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Subcellular Partitioning of MRP RNA Assessed by Ultrastructural and Biochemical Analysis

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Abstract. A small RNA encoded within the nucleus is an essential subunit of a RNA processing endonuclease (RNase MRP) hypothesized to generate primers for mitochondrial DNA replication from the heavy strand origin of replication. Controversy has arisen, however, concerning the authenticity of an intramitochondrial pool of MRP RNA, and has called into question the existence of pathways for nucleo-mitochondrial transport of nucleic acids in animal cells. In an effort to resolve this controversy, we combined ultrastructural in situ hybridization and biochemical techniques to assess the subcellular partitioning of MRP RNA. Cryosections of mouse cardiomyocytes were hybridized with biotin-labeled RNA probes complementary to different regions of MRP RNA and varying in length from 115 to 230 nucleotides, followed by immunogold labeling. In addition, we transfected mouse C2C12 myogenic cells with constructs bearing mutated forms of the mouse MRP RNA gene and compared the relative abundance of the resulting transcripts to that of control RNAs within whole cell and mitochondrial fractions. In the former analysis we observed preferential localization of MRP RNA to nucleoli and mitochondria in comparison to the nucleoplasm and cytoplasm. In the latter series of studies we observed that wild-type MRP RNA partitions to the mitochondrial fraction by comparison to other RNA transcripts that are localized to the extramitochondrial cytoplasmic space (28S rRNA) or to the nucleoplasm (U1 snRNA). Deletions within 5′ or 3′ regions of the MRP RNA gene produced transcripts that remain competent for mitochondrial targeting. In contrast, deletion of the midportion of the coding region (nt 118 to 175) of the MRP RNA gene resulted in transcripts that fail to partition to the mitochondrial fraction. We conclude that an authentic intramitochondrial pool of MRP RNA is present in these actively respiring cells, and that specific structural determinants within the MRP RNA molecule permit it to be partitioned to mitochondria.

MITOCHONDRIAL biogenesis requires the participation of two distinct genetic compartments: the nuclear genome that contributes the vast majority of mitochondrial proteins, and the mitochondrial genome that contributes 13 protein subunits to inner membrane enzymes of the respiratory chain (Anderson et al., 1981). With the exception of two ribosomal RNA subunits and a complete set of tRNA species, the gene products necessary for replication, transcription, and translation of mitochondrial genes in cells of higher eukaryotes are derived entirely from the nucleus (Kruse et al., 1989; Parisi and Clayton, 1991; Attardi and Schatz, 1988).

Interestingly, the set of nuclear genes required for replication and expression of the mitochondrial genome may include not only protein-coding loci, but genes that encode small RNA transcripts. Nuclear-encoded tRNAs are present in mitochondria from protozoa (Mottram et al., 1991; Lye et al., 1993; Nagley, 1989) and plants (Marechal-Drouard et al., 1988; Small et al., 1992). In animal cells, however, the existence of a pathway for nucleomitochondrial transport of nucleic acids has been controversial. Mitochondrial RNase P activity in mammalian cells may require a nuclear-encoded RNA subunit (Doersen et al., 1985), but potential participation of RNA transcripts of nuclear origin in essential mitochondrial functions in mammalian cells has been studied most intensively with respect to the RNA subunit of RNase MRP.[1] This site-specific endoribonuclease was isolated originally by Chang and Clayton from mitochondrial fractions of mouse LA9 cells on the basis of its ability to cleave an RNA substrate representing primary transcripts from the

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1. *Abbreviations used in this paper*: Pol III, RNA polymerase III; RNase MRP, mitochondrial RNA processing endonuclease.

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mitochondrial light strand promoter. The 3′ ends of RNA fragments generated by this reaction corresponded closely to the 5′ ends of nascent replication products of mitochondrial DNA in vivo (Chang et al., 1985), leading to the hypothesis that RNase MRP generates primers for mitochondrial DNA replication (Chang and Clayton, 1987*a*, *b*; Stohl and Clayton, 1992). This concept was supported by the observation that substrate recognition in vitro was dependent on complementarity of a segment of the MRP RNA sequence to conserved sequences of the mitochondrial RNA substrate (Bennett and Clayton, 1990). Selectivity has been conserved throughout evolution: human RNase MRP complex will cleave a substrate derived from the D-loop region of mouse mitochondria (Bennett and Clayton, 1990), and yeast MRP complex can process both mouse and human substrates (Stohl and Clayton, 1992). Activity of RNase MRP was found to require an RNA subunit (MRP RNA) encoded by a single copy nuclear gene that is highly conserved among mammalian species (Chang and Clayton, 1989; Yuan et al., 1989; Gold et al., 1989).

Although MRP RNA was identified originally in mitochondrial fractions of mammalian cells (Chang and Clayton, 1987*a*), MRP RNA is not limited to mitochondria (Karwan et al., 1991; Yuan et al., 1989; Gold et al., 1989). A ribonucleoprotein complex that contains MRP RNA and at least 10 protein components is immunoprecipitated from nuclear extracts of human cells by an antinucleolar antibody present in sera of patients with autoimmune disorders (Yuan et al., 1991). Th/To antigen, a 40-kD protein, constitutes the major antigenic determinant of this complex (Yuan et al., 1991), and immunoelectron microscopy has confirmed the nucleolar location of this protein (Reimer et al., 1988). No previous ultrastructural analyses, however, have addressed directly the subcellular partitioning of MRP RNA.

Other recent findings have cast doubt on the existence of an authentic intramitochondrial pool of MRP RNA in mitochondria of animal cells (Kiss and Filipowicz, 1992). Specifically, the abundance and nuclease sensitivity of MRP RNA in highly purified mitochondrial fractions prepared from HeLa cells were similar to several small nuclear RNA transcripts. Accordingly, the previously reported association of MRP RNA with mitochondria was ascribed to contamination from the abundant nuclear pool of MRP RNA.

The present report describes detection of MRP RNA by in situ hybridization in tissue sections with relatively undistorted cellular ultrastructure. Glutaraldehyde-paraformaldehyde fixation of mouse hearts before sectioning minimized dislocation of RNA during preparation of ultracryosections. After hybridization of biotinylated RNA probes and posthybridization washes, RNA-RNA hybrids were detected with immunoelectron microscopy, using a gold-conjugated secondary antibody. We employed three separate anti-sense RNA probes complementary to different regions of MRP RNA (see Fig. 5) and a variety of control probes. The subcellular localization of hybrids formed with each probe was defined by an unbiased quantitative analysis. The results indicate that MRP RNA partitions both to mitochondria and to nucleoli of these actively respiring cells.

We also constructed plasmids that express deleted forms of mouse MRP RNA, and analyzed the partitioning of these transcripts to mitochondrial fractions after transfection of mouse C2C12 myogenic cells. We observed that disruption of a small region from nt 118 to nt 175 within the mouse MRP RNA gene has little or no effect on accumulation of the resulting RNA transcript within the whole cell RNA pool, but severely inhibits its partitioning to mitochondria. Other mutations that delete or substitute for sequences within the 5′ or 3′ regions of the gene resulted in transcripts that partition to the mitochondrial fraction in a manner identical to native MRP RNA. These results provide further evidence for the existence of a pathway for nucleomitochondrial transport of RNA in animal cells, and suggest that specific structural determinants within the MRP RNA molecule promote recognition by carrier proteins or receptors that participate in this process.

Materials and Methods

Preparation of RNA Probes for In Situ Hybridization

The MRP RNA gene was cloned by the PCR using mouse genomic DNA and primers based on the published sequence (Chang and Clayton, 1989), subcloned into a pGEM vector (Promega Corp., Madison, WI), and the sequence confirmed. The plasmid was linearized by restriction digestion to generate DNA templates for in vitro transcription of the MRP-230 and MRP-115 riboprobes using T7 RNA polymerase. MRP-215 was transcribed using SP6 polymerase and a DNA template generated by the PCR from a construct in which nucleotides from positions 116 to 164 were deleted. The Ula1 gene was obtained from Yang Cheng (U. Wisconsin) and cloned into a Bluescript vector (Stratagene, La Jolla, CA). The PCR, using primers based on the sequence published by Manser and Gesteland (1982) was used to generate a full-length (165 nt) DNA template that included a T7 RNA polymerase promoter. A 503 nt RNA probe transcribed from a T7 promoter and corresponding to the sense strand of Exon 3 of the mouse myoglobin gene (Blanchetot et al., 1986) was used as a measure of non-specific binding. Probes representing MRP RNA sense strand sequences were inappropriate as controls for non-specific binding because of possible complementarity to transcripts generated in vivo from the mitochondrial light strand promoter (Bennett and Clayton, 1990). Oligonucleotides were generated on a synthesizer (Appl. Biosystems Inc., Foster City, CA).

Biotin-labeled probes were transcribed in a reaction (20 μl) containing 40 mM Tris Cl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 40 U RNAsin, 0.5 mM rATP, 0.5 mM rGTP, 0.5 mM rCTP, 1 mM biotin-11-UTP (Enzo), 1-2 μg DNA template, and 40 U SP6 or T7 RNA polymerase. All reaction components except biotin-11-UTP were from Promega. After 2-h incubation at 37°C, 1 μl/μg DNA of RQ1 RNAse-free DNAse (Promega) was added and the incubation extended 15′. RNA was precipitated by addition of 0.3 M NaOAc and ethanol; the dried precipitate was resuspended in 20 μl dH_2O denatured at 72°C, and analyzed for size and abundance by agarose gel electrophoresis. RNA standards (Bethesda Research Laboratories) were included with each gel. All dH_2O used throughout the entire experiment was treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved.

Preparation of Tissue

Adult mice were perfusion-fixed with heparinized PBS followed by 0.5% glutaraldehyde/4% paraformaldehyde (5′) and by 4% paraformaldehyde (30′). All protocols that employed animals were approved by the Institutional Review Board for Animal Research and followed guidelines published by the National Institutes of Health ("Guide for the care and use of laboratory animals").

Tissues were trimmed and immersed in 4% paraformaldehyde (2 h) followed by immersion in 2.3 M sucrose/0.2 M Tris pH 7.4 for 2 h. Tissue blocks were oriented on specimen pins and submerged in liquid nitrogen. 100-nm thick sections of tissue blocks were cut on an ultracryomicrotome (Reichert-Jung) cooled to −90°C and transferred to formvar-coated gold grids (Pelco, 100-mesh). Grids were transferred to 2.3 M sucrose/0.2 M Tris pH 7.4 droplets. Immediately before in situ hybridization, grids were transferred to 0.1 M glycine/0.2 M Tris pH 7.4 (10′, r.t.) to quench free aldehydes. Using baked nichrome wire loops (Pella), grids were transferred to

prewarmed 2× standard saline citrate (SSC)/50% deionized formamide at 65°C for 10′ and cooled to 37°C.

Hybridization

Probe was added to hybridization mix (1 ng/μl) and denatured by heating to 65°C for 5′. After cooling to 37°C, DTT was added to 10 mM. Hybridization mix consisted of 50% deionized formamide pH 7.5, 0.3 M NaCl, 20 mM Tris Cl pH 8.0, 5 mM EDTA, 10 mM sodium phosphate pH 8.0, 10% dextran sulfate, 1× Denhardt's solution, 0.05 mg/ml yeast rRNA. Using anti-capillary tweezers, gold grids were transferred to 40 μl droplets of hybridization mix containing probe on 37°C silicon mats (Pella) that had been previously soaked in 0.1% DEPC and autoclaved. Mats were placed in a humid chamber and incubated overnight at 37°C.

After hybridization, washes were performed at 37°C unless otherwise indicated. Grids were transferred to HS buffer (0.1 M DTT, 2× SSC, 50% deionized formamide; 5′, 2×), to HS buffer at 60°C (15′), then to NTE buffer (0.5 M NaCl, 10 mM Tris pH 8.0, 5 mM EDTA) (5′, 3×). Excess probe was digested by 20 μg/ml RNAse A (Boehringer-Mannheim) and 1 U/ml RNAse T_1 (Bethesda Research Laboratories) in NTE buffer, 30′. Grids were washed with NTE (5′) and transferred to HS buffer at 60°C (15′). Final washes consisted of 2× SSC (15′) and 0.1× SSC (15′). RNAse H digestion, when used, preceded after hybridization washing and consisted of transfer of grids to HS buffer (5′, 2×), and KMD (5′, 2×) followed by transfer to droplets containing 0.6 μ RNAse H (Progmega) in KMD and incubation (1 h at 37°C). KMD consisted of 20 mM Tris pH 8.0, 100 mM KCl, and 10 mM $MgCl_2$, 1 mM DTT.

Immunogold Detection of Probe

All procedures were performed at room temperature on a parafilm surface. Using nichrome loops, grids were transferred to large droplets of 1% BSA/0.5 M NaCl/PBS pH 7.2 (BNP), 10′, blocked with normal goat serum (Vecta-Stain), 10′, and, using anti-capillary tweezers, transferred to droplets of rabbit anti-biotin (Enzo) diluted 1:25 in BNP (30′). Grids were washed on large droplets of BNP and incubated with 1:25 dilution in BNP of goat anti-rabbit IgG conjugated to 5-nm or 10-nm gold particles (Auroprobe, Amersham), 30′. After transfer through PBS (5′, 3×), the grids were transferred to 1% glutaraldehyde/PBS (10′) and washed 3× on dH_2O. Grids were transferred to 2% methylcellulose/0.02% uranyl acetate on ice (10′), mounted in 5-mm nichrome loops, and excess methylcellulose removed before drying. Grids were viewed on a JEOL 1200 electron microscope, operated at 80 kV–120 kV.

Quantitative Analysis

Twenty-five electron micrographs (25,000× magnification) of tissue sections mounted on grids were prepared using a systematic, bias-free sampling method to determine the grid sites recorded. The film negative sets were encoded, viewed under magnification on a transilluminator, and scored by an observer with no knowledge of the code. The negative was overlaid by a transparency containing 12 random dots and the number of dots falling over each intracellular compartment (nucleoplasm, nucleolus, myofibrils, and mitochondria) was counted. The number of gold particles within each compartment was determined. The ratio of gold particles to random dots for each compartment was calculated, and converted to gold particles per μm^2 surface area of each compartment using the following formula: $GP/[(R/R_t)\ (1 \times w/25{,}000^2)\ (N)]$ where GP = total number of gold particles within a subcompartment, R = total number of random dots falling over each subcompartment, R_t = total number of random dots for all negatives within a set, $l \times w$ = dimensions of the film negative in mm, N = total number of negatives in a set, and 25,000 = magnification of each micrograph negative. The resulting ratio corresponds to a measure of the abundance of each probe target relative to the fractional area of each subcellular compartment.

Construction of Mutated MRP RNA Genes

The MRP RNA gene construct pMRP-A consists of 273 bp of coding region, 700 bp of 5′ flanking DNA and the 3′ transcriptional termination sequence. A unique Bgl II site was engineered near to the 3′ terminus of the coding region using PCR primers containing a Bgl II linker (Fig. 5, pMRP-A). Deletion mutants were generated by PCR primer-guided synthesis and Acc I and Esp I restriction to remove selected segments of the MRP RNA coding region. All the constructs were cloned into pBluescript KS (Stratagene) and verified by restriction mapping and sequencing.

Cell Culture and Transfection

Mouse C2C12 myoblast cells were grown in DMEM with 10% FCS, 5% chick embryo extract and 20 U/ml penicillin-streptomycin. Calcium phosphate transfections were performed as described previously (Li et al., 1990). Thirty micrograms of plasmid DNA were added to each 100-mm dish. For transcriptional analysis, the duplicate plates of transfected cells were mixed. One half was used to extract transfected plasmid (Hirt, 1967) as a control for the efficiency of transfection, and the other half was employed for RNA isolation and Northern blot hybridization.

In Vitro Transcription

Nuclear and cytosolic extracts were prepared from Hela cells as described previously (Dignam et al., 1983). The in vitro transcription reactions were performed in 30 μl of reaction volume containing 600 ng plasmid DNA and 7.5 μl each of nuclear and cytosolic extract using a modified procedure (Ullu and Weiner, 1984). The reaction products were resolved by electrophoresis in 6% urea-polyacrylamide gels.

Isolation of Mitochondria and Nuclei

For each experiment, 20 plates of C2C12 cells were transfected with a mutated plasmid. One thirtieth of harvested cells were lysed directly to isolate total cellular RNA. The remaining cells were permeabilized with digitonin to facilitate isolation of mitochondria (Moreadith and Fiskum, 1984; Howell et al., 1986). Briefly, the cells were suspended in 4 vol of mitochondrial homogenization buffer (MTHB: 210 mM mannitol, 70 mM sucrose, 5 mM Hepes pH 7.3, 0.5% BSA) after three washes. Digitonin (5%) was added to a final concentration of ∼0.5 mg/ml. This concentration of digitonin partially disrupts the outer mitochondrial membrane and was used to reduce contamination of the mitochondrial fractions with cytoplasmic RNAs that may be adherent to the outer membrane. The cells were washed once, resuspended in 4 vol of MTHB and homogenized with a Dounce homogenizer (6–15 strokes). The lysate was diluted to 0.25× MTHB and centrifuged at 8,000 g for 10 min. After resuspending the pelleted mitochondria, nuclei, and cell debris in 15 vol of MTHB, the lysate was subjected to three consecutive centrifugations at 1,000–1,080 g for 5 min each (2,100–2,200 rpm, Sorvall RT6000B centrifuge, H1000B rotor). An aliquot was removed from the supernatant of each low speed centrifugation step and centrifuged at 8,000 g for 10 min to produce increasingly purified mitochondrial fractions for RNA extraction.

RNA Isolation and Northern Blot Hybridization

RNA was isolated by a modification of the guanidinium thiocyanate procedure (Sambrook et al., 1989). RNA samples were electrophoresed in 1.5% (for U1 and MRP RNAs) and 1.1% (for ribosomal RNAs) agarose gels under denaturing conditions. The gel buffer contained 20 mM Hepes pH 7.5, 5 mM NaCl, 1 mM EDTA and 2.2 M formaldehyde. After transfer to nylon membranes, Northern blot hybridizations were performed by standard techniques (Overhauser et al., 1987). Sense strand MRP RNAs were synthesized in vitro from Hind III-linearized pMRP-A using T7 RNA polymerase, yielding ∼1 kb RNA transcripts that were used as positive controls for Northern hybridizations.

Synthetic oligonucleotides were used as probes specific for detection of MRP RNA, U1 RNA, 28S cytoplasmic ribosomal RNA, and 16S mitochondrial ribosomal RNA. Three different oligonucleotide probes (see Fig. 5) were used to detect mutated and endogenous MRP RNA transcripts: MRP probe 1,5′-GAATGAG<u>atc</u>TGTGGTTGGTGCG (mutated); MRP probe 2,5′-CATGTCCCTCGTATGTAGCCTAG (wild type); MRP probe 3,5′-GAGAATGAG<u>CCCCG</u>TGTGGTTG (wild type). The five bases underlined in probe 3 were replaced in mutated constructs by the three bases underlined and indicated in lower case in probe 1. This maneuver permitted probe 1 to hybridize exclusively to transcripts from transfected plasmid constructs without cross-hybridization to endogenous MRP RNA under the high stringency conditions that were employed. Likewise, probe 3 hybridized exclusively to endogenous MRP RNA without cross-hybridization to transcripts derived from the transfected genes. Probe 2 hybridized indiscriminately to both endogenous and foreign MRP RNA transcripts, with the exception of products transcribed from pMRP-D, which lack sequences complementary to probe 2.

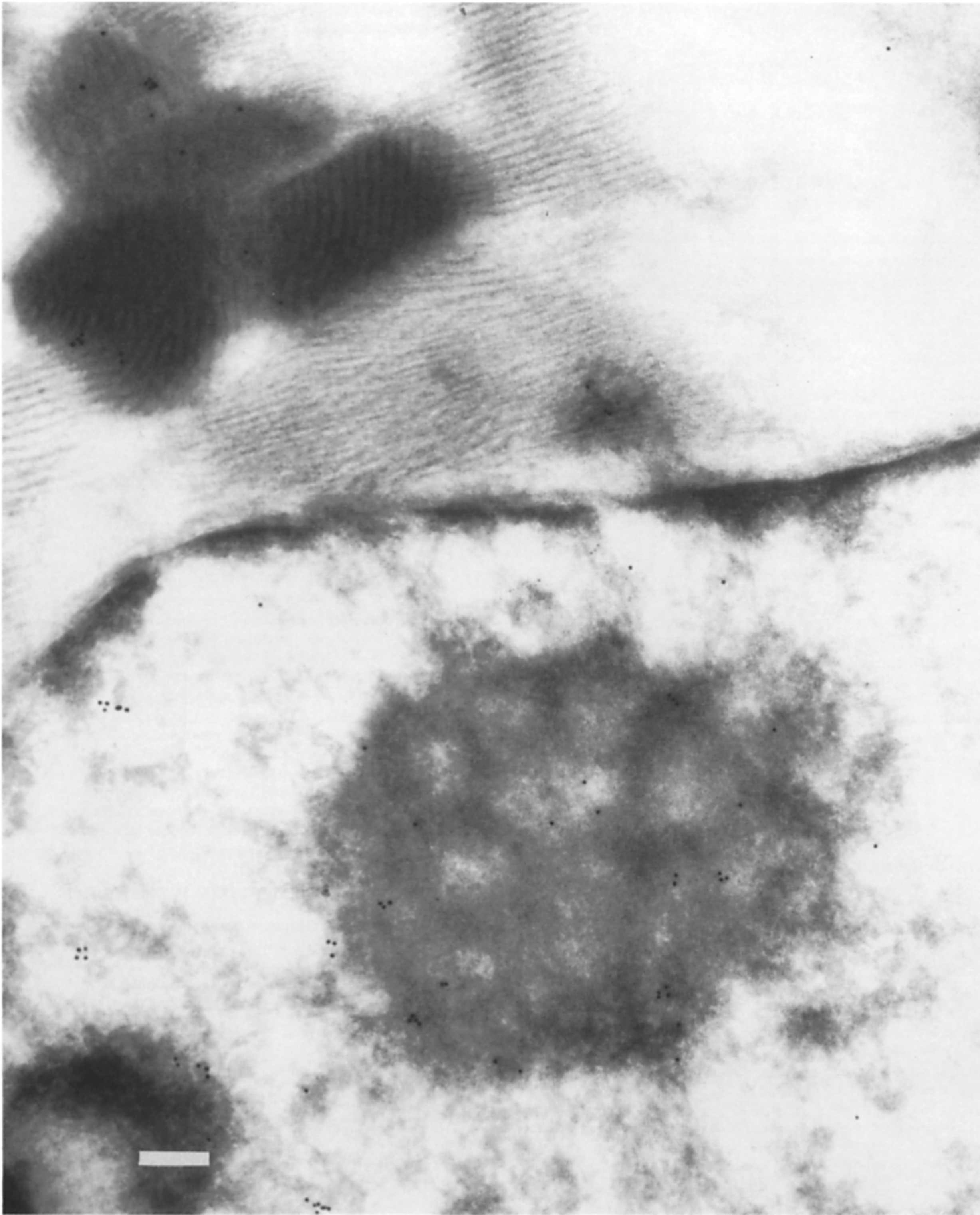


Figure 1. Immunogold detection of MRP RNA within a nucleolus and mitochondria. Photomicrograph of in situ hybridization of the MRP-230 anti-sense RNA probe to an ultracryosection of a cardiac ventricular myocyte. Hybrids formed with this probe are most abundant within the granular segment of a nucleolus (*center*) and within mitochondria (*upper left corner*). Magnification, 25,000×. Bar, 200 nm.

RNA Quantitation and Data Analysis

Hybridization of ^{32}P-labeled probes to specific bands in Northern blots was measured quantitatively using ImagerQuant or PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Mitochondrial targeting of mutated MRP RNA transcripts was calculated as follows:

$$\text{Mitochondrial partitioning ratio} = \frac{\text{mito/total mutated MRP RNA}}{\text{mito/total endogenous MRP RNA}}$$

Results

Nucleolar and Mitochondrial Localization of MRP RNA

Within the nucleolar compartment of the cardiomyocyte nucleus, all three MRP RNA anti-sense probes formed hybrids within the finely grained, electron lucent regions (Figs. 1 and 2) that correspond to extended chromosomal tips undergoing transcription of rDNA genes (Raska et al., 1990). The electron dense, coarsely grained nucleolar regions representing condensed heterochromatin did not hybridize to probes complementary to MRP RNA. For quantitation, the density of gold particles within specific subcellular regions was scored in electron micrographs of ultracryosections using the procedure described in Materials and Methods. Hybridization of the MRP-230 antisense probe was calculated as 20.3 gold particles per μm^2 nucleolar surface area (Fig. 4, Table I). Preferential localization of the MRP-230 riboprobe to mitochondria was observed using the same method (Figs. 1 and 3). Binding of the MRP-230 probe resulted in 7.5 gold particles per μm^2 mitochondrial surface area. This probe formed hybrids much less often within the nucleus exclusive of nucleoli (nucleoplasm) or within the cytoplasm exclusive of mitochondria (myofibrils) (Fig. 4, Table I).

To discriminate between RNA-RNA and RNA-DNA hybrids formed between anti-sense MRP RNA riboprobes and endogenous targets, separate experiments using the MRP-230 probe were conducted under identical conditions, except that the sections were treated with RNAse H for 1 h after hybridization to eliminate RNA-DNA hybrids. The possibility of hybridization of anti-sense MRP RNA to mitochondrial DNA was of concern, since MRP RNA contains a region of complementarity to the D loop region of the mitochondrial genome (Chang and Clayton, 1989). Under the conditions used in our experiments, however, no decrease in mitochondrial binding of the MRP-230 probe was observed after RNAse H treatment (7.7 gold particles per μm^2 surface area), confirming that the iμmunogold labeling was derived from RNA/RNA hybrids. Nucleolar binding of the MRP-230 probe after RNAse H digestion was reduced somewhat from that observed in the absence of RNAse H, but remained well above background levels (7.9 gold particles per μm^2 surface area). In subsequent experiments using the MRP-215 and MRP-115 riboprobes, all grids were treated with RNAse H after hybridization. These probes also demonstrated preferential binding to nucleoli and mitochondria in a manner similar to results obtained with the MRP-230 probe. Binding of the MRP-215 and MRP-115 riboprobes was calculated as 6.6 and 8.3 gold particles bound per μm^2 mitochondrial surface area, and 11.2 and 8.0 gold particles per μm^2 nucleolar surface area, respectively. Also, like MRP-230, these shorter probes were detected only at back-

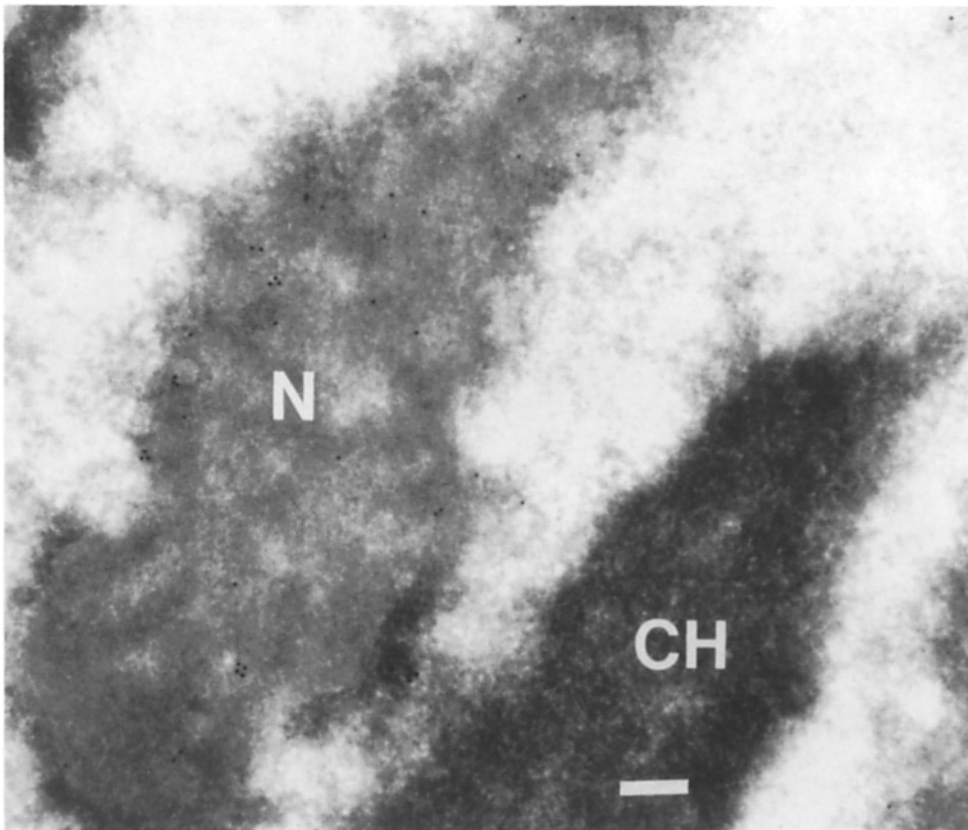


Figure 2. Localization of MRP RNA to the granular region of the nucleolus. Immunogold detection of MRP RNA within a nucleolus after in situ hybridization of the MRP-230 anti-sense RNA probe to an ultracryosection of a cardiac ventricular myocyte. Gold particles are evident within the actively transcribed nucleolar region (*N*) but do not appear in an adjacent region of condensed heterochromatin. (*CH*). Magnification, 25,000×. Bar, 200 nm.

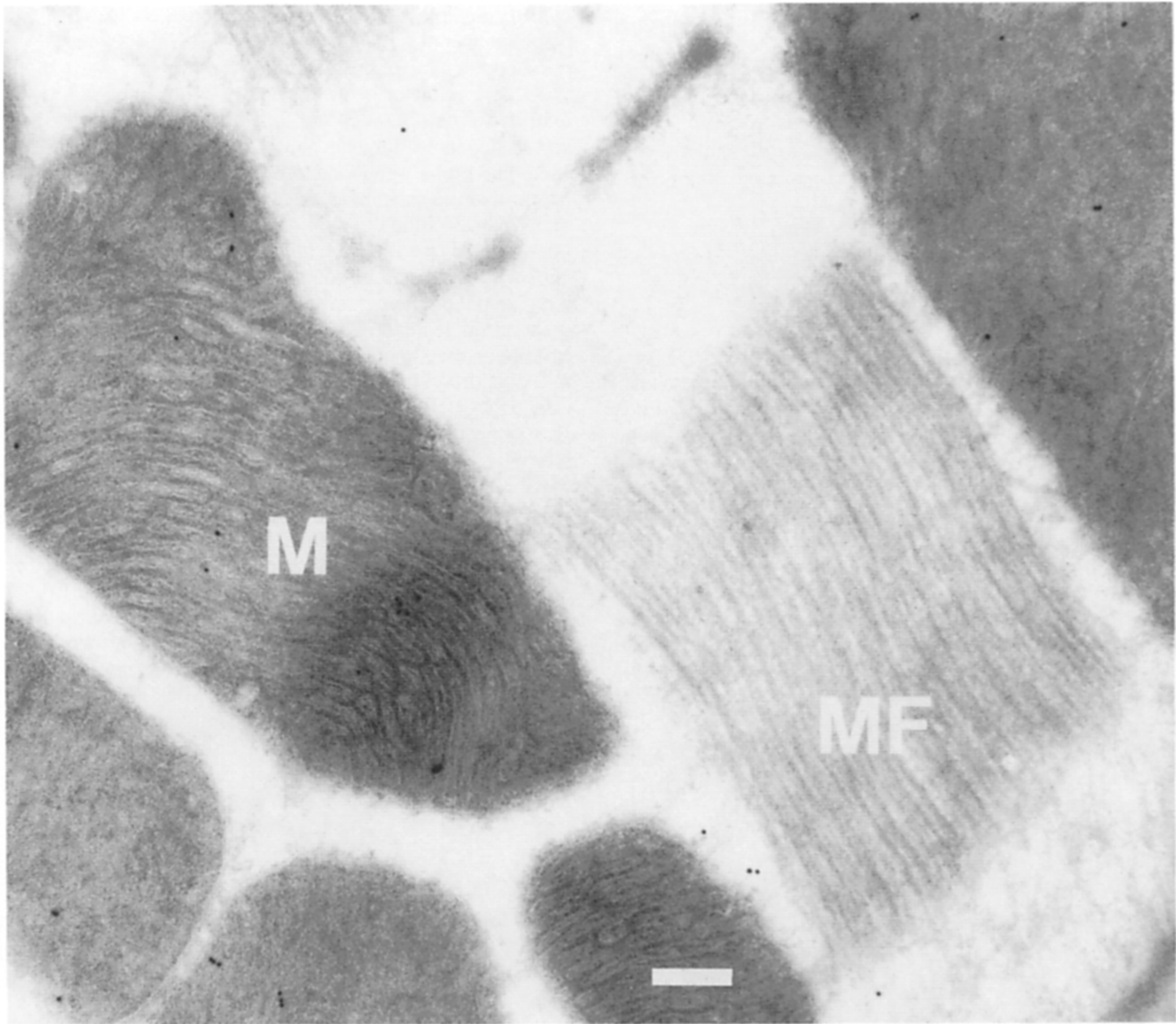


Figure 3. MRP RNA within mitochondria. Immunogold particles representing hybrids formed with the biotinylated MRP-230 anti-sense RNA probe within cardiomyocyte mitochondria after in situ hybridization. *MF*, myofibrils. Magnification, 25,000×. Bar, 200 nm.

ground levels in the nucleoplasm or myofibrillar compartments as well (Fig. 4, Table I). The density of binding of antisense riboprobes complementary to MRP-RNA within mitochondria is similar to that described previously in ultrastructural analyses using probes complementary to mRNA transcripts derived from mitochondrial genes that encode subunits II and III of cytochrome oxidase (Escaig-Haye et al., 1991).

Control Probes in Ultrastructural Analysis

Hybridization of probes complementary to MRP RNA was interpreted by comparison to two control probes. A biotinylated RNA probe complementary to 165 nt of the U1 small nuclear (sn) RNA formed hybrids preferentially within the nucleoplasm (Fig. 4, Table I), in distinct contrast to the preferential binding of MRP RNA antisense probes to nucleoli and mitochondria. A similar subcellular location for U1 snRNA within the nucleoplasm was observed in previous studies based on immunogold detection of a biotinylated DNA probe (Visa et al., 1993). Non-specific binding was assessed by hybridization of a 503-nt riboprobe corresponding to the sense strand from exon 3 of the human myoglobin gene (MBE_3). No known RNA transcripts within mammalian cells are complementary to this probe, which we have used to estimate background for in situ hybridizations in previous studies (Parsons et al., 1993). Binding of MBE_3 ranged from 0.5 to 1.7 gold particles per μm^2 surface area in each of the subcellular compartments. These background values were 4–40-fold below the binding of MRP RNA probes within nucleoli and mitochondria, but in a similar range to binding of MRP RNA probes within the nucleoplasm and extramitochondrial cytoplasmic space (Fig. 4, Table I).

The conventional control based on hybridization of a sense strand sequence to assess non-specific binding was unsuitable, due to potential complementarity of MRP RNA to its substrate transcribed from the mitochondrial light strand

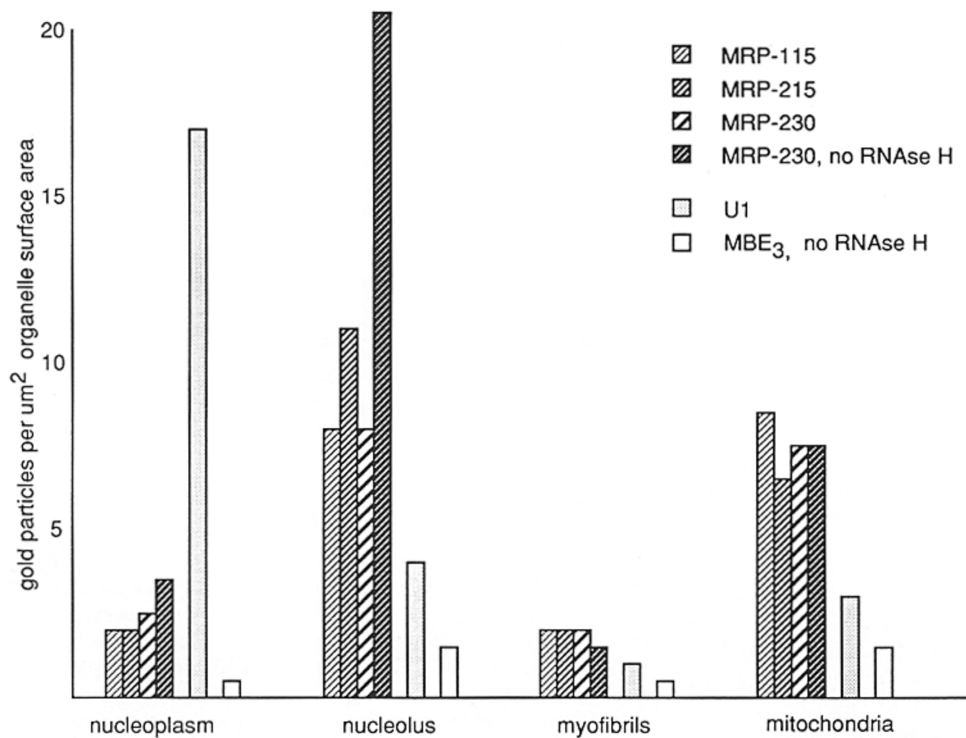


Figure 4. Subcellular distribution of hybrids formed with anti-sense MRP RNA and control probes. Quantitative assessment of gold particles per μm^2 surface area (*GP/* μm^2) within nuclear, nucleolar, myofibrillar, and mitochondrial subcompartments of cardiac myocytes after in situ hybridization with biotin-labeled MRP-115, MRP-215, MRP-230, U1, and MBE_3 probes, as described in Materials and Methods. Except as indicated, tissue sections were treated with RNase H after hybridization.

promoter (Bennett and Clayton, 1990). In pilot experiments, an MRP sense strand probe (230 nt) exhibited preferential binding to mitochondria (approximately threefold over background) that was insensitive to RNAse H. Hybrids formed between this MRP RNA sense strand probe and an endogenous mitochondrial RNA were eliminated, however, by inclusion of a 24-fold molar excess of a single-stranded 39 nt deoxyoligonucleotide corresponding to bases 16085-16123 of the mouse mtDNA (Bibb et al., 1981), the region of potential complementarity between MRP RNA and its putative intramitochondrial substrate.

Foreign MRP RNA Transcripts Target to Mitochondrial Fractions in Parallel to the Endogenous Gene Product

To complement the ultrastructural analysis of subcellular localization of MRP RNA, we also performed biochemical analyses of the partitioning of native and mutated forms of MRP RNA to purified mitochondrial fractions of murine cells. A linker mutation plasmid, pMRP-A, was engineered with a 3-bp insertion and a 5-bp deletion at nt 251-255, yielding a 273-nt RNA transcript, two nucleotides shorter than wild-type MRP RNA. Three other plasmid constructions carried internal deletions within the coding region. The deletion in pMRP-B removed most of the 3′ end of the gene from nt 181 to nt 255, while most of the 5′ region (nt 6 to nt 115) was removed in pMRP-D, including the To/Th antigen-binding domain (Yuan et al., 1991). The deletion in pMRP-F extended from nt 118 to nt 175 and disrupted a sequence resembling an intragenic transcriptional control region (Box A) found within some small RNA genes transcribed by RNA polymerase III (Pol III) (Sakonju et al., 1980; Galli et al., 1981; Ullu and Weiner, 1985). All the mutated plasmids contained 700 nt of 5′ flanking sequence that includes essential upstream activation sequences (Ordway et al., 1993) and 3′ flanking signals for transcriptional termination identical to the endogenous MRP RNA gene (Fig. 5).

A number of small RNA genes transcribed by Pol III require internal regulatory elements for efficient transcription (Bogenhagen et al., 1980; Sakonju et al., 1980; Galli et al., 1981; Hofstetter et al., 1981; Ullu and Weiner, 1985). Transcription by Pol III of other genes encoding small RNAs is dependent only on the presence of an upstream promoter (Murphy et al., 1987). Because in vitro transcription assays indicated that MRP RNA also was transcribed by Pol III (Yuan and Reddy, 1991), our initial objective was to test whether internal deletions would influence transcription. After transfection of mutated MRP RNA plasmids into C2C12 myogenic cells, their transient expression was detected by Northern blot hybridization with specific oligonucleotide probes. Probe 1 hybridizes selectively to mutated MRP RNAs transcribed from pMRP-A, pMRP-D, and pMRP-F, but not to endogenous MRP RNA (Fig. 6). Probe 2 hybridizes to both mutated and endogenous MRP RNAs, but can distinguish deleted forms by differences in size. The

Table I. Distribution of Gold Particles after In Situ Hybridization with Biotinylated RNA Probes

	Gold particles per μm^2 surface area			
Probe	Nucleoplasm	Nucleolus	Myofibrils	Mitochondria
MRP-230	3.6	20.3	1.4	7.5
MBE_3	0.6	1.3	0.5	1.7
RNase H				
MRP-230	2.7	7.9	2.1	7.7
MRP-215	2.0	11.2	1.8	6.6
MRP-115	2.0	8.0	2.0	8.3
U1	17.1	4.0	0.8	2.8

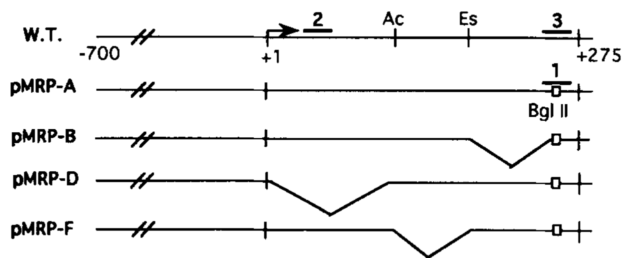


Figure 5. Schematic representation of mutated MRP RNA constructs and probes for Northern hybridizations. Numbers beneath the wild-type (*W.T.*) MRP RNA gene reflect the 5′ flanking sequences included in the plasmid constructs (*−700*), the start site for transcription (*+1 and arrow*), and the termination site (*+275*), respectively. The positions complementary to three oligonucleotide probes (*1*, *2*, *3*) are shown by corresponding numbers and bars. The box represents the engineered Bgl II site, and deletions are indicated by gaps. Restriction sites employed to generate internal deletions are shown. *Ac*, Acc I; *Es*, Esp I.

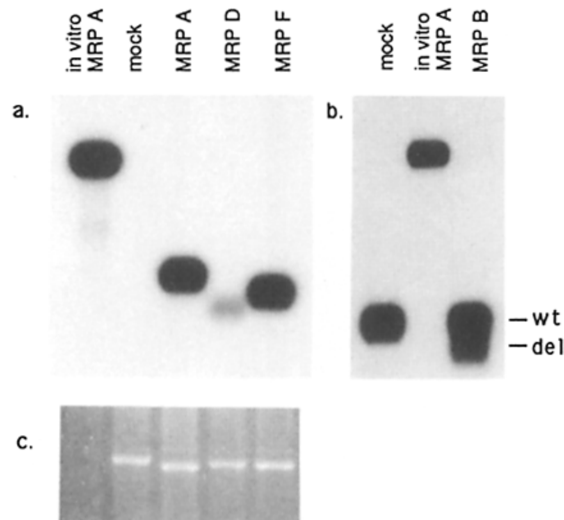


Figure 6. Expression of mutated MRP transcripts in the total pool of cellular RNA after transfection of C2C12 cells. (*a*) RNA blot hybridized with MRP probe 1, which detects all of the mutated forms of MRP RNA but not the endogenous gene product. (Lane *1*) 3 ml of 1,000-fold dilution of RNA transcribed in vitro from pMRP-A (positive control). (lane *2*) 5 μg of total RNA from mock transfection. There is no hybridization of MRP probe 1 to endogenous MRP RNA in this sample. (Lanes *3–5*) 5 μg of total RNA each from cells transfected with pMRP-A, pMRP-D, and pMRP-F, respectively. (*b*) RNA blot hybridized with MRP probe 2, which detects the endogenous gene product and transcripts of pMRP-A and pMRP-B, as shown. (lane *1*) 5 μg of total RNA from mock transfection. (lane 2) In vitro transcript of pMRP-A. (lane *3*) 5 μg of total RNA from cells transfected with pMRP-B. *wt*, endogenous MRP RNA. *del*, deleted form of MRP RNA (pMRP-B). (*c*) Plasmid extraction from the transfected cells. (lane *1*) Mock transfection. (lanes *2–5*) Plasmids pMRP-A, pMRP-D, pMRP-F, and pMRP-B from transfected cells. One half of the preparation (representing an equal number of cells) was loaded in each lane.

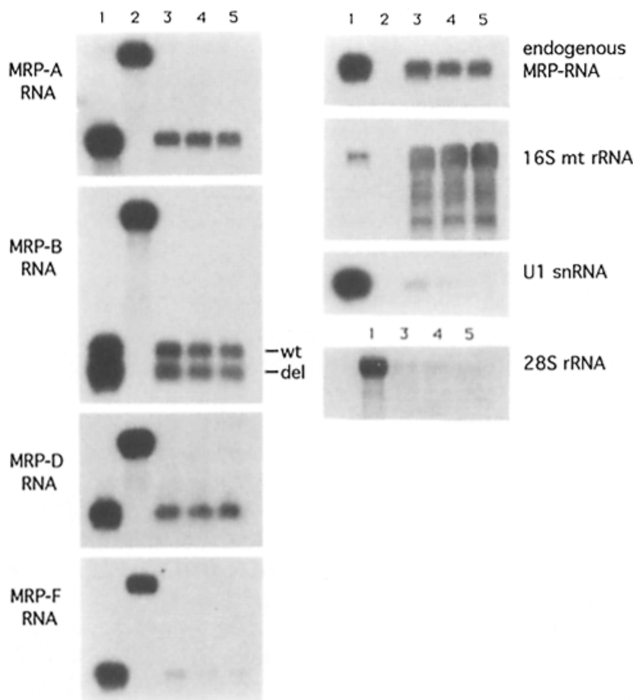
results indicated that all of the mutated MRP RNA constructs were transcribed. Transfection of plasmids pMRP-A, pMRP-F, and pMRP-B produced transcript levels similar to each other and to endogenous MRP RNA. Disruption of the Box A-like element by the deletion in pMRP-F had no effect on the relative abundance of the resulting transcript relative to other constructs in which the Box A-like element was undisturbed. However, the deletion in pMRP-D resulted in reduced levels of transcript in the total cellular RNA pool (Fig. 6). This difference was not attributable to reduced efficiency of transfection (Fig. 6). All of the mutated constructs were transcribed in vitro at an equivalent rate using HeLa cell nuclear extracts as the source of Pol III and relevant transcription factors (data not shown). Accordingly, we reasoned that the reduced abundance of MRP-D transcripts after transfection of C2C12 cells results from more rapid degradation rather than from disruption of an internal control element important for transcription.

For assessment of mitochondrial partitioning of MRP RNA, mouse C2C12 myoblast cells were chosen because of their high mitochondrial content relative to other cell lines. The mitochondrial partitioning of heterologous MRP RNA was examined through three sequential mitochondrial preparations segregated from nuclei and cytosol by low speed centrifugations. The final mitochondrial fraction was devoid of contamination by intact nuclei as assessed by staining with Trypan blue. A series of controls confirmed the authenticity of the mitochondrial fractions isolated from these cells. As shown in Fig. 7, U1 RNA, chosen as a nuclear marker (Carmo-Fonseca et al., 1991), and thereby as a negative control for mitochondrial targeting, was abundant in the whole cell homogenate but was depleted during purification of mitochondria. Likewise, cytosolic (28S) ribosomal RNA was absent from the mitochondrial fractions. This result indicates the success of the digitonin-based fractionation procedure in removing cytoplasmic RNAs that may be adherent to the outer mitochondrial membrane. In contrast, 16S mitochondrial ribosomal RNA, an unambiguous mitochondrial marker, was enriched in the mitochondrial fractions.

To examine mitochondrial targeting of foreign RNA sequences, we first compared the partitioning of MRP-A transcripts with that of endogenous MRP RNA. The small linker mutation within MRP-A permitted it to be distinguished readily from the endogenous gene product while introducing only minor alterations in the sequence of the transcript. We reasoned that such minimal deviation from the endogenous sequence would be unlikely to interfere with mitochondrial targeting of the pMRP-A transcript, and would permit this construct to serve as a positive control for mitochondrial partitioning of products of other mutated MRP RNA genes. Fig. 7 demonstrates that the foreign MRP-A transcript partitions to mitochondria fractions in parallel with endogenous MRP RNA.

Deletion from nt 118 to nt 175 Impairs Partitioning of MRP RNA to Mitochondria

Using the same assay system, we examined mitochondrial partitioning of deleted forms of MRP RNA. Transcripts derived from pMRP-B and pMRP-D partitioned to mitochondrial fractions in a manner indistinguishable from pMRP-A (Fig. 7), indicating that sequences from the 5′ (nt 6 to nt 115) or 3′ (nt 181 to nt 255) regions of MRP RNA are not essential for mitochondrial targeting. The reduced accumulation of pMRP-D transcripts in the whole cell pool (Fig. 6) did not



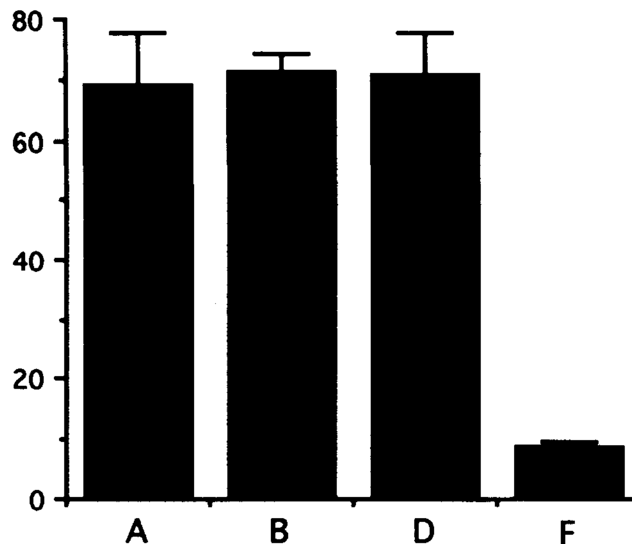


Figure 7. Northern blot hybridization showing mitochondrial partitioning of transcripts derived from pMRP-A, -B, -D, and -F. Blots were prepared using RNA samples extracted from cells transfected with each form of MRP RNA, as indicated in the left column. Hybridizations were performed using synthetic oligonucleotide probes illustrated in Fig. 5 complementary to transgene products (probe 1 to detect transcripts of pMRP-A, -D, and -F; probe 2 to detect transcripts of pMRP-B (del) as well as endogenous MRP RNA (*wt*) in the same blot (*left column*), and probe 3 to detect endogenous MRP RNA (*right column*). Identical blots prepared from the same batch of cells also were hybridized to probes complementary to Ul snRNA (*nuclear marker*), 16S mt rRNA (*mitochondrial marker*), or 28S rRNA (*cytoplasmic markers*) as controls for the integrity and purity of the mitochondrial fractions. (lane *1*) Total cellular RNA (10 μg). (lane 2) In vitro transcripts of pMRP-A and pMRP-F, respectively. (lanes *3*, *4*, and *5*) Mitochondrial RNAs (5 μg) isolated after 1st, 2nd, and 3rd fractionation steps, respectively (see Materials and Methods).

Figure 8. Mitochondrial partitioning ratios of mutated MRP RNA transcripts. The relative partitioning to mitochondria of transcripts derived from each mutated MRP RNA gene (Fig. 5) were calculated (see Materials and Methods) from data derived from the most highly purified mtRNA fraction as assessed in Northern hybridizations. Mean values ($\pm$SE) from two independent transfection experiments are presented. *A*, *B*, *D*, and *F* represent transcripts from pMRP-A, pMRP-B, pMRP-D, pMRP-F plasmids, respectively.

compromise partitioning to the mitochondrial compartment (Fig. 7).

In contrast, the deletion present in transcripts derived from pMRP-F severely impaired its partitioning to mitochondrial fractions (Fig. 7). Transcripts from pMRP-F accumulated to high levels within the total cellular RNA pool, but its fractionation pattern was similar to that of Ul snRNA and quite distinct from either endogenous MRP RNA or the other mutated transcripts. These results are summarized in a quantitative manner in Fig. 8.

Discussion

Our data provide the first direct ultrastructural evidence for localization of MRP RNA to both nucleoli and mitochondria of mammalian cells. The authenticity of a pathway for nucleomitochondrial transport of MRP RNA in animal cells is supported further by biochemical evidence that specific structural determinants with the MRP RNA molecule are necessary for mitochondrial partitioning.

MRP RNA was previously shown to be abundant in nuclear fractions isolated from human cells (Karwan et al., 1991) and present in complexes that include a 40-kD nucleolar (Th/To) antigen (Yuan et al., 1991). Our present demonstration by ultrastructural in situ hybridization of an abundant nucleolar pool of MRP RNA was, therefore, an anticipated finding, and serves as a positive internal control for examination of the same sections for the presence of MRP RNA in other cellular compartments. In contrast to this consensus concerning nucleolar localization of MRP RNA, mitochondrial compartmentation of this small RNA has been the subject of recent controversy (Kiss and Filipowicz, 1992; Topper et al., 1992). Our current findings provide the first direct ultrastructural evidence for the presence of an authentic intramitochondrial pool of MRP RNA, at least in actively respiring cells such as cardiomyocytes that are characterized by abundant mitochondria and high concentrations of mitochondrial DNA (Williams, 1986; Annex and Williams, 1990).

Evidence provided by ultrastructural in situ hybridization is buttressed by the results of gene transfer experiments in which the partitioning of mutated forms of MRP RNA into a purified mitochondrial fraction was examined in comparison to native endogenous MRP RNA and to other RNA species with unambiguous subcellular localization to the nucleus, cytoplasm, or mitochondrial matrix. Using this biochemical approach, mitochondrial partitioning of native MRP RNA can be clearly distinguished from that of Ul snRNA and cytoplasmic 28S rRNA. In addition, a mutated form of MRP RNA was identified that accumulates to high levels in the whole cell RNA pool, but fails to partition to

the mitochondrial fraction. Since other mutations in MRP RNA produced transcripts that remained competent for mitochondrial partitioning, we conclude that specific structural determinants within the molecule are necessary for recognition by components of a nucleomitochondrial transport pathway. In conjunction with the ultrastructural data, these results corroborate the existence of an authentic intramitochondrial pool of MRP RNA.

Since MRP-RNA is encoded by a single nuclear gene (Chang and Clayton, 1989; Hsieh et al., 1990), partitioning of this transcript to both nucleolar and mitochondrial compartments of mammalian cells, as demonstrated by our current findings, raises interesting questions concerning the molecular mechanisms by which such complex intracellular trafficking is accomplished. Recent studies have defined features of nucleocytoplasmic shuttling mechanisms (Nigg et al., 1991), including discrete "tracks" for transport of mRNAs (Xing et al., 1993; Carter et al., 1993). Resinless section electron microscopy of chromatin-extracted nuclei has identified 10-nm filaments that extend from nucleoli to the nuclear lamina (Fey et al., 1986), suggesting a cytoskeletal basis for macromolecular tracking phenomena. Some RNAs are exported from the nucleus in association with proteins (Schmidt-Lachmann et al., 1993). Electron microscope tomography has shown that translocation of a premessenger RNA ribonucleoprotein particle through the nuclear pore of *Chironomous tetanus* is accomplished by unfolding of a ribonucleoprotein ribbon from an original ring-like structure (Mehlin et al., 1992). In animal cells, subcellular organelles known as vaults can be visualized in ultrastructural studies and have been isolated by biochemical fractionation and shown to be comprised of ribonucleoprotein complexes (Chugani et al., 1993). Other studies have identified features of pathways for reuptake of snRNAs from the cytoplasmic space to the nucleus (Baserga et al., 1992).

The structural and biochemical features of pathways by which RNA may gain access to the mitochondrial matrix are, however, entirely unknown at this time. The existence of pathways for nucleomitochondrial transport of RNA is supported by reports that the mitochondrial genomes of certain plant species and protozoa lack sequences encoding tRNAs, which must, therefore, be imported from the nucleus (Small et al., 1992; Marechal-Drouard et al., 1988; Lye et al., 1993). In yeast, mitochondrial RNAse P requires an RNA subunit encoded within the mitochondrial genome (Hollingsworth and Martin, 1986). The apparent absence of a gene within mitochondrial DNA of mammalian cells encoding an RNAse P RNA subunit suggests that mammalian RNAse P, like RNAse MRP, is dependent on import of an RNA subunit from the cytoplasmic space (Doersen et al., 1985).

At this time we can only speculate as to what specific steps and molecular mechanisms are required for RNA transcribed from nuclear genes to be directed to the mitochondrial compartment. Nucleocytoplasmic trafficking of proteins and import of polypeptides to mitochondria has been intensively studied (Meier and Blobel, 1992; Attardi and Schatz, 1988; Douglas et al., 1991; Manning-Krieg et al., 1991; Sequi-Real et al., 1992) and analogous processes may be involved in RNA transport. Accordingly, we hypothesize that import of MRP RNA to mitochondria requires selective recognition by carrier or chaperone proteins and/or by a mitochondrial surface receptor. Potential analogies between mechanisms of protein import and RNA partitioning to mitochondria should not, however, be drawn too literally. It is likely, for example, that structural requirements within RNA molecules that permit recognition by carrier or receptor proteins are more complex than the modular presequences that target proteins for mitochondrial import.

A logical step towards greater understanding of mitochondrial partitioning of RNAs would be the identification of cytoplasmic and mitochondrial proteins that associate specifically with MRP RNA. The Th/To antigen (Reimer et al., 1988; Yuan et al., 1991) is present in ribonucleoprotein complexes within nucleoli that contain MRP RNA, but other proteins with which MRP RNA may associate are uncharacterized at this time.

Further study of the molecular mechanisms that govern transport of nucleic acids to mitochondria has potential medical, as well as scientific, importance. Several maternally inherited human diseases are associated with deletions and point mutations in the mitochondrial genome (Holt et al., 1988; Wallace et al., 1988; Shoffner et al., 1990; Goto et al., 1990). For example, myoclonic epilepsy and ragged-red fiber disease (MERRF) and mitochondrial myopathy, encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS) are attributable to single base substitutions in $tRNA^{Lys}$ and $tRNA^{Leu}$, respectively (Shoffner et al., 1990; Goto et al., 1990). The $tRNA^{Lys}$ mutation causes a general reduction in mitochondrial protein synthesis (Chomyn et al., 1991). Prospects for gene therapy directed at these mitochondrial gene defects are limited currently by the absence of methods for efficient introduction of foreign genetic material into mitochondria of mammalian cells (discussed by Lander and Lodish, 1990). Our current findings illustrate that RNA transcripts derived from nuclear trans-genes can partition to the mitochondrial compartment. While it may be difficult to engineer chimeric RNA molecules that retain the mitochondrial import signal, the ability to direct foreign RNA sequences to mitochondria raises the possibility, in principal, of functional complementation of mitochondrial gene defects without a requirement for direct genetic transformation of mitochondria with exogenous DNA.

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