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ARTICLE *in* BIOCHEMISTRY · SEPTEMBER 2009

Impact Factor: 3.02 · DOI: 10.1021/bi901236g · Source: PubMed

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Biochemistry **2009,** *48,* 9959–9968 9959
DOI: 10.1021/bi901236g

Translation Initiation from Two In-Frame AUGs Generates Mitochondrial and Cytoplasmic Forms of the p43 Component of the Multisynthetase Complex†

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Received July 20, 2009; Revised Manuscript Received September 7, 2009

ABSTRACT: In humans, nine aminoacyl-tRNA synthetases form a stable multiprotein complex with the three auxiliary proteins p18, p38, and p43. The N-terminal moiety of p43 is involved in its anchoring to the complex, and its C-terminal moiety has a potent tRNA binding capacity. The p43 component of the complex is also the precursor of p43(ARF), an apoptosis-released factor, and of p43(EMAPII), the endothelial-monocyte activating polypeptide II. Here we identified a new translation product of the gene of p43, which contains nine additional N-terminal amino acid residues. This gene product is targeted to the mitochondria and accounts for 2% of p43 expressed in human cells. The cytoplasmic and mitochondrial species of p43 are produced from the same mRNA by a mechanism of leaky scanning of the AUG codon at position −27, which is in an unfavorable sequence context for translation initiation. The finding that a mitochondrial species of p43 exists in human cells further exemplifies the multifaceted implications of p43 and opens new perspectives for the understanding of the role of p43 in the apoptotic cell.

In higher eukaryotic organisms belonging to the metazoan species of coelomates, from *Drosophila* to mammals, the nine aminoacyl-tRNA synthetases specific for amino acids Glu, Pro, Ile, Leu, Met, Gln, Lys, Arg, and Asp form a multi-aminoacyl-tRNA synthetase complex (MARS)[1] with the three auxiliary proteins p18, p38, and p43 (*1*). The p43 subunit of MARS is a structurally important building block of the complex. This dimeric protein of 312 amino acid residues interacts with GlnRS and ArgRS to form a subcomplex of MARS, and this subcomplex is anchored to MARS via p43−p38 interaction (*2*, *3*). The N-terminal moiety of p43 can replace full-length p43 for this function. It associates with the leucine zipper motif of p38 (*4*). The p43 subunit is also a RNA-binding protein (*5*, *6*) organized around a pseudodimeric OB fold-based domain (*7*). It occupies a central position within the multisynthetase complex (*8*). It has been proposed that p43 might play a role of a cofactor for aminoacylation (*9*), but this function remains a subject of controversy (*10*). The presence of p43, or of p43-related proteins, in the cytoplasm but also in the nucleus of rabbit kidney cells has been observed by immunoelectron microscopy (*11*). It has been shown that an N-terminally truncated form of p43, but not the full-length species, can localize to the nucleus (*5*).

The p43 protein is certainly among the most ancient proteins of the translation apparatus. Homologues of p43 have been described in all living kingdoms, suggesting that it fulfills an essential function in translation, in relation to its tRNA binding capacity, a property common to all p43-related proteins. The C-terminal moiety of p43 is recovered in a bacterial tRNA binding protein, Trbp111, that may form a ternary complex with a tRNA and an aminoacyl-tRNA synthetase (*12*). A similar domain is also associated in cis with other aminoacyl-tRNA synthetases: with bacterial and plant MetRS where it acts as a cofactor for aminoacylation (*13*), with human TyrRS where it may direct tRNA to the active site of the enzyme (*14*), or with bacterial PheRS where it contributes to the editing of noncognate aminoacyl-tRNA (*15*). In the yeast *Saccharomyces cerevisiae*, Arc1p, a p43-like protein, associates with MetRS and GluRS (*16*), acts as a cofactor of these enzymes for aminoacylation (*17*, *18*), and sequesters tRNA and aminoacyl-tRNA synthetases in the cytoplasm (*19*, *20*). However, more recent data showed that in mammals this protein is also involved in cellular mechanisms beyond translation, suggesting that p43 may have evolved additional functions.

The p43 component of MARS is also the precursor of the endothelial-monocyte activating polypeptide II isolated from methylcholanthrene A-induced fibrosarcoma cells, a cytokine generated during apoptosis (*21*−*23*). The mature p43(EMAPII) has been ascribed to a proinflammatory cytokine, which stimulates chemotactic migration of polymorphonuclear granulocytes and mononuclear phagocytes and induces tissue factor activity on endothelial cells. The C-terminal, p43-like domain of human TyrRS was also shown to have cytokine activities similar to that of p43(EMAPII) (*24*). Whether p43(EMAPII) or its precursor, the p43 component of MARS, is the real cytokine remains controversial (*21*, *23*, *25*). Stable overexpression of p43, of

†This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS) and the Association pour la Recherche sur le Cancer (ARC). V.S. was the recipient of postdoctoral fellowship from the Agence Nationale de Recherches sur le SIDA, and M.K. was the recipient of postdoctoral fellowships from the Fondation pour la Recherche Médicale and Association pour la Recherche sur le Cancer.

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[1]Abbreviations: MARS, multi-aminoacyl-tRNA synthetase complex; ARF, apoptosis-released factor; EMAPII, endothelial-monocyte activating polypeptide II.

Published on Web 09/23/2009

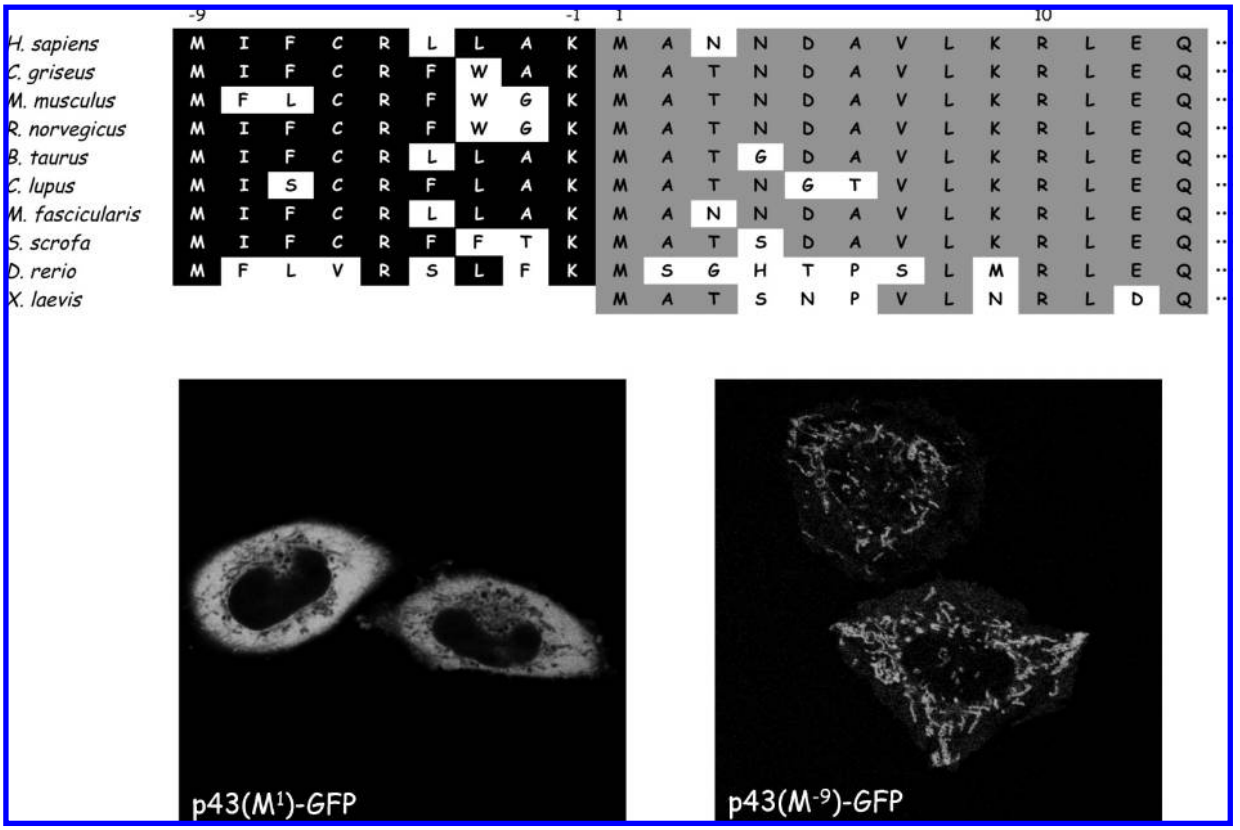

FIGURE 1: Differential subcellular localization of the two forms of p43 in human cells. The putative N-terminal amino acid sequences of the p43 proteins from various species were deduced from the most 5′-upstream sequences of the known cDNA. The sequences of p43 from human (*Homo sapiens*, NP_001135888), hamster (*Cricetulus griseus*, AAB95207), mouse (*Mus musculus*, NP_031952), rat (*Rattus norvegicus*, NP_446209), cattle (*Bos taurus*, NP_001030190), dog (*Canis lupus*, XP_545016), macaque (*M. fascicularis*, BAE90551), pig (*Sus scrofa*, NP_001107755), zebrafish (*Danio rerio*, NP_001039316), and frog (*X. laevis*, NP_001080110) are aligned. HeLa cells were transiently transfected with pEGFP-N1 plasmids that expressed fusion proteins with p43 starting at Met^{1} or at Met^{-9}. The cellular localization of the GFP fusion proteins was analyzed by confocal laser scanning microscopy.

p43(EMAPII), or of p43(ARF) in human HeLa of U937 cells was not lethal for the cells, which did not enter apoptosis in the absence of other stimuli (*2*, *5*). When p43(ARF) or p43(EMAPII) was overexpressed in U937 cells induced in apoptosis by serum starvation, the time course of apoptosis development was not significantly modified (*5*). p43 as well as p43(EMAPII) has also been reported to have anti-angiogenic properties that would limit establishment of neovasculature and thus would suppress tumor growth (*26*, *27*). This cytokine is believed to be involved in many other physiological processes and pathological disorders (see ref *28* for a review). However, in most of these studies, the real cytokine associated with these pathologies is not well-identified. Indeed, using immunohistology, antibodies directed to p43-(EMAPII) cannot discriminate between the full-length precursor protein, the p43 component of MARS ubiquitous to all cell types, and the mature p43(EMAPII) or p43(ARF) molecules (*6*, *29*). We now show that the diversity of p43 proteins is even more puzzling than previously thought.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Expressing GFP Fusion Proteins. The complete cDNAs encoding human p43 starting from Met^{1} or Met^{-9} to Lys^{312} were amplified by PCR and introduced between the *Eco*RI and *Bam*HI sites of pEGFP-N1 (BD Biosciences) to express p43(M^{1})−GFP or p43(M^{-9})−GFP fusion protein, respectively. The cDNAs encoding p43 from Met^{-9} to Ser^{147}, from Met^{-9} to Phe^{80}, from Met^{-9} to Lys^{312} with a mutation of Met^{1} to Ile (AUG to AUU mutation), or from Met^{-9} to Lys^{312} with a mutation of Ile^{-8} to Ala (AUU to GCG mutation) were produced by PCR and introduced between the *Eco*RI and *Bam*HI sites of pEGFP-N1, to express p43(M^{-9}:S^{147})−GFP, p43(M^{-9}:F^{80})−GFP, p43(M^{-9}.M^{1}I)−GFP and p43(M^{-9}.I^{-8}A)−GFP proteins. To express p43(M^{-9}:K^{-1})−GFP (encoding p43 from Met^{-9} to Lys^{-1}), p43(M^{-9}:M^{1}I:D^{5})−GFP [encoding p43 from Met^{-9} to Asp^{5}, with a mutation of Met^{1} to Ile (AUG to AUU mutation)], p43(M^{-9}:M^{1}I:R^{10})−GFP (encoding p43 from Met^{-9} to Arg^{10}, with a mutation of Met^{1} to Ile), or p43(M^{-9}:I^{-8}A:K^{-1})−GFP [encoding p43 from Met^{-9} to Lys^{-1}, with a mutation of Ile^{-8} to Ala (AUU to GCG mutation)] protein, oligonucleotide duplexes were introduced between the *Eco*RI and *Bam*HI sites of pEGFP-N1. All constructs were verified by DNA sequencing.

Confocal Imaging. HeLa cells were grown in F12 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 μg/mL penicillin and streptomycin. Cells were transfected with Effectene (Qiagen). For localization experiments, cells were cotransfected with a pEGFP-N1 derivative and with pDsRed2-Mito (BD Biosciences). Cells were grown in eight-well Lab-Tek II chambers (Nalge Nunc International) and observed by confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope equipped with a DD488/543 mirror. GFP was excited using a 488 nm laser line of an Ar laser and detected at 500−535 nm. DsRed was excited at 543 nm with a He−Ne laser and detected at 584−659 nm. Imaging of GFP and DsRed fluorescence was performed in a sequential manner. The expression and stability of the fusion proteins in HeLa cells

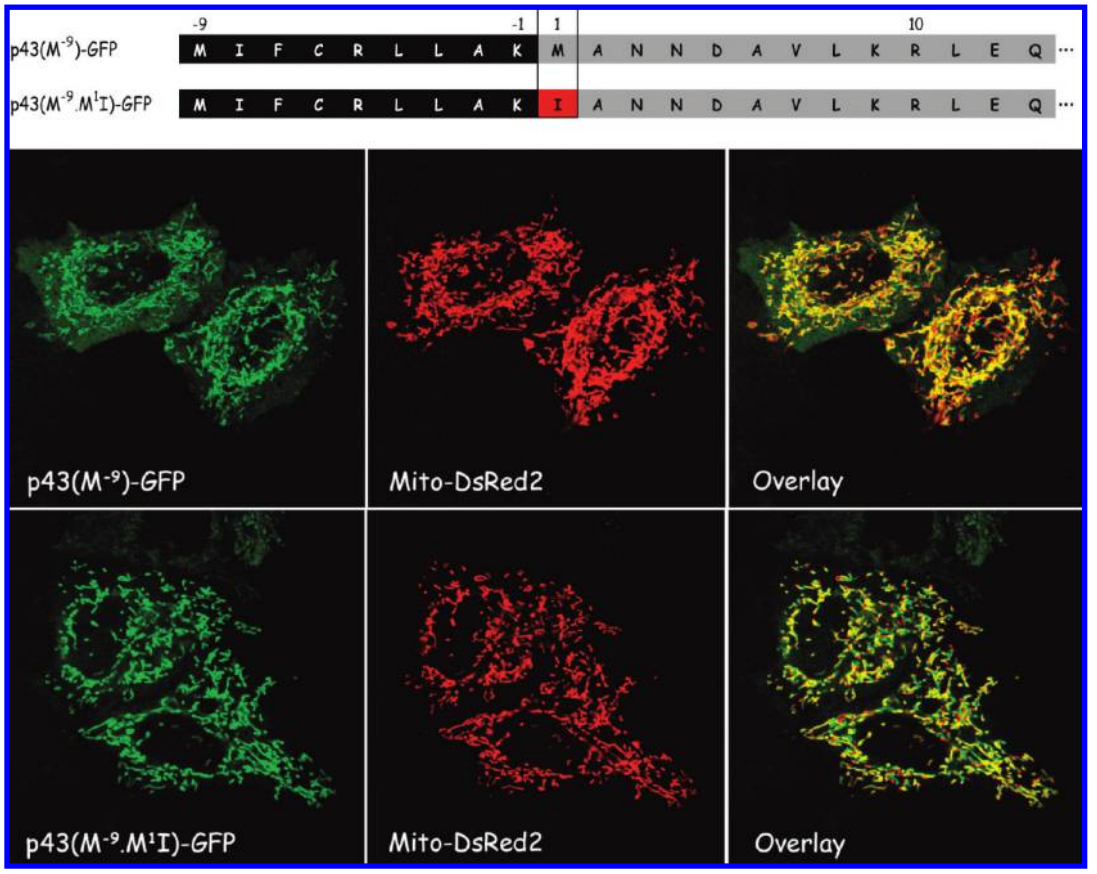

FIGURE 2: Colocalization of p43(M^{-9}) with mitochondria. HeLa cells were cotransfected with plasmids expressing the p43(M^{-9})−GFP or p43(M^{-9}.M^1I)−GFP fusion protein and with pDsRed2-Mito, a plasmid that expresses a mitochondrial protein marker. Cellular localization of the fusion proteins was analyzed by confocal microscopy. The overlay shows a perfect colocalization of the p43 proteins and of the mitochondrial marker, and a decreased green cytoplasmic background when Met^1 is mutated to Ile.

were checked by Western blot analysis with anti-GFP and anti-DsRed antibodies.

Mapping the 5′-Ends of p43 Transcripts. Determination of the 5′-ends of the transcripts encoding p43 was conducted by rapid amplification of cDNA ends (5′-RACE, Roche). Poly(A^+) mRNA isolated from the U937 human cell line with Oligotex (Qiagen) was reverse transcribed into single-stranded cDNA using oligonucleotide 5′-GCTTAGAGTCGGCACTTCC-3′, complementary to nucleotides 445 to 427 of p43 mRNA (+1 corresponds to A of the AUG codon specifying the cytoplasmic protein). The first strand cDNA was dA-tailed and amplified by PCR between oligo d(T)-anchor primer (Roche) containing a *Cla*I site at the 5′-end and oligonucleotide 5′-GGGGGATCC-GGTATTTGCTTCACTCCATT-3′, complementary to nucleotides 236 to 217 of p43 mRNA and containing a *Bam*HI site at the 5′-end. PCR products were cloned and sequenced.

Antibodies and Western Blot Analysis. Monoclonal antibody directed to GFP or polyclonal antibody to DsRed was from BD Biosciences, and monoclonal antibody to human cytochrome *c* was from PharMingen. Polyclonal anti-p43, anti-LysRS, and anti-AspRS antibodies have been described previously (*30*). Western blot analyses were conducted with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with peroxidase (Chemicon) and the ECL detection reagents (Amersham Biosciences).

Isolation of Mitochondria. U937 cells were grown in suspension in RPMI medium (Invitrogen) supplemented with 10% FCS. Subcellular fractionation of U937 cell extracts was conducted essentially as described previously (*31*). U937 cells (100×10^6 cells) were harvested by centrifugation at 600*g* for 10 min at 4 °C, washed once with 10 mL of ice-cold PBS, and resuspended in 2 mL of buffer MitoA [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, and 1 mM DTT]. All subsequent steps were conducted at 4 °C. After incubation on ice for 10 min, cells were pelleted at 600*g*, resuspended in 550 μL of buffer MitoA, and incubated for 10 min. Cells were lysed with 30−50 strokes of a Teflon homogenizer (Kontes) and diluted with 1 volume of buffer MitoA. The lysate was centrifuged at 750*g* for 10 min and then at 900*g* for 10 min to remove cell debris and nuclei. After another centrifugation at 5500*g* for 10 min, the supernatant was recovered and centrifuged at 8000*g* for 10 min to remove residual mitochondria, and the resulting supernatant was termed the cytoplasmic fraction. The pellet from the centrifugation at 5500*g*, containing mitochondria, was resuspended with 0.5 mL of buffer MitoA, centrifuged at 5500*g* for 10 min, resuspended with 0.5 mL of buffer MitoA, and centrifuged at 8000*g* for 10 min. The resulting pellet was termed the mitochondrial fraction.

RESULTS

A Mitochondrial Species of p43 Is Encoded from an Upstream AUG Codon. The p43 protein is a ubiquitous component of all multi-aminoacyl-tRNA synthetase complexes isolated so far. Its N-terminal domain mediates its association with the arginyl- and glutaminyl-tRNA synthetase components of the complex, and with p38, the scaffold protein of MARS (*2*). Two human p43 species are registered on the NCBI Web site. Entry NP_004748.2, annotated "small inducible cytokine subfamily E,

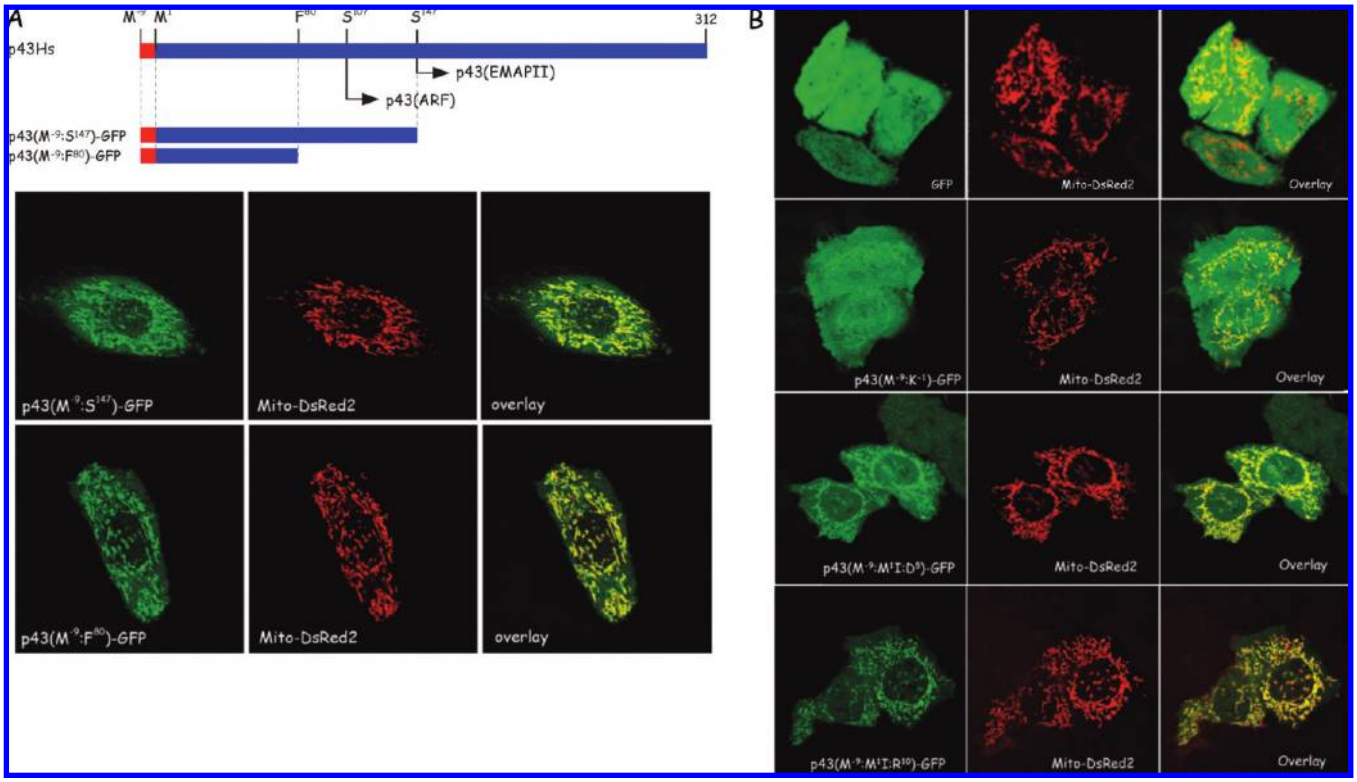

FIGURE 3: Identification of the minimal peptide required for mitochondrial targeting of p43(M^{-9}). (A) The mitochondrial p43 protein is composed of two conserved domains (from Met^1 to Phe^{80} and from Ser^{147} to Lys^{312}), a highly charged and more variable linker domain (from Phe^{80} to Ser^{147}), and a N-terminal sequence of nine amino acid residues specific for the mitochondrial protein. HeLa cells were transiently transfected with pDsRed2-Mito and with pEGFP-N1 plasmids that expressed GFP fusion proteins containing p43 sequences from Met^{-9} to Ser^{147} [p43(M^{-9}:S^{147})−GFP] or to Phe^{80} [p43(M^{-9}:F^{80})−GFP]. The colocalization of the GFP fusion proteins and of the mitochondrial marker was analyzed by confocal laser scanning microscopy. (B) The N-terminal nine-amino acid sequence specific to mitochondrial p43 was fused to GFP without [p43(M^{-9}:K^{-1})] or with 5 [p43(M^{-9}:M^1I:D^5)] or 10 [p43(M^{-9}:M^1I:R^{10})] additional amino acid residues of p43. The two last constructs also display a Met^1 to Ile mutation, to prevent translation initiation at the level of the Met codon specifying the cytoplasmic protein. As a control, localization of GFP alone is shown in the top panels. Localization of fluorescent proteins was analyzed by confocal laser scanning microscopy.

member 1 isoform a precursor", corresponds to the 312-amino acid protein that has been initially described as the precursor of the EMAPII-cytokine (*21*). It corresponds to the p43(M^1) species described below. When additional genomic sequences became available, a putative 336-amino acid p43 species was indexed as "small inducible cytokine subfamily E, member 1 isoform b precursor", described in entry NP_001135888.1. This p43 species would be encoded by a longer mRNA species containing two putative upstream AUG codons, but no experimental data have been provided to date to ascertain that these two upstream AUG codons are indeed functional.

Comparison of the amino acid sequences potentially encoded by the 5′-ends of p43 cDNA from different species revealed that a putative nine-amino acid polypeptide sequence is encoded upstream from the AUG codon specifying the cytoplasmic form of p43, p43(M^1). This peptide is highly conserved in p43(M^{-9}) forms from humans to fish (Figure 1). By contrast, the sequence data available for the p43 cDNA from *Xenopus laevis* suggest that it does not encode an additional N-terminal, nine-amino acid sequence. With regard to the most upstream AUG codon found on the longer mRNA, the finding that it is not recovered in p43 from other species of primates such as *Pan troglodytes* (chimpanzee), *Maccaca fascicularis* or *Maccaca mulatta*, and *Pongo abelii* (orangutan) indexed in the data libraries, proteins that are 96% identical with human p43, suggests that it might not be functional. Further analyses are required to validate this most upstream AUG codon as a functional one.

To gain some insight into the physiological significance of the p43(M^{-9}) species, the cellular localization of the two human p43 species, p43(M^1) and p43(M^{-9}), was determined by confocal microscopy of human cells transformed with GFP fusion proteins (Figure 1). The p43(M^1)−GFP fusion protein exhibited a diffuse fluorescence pattern extending throughout the cytoplasm, with a distinct exclusion of the nucleus (Figure 1), as reported previously (*5*). By contrast, the p43(M^{-9})−GFP fusion protein revealed a punctuate pattern within the cytoplasm, superimposed to a faint diffuse labeling of the whole cytoplasm.

The punctuate pattern displayed by the p43(M^{-9})−GFP fusion protein suggested that this protein could be localized into the mitochondria. To test this hypothesis, the p43(M^{-9})−GFP fusion protein was coexpressed in HeLa cells with a mitochondrial marker Mito-DsRed2, a DsRed fluorescent protein expressed with the mitochondrial targeting sequence of human cytochrome *c* oxidase. As shown in Figure 2, the green fluorescence labeling observed with the p43(M^{-9})−GFP fusion protein superimposed with the red fluorescence labeling observed with Mito-DsRed2. Thus, the nine additional amino-terminal residues found in p43(M^{-9}) are involved in mitochondrial targeting of p43. To ascertain that the AUG codon encoding methionine at position −9 was used as the initiation codon for the p43-(M^{-9})−GFP fusion protein, the second AUG codon encoding methionine at position 1 was mutated into an AUU codon (Ile). The p43(M^{-9}.M^1I)−GFP fusion protein was also directed to the mitochondria (Figure 2). It should be noticed that in the presence

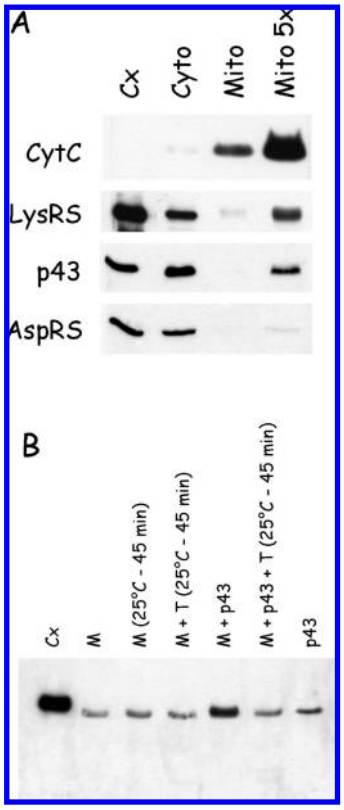

FIGURE 4: Detection of a p43-related polypeptide in isolated mitochondria. (A) The cytoplasmic (Cyto) or mitochondrial (Mito) fractions obtained after subcellular fractionation of a U937 cell extract were analyzed by Western blotting using antibodies directed to the LysRS, p43, or AspRS component of the multi-aminoacyl-tRNA synthetase complex (Cx) or to cytochrome *c* (CytC). Lanes Cyto and Mito contained equivalent amounts of the initial extract of U937 cells. Lane Mito 5× contained a 5-fold excess of the Mito fraction. (B) Mitochondria (M) purified from human U937 cells were incubated at 25 °C for 45 min in the absence or presence (M+T) of trypsin at a concentration of 0.1 mg/mL, and in the absence or presence (M+p43) of purified human cytoplasmic p43 added at a concentration of 1 μg/mL. Samples were heated for 5 min at 100 °C in 62.5 mM Tris-HCl (pH 7.5), 2% SDS, 5 M urea, 100 mM DTT, and 0.002% bromophenol blue and analyzed by Western blotting with anti-p43 antibody. The purified multi-aminoacyl-tRNA synthetase complex (Cx) and p43 protein (p43) were used as controls.

of the M^1I mutation, the green fluorescence cytoplasmic background observed with the p43(M^{-9})−GFP fusion protein is now virtually absent. Thus, the translation product starting at Met at position −9 is exclusively targeted to the mitochondria, and the AUG codon at position 1 is solely used for translation of the cytoplasmic form of p43.

The 19 N-Terminal Amino Acid Residues of p43 Specify the Mitochondrial Targeting Sequence. The mitochondrial targeting signals are generally located at the N-terminus of the imported proteins, but in some instances, mitochondrial precursors display internal or C-terminal targeting signals (*32*). When the C-terminal region of p43 was removed, from residue 148 to 312 or from residue 81 to 312, the polypeptides initiating at Met^{-9} were still directed to the mitochondria (Figure 3A), suggesting that the sequence elements responsible for mitochondrial targeting are localized only in the N-terminal region of p43(M^{-9}). To identify the minimal polypeptide that constitutes the mitochondrial targeting sequence of p43, N-terminal peptides of p43(M^{-9}) of different lengths were fused to GFP. When the cDNA sequences encoding the amino acid residues located upstream of Met^1, from Met^{-9} to Lys^{-1}, were fused to GFP to give the p43(M^{-9}:K^{-1})−GFP fusion protein, no labeling of mitochondria could be observed (Figure 3B). The labeling was very similar to that obtained with GFP alone (top of Figure 3B).

Thus, the additional sequences found in p43(M^{-9}) as compared to p43(M^1) are not sufficient to trigger mitochondrial localization of GFP. When five additional amino acid residues of p43 were fused to GFP, the p43(M^{-9}:M^1I:D^5)−GFP fusion protein, containing p43 sequences from Met^{-9} to Asp^5, including the M^1I mutation, revealed a clear mitochondrial labeling (Figure 3B). However, the cytoplasmic background was high, suggesting that these sequences contain most but not all the sequence information necessary for targeting. A nearly complete mitochondrial targeting of GFP was observed with the p43(M^{-9}:M^1I:R^{10})−GFP fusion protein (Figure 3B), suggesting that the 19 amino-terminal residues of p43(M^{-9}) are necessary and sufficient to trigger mitochondrial localization and account for its mitochondrial targeting signal.

p43(M^{-9}) Is Translocated into the Mitochondria. To ascertain that the p43(M^{-9}) polypeptide is not merely loosely interacting at the surface of the outer mitochondrial membrane but is translocated into the matrix of the mitochondria, we first isolated mitochondria from human cells in culture and checked for the presence of the p43-related polypeptide in an extract of purified mitochondria. The relative amount of the cytoplasmic and mitochondrial species of p43 was determined by Western blotting with an anti-p43 antibody directed to the common region of p43(M^1) and p43(M^{-9}). After subcellular fractionation of a U937 cell extract, antibodies to cytochrome *c* and to the cytoplasmic species of aspartyl-tRNA synthetase, representative of a mitochondrial and of a cytoplasmic protein, respectively, were used as markers of the subcellular fractions (Figure 4A). A trace amount of AspRS was observed in the mitochondrial fraction, showing a slight cytoplasmic contamination. By contrast, a clear signal was observed in the mitochondrial fraction when anti-p43 antibody was used (Figure 4A). Similarly, antibody directed to LysRS also showed the presence of LysRS in the mitochondrial fraction, as observed previously (*31*). The intensities of the signals obtained by Western blotting were quantified; the fractions of the mitochondrial species of p43 and LysRS were found to represent ~1−2% of the total. Interestingly, the amount of mitochondrial p43 is similar to that determined for mitochondrial LysRS, the only LysRS species that sustains mitochondrial translation. This observation is consistent with a functional role of mitochondrial p43.

If p43(M^{-9}) is translocated into the mitochondrial matrix, then it should not be accessible to proteases added to purified mitochondria. When isolated mitochondria were incubated at 25 °C, in the presence or absence of trypsin, the polypeptide corresponding to p43 was not degraded (Figure 4B). When an equal amount of purified, cytoplasmic p43 was added to purified mitochondria, the exogenously added p43 was completely degraded after incubation in the presence of trypsin (Figure 4B). These results are consistent with the import of p43(M^{-9}) into the mitochondrial matrix.

Mapping the 5′-Ends of p43 cDNA. The gene encoding the human, cytoplasmic, and mitochondrial species of p43 is located on chromosome 4, at position 4q24. It is made of seven exons that encompass 31898 nucleotides (Figure 5A). The first two exons are separated by a long intron of 8390 nucleotides. The AUG initiation codon for p43(M^1) is located within exon 2 (Figure 5A). The AUG initiation codon for p43(M^{-9}) is split by this large

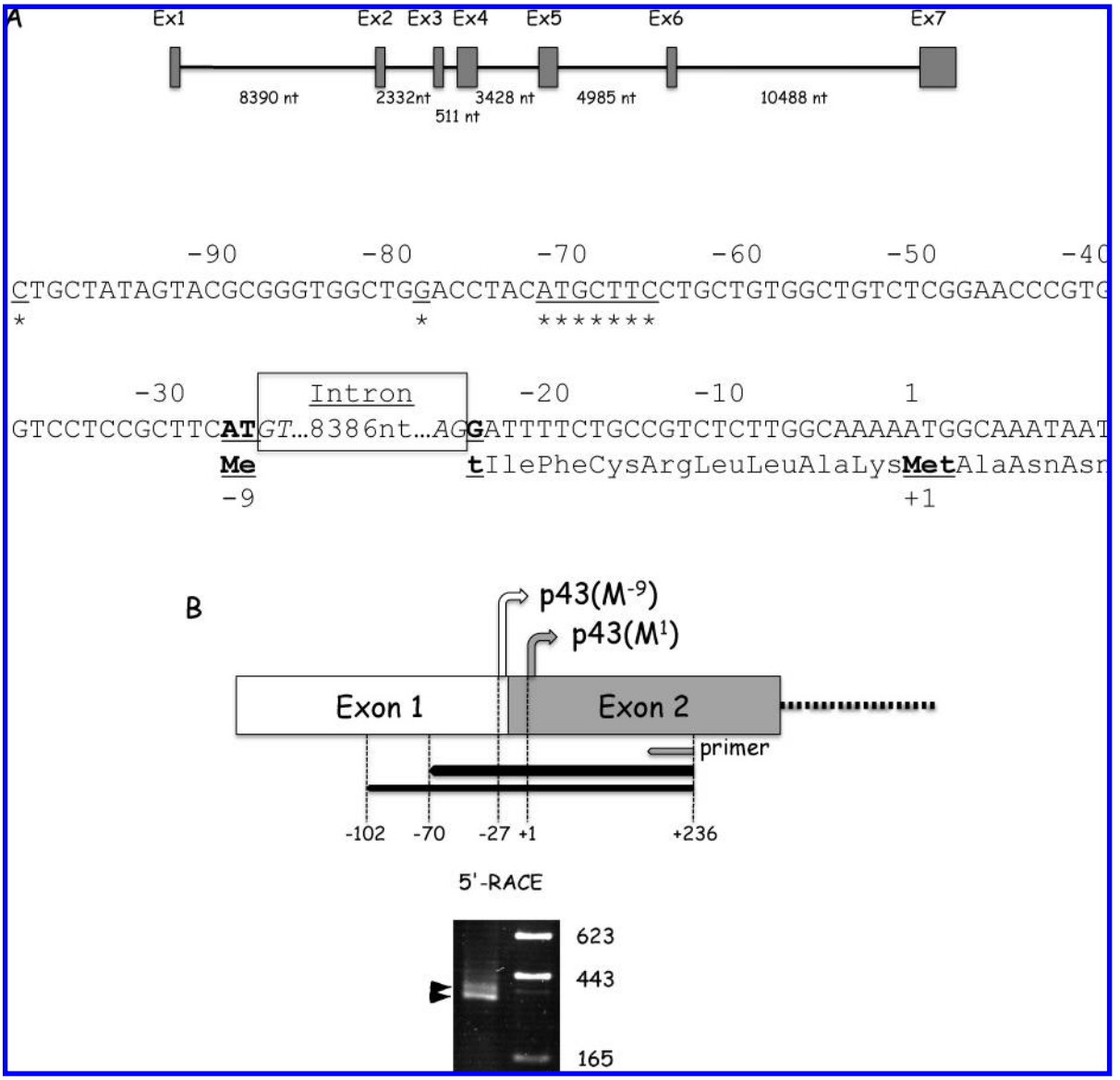

FIGURE 5: Mapping of the 5′-ends of p43 transcripts. (A) The top panel shows a schematic view of the gene encoding human p43, made of seven exons (boxes) linked by six introns (lines). The bottom panel shows the 5′-end DNA sequence of the gene. Sequences located at the 3′-end of exon 1 and at the 5′-end of exon 2 are indicated. Met residues at positions +1 and −9 correspond to the initiating methionine for the cytoplasmic and mitochondrial species of p43, respectively. The nucleotide at position 1 corresponds to the A residue of the ATG encoding cytoplasmic p43. The sequence of the ATG codon for Met^{-9} is interrupted by a long intron. The 5′-ends of the transcripts determined by 5′-RACE are denoted (asterisks). (B) The 5′-end of the spliced mRNA containing exons 1 and 2 is schematized. The two cDNA products obtained by 5′-RACE with the primer at position 236 (gray arrow) are indicated by arrowheads (left lane), and their sizes are compared with a DNA marker (right lane). The major cDNA extends up to nucleotides around position −70 (thick black arrow) and the minor one to position −102 (thin black arrow).

intron; the two first nucleotides are located at the 3′-end of exon 1, while the G of the AUG codon is provided by exon 2 after splicing of the intron. The formation of the AUG codon for p43(M^{-9}) requires that splicing occur at this position. Thus, we considered the possibility that two different mRNAs could be produced by this gene by a mechanism of alternative splicing.

To test this possibility, we performed a 5′-RACE experiment using a specific primer localized in exon 2 (Figure 5B). Two products of ~300−350 nucleotides, corresponding to two putative mRNAs, were obtained. The smaller PCR product was the most abundant. Quantitative PCR experiments revealed that the longest mRNA is 4-fold less abundant than the short one (result not shown). To identify the 5′-ends of the PCR products, they were cloned and their nucleotide sequences determined. The 5′-ends of the smaller product fell in the region of nucleotides −66 to −72, as compared to the AUG codon for p43(M^1). Thus, the AUG codon at position −27, encoding p43(M^{-9}), is also carried by the shortest mRNA. The longer mRNA extended up to nucleotide −102 in exon 1 (Figure 5B). Thus, the two AUG codons at positions −27 and +1 are both present on the two mRNA species, and no evidence for a mechanism of alternative splicing of the first intron was observed.

Analysis of the Sequence Context of the AUGs. According to the proposed mechanism of ribosome scanning for translation initiation in mammals (*33*, *34*), the sequence context surrounding the AUG codons determines whether the putative initiation site is strong or weak. The optimal context corresponds to the sequence gcc(A/G)ccAUGG, where a purine at position −3 and a G at position +4 are the most important determinants for a strong context in mammals. The sequence context around the AUG at position −27, for initiation of translation of p43-(M^{-9}), with a U at position −3 and an A at position +4,

	-3	+1	+4
Favorable context : g c c	A/G	c c A U G	G
p43(M[1]) : g c a	A	a a A U G	G
p43(M[-9]) : c g c	U	u c A U G	A

FIGURE 6: AUG contexts of p43(M^1) and p43(M^{-9}). The nucleotides surrounding the AUG codons specifying the cytoplasmic [p43(M^1)] or mitochondrial [p43(M^{-9})] species of p43 are aligned with the sequence of a canonical AUG codon in a favorable sequence context for translation initiation. The A of the AUG codons is numbered +1. Nucleotides at positions −3 and +4 are boxed.

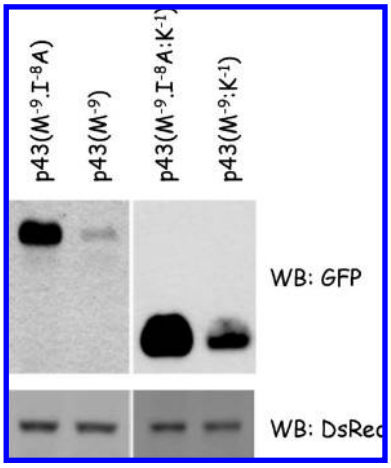

FIGURE 7: AUG codon specifying mitochondrial p43 in an unfavorable sequence context. The levels of expression of p43−GFP fusion proteins with an A [p43(M^{-9}) and p43(M^{-9}:K^{-1})] or a G [p43(M^{-9}.I^{-8}A) and p43(M^{-9}.I^{-8}A:K^{-1}] at position +4 were determined by Western blotting with anti-GFP antibody after cotransformation of HeLa cells with pEGFP-N1 plasmids expressing the p43-GFP fusion proteins and with pDsRed2-Mito. Expression of DsRed-Mito was monitored by Western blotting with an anti-DsRed antibody.

significantly deviates from the consensus for a favorable context (Figure 6). By contrast, the AUG codon for p43(M^1) displays a consensus context for a strong initiation site (A at position −3 and G at position +4). Thus, a mechanism of leaky scanning would allow the initiation complex to frequently bypass the AUG at position −27, to favor translation initiation at the level of the second AUG.

All the constructs used in this study were expressed from a plasmid carrying an ATA sequence upstream of the AUG initiation codon, thus with an A at position −3. To test the importance of the context of the AUG codon on the level of expression of the constructs, a derivative of the p43(M^{-9})−GFP fusion protein was expressed in HeLa cells after replacement of the AUU codon encoding Ile^{-8} by a GCG codon encoding Ala^{-8}. Thus, the AUG initiation codon of the p43(M^{-9}.I^{-8}A)−GFP fusion protein is placed into a favorable context, with a G at position +4 and an A at position −3. The level of expression of the p43(M^{-9}.I^{-8}A)−GFP fusion protein was found to be ~5-fold higher as compared to that of the p43-(M^{-9})−GFP fusion protein (Figure 7). The two fusion proteins were targeted to the mitochondria (not shown).

To further assess the role of the nucleotide at position +4 on the expression level of the protein, the level of expression of the cytoplasmic p43(M^{-9}:K^{-1})−GFP fusion protein (Figure 3B), containing p43 sequences from Met^{-9} to Lys^{-1}, was compared to that of the p43(M^{-9}.I^{-8}A:K^{-1})−GFP fusion protein, containing the AUU to GCG mutation at the second codon. The GFP fusion protein with the AUG codon in a favorable context, p43(M^{-9}.I^{-8}A:K^{-1})−GFP, was expressed much more efficiently than the p43(M^{-9}:K^{-1})−GFP fusion protein (Figure 7). Thus, a mitochondrial [p43(M^{-9})−GFP] or cytoplasmic [p43(M^{-9}:K^{-1})−GFP] fusion protein with an AUG initiation codon in a nonfavorable context, mimicking that of the endogenous p43-(M^{-9}) protein, is poorly expressed.

DISCUSSION

The low steady-state level of mitochondrial proteins involved in translation and in particular of the mitochondrial species of p43 precluded its identification when a crude extract of human cells was analyzed by molecular sieve chromatography (*23*), leading to the assumption that a single p43 species is present in the cell, as a component of the MARS complex. This protein has the capacity to bind tRNA via its C-terminal moiety (*5*) and to participate in the assembly of the MARS complex via its N-terminal moiety (*2*). The mitochondrial species of p43 identified in this study is identical to the p43 component of MARS but is expressed as a precursor possessing a short, nine-amino acid additional sequence at its N-terminus. Thus, the mitochondrial species of p43, mp43, should be able to bind tRNA as efficiently as its cytoplasmic counterpart, cp43. In addition, because mp43 and cp43 share the same N-terminal protein−protein interaction domain involved in the association of cp43 with GlnRS, ArgRS, and p38 (*2*), mp43 should also have the intrinsic capacity to bind to these components of MARS. However, all the components of MARS are confined to the cytoplasm (*35*). The only known exception is LysRS for which a cytoplasmic species and a mitochondrial species are encoded by the same gene by means of alternative splicing (*31*, *36*). Thus, mp43 certainly fulfills in the mitochondria a role distinct from its function in the cytoplasm.

Because the AUG codon specifying mp43 is interrupted by an intron, a possible mechanism to account for expression of mp43 and cp43 from the same gene was to consider that two distinct mRNA species were produced by a mechanism of alternative splicing of the single gene identified in the human genome. However, our data clearly show that mp43 and cp43 are two translation products of the same mRNA. Several examples of a single gene producing two translation products have been described for aminoacyl-tRNA synthetases, in yeast, plants, or humans, but different mechanisms could be involved. In the yeast *S. cerevisiae*, the cytoplasmic and mitochondrial HisRS and ValRS are encoded by two distinct mRNAs of different lengths transcribed from a single gene (*37*, *38*). The longer messages, translated from an upstream AUG codon, encode the mitochondrial enzymes. In yeast, a single gene also encodes the cytoplasmic and mitochondrial forms of AlaRS and GlyRS, which are produced from a single mRNA (*39*, *40*). The longer mitochondrial isoforms are translated from upstream non-AUG initiation codons. In the plant *Arabidopsis thaliana*, five aminoacyl-tRNA synthetases are shared between cytosol and mitochondria and 15 are shared between the two organelles, mitochondria and chloroplasts (*41*). In humans, the cytoplasmic and mitochondrial GlyRS and LysRS are encoded by a single gene. Whereas the two LysRS species are produced from two mRNAs arising by alternative splicing of the unique KARS gene (*31*, *36*), the two GlyRSs are believed to be translated from a single mRNA from two in-frame initiation codons (*42*). Similarly, two forms of human ArgRS are also translated from two in-frame initiation codons (*43*, *44*). The longest species corresponds to the cytoplasmic enzyme associated within MARS, but the function of the shortest

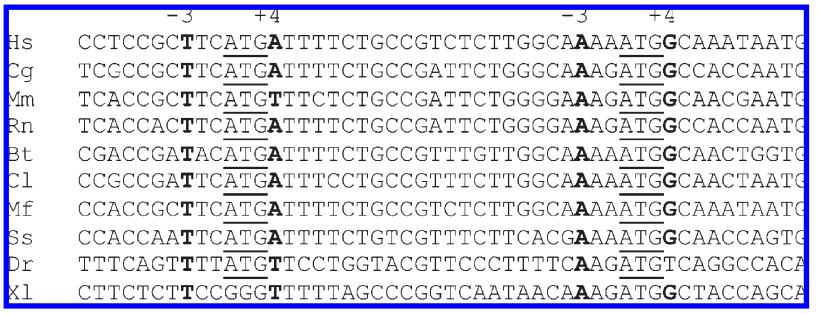

FIGURE 8: Sequence context of the AUG codon specifying the mitochondrial and cytoplasmic p43 from different species. The nucleotide sequences surrounding the AUG initiation codons for p43 starting at Met^{-9} or at Met^{1} are indicated for human (Hs), hamster (Cg), mouse (Mm), rat (Rn), cattle (Bt), dog (Cl), macaque (Mf), pig (Ss), zebrafish (Dr), and frog (Xl). Nucleotides at positions −3 and +4 of the two AUG are indicated in bold.

form remains hypothetical. The mitochondrial species of ArgRS is encoded by a separate gene (*45*).

The finding that the precursor of mp43 is produced from the upstream AUG codon found in an unfavorable sequence context, by a mechanism of leaky scanning of the mRNA, is in agreement with the lower expression level of the mitochondrial species of p43, which represents only 1−2% as compared to the cytoplasmic species. When comparing the sequence contexts of the AUG encoding the mitochondrial and cytoplasmic species of p43 from the different species listed in Figure 1, which display a mRNA with two putative translation initiation codons, similar to that of human p43 analyzed in this study, we find it appears that the first AUG codon is always located in an unfavorable sequence context (T at position −3 and, in most cases, A at position +4) as compared to the second AUG codon (A at position −3 and, in most cases, G at position +4) (Figure 8). The first AUG codon is always preceded by three pyrimidine nucleotides and the second by three purine nucleotides, positions that are the most relevant to determining the efficiency of an AUG codon. Thus, the expression pattern of p43 is certainly similar in many metazoan species. The putative role of the two mRNA species of different lengths on the expression of the two p43 translation products remains to be established.

The existence of a p43 protein within the mitochondria raises the question of its functional role in this organelle. Previous experiments have shown that the knock-down of p43 with a siRNA targeted to the sequences common to the cytoplasmic and mitochondrial p43 species is not lethal for human cells in culture (*2*). Thus, neither cp43 nor mp43 is essential in the context of cultured cells.

Several putative roles may be envisioned. In this regard, it should be recalled that truncated derivatives of p43, p43(ARF) and p43(EMAPII), have been identified in apoptotic cells. It was shown that overexpression or p43(ARF) or p43(EMAPII) in the cytoplasm of human cells did not stimulate apoptosis (*5*). It has been proposed that cleavage of the p43 component of MARS may have some negative effect on the efficiency of the translation process and, thus, may be one of the mechanisms leading to arrest of translation after the onset of apoptosis. However, because mitochondria are essential to many aspects of the apoptotic process, we examined the possibility that mp43 or one of its truncated derivatives identified in apoptotic cells may have a direct effect on apoptosis when targeted to the mitochondria. Overexpression of mp43 did not induce apoptosis on HeLa or U937 cells (unpublished results). Similarly, when p43(ARF) or p43(EMAPII) was overexpressed and targeted to the mitochondria after fusion to N-terminal sequence M^{-9}:M^{1}I:D^{5} or M^{-9}:M^{1}I:R^{10} of mp43, the two proteins were localized to the mitochondria but did not induce apoptosis (unpublished results). Thus, a putative link between mp43 and apoptosis remains to be established.

Whether mp43 itself is important for the biology of mitochondria or whether it mediates the mitochondrial import of other components essential for this organelle remains to be deciphered. The C-terminal domain of p43 is a classical OB fold (*7*), similar to that determined for the bacterial Trbp111 protein. This ancient tRNA-binding protein has been described as a tRNA-specific chaperone which may stabilize the L-shaped fold of the tRNA molecule (*46*). One possibility is that mp43 acts in the folding of some mitochondrial tRNAs which lack canonical elements important in assisting in the proper folding of the tRNA molecules (*47*). Alternatively, the tRNA binding capacity of mp43 could be used to target tRNA molecules to the mitochondria. Import of tRNA into mitochondria is a complex process that differs from one species to another (*48*). No unifying mechanism has emerged from the many studies performed in different organisms, but it is clear that cytosolic factors could be involved in this process. For instance, in yeast, both cytoplasmic and mitochondrial LysRS are involved in import of a cytoplasmic $tRNA^{Lys}$ in mitochondria (*49*).

Finally, mp43 might be used to trigger mitochondrial localization of cytoplasmic proteins. The N-terminal domain of mp43, from Met^{1} to Ser^{106}, is identical to the N-terminal domain of cp43 that is involved in the assembly of MARS via its interaction with GlnRS, ArgRS, and p38 (*2*). Recent studies showed the presence of nucleus-encoded $tRNA^{Gln}$ and of cytoplasmic GlnRS in the mitochondria of *S. cerevisiae* (*50*). Because no mitochondrial GlnRS could be identified in the yeast genome, it was suggested that Gln-$tRNA^{Gln}$ synthesis in mitochondria uses the components imported from the cytoplasm. If such a mechanism also prevails in humans, association of human, cytoplasmic GlnRS with mp43 may be a means of importing the glutaminylation pathway into human mitochondria. Alternatively, recent results also demonstrated that the transamidation pathway may be the pathway by which *S. cerevisiae* mitochondria synthesize its Gln-$tRNA^{Gln}$, using mitochondrial $tRNA^{Gln}$ first aminoacylated with Glu by a GluRS imported from the cytoplasm (*19*). Whether mp43 could be involved in the pathway of Gln-$tRNA^{Gln}$ formation in human mitochondria is an intriguing possibility.

The finding that a p43 species is targeted to human mitochondria opens new perspectives for the understanding of several physiological processes. It is also an interesting example of a protein that is targeted to a multienzyme complex in the cytoplasm and could fulfill additional functions when targeted

to the mitochondria. Future experiments should address this important issue.

ACKNOWLEDGMENT

We thank Spencer Brown, Susanne Bolte, and Marie-Noëlle Soler for access to the confocal microscope facility (ISV, Gif-sur-Yvette, France).

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