

Dual Sites of Protein Initiation Control the Localization and Myristoylation of Methionine Sulfoxide Reductase A*

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Methionine sulfoxide reductase A is an essential enzyme in the antioxidant system, which scavenges reactive oxygen species through cyclic oxidation and reduction of methionine and methionine sulfoxide. In mammals, one gene encodes two forms of the reductase, one targeted to the cytosol and the other to mitochondria. The cytosolic form displays faster mobility than the mitochondrial form, suggesting a lower molecular weight for the former. The apparent size difference and targeting to two cellular compartments had been proposed to result from differential splicing of mRNA. We now show that differential targeting is effected by use of two initiation sites, one of which includes a mitochondrial targeting sequence, whereas the other does not. We also demonstrate that the mass of the cytosolic form is not less than that of the mitochondrial form; the faster mobility of cytosolic form is due to its myristoylation. Lipidation of methionine sulfoxide reductase A occurs in the mouse, in transfected tissue culture cells, and even in a cell-free protein synthesis system. The physiologic role of myristoylation of MsrA remains to be elucidated.

All organisms living in an aerobic atmosphere are subjected to oxidative stress from reactive oxygen and nitrogen species. Proteins are a notable target of these species, and mechanisms have evolved to intercept them and to cope with proteins that do undergo oxidative modification (1). If the covalent modification is not reversible then the protein is targeted for selective degradation. Repair systems have evolved to cope with the reversible modifications, including oxidation and reduction of the sulfur-containing amino acids cysteine and methionine. The latter is relatively readily oxidized to methionine sulfoxide (MetO).² Virtually all organisms from bacteria to mammals have several methionine sulfoxide reductases (Msr), which catalyze the reduction of MetO to Met (2). Reduction of methionine residues in proteins allows them to react again with reactive species, creating a system with catalytic efficiency in scavenging potentially damaging species. Thioredoxin and thioredoxin reductase act sequentially to regenerate active Msr, with the net result being the catalytic scavenging of reactive

oxygen species at the expense of NADPH. A scheme of the system is shown in Fig. 1 of Ref. 3.

Oxidation of Met to MetO creates a chiral center, and the *S*- and *R*-epimers are substrates for specific Msr (2, 4). In most organisms, the *S*-epimer is reduced by MsrA, and the *R*-epimer is reduced by MsrB, both using thioredoxin as the source of reducing equivalents. MsrA was described well before MsrB so that the majority of the studies in the literature were performed with MsrA. Those studies provide strong evidence supporting the physiological importance of cyclic oxidation and reduction of Met residues. Knocking out MsrA caused increased susceptibility to oxidative stress in mice (5), yeast (6), and bacteria (7–9). Conversely, overexpressing MsrA conferred increased resistance to *Drosophila* (10), *Saccharomyces* (11), *Arabidopsis* (12), PC-12 cells (13), and human T cells (11). Bacteria grow well when methionine is replaced by its carbon-containing analogue, norleucine, but the cells are much more susceptible to killing by oxidative stresses (3). Several investigations demonstrated that modulation of MsrA activity altered the level of reactive oxygen species; overexpression lowered the level in PC-12 cells (13), whereas lens cells lacking MsrA had an increased level (14). Increased expression of either MsrA in WI-38 fibroblasts or MsrB in MOLT-4 leukemia cells reduced the steady state level of oxidized proteins after a hydrogen peroxide challenge (15, 16).

Accumulation of oxidatively damaged proteins has been hypothesized to be an important mechanism of the aging process (17), and it is notable that overexpression of MsrA in *Drosophila* doubled the life span of the flies (10). We wished to determine whether the life extension observed in flies would also occur in mammals overexpressing MsrA and, if it did, whether the subcellular localization of MsrA were important. MsrA in mammals is encoded by a single gene but is found both in the cytosol and mitochondria (18, 19). As shown in Fig. 1, the first 20 residues encode a classical mitochondrial targeting sequence, which has been shown experimentally to direct MsrA to the mitochondria (18). Just following the targeting sequence, there is a Met at residue 21. If protein synthesis could be initiated there, it would produce a product lacking the targeting sequence and should therefore be cytosolic. The sequences of the cytosolic and mitochondrial forms would thus be the same after post-translational processing, except perhaps for an amino-terminal methionine residue that might be present in the mitochondrial form. However, on SDS-gel electrophoresis, the cytosolic form has a faster mobility than the mitochondrial form (*i.e.* the cytosolic form appears to be of lower molecular weight than the mitochondrial form) (19, 20). Based on

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² The abbreviations used are: MetO, methionine sulfoxide; Lys-C, lysyl endopeptidase; Msr, methionine sulfoxide reductase(s); MsrA, methionine sulfoxide reductase A; HPLC, high pressure liquid chromatography.

Methionine Sulfoxide Reductase A

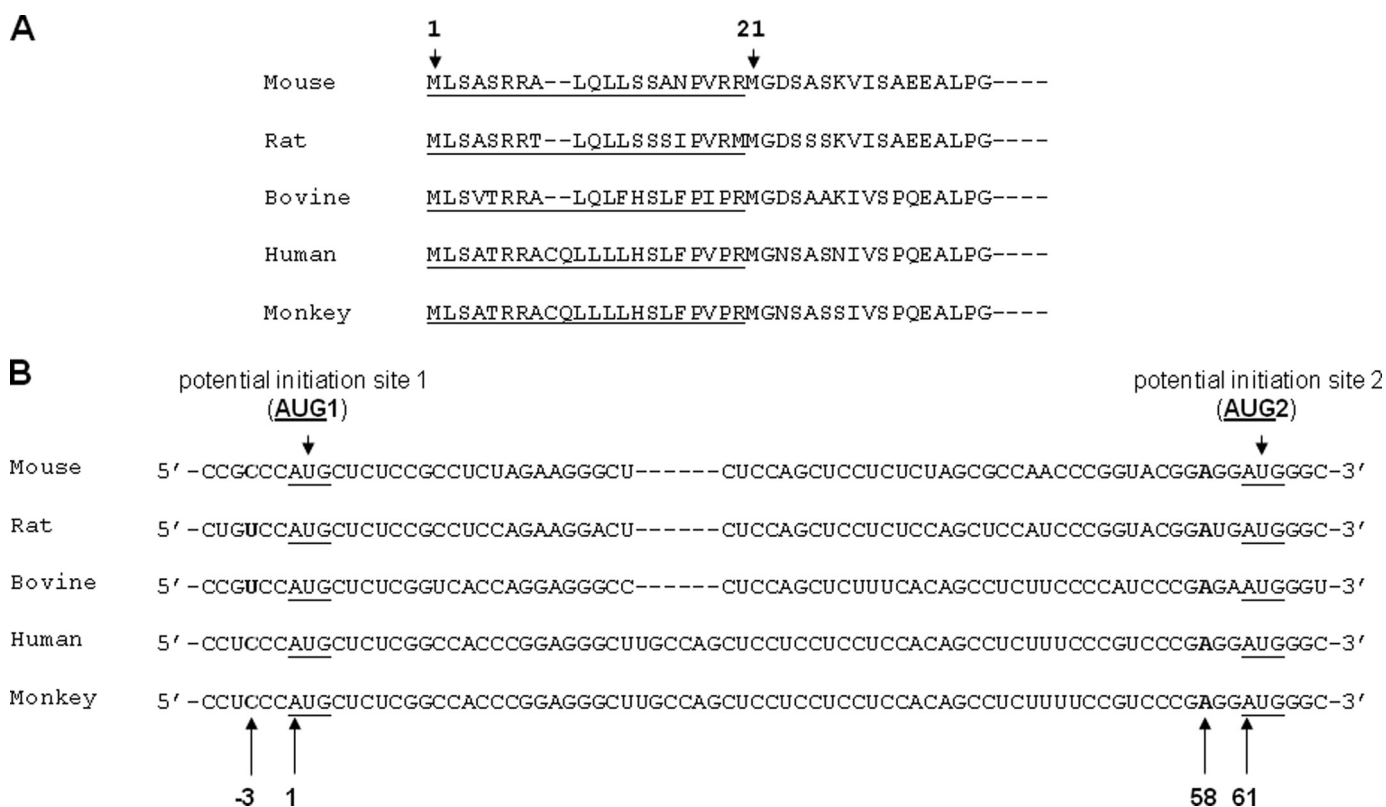


FIGURE 1. **Sequence of mammalian MsrA proteins and genes.** *A*, the amino-terminal sequence of MsrA proteins. Note the methionine residues at position 1 and 21. The mitochondria leader sequence is *underlined*. *B*, sequence of mammalian MsrA mRNAs. The two potential translation initiation sites (AUG1 and AUG2) are *underlined* and marked by *short arrows*. The *long arrows* point out nucleotides at the -3- and 1-positions of these potential initiation sites and are discussed under "Results." The accession numbers for protein and mRNA sequences are as follows: mouse, NP080598 and NM_026322; rat, NP445759 and NM_053307; cow, AAI02981 and NM_174114; human, AAH54033 and NM_012331; monkey, BAE72974.1 and AB220441.

reported expressed sequence tags, previous investigators concluded that the cytosolic form was generated by alternative splicing within the first exon (20), so that the cytosolic form would contain 191 residues compared with 212 for the mitochondrial form after removal of the targeting sequence. Before attempting to construct transgenic mice expressing MsrA targeted to one or the other compartment, we investigated further the nature of the MsrA in both compartments. We show that the cytosolic and mitochondrial forms of MsrA are almost the same length, being generated from two initiation sites. The faster mobility of the cytosolic form is a result of its myristoylation.

EXPERIMENTAL PROCEDURES

Expression of Mouse MsrA in Escherichia coli and HEK293 Cells—Mouse MsrA (GenBankTM accession number NP080598) was amplified by PCR from a mouse kidney cDNA library (Clontech catalog no. ML5010t). Full-length (MsrA(1–233)) and cytosolic (MsrA(21–233)) sequences were generated with NdeI and BamHI cleavage sites at the 5'- and 3'-ends, respectively. The PCR products were digested with restriction enzymes, cloned into the pET17b plasmid (Novagen), and expressed in *E. coli* strain BL21 (DE3). Myristoylated cytosolic MsrA was prepared by co-expressing *N*-myristoyltransferase (21), kindly provided by Dr. Julie Donaldson (NHLBI, National Institutes of Health). The recombinant proteins were purified by sequential chromatography on anion exchange (TosoHass

DEAE-5PW) and hydrophobic interaction (TosoHass Phenyl-5PW) columns as described previously (22).

For expression in HEK293 cells (strain AAV293; Stratagene, catalog no. 240073), the PCR product was cloned into two eukaryotic expression vectors, pDRIVE (InvivoGen, catalog no. pdrive-cag) and pCR3.1 (Invitrogen, catalog no. K3000-01). The fidelity of the expressed proteins was confirmed by measuring their mass with HPLC-mass spectrometry using cell lysate prepared from transiently transfected cells without further purification (23).

Fluorescence Confocal Microscopy—COS cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) in an incubator containing 95% air, 5% CO₂. Cells were transiently transfected with Fugene 6 (Roche Applied Science) and incubated for 16–30 h at 37 °C. Staining of cells was performed as described (24). Cells were fixed in 3.7% formaldehyde for 20–30 min at room temperature in 0.1 M sodium phosphate, pH 7.2, 150 mM NaCl. Stock 37% formaldehyde solutions (Sigma, catalog no. 252549) were deionized using mixed bed resin (Sigma, catalog no. M8032) until no color change occurred. Immunofluorescence staining was performed as described (24). Antibodies to MsrA (1:1000) and cytochrome *c* (1:500) were used for cell staining.

Subcellular Fractionation—For subcellular fractionation of transiently transfected HEK293 cells, cells were washed with cold phosphate-buffered saline three times and ruptured in

homogenization buffer (5 mM Hepes, pH 7.5, 210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 1 mM diethylenetriamine pentaacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) protease inhibitor mixture (Sigma, catalog no. P2714; stock prepared according to the supplier's directions)) by passage 15 times through a 26-gauge syringe needle. The broken cells were centrifuged at $800 \times g$ for 8 min to separate the pellet (low spin pellet) and the supernatant. The supernatant was removed carefully without disrupting the low spin pellet and centrifuged at $9,000 \times g$ for 15 min. The resulting supernatant was saved as the cytosolic fraction. The high spin pellet was washed twice with homogenization buffer and centrifugation at $9,000 \times g$ for 4 min, resuspended in homogenization buffer, and saved as the mitochondrial fraction. The low spin pellet was washed with phosphate-buffered saline three times and then sonicated to give the "insoluble fraction."

A Dounce homogenizer (Wheaton) was used for homogenization of mouse tissue; other styles of pestles tended to disrupt mitochondria, thus contaminating the cytosol fraction with mitochondrial components. All procedures were performed on ice. Mouse tissues (strain C57BL/6) were excised, rinsed with homogenization buffer, minced, and then homogenized with 10 gentle strokes of the Dounce homogenizer and then fractionated as for HEK293 cells. Fractions were analyzed for purity by Western blot to detect α -tubulin as a cytosolic protein marker and either porin or mitochondrial HSP70 as mitochondrial protein markers. Anti- α -tubulin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-porin and anti-mitochondrial HSP70 were from Affinity BioReagents.

In Vitro Synthesis of MsrA—The *in vitro* transcription and translation kit was from Ambion (catalog no. AM1281), and it was employed according to the manufacturer's directions. Constructs for *in vitro* translation were prepared in pCR3.1, which contains a T7 RNA polymerase promoter sequence upstream of the coding region. The constructs were incubated in the *in vitro* transcription reaction mixture at 30 °C for 1 h. The transcript was immediately translated with the rabbit reticulocyte lysate at 30 °C for 1 h, and the products were analyzed by Western blotting for MsrA.

Western Blotting—HEK293 cells were transiently transfected with expression vectors, harvested 26–36 h later, and then subjected to subcellular fractionation. Protein concentrations were measured with the Coomassie Blue method (25) (Bio-Rad). The cytosolic (3.34 μ g of protein) and mitochondrial (1.0 μ g of protein) fractions were reduced in SDS-PAGE buffer containing 0.5% β -mercaptoethanol with heating at 95 °C for 5 min. Gel electrophoresis was performed on 10–20% Tris/glycine gels (15-well, 1.5 mm; Invitrogen, catalog no. EC61385BOX). A constant output power of 230 V was applied at room temperature for 90 min. The proteins were transferred at 1.5 A for 1 h onto nitrocellulose membrane (Invitrogen). The membrane was incubated with the primary antibody and then with the secondary antibody (Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen, catalog no. A21109) or IRDye800CW (Rockland)), each for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20 in phosphate-buffered saline and then quantitatively visualized by scanning with an Odyssey infrared scanner (Li-Cor Biosciences).

Immunoprecipitation and In-gel Digestions—For immunoprecipitation of mouse tissue MsrA, kidney was disrupted in homogenization buffer, with all procedures performed on ice. Mouse tissue was excised, rinsed with buffer, minced, and homogenized with a Dounce homogenizer using 10 gentle strokes. Homogenates were clarified by centrifugation at $100,000 \times g$ for 1 h at 4 °C. The supernatant was used for immunoprecipitation with anti-MsrA antibody. The immune complexes were collected with protein G-agarose beads (Sigma) and washed three times with buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Sigma proteinase inhibitor mixture), and the proteins were eluted from the beads by boiling for 5 min in SDS-PAGE sample buffer without reducing agent. The proteins were separated by non-reducing SDS-PAGE and visualized by Coomassie Blue staining.

Gels were destained with 50% methanol and 7% acetic acid, and the protein bands were excised. Each band was cut into small pieces, washed twice for 30 min with 40% acetonitrile, 25 mM Tris, pH 8.5, to extract SDS, and dried in a vacuum concentrator (Thermo Savant). Disulfide bonds were reduced by incubation with 50 μ l of 25 mM dithiothreitol, 25 mM Tris, pH 8.5, for 30 min at 56 °C. The solution was aspirated and discarded after this and each subsequent step until the addition of Lys-C. Cysteines were alkylated by incubation at room temperature in the dark with 50 μ l of 55 mM iodoacetamide in 25 mM Tris, pH 8.5. The gel pieces were then washed with 200 μ l of water for 15 min in a thermomixer at 300 rpm, equilibrated with 200 μ l of 25 mM Tris, 1 mM diethylenetriamine pentaacetic acid, pH 8.5, for 10 min, dehydrated by three 10-min incubations with 200 μ l of 50% acetonitrile, 50% 25 mM Tris, 1 mM diethylenetriamine pentaacetic acid, pH 8.5, and then dried in the vacuum concentrator. A solution of 25 μ l of 25 mM Tris, pH 8.5, 1 mM diethylenetriamine pentaacetic acid containing 0.5 μ g of Lys-C (lysyl endopeptidase; Wako, catalog no. 125-02543) was added, and samples were incubated for 18 h at 37 °C with gentle rocking. The tubes were centrifuged, and the supernatant was transferred to glass autosampler vials (Sun-SRI). Gel pieces were further extracted twice for 1 h with 200 μ l of 60% acetonitrile, 0.1% trifluoroacetic acid at room temperature. These extracts were added to the glass vial, and the contents were dried in the vacuum centrifuge. Samples were redissolved in 12 μ l of 5% acetonitrile in 0.05% trifluoroacetic acid and analyzed by HPLC-mass spectrometry.

Liquid Chromatography-Mass Spectrometry—Reverse phase analyses and molecular weight determinations of proteins were carried out on an Agilent 1100 series high pressure liquid chromatograph with electrospray ionization and detected by a model G1946 mass spectrometer equipped with a time-of-flight detector (Agilent), using columns and conditions as described (23, 26). Peptide separation and sequencing by tandem mass spectrometry were conducted with the same methods except that the mass spectrometer was an Agilent 6300 ion trap. *De novo* sequence analysis was performed with PEAKS software (Bioinformatics Solutions).

Assay for Myristoylation by In-gel Limited Acid Hydrolysis—Goddard and Felsted (27) demonstrated that myristoylated proteins could be identified by taking advantage of the fact that

Methionine Sulfoxide Reductase A

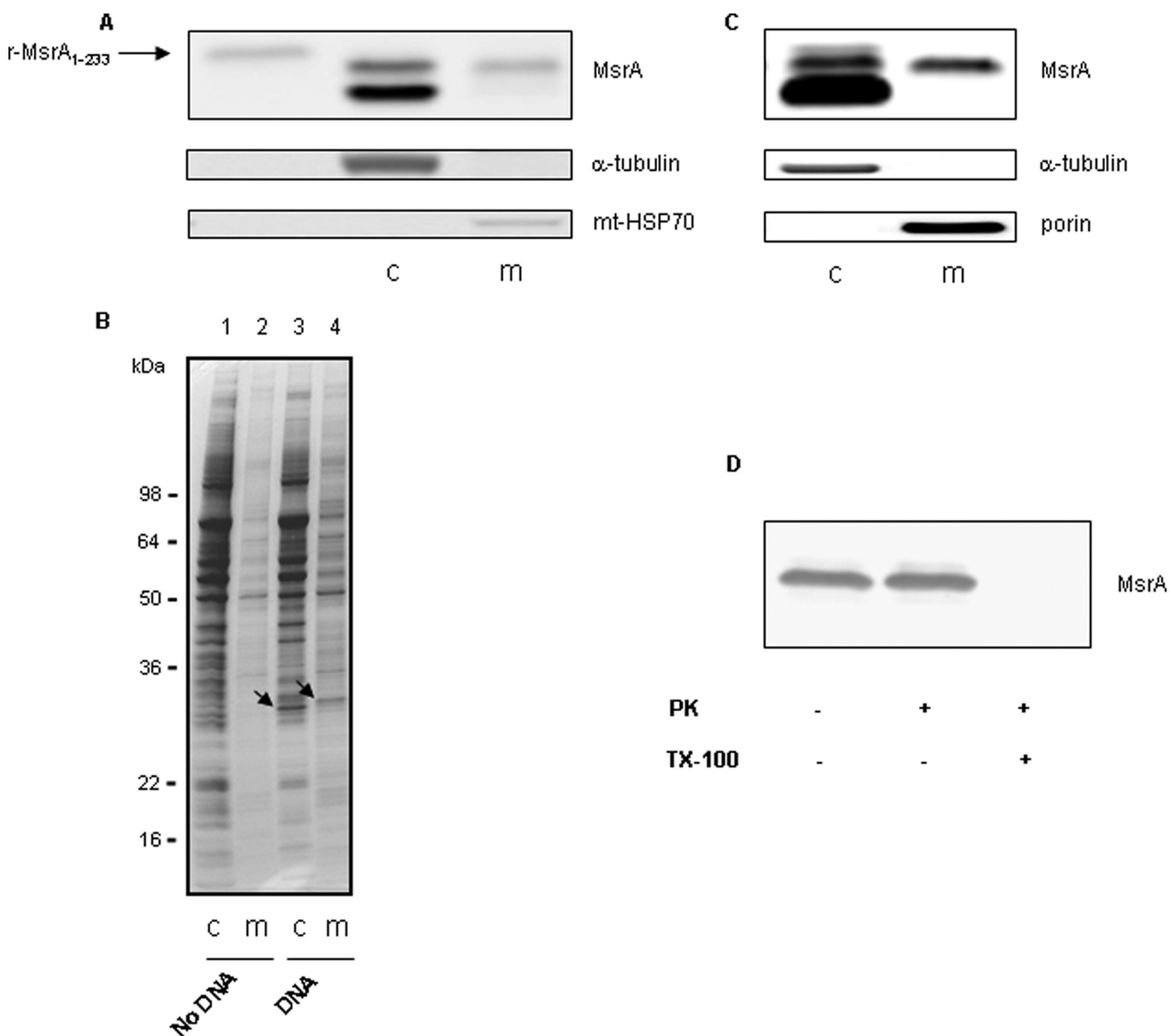


FIGURE 2. Mobility and distribution of mouse MsrA. *A*, immunoblotting detection of MsrA in the subcellular fractions of mouse liver MsrA after SDS-gel electrophoresis. The cytosol (*c*) contains two forms, whereas the mitochondria (*m*) contain 1. Both forms have faster mobility than full-length recombinant MsrA, which includes the mitochondrial targeting sequence. *B*, Coomassie Blue-stained SDS gel from subcellular fractions of HEK293 cells at 26 h following transfection with full-length mouse MsrA. The arrows point to MsrA. *C*, Western blot from the gel shown in *B*. *D*, MsrA in the mitochondrial fraction is resistant to proteinase K (PK) unless mitochondria are disrupted by Triton X-100 (TX-100), establishing that MsrA is within the mitochondria.

mild acid hydrolysis releases *N*-myristoylated glycine from the amino terminus. After SDS-PAGE and Coomassie Blue staining, the putatively myristoylated MsrA band was cut from the gel and placed in a glass vial. The gel piece was covered with 100 μ l of 50 mM HCl, and the vial was tightly closed with a Teflon-lined screw cap (Sun SRI) and heated at 100 $^{\circ}$ C for 4 h. The vial, with the gel left in place, was dried in the vacuum centrifuge. To remove residual acid, 100 μ l of water was added and dried in the vacuum centrifuge. This was repeated one more time. The gel and tube were extracted twice with 100 μ l of methanol, which we found readily dissolved authentic *N*-myristoylated glycine (Bachem, catalog no. F-2550). The two extracts were combined in a low volume autosampler tube (Waters, catalog no. 186000384c) and dried. The residue was blue because Coomas-

TABLE 1
Masses of MsrA

Fraction	Observed mass	Calculated mass	Assignment
	<i>Da</i>	<i>Da</i>	
Mitochondrial	23,779.2	23,779.8	Met ²¹ -Lys ²³³
Cytosolic	23,858.7	23,859.0	<i>N</i> -Myristoylated Gly ²² -Lys ²³³

ie Blue was also extracted. The dried sample was redissolved in 7.5 μ l of acetonitrile containing 0.05% trifluoroacetic acid. 7.5 μ l of water was added to give 50% acetonitrile; *N*-myristoylated glycine binds to the C₁₈ column in 50% acetonitrile. Up to 8 μ l was injected onto the same HPLC-mass spectrometry system described above for tandem mass spectrometry, except that the

gradient was from 50 to 100% acetonitrile with 0.05% trifluoroacetic acid, ramped at 10%/min. Authentic *N*-myristoylated glycine eluted at 7 min in this system. Its parent ion had an *m/z* of 286.2, and the tandem mass spectrometric spectrum had a peak at 211 from the singly protonated myristic acid.

Enzyme Assay—MsrA activity was measured using *L*-MetO (Sigma) as substrate. The reaction mixture for activity assay was 10 mM *L*-MetO, 10 mM dithiothreitol, 50 mM sodium phosphate buffer, pH 7.4. The reaction was carried out at 37 °C for 30 min

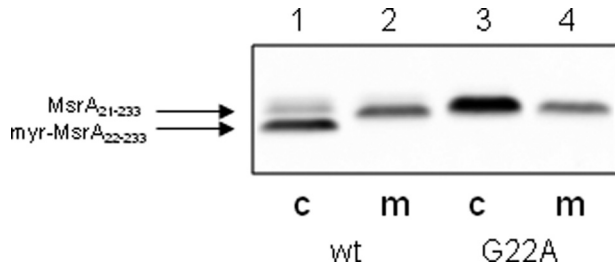


FIGURE 3. Myristoylation of cytosolic MsrA requires Gly at residue 22. HEK293 cells were transfected with full-length MsrA (residues 1–233) containing either the wild-type (*wt*) Gly or Ala (*G22A*) at position 22. The cells were subjected to subcellular fractionation, SDS-gel electrophoresis, and then Western blotting to detect MsrA. The wild-type cytosolic MsrA is primarily the myristoylated form with faster mobility (*myr-MsrA*(22–233)). Substitution of Gly by the non-myristoylatable Ala eliminated the faster mobility form from the cytosolic fraction. Mutation of Gly²² to Ser also eliminated myristoylation (not shown). *c*, cytosol; *m*, mitochondria.

and stopped by bringing the solution to 0.2% trifluoroacetic acid with one-fiftieth volume of 10% trifluoroacetic acid. The reaction product, methionine, was analyzed on an Agilent 1100 high pressure liquid chromatograph as described (3).

RESULTS

Molecular Weight of the MsrA Forms—We confirmed the presence of two forms of MsrA from mouse liver tissue (18, 19) by Western blotting after SDS electrophoretic separation (Fig. 2A). Subcellular fractionation also confirmed that the faster migrating form was cytosolic, whereas the slower form was mitochondrial. Both forms have faster mobility than a full-length recombinant with residues 1–233 (*rMsrA*(1–233)). The cytosolic fraction was the supernatant from a 9,000 × *g* centrifugation, leaving open the possibility that MsrA was membrane-bound, perhaps to microsomes. However, after 1 h of centrifugation at 100,000 × *g*, 90% of the MsrA remained soluble and was thus cytosolic. In addition to the faster migrating band, Fig. 2A also shows a lighter cytosolic band (32%) with the same mobility as the mitochondrial form (68%). The subcellular markers demonstrate that the cytosolic fraction was not contaminated by mitochondria, indicating that the cytosol also contains non-myristoylated MsrA; it may be acetylated (see “The Faster Migrating MsrA Is Myristoylated” and “Discussion”).

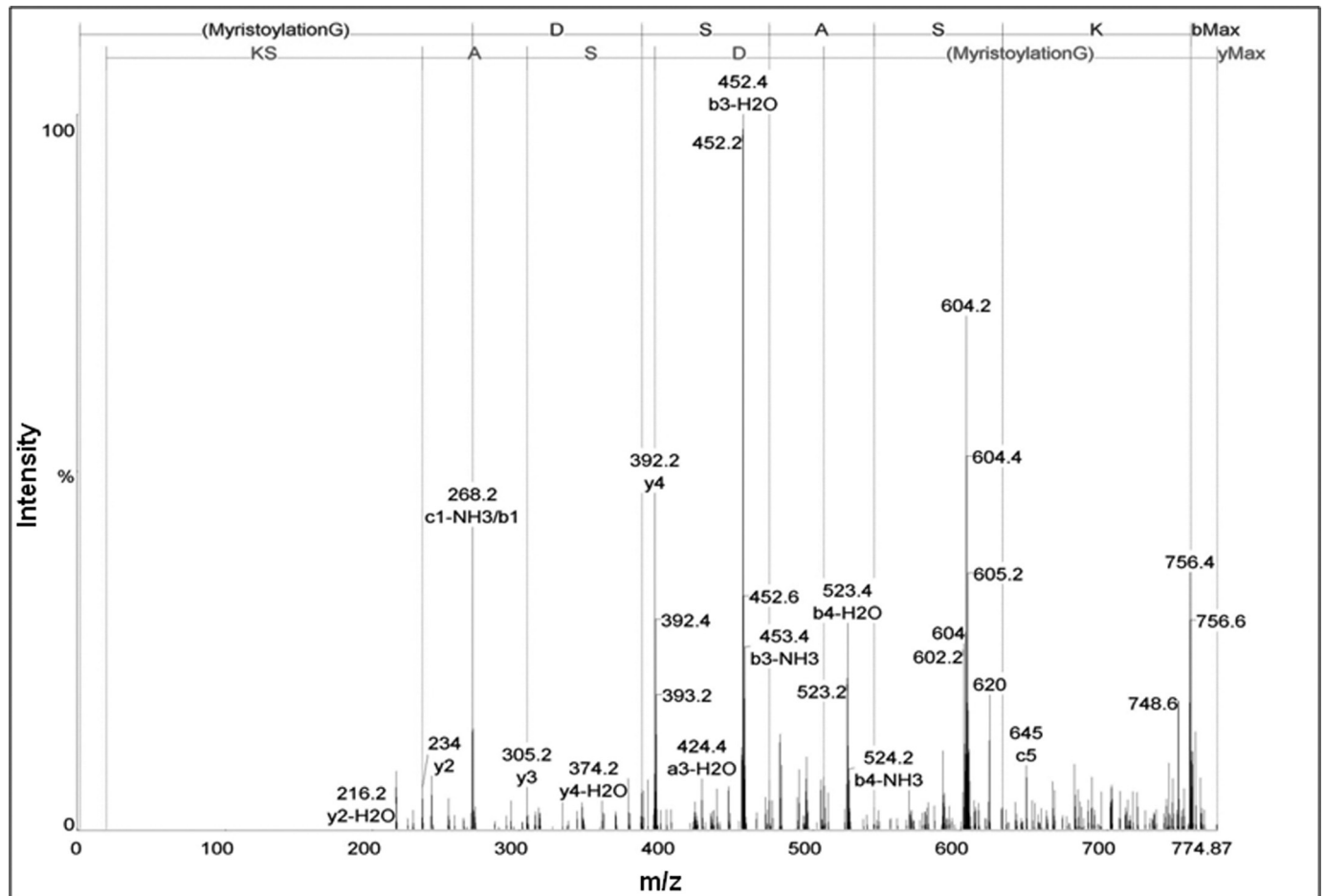


FIGURE 4. The amino-terminal Gly of mouse kidney MsrA is myristoylated. Shown is the tandem mass spectrometer chromatogram of the amino-terminal 6-residue Lys-C-generated peptide. The sequence shown was determined *de novo* with the PEAKS software program. The amino-terminal Gly is myristoylated.

Methionine Sulfoxide Reductase A

We employed mass spectrometry to obtain accurate mass determinations of the two forms in order to determine the putative cleavage sites giving rise to the processed forms. The AAV293 strain of HEK293 cells was employed because we found that it had no detectable endogenous MsrA. Cells were transiently transfected with a construct containing the full-length MsrA(1–233). The recombinant proteins were readily visualized on SDS gels with Coomassie Blue staining (Fig. 2B) and were reactive with anti-MsrA antibody (Fig. 2C). Proteinase K exposure established that the mitochondrially localized MsrA was within the mitochondria (Fig. 2D). On reverse phase HPLC-mass spectrometry, the mitochondrial form was eluted by ~37% acetonitrile, whereas the cytosolic form required ~41%, implying that the faster migrating cytosolic form was more hydrophobic than the mitochondrial form. MsrA was previously reported to possess a mitochondrial targeting sequence (18, 19). The mitochondrial form would be expected to be produced by cleavage of the mitochondrial leader sequence between Arg²⁰ and Met²¹, with a calculated mass of 23,779.2 Da. The measured mass was 23,779.8 Da, confirming the expected cleavage site (Table 1). However, the measured mass of the faster migrating cytosolic form was 23,858.7 Da, greater than the mitochondrial form. If this form had been produced by the previously reported expressed sequence tag (20), it would have only 191 residues and a mass distinctly less than that of the mitochondrial form. If the cytosolic form were produced by initiation at Met²¹, its unmodified mass would be 23,648.6 Da. The observed mass was 210 Da higher, which is exactly the increase in mass caused by *N*-myristoylation.

The Faster Migrating MsrA Is Myristoylated—The sequence beginning at Met²¹ is Met-Gly-Asp-Ser-Ala-Ser-Lys, which matches the classic myristoylation signature sequence Met-Gly-X-X-X-Ser-Lys, although an aspartate at position 3 could favor acetylation over myristoylation (28, 29). To obtain sufficient material for additional biochemical analysis, we expressed in HEK293 cells a construct lacking the first 20 amino acids comprising the mitochondrial targeting sequence. As for MsrA from mouse tissues, two cytosolic forms were separable by either SDS-gel electrophoresis or reverse phase chromatography. The slower migrating form had a mass identifying it as the *N*-acetylated rMsrA(22–233) (calculated, 23,690.7 Da; observed, 23,690.8 Da). The mass of the faster migrating form was that expected for the myristoylated form rMyr-MsrA(22–233) (calculated, 23,859.0 Da; observed, 23,858.4 Da).

We confirmed that the mass increase was actually due to myristoylation by two different analytical methods, an adaptation of the Goddard and Felsted (27) assay for *N*-myristoylated glycine and by tandem mass spectrometric sequencing of an in-gel Lys-C digest. When we expressed the full-length MsrA(1–233) sequence, both the mitochondrial and myristoylated cytosolic forms were observed, but when Gly²² was mutated to non-myristoylatable Ala, the cytosolic form was no longer myristoylated (Fig. 3).

Mouse MsrA Is *N*-Myristoylated *in Vivo*—We next determined whether the faster migrating form was actually myristoylated *in vivo*. MsrA was immunoprecipitated from a homogenate of mouse kidney and then subjected to SDS-gel

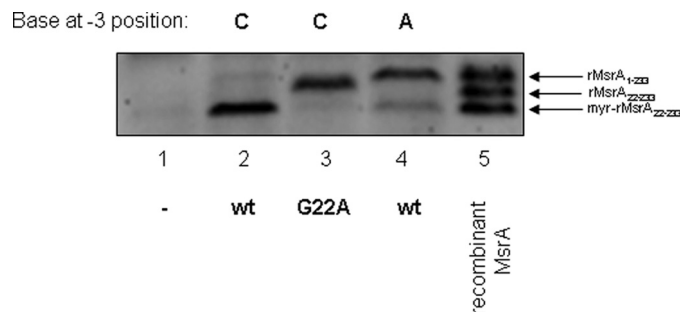


FIGURE 5. Cell-free synthesis of MsrA detected by Western blotting. Wild type (wt) or mRNA with Ala substituted for Gly at position 22 was added to a rabbit reticulocyte lysate. Lane 1 had no mRNA added, and lane 5 contained recombinant proteins purified from *E. coli*. The constructs used in lanes 2 and 3 contained C at position –3, as occurs normally in the mammalian gene (Fig. 1). As further discussed under “Results,” the construct used for lane 4 had the C changed to A, rendering it a better initiation site.

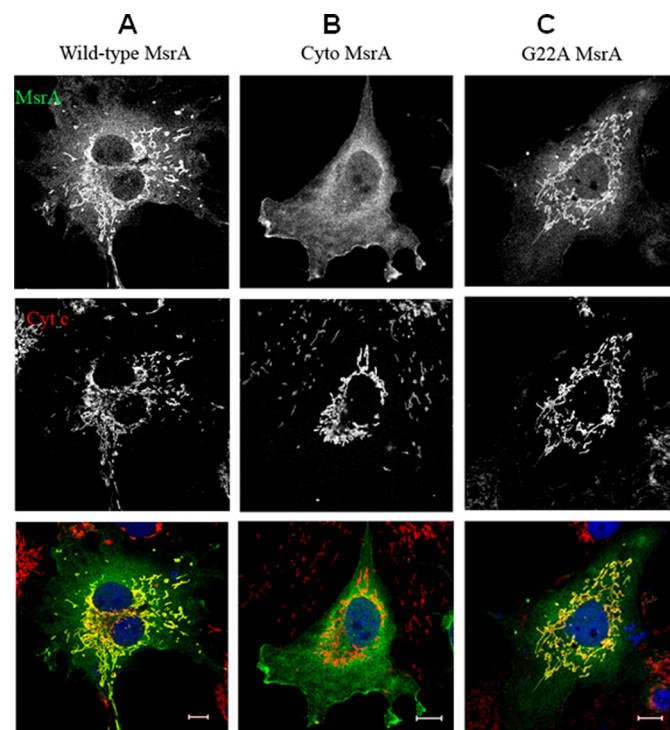


FIGURE 6. Myristoylation does not affect subcellular localization of MsrA. COS cells were transiently transfected and then examined by immunofluorescence for the localization of MsrA (top panels) and of the mitochondrial marker cytochrome *c* (middle panels). Merged images are shown in the bottom panels. Wild-type MsrA was visualized in the mitochondria and the cytosol in column A; the latter form is myristoylated. The same distribution was observed in column C with the full-length MsrA in which Gly²² was mutated to Ala to prevent myristoylation. When the mitochondrial targeting sequence was omitted (residues 1–20), MsrA was exclusively cytosolic, as shown in column B. Bar, 10 μ m.

electrophoresis. The faster migrating band was excised and digested with Lys-C, and the peptides were analyzed by HPLC-mass spectrometry. The amino-terminal peptide was very hydrophobic, eluting at 36% acetonitrile. Its mass spectrum is shown in Fig. 4, establishing that its sequence is *N*-myristoylated Gly-Asp-Ser-Ala-Ser-Lys. *In vivo* myristoylation was also demonstrated for mouse liver MsrA (not shown). Thus, the faster migrating band of MsrA from the cytosol is myristoylated on Gly²², leading to the conclusion that translation of this form is initiated at the codon for Met²¹.

A less likely mechanism for myristoylation would be processing from the full-length MsrA(1–233). This would require partial import of the protein into mitochondria, cleavage of the mitochondrial targeting peptide, and then release of the trimmed protein into the cytosol, where it would be myristoylated. Such mitochondrial trimming has been proposed to occur with yeast fumarase (31). We ruled out this mechanism through *in vitro* synthesis of MsrA with a rabbit reticulocyte system devoid of mitochondria. We note that rabbit reticulocyte lysates contain *N*-myristoyltransferase and are capable of myristoylating *in vitro* synthesized proteins (21). When mRNA encoding the full-length protein was added to the reticulocyte lysate, synthesis was observed of both the full-length protein with residues 1–233 and the shorter, myristoylated protein with residues 22–233 (Fig. 5, lane 2). We conclude that mito-

chondrial processing of full-length protein is not required for generation of the myristoylated form.

Characterization of Myristoylated and Non-myristoylated MsrA—Although many myristoylated proteins are constitutively membrane-bound, structural roles have also been established for myristoylation (32). We compared the distribution of MsrA in COS cells transfected with either wild-type or the non-myristoylatable G22A MsrA. Both were localized to the cytosol and mitochondria, and no enrichment in the plasma membrane was observed by fluorescent microscopy (Fig. 6). Subcellular biochemical fractionation allowed estimation of the distribution between the cytosolic and mitochondrial fractions, which was ~75% cytosolic and ~25% mitochondrial for both constructs. Thus, myristoylation does not affect subcellular localization under our cell culture conditions.

Myristoylation is required for membrane binding of certain proteins, but it is not sufficient to direct them to the membrane (32). A second structural element is required, and if that element can be modulated, then membrane binding of the protein becomes reversible. Two second elements that can join with myristoylation to direct membrane binding are palmitoylation and an exposed cluster of basic residues. Palmitoylation is reversible so that proteins utilizing myristoylation plus palmi-

TABLE 2
Kinetic parameters of myristoylated and non-myristoylated recombinant MsrA

MsrA	K_m^a for (S)-MetO	V_{max}	K_{cat}
	mM	$\mu\text{mol}/\text{min}/\text{mg}$	s^{-1}
Non-myristoylated	2.8	6.5	2.6
Myristoylated	2.6	6.0	2.4

^a The K_m was calculated for (S)-MetO because MsrA is specific for this epimer (30).

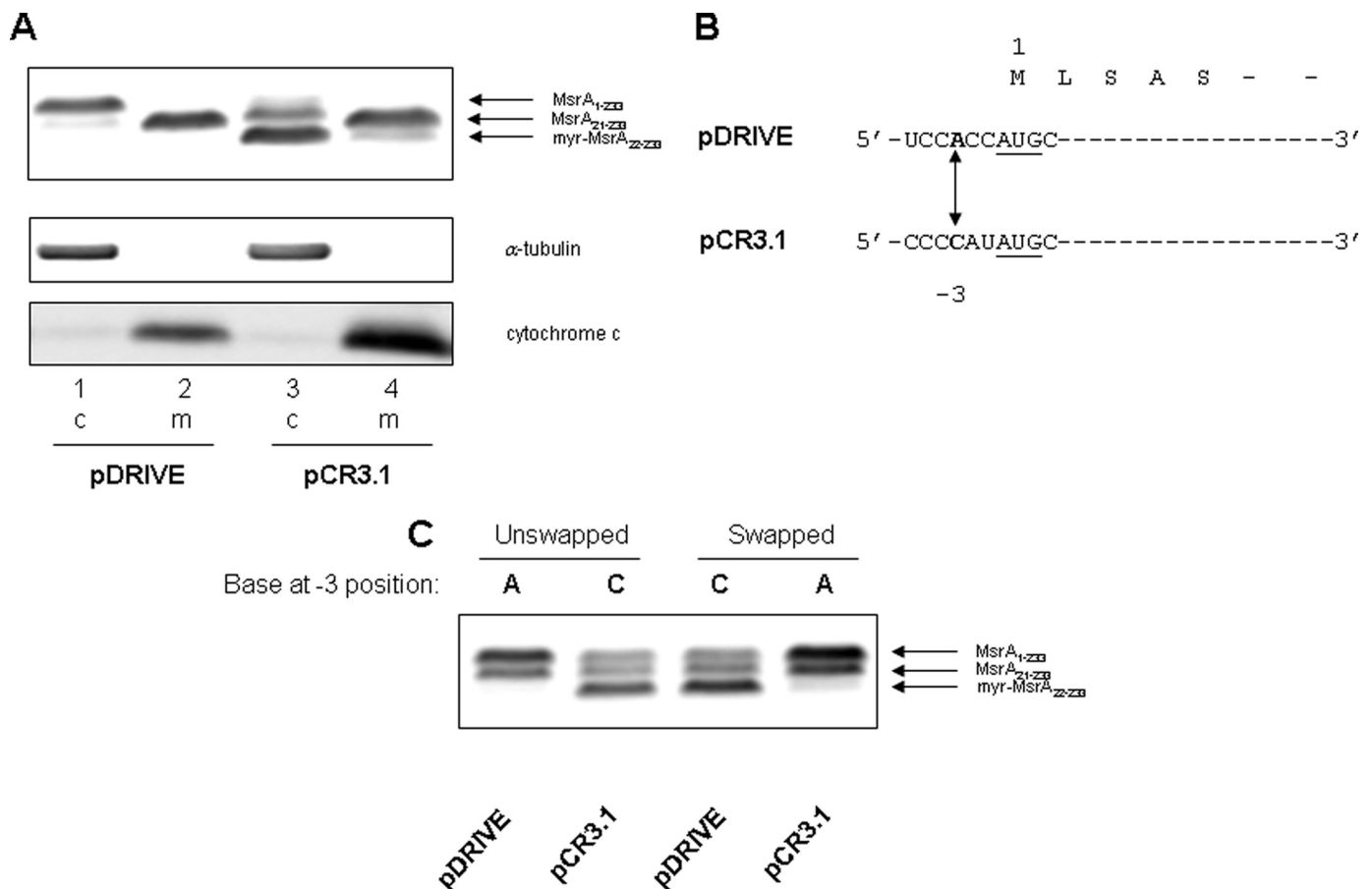


FIGURE 7. Effect of vector and of the base at position -3 on choice of initiation site and consequent myristoylation. A, wild-type, full-length MsrA was cloned into either a pDRIVE or pCR3.1 vector and expressed in HEK293 cells. Cells were subjected to subcellular fractionation, and MsrA was probed by Western blotting. The mobility of recombinant marker proteins is indicated by arrows on the right. The cytosolic MsrA produced by pDRIVE was almost all full-length MsrA(1–233), whereas pCR3.1 produced mainly myristoylated myr-MsrA(22–233). B, sequence of the vectors upstream of the first initiation site. Note that the base at position -3 is A in pDRIVE and C in pCR3.1. C, the effect of swapping A and C nucleotides at position -3 in the vectors is to also swap the pattern of MsrA production.

Methionine Sulfoxide Reductase A

toylation can reversibly associate with the membrane. Reversibility for proteins utilizing the myristoylation plus basic cluster requires that one or the other domain be sequestered, and examples of both are known. We reasoned that MsrA might be reversibly translocated to a membrane, perhaps in cells under oxidative stress, because MsrA is a component of an antioxidant defense system. Taking care to avoid artifactual pitfalls (24), we looked for translocation by fluorescent microscopy and in some cases also by subcellular biochemical fractionation in transfected cells exposed to oxidative stresses. We separately tested hydrogen peroxide, glucose plus glucose oxidase, paraquat, rotenone, antimycin A, and hypoxia (3% oxygen), followed by reoxygenation in room air. No change in subcellular distribution of MsrA was observed with any of these challenges (not shown).

Published studies report that non-myristoylated MsrA has a rather high K_m for MetO and a relatively low specific activity. We considered the possibility that myristoylation might enhance the catalytic efficiency of MsrA. We were able to produce recombinant myristoylated MsrA in *E. coli* by co-expressing mammalian *N*-myristoyltransferase and adding myristate to the culture medium (33). Myristoylation did not affect the kinetic parameters of the enzyme (Table 2).

Choice of Initiation Site for MsrA—The experimental results shown thus far demonstrate that the mitochondrial form of MsrA is produced by initiation at Met¹, and the myristoylated cytosolic form is produced by initiation at Met²¹. During the course of other studies on MsrA, we noted that the cytosolic form produced depended on whether we used the pDRIVE or pCR3.1 expression vector containing the same coding sequence for MsrA(1–233) (Fig. 7A). The mitochondrial production was the same for both vectors, but only pCR3.1 produced the myristoylated form. The cytosolic form produced by pDRIVE was the full-length MsrA(1–233), probably trapped in the cytosol because it folded before mitochondrial import could occur. Thus, pDRIVE preferentially initiates synthesis at the first AUG encoding Met¹, whereas pCR3.1 preferentially initiates at the second AUG encoding Met²¹. The nucleotides preceding the initiating AUG are known to affect the efficiency of initiation, especially that at the –3-position (34). Comparison of the sequences of the two vectors revealed that the –3-position is an A in pDRIVE and a C in pCR3.1 (Fig. 7B). C is also present at the –3-position in the mammalian genes (Fig. 1), making the first AUG a poorer initiation site and thus favoring initiation at the second AUG, located immediately after the mitochondrial targeting sequence (Met²¹). The A in pDRIVE makes the first AUG a good initiation site, thereby favoring initiation at Met¹. We experimentally confirmed this by transfecting cells with the two vectors containing either A or C at the –3-position. Swapping nucleotides reversed the expression pattern (Fig. 7C). Pattern swapping was also observed with the *in vitro* protein synthesis (lanes 2 and 4 of Fig. 5A).

We further examined the cellular preference for the two initiation sites by mutating either Met¹ or Met²¹ to Leu to abolish initiation. We quantitated the cellular MsrA content for each construct, and the results are shown in Fig. 8. When Met¹ was changed to Leu, there was little difference in the cellular MsrA compared with the wild type. Changing Met²¹ to Leu decreased

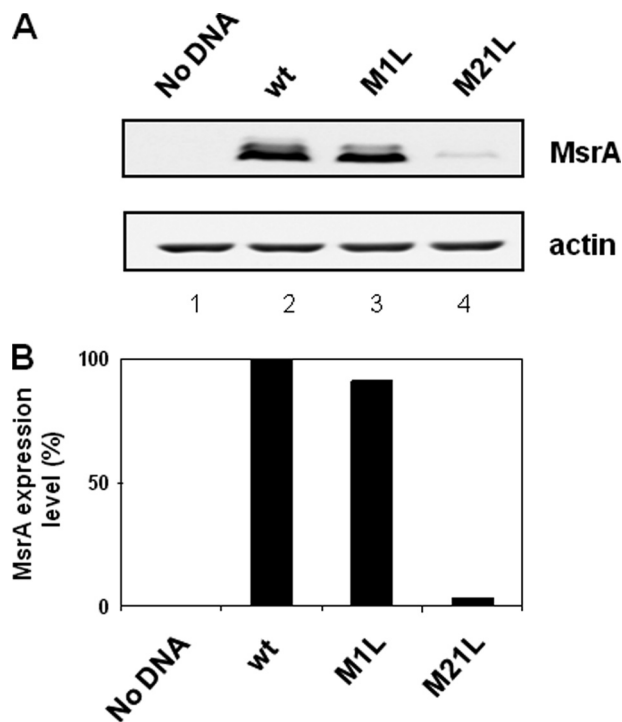


FIGURE 8. Effect of mutating initiating Met to Leu. A, Western blot of the HEK293 cells expressing wild type (wt), M1L, or M21L with actin loading controls. The vector for these constructs was pCR3.1. B, quantitation of the MsrA content from the Odyssey near-infrared fluorescent scanner. Prevention of initiation at the first site has little effect on the cellular content of MsrA, whereas mutation of the second initiation site almost eliminates expression.

cellular MsrA to less than 5% that of the wild type. Deletion of the Met²¹ codon also decreased cellular MsrA to less than 5% of the wild-type (not shown). We conclude that the second AUG is the preferred initiation site.

DISCUSSION

Two feasible proposals have been advanced to explain how the one *MsrA* gene encodes proteins targeted to two subcellular compartments, the cytosol and the mitochondria: initiation at codon 1 or 21 (18) or differential splicing of mRNA (20). The latter would produce a cytosolic form whose molecular weight was ~2,100 less than the mitochondrial form. On SDS electrophoresis, the cytosolic form does have faster mobility than the mitochondrial form, and the difference is consistent with the molecular weight difference expected. However, the results presented in our current studies establish that the cytosolic form is produced by initiation of synthesis at codon 21. Its faster mobility is due to the unexpected finding that the cytosolic form is myristoylated (Fig. 9). Mammalian MsrA joins the list of dually localized proteins that are differentially targeted by the presence of two functional sites of protein initiation. These include proteins present in both the nucleus and mitochondria (35–37), in the cytosol and mitochondria (38–43), and in other pairs of compartments (44–46). The relative distribution of many of these proteins is regulated by the comparative efficiency of initiation of the two sites, which was also the case for MsrA. Cytosolic MsrA constitutes ~75% and mitochondrial MsrA ~25% of the cellular content. The predominance of the cytosolic form requires the presence of C at the –3-position of

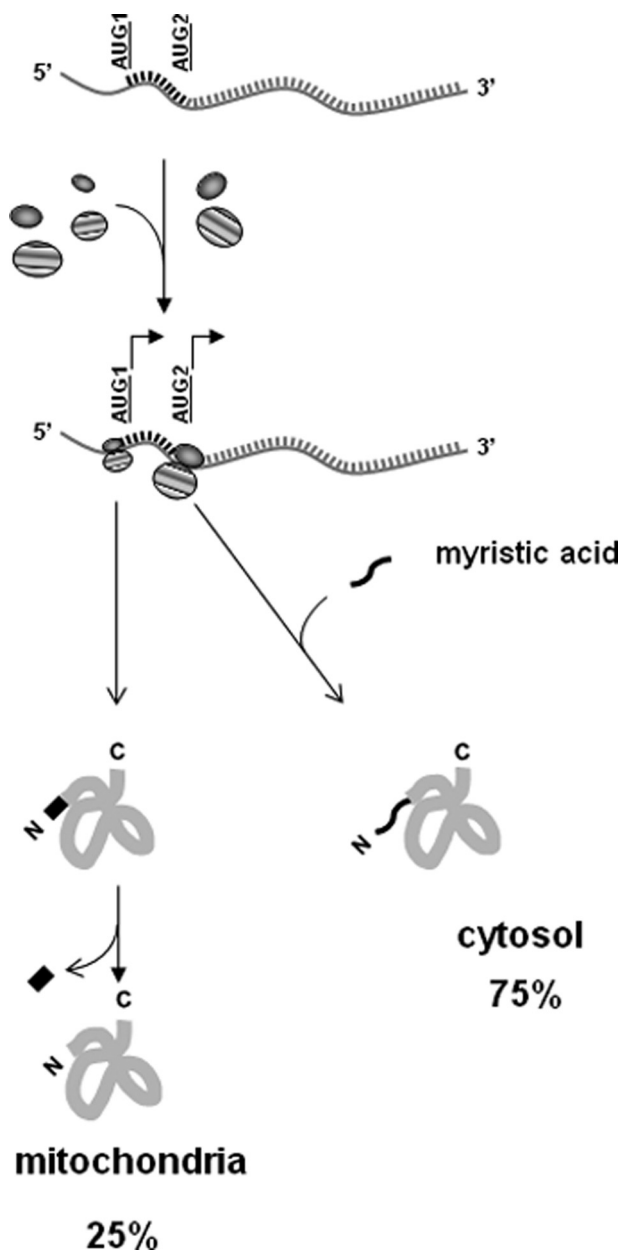


FIGURE 9. Scheme summarizing the mechanism of subcellular localization and myristoylation from one gene. Initiation at the first AUG will include the mitochondrial targeting sequence, shown as a black box. Initiation at the second AUG produces a protein lacking the targeting sequence but with an amino-terminal myristoylation sequence.

the first initiation site, creating a relatively poor Kozak sequence (34). Mutation to the favored A greatly increased synthesis of the longer form containing the mitochondrial targeting sequence. This longer form tended to accumulate in the cytoplasm rather than being imported into mitochondria, presumably because the protein folds before import can be accomplished, thus masking the targeting sequence.

The finding that the faster migration of the cytosolic form is a consequence of myristoylation is a reminder that not all that runs more quickly during SDS gel electrophoresis is smaller. Myristoylation was observed *in vivo*, in transiently transfected HEK293 cells, and in cell-free protein synthesis systems. Myristoylated proteins are often targeted to the plasma membrane.

When we examined the subcellular localization of myristoylated MsrA in transfected HEK293 and CHO cells, both biochemical fractionation and immunohistology revealed a cytosolic distribution without concentration in membranes. This is not unusual because myristoylation alone is not sufficient for membrane association (47); a basic domain is generally required as well. If that domain can undergo conformational changes that expose it or bury it, then membrane binding can be switched on and off (47). Protein-protein interactions can also act as a switch for myristoylated proteins, as they do for protein kinase A (48). Exposure of an amino-terminal myristoylation sequence by proteolytic clipping of BID causes translocation from the cytosol to mitochondria (49). We considered the possibility that oxidative stress might induce translocation to a membrane, but application of various oxidative stressors failed to alter distribution (24). The role of myristoylation remains to be elucidated.

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Methionine Sulfoxide Reductase A

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