Expression of the Gene for Mitoribosomal Protein S12 Is Controlled in Human Cells at the Levels of Transcription, RNA Splicing, and Translation*

(Received for publication, March 29, 1999, and in revised form, July 7, 1999)

Paolo Mariotti‡, Zahid H. Shah§, Janne M. Toivonen§, Claudia Bagni¶, Johannes N. Spelbrink§, Francesco Amaldi§, and Howard T. Jacobs§**

From the ‡Department of Biology, Università di “Roma Tre,” Rome, I-00146, Italy, the §Institute of Medical Technology and Tampere University Hospital, University of Tampere, Tampere, Finn-33101, Finland, the ¶Department of Biology, Università di Roma “Tor Vergata,” Rome, I-00133, Italy, and the ¶Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, Scotland, United Kingdom

The human gene RPMS12 encodes a protein similar to bacterial ribosomal protein S12 and is proposed to represent the human mitochondrial orthologue. RPMS12 reporter gene expression in cultured human cells supports the idea that the gene product is mitochondrial and is localized to the inner membrane. Human cells contain at least four structurally distinct RPMS12 mRNAs that differ in their 5′-untranslated region (5′-UTR) as a result of alternate splicing and of 5′ end heterogeneity. All of them encode the same polypeptide. The full 5′-UTR contains two types of sequence element implicated elsewhere in translational regulation as follows: a short upstream open reading frame and an oligopyrimidine tract similar to that found at the 5′ end of mRNAs encoding other growth-regulated proteins, including those of cytosolic ribosomes. The fully spliced (short) mRNA is the predominant form in all cell types studied and is translationally down-regulated in cultured cells in response to serum starvation, even though it lacks both of the putative translational regulatory elements. By contrast, other splice variants containing one or both of these elements are not translationally regulated by growth status but are translated poorly in both growing and non-growing cells. Reporter analysis identified a 26-nucleotide tract of the 5′-UTR of the short mRNA that is essential for translational down-regulation in growth-inhibited cells. Such experiments also confirmed that the 5′-UTR of the longer mRNA variants contains negative regulatory elements for translation. Tissue representation of RPMS12 mRNA is highly variable, following a typical mitochondrial pattern, but the relative levels of the different splice variants are similar in different tissues. These findings indicate a complex, multilevel regulation of RPMS12 gene expression in response to signals mediating growth, tissue specialization, and probably metabolic needs.

In mammals, mitochondrial DNA encodes 13 polypeptide subunits of the mitochondrial respiratory chain and oxidative phosphorylation system (1). Expression of these genes depends upon a dedicated apparatus of transcription, RNA processing, and translation, mainly encoded by nuclear genes. Typically these exhibit clear eubacterial affinities compared with their counterparts that specify cytosolic or nuclear isologues.

Genes encoding components of the apparatus of mitochondrial gene expression are regulated in a variety of ways. For example, the mRNA for the mitochondrial (mt) transcription factor-A is present at different levels in different tissues (2), and its expression is in turn regulated by transcription factors of the nuclear respiratory factor group (3). Despite examples such as this, plus some knowledge obtained from studies in yeast (4), rather little is known of how genes for the apparatus of mitochondrial gene expression are regulated in mammalian cells. In principle this may have both developmental and physiological aspects and would be expected to involve regulation at different levels in the pathway of gene expression.

We have set out to study a gene encoding a key mitochondrial ribosomal protein, the homologue of Escherichia coli ribosomal protein S12 (rps12). Tentative identification of the single copy gene designated RPMS12 (5, 6) as encoding the mitochondrial isologue of rps12 in humans was based on phylogenetic analysis of the encoded polypeptide, which groups it with homologues found in eubacteria and plant/protistan organelles, but correspondingly distant from the identified cytosolic homologue in yeast (Rps28p). Moreover, compared with the Rps12 proteins of eubacteria, or those encoded by organelle DNAs, the human gene product shows an N-terminal extension exhibiting features similar to those of mitochondrial targeting peptides. However, formal proof that the RPMS12 gene product is mitochondrially localized is lacking. We therefore set out initially, using a reporter gene approach, to verify that RPMS12 encodes a mitochondrial protein.

Ribosomal protein genes are regulated in unusual ways, perhaps not surprisingly, given their central importance in biosynthesis. In eubacteria and archaea, operons containing ribosomal protein genes are typically autoregulated by one of the proteins they encode (7). The 11 gene S10 operon of E. coli, for example, is regulated by ribosomal protein L4 binding to the S10 leader and simultaneously repressing translation and also promoting transcriptional attenuation (8). In yeast, ribosomal

* This work was supported by CNR Grant 98.59.PF31, Ministero Universitá e Ricerca Scientifica e Tecnologia (Print Project), the Finnish Academy, Juselius Foundation, Tampere University Hospital Medical Research Fund, and the European Union. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
** To whom correspondence should be addressed: Institute of Medical Technology, University of Tampere, P. O. Box 607, 33101 Tampere, Finland. Tel.: +358-50-341-2894; Fax: +358-3-215-7710; E-mail: howy.jacobs@uta.fi.

1 The abbreviations used are: mt, mitochondrial; uORF, upstream open reading frame; UTR, untranslated region; nt, nucleotide(s); mRNP, messenger ribonucleoprotein; TOF, terminal oligopyrimidine; BT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; bp, base pair; PBL, peripheral blood lymphocyte; kb, kilobase pair; np, nucleotide pair.
protein expression does not appear to be controlled by such a translational feedback mechanism (9), although a distinct type of post-transcriptional regulation in which Rps14p binds to its own pre-mRNA has been documented (10).

In vertebrate cells, cytosolic ribosomal protein mRNAs are translationally regulated in response to growth status (Ref. 4 and references therein). Such regulation has been demonstrated in response to a number of physiological stimuli, such as serum starvation (11–13), growth factor or mitogen stimulation (14–16), dexamethasone treatment (17), and contact inhibition (18). In all these cases the fraction of ribosomal protein mRNA associated with polysomes varies according to cellular need, a higher proportion being loaded on polysomes (translationally active state) in rapidly growing cells as opposed to resting cells, in which most of it is stored in the subpolysomal or mRNP fraction (translationally inactive state, Ref. 19). In all cases so far analyzed, a 5' UTR containing a terminal oligopyrimidine (TOP) tract plays a critical role in the translational control of such genes (20, 21). This sequence element is believed to act via specific interactions with one or more regulatory proteins (13, 22–24).

Little is known of how or even in what context the synthesis of mitochondrial ribosomal proteins is regulated. It is probable that, like their cytosolic counterparts, mitochondrial ribosomal protein genes are regulated in respect to cellular growth, although other types of regulation are also to be expected, for example in response to cellular bioenergetic state, the availability of different kinds of substrate and cell differentiation. Tissues such as heart, skeletal muscle, or pancreas, which are highly dependent on mitochondrial ATP synthesis or in which rapid response of the bioenergy-generating system is needed, may be predicted to exhibit specific modes of regulation of the mitochondrial translational apparatus. The mRNA for one mitochondrial ribosomal protein (MRPL12) is known to be regulated in mouse cells by growth induction but at the transcriptional rather than the translational level (25, 26).

Sequence analysis of a full-length or nearly full-length cDNA derived from the human RPSM12 gene (5) revealed a long (>300 nt) 5' UTR that contains two features strongly suggestive of translational regulation. A short, upstream open reading frame (uORF), potentially encoding the pentapeptide MRACG, is located near the middle of the 5' UTR. Such uORFs have been demonstrated to play a role in translational regulation of many genes in both fungi and vertebrates (27–33). They have been demonstrated to play a role in translational regulation of many genes so far analyzed, a 5' UTR containing a terminal oligopyrimidine tract (TOP) plays a critical role in the translational control of such genes (20, 21). This sequence element is believed to act via specific interactions with one or more regulatory proteins (13, 22–24).

In vertebrate cells, cytosolic ribosomal protein mRNAs are translationally regulated in response to growth status (Ref. 4 and references therein). Such regulation has been demonstrated in response to a number of physiological stimuli, such as serum starvation (11–13), growth factor or mitogen stimulation (14–16), dexamethasone treatment (17), and contact inhibition (18). In all these cases the fraction of ribosomal protein mRNA associated with polysomes varies according to cellular need, a higher proportion being loaded on polysomes (translational active state) in rapidly growing cells as opposed to resting cells, in which most of it is stored in the subpolysomal or mRNP fraction (translational inactive state, Ref. 19). In all cases so far analyzed, a 5' UTR containing a terminal oligopyrimidine (TOP) tract plays a critical role in the translational control of such genes (20, 21). This sequence element is believed to act via specific interactions with one or more regulatory proteins (13, 22–24).

Our findings indicate a complex multilevel regulation of the expression of a mitochondrial ribosomal protein gene in response to various physiological and developmental stimuli.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293-EBNA cells were maintained in Dulbecco's modified Eagle's medium, HyClone), 10% fetal calf serum, supplemented with 50 μg/ml uridine, 1 mM glutamine, and 1 mM sodium pyruvate plus 100 units/ml penicillin and 100 μg/ml streptomycin. HaLa, HEK293, and Xenopus kidney B3.2 cultured cells were grown in Dulbecco's modified Eagle's medium (Sigma), 10% fetal calf serum containing 50 μg/ml gentamicin and 2 mM glutamine. To induce a "downshift" to serum starvation conditions, cells were rinsed twice with PBS and detached with a limited amount of trypsin. After resuspension in PBS to dilute the trypsin, cells were centrifuged at 2000 × g max for 5 min at 4 °C, resuspended in serum-free medium, and incubated at 37 °C for a further 4 h.

DNA Transfection of Cultured Cells—Cells were grown to 80% confluence on 100-mm plates and transfected in 6 ml of Opti-MEM serum-free medium (HyClone) with 10 μg of plasmid DNA plus 40 μg of LipofectAMINE reagent (Life Technologies, Inc.), following the manufacturer's recommendations. After 5 h of incubation 6 ml of Dulbecco's modified Eagle's medium (HyClone) containing 20% fetal calf serum was added. For serum starvation conditions no serum was added in this added medium. Cells were harvested 24 h after the start of transfection. Smaller scale transfections on 35-mm plates used 1 μg of plasmid DNA plus 10 μg of LipofectAMINE.

Subcellular Fractionation—Cytosolic extracts for Western analysis were prepared from transfected HEK293-EBNA cells grown on 35-mm plates, collected by pipetting up and down in 500 μl of PBS, and centrifugation at 12,000 × g max for 5 min. The cell pellet was vortexed and lysed in 50 μl of PBS containing 1.5% (w/v) lauryl maltoside and 2 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min, and then centrifuged at 16,000 × g max for 5 min at 4 °C. Ten μl of the supernatant was used immediately for SDS-PAGE. Finner subcellular fractionation of transfected cells was carried out using a standard procedure for mitochondrial isolation (Ref. 36, adapted from Ref. 37). Essentially, transfected cells from each 100-mm plate were washed once with PBS, dislodged from the plate by pipetting up and down in 1.3 ml of ice-cold PBS, and centrifuged at 3000 × g max for 10 min at 4 °C. The cell pellet was resuspended by gentle pipetting in 10 volumes of ice-cold 0.133 M NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 25 mM Tris-HCl, pH 7.5, and centrifuged again at 3000 × g max for 10 min at 4 °C. The cell pellet was resuspended by pipetting up and down in 500 μl of ice-cold 10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-HCl, pH 7.5, kept on ice for 15 min, and then homogenized in a glass homogenizer with 18–25 strokes of a tight fitting pestle. Disruption of the cells was monitored by microscopy. An equal volume of ice-cold 0.68 M sucrose, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, was added, and nuclei and cell debris were pelleted by two sequential centrifugations for 10 min at 4 °C. The combined pellets ("nuclear fraction") were washed once with 1 ml of ice-cold PBS, resuspended in a final total volume of 300 μl of ice-cold PBS, and repeatedly passed through a syringe needle to shear chromatin. Mitochondria from the post-nuclear supernatants of four 100-mm plates of transfected cells were recovered by centrifugation at 16,000 × g max for 30 min at 4 °C. The supernatant from this step was saved as the cytosol fraction. Mitochondria were washed once with 1 ml of ice-cold PBS and finally resuspended by gentle pipetting in 160 μl of ice-cold PBS. From this suspension 20 μl was saved as the mitochondrial fraction, the remaining being further processed to yield various sub mitochondrial fractions, essentially as described in Ref. 36. Briefly, the mitochondrial suspension was adjusted to 350 μl with ice-cold PBS, and an equal volume of ice-cold digitonin solution (4 mg/ml in PBS) was added. The suspension was kept on ice for 5 min, after which 700 μl of ice-cold PBS was added, and mitochondria were recovered by centrifugation at 16,000 × g max for 10 min at 4 °C. The supernatant was ultracentrifuged at 144,000 × g max for 50 min at 5 °C to separate intermembrane space (supernatant) and outer membrane (pellet) fractions. The outer membrane fraction was resuspended in 20 μl of ice-cold PBS. Mitoplasts were resuspended in 140 μl of ice-cold PBS, and 20 μl was saved as the mitoplast fraction. The remainder was sonicated in a total volume of 300 μl on ice, 3 times for 2 s with 30-s intervals, using a VibraCell High Intensity Ultrasonic Processor (Sonics & Materials, Inc.), fitted with the manufacturer's micro-tip. The suspension was then ultracentrifuged at 144,000 × g max for 50 min at 5 °C to separate matrix (supernatant) and inner membrane (pellet) fractions. The matrix frac-
Regulation of Mitoribosomal Protein S12 Expression

...mu1 of each supernatant (in vitro translate and mitochondrial lysate) were used immediately for SDS-PAGE, in adjacent tracks of a 12% polyacrylamide gel. The gel was blotted to HybondTM-C extra nitrocellulose membrane (Amersham Pharmacia Biotech) as above, autoradiographed to detect the [35S]-labeled translation product, and then probed with the monoclonal antibody to Myc-His encoded by plasmid RPMS12-Myc. The membrane was then exposed and developed as described above.

Oligonucleotides and PCR—Sequences of oligonucleotides used to design PCR primers for both Northern blot analysis and RT-PCR analysis of RPMS12 mRNAs were as shown in the figures and legends. Those used for rP4 transcription analysis were rP4-A (CATCGTATGAGGAGTTC-C), representing nucleotide pair (np) 439–458 of the rP4 DNA (42), and rP4-B (TGTCGGTTGTGCTC), corresponding with np 927–949, for β-actin transcription analysis β-ACT-A (CGCTGGTCTGCTGACAAGC) and β-ACT-B (AGGTTCAAAATGCATGCTT), which amplify a 360-bp PCR product (43). For the control rP22 transcript used in RT-PCR assays, the primers were rP22 (CGTGGGACACCTGAGCTC), (np 57–75, Ref. 44) and the KS vector primer (Promega), which amplify a 600-bp PCR product encompassing the cloned cDNA and a small portion of the pBS KS (Promega), thus avoiding the amplification of the endogenous rP22 mRNA. Probes for Northern analysis of RPMS12 mRNAs were derived by PCR amplification of a full-length RPMS12 cDNA clone and were gel-purified using the QIAquick gel extraction kit (Qiagen) before radiolabeling. PCR was carried out on HeLa cell genomic DNA (200 ng) to check the specificity of the various primer pairs designed for transcript analysis. Products were purified using either the QIAQuick PCR purification or QIAquick gel extraction kit (Qiagen) and were sequenced by means of the Amplitape sequencing kit (Perkin-Elmer). For RT-PCR total RNA samples were reverse-transcribed into cDNA by the random hexanucleotides technique (39) using 200 units of M-MLV reverse transcriptase (RNase H-, recombinant). Reactions were carried out at 37 °C for 90 min. Four of the 20 μl of the reverse transcriptase reaction were PCR-amplified in a final volume of 50 μl, using 20 μl of each specific primer, 200 μM of each dNTP, and 0.5 units of Taq DNA polymerase (Amersham Pharmacia Biotech). To perform quantitative RT-PCR analysis on RNA extracted from polysonic gradient fractions, each sample was reverse-transcribed into cDNA together with 15 μg of an in vitro transcribed RNA for rP22, included as an internal control to confirm that the amount of product was not influenced by experimental variations in the reactions. For each PCR, different cycles and template amounts were tested, in order to avoid conditions of saturation.

Extraction and Analysis of RNA—Total RNA was extracted from gradient fraction pellets by the proteinase K method (19). For Northern blot analysis RNA was fractionated on formaldehyde-agarose gels and transferred to GeneScreen Plus membrane (NEN Life Science Products). Northern and Western hybridization were carried out essentially according to the manufacturer’s instructions. Northern hybridization to human cell line and tissue RNAs used filters purchased from CLONTECH. Radioactive probes were prepared by the random priming technique (39) using as templates the inserts of plasmids containing cDNAs for human RPMS12 (5) and rP4 (42), plus chicken β-actin (45) or PCR products for specific regions of the human RPMS12 mRNA 5′-UTR (see legends to Figs. 4–6). Probes were synthesized in the presence of [α-32P]dCTP (Amersham Pharmacia Biotech, 3000 Ci/mmole). Standard hybridization conditions were used (35), with final washes generally at 55 °C in 0.1× SSC, 0.1% SDS. Re-washing at higher temperatures up to 65 °C gave indistinguishable results.

Sequence Analysis—Analysis of database sequences used the GCG package (46) (UK HGMP Resource Center, Cambridge) and on-line facilities of the NCBI.

RESULTS

RPMS12 Encodes a Mitochondrially Targeted Polypeptide—The mitochondrial localization of the RPMS12 gene product was investigated by means of a reporter fusion to an epitope from the human c-MYC protein, for which a monoclonal antibody is available. Transient expression of the RPMS12-Myc fusion peptide was monitored by Western blotting, 24 h following lipofection into cultured HEK293 ERNA cells (Fig. 1). In unfraccionated cytoplasmic extracts solubilized by lauryl maltoside treatment, a prominent, Myc antibody-reactive polypeptide of approximately 21 kDa was detected in cells transformed with the RPMS12-Myc construct (Fig. 1a) but not in cell extracts from cells transfected with Myc fusion constructs for TUFM or LacZ or from mock-transfected cells. Subcellular
fractionation revealed that whereas the LacZ-Myc control polypeptide was mainly in the cytosol, the RPMS12-Myc polypeptide was localized to mitochondria (Fig. 1b). RPMS12-Myc was resistant to trypsin digestion in intact mitochondria (Fig. 1f), but lauryl maltoside solubilization rendered it trypsin-digestible. Further subfractionation confirmed that it was present in mitoplasts and exclusively in the inner membrane fraction after sonication (Fig. 1, c−e), colocalizing with a subunit of the respiratory chain (cytochrome oxidase subunit II), whereas glutamate dehydrogenase partitioned mainly to the matrix fraction. Careful alignment revealed that the electrophoretic mobility of the mitochondrially localized product on SDS-PAGE gels was less than that of the in vitro translated RPMS12-Myc polypeptide (Fig. 1g), indicating post-translational proteolytic processing upon mitochondrial import. A faint band corresponding to the unprocessed precursor is just visible in mitochondrial extracts.

**RPMS12 mRNA Is Alternately Spliced within the 5′-UTR**—Cyberscreening of dbEST followed by alignment into cDNA contigs revealed the existence of three distinct classes of transcript of the RPMS12 gene, with alternate splicing in the 5′-UTR (Table I and Fig. 2). The isoforms differ in respect to the two previously noted sequence elements suggestive of translational regulation, namely the uORF and oligopyrimidine tracts, as illustrated in Fig. 3. The long isoform a remains unspliced within the 5′-UTR, whereas isoform b is spliced to remove 101 nt including the oligopyrimidine tract, which could function as part of the splice-acceptor recognition sequence (Fig. 2). The splice sites respect the conventional GT...AG rule. The uORF remains intact in isoform b mRNA, with the splice donor site located 11 nt beyond the uORF stop codon. All 15 cDNAs representing isoforms a and b that extended over the coding region splice site were correctly spliced at that position, indicating that these isoforms do not represent unprocessed nu-

---

**TABLE I**

| mRNA isoform | Number of cDNAs | GenBank entries |
|--------------|-----------------|-----------------|
| a            | 7               | AA257081, AA770435, AA587905, AA336097, AA256974, AI206816, N77629† |
| b            | 10              | AI215059, AI004808, AA77323, AA911107, AA612835, AA568553, AA716415, AA888859, AA513737, AA483273 |
| c            | 9               | HT2224, AA300997, AA148039, AA379276, AA379723, AA158941, AA047428, AI168197†, AA725879† |

† GenBank entries H63370 and AA086145 do not fit this scheme and appear to represent aberrant splice products lacking substantial portions of the RPMS12 coding sequence.

‡ Shows numerous discrepancies from the other sequences, especially after np 250. We have independently resequenced this cDNA (Image Clone 247801, GenBank™ Y116881, Ref. 5) finding all these discrepancies to be sequencing errors. Another GenBank™ entry, AA806508, also shows many discrepancies that are probably errors.

§ These cDNAs extend to a putative upstream transcriptional start site, as shown by the dashed line in Fig. 3a.

---

This is also consistent with the results of Northern analysis.
Regulation of Mitoribosomal Protein S12 Expression

Transcripts are shown in italics and denote sequence found only in a minor fraction of ESTs with the double arrowhead shown alongside the corresponding amino acid sequence (one-letter code). No consensus 3'-UTR. These were sequenced using probes for successively more inclusive portions of the RPMS12 5'-UTR. These were designed to detect the three isoform-related sequences in RPMS12 mRNAs Are Tissue Differentially Expressed—The isoforms of RPMS12 mRNA were represented in the different cell lines tested in very variable relative amounts. In order to investigate their expression in vivo, the same probes were hybridized sequentially to Northern blots of RNA from human tissues, as documented in Fig. 4. The pattern of relative abundance between tissues was similar for all three of the 5'-UTR splice variants, although the unspliced, 5'-truncated isoform d, seen prominently in some cultured cells, was not detected. The unspliced isoform a was detected in heart (Fig. 4d), but only weakly in most other tissues, and in many cases a larger transcript of approximately 2 kb was also detected by the isoform a-specific probe, possibly representing unspliced nuclear RNA from which the coding region intron had also not been removed. The isoform b splice variant, which retains the uORF, was more highly represented in all tissues but was especially prominent in heart, skeletal muscle, and kidney (Fig. 4, b and c). A similar pattern of hybridization was seen using the full-length probe (Fig. 4c), which detects also (and mainly) the shorter, fully spliced isoform c. Some minor differences can be discerned, for example isoform c was detected more strongly between the cell types tested. The shorter transcripts are proposed to represent the 5'-truncated isoform d, cyberscreening having revealed no evidence for 3' heterogeneity, nor any reasonable match to the consensus poly(A) addition signal located elsewhere in the 3'-UTR. Even if the length of the poly(A) tail in these shorter transcripts is much reduced, their overall size precludes that they contain the full 5'-UTR of isoform a. 5'-Truncated transcripts containing the oligopyrimidine tract unique to isoform a are evident in dbEST (e.g. GenBank™ entry AA257081), although they do not constitute a coherent class indicating a specific 5' end. Our best guess is that they are heterogeneous, as indexed by the dashed line in Fig. 3a. It is unclear whether these transcripts indicate a downstream transcriptional start region or else 5' truncation in vivo. The shorter transcripts were prominent in A549 lung carcinoma cells but almost undetectable in the K-562 leukemia cell line and were also absent from solid tissues (see below).

After stripping and reprobing for a region contained within both isoforms a and b, but not c, an additional, prominent transcript of intermediate size was detected (Fig. 3, b and c), estimated at 1.15 kb. Both transcript classes detected by the earlier probe were recognized only weakly by the second probe, indicating that the intermediate sized transcripts, which must represent isoform b, are considerably more abundant in all cell lines tested than those of isoforms a or d. Blots were stripped and reprobed again for the full-length RPMS12 mRNA, detecting all classes of transcript. In this case, a prominent additional species was detected in the 1.0-kb size range, i.e. migrating faster than the isoform b transcripts, which must represent the fully spliced isoform c not detected by other probes. This transcript appears to be the major isoform in all cells tested. Larger transcripts, which would be derived from a far upstream start such as tentatively inferred from cyberscreening, were present only at very low abundance. The ratio of isoforms (a + d), b and c appears to be similar in all cell lines studied.

RPMS12 mRNAs Are Tissue Differentially Expressed—The isoforms of RPMS12 mRNA were represented in the different cell lines tested in very variable relative amounts. In order to investigate their expression in vivo, the same probes were hybridized sequentially to Northern blots of RNA from human tissues, as documented in Fig. 4. The pattern of relative abundance between tissues was similar for all three of the 5'-UTR splice variants, although the unspliced, 5'-truncated isoform d, seen prominently in some cultured cells, was not detected. The unspliced isoform a was detected in heart (Fig. 4d), but only weakly in most other tissues, and in many cases a larger transcript of approximately 2 kb was also detected by the isoform a-specific probe, possibly representing unspliced nuclear RNA from which the coding region intron had also not been removed. The isoform b splice variant, which retains the uORF, was more highly represented in all tissues but was especially prominent in heart, skeletal muscle, and kidney (Fig. 4, b and c). A similar pattern of hybridization was seen using the full-length probe (Fig. 4c), which detects also (and mainly) the shorter, fully spliced isoform c. Some minor differences can be discerned, for example isoform c was detected more strongly in peripheral blood lymphocytes (PBLs) than in colonic mucosa or thymus, and at about the same level as in testis, whereas isoform b was more prominent in colonic mucosa, thymus, and testis than in PBLs (Fig. 4c). In general, isoform b appeared to show more pronounced differences between tissues than either of isoforms a or c. Higher molecular weight transcripts that could correspond with the use of a far upstream transcriptional start site were not detected, except in PBLs, where a (~2.5 kb) species of unknown origin was detected by the isoform b probe.

The Major RPMS12 mRNA Splice Variant Is Translationally...
Regulated—In order to determine whether RPSM12 mRNAs were translationally regulated in a manner akin to the TOP mRNAs, sucrose density gradient centrifugation was used to separate post-mitochondrial supernatants from cell lysates into polysomal and subpolysomal (mRNP) fractions. Such gradients were prepared from HeLa and HEK293 cells cultured under standard conditions and under serum starvation. RNA was extracted from successive fractions across the gradients and probed for RPSM12 by Northern hybridization. The blots were then reprobed for two control mRNAs, β-actin, which is efficiently translated in both growing and non-growing cells, and rpL4, a cytosolic ribosomal protein encoded by a typical,
the five primers used for RT-PCR. Reactions used a common 3′ primer R5, with each of four 5′ primers R1 through R4, as shown in Fig. 4. b, Northern blot strips of RNA extracted from gradient fractions, as indicated by A260 traces, probed successively for β-actin, rpL4, and RPMS12 (full-length probe). c, RT-PCR reactions, using various RPMS12 primer pairs, alongside reference reactions using primers for rpL4 and β-actin. Also shown is a quantitation control (15 pg of an in vitro synthesized RNA for rpL22, see “Experimental Procedures”). The agarose gel photographs are aligned with the A260 traces in b above.

translationally regulated TOP mRNA. As seen in Fig. 5b, RPMS12 mRNA behaved qualitatively like a typical TOP mRNA in HeLa cells, being mainly polysomal in growing cells but mainly non-polysomal in serum-starved cells. The effect was less dramatic than for rpL4 mRNA, mainly due to the fact that even in growing cells most of the RPMS12 mRNA seemed to be translated on rather small polysomes. Similar results were obtained for HEK293 cells, and also, via inter-specific cross-hybridization, for Xenopus B3.2 cells (data not shown).

In order to determine which splice variants of RPMS12 mRNA were translationally regulated in this fashion, a quantitative RT-PCR approach was adopted, using primer pairs capable of generating specific products diagnostic for each splice variant, plus primers for β-actin and rpL4 as controls. Direct sequencing was used to confirm the identity of the major RT-PCR products synthesized from total RNA using all four primer pairs. Primer pairs R1/R5 and R2/R5 generated, respectively, 86- and 72-bp products corresponding to the fully spliced isoform c. Primer pair R3/R5 generated a major product of 74 bp, representing isoform b, whereas primer pair R4/R5 gave a product of 76 bp containing the oligo(Y) tract found only in isoforms a and d. PCR on genomic DNA using these primer pairs (not shown) yielded products identical in sequence to cDNA clones for isoform a (Fig. 2), confirming that the latter is colinear with the genome.

The RT-PCR products generated by primer pairs R1/R5 and R2/R5, representing the fully spliced mRNA isoform c, showed a similar pattern of distribution between polysomal and mRNP fractions as did the total RPMS12 mRNA on Northern blots (Fig. 5b). Although quantitatively less dramatic than for rpL4, isoform c was thus found to be translationally regulated, being mainly polysomal in growing cells, but predominantly non-polysomal in resting (serum-starved) cells. This is not unexpected, since isoform c was the mRNA variant predominantly detected on Northern blots (Fig. 3). By contrast, the RT-PCR product generated by primer pair R3/R5, representing mRNA isoform b, showed identical distributions in growing and serum-starved cells, being found in both cases associated with monoribosomes and short polysomes. It behaved similarly to the β-actin control, found mainly on large polysomes, which showed no translational response to serum starvation. The RT-PCR product from primer pair R4/R5, representing isoforms a and d, was distributed approximately equally between the polysome and mRNP fractions in both growing and non-growing cells, indicating that these isoforms were also not translationally regulated. Interestingly, the distribution of this RNA in the gradient was wider than for isoform b, encompassing both the postribosomal and larger polysomal fractions.

Thus, the only RPMS12 mRNA variant that was found clearly to be regulated translationally in response to growth status is the one in which both of the putative elements originally hypothesized to be involved in translational control are removed by splicing. Furthermore, the translational properties of each of the 3 splice variants appear to be distinct.

**Sequences in the RPMS12 mRNA 5′-UTR Mediate Its Translational Regulation**—In order to localize the signals responsible for translational regulation of RPMS12 mRNAs, we compared the effects of serum deprivation on the transient expression of two RPMS12-Myc fusion constructs in HEK293-EBNA cells. The first construct was the one referred to above (RPMS12-Myc/S), which includes a 155-nt stretch of vector-derived RNA at its 5′ end, fused directly to the RPMS12 coding sequence preceded by just 24 nt of untranslated sequence (Fig. 6a). The second construct includes the same vector sequence, but followed by essentially the entire 5′-UTR of RPMS12, commencing in the region of the major 5′ end found in the short (fully spliced) mRNA isoform c and including the splice donor and acceptor sites between which are located the uORF and oligopyrimidinetract. The latter construct (RPMS12-Myc/B) is predicted to give rise to a major spliced transcript that has the 155-nt vector sequence, followed by the 5′-UTR of the “short” RPMS12 mRNA isoform c. RT-PCR analysis (Fig. 6b) confirmed that the transiently expressed constructs gave rise to the predicted transcripts. Western analysis (Fig. 6c) showed that both constructs were efficiently expressed in the presence of serum. However, under serum starvation conditions, a much lower amount of product was expressed reproducibly from construct B, whose transcript is spliced in the isoform c-specific mode, whereas the expression of construct S or the LacZ-Myc control was only slightly affected by serum starvation. In other words, despite the presence of an additional 155 nt of vector sequence, the fully spliced RNA was regulated by serum in a
been proteolytically processed, and was localized to the inner membrane. This is further supported by the fact that the mitochondrially expressed gene product, whose structure was confirmed by direct sequencing. Construct S gives a 170-bp product that is colinear with the DNA.

**FIG. 6.** Reporter analysis of 5'-UTR signals. **a,** sequences of the predicted 5'-UTRs of the mRNAs encoded by the two reporter constructs RPMS12-Myc/B and RPMS12-Myc/S. The 155 nt of vector sequence (not shown) are common to both. Most RNA made from construct B is spliced to remove the intron that contains the uORF and oligo(Y) as shown and as confirmed by the experiment shown in b. The two constructs share 21 nt of sequence located immediately upstream of the start codon. Construct B contains an additional 26 nt of RPMS12 sequence from the other side of the splice junction unique to isoform c, in the place of which construct S has 3 nt of intron-derived sequence but not the full splice acceptor. RT-PCR analysis of transgene-specific transcripts confirms that the 5'-UTR of the longer isoforms, containing the uORF and oligopyrimidine tract, is a negative element for translation. **b,** expression of a LacZ-Myc control in transfected cells grown with or without serum. Shown alongside is the cytosolic product, whose structure was confirmed by direct sequencing. Construct S gives a 170-bp product that is colinear with the DNA. **c,** Western analysis shows that the 5'-UTR signals were efficiently expressed under both conditions tested is consistent with the earlier inference that the 5'-UTR of the longer isoforms, containing the uORF and oligopyrimidine tract, is a negative element for translation.

**DISCUSSION**

These findings support the identification of the RPMS12 gene product as a mitochondrial ribosomal protein, reveal unexpected complexity in the regulation of its expression, and identify specific regions of the 5'-UTR involved in translational control. We now address the implications of these findings.

**Mitochondrial and Submitochondrial Localization of the RPMS12 Gene Product**—The targeting to mitochondria of the RPMS12-Myc reporter protein strongly supports the previous assignment of RPMS12 as encoding the mitochondrial isologue of E. coli ribosomal protein S12. The protein is a member of a well characterized and conserved family of ribosomal proteins (5) and in most plants is even mitochondrially encoded (47–49). Comparable experiments with an RPMS12-GFP reporter fusion also showed colocalization to mitochondria. The assignment is further supported by the fact that the mitochondrially localized fusion protein was inaccessible to external protease, i.e. had been imported into the mitochondria, appears to have been proteolytically processed, and was localized to the inner mitochondrial membrane. In yeast, the inner membrane is the site of productive synthesis of the hydrophobic, mtDNA-encoded mitochondrial proteins that contribute to the respiratory chain (50). It is therefore logical that an epitope-tagged mitochondrial ribosomal protein will be localized there.

**Multilevel Control of RPMS12 Gene Expression**—The above findings lead to the conclusion that RPMS12 is regulated at the levels of transcription, RNA processing, and translation. The generation of mRNA isoforms with different patterns of translational behavior would seem to be the major outcome of the alternate synthetic pathways.

Each of the isoforms of RPMS12 mRNA is clearly represented in highly tissue-variable amounts. We were unable to perform a meaningful loading control hybridization with the commercially supplied Northern blot membranes, since the final probe could not be completely stripped. However, the claim of the manufacturer that the lanes are evenly loaded with 2 μg of poly(A) RNA and quality controlled to check RNA integrity means that minor variations in loading cannot account for the order of magnitude differences in signal seen between the highest and lowest expressing tissues. The pattern of relative abundance between tissues represents a typical pattern for a gene involved in mitochondrial respiratory function, with prominent expression in tissues highly dependent on oxidative metabolism such as heart, skeletal muscle, kidney, and to a lesser extent brain, liver, testis, and pancreas. The gene encodes a conserved ribosomal protein indispensable for translation; hence, its expression is expected to follow a similar pattern to that of the mtDNA-encoded mRNAs that are translated by mitoribosomes. The fact that the various mRNA isoforms show similar patterns of tissue distribution as one another suggests that differential RNA processing is not of major importance in generating these different tissue levels of expres-

---

2 Z. H. Shah, unpublished data.
sion. Instead the gene is most likely transcribed at different rates in different tissues, although a contribution from RNA stability cannot be ruled out.

Transcription may also be regulated in another way, via the selection of alternate start sites. Tentative evidence for a far upstream start active in at least some tissues was obtained by cyberscreening of dbEST and is also suggested by the detection in PBLs of a larger transcript carrying the b isoform splice pattern. More convincingly, the 5′-truncated isoform d detected in cultured cells suggests strongly the use of a downstream initiation site. Although 5′ truncation by an exonucleolytic activity in vivo cannot be ruled out, the fact that the isoform d transcripts detected on Northern blots constitute a discrete size class argues strongly that they derive instead from the use of a separate initiation site, which would be located just upstream of the oligopyrimidine tract, based on the transcript size. The physiological significance of alternate 5′ termini in RPMS12 mRNAs remains unknown. It may relate to translational regulation, as discussed further below. The expression of a minor but constitutively translated form of RPMS12 mRNA in the stem cell compartment which cultured cells represent makes obvious sense.

Alternate splicing in a 5′-UTR is relatively uncommon. It has been reported, for example, in the bovine gene encoding connexin-32 (51) and the human genes encoding reduced folate carrier (52) and thrombopoietin (53). In principle, 5′ splice heterogeneity inferred from cyberscreening could be due to the presence in the data base of sequences derived from partially processed nuclear RNA. In the case of RPMS12 this is highly unlikely. Essentially all of the RPMS12 cDNA sequences deposited in dbEST represent transcripts from which the coding region intron has been correctly spliced out, yet a clear majority of them are unspliced or partially spliced within the 5′-UTR. Moreover, if the oligo(Y) tract is essential for recognition of the splice acceptor site just upstream of the RPMS12 start codon, then isoform b could not be efficiently processed further to isoform c. In addition, unspliced or partially spliced 5′-UTR variants were found at least partly in the polysomal fraction in cultured cells. Alternate splicing must therefore give rise to several different forms of translatable mRNA. Only in those human tissues showing low expression (9) were various mRNAs detected in cultured cells suggests strongly the use of a downstream initiation site. Although 5′ truncation by an exonucleolytic activity in vivo cannot be ruled out, the fact that the isoform d transcripts detected on Northern blots constitute a discrete size class argues strongly that they derive instead from the use of a separate initiation site, which would be located just upstream of the oligopyrimidine tract, based on the transcript size. The physiological significance of alternate 5′ termini in RPMS12 mRNAs remains unknown. It may relate to translational regulation, as discussed further below. The expression of a minor but constitutively translated form of RPMS12 mRNA in the stem cell compartment which cultured cells represent makes obvious sense.

TOP mRNAs are believed to be controlled via specific interactions between the TOP tract and regulatory proteins mediating mRNA recruitment to ribosomes (23, 24). The exact properties of these proteins and the machinery with which they interact have not yet been characterized. It seems logical to postulate that the regulatory sequence unique to isoform c interacts specifically with a negative regulatory protein that is only present in an active state in growth-arrested cells. The same protein may independently interact with TOP mRNAs. Alternatively, different proteins may bind to RPMS12 and TOP mRNAs, independently influencing their ability to interact with a common component of the translational recruitment machinery. The fact that the translational regulation of RPMS12 mRNA is less dramatic than for some TOP mRNAs with an “optimal” oligo(Y) tract at the 5′ end recalls the behavior of some mRNAs bearing poor pyrimidine tracts, whose translational regulation is less evident and/or dependent on the cellular context (57, 58).

The synthetic reporter construct RPMS12-Myc/S, which lacks both the uORF and oligo(Y) motifs, was expressed as efficiently as the LacZ-Myc control. This supports the inference that the uORF and/or oligo(Y) tracts are negative regulatory elements for translation, at least in cultured cells. One possibility is that the negative effect of the uORF is disabled where mitochondrial function must be rapidly enhanced, for example in response to bioenergetic needs or developmental signals. Our data suggest that isoform b is likely to be the mRNA variant responsive to such signals. It is significantly more abundant than isoform a in all tissues but especially in oxidative tissues showing high overall expression, such as heart and skeletal muscle. Moreover, the profile of its polysome distribution in cultured cells, where it is found mainly associated with monoribosomes and small polysomes, suggests that it is indeed susceptible to futile initiation at the uORF, with rather few ribosomes traversing the genuine RPMS12 coding sequence. By contrast, the mRNAs containing the oligo(Y) tract (i.e. isoforms a and d) are distributed quite differently, being more prominent in both the overtly post-polysomal fraction and in larger polysomes than isoform b. This suggests that the isoform a and d mRNAs are regulated in a quite different fashion, being only inefficiently recruited into polysomes, but once there are less subject to futile initiation at the uORF. Our findings prompt a more thorough investigation of the effects on translation in different cellular contexts of the various 5′-UTR elements.

The presence of multiple isoforms of RPMS12 mRNA in all
tissues potentially allows each cell type to respond to a variety of intra- and intercellular signals, to enhance the biosynthesis of mitoribosomes according to cellular needs. The complex pattern of RPMS12 mRNAs seen in humans is not shared with the mouse, however, where all cDNA sequences from the orthologous gene represented in dbEST form a single contig, with a relatively short 5'-UTR (approximately 90 nt), no uORF, and only weak evidence for 5'-heterogeneity. Regulation of mitoribosome biosynthesis in the mouse might therefore employ different mechanisms than in humans. By contrast, the presence of a uORF may be a common feature in human mitoribosomal protein mRNAs, as it has been found in the one example studied in detail, MRPL12 (25).

Further analysis of the regulatory roles of the different 5'-UTR elements of RPMS12 mRNA will enable the components of the regulatory machinery to be identified. Further clues will also doubtless emerge from studies of other mRNAs encoding mitochondrial ribosomal proteins and other components of the mitochondrial translational apparatus, both in humans and in other vertebrates.

Acknowledgments—We thank Richard Jackson for useful discussions. We are grateful to Liliana Mannucci and Pietro Pilo-Boy for assistance in the cell culture experiments and to Claudia Crosio for unpublished results.

REFERENCES

1. Schatz, G., and Attardi, G. (1986) Annu. Rev. Cell Biol. 4, 289–333
2. Larsson, N. G., Garman, J. D., Oldfors, A., Barsh, G. S., and Clayton, D. A. (1996) Nat. Genet. 13, 296–302
3. Scarpulla, R. C. (1997) J. Bioenerg. Biomembr. 29, 109–119
4. Graack, H. R., and Wittmann-Liebold, B. (1998) Biochem. J. 329, 433–448
5. Shah, Z. H., O’Dell, K., Miller, S. C. M., An, X., and Jacobs, H. T. (1997) Gene (Amst.) 204, 55–62
6. Shah, Z. H., Migliosi, V., Miller, S. C. M., Wang, A., Friedman, T. B., and Jacobs, H. T. (1998) Genomics 48, 384–388
7. Zengel, J. M., and Lindahl, L. (1994) Prog. Nucleic Acids Res. Mol. Biol. 47, 351–370
8. Freedman, L. P., Zengel, J. M., Archer, R. H., and Lindahl, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6516–6520
9. Tsay, Y. F., Thompson, J. R., Rotenberg, M. O., Larkin, J. C., and Woolford, J. L. (1988) Genes Dev. 2, 664–676
10. Fewell, S. W., and Woolford, J. L., Jr. (1999) Mol. Cell. Biol. 19, 826–834
11. Geyer, P. K., Meyuhas, O., Perry, R. P., and Johnson, L. F. (1982) J. Biol. Chem. 257, 508–514
12. Kaspar, R. L., Rychlik, W., White, M. W., Rhoads, R. E., and Morris, D. R. (1990) J. Biol. Chem. 265, 3619–3622
13. Loreni, F., and Amaldi, F. (1997) Mol. Gen. Genet. 255, 1027–1032
14. Agrawal, M. G., and Bowman, L. H. (1987) J. Biol. Chem. 262, 4688–4695
15. Hammond, M. L., and Bowman, L. H. (1988) J. Biol. Chem. 263, 17785–17791
16. Kaspar, R. L., Kakegawa, T., Cranston, H., Morris, D. R., and White, M. W. (1992) J. Biol. Chem. 267, 508–514
17. Meyuhas, O., Thompson, A. E., Jr., and Perry, R. P. (1987) Mol. Cell. Biol. 7, 2691–2699
18. Avni, D., Shama, S., Loreni, F., and Meyuhas, O. (1994) Mol. Cell. Biol. 14, 3822–3833
19. Meyuhas, O., Biberman, Y., Pierandi-Amaldi, P., and Amaldi, F. (1996) in A Laboratory Guide to RNA: Isolation, Analysis and Synthesis (Krieg, P. K., ed) pp. 65–81, Wiley-Liss, Inc., New York.
20. Amaldi, F., and Pierandi-Amaldi, P. (1997) Proc. Mol. Subcell. Biol. 18, 1–17
21. Meyuhas, O., Avni, D., and Shama, S. (1996) Translational Control, pp. 363–388, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY