Identification and analysis of the promoter region of the human methionine sulfoxide reductase A gene

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MsrA (methionine sulfoxide reductase A) is an antioxidant repair enzyme that reduces oxidized methionine to methionine. Moreover, the oxidation of methionine residues in proteins is considered to be an important consequence of oxidative damage to cells. To understand mechanisms of human *msrA* gene expression and regulation, we cloned and characterized the 5' promoter region of the human *msrA* gene. Using 5'-RACE (rapid amplification of cDNA ends) analysis of purified mRNA from human cells, we located the transcription initiation site 59 nt upstream of the reference MsrA mRNA sequence, GenBank® accession number BC 054033. The 1.3 kb of sequence located upstream of the first exon of *msrA* gene was placed upstream of the luciferase reporter gene in a pGL3-Basic vector and transfected into different cell lines. Sequentially smaller fragments of the *msrA* promoter region were generated by PCR, and expression levels

were monitored from these constructs within HEK-293 and MCF7 human cell lines. Analysis of deletion constructs revealed differences in promoter activity in these cell lines. In HEK-293 cells, the promoter activity was constant from the minimal promoter region to the longest fragment obtained. On the other hand, in MCF7 cells we detected a down-regulation in the longest fragment. Mutation of a putative negative regulatory region that is located between –209 and –212 bp (the CCAA box) restored promoter activity in MCF7 cells. The location of the *msrA* promoter will facilitate analysis of the transcriptional regulation of this gene in a variety of pathological contexts.

Key words: methionine sulfoxide reductase, promoter, reactive oxygen species, transcriptional start site.

INTRODUCTION

The overproduction of ROS (reactive oxygen species) can result in various deleterious effects. In order to protect against these harmful ROS, aerobic organisms have developed a number of cellular defences [1]. The classical cellular defence against ROS is represented by enzymes such as catalase, superoxide dismutases and glutathione peroxidase, which destroy the ROS [2]. In the recent years, to this important 'antioxidant triad' one can add MSRs (methionine sulfoxide reductases), which can repair oxidative damage to proteins. ROS can oxidize methionine residues in proteins to Met(O) (methionine sulfoxide). The MSR family contain proteins that can reduce both free and protein-linked oxidized methionine residues, and this system is now considered an important defence mechanism against oxidative damage [3–6]. To date, the MSR proteins that reduce Met(O) in proteins are referred to as MsrA and MsrB, catalysing the reduction of the two different epimeric forms, methionine *S*-sulfoxide and methionine-*R*-sulfoxide (Met-*S*-O and Met-*R*-O), respectively [3,7,8]. It is believed that MsrA and MsrB require reduced thio-redoxin as the natural reducing system, although dithiothreitol can be used *in vitro* [3,8,9].

MsrA was first identified several years ago in studies on the biological activity of the *Escherichia coli* ribosomal protein L12 [9,10]. This protein lost its biological activity during the oxidation of Met residues to Met(O) [11]. *E. coli* extracts possessed an enzymatic activity that was able to repair this damage by reducing the Met(O) in L12 protein to methionine [9]. The enzyme was initially called peptide-MsrA, but is now simply called MsrA, and is able to reduce the free Met(O) and other compounds containing

a methyl sulfoxide moiety [8,12]. To date, it is not clear how prokaryotic and eukaryotic MSR expression is regulated. Bacterial MsrA appears to be modulated by phenolic compounds simulating chemical stress conditions [13], and recently it was reported that the increase in expression of MsrA in *Ochrobactrum anthropi* is co-related with the overproduction of ROS caused by aromatic substrates [14].

In animal systems, the *msrA* gene has been either overexpressed or knocked out, and the importance of methionine oxidation in some age-related diseases is highlighted by several findings. In fact, neuronal PC12 cells, which overexpress MsrA, had lower levels of ROS after hypoxia and reoxygenation than control cells [15]. Moreover, *msrA*-knockout mice had a shorter life span, were more sensitive to hyperbaric oxygen and had a neurological defect that resulted in abnormal walking [16]. Overexpression of MsrA in *Drosophila melanogaster* leads to nearly a doubling of the life span [17].

Methionine oxidation is often discussed as being one of the sources for physiological dysfunction in several age-associated changes and degenerative diseases. Oxidatively modified calmodulin, which has been shown to accumulate in the senescent brain [18], was incubated with MsrA. This treatment restored the functional activity of the protein [19]. It has been reported that MsrA activity, as well as gene and protein expression, are decreased as a function of age [20,21], but the transcriptional mechanisms involved in this process are not known. Moreover, very little is known about how *msrA* expression is regulated, and few data are available on the transcriptional regulation of this important antioxidant gene in mammals. Until now, the only data available on the *msrA* eukaryotic promoter were obtained

Abbreviations used: CBF, CCAAT-binding factor; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift analysis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; MetO, methionine sulfoxide; MsrA, methionine sulfoxide reductase A; (RLM)-RACE, (RNA ligase-mediated) rapid amplification of cDNA ends; ROS, reactive oxygen species; RT, reverse transcriptase; TBE, Tris/borate buffer; TBS, Tris-buffered saline; TGF, transforming growth factor; TSS, transcription start site; UTR, untranslated region.

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Table 1 Sequences of all the primers used

See the Experimental section for further details.

Name	Sequence
RACE	5'-GCTGCTGGCGGGGCTGGAGG-3'
RACE-1	5'-GCTGGAGGCGCCGCGTAATCG-3'
P1.3	5'-CTGGAGCACAGCAGTGTG-3'
REV	5'-GTTCTGAGGTTCTCTCGG-3'
P1.3-Kpn	5'-GGGGTACCCTGGAGCACAGCAGTGTG-3'
REV-Kpn	5'-GGGGTACCGTTCTGAGGTTCTCTCGG-3'
P0.3	5'-GGGGTACCGAATTCATCTCCCTATTCCG-3'
P0.15	5'-GGGGTACCCACTGCTAACTGCG-3'
P0.11	5'-GGGGTACCTTTAAATCAAACGCATCC-3'
P0.03	5'-GGGGTACCGTTCTCTGTCGAGAAC-3'
Emsa 1F(wt)	5'-CATGACAATGTCTCCAATAAACTGACAG-3'
Emsa 2F(mut)	5'-CATGACAATGTCTAGGTTAAAACTGACAG-3'
F1MsrA	5'-GACCACCCTTCGGCTGG-3'
R1MsrA	5'-CCACCATGTGGGGAGCAA-3'
GAPDHf	5'-CCACCATGTGGCAAATTCATGGCA-3'
GAPDhr	5'-TCTAGACGGCAGGTACAGTCCACC-3'
F	5'-CGGGATCATGCTCTCGGCCACCC-3'
R	5'-CGGAATCTTATTTTAAATCCCACTGG-3'

with a yeast model, in which it has been reported that calcium phospholipid-binding protein (CPBP) is a component of a complex that binds the *msrA* promoter [22].

In the present study, we determined the TSS (transcription start site) of human *msrA*, isolated the 5'-flanking region of human *msrA* starting -1341 bp upstream of the TSS, and prepared a set of deletion plasmids to identify transcription-factor-binding sites involved in the promoter activity. By site-directed mutagenesis and EMSA (electrophoretic mobility-shift analysis), we identified a *cis*-element implicated in cell-specific *msrA* regulation.

EXPERIMENTAL

Cell cultures

HEK (human embryonic kidney)-293 cells and human breast carcinoma MCF7 cells were grown in a humidified atmosphere containing 5% CO₂ at 37 °C in DMEM (Dulbecco's modified Eagle's medium) containing a high glucose concentration (4.5 g/l at 25 mM) and supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin and 10% (v/v) fetal bovine serum. Immediately before transfection, the medium was removed and replaced with fresh medium without serum.

Determination of the 5'-terminal cDNA sequence

To map the TSS of *msrA*, an RLM-RACE (RNA ligase-mediated rapid amplification of 5'-ends) strategy was used to obtain the full-length cDNA sequence at the 5' end using a GeneRacer™ (Invitrogen) kit. Briefly, 5 µg of total RNA from HeLa cells, provided with the kit, were used to prepare 5'-racing cDNA: truncated mRNA was eliminated from ligation with the GeneRacer RNA Oligo™ by using calf intestinal phosphatase and tobacco acid pyrophosphatase. Reverse transcription of adapter-ligated mRNAs was performed by using oligo(dT)₂₀ and SuperScript™ III RT (reverse transcriptase) (Life Technologies). To obtain 5' ends, the first-strand cDNA was amplified by using a reverse gene-specific primer RACE (see Table 1 for details), and the GeneRacer™ 5' Primer (identical with the GeneRacer RNA Oligo). In addition to the template (first-strand cDNA) and primers, the 50 µl reaction mixture contained 0.2 mM dNTPs, 5% (v/v) DMSO, *Taq* Gold polymerase buffer and 5 units of *Taq*

Gold polymerase (Applied/PE Biosystems), and was incubated for 10 min at 95 °C followed by five cycles of amplification (60 s at 95 °C, 60 s at 68 °C and 60 s at 72 °C), and then by 30 cycles of amplification (60 s at 95 °C, 60 s at 66 °C and 60 s at 72 °C). An aliquot of reaction products generated with a primer binding at the RACE site was used as the template for a nested reaction by using the RACE-1 reverse gene-specific primer and the GeneRacer™ 5' Nested Primer. As mentioned above, in addition to the template (first-strand cDNA) and primers, the 50 µl reaction mixture contained 0.2 mM dNTPs, 5% (v/v) DMSO, *Taq* Gold polymerase buffer and 5 units of *Taq* Gold polymerase (Applied/PE Biosystems), and was incubated for 10 min at 95 °C followed by five cycles of amplification (60 s at 95 °C, 60 s at 68 °C and 60 s at 72 °C), and then by 30 cycles of amplification (60 s at 95 °C, 60 s at 66 °C and 60 s at 72 °C). PCR products were analysed on a 3% agarose gel, cloned into pCR4-TOPO vector and analysed by sequencing.

Construction of luciferase deletion plasmids

Genomic DNA was prepared from the HEK-293 cells by using Wizard Genomic DNA Purification Kit (Promega). Two oligonucleotides, P1.3 and REV, were designed (see Figure 4 and Table 1 for details) on the basis of genomic DNA sequence of the 5'-flanking region of the *msrA* gene to amplify a portion of DNA starting -1341 upstream of the identified transcription start site (+1). In addition to the template (genomic DNA) and primers P1.3 and REV, the 50 µl reaction mixture contained 0.2 mM dNTPs, *Pfu* DNA polymerase buffer and 5 units of *Pfu* DNA polymerase (Promega), and was subjected to 35 cycles of amplification (60 s at 94 °C, 60 s at 55 °C and 120 s at 72 °C). The PCR product was recovered from the low-melting agarose gel and used as the template in PCR reaction. In this PCR amplification, we used the primers P1.3-Kpn and REV-Kpn tailed at the 5' end with a KpnI restriction endonuclease recognition sequence (see Table 1 for details). The 50 µl reaction mixture contained the template, obtained as described above, primers P1.3-Kpn and REV-Kpn, 0.2 mM dNTPs, *Pfu* DNA polymerase buffer and 5 units of *Pfu* DNA polymerase (Promega). The reaction was subjected to 30 cycles of amplification (60 s at 94 °C, 60 s at 54 °C and 120 s at 72 °C). The PCR product was loaded on to a 1.5% low-melting agarose gel, recovered from the gel, purified and ligated into KpnI-digested pGL3-Basic reporter vector and dephosphorylated; the resulting plasmid was designated P1.3. Insertion into the pGL3-Basic reporter vector and the correct orientation were verified by DNA sequencing. A nested set of *msrA* promoter reporter constructs containing the 5' deletion was prepared by PCR using specific 5' primers (P0.30, P0.15, P0.11, and P0.03; see Table 1 for details), and a common 3' primer (REV-Kpn) and P1.3 as template. PCR-amplification products were loaded on to a 2% low-melting agarose gel, recovered from the gel, purified and ligated into KpnI-digested pGL3-Basic reporter vector. The resulting plasmids, P0.30, P0.15, P0.11 and P0.03, were analysed by DNA sequencing to ensure the fidelity of amplification and the correct orientation.

Site-directed mutagenesis

The 300 bp promoter region (-309 to +11 bp) incorporated into the P0.30 construct was subjected to site-directed mutagenesis to eliminate cores of putative transcription-factor-binding sites by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The two mutagenic primers containing the mutation (shown in bold type) were as follows: 5'-GTCATGACAATGTCT-**AGGTTAAAA**ACTGACAGTC-3' and 5'-GACTGTCAGTTT-TTAACCTAGACATTGTCATGAC-3'. Both primers annealed to

the same target sequence on opposite strands of P0.3. The 150 bp promoter region (–155 to +11 bp) extended into the P0.15 construct, containing the putative TATA element, was subjected to site-directed mutagenesis. The two mutagenic primers containing the mutation (shown in bold type) were as follows: 5'-CACGGC-AGCAACTTT**CG**CATCAACCGCATCCC-3' and 5'-GGGAT-GCGGTTT**GATGCG**AAAGTTGCTGCCGTG-3'. Site-directed mutagenesis was performed as described by the manufacturer. The resulting plasmids were designated respectively P0.3-mut and P0.15-TATAmut; both constructs were confirmed by DNA sequencing.

Transient transfections and luciferase assays

HEK-293 and MCF7 cells were plated in 24-well plates 2 days before transfection. On the day of transfection, cells were washed twice with PBS solution and replaced with serum-free DMEM media (not containing penicillin/streptomycin). Cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.8 µg of P1.3 (the longest luciferase construct) and equimolar amounts for other plasmids were used. HEK-293 and MCF7 cells were co-transfected with 50 ng of pcDNA3.1 (Invitrogen; a plasmid containing the cytomegalovirus promoter upstream of the β -galactosidase gene) to normalize for transfection efficiencies. After 4 h of incubation at 37°C, the transfection solution was withdrawn and replaced with the complete medium described above, and cultivated for an additional period of 48 h at 37°C. Transfections were performed in duplicate, and repeated at least 5 times. Measurement of luciferase activity was performed 48 h after transfection using the Luciferase Assay Kit (Promega) according to the manufacturer's protocol. Briefly, cells were washed with PBS solution, 100 µl of Passive Lysis Buffer (Promega) was added to each well, and cells were incubated for 10 min at room temperature in an orbital shaker. The lysates were centrifuged at 12000 *g* for 2 min to remove cell debris. Luciferase assays were performed using 10 µl aliquots of supernatant in a Lumat Luminometer (LB9501, Berthold, Bundoora, Australia). Each lysate was measured twice. Luciferase activities were normalized for β -galactosidase activity in each extract to correct for transfection efficiency, and reporter gene expression was expressed as relative light units. The luciferase activity of each construct was compared with that of the promoterless pGL3-Basic vector.

EMSA

Nuclear extracts from MCF7 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Approx. 2×10^6 cells were trypsinized, collected and washed once in 5 ml of PBS. The cell pellet was then processed as described by the manufacturer. The protein concentration of the nuclear extract was determined using the BCA (bicinchoninic acid) assay (Pierce), with BSA as the standard. The nuclear proteins were stored at –80°C and used in band-shift assays.

The sense sequences of the oligonucleotides used [EMSA 1F(wt) and EMSA 2F(mut)] are shown in Table 1. To prepare the double-stranded oligonucleotides, single-stranded forward and reverse oligonucleotides were annealed by heating to 95°C and cooling slowly to room temperature in TE buffer (10 mM Tris/1 mM EDTA).

Binding was tested by using the LightShift® Chemiluminescent EMSA Kit (Pierce). The 20 µl aliquots of binding solution contained 3 µg of nuclear extract with 20 fmol of 5'-end-biotinylated DNA target (see Table 1 for details) in the presence

or absence of competitor, 2.5% (v/v) glycerol, 5 mM MgCl₂, 50 ng/µl poly(dI-dC), 0.05% (v/v) Nonidet P40 and the binding buffer provided with the kit. Binding reactions were incubated at room temperature for 20 min. Samples (5 µl) of 5 × loading buffer were then added to each 20 µl binding reaction. The complex was separated on a 6% non-denaturing polyacrylamide gel in 0.5 × TBE (Tris/borate buffer) for 1.5 h at 100 V at room temperature. Complexes were then transferred to a nylon membrane (Biodyne® B; Pierce) using an electrophoretic transfer unit (Bio-Rad), according to the manufacturer's instructions, in 0.5 × TBE cooled to 4°C for 1 h at 380 mA. DNA was cross-linked to the membrane with a UV-light cross-linker instrument. Complexes were then detected by following the LightShift® Chemiluminescent EMSA Kit (Pierce) instructions. Samples (20 ml) of blocking buffer were added to the membrane, and this was incubated at room temperature for 15 min. This buffer was then replaced with blocking buffer containing stabilized streptavidin-horseradish peroxidase conjugate, and the membrane was incubated for a further 15 min at room temperature. The blot was washed five times (15 min each wash) with 20 ml of 1 × Wash solution, and then incubated in 30 ml of Substrate Equilibration Buffer for 5 min. The membrane was placed in Substrate Working Solution for 5 min and then placed in a film cassette and exposed to X-ray film. EMSA experiments were repeated at least three times.

For the antibody-supershift assay, 2 and 4 µg of anti-CBF-A/NF-YB (C-20), anti-CBF-B/NF-YB (C-18) and anti-CBF-C/NF-YC (C-19) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixture before the addition of the probes and incubated at room temperature for 30 min. Protein–DNA complexes were fractionated in a 6% non-denaturing polyacrylamide gel in 0.5 × TBE buffer at room temperature for 1.5 h at 100 V.

Evaluation of MsrA mRNA levels

Total RNA was isolated from 80% confluent HEK-293 and MCF7 cells using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RT-PCR was performed using 100 ng of total RNA. Reverse transcription reactions were performed in a final volume of 20 µl using MMLV (Moloney-murine-leukaemia virus)-RT (Sigma) and poly(dT) primers, as recommended by the manufacturer. F1MsrA and R1MsrA primers (see Table 1 for details) were designed to amplify a portion of 752 bp located at the 5'- and 3'-UTRs (untranslated regions) of *msrA* gene in a PCR-amplification reaction. In addition to primers and 2 µl of template (cDNAs obtained as reported above), the 50 µl reaction mixture contained 0.2 mM dNTPs, *Taq* DNA polymerase buffer and 5 units of *Taq* Gold DNA polymerase (Applied/PE Biosystems), and was incubated for 10 min at 95°C, followed by 30 cycles of amplification (60 s at 95°C, 60 s at 54°C and 60 s at 72°C). PCR products were verified by DNA sequencing. To provide further confidence in the data, 598 bp of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified by using primers GAPDHf and GAPDhr (see Table 1 for details). The templates used in the PCR reactions were the same cDNAs obtained as described above. Products were separated by gel electrophoresis on 1.5% agarose gels, visualized by ethidium bromide staining and analysed using the Chemi Doc System (Bio-Rad). RT-PCR experiments were performed at least three times.

The abundance of PCR products was evaluated by densitometric scanning of the ethidium-bromide-stained agarose gels using the Chemi Doc System, and the cDNA fragments corresponding to each amplified gene were compared between HEK-293

and MCF7 cells. PCR signals for the *msrA* gene were normalized to the GAPDH signals.

Western blot analysis

On the basis of the ORF coding for MsrA, two oligonucleotide primers were designed as follows: primer F (carrying a 5' recognition site for the restriction enzyme BamHI, shown underlined), 5'-CGGGATCCATGCTCTCGGCCACCC-3' (sense), and primer R (carrying a 5' recognition sites for the restriction enzyme EcoRI, shown underlined), 5'-CGGAATTCTTATTTTAAATACCACTGG-3' (antisense). PCR was performed to obtain the *msrA* gene; in addition to the template, obtained as described for the RT-PCR experiments above, and the primers (F and R), the 50 µl reaction mixture contained 0.2 mM of each dNTP, *Pfu* DNA polymerase buffer and 2.5 units of *Pfu* DNA polymerase (Promega). The PCR reaction was subjected to six cycles of amplification (60 s at 94°C, 60 s at 50°C and 60 s at 72°C) followed by 30 cycles of amplification (60 s at 94°C, 60 s at 55°C and 60 s at 72°C). Both the amplified products and the pGEX-4T-1 expression vector (Pharmacia) were digested with BamHI and EcoRI. The PCR fragment encompassing the complete MsrA-coding region was ligated into the restricted pGEX-4T-1 vector by using T4 DNA ligase (Boehringer Mannheim); the resulting plasmid was designated pGEX-MsrA. An overnight culture of *E. coli* BL21pLys cells transformed with pGEX-MsrA was diluted 1:10 and allowed to grow until the D_{600} reached 0.4. To induce gene transcription, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM and the incubation was extended for a further 5 h. The cells were collected by centrifugation (10 000 g for 15 min), suspended in PBS and disrupted by cold sonication. MsrA protein was obtained as previously described [23]. The MsrA recombinant protein was cleaved by treatment with thrombin, as described by the manufacturer (Pharmacia). The purity of the protein was analysed using SDS/PAGE (12.5 % polyacrylamide gels), and proteins were detected by silver staining. PBS containing 100 µg of recombinant MsrA was emulsified with Freund's complete adjuvant (1:9, v/v) and subsequently injected into a New Zealand White rabbit. After primary immunization, the animal was given 500 µl of emulsion containing 100 µg of antigen on days 14, 21 and 42; it was then bled 42 days later, and the antiserum was then collected and used for immunoblotting. Protein extracts were obtained from 80 % confluent HEK-293 and MCF7 cells by using Passive Lysis Buffer (Promega). The particulate material was removed by centrifugation at 10 000 g for 30 min, and the protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad), with BSA as the standard. Crude extracts (20 µg) were analysed by SDS/PAGE on 15 % (w/v) polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose (for 120 min at 0.3 A) using an immunoblot transfer apparatus (Bio-Rad). After transfer, the nitrocellulose was incubated overnight at 4°C in 10 % (w/v) non-fat milk in TBS (Tris-buffered saline; 500 mM NaCl and 20 mM Tris/HCl, pH 7.5), supplemented with 0.05 % (v/v) Tween 20 to block non-specific binding. The blot was incubated for 90 min at room temperature with 10 % non-fat milk in TBS, supplemented with 0.05 % (v/v) Tween 20 containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.05 % (v/v) Tween 20, the blot was incubated for 60 min at room temperature with peroxidase-conjugated goat anti-rabbit IgG (Calbiochem) at a dilution of 1:4000 in 10 % non-fat milk in TBS, supplemented with 0.05 % (v/v) Tween 20. The blot was again washed three times with TBS containing 0.05 % (v/v) Tween 20. Antibodies were visualized using a chemiluminescence

Figure 1 5'-RACE analysis of the *msrA* gene

Agarose gel electrophoresis of size marker (M) and *msrA* 5'-RACE product from purified total RNA using primer RACE-1 (see Table 1 and Figure 4 for primer details). The 5' terminus of the sequence of the band from the RACE-1 primer reaction is reported. Nucleotides shared with the reference sequence BC054033 are shown in uppercase, the upstream sequence from the fragment cloned from the band is shown in lowercase, and the terminal adenine residue is shown in uppercase and underlined.

detection system (Western Blotting Luminol Reagent; Santa Cruz Biotechnology).

RESULTS

Identification of the TSS

To identify the TSS of the *msrA* gene, we performed 5' RACE analysis using total RNA isolated from HeLa cells. RLM-RACE is a technique taking advantage of the presence of the cap structure. It offers the selective amplification of full-length transcripts discriminating against truncated messages. The analysis was performed using two reverse oligonucleotides derived from the 5'-UTR region (see Table 1 and Figure 4 for details) and two adapter primers provided with the kit for the primary and nested PCR. A weak smear on an agarose gel was observed in the first PCR with the outer primer GeneRacer™ 5' Primer and reverse primer RACE, by using the total RNA (results not shown). The primary PCR products were used as a template for nested PCR by using GeneRacer™ 5' Nested Primer and RACE-1 nested primer. Agarose gel electrophoresis resolved the 5'-RACE-nested reaction product into a single DNA fragment, shown in Figure 1, which was then cloned. The sequencing of five different clones, randomly selected, showed that they have the same sequence reported in Figure 1. The TSS was identified by sequence analysis, and mapped 59 bp upstream of the 5' end of the BC054033 MsrA cDNA clone, containing the longest 5' UTR among other human MsrA cDNA sequences deposited in the National Center for Biotechnology Information library (see Figure 2B).

Isolation and analysis of the 5'-flanking region of the *msrA* gene

On the basis of the TSS position, a 1352 bp fragment of human genomic DNA (−1341/+11 bp) was obtained by PCR. The sequence of the 5'-flanking region of the *msrA* gene was analysed by using MATINSPECTOR V2.2 software for the presence of transcription-factor-binding sites (solution parameters: core similarity 1.0; matrix-optimized). The *msrA* 5'-flanking region revealed a variety of putative transcription-factor-binding sites (results not shown).

Analysis of promoter activity of the 5'-flanking region

To map promoter activity of the 5'-flanking region of the *msrA* gene, various 5' progressive deletions starting from −1352 bp were prepared (Figure 2A), and their activities were measured.

Figure 2 Schematic representation of the different constructions employed in the promoter analysis

Numbering for each construction is based on the TSS (+1). **(A)** Positions of the 5' and 3' ends relative to TSS for each fragment are indicated. **(B)** The position of the identified TSS in the proximal promoter sequence relative to the highest cDNA previously reported (BC054033) is indicated as +1. Luc, luciferase.

The 1352 bp DNA fragment was subcloned, sequenced and used as template to make PCR products of lengths between -1341/+11 bp and -31/+11 bp. These DNA fragments were subcloned into the promoterless pGL3-Basic reporter plasmid and the resulting constructs P1.35, P0.30, P0.15, P0.11, P0.03, and R1.35 were analysed by sequencing to ensure fidelity of amplification. The plasmid R1.35 contains the longest putative promoter fragment cloned in the reverse orientation. The human HEK-293 and MCF7 cells were chosen as cell models to investigate transcriptional activity. These cell lines consistently express high levels of MsrA (see Figure 7). Figure 3 shows the relative luciferase activities of reporter constructs, harbouring 0.03–1.3 kb of sequence upstream of the TSS in MsrA, after transient transfection into MCF7 or HEK-293 cells. The construct named P0.03 did not possess promoter activity in either HEK-293 or MCF7 cells, indicating that a core promoter is located between nt -117 and +11. The activities of the constructs in the forward orientation were 30–60 times higher than the activity of the construct possessing the reverse orientation (P1.35). The construct containing 117 nt (P0.11) had the highest activity in HEK-293 cells, whereas the construct showing the highest activity in MCF7 was that containing 155 nt (P0.15). Moreover, the promoter activity detected using the different constructs (P0.11 through to P1.35) was of the same order of magnitude in the HEK-293 cells (Figure 3). On the other hand, in MCF7 cells there was a decrease in luciferase activity as the insert size increased. These data suggest the presence of a putative *cis*-element located between -155 and -309 bp upstream of the TSS recognized by cellular specific repressor(s).

Identification of critical transcription-factor-binding sites

As shown in Figure 4, the only putative TATA element is located at -112 bp. Site-directed mutagenesis experiments were performed to obtain a P0.15 construct without this element. No changes in promoter activity were detected transfecting either HEK-293 or MCF7 cells with P0.15 mutated construct compared with P0.15 wild-type (results not shown).

Two putative transcription-factor-binding sites as shown in Figure 4, CCAAT and FAST-1, located at the optimal regulatory

Figure 3 Promoter activity of *msrA* deletion constructs

Data are shown for HEK-293 cells (white bars) and MCF7 cells (grey bars). Cells were transfected with luciferase reporter constructs containing different sized promoter fragments. Luciferase activity is expressed as the magnitude of normalized luciferase activity relative to the pGL3-Basic promoterless negative control vector. The results are expressed as means $\pm$ S.D. for at least five different experiments, in duplicate for each construct.

distance from the TSS were identified by using MATINSPECTOR V2.2 software in the DNA region -155/-309 bp. To understand better the critical responsive elements involved in the promoter activity inhibition, additional reporter constructs were prepared by site-directed mutagenesis, in which target boxes were mutated (Figure 5A). To eliminate the core sequence common to both putative transcription-factor-binding sites [i.e. for CBF (CCAAT-binding factor) and FAST-1 SMAD-interacting protein], we mutated the CCAA box (to a sequence of AGGT). The mutation

Figure 4 Analysis of the human *msrA* promoter sequence

The sequence shows 1.35 kb of the human *msrA* promoter and the 5'-UTR up to the start codon (atg, which is shown in boldface type). Primers used for the luciferase reporter constructs are underlined; the cores of binding sites for the transcriptional factors CBF and FAST-1 are shown enclosed in boxes; the circumscribed boxes for RACE-1 and RACE indicate the reverse primers for TSS identification. The arrowhead indicates the TSS position (+1).

of the CCAA box did not introduce any new binding sites, as confirmed by sequence analysis with MATINSPECTOR V2.2 software. Clones obtained by site-directed mutagenesis were analysed by sequencing to ensure fidelity of amplification. Figure 5(B) shows that the mutation increased the promoter activity in MCF7 cells, restoring the same order of magnitude detected by using the P0.15 construct, whereas no changes in promoter activity were detected on transfection of HEK-293 cells with the same mutated construct. These data suggest that the CCAA box identified could represent a binding site recognized by cellular, specific negative regulatory elements.

EMSA of the putative transcription-factor-binding site in *MsrA* promoter

The analysis of the promoter activity of the 5'-flanking region deletion constructs suggests the presence in MCF7 cells of cellular specific repressor(s) that bind *cis*-element(s) located between

– 155 and – 309 bp upstream of the TSS. We performed EMSA to investigate whether transcription factors from MCF7 cells could specifically bind *msrA* promoter fragments containing the DNA region responsible for the difference in promoter activity detected in MCF7 as compared with HEK-293 cells (Figure 3). As shown in Figure 6, a specific complex was formed between 5'-biotinylated wild-type probe and nuclear extract proteins from MCF7 cells. This binding was specific, since complex formation was inhibited by adding a 200-fold molar excess of unlabelled wild-type probe. Moreover, a lower level of DNA–protein complex was observed using the 5'-biotinylated probe carrying the CCAA box mutated to AGGT compared with the complex obtained by using wild-type probe. As described above, the CCAA box identified by site-directed mutagenesis is the core sequence recognized by FAST-1 and/or CBF, and it is essential for the binding of these factors. The finding that mutation of the CCAA box did not abolish complex formation may suggest that the box identified is recognized by novel repressor element(s). To eliminate entirely the possibility

Figure 5 Effect of CCAA-box mutation

(A) Circumscribed boxes indicate the cores of binding sites for putative transcriptional factors CBF and FAST-1, which were mutated in reporter constructs. (B) Effects of core mutations for CBF and FAST-1 factors on activity of P0.3 constructs. HEK-293 or MCF7 cells were transfected with P0.3 luciferase constructs containing core mutations of the CBF- and/or FAST-1-binding sites (shaded bars). Luciferase activity of the constructs containing mutations was compared with the wild-type (white bars). The results are expressed as means $\pm$ S.D. for at least five different experiments, in duplicate for each construct.

of CBF involvement, supershift assays were performed. No effect in migrating species was detected when CBF antibodies were added to the samples analysed by gel-shift experiments (results not shown).

MsrA transcript and protein are expressed in both HEK-293 and MCF7 cell lines

In order to evaluate the relative levels of MsrA transcript in both HEK-293 and MCF7 cells, RT-PCR was conducted with total RNA obtained from these cells. Using the same samples of cDNAs, we examined the expression levels of GAPDH: this gene was expressed similarly in both HEK-293 and MCF7 cells (Figure 7A). As shown in Figure 7(A), a lower level of MsrA transcript was detected in RT-PCR products obtained by using total RNAs extracted from MCF7 cells compared with those obtained from HEK-293 cells. Levels of MsrA protein detected by Western blot analysis were consistent with the MsrA transcript data (Figure 7B). In fact, a lower level of MsrA protein was detected in crude extracts obtained from MCF7 cells as compared with HEK-293 cells. These data suggest a difference in transcriptional regulation between these human cell lines.

DISCUSSION

The importance of MsrA in a multiplicity of biological and pathological contexts is becoming increasingly evident and an understanding of the mechanisms controlling its synthesis ever more important. Methionine oxidation has been implicated in different age-related pathological contexts, such as cataract formation [24], emphysema [25,26], and several neurodegenerative diseases, such as Alzheimer's [27] and Parkinson's [28,29] diseases. MsrA is one of the enzymes that is able to reduce Met(O)

Figure 6 Gel mobility-shift analysis of protein complexes formed with oligonucleotides containing the CCAA box

(A) The presence of nuclear extracts and competing probes are as shown at the top of the gel. wt, the 5'-end-biotinylated DNA wild-type probe containing the CCAA box; mut, the 5'-end-biotinylated DNA mutated probe (where the CCAA box has been mutated to sequence AGGT). (B) Densitometric evaluation of the EMSA blots. Values are expressed as percentages (referred to wt densities). The data are the means $\pm$ S.D. for three independent experiments. Results were compared using Student's *t* test analysis ($P < 0.01$).

to methionine, restoring function to specific proteins, which may be an important defence mechanism against oxidative damage. In addition, the transcriptional regulation of MsrA has potential significance in aging processes. It has been reported that levels of MsrA transcript and protein decrease with age in rat organs [20] and in senescent fibroblasts [21]. The mechanisms by which MsrA expression is regulated during aging remain unknown, making it difficult to ascertain whether MsrA down-expression is a cause or an effect of the aging process. Thus knowledge concerning the molecular mechanisms involved in *msrA* gene expression is of significant importance.

In the present study, we elucidate for the first time, to our knowledge, the transcriptional regulation of the human *msrA* gene by identifying its promoter region, the TSS and a *cis*-acting element involved in cellular specific MsrA regulation.

To study the regulation of MsrA expression using the identified TSS, we isolated and cloned the human *msrA* 5'-flanking promoter region ($-1341/+11$ bp). Using the 1.35 kb *msrA* promoter, we have provided evidence that this promoter fragment drives expression of luciferase reporter constructs in several human cell lines, and it is differentially regulated in MCF7 breast cancer

Figure 7 Analysis of *msrA* transcript and protein levels in MCF7 and HEK-293 cells

(A) Ethidium-bromide-stained gels showing the levels of *msrA* transcripts between MCF7 and HEK-293 cells. GAPDH was used as the control. The data are the means $\pm$ S.D. for three independent experiments. Results were compared using Student's *t* test analysis ($P < 0.01$). (B) Immunoblotting of MCF7 (lane 2) and HEK-293 (lane 3) extracts with a MsrA antiserum using 20 μ g of protein. Recombinant human MsrA (50 ng) was loaded as the control (lane 1). The observed increase in the MsrA level in HEK-293 cells was analysed using the Chemi Doc System (Bio-Rad) and normalized with β -actin signal intensities of the corresponding lanes. Values are the means $\pm$ S.D. for at least five different immunoblotting experiments.

cells as compared with HEK-293 cells. Functional analysis of constructs containing different sized fragments of the 5'-flanking region demonstrated a variable profile of promoter activity. In fact, there was suggestive evidence for a significant decrease in luciferase activity of constructs P0.30 and P1.35 compared with smaller fragments in MCF7 cells, suggesting the presence of repressor(s) able to bind the DNA region located between P0.15 and P0.30. On the other hand, in HEK-293 cells the promoter activity was constant from P0.11 to the longest construct. Detailed sequence analysis of the DNA sequence spanning the P0.15 and P0.30 constructs revealed several putative binding sites. Among these sites were overlapping FAST-1 and CCAAT sites (Figure 4), which have been shown to play an important role in the transcription of a wide variety of eukaryotic genes. Functional analysis using a luciferase reporter construct carrying a core mutation in the DNA region containing the putative binding site recognized by CBF and/or FAST-1 in the P0.30 construct demonstrated that the integrity of this DNA region, indicated by the CCAAT box, is necessary to provide the inhibitory effect of the promoter detected only in MCF7 cells (Figure 3, lower panel).

Several findings support the hypothesis that neither CBF nor FAST-1 are involved in MsrA regulation. In fact, addition of CBF antibodies had no effect on migrating species. Furthermore, to investigate the possible involvement of CBF in the regulation of MsrA expression, we used the phytoestrogen genistein, which

abolishes CBF binding to the CCAAT box by inhibiting tyrosine phosphorylation of the protein [30]. Genistein treatment of MCF7 had no effect on expression of MsrA, as detected by RT-PCR and Western blot analysis (results not shown).

FAST-1 has been shown to associate with Smad-2 and Smad-4, transducers of TGF (transforming growth factor)- β superfamily signals, in response to stimulation by several TGF- β superfamily ligands [31]. Treatment of MCF7 with several concentrations of TGF- β did not modulate the expression of MsrA (results not shown).

To verify a possible DNA-protein interaction responsible for the inhibitory effect observed by transfection analysis in MCF7 cells, we performed EMSA. The results obtained strongly suggested that transcription factor(s) from MCF7 cells could specifically bind to a biotinylated DNA probe containing the CCAAT box. To verify the importance of this sequence box in the DNA-protein complex formation, we used a biotinylated DNA probe in which we mutated the CCAAT box to AGGT. This mutation reduced, but did not abolish, DNA-protein complex formation. These findings suggest that the CCAAT box could either represent, or be a part of, the core sequence recognized by a new putative cellular-specific transcriptional factor.

The data obtained from promoter construct transfections seem to suggest a differential MsrA expression in the two human cell lines used. Therefore we evaluated MsrA expression levels by RT-PCR and Western blotting in both MCF7 breast cancer and HEK-293 human cells, and observed different levels of expression for both. In fact, we detected a significant lower enzyme expression level in MCF7 cells compared with HEK-293 cells. This difference in MsrA expression suggests that cell-specific mechanisms regulate transcription of this gene.

In summary, we have performed 5'-RACE analysis, and identified the TSS of human MsrA located 59 nt upstream of the reference human MsrA mRNA sequence, GenBank[®] accession number BC 054033, representing to date the longest 5'UTR identified for hMsrA. We have also identified and characterized the promoter region regulating the expression of human MsrA. No canonical TATA box was found in the vicinity of the TSS. The only putative TATA element was located at -112 bp, but it did not have an influence on promoter activity: thus we concluded that human MsrA is a TATA-less promoter. Mutagenesis experiments revealed the presence of a *cis*-element recognized by a putative cellular specific regulatory transcriptional factor(s) that will be subject of our future investigations.

The current identification and characterization of the promoter sequence of human *msrA* provides the basis for further studies of gene regulation during the development of several disease states.

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