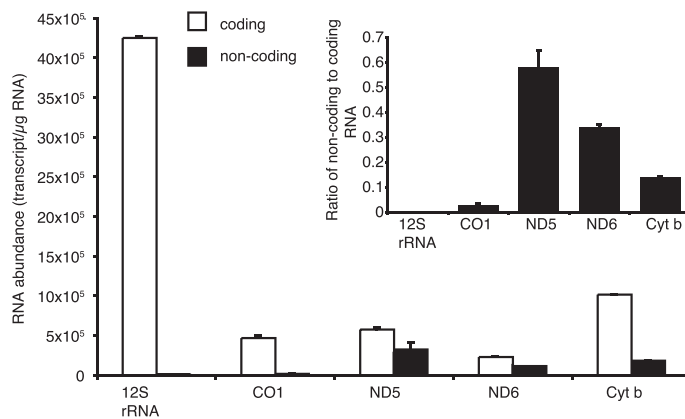
A**B****C**

FIGURE 1. Long noncoding RNAs are generated from the light strand of the mitochondrial genome. (A) Noncoding RNAs make up 15% of the human mitochondrial transcriptome, excluding rRNAs and tRNAs. (B) Deep sequencing detected the existence of mitochondrial *ND5*, *ND6*, and *Cyt b* lncRNAs. PARE analyses detect the 5' ends of mitochondrial transcripts and poly(A) analyses detect the polyadenylated 3' ends of mitochondrial transcripts. (C) Abundance of the noncoding *ND5*, *ND6*, and *Cyt b* RNAs compared with coding mRNAs and the 12S rRNA determined by strand-specific qRT-PCR.

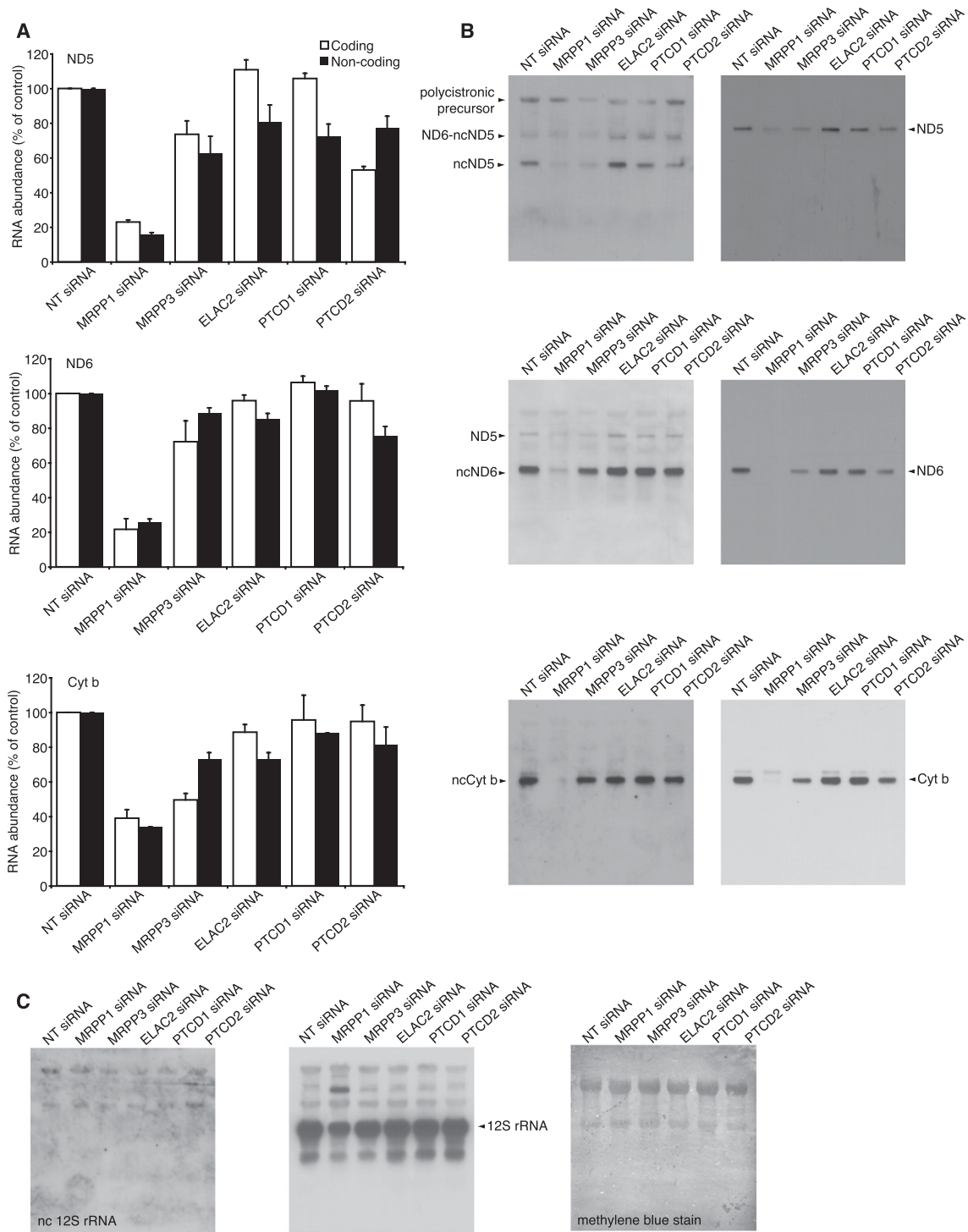


FIGURE 2. Mitochondrial lncRNAs are regulated by nuclear-encoded proteins involved in mitochondrial RNA processing. **(A)** Strand-specific qRT-PCR showing the effects of mitochondrial RNA processing proteins on lncRNAs. The effects of ELAC2, MRPP1, MRPP3, PTCD1, and PTCD2 knockdown in HeLa cells were analyzed on mitochondrial transcripts by ss qRT-PCR. **(B)** RNA isolated from cells transfected with ELAC2, MRPP1, MRPP3, PTCD1, or PTCD2 siRNA and nontargeting (NT) siRNA was analyzed for mitochondrial noncoding and complementary coding RNA abundance by Northern blotting. **(C)** The 12S rRNA and the complementary noncoding region of 12S rRNA were probed as controls. The data are typical of results repeated on four separate RNA preparations. Each Northern blot was probed for all transcripts by stripping the blot and reprobing. The methylene blue-stained blot shows equal loading of RNA to each well.

on the mitochondrial lncRNAs. Interestingly, we found that PTC2 decreased the levels of *ND5* lncRNA and the lnc*ND6* RNA, which can form the 3' UTR of the *ND5* mRNA (Fig. 2A). These observed changes suggest an important contribution of the five nuclear-encoded proteins to the regulation of mitochondrial gene expression, possibly reflecting their roles in processing the precursor transcripts from which both coding and noncoding RNAs are derived.

MRPP1 is necessary for the accumulation of mitochondrial lncRNAs

We used Northern blotting to detect and compare the abundance of the mitochondrial ncRNAs and their complementary mRNAs and rRNA (Fig. 2B). We show that the lnc*ND5*, lnc*ND6*, and lnc*Cyt b* RNAs are abundant in mitochondria. In contrast, overexposure of the blot probed for the noncoding transcript of the *12S* rRNA showed that there were no abundant RNAs corresponding to this region (Fig. 2C), indicating that the *ND5*, *ND6*, and *Cyt b* lncRNAs are specifically enriched in mitochondria. We further investigated the effects of knocking down the RNA processing proteins on the levels of *ND5*, *ND6*, and *Cyt b* lncRNAs and their complementary mRNAs. We found that MRPP1, and in most cases, MRPP3, are important for the abundance of the lncRNAs (Fig. 2B). Knockdown of PTC2 caused a specific decrease of the *ND5* lncRNA. We used a probe that was complementary to the 3' UTR alone to detect the *ND6* lncRNA. We were surprised to detect that the 3' UTR of *ND5* was also present independent of the mature *ND5* mRNA, which we describe as lnc*ND6* (Fig. 2B). We show that lnc*ND6* is an abundant transcript that has previously not been annotated or investigated in Northern blots, most likely because probes that were complementary to this region of the *ND5* mRNA have not been used. We observe that in addition to the lnc*ND6*, we also detect the mature *ND5* mRNA that includes the 3' UTR or lnc*ND6*. Interestingly, we do not observe the presence of an *ND5* mRNA that is missing the 3' UTR, suggesting that it may be required for the mature *ND5* mRNA (Fig. 2B). MRPP1 or MRPP3 knockdown lowers the abundance of the *ND5*, *ND6*, and *Cyt b* mRNAs and lncRNAs in a similar manner to that observed with ss qRT-PCR (Supplemental Fig. 2B). In addition, MRPP1 knock down decreases the abundance of the *12S* rRNA and causes the accumulation of a larger mitochondrial precursor transcript containing the *12S* rRNA (Fig. 2C).

Mitochondrial ncRNAs are specifically processed at defined sites

We investigated the effects of protein knockdown on the processing of the 5' and 3' ends of the mitochondrial lncRNAs. We used rapid amplification of cDNA ends (RACE) to show that MRPP1 is responsible for processing of the 5' and 3' ends of the lncRNAs, with the exception of

the 5' end of the *ND6* lncRNA (Fig. 3A). In addition, we observed that MRPP3 and PTC1 knockdown cause a decrease in the processing of the 5' end of the *ND5* lncRNA, whereas ELAC2 has a minor effect on the processing of the 3' end of the *ND6* lncRNA (Fig. 3A). We observed the enrichment of an additional transcript end when ELAC2 is depleted in cells, which is that of a preprocessed *ND6-tRNA^{Glu}* transcript, further supporting our previous findings that ELAC2 processes the 3' end of mitochondrial tRNAs (Lopez Sanchez et al. 2011). Furthermore, we observed that the lnc*ND5* RNA was occasionally found as a 3' UTR of the *ND6* mRNA, as well as an independent lnc*ND5* RNA (Fig. 3A–C). This was also observed in the Northern blot of the lnc*ND5* RNA (Fig. 2B). However, it remains to be determined whether these two isoforms provide an opportunity to differentially regulate *ND6* expression.

Next, we identified the exact transcript ends of the mitochondrial lncRNAs by Sanger sequencing of the RACE products (Fig. 3B). We found that the mitochondrial lncRNAs have a defined length and transcript ends (Fig. 3C). Here we have observed cleavage by the mitochondrial RNase P adjacent to antisense or mirror tRNAs (Fig. 3B), indicating that this enzyme may recognize them as substrates, suggesting that mirror tRNAs may have a functional role as previously suggested (Seligmann 2010a,b). Interestingly, we observed several different transcript ends for some of the lncRNA ends (Fig. 3B), indicating the occasional promiscuity of the mitochondrial RNase P. Recently, it has been shown that the mitochondrial RNase P is an unconventional RNase P in that it lacks an RNA component (Holzmann et al. 2008). Our findings that the mitochondrial RNase P cleaves adjacent to mirror tRNA sequences with imprecise substrate selection at certain sites provides further evidence of the enzyme's unconventional characteristics and may explain why mammalian mitochondrial RNase P has two additional protein subunits (Holzmann et al. 2008) relative to plants, where the mitochondrial RNase P fulfills a more canonical role in tRNA processing similar to nuclear RNase P (Gobert et al. 2010). Some of the different consequences of MRPP1 and MRPP3 knockdown might reflect the distinct activities of each RNase P protein. For example, in addition to its requirement for RNase P activity, MRPP1 has been shown to have tRNA methyltransferase activity (Lopez Sanchez et al. 2011).

Mitochondrial ncRNAs form intermolecular duplex structures

We investigated whether the three mitochondrial lncRNAs were single or double stranded by ss qRT-PCR using mitochondria isolated from cells, lysed, and treated either with RNase A, an RNA endonuclease that cleaves after cytosine and uracil residues, or with RNase I^f, a single-strand-specific RNA endonuclease. RNase A degraded both the sense and lncRNAs almost completely and to similar extents (Fig. 4A).

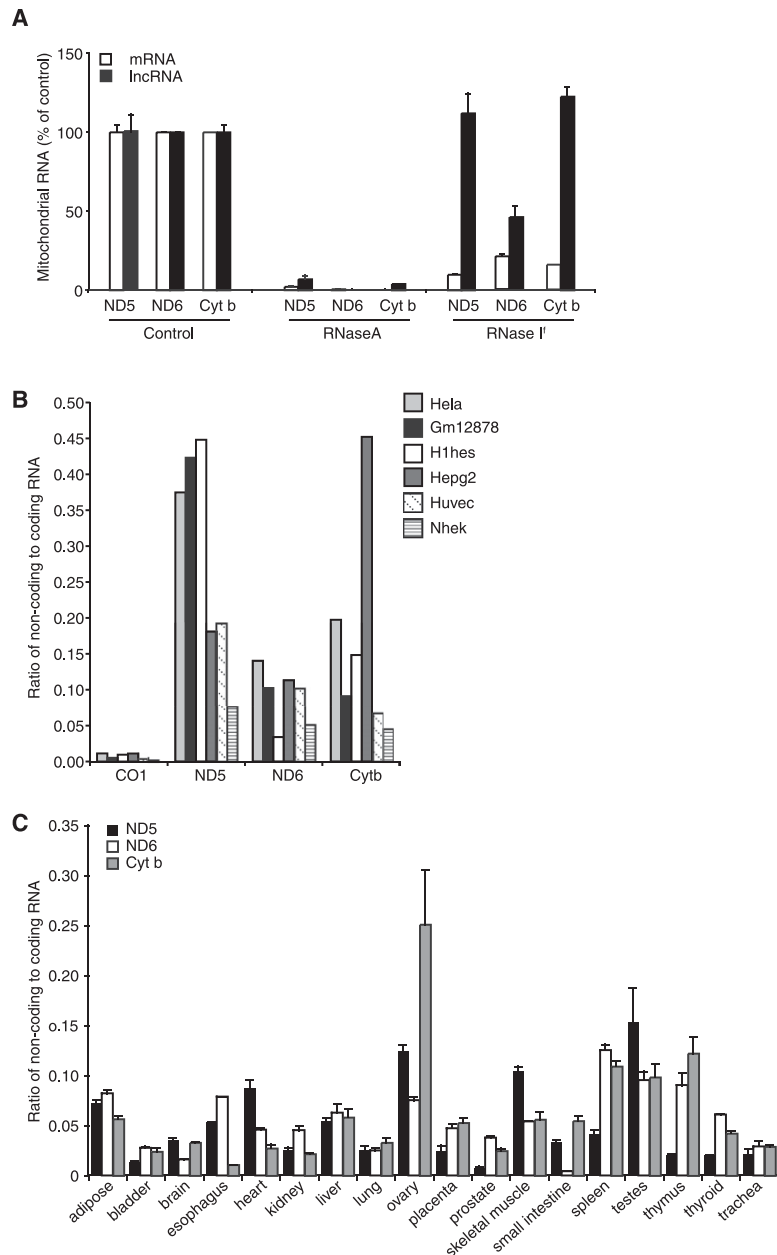


FIGURE 4. Mitochondrial ncRNAs are predominantly found as duplexes and show cell-specific expression. (A) Mitochondria isolated from HeLa cells were lysed and treated with either RNase A or RNase I¹, and lncRNAs and mRNAs were measured by ss qRT-PCR. Mitochondria not treated with RNases were used as a control. Differential abundance of lncRNAs compared with their coding counterparts in different cell lines determined from deep-sequencing data sets (B) and in 18 different human tissues (C) determined by ss qRT-PCR, suggesting that they may have a role in the regulation of mitochondrial gene expression.

stabilize *ND5*, *ND6*, and *Cyt b* mRNAs or regulate their expression. The *ND6* mRNA is the only mitochondrial mRNA that is not polyadenylated (Temperley et al. 2010; Mercer et al. 2011), and the presence of the lnc*ND6* transcript may play a role in stabilizing this mRNA. Moreover, the double-stranded nature of these lncRNAs may impair the translation of their corresponding mRNAs, potentially

explaining why the *ND6* protein is the least abundant mitochondria encoded protein.

Cell and tissue-specific expression of mitochondrial lncRNAs

Lastly, we investigated whether the abundance of mitochondrial lncRNAs varies in different cells in strand-specific deep-sequencing data sets and by ss qRT-PCR of RNA from tissues. We observed a large variation in the abundance of the three lncRNAs in different cell lines (Fig. 4B). In addition, we found that there was variation in the abundance of the three lncRNAs across 18 different human tissues, and lnc*ND5* RNA was the most abundant of the three ncRNAs (Fig. 4C). Interestingly, the lncRNAs are most abundant in reproductive tissues such as ovaries and testes. To investigate whether mitochondrial lncRNAs might have a regulatory role, we tested the ratios of lncRNAs compared with adjacent mRNAs from opposite strands and found that the lncRNA ratios are different compared with the mRNAs that are on the opposite strand, suggesting that the regulation of the lncRNAs may be independent from that of coding transcripts (Supplemental Fig. 3). These data further suggest that the regulation of mitochondrial gene expression varies depending on specific cell-type requirements, and implies that the identified lncRNAs may contribute to the regulation of mitochondrial gene expression.

MATERIALS AND METHODS

Cell culture

HeLa human cervical cancer cells were cultured at 37°C under humidified 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing glucose (4.5 g.l⁻¹), 1 mM pyruvate, 2 mM glutamine, penicillin (100 U.mL⁻¹), streptomycin sulfate (100 μg.mL⁻¹), and 10% fetal bovine serum (FBS).

Transfections

Cells were plated at 60% confluence in 6-well plates or 10-cm dishes and transfected with annealed siRNAs in OptiMEM media (Invitrogen). A total of 125 nM (for 6-well plates) or 145 nM

(for 10-cm dishes) of ELAC2, MRPP1, MRPP3, PTCD1, PTCD2, or control off-target siRNAs (Dharmacon) were transfected using Lipofectamine 2000 (Invitrogen). Cell incubations were carried out for up to 3 d and the cells were retransfected and incubated for a further 3 d following transfection.

Mitochondrial isolation and RNase treatments

Mitochondria were prepared from 15×10^6 cells grown overnight in 15-cm dishes. Cells in PBS were sedimented (150g for 5 min at 4°C), resuspended in 4 mL of ice-cold 10 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl (pH 7.5). Cells were allowed to swell for 5 min on ice and briefly homogenized with a 7-mL glass homogenizer. The sucrose concentration was then adjusted to 250 mM by adding 2 M sucrose in 10 mM Tris-HCl and 1 mM EDTA (pH 7.6) (T₁₀E₂₀ buffer). The nuclei from this suspension were sedimented (1300g for 3 min at 4°C), and the centrifugation step was repeated for the supernatant once more. Mitochondria were sedimented from the supernatant by centrifugation (15,000g for 15 min at 4°C). The mitochondrial suspension was layered on a discontinuous sucrose gradient (1.0 and 1.7 M) in T₁₀E₂₀ buffer and centrifuged at 70,000g for 40 min at 4°C. The mitochondrial fraction was recovered from the interface between the two sucrose cushions, diluted with 400 μL of 250 mM sucrose in T₁₀E₂₀ buffer, and washed once in the same solution. The final mitochondrial pellet was resuspended in 100 μL of 250 mM sucrose in T₁₀E₂₀ buffer and protein concentration as determined by the bicinchoninic acid (BCA) assay (Smith et al. 1985) using bovine serum albumin (BSA) as a standard. For RNase sensitivity assays, mitochondria were lysed in ShortCut reaction buffer (NEB) containing 20 mM MnCl₂ and 0.1% Triton X-100 for 15 min at 37°C in the presence or absence of either 100 μg.mL⁻¹ RNase A or 50 U of RNase I.

RNA isolation and strand-specific quantitative RT-PCR

RNA was isolated from highly purified, mitochondria using the miRNeasy Mini kit according to the manufacturer's instructions (Qiagen). RNA from tissues was purchased from Ambion. Contaminating genomic DNA was eliminated by an on-column RNase-free DNase treatment. To quantify the levels of mitochondrial RNA produced from a specific strand of mitochondrial DNA, total cellular RNA was denatured at 65°C for 5 min and reverse transcribed using SuperScript III (Invitrogen) at 55°C for 1 h in the presence of a gene-specific primer that incorporates additional adaptor sequence. The reverse transcriptase was inactivated by incubation at 70°C for 15 min. qRT-PCR was performed with a gene-specific primer designed to anneal at the adjacent side of the cDNA of interest and another primer complementary to the additional adaptor sequence incorporated in the primer used for reverse transcription. PCR was performed with a Corbett Rotorgene 6000 using Platinum UDG SYBR Green mastermix (Invitrogen) and normalized to 18S rRNA. Control reactions were performed by omitting the reverse transcriptase in the cDNA synthesis; qRT-PCR using this material as a template revealed no detectable amplification.

Deep-sequencing analyses

RNA seq data were downloaded from ENCODE and used in strict accordance with the ENCODE Data Release policy (<http://genome.ucsc.edu/ENCODE/index.html>). Directional RNA sequenced reads were used to identify sites of nontemplate poly(A)

additions to the mitochondrial transcriptome using the following approach. RNA sequencing data from HeLa and cell lines data were generated as part of the ENCODE project (Birney et al. 2007) and used in strict accordance with the Data Release policy (<http://genome.ucsc.edu/ENCODE/index.html>). Raw data reads are available from: <http://hgdownload.cse.ucsc.edu/goldenPath/hg18/encodeDCC/wgEncodeCaltechRnaSeq>. Sequenced reads were aligned to the human genome requiring exact matching. Those reads that did not map exactly to the genome were then trimmed of three or more –A nucleotides from the 3' end. Trimmed reads longer than 15 nt were then realigned to the human genome again with those reads that mapped uniquely and exactly following 3' poly(A) trimming were indicative of polyadenylation sites. Nucleotide sites were additionally required to have evidence of 10 or more aligned reads for consideration in analysis. PARE sequencing data were generated from HeLa cell lines (Shin et al. 2010). Raw data reads are accessible from GEO (Accession ID GSE22068): <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22068>. Sequenced reads were initially trimmed of adaptor sequence and aligned uniquely and exactly to the human mitochondrial genome with ZOOM (Lin et al. 2008) with up to two mismatches, permitting sequenced reads to overlap SNPs or modified nucleotides. Read mapping was converted into .bed and .wig format for visualization on the UCSC genome browser (genome.ucsc.edu).

Rapid amplification of cDNA ends (RACE)

We polyadenylated the 3' ends of RNAs isolated from HeLa cells where MRPP1, MRPP3, ELAC2, PTCD1, and PTCD2 were knocked down with *Escherichia coli* poly(A) polymerase (NEB) ligated RNA adaptors to the RNA 5' ends, and reverse transcribed with an oligo d(T) primer. Rapid amplification of cDNA ends (RACE) was then performed to identify the 5' or 3' ends of the cDNA of interest.

Northern blotting

RNA (4 μg) was resolved on 1.2% agarose formaldehyde gels, then transferred to 0.45 μm of Hybond-N⁺ nitrocellulose membrane (GE Lifesciences) and hybridized with biotinylated oligonucleotide probes specific to mitochondrial RNAs. The hybridizations were carried out overnight at 50°C in 5x SSC, 20 mM Na₂HPO₄, 7% SDS, and 100 μg.mL⁻¹ heparin, followed by washing. The signal was detected using a streptavidin-linked horse radish peroxidase (diluted 1:2000 in 3x SSC, 5% SDS, 25 mM Na₂HPO₄ at pH 7.5) by enhanced chemiluminescence (GE Lifesciences).

Immunoblotting

Specific proteins were detected using rabbit αELAC2, αPTCD1 (Sigma, diluted 1:500), αMRPP1, αMRPP3, αPTCD2 (Abcam, diluted 1:500) antibodies, and mouse αporin antibody (MitoSciences, diluted 1:2000) in 1% skim milk powder in PBS (4.3 mM sodium phosphate, dibasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate, monobasic, PBS). The primary antibodies were detected using goat anti-mouse or goat anti-rabbit horse radish peroxidase (Biorad, diluted 1:10,000).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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