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# A Delayed-early Response Nuclear Gene Encoding MRPL12, the Mitochondrial Homologue to the Bacterial Translational Regulator L7/L12 Protein\*

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**We have characterized a new delayed-early response mRNA encoding a 21-kDa product (MRPL12) that accumulates during the G<sub>1</sub> phase of growth-stimulated cells. MRPL12 is the mammalian homologue to chloroplastic and bacterial L12 ribosomal proteins. Immunofluorescence microscopy and cell fractionation indicate a predominant mitochondrial localization in various mammalian cell lines. The NH<sub>2</sub>-terminal 49 amino acids are necessary and sufficient to target the protein within the mitochondria and are probably cleaved off during import. MRPL12 proteins associate *in vitro* and cofractionate with ribosomal structures, as is the case for prokaryotic L12 proteins. Expression of a dominant inhibitory truncated protein leads to a severe reduction in cell growth by inhibiting mitochondrial ATP production. MRPL12 is the first mammalian mitochondrial ribosomal protein to be characterized.**

Addition of growth factors to a resting cell culture elicits a wide variety of biochemical and genetic responses. The early biochemical events trigger the cytoplasmic accumulation of newly transcribed RNAs (primary or immediate-early response RNAs), which does not require *de novo* protein synthesis. In fibroblastic cells, a large number of genes have been characterized as being transiently induced within 4 h of growth stimulation (1, 2). They encode various types of products, such as cytokines, structural proteins, metabolic enzymes, protein phosphatases, and transcription factors (3–6). However, activation of immediate-early response genes is not sufficient *per se* for eliciting DNA synthesis, which requires long-term growth factor exposure and active protein synthesis for at least 10 h (7, 8). Stimulated cells may also be blocked in late G<sub>1</sub> upon transforming growth factor- $\beta$  or glucocorticoid treatment without affecting the early biochemical and genetic events (9–11). A limited number of genes activated during late G<sub>1</sub> have been characterized as (i) early-early response mRNAs, the accumulation of which, like immediate-early response mRNAs, does not require *de novo* protein synthesis. They encode products such as p53 (12), cyclin genes (13), Ras-like proteins (14), and biosynthetic enzymes or secreted proteases (4, 15–17). A lim-

ited number of genes have also been characterized as (ii) delayed-early response RNAs, which accumulate upon growth stimulation only when protein synthesis is active. They were found to encode various types of products, such as structural proteins, non-histone chromosomal proteins, extracellular matrix proteins, and transcription factors (18, 19).

Although mitochondrial activity is not necessary for cell growth in defined cultured conditions (20), several nuclear genes encoding mitochondrial proteins have also been described as being growth-regulated. These include the mitochondrial chaperonin hsp60 (21), the Fos transformation effector (22), an ADP/ATP carrier (23), and a proton/phosphate symporter (19).

We report here the characterization of the mammalian mitochondrial ribosomal (mitosomal) MRPL12 protein as being encoded by a delayed-early response mRNA. MRPL12 mRNA accumulation in growth-stimulated cells occurs in proportion to the mitogenic strength and is paralleled by the accumulation of the corresponding protein. MRPL12 protein is phylogenetically related to the chloroplastic and bacterial L12 ribosomal proteins, which control mRNA translation. Immunofluorescence microscopy and cell fractionation indicate a predominant mitochondrial localization of the protein in various mammalian cell lines. The amino-terminal region is necessary and sufficient to promote a mitochondrial targeting of exogenous proteins. MRPL12 proteins associate *in vitro* as dimers and cofractionate with ribosomal structures from mitochondrial extracts. Expression of a truncated MRPL12 protein acting as a dominant inhibitor affects cell growth and mitochondrial ATP synthesis.

## MATERIALS AND METHODS

**Cell Culture**—CCL39, COS-7, NIH3T3, and HeLa cells were grown, respectively, in Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% calf serum (FCS).<sup>1</sup> For serum starvation, confluent cells were incubated for 20 h with plain Dulbecco's modified Eagle's medium. Renewed growth was stimulated by addition of 10% FCS,  $\alpha$ -thrombin (1 unit ml<sup>-1</sup>), or epidermal growth factor (EGF; 35 ng ml<sup>-1</sup>) in combination with insulin (10  $\mu$ g ml<sup>-1</sup>). Concentrations were 1 mM for 8-bromo-cAMP and 10  $\mu$ g ml<sup>-1</sup> for cycloheximide.

**RNA and Protein Analysis**—RNA extraction and analysis were carried out as described (24). The hybridization mixture contained 50% formamide, 5  $\times$  Denhardt's solution, 10 mM phosphate buffer (pH 7.0), 0.75 M NaCl, 0.1% SDS, 10% dextran sulfate, and 100  $\mu$ g ml<sup>-1</sup> denatured salmon sperm. Hybridization was at 42 °C for 12–24 h using a probe concentration of 2  $\times$  10<sup>6</sup> cpm ml<sup>-1</sup>. Filters were washed twice in 2  $\times$  SSC, 0.1% SDS at room temperature and once in 0.2  $\times$  SSC, 0.1% SDS at 65 °C. Signals were quantified using either an image processing workstation (BioImage, Millipore Corp.) or a PhosphorImager (Molecular Dynamics, Inc.). Proteins were prepared from cells lysed for 1 h at

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X79864 and X79865.

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<sup>1</sup> The abbreviations used are: FCS, fetal calf serum; EGF, epidermal growth factor; HA, hemagglutinin; PCR, polymerase chain reaction; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

4 °C in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, and 1% Triton X-100). Cells extracts were cleared by centrifugation (15 min at 10,000 × *g*). Protein detection on Western blots was performed using the ECL detection kit (Amersham Corp.).

**Isolation of Full-length cDNA Clones**—The construction of cDNA libraries was as described (14). Libraries from hamster CCL39 cells growth-stimulated for 5 h and exponentially growing HeLa cells were screened at low density on nylon membranes (Hybond N, Amersham Corp.). DNA was extracted from pools of colonies isolated from positive areas and restricted with *Bam*HI and *Hind*III enzymes, and insert size was monitored by Southern blotting. Pools harboring inserts larger than 1 kilobase pair (*i.e.* corresponding to the size of mRNA) were plated at a lower density, and individual positive clones were selected after a second round of screening.

**cDNA Sequencing and Analysis**—DNA sequence was determined on crude double-stranded plasmid DNA (T7 sequencing kit, Pharmacia Biotech Inc.). The complete hamster and human sequences were obtained on both strands by subclones generated by restriction cleavage. Due to their high G + C content, the sequence corresponding to the 5'-end of both RNAs was determined using M13 subcloning and manual and automatic sequencing using an Applied Biosystems 373A sequencer. Data base searches and sequence analysis were worked out by using the facilities of the Centre Inter-universitaire de Traitement de l'Information (Paris) (25).

**Construction of Expression Vectors**—For eukaryotic expression, pcDNA1 (Invitrogen) was modified as follows. A double-stranded oligonucleotide containing a canonical ribosome-binding sequence and a translation initiator (5'-(*Hind*III)-CCCCGCCTCGGAGCCGCCAC-CATGCC-(*Bam*HI)-3') was inserted in the *Hind*III and *Bam*HI sites of pTT7318U (Pharmacia Biotech Inc.). Oligonucleotides containing the hemagglutinin (HA) epitope (5'-TACCCATACGATGTTCCGGAT-TACGCTAGCCTC-3') were then inserted in the *Nco*I and *Eco*RI sites. A *Hind*III/*Sca*I fragment containing the ribosome-binding sequence with the HA epitope was inserted in the *Hind*III and *Eco*RV sites of the pcDNA1 vector, yielding pcDNAFT. p(FT)L12 is pcDNAFT containing the PCR-amplified human MRPL12 ORF. pΔL(FT)L12 contains a DNA fragment encoding amino acids 51–191 that was PCR-amplified and subcloned in the *Eco*RI and *Xho*I sites of pcDNAFT (see above). A PCR-amplified DNA fragment corresponding to the amino-terminal region of human MRPL12 (amino acids 1–48) was subcloned in the *Hind*III and *Eco*RI sites of pcDNA3 (Invitrogen). A fragment encoding a HA-tagged version of RhoG (14) was then inserted 3' of the MRPL12 fragment, yielding pL(FT)RhoG. pL12Δ(FT) was obtained by cloning the human MRPL12 ORF in pcDNA1 and replacing the *Eco*RV/*Xba*I fragment corresponding to amino acids 123–199 with a double-stranded oligonucleotide encoding the HA epitope followed by a stop codon.

**In Vitro Translation and Assay for Interaction**—RNAs were *in vitro* transcribed from the T3 promoter and translated in rabbit reticulocyte extracts according to the suppliers' recommendations (Pharmacia Biotech Inc. and Amersham Corp.). For interaction assays, wild-type MRPL12 and HA-tagged L12Δ(FT) proteins were cotranslated, immunoprecipitated with 12CA5 antibodies, and separated by SDS-PAGE. Signal for MRPL12 (A) was quantified on PhosphorImager screens. The efficiency of interaction was monitored as follows. Assuming an interaction rate  $\delta$  and  $p$  and  $(1 - p)$  representing the proportion of wild-type L12 and L12Δ(FT) proteins after translation, respectively, the relative amount of interacting molecules is  $2 \times \delta \times p(1 - p)$ . This value was corrected by the efficiency of immunoprecipitation ( $\rho$ ) and the unspecific background ( $B$ ), estimated by immunoprecipitation of HA-tagged L12Δ(FT) products and of wild-type L12, respectively. Estimation of  $\delta$  is given by  $\delta = (A - B)/2 \times \rho \times p(1 - p)$ .

**Preparation of Glutathione S-Transferase-MRPL12 and Maltose-binding Protein-MRPL12 Fusion Proteins**—The human ORF (positions +138 to +735) was amplified by PCR, subcloned in pUC18, and sequenced. It was then cloned in pMalcR1 (New England Biolabs, Inc.) and pGEX2T (Pharmacia Biotech Inc.) vectors. Maltose-binding protein and glutathione S-transferase fusion proteins were expressed in TG1 bacteria upon addition of 0.3 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 4 h or 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 4.5 h, respectively. Recombinant maltose-binding proteins were purified on amylose columns as described by the supplier (New England Biolabs, Inc.).

**Preparation of Specific Antibodies**—Maltose-binding protein-P2A1 fusion protein (100  $\mu$ g) was used for subcutaneous injection in rabbits. Five rounds of boost were performed, and the immune response was monitored by Western blotting of the bacterial protein. Two independent antisera were raised, termed a179 and a180. Specific antibodies were affinity-purified against 50  $\mu$ g of glutathione S-transferase-P2A1

fusion protein immobilized by Western blotting on a nitrocellulose membrane. A nitrocellulose slice (3 × 0.2 cm) containing the fusion protein was incubated overnight at 4 °C in a 5-ml test tube with 500  $\mu$ l of PBS containing 0.2% gelatin and 50  $\mu$ l of a180 antiserum. The membrane was washed for 30 min with cold PBS/Tween (0.2%) and then with cold PBS, and antibodies were eluted by adding 400  $\mu$ l of 100 mM glycine (pH 2.8). The solution was immediately neutralized in 3 M Tris-HCl (pH 8.8). Antibodies to the HA epitope were prepared from the supernatant medium of the 12CA5 hybridoma cell culture and purified on a protein A-Sepharose affinity column.

**Immunolocalization**—Hamster CCL39, human HeLa, and monkey COS-7 cells were grown in coated 35-mm plastic tissue culture dishes. Cell cultures were fixed with 3% formaldehyde in PBS for 30 min at 4 °C, washed twice for 10 min with 50 mM NH<sub>4</sub>Cl in PBS and once for 10 min with PBS, and permeabilized for 20 min at 4 °C with 0.1% Triton X-100 in PBS. Cells were then washed for 10 min at 4 °C with PBS containing 0.2% gelatin and incubated with affinity-purified rabbit anti-MalE/P2A1 (a180, at a 1:100 dilution in PBS/gelatin; see "Preparation of Specific Antibodies") in PBS for 1 h at 22 °C. Affinity-purified goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (1:200; Sigma) or biotinylated anti-rabbit IgG (1:200; Amersham Corp.) was detected by incubating cells for 15 min with Texas Red-streptavidin (1:400; Amersham Corp.). For mitochondrial labeling of living cells, cells were incubated for 30 min at 37 °C in 5% CO<sub>2</sub> in the presence of 10  $\mu$ g ml<sup>-1</sup> rhodamine 123, followed by four rounds of washings (10 min at 37 °C) with fresh Dulbecco's modified Eagle's medium. Cell cultures were visualized with a Zeiss Axiophot epifluorescence or confocal microscope.

**Mitochondrial Extract Analysis**—Cells were washed in TTM buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 15 mM MgCl<sub>2</sub>) and incubated for 20 min at 4 °C. Cells were broken with a Dounce homogenizer, and the lysate was centrifuged for 5 min at 900 × *g*. The pellet was resuspended in TTM buffer containing 0.25 M sucrose and 1 mM EDTA and centrifuged for 15 min at 13,000 × *g*. Pelleted mitochondria were resuspended in TTM buffer containing 0.25 M sucrose and centrifuged on sucrose cushions (1.5 M:1 M) for 20 min at 20,000 × *g*. Mitochondria were collected from the interphase, diluted in TTM buffer, and centrifuged for 20 min at 20,000 × *g*. For mitochondrial extract fractionation, purified mitochondria were resuspended in 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 30 mM MgCl<sub>2</sub>, and 6 mM  $\beta$ -mercaptoethanol and then incubated for 15 min at 4 °C after addition of 1% Triton X-100. Mitochondrial lysates were cleared by centrifugation at 60,000 × *g* for 10 min and then fractionated by centrifugation on a 15–30% sucrose gradient (60,000–140,000 × *g* for 4 h at 3 °C). 16 S rRNA distribution was determined by Northern blot analysis (see above). The probe was a 2-kilobase pair-long *Apal*/*Sac*I fragment isolated from *Camelus bactrianus* (kindly supplied by Emmanuel Douzery, Institut des Sciences de l'Evolution de Montpellier, Montpellier, France). Washing conditions were 0.1 × SSC, 0.1% SDS at 65 °C for 30 min.

## RESULTS

**Mitogen Induction of P2A1 Expression**—We previously reported the isolation of a set of cDNA clones corresponding to mRNAs that accumulate in mid-G<sub>1</sub> of resting CCL39 fibroblastic cells stimulated to grow (19). One of these, termed P2A1, revealed on Northern blotting a 1.1-kilobase pair mRNA species, whose induction was strictly dependent on active protein synthesis. As shown Fig. 1A, the P2A1 mRNA level decreased when exponentially growing cells (*lane E*) were serum-starved for 24 h (*lane Q*) or 48 h (*lane C*). Following serum stimulation of cells (*lane Q*) for the indicated times, the P2A1 mRNA level was found to increase up to 12 h (*FCS lanes*). When cycloheximide was added in combination with FCS, mRNA accumulation was impaired (*FCS+CHX lanes*). This inhibition does not reflect a long-range side effect of cycloheximide, as a lag period of 1.5–2 h between serum stimulation and cycloheximide treatment was sufficient for P2A1 mRNA accumulation to resume (*FCS/FCS+CHX lanes*). This indicates that protein synthesis is required during the first 2 h of stimulation for P2A1 mRNA accumulation. To obtain information on the biochemical pathways involved in increased P2A1 mRNA expression, we then examined resting cells for their response to various purified growth factors, following distinct biochemical pathways for transducing their mitogenic trigger, either through receptor

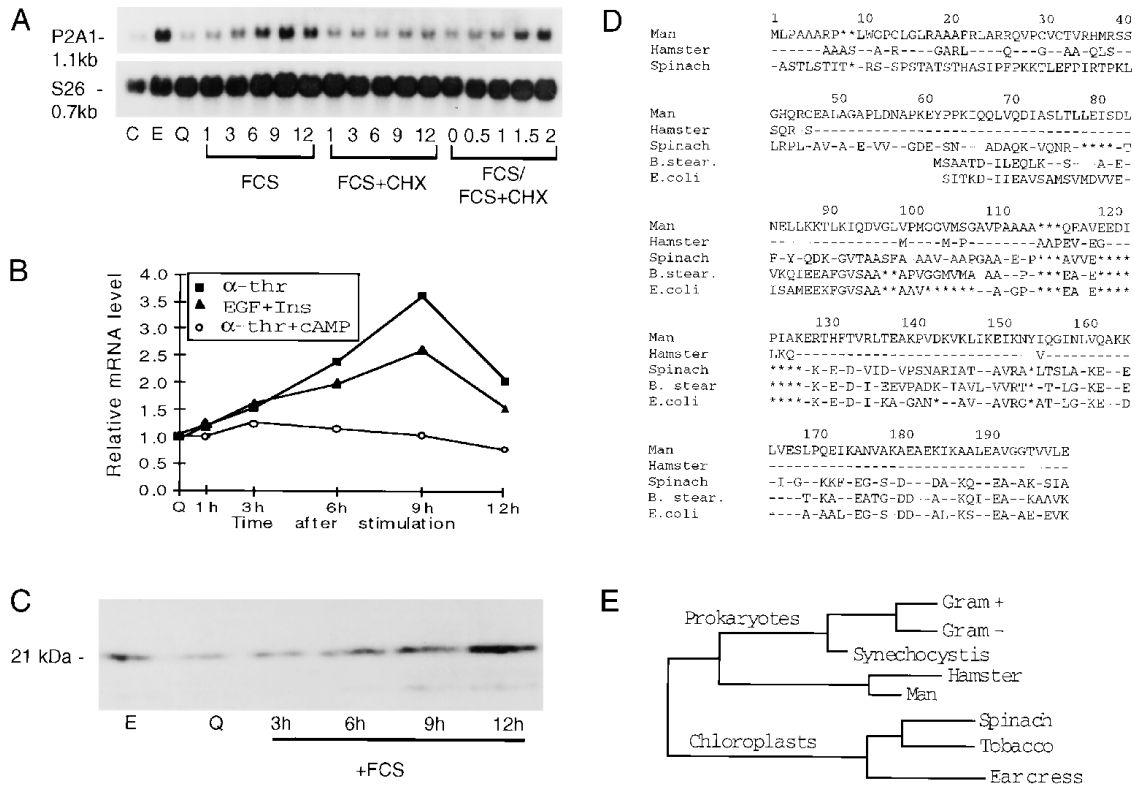


FIG. 1. A, RNA was extracted from CCL39 cells serum-starved for 48 h (lane C), exponentially growing cells (lane E), or cells serum-starved for 24 h (lane Q) and then stimulated for 1, 3, 6, 9, and 12 h with 10% serum alone (FCS), for 1, 3, 6, 9, and 12 h with FCS in combination with 10  $\mu\text{g ml}^{-1}$  cycloheximide (FCS+CHX), or for 0, 0.5, 1, 1.5, and 2 h with FCS and then for 6, 5.5, 4, 4.5, and 4 h with FCS + cycloheximide (FCS/FCS+CHX). 10  $\mu\text{g}$  of total RNA was electrophoresed and transferred to nylon membranes. Filters were sequentially probed with  $^{32}\text{P}$ -labeled P2A1 and S26 cDNA (65 inserts). B, shown is the effect of purified growth factors on P2A1 mRNA accumulation. Total RNA was extracted from cells stimulated for the indicated times (in hours) with 1 unit  $\text{ml}^{-1}$   $\alpha$ -thrombin ( $\alpha$ -thr),  $\alpha$ -thrombin plus 1 mM 8-bromo-cAMP ( $\alpha$ -thr+cAMP), or 35  $\text{ng ml}^{-1}$  EGF plus 10  $\mu\text{g ml}^{-1}$  insulin (EGF+Ins). 10  $\mu\text{g}$  of total RNA was analyzed by Northern blotting, and autoradiographic signals were quantified by using a Millipore BioImage workstation. C, total proteins were extracted from exponentially growing cells (lane E), serum-starved cells (lane Q), or cells stimulated for the indicated times (in hours) with 10% fetal calf serum. Proteins (500  $\mu\text{g}$ ) were analyzed by Western blotting, and P2A1 peptide was revealed using polyclonal a180 antibodies. D, shown is a sequence comparison of human and hamster MRPL12 proteins with chloroplastic proteins (ear cress (28) and spinach (29)) and prokaryotic proteins (*B. stearothersophilus* (32) and *E. coli* (33)). Protein sequences were aligned using TREEALIGN software (36). Identical amino acids (-) and deletions (\*) are indicated. E, shown is the evolutionary tree of L12 proteins. Distances using the TREEALIGN package (36) were used to trace an evolutionary tree. Branch length is proportional to the computed distances. The tree is unrooted.

TABLE I  
Nucleotide sequence of the human 5'-noncoding region

The ATG initiators (boldface letters) are located within a repeated sequence (underlined).

10	20	30	40	50	60
CCGAATTTTCGGCTCGA <b>ATG</b> CCC GGC AGC CGT GGC GGC TAG AGCGTTCCTCCCCAGCTC	M P G S R G G *				
70	80	90	100	110	120
<b>GAATG</b> CCC GGC GGC CGA GGC GGC TAG AGCGTCGCCTCCTCCGGGGAACCGCGTGTGACC	M P G G R G G *				
130	140	150	160	170	
TTCAGCCCGCGACCG <b>ATG</b> CTG CCG GCG GCC GCT CGC CCC CTG TGG GGG	M L P A A A R P L W G				

tyrosine kinase (EGF) or G-protein-coupled receptor ( $\alpha$ -thrombin) (26, 27). Insulin is not mitogenic on CCL39 cells, but enhances the effect of EGF. Total RNA prepared from resting CCL39 cells stimulated for 1, 6, and 12 h was analyzed by Northern blotting. Signal quantification is shown Fig. 1B.  $\alpha$ -Thrombin led to a 3.5-fold increase in P2A1 mRNA 9 h after stimulation, whereas a combination of EGF and insulin led to a 2.5-fold induction. To ensure that P2A1 mRNA accumulation correlated with the mitogenic response induced by growth factors, we tested the antimetabolic effect of 8-bromo-cAMP on  $\alpha$ -thrombin stimulation. The accumulation of P2A1 mRNA was inhibited >80% when 8-bromo-cAMP was added in conjunction with  $\alpha$ -thrombin, indicating that biochemical pathways associ-

ated with DNA synthesis are responsible for the increase in the P2A1 RNA level. Identical cAMP-mediated inhibition was observed in cells treated with EGF or EGF + insulin (data not shown).

To demonstrate that P2A1 activation also occurs at the protein level, Western blots of proteins prepared from cells in various physiological states were incubated with affinity-purified rabbit polyclonal antibodies (a183) to the bacterially expressed human protein. As observed in Fig. 1C, a single 21-kDa peptide was revealed, whose level decreased in resting *versus* exponentially growing cells (compare lanes E and Q) and increased when cells were stimulated to grow.

*P2A1 Encodes a Protein Homologous to L12 Ribosomal Pro-*

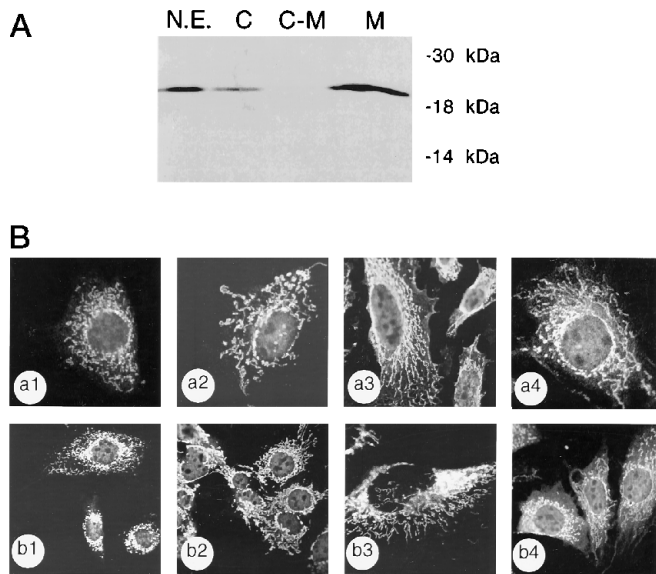


FIG. 2. *A*, subcellular fractionation of MRPL12. Nuclei and membranes (*lane N.E.*) were separated from cytoplasmic extracts (*lane C*) by low speed centrifugation of mechanically broken CCL39 cells. The cytosolic extracts (*lane C-M*) were depleted of mitochondria (*lane M*) by high speed centrifugation. Aliquots of either fraction were separated by SDS-PAGE, and the amount of MRPL12 protein was detected by immunostaining of Western blots using the affinity-purified a180 antibody. *B*, immunolocalization of MRPL12. Exponentially growing CCL39 cells (*panel 1*), COS-7 cells (*panel 2*), HeLa cells (*panel 3*), and NIH3T3 cells (*panel 4*) were incubated for 30 min with rhodamine 123, and mitochondria were observed under confocal microscopy (*row b*). Alternately, cells were fixed and incubated in the presence of the affinity-purified a180 antibodies (*row a*).

*tein*—The size of the insert in the original clone was 0.5 kilobase pair. Full-length cDNAs were then isolated from hamster and human libraries, and complete sequences were determined (data not shown). Searching for homologous sequences in the GenBank™ Data Bank revealed no match with known DNA sequences except for several randomly sequenced cDNAs (GenBank™ accession numbers H51431, H69197, H51739, R10227, and R10550). The largest ORFs contained in hamster and human cDNAs encode proteins of 203 and 198 amino acids, respectively. Two additional AUG initiator codons were found upstream of the human ORF, at positions 19 and 63 (Table I). The codon at position 63 is in frame with the main ORF (starting at position 138). Both codons are included within a 34-nucleotide-long tandem repeat (positions 13–47 and 57–91) and are followed by stop codons (positions 40 and 84). Protein sequences derived from hamster and human main ORFs were compared against NBRF, GenPro, and SwissProt data bases using the BLAST program, which revealed that their carboxyl-terminal ends displayed a significant similarity to chloroplast and bacterial L7/L12 proteins (smallest Poisson probability ranging from  $10^{-12}$  to  $10^{-3}$ ) (Fig. 1D). We investigated more precisely the relationships between P2A1 and L7/L12 proteins from different sources: chloroplasts from *Arabidopsis thaliana* (ear cross) (28), *Spinacia oleracea* (spinach) (29), *Nicotiana tabacum* (common tobacco) and *Nicotiana sylvestris* (wood tobacco) (30, 31), and prokaryotes (*Bacillus stearothermophilus* and *Bacillus subtilis* (32), *Escherichia coli* (33), *Salmonella typhimurium* (34), and *Synechocystis* sp. (35)). Sequences were first aligned with the TREEALIGN package (36). Multiple sequence alignments were then used to compute identity and distance scores between members of each pair and to trace the evolution of RL7/RL12 proteins, presented in Fig. 1E. Proteins from Gram-positive and Gram-negative bacteria are correctly clustered, as are chloroplastic proteins. The tree consists of

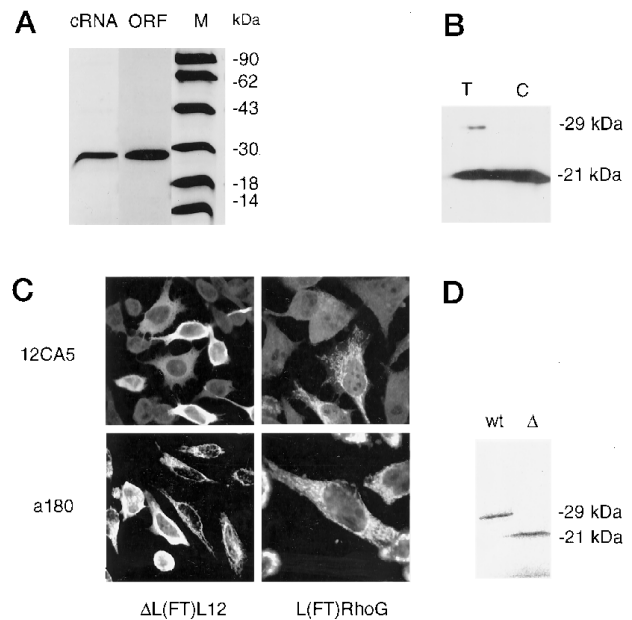
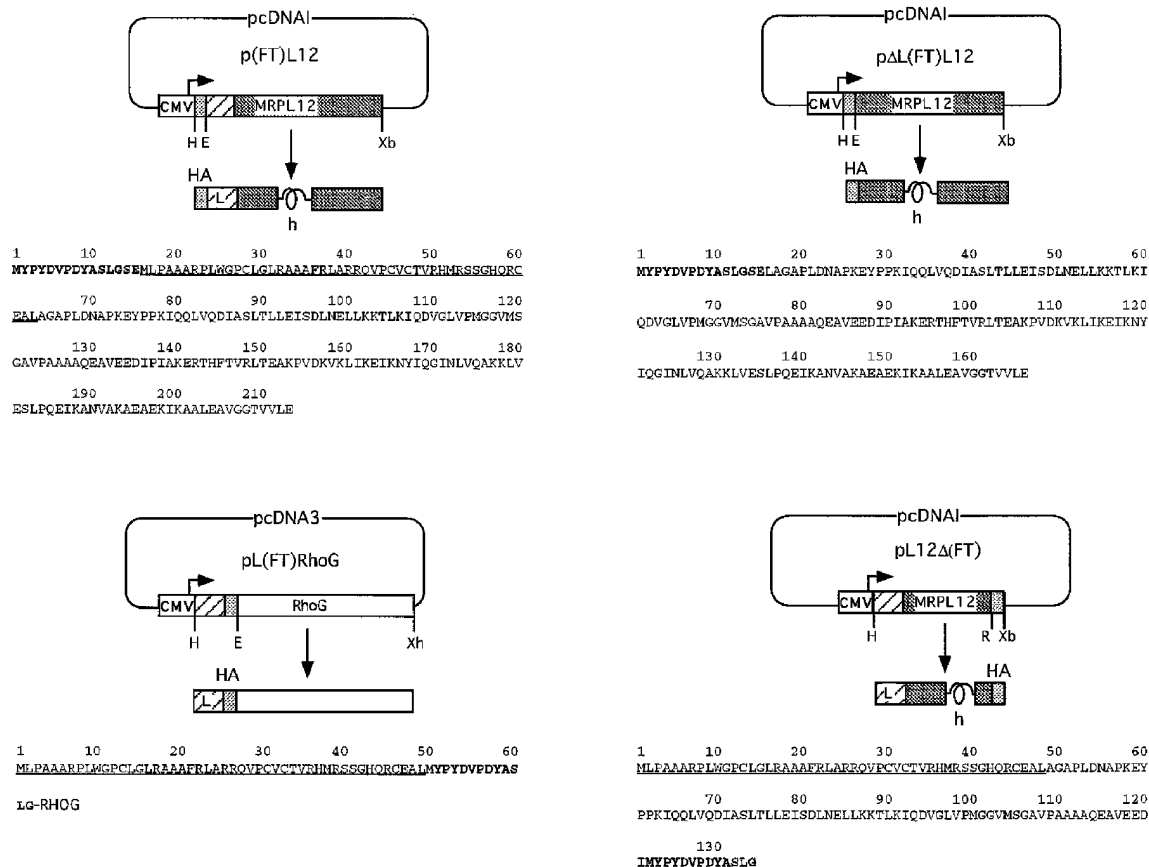


FIG. 3. *A*, comparison of electrophoretic mobilities of *in vitro* translated human products on SDS-PAGE. Proteins were translated *in vitro* from the full-length cRNA or from RNA corresponding to the largest ORF and separated by SDS-PAGE. Size markers are indicated on the right (*lane M*). *B*, detection of the unprocessed MRPL12 product. Extracts from HeLa cells transfected with the p(FT)L12 construct (*lane T*) or from control cells (*lane C*) were analyzed by Western blotting using the a180 antibodies. *C*, immunolocalization of  $\Delta$ L(FT)L12 and L(FT)RhoG proteins. HeLa cells were transiently transfected with p $\Delta$ L(FT)L12 and pL(FT)RhoG (see "Materials and Methods" and Fig. 4). 24 h after transfection, cells were fixed and incubated in the presence of immunopurified 12CA5 antibodies or affinity-purified a180 antibodies. *D*, *in vitro* translation of  $\Delta$ L(FT)L12 protein. RNA was synthesized and translated *in vitro* in the presence of [ $^{35}$ S]methionine. Labeled products were separated by SDS-PAGE. *Lane wt*, (FT)L12 protein; *lane  $\Delta$* ,  $\Delta$ L(FT)L12 protein.

three main distinct stems, corresponding to prokaryotic, chloroplastic, and P2A1 proteins, the latter having diverged earlier from chloroplastic proteins than from prokaryotic proteins. In addition to primary sequence similarity, secondary structures (37) of human and hamster putative proteins exhibit features very close to those of prokaryotic L7/L12 proteins (38): a helical region spanning amino acids 45–100 and a highly flexible glycine/alanine-rich hinge region (amino acids 100–113), followed by two large helical  $\alpha$ - and  $\beta$ -segments separated by a "turn" region, constituting a globular domain. The amino-terminal regions (amino acids 1–45) of mammalian and chloroplastic proteins also share common features, *i.e.* two short helical segments separated by coiled structures.

*P2A1 Protein Colocalizes with the Mitochondria*—The topology of the evolutionary tree suggested that P2A1 might be the mitochondrial homologue of L12 proteins. To address this hypothesis, a180 antibodies were used in Western blotting of subcellular extracts (Fig. 2A) and immunolocalization (Fig. 2B). Exponentially growing HeLa cells were lysed, and cytoplasmic extracts (Fig. 2A, *lane C*) were separated from nuclei and membranes (*lane N.E.*) by low speed centrifugation. Mitochondria (*lane M*) were then fractionated from the cytosol (*lane C-M*) by centrifugation on sucrose cushions. A single specific 21-kDa protein was detected in the mitochondrial fraction, whereas no signal remained associated with the cytosolic fraction. A significant amount of protein was also detected in the nuclei and membranes, which probably results from mitochondria trapped within the perinuclear microtubule network when cells are broken in the absence of detergent. To confirm these observations, immunofluorescence studies using a180 antibod-

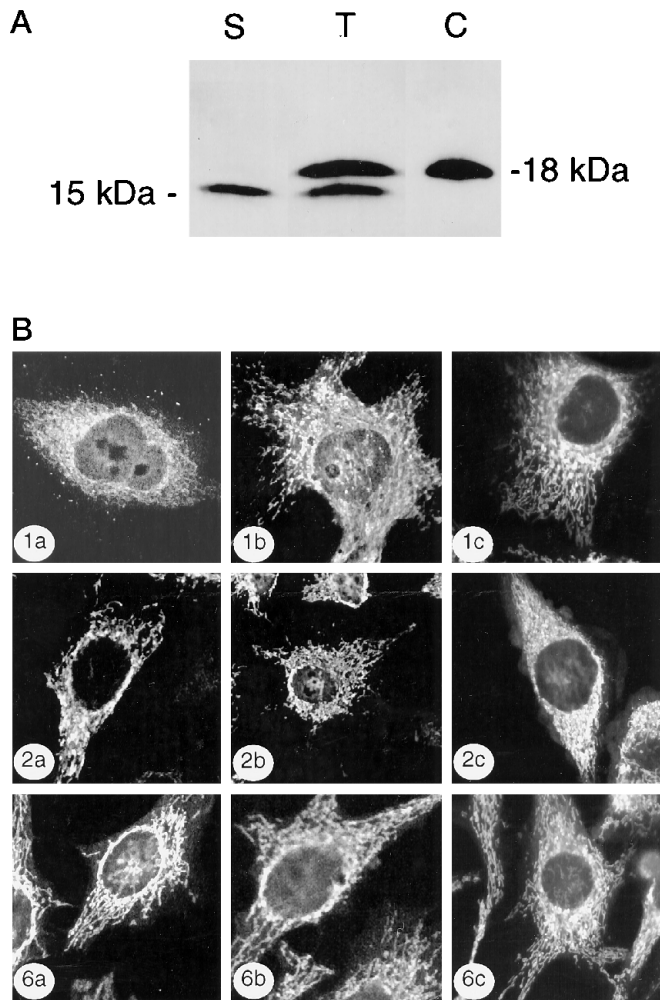


**FIG. 4. Constructs expressing various modified MRPL12 proteins.** The corresponding amino acid sequences are indicated below each map. The HA epitope sequence is indicated in *boldface*, and the mitochondrial targeting peptide is *underlined*. *A*, p(FT)L12, derived from pcDNA1, contains the complete ORF (MRPL12) fused to the HA epitope. *B*, pΔL(FT)L12, derived from pcDNA1, contains the ORF from which a portion coding for the NH<sub>2</sub>-terminal 48 amino acids was deleted. *C*, pL(FT)RhoG, derived from pcDNA3, contains a HA-tagged version of the human RhoG ORF fused to a fragment coding for the NH<sub>2</sub>-terminal 49 amino acids of MRPL12. *D*, pL12Δ(FT), derived from pcDNA1, contains the MRPL12 ORF from which a fragment coding for the carboxyl-terminal 77 amino acids was replaced with the HA epitope. *CMV*, cytomegalovirus; *H*, *Hind*III; *E*, *Eco*RI; *Xb*, *Xba*I; *Xh*, *Xho*I; *R*, *Eco*RV.

ies (Fig. 2*B*, row *a*) were carried out in hamster CCL39 cells (panel 1), monkey COS-7 cells (panel 2), human HeLa cells (panel 3), and murine NIH3T3 cells (panel 4). In all cell lines studied, most of the fluorescence appeared as cytoplasmic punctations or short filaments, reminiscent of mitochondrial distribution. This was further confirmed by specific mitochondrial staining of living cells with rhodamine 123 (Fig. 2*B*, row *b*). No signal was obtained with the preimmune serum (data not shown). Taking into account its similarity to the bacterial and chloroplastic L12 proteins and its mitochondrial localization, this protein will be termed MRPL12 for mitochondrial ribosomal protein L12.

**Characterization of MRPL12 Mitochondrial Targeting Sequences**—To confirm the efficient use of the third AUG codon, the human ORF was selectively PCR-amplified, and *in vitro* translated products were compared with those synthesized from the complete cDNA by SDS-PAGE (Fig. 3*A*). Proteins translated from both substrates displayed an identical apparent molecular mass of 29 kDa, indicating that the third AUG codon is efficiently used. However, the *in vitro* translated peptide exhibited a SDS-PAGE mobility 7–8 kDa higher than that of the MRPL12 protein (21 kDa) extracted from hamster or human cells. To rule out any artifactual modifications during *in vitro* translation, we first transiently transfected human 293 cells with the p(FT)L12 plasmid (Fig. 4*A*), a construct expressing the complete human MRPL12 protein fused to the HA epitope. A single product was specifically detected in transfected cells (Fig. 3*B*), whose apparent mobility (29 kDa) was

identical to that of the *in vitro* translated peptide. We next examined the amino-terminal sequence of both hamster and human proteins, which revealed classical features of a mitochondrial sequence leader (39, 40): (i) absence of acidic residues; (ii) a high content of arginine, leucine, and serine residues (34% (man) and 44% (hamster)); (iii) a high content of positively charged amino acids (23% (man) and 18% (hamster)); and (iv) a high hydrophobic helical moment. A potential cleavage site (R $XZ(X)_2$ (S/T/G)(X)<sub>4</sub>R, where *Z* is any hydrophobic residue) for mitochondrial processing peptidase (39, 40) was found at positions 45 (man) and 47 (hamster), as shown in Fig. 1*B*. To address the role of the amino-terminal sequence, we transiently transfected human HeLa cells with the pΔL(FT)L12 construct (Fig. 4*B*), expressing a HA-tagged truncated version of human MRPL12 lacking 48 amino acids in its amino-terminal part. *In vitro* translation yielded a product of 21 kDa, in agreement with the size of the endogenous protein (Fig. 3*D*). Immunolocalization of the ΔL(FT)L12 protein with both anti-HA monoclonal 12CA5 antibodies and anti-MRPL12 polyclonal a180 antibodies revealed a distribution throughout the cytoplasm (Fig. 3*C*), indicating that at least part of the targeting signal has been deleted from the protein. We next transiently transfected the pL(FT)RhoG construct (Fig. 4*C*), which drives the expression of a HA-tagged mutated version of RhoG protein, normally localized in the perinuclear endoplasmic reticulum (41), fused to the first 49 amino acids of MRPL12 leader sequence. At variance with the pΔL(FT)L12 construct, staining of cells transfected with the pL(FT)RhoG construct



**FIG. 5. Characterization of clones expressing MRPL12 $\Delta$  protein.** A, HeLa cells were transfected with pL12 $\Delta$ (FT), and total protein extracts were analyzed by Western blotting using 12CA5 antibodies. Lane S, stably transfected cells; lane T, transiently transfected cells; lane C, *in vitro* translated peptide. B, cells from stably transfected independent HeLa clones (1, 2, and 6) were fixed and incubated in the presence of immunopurified 12CA5 antibodies (row a) or affinity-purified a180 antibodies (row b) or were stained in the presence of rhodamine 123 (row c). Cells were visualized under confocal microscopy. Signals corresponding to full cell sections are shown.

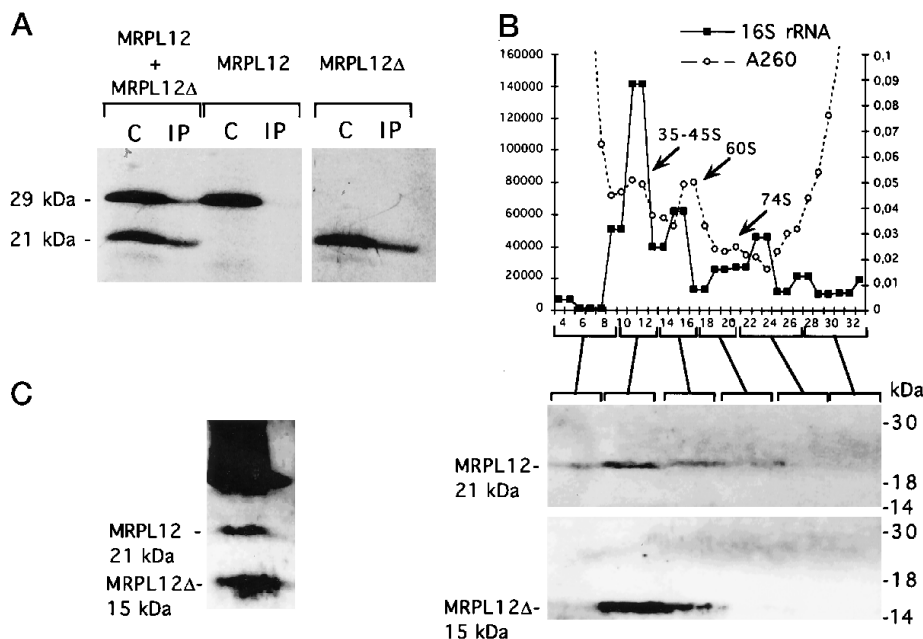
with 12CA5 antibodies showed a typical mitochondrial distribution. These data establish that the NH<sub>2</sub>-terminal 49 amino acids are necessary and sufficient for mitochondrial targeting and suggest that the leader peptide is cleaved off during MRPL12 mitochondrial import.

**Expression of MRPL12 $\Delta$ , a Truncated Version of MRPL12 Protein**—Prokaryotic L7/L12 proteins are associated with ribosomes as structures made of two homodimeric complexes bound to a single molecule of L10 protein (42). The protein segments involved in homodimer formation span the amino-terminal helix, corresponding to amino acids 46–91 in the human sequence, while the region spanning amino acids 119–198 displays a basic helix-turn-helix structure, required for binding to ribosomal RNA (43–45). To inhibit the activity of endogenous MRPL12, we expressed a MRPL12 protein lacking its RNA binding activity upon deletion of 76 amino acids in its carboxyl-terminal tail (pL12 $\Delta$ (FT) construct) (Fig. 4D), but retaining its ability to be targeted to the mitochondria and to interact with the endogenous protein. The correct processing and targeting of the exogenous protein were monitored by analyzing the apparent mobility and the localization of the truncated protein in

transiently or stably transfected HeLa cells (Fig. 5A). 18-kDa (*i.e.* the same size as the *in vitro* translated product (lane C)) and 15-kDa peptides were detected with 12CA5 antibodies in cellular extracts from transiently transfected cells (lane T), while a single band of 15 kDa was observed in extracts from stably transfected cells (lane S). Although the mobility shift approximates 3 kDa, this indicates that stably transfected cells express prominently the processed protein. We next isolated six independent stably transfected clones and compared the subcellular distribution of the truncated protein with that of endogenous MRPL12 by immunofluorescence analysis. All cell clones elicited a strong signal for the truncated construct, corresponding to a mitochondrial distribution, as illustrated in Fig. 5B.

**In Vitro Interaction between MRPL12 and MRPL12 $\Delta$** —We then examined whether wild-type MRPL12 and MRPL12 $\Delta$  proteins were able to associate. mRNAs transcribed from a wild-type MRPL12 cDNA and the pL12 $\Delta$ (FT) construct were cotranslated *in vitro* in the presence of [<sup>35</sup>S]methionine, and translation products were immunoprecipitated using 12CA5 antibodies (Fig. 6A). Equal amounts of nonimmunoprecipitated (lanes C) and immunoprecipitated (lanes IP) reactions were analyzed by SDS-PAGE. Under these conditions, ~19% of the 18-kDa MRPL12 $\Delta$  protein immunoprecipitated (MRPL12 $\Delta$ , lane IP versus lane C), while 12CA5 antibodies gave a background of 0.05% with the untagged 29-kDa complete protein (MRPL12, lane IP versus lane C). After cotranslation, addition of 12CA5 antibodies led to the immunoprecipitation of 5% of the untagged 29-kDa protein (MRPL12+MRPL12 $\Delta$ , lane IP versus lane C). According to the relation described under “Materials and Methods” and given that the relative amounts of the proteins synthesized during cotranslation were 68% (29 kDa) and 32% (18 kDa) (MRPL12+MRPL12 $\Delta$ , lane C), this indicates that ~60% of the translated proteins have interacted, probably as heterodimers, as shown for the bacterial L7/L12 proteins (42).

**Cofractionation of Wild-type and Mutant MRPL12 Proteins**—The previous experiments showed that the truncated version of MRPL12 protein was processed and targeted to the mitochondrion and could associate *in vitro* with the wild-type protein. As bacterial L12 proteins were shown to associate with ribosomes, we next examined the submitochondrial localization of both endogenous and truncated proteins in stably transfected HeLa cells. Lysates from purified mitochondria were fractionated on a 15–30% sucrose gradient, and aliquots of the collected fractions were analyzed by Western blotting for their content in MRPL12 and MRPL12 $\Delta$  proteins, by Northern blotting for their content in 16 S ribosomal RNAs, and by spectrophotometry for their content in nucleic acids (Fig. 6B). The overall distribution of nucleic acids (*upper panel, dashed line*) is in agreement with published data (46), exhibiting three peaks corresponding to 35–45 S (ribosomal subunits), 60 S (monomeric ribosome), and 74 S (polysomal structures). 16 S rRNA is mainly distributed within all ribosomal structures (*filled squares*). Western analysis of MRPL12 proteins shows a loose distribution of the endogenous protein centered at 35–45 S (Fig. 6B), while the truncated protein exhibits a much tighter distribution on the same ribosomal structure (Fig. 6B). This establishes that both endogenous and truncated MRPL12 proteins cofractionate with ribosomal subunits. As the truncated protein has lost its basic helix-turn-helix RNA-binding domain, this also suggests that both wild-type and truncated proteins are present in the same protein complex. To address this latter issue, mitochondrial extracts were immunoprecipitated using anti-HA 12CA5 antibodies. Immunoprecipitated products were analyzed by Western blotting using anti-MRPL12 a180 anti-



**FIG. 6. Dimerization and submitochondrial distribution of MRPL12 and MRPL12 $\Delta$  proteins.** *A*, MRPL12 (29 kDa) and HA-tagged MRPL12 $\Delta$  (21 kDa) proteins were synthesized alone or in combination from *in vitro* translation mixtures in the presence of  $^{35}\text{S}$ -labeled methionine and immunoprecipitated using anti-HA 12CA5 antibodies. Aliquots from immunoprecipitated proteins (*lanes IP*) or from the translation mixture (*lanes C*) were separated by SDS-PAGE and exposed to autoradiography. *B*, mitochondria were purified from stably transfected cells (clone 6). After lysis and membrane removal, mitochondrial matrix extracts were fractionated on a 15–20% sucrose gradient. Fractions were collected from the top (fraction 1) to the bottom (fraction 32) of the gradient. *Upper panel*, nucleic acid content was estimated by  $A_{260}$  spectrophotometry (empty circles), and assay for 16 S rRNA distribution (filled squares) was performed by Northern analysis of an aliquot of each fraction. *Lower panel*, fractions were pooled, and proteins were extracted and analyzed on Western blots. Membranes were incubated with a180 antibodies or with 12CA5 antibodies to detect the endogenous protein (21 kDa) or the exogenous protein (15 kDa). Molecular mass markers are indicated on the right. *C*, extracts from the mitochondrial matrix were immunoprecipitated with 12CA5 antibodies. Immunoprecipitates were analyzed by Western blotting, and proteins were revealed using a180 antibodies. The strong signal at the top of the gel corresponds to mouse IgG heavy chain detected by goat antibodies to rabbit IgG.

bodies (Fig. 6C). Two peptides of 21 and 15 kDa were detected, indicating that both proteins are associated with the same structures.

**Expression of MRPL12 $\Delta$  Inhibits Cell Growth and Mitochondrial ATP Production**—We next examined transfected HeLa clones for their physiological properties. We first compared the growth kinetics of six independent transfected clones. Cells transfected with wild-type pcDNA1/Neo were included as a control. Cells were seeded at  $20 \times 10^3$  cells  $\text{ml}^{-1}$  in RPMI 1640 medium supplemented with 10% FCS, and cell populations were estimated up to 172 h after seeding. As observed in Fig. 7, all transfected clones exhibit a reduced growth rate (average doubling times ranging from 26 to 28 h for clone 2 to 34 to 36 h for clones 1 and 5) compared with the control cells (average doubling time of 18–20 h). As the mitochondrial genome codes exclusively for proteins involved in oxidative phosphorylation, we analyzed the transfected clones for their mitochondrial ATP production. Three clones exhibiting different ranges of growth inhibition were compared for their behavior with regard to the absence of glycolysis by incubating cells in glucose-free medium and with regard to mitochondrial ATPase inhibition upon addition of  $10 \mu\text{g ml}^{-1}$  oligomycin (Fig. 7B). In the presence of oligomycin, the doubling time of control cells increased moderately (20–22 h), while the doubling times of MRPL12-transfected cells remained similar to those observed in Fig. 7A, indicating that inhibition of mitochondrial ATPase in the transfected clones has no effect on their growth properties. At variance, glucose starvation led to an expected reduction in the growth rate of control cells (doubling time of 28–30 h), but to a rapid death of the transfected clones, confirmed by trypan blue staining (data not shown). Identical cell death was observed in control cells upon inhibition of both pathways. These data demonstrate that mitochondrial ATPase activity is inhibited or

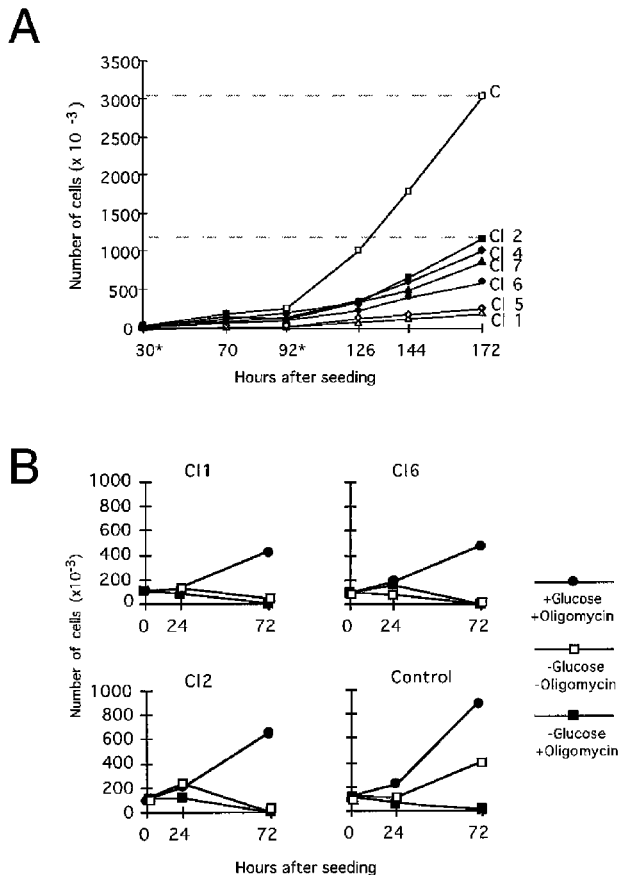
severely reduced in the transfected clones and that their ATP supply derives exclusively from glycolysis.

#### DISCUSSION

To get a better understanding of the molecular events associated with cell proliferation, several groups have initiated an overall survey of mRNAs that accumulate in late  $G_1$  of serum-stimulated cells. We previously characterized a subset of cDNA clones complementary to serum-inducible RNAs, derived from hamster CCL39 fibroblastic resting cells stimulated with serum for 5 h. Three of them were shown to participate in the delayed-early response (19). Sequence analysis showed that two delayed-early response mRNAs encoded the plasminogen activator inhibitor (type I) (19) and the immunophilin-binding FKBP59 protein (41). We report here the characterization of the third delayed-early response mRNA, originally termed P2A1 (19), as well as its human counterpart.

This represents the first cloning and characterization of a mammalian mitochondrial ribosomal protein encoded by the nucleus. Previous reports led to the identification of various mammalian L12 ribosomal proteins related to prokaryotic L11 and yeast L15 proteins (47–49) that were shown to bind 28 S rRNA and to be cross-linked to elongation factors  $1\alpha$  and 2 (50–52). Although attempts were made to relate these proteins to prokaryotic L12 (30), our data unambiguously demonstrate that P2A1 is the mitochondrial homologue of prokaryotic L7/L12 ribosomal protein, which will now be termed MRPL12. Although sharing a low level of identity in their primary structures, MRPL12 and bacterial L12 proteins exhibit a conserved overall secondary structure (two helical structures separated by a flexible hinge region). In bacteria, the  $\text{NH}_2$ -terminal helix is involved in dimerization, while the  $\text{COOH}$ -terminal helix displays a helix-turn-helix structure, involved in binding to





**FIG. 7. Growth properties of HeLa clones expressing MRPL12 $\Delta$ .** A, cells transfected with pcDNAI (C) or cells from six independent clones (C1, C2, C4, C5, C6, and C7) were seeded at a density of  $2 \times 10^4$  ml $^{-1}$  and grown for the indicated times in RPMI 1640 medium supplemented with 10% FCS. Asterisks indicate times at which cells were refed with fresh medium. B, cells transfected with pcDNAI (Control) or cells from three independent clones (C1, C2, and C6) were seeded at  $10^5$  cells ml $^{-1}$  in minimum RPMI 1640 medium supplemented with 10% FCS and 2 g liter $^{-1}$  glucose. 24 h later, cells were cultured for the indicated times in the absence (empty symbols) or presence (filled symbols) of 10  $\mu$ g ml $^{-1}$  oligomycin, in the absence (squares) or presence (circles) of 2 g liter $^{-1}$  glucose.

ribosomal RNA. Assuming similar properties for MRPL12 protein, we showed that the expression of a protein potentially devoid of RNA binding activity impairs the normal function of the protein complex, probably due to reduced translation, leading to inhibition of oxidative phosphorylation.

Mitochondria are organelles whose main function is ATP production, but that also contribute to the biosynthesis of many metabolites such as pyrimidines, amino acids, and phospholipids. Although not essential for *in vitro* cell cultures (20), the mitochondrial activity is regulated at developmental and physiological levels (53, 54), and its failure is probably involved in numerous degenerative diseases (55–57). Several nuclear genes activated by growth factors or hormones were shown to encode proteins controlling various aspects of mitochondrial metabolism: energy production (the adenine nucleotide translocator ANT2 (23), a proton/phosphate symporter (19), or cytochrome *c* (58)), lipid biosynthesis (the p90 glycerol-3-phosphate acyltransferase (59) or the uncoupling protein (60)), and protein import (the chaperonin hsp60 (61) or Fos effector protein-1, similar to a yeast mitochondrial protein import (22)).

In mammalian cells, the mitochondrial genome encodes a limited number of proteins involved in oxidative phosphorylation. Mitochondrial RNAs are transcribed as polycistronic primary transcripts from a unique bidirectional promoter, imply-

ing that regulation of gene expression mainly relies on post-transcriptional mechanisms such as RNA or protein turnover and translational control (62–64). As L7/L12 proteins have been shown to control the efficiency of mRNA translation in *E. coli*, it is tempting to speculate a similar function for MRPL12 in the mitochondria. Our data establish that MRPL12 mRNA and protein accumulate in response to growth factor stimulation and that mRNA induction is inhibited when cells are treated with antimitogenic agents such as cAMP. Growth regulation of MRPL12 protein could then represent a direct regulatory mechanism for increasing the amount of proteins involved in mitochondrial metabolic pathways in response to external stimulation.

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**A Delayed-early Response Nuclear Gene Encoding MRPL12, the Mitochondrial Homologue to the Bacterial Translational Regulator L7/L12 Protein**

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