Assignment of 2′-O-Methyltransferases to Modification Sites on the Mammalian Mitochondrial Large Subunit 16 S Ribosomal RNA (rRNA)*

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Summary: RNA (rRNA) methylation is required for ribosome assembly and/or function. Knockdown of MRM1, MRM2, and RNMTL1 increases susceptibility of mitochondrial 16 S rRNA to site-specific cleavage by DNAzymes. The enzymes responsible for all known 2′-O-ribose methylations of 16 S rRNA are now identified. Assignment of rRNA modifications to nucleoid-associated proteins implies that mitochondrial ribosome biogenesis begins at the nucleoid.

Background: rRNA methylation is required for ribosome assembly and/or function. Knockdown of MRM1, MRM2, and RNMTL1 increases susceptibility of mitochondrial 16 S rRNA to site-specific cleavage by DNAzymes. The enzymes responsible for all known 2′-O-ribose methylations of 16 S rRNA are now identified. Assignment of rRNA modifications to nucleoid-associated proteins implies that mitochondrial ribosome biogenesis begins at the nucleoid.

Results: Knockdown of MRM1, MRM2, and RNMTL1 increases susceptibility of mitochondrial 16 S rRNA to site-specific cleavage by DNAzymes. The enzymes responsible for all known 2′-O-ribose methylations of 16 S rRNA are now identified.

Conclusion: The enzymes responsible for all known 2′-O-ribose methylations of 16 S rRNA are now identified.

Significance: Assignment of rRNA modifications to nucleoid-associated proteins implies that mitochondrial ribosome biogenesis begins at the nucleoid.

Advances in proteomics and large scale studies of potential mitochondrial proteins have led to the identification of many novel mitochondrial proteins in need of further characterization. Among these novel proteins are three mammalian rRNA methyltransferase family members RNMTL1, MRM1, and MRM2. MRM1 and MRM2 have bacterial and yeast homologs, whereas RNMTL1 appears to have evolved later in higher eukaryotes. We recently confirmed the localization of the three proteins to mitochondria, specifically in the vicinity of mtDNA nucleoids. In this study, we took advantage of the ability of 2′-O-ribose modification to block site-specific cleavage of RNA by DNAzymes to show that MRM1, MRM2, and RNMTL1 are responsible for modification of human large subunit rRNA at residues G1445, U1369, and G1370, respectively.

Mitochondria are known for their roles in generating energy and programmed cell death. Although mammalian mitochondrial proteins are mostly encoded by the nuclear genome and imported into the organelle, mitochondria maintain a compact genome encoding 13 mRNAs, 2 rRNAs, and 22 tRNAs completely dedicated to the synthesis of a minor fraction of the subunits of the electron transport chain. Mitochondrial dysfunction is implicated in aging, as well as a broad range of diseases including diabetes, cancer, Parkinson disease, Alzheimer disease, Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), Leber’s hereditary optic neuropathy (LHON), and progressive external ophthalmoplegia (PEO) (1, 2). Many of the disease mutations are found in tRNA or mRNA sequences; however, some have been found in the 12 S and 16 S rRNAs (3–6) and nuclear-encoded mitochondrial ribosomal proteins (MRPs)2 (7–9). Mutations in AFG3L2 or paraplegin, subunits of an AAA protease that processes mitochondrial ribosomal protein MRPL32 and functions in the assembly of the mitochondrial ribosome (mitoribosome), are associated with hereditary spastic paraplegia, spinocerebellar ataxia type 28, and spastic ataxia-neuropathy syndrome, highlighting the importance of proper mitochondrial protein synthesis in mammals (10, 11).

The mammalian mitoribosome (55 S) consists of a small subunit (SSU; 28 S) composed of 12 S rRNA and 31 MRPs, and a large subunit (LSU; 39 S) composed of 16 S rRNA and 51 MRPs. The mitoribosome has many similarities to its ancestral prokaryotic ribosome, although only 14 of the SSU MRPs and 28 of the LSU MRPs are conserved in bacteria. The reduced lengths of mitochondrial rRNAs are accomplished by multiple small deletions that leave a core structure similar to that of the bacterial RNA (12, 13), conserving the regions participating in the peptidyl transferase center. Bacterial and mitochondrial ribosomes also share susceptibility to certain antibiotics including chloramphenicol, tetracyclines, and linezolid.

Proteomics has identified a nearly complete set of mitochondrial ribosomal proteins (14–21), some of which appear to be multifunctional (DAP3, AURKAIP, CRIF1, ICT1) (18, 22, 23). Our understanding of the mitoribosome structure is improving with advancements in cryo-EM technology (12, 24–28), but the assembly process has received little attention. We have recently used microscopy and stable isotope labeling by/with amino acids in cell culture (SILAC) proteomic analysis to show that a subset of newly synthesized mitochondrial ribosomal proteins participates in the early stages of ribosome assembly at the nucleoid (29).

An important step in mitoribosome assembly is the modification of rRNA at conserved regions, often in catalytic domains. The small subunit 12 S rRNA is dimethylated by TFB1M at two adjacent adenosines, 936 and 937 (30). Recently, NSUN4 has been described as a cytosine 5-methyltransferase, acting with adjacent adenosines, 936 and 937 (30). Recently, NSUN4 has been described as a cytosine 5-methyltransferase, acting with MRPL32 and functions in the assembly of the mitochondrial ribosome (mitoribosome), which is involved in the localization of the three proteins to mitochondria, specifically in the vicinity of mtDNA nucleoids. In this study, we took advantage of the ability of 2′-O-ribose modification to block site-specific cleavage of RNA by DNAzymes to show that MRM1, MRM2, and RNMTL1 are responsible for modification of human large subunit rRNA at residues G1445, U1369, and G1370, respectively.

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bose methylations at G$^{1145}$, U$^{1369}$, and G$^{1370}$ and a pseudouridylation at U$^{1397}$. We have identified RNMTL1 as a novel mitochondrial rRNA methyltransferase localized at nucleoids involved in the 2'-O-methylation of G$^{1370}$, a unique site that is not methylated in bacterial or yeast mitochondrial rRNA (38). Studies in yeast mitochondria suggest that human homologs of MRM1 and MRM2, both of which we found to co-localize with nucleoids, are involved in 16 S rRNA methylation. These modifications occur at sites that are conserved in bacteria and yeast mitochondria, and contribute to the catalytic domain of the mitoribosome, the peptidyl transferase center, suggesting that they are important for function, yet the exact role of each modification is still unclear (39–42).

There are several established methods of detecting 2'-O-ribose methylation on RNA, including resistance to RNase H when hybridized to a chimeric oligonucleotide (43), splint ligation (44), reverse transcription coupled to PCR (45), mass spectrometry, two-dimensional TLC, boronate affinity chromatography, and other chemical tests (46). One of the most common methods involves inhibition of reverse transcriptase at low deoxynucleotidetriphosphate levels (47), as we have recently applied (38). However, a method that has not received much attention is the use of DNAzymes, deoxyoligonucleotides that can anneal to RNA and direct cleavage at a specific unmodified sequence. DNAszymes were selected in a self-amplifying screen (48) and applied to detecting 2'-O-methylation of yeast nucleo-cytosolic rRNA (34). Here, we combine the use of DNAszymes and Northern blotting for increased sensitivity and specificity to show that MRM1, MRM2, and RNMTL1 are responsible for the 2'-O-ribose methylation of G$^{1145}$, U$^{1369}$, and G$^{1370}$ on 16 S rRNA, respectively.

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**HeLa and HEK293 cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Silencer® Select siRNA (Ambion) sequences are as follows: siMRM1-A (5’-CCAGCCCAUGUUUAGUA-3’), siMRM1-B (5’-GAAAGUGAGGGAAAGU-UU-3’), siMRM2-A (5’-ACAUCUGAGCCCCAAGU-3’), and siMRM2-B (5’-GAAAGUGAAGGAUCUAAA-3’). HeLa cells were reverse-transfected with 3 or 6 nM siRNA and Lipofectamine® RNAiMAX (Life Technologies) for 3 days, as described (38). Antibodies are listed with their suppliers and dilutions: MRM1 (Sigma-Aldrich, 1:3,000), MRM2 (Abcam; 1:1,000), and succinate dehydrogenase subunit A (SDHA, MitoSciences, 1:10,000).

**RNA Isolation—**HeLa cells treated with siRNA were trypsinized and collected by centrifugation at 500 × g for 5 min at 4 °C. Cells were resuspended in PBS and repelleted. 1 ml of TRizol® was used to lyse the cells, and RNA was isolated according to the manufacturer’s instructions. The RNA was subject to an additional phenol-chloroform extraction and diethyl ether extraction to obtain highly purified RNA. The concentration and purity of RNA was detected by a NanoDrop 1000 spectrophotometer.

**DNAszyme-mediated RNA Cleavage—**The 8-17-type DNAszyme sequences used for each site are: G1144 (5’-TCCGAGGTCCGCTATTAGCAATACGAAACCGAAATTTTTT-3’), G1145 (5’-TCAGGAGATCGATAGCAATACGAAACCGAAATTTTTT-3’), and U1369 (5’-TATACTGGTAATATTACGATAACGAAACCGAAATTTTTA-3’), and the 10-23-type DNAszyme sequence for G1370 was (5’-TATTAATCTGGTAGAG-GCTAGTACACCAAGAAACCGAAACCGTTTTA-3’). The underlined sequences correspond to the catalytic region of the DNAszyme that is flanked by targeting sequences to direct the site of cleavage. The reactions were carried out as in Refs. 34 and 48, with minor modifications. 8-17-type reactions were performed by mixing 0.75–4 μg of RNA with 400 pmol of DNAszyme and supplementing with DEPC-treated water to 32 μl. The nucleic acids were boiled in a water bath for 2 min and then cooled to room temperature for 10 min followed by the addition of an equal volume of 2× buffer (200 mM KCl, 800 mM NaCl, 100 mM Heps, pH 7.5, 15 mM MgCl2, 15 mM MnCl2). The reaction was incubated at 37 °C for 1 h and then stopped by the addition of 3 μl of DEPC-treated 0.25 M EDTA, 1 μl of 10 mg/ml glycerol as carrier, 100 μl of DEPC-treated 0.3 M sodium acetate in TE (10 mM Tris, pH 7.5, 1 mM EDTA) buffer, and 2.5 volumes of 100% ethanol. The RNA was precipitated at −20 °C overnight and spun in a desktop centrifuge at 17,000 × g for 10 min at 4 °C. The supernatant was discarded, and the pellet was washed in 70% ethanol, spun again, and dried in a vacuum centrifuge. The RNA was resuspended in a small volume of TE, typically 6 μl. The 10-23-type reactions were performed by mixing RNA with 400 pmol of DNAszyme supplemented with DEPC-treated water to 12 μl and an equal volume of 2× buffer (20 mM NaCl, 8 mM Tris, pH 8) and boiling for 3 min, chilled on ice for 5 min, and then incubated at room temperature for 10 min. 6 μl of 5× buffer (750 mM NaCl, 200 mM Tris, pH 8) and 2 μl of 300 mM MgCl2 were added. The mixture was incubated at 37 °C for 1 h and stopped as with the 8-17-type reactions. Control reactions used water in place of DNAszyme.

**RNA Analysis and Northern Blotting—**Denaturing agarose gels were performed as (49) with minor modifications. 9 μl of RNA loading solution (80% deionized formamide, 5% formaldehyde, 20 mM EDTA, 40 μg/ml ethidium bromide, 0.05% bromphenol blue, 0.05% xylene cyanol) were added to 6 μl of the resuspended RNA and heated in a water bath at 65 °C for 5 min and then loaded onto a 2% agarose/MOPS gel with no formaldehyde. The gel was run in 1× MOPS, pH 7.0, for 2.5 h at 83 V with a buffer exchange system. The ethidium bromide-stained gels was photographed and the RNA was then transferred to a Hybond-N+ membrane (GE Healthcare) in 10× SSC (1.5 mM NaCl, 150 mM sodium citrate) overnight by capillary action. The membrane was auto-cross-linked by a UV Stratallinker (Stratagene) and baked in a vacuum at 80 °C for 2 h. ULTRAhyb prehybridization buffer (Ambion) was applied according to the manufacturer’s instructions, and a biotinylated RNA probe was hybridized overnight at 68 °C. The membrane was then washed two times in 2× SSC, 0.5% SDS, and then two times in 0.2× SSC, 0.5% SDS for 10 min each at 65 °C. RNA was detected by streptavidin linked to alkaline phosphatase (BrightStar® BioDetect kit; Ambion) according to the manufacturer’s instructions. The biotinylated probe was generated in *in vitro* T7 RNA polymerase transcription of a sequence complementary to residues 142–458 of 16 S rRNA that was cloned into pBS − plasmid in the presence of biotinylated CTP. *In vitro*-synthesized full-
length 16 S RNA was transcribed from a pBSKII insert as described with a 16 S RNA insert as described (38).

**RESULTS**

DNAzymes Can Target the Cleavage of Unmodified RNA at Specific Sites—Baer and Dubin (35) and Dubin et al. (50) used radioactive labeling and RNA fingerprinting to identify the methylation sites on mammalian (hamster) mitochondrial large and small subunit rRNA. The large subunit rRNA methylation sites all occur on the ribose 2'-OH, whereas the small subunit rRNA methylation sites all occur at the nucleotide base. The location of the 2'-O-methylation sites corresponds to homologous sites in bacteria that participate in the peptidyl transferase center of the ribosome. These residues are identified within 16 S rRNA structures similar to the well described A-loop and P-loop regions shown with those of the mammalian mitochondrial large ribosomal subunit 16 S rRNA.

**Assignment of Mitochondrial Methyltransferases to Target Sites**—We have previously used siRNA to significantly reduce the levels of RNMTL1 protein in HeLa cells (38). In this analysis, primer extension in the presence of limiting deoxynucleotidate triphosphate concentrations was used to conclude that RNMTL1 methylates residue G\textsuperscript{11032} of 16 S rRNA, but this analysis was complicated somewhat by methylation of the adjacent U\textsuperscript{1369} residue. In our present work, we treated HeLa and HEK293 cells with siRNA targeting a negative control scrambled (siScr), RNMTL1, MRM1, or MRM2 for 3 days. Two different siRNA sequences were each tested for reduction of MRM1 and MRM2 protein levels. Although both were effective, later experiments were conducted with the siRNAs causing a greater effect, siMRM1-B and siMRM2-B (Fig. 1, D and E). Efficacy of the RNMTL1 siRNA was established previously (38). Whole cell RNA was isolated from HeLa and HEK293 cells that were transfected with siRNA. 2 μg of each RNA sample were treated with each DNAzyme, and the products were sep-
Hepatitis B virus ribonucleoprotein particle (RNP) and hepatitis B virus core antigen (HBcAg) were assayed by western blot analysis. Overall, HBV-RNP RRM consumers were treated with 8-14 and 10-23 type DNAzymes target to Hybond-N membrane. The RRM cleavage of a 2’-O-methylation, which is difficult to interpret quantitatively due to variable polymerase bypass of the modified residue. The primer extension method is also complicated by local RNA sequence features. In our previous work (38), we found that the GGGG sequence encompassing G1145, which is known not to be methylated. This control confirmed essentially complete cleavage by the DNAzyme at this site on 16S rRNA. The cleavage at residue U1369 was increased more than 2-fold, clearly implicating MRM2 in modification of U1369. Treatment with either 3 nM or 6 nM siRNA directed against MRM2 resulted in a general suppression of cell growth. The total cell number after 3 days of siRNA targeting MRM2 was decreased to 72% of the control using siRNA targeting a scrambled sequence.

**DISCUSSION**

Mammalian mitochondria possess three well defined 2’-O-methyltransferase family members. We used the DNAzyme and Northern blotting approach to confirm our previous indication that RNMT1 is required for the methylation at G1370 of 16S rRNA (38). We also provide novel evidence that MRM1 and MRM2 are required for the methylation at G1145 and U1369, respectively, which is consistent with the conservation of these methyltransferases and target residues in bacteria and yeast mitochondria. While this manuscript was under review an article appeared on-line supporting our previous observation that RNMT1 is responsible for methylation of G1370 and suggesting that MRM2 was involved in modification of U1369 (53). This study, like that of Lee et al. (38), used a primer extension approach to detect 2’-O-methylation, which is difficult to interpret due to variable polymerase bypass of the modified residue. The primer extension method is also complicated by local RNA sequence features. In our previous work (38), we found that the GGGG sequence encompassing G1145 has a tendency to block primer extension by reverse transcriptase. Second, detection of methylation sites using the primer extension method can be obscured by modification of a closely spaced residue, as in the case of two adjacent methylations occurring at U1369 and G1370. The DNAzyme approach circumvents these limitations of the primer extension assay.

The DNAzyme-mediated RNA cleavage approach described here is not limited to cases where modified RNA sites and RNA-modifying suspects have already been identified. One may apply DNAzymes to scan transcripts for 2’-OH modifications by shifting the annealing arms to redirect the interrogation site.
Because the annealing arms will likely limit the analysis of modifications near the 5′ or 3′ end of transcripts, one may consider ligating single-stranded RNA to the ends or circularizing the RNA. DNAzymes have been developed to cleave at nearly all dinucleotide RNA junctions; thus they are not limited by the target RNA sequence. DNAzymes can also detect pseudouridylation sites, although less effectively than 2′-O-methylation sites (34).

The occurrence of adjacent 2′-O-methylation sites in mammalian 16 S rRNA seems unusual, given that the Saccharomyces cerevisiae LSU mitochondrial rRNA lacks one of these modifications. However, the corresponding G residue in the A-loop of yeast cytoplasmic 23 S rRNA is modified by 2′-O-methylation (54). A comprehensive evolutionary analysis of the conservation of the three mitochondrial RNA methyltransferases has recently appeared (53). Because 16 S rRNA from cells with normal levels of mitochondrial methyltransferases has some susceptibility to DNAzyme cleavage at G1445 and U1369, it is likely that at steady state, a significant fraction of 16 S rRNA molecules is not completely methylated and/or there is a structured order of methylation events, as with nucleo-cytoplasmic ribosomes (55, 56). It is possible that modification of either U1369 or G1370 may be sufficient to support mitoribosome assembly and translation, so that there may be some redundancy in the retention of both enzymes. We have not ruled out the possibility that some of the methylations may be dynamic (reversible) as with m6A in cytoplasmic mRNA (57–59).

Our in vitro methylation assays with purified protein and in vitro-synthesized RNA have not been successful (38). Although technical and biological explanations for this exist, we have not ruled out the remote possibility that RNMTL1, MRMI, and MRM2 act by an indirect mechanism. However, it is likely that the mitochondrial methyltransferases resemble bacterial methyltransferases by acting as stand-alone proteins that recognize and modify specific sites on RNA, in contrast to eukaryotic nucleo-cytoplasmic ribosomes, which require guide RNAs within small nucleolar ribonucleoprotein complexes to specify the modification sites.

Although the mechanism of mitoribosome assembly is not well understood, we are beginning to accumulate evidence that mitoribosome biogenesis begins at the nucleoid on nascent transcripts by incorporating newly synthesized MRPs. In addition to finding methyltransferases localized near nucleoids, we have recently shown that newly synthesized MRPs are localized in the nucleoid complex to encounter newly synthesized rRNA using pulse-chase stable isotope labeling by/with amino acids in cell culture labeling (29). It is also known that GTases are important for mitoribosome assembly, just as they are for bacteria (60). Human NOA1 (hNOA1), also known as MTG3 or C4orf14, is associated with the nucleoid and is necessary for SSU assembly (61–63). We have also identified ERAL1 in nucleoids (29, 64). ERAL1 is a GTPase that functions as an RNA chaperone, binding to the TF81M-mediated dimethylation region of 12 S rRNA (65, 66), potentially folding the RNA into the proper conformation needed for SSU assembly. The relationship between the nucleoid and ribosome assembly is becoming clearer, and more experiments in the future will solidify this relationship. It is also noteworthy that ~30 bacte-rial rRNA modifications and modifying proteins have not been retained in mitochondria (67). The lack of modifications may be explained by an expansion of mitochondrial ribosomal proteins and truncation of rRNA structures. The retained modifications and modifying enzymes that have not been eliminated through evolution most likely play important roles in mitoribosome assembly and function.

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