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Structure and Expression of the Human SM22α Gene, Assignment of the Gene to Chromosome 11, and Repression of the Promoter Activity by Cytosine DNA Methylation[1]

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To investigate the molecular mechanisms that control expression of smooth muscle cell (SMC) differentiation genes, we have isolated the human SM22α gene, which is composed of five exons and four introns, spanning an approximately 6-kilobase (kb) genomic DNA at chromosome region 11q23.2. Expression of the SM22α messenger RNA was detected in serum-stimulated cell cultures including SMC, undifferentiated skeletal muscle-lineage cells, and fibroblasts, and it was down-regulated in SMC of balloon-injured atheromatous human vessels. A major transcription start site of the SM22α gene is located at 75 base-pairs (bp) upstream of the ATG start codon. Analysis of the 2.6 kb 5′-upstream sequence demonstrated that two CArG/SRF-boxes and two GC-box/Sp1-binding sites were present at bp −147 and −274, and at bp −233 and −1635, respectively. The nucleotide sequences of the two CArG/SRF-boxes and the proximal GC-box/Sp1-binding site are 100% conserved with those of the murine SM22α genes [Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morriesey, E.E., Ip, H.S., and Parmacek, M.S. (1995) *J. Biol. Chem.* 270, 13460-13469; Kemp, P.R., Osbourn, J.K., Grainger, D.J., and Metcalf, C. (1995) *Biochem. J.* 310, 1037-1043]. Cell transfection assays using a luciferase reporter gene construct containing the 455-bp 5′-flanking region (positions −26 to −480) showed that methylation of the CpG dinucleotides within this segment reduces its transcriptional activity. The results imply a novel mechanism for transcriptional control of the SMC differentiation-specific gene promoter.

Key words: α-actin, calponin, CArG box, CpG, differentiation, DNA methylation, smooth muscle, SM22α gene.

Induction of the SMC differentiation features in mesenchymal cells has been identified in the development of cardiovascular tissues during embryogenesis, as well as in many pathologic settings characterized by tissue remodeling and fibrosis (*1*). A critical to understanding SMC differentiation and development is to identify the key environmental signals and ligands that induce or maintain the differentiated state of SMC, and to determine the molecular genetic mechanisms that control expression of the SMC differentiation-specific genes (*2*). Recent studies demonstrate that members of the TGF-β superfamily of growth factors promote differentiation of the neural crest stem cells to SMC with induction of the SMC lineage-specific genes, α-SMC actin, and calponin (*3*). To date, however, relatively little is known about the myogenic program that regulates transcription of the SMC differentiation-specific gene promoter.

Mammalian SM22α gene encodes an actin-associated protein with 201 amino acids which shares sequence homology with calponin (*4-7*). The SM22α gene is expressed in smooth, cardiac, and skeletal muscle-lineage cells during embryogenesis (*8*), and is one of the earliest markers of differentiated SMC (*9*). Previous studies showed that approximately 400-bp 5′-flanking sequence of the mouse and rat SM22α gene promoter was sufficient to direct the specific expression of reporter transgenes in cultured SMC and undifferentiated skeletal muscle-lineage cells (*5, 6, 10*), as well as in the embryos of transgenic mice (*11*).

To define the mechanisms that regulate SMC-specific gene transcription, we isolated and structurally characterized the human SM22α gene. We analyzed regulation of the promoter activity in SMC and undifferentiated skeletal muscle-lineage cells, and found that methylation of the CpG dinucleotides within the 455-bp sequence preceeding the transcription initiation site reduces the transcriptional activity of this gene.

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EXPERIMENTAL PROCEDURES

Antibodies—A polyclonal antibody against the chicken gizzard SM22α protein (*12*) was generated in rabbits according to the reported method (*13*). Antibody that does not cross-react with calponin was prepared by antigen-affinity chromatography after passage through a calponin-Sepharose 4B affinity column. Anti-SM α-actin monoclonal antibody was purchased from Sigma (St. Louis, USA). The specificities of the antibodies were verified by the immunoblot analysis (*14*) of total homogenates of chicken gizzard and human aortic smooth muscles.

Isolation of Human SM22α cDNA and Bacterial Protein Expression—The SM22α cDNAs were isolated from a λgt11 human aorta cDNA library (Clontech Laboratories, Palo Alto, USA) using the 5′-end ^{32}P-labeled anti-sense oligonucleotides 5′-CAT GAA CCA (G/A)TT GGG (G/A) TC GCC (G/A)TG (G/A)TA (G/A)TG GCC (G/A)TC-3′ designed from the amino acid sequence between Asp^{141} and Met^{152} of chicken gizzard SM22α (*15*). Prehybridization and hybridization were performed in a solution of 25 mM sodium-phosphate buffer (pH 6.5) containing 0.1% (w/v) SDS, 1×Denhardt (1×Denhardt=0.1% BSA (Fraction V), 0.1% Ficoll, 0.1% polyvinyl pyrrolidone), 6×SSC (1×SCC=15 mM sodium citrate buffer, pH 7.0, containing 0.15 M NaCl), and 100 μg/ml calf thymus DNA at 37°C for 16 h. Final washes were done in 0.5×SSC and 0.1% SDS (w/v) at 38°C for 1 h. Screening of 8×10^5 plaques resulted in the isolation of 18 positive clones, which on the basis of the insert size, fell into two categories (1,064-bp and 838-bp (nt No. 225 to 1,064)). Representatives of each class of clones were isolated, subcloned into the pBluescript SK(−) vector (Stratagene, La Jolla, USA) and then sequenced. The nucleotide sequence of the cloned cDNAs was determined on both strands using an Applied Biosystem Model 373A DNA sequencer according to the manufacturer's instructions.

The *Msc*I/*Pst*I-digested fragment of the full length human SM22α cDNA (bp 52 to 553) was first subcloned into the *Sma*I/*Pst*I-digested pBluescript vector (pBl-M/PSM22). Next, the *Pst*I/*Eco*RV-digested subfragment (including the SM22α sequence spanning from bp 554 to 1,064) was ligated to the *Pst*I/*Eco*RV-digested pBl-M/PSM22. The *Bam*HI/*Msc*I fragment (including bp 52 to 757) was excised and subcloned into the bacterial expression vector pGEX 3X (Pharmacia Biotech., Uppsala, Sweden). Its correct framing as well as orientation relative to the glutathione S-transferase (GST) gene was confirmed by DNA sequence analysis. The plasmid was introduced into *Escherichia coli* DH5α cells and protein expression was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The human SM22α polypeptide was purified by digestion with Factor Xa (Boehringer Mannheim, Mannheim, Germany) following the glutathione-Sepharose 4B affinity chromatography (Pharmacia Biotech), according to the manufacturer's instruction. Cross-reactivity of the polyclonal anti-gizzard SM22α antibody to the recombinant human SM22α protein was verified by SDS-PAGE and immunoblot analysis.

RNA Preparation and Northern Blot Analysis—Total RNA was extracted from cultured cells (60–70% confluency for growing culture) according to the method described by Stallcup and Washington (*16*), and from tissue samples by the ISOGEN RNA extraction kit (Nippon Gene, Toyama). Agarose (1%) gel electrophoresis, transfer to nitrocellulose, and hybridization to ^{32}P-labeled cDNA probe were as described previously (7). The 1,064-bp full-length SM22α insert was labeled with [α-^{32}P]dCTP by random oligomer priming and used at 6×10^6 cpm/ml. Prehybridization and hybridization were performed at 43°C in a solution of 25 mM sodium phosphate buffer (pH 6.5) containing 50% formamide (v/v), 0.2% SDS (w/v), 2×Denhardt, 6×SSC, and 100 μg/ml calf thymus DNA. Final washes were carried out in 0.2×SSC and 0.1% SDS (w/v) at 50°C for 1 h.

Immunofluorescence Microscopy—Human coronary artery tissue was obtained from autopsied patients 6 days after balloon angioplasty. The specimens were rinsed with PBS and mounted in O.C.T. compound (Miles, Elkhart, USA), and then frozen using liquid nitrogen. The frozen sections were cut with a cryostat (5 μm thick), mounted on glass slides, air-dried, and then fixed in 100% acetone at −20°C for 7 min. After being rinsed in PBS for 15 min, the sections were incubated with 1% (v/v) goat serum/PBS for 1 h at room temperature, washed in PBS, and incubated with the polyclonal anti-SM22α antibody in 2% (w/v) BSA/PBS overnight at 4°C. They were then washed 5 times with 0.005% (v/v) Tween 20/PBS, followed by incubation with the rhodamine-conjugated goat anti-rabbit or anti-mouse IgG (TAGO Immunologicals, Camarillo, USA) in 2% (w/v) BSA/PBS for 1 h at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, they were examined under a fluorescence microscope (Olympus Vanox-S).

Isolation of Human SM22α Genomic Clones—An EMBL3 human genomic library (*17*) (Japanese Cancer Resources Bank, Tokyo) constructed from peripheral blood cells was screened with a full length human SM22α cDNA (1,064 bp) as a probe. The probe was labeled with digoxigenin (DIG) using the DIG-High Prime labeling mixture (Boehringer Mannheim). Prehybridization and hybridization were performed in a solution of 250 mM sodium-phosphate buffer (pH 7.2) containing 20% (w/v) SDS, 1 mM EDTA, and 0.5% (w/v) blocking reagent (Boehringer Mannheim) at 60°C for 12 h. The filters were washed twice at 68°C for 1 h in a solution comprising 0.1×SSC and 1% SDS before exposure to Fuji RX X-ray film. Screening of 5.1×10^5 plaques of the human genomic library resulted in the isolation of two distinct classes of overlapping clones on the basis of the insert size. A representative of each class of clones was isolated and subcloned into the pUC18 vector (Clontech Laboratories, Palo Alto, USA) for restriction endonuclease mapping and sequencing. A 9.5 kb DNA fragment containing the 5′-flanking region and the entire coding sequence of the human SM22α gene was sequenced through the construction of restriction endonuclease fragment subclones as well as synthetic oligonucleotide primers. The intron-exon boundaries were consistent with the AG/GT splicing consensus sequence.

All restriction and DNA modifying enzymes were purchased from New England Biolabs (Beverly, USA), Takara Shuzo (Otsu), and Boehringer Mannheim.

Determination of the Transcription Start Site—The oligo-capping protocol (*18*) was used to identify the transcription initiation site of the human SM22α gene.

Poly(A)$^+$ RNA (5 μg) was purified using the QuickPrep mRNA Purification Kit (Pharmacia Biotech.) from primary cultured human aortic SMC (clone HS4001) (Kurabo Biomedical Business, Osaka) and liquid nitrogen-frozen adult human aortic smooth muscle tissues, and then was oligo-capped with an RNA oligonucleotide (5′-ACC GGU UGU UGC AGC GGA GG-3′). A 19-nucleotide (nt) oligonucleotide primer (5′-CTG CTG CCA TGT CTT TGC C-3′) was synthesized as the antisense sequence of human SM22α cDNA (nt No. 423 to 405). Reverse transcription (RT) was carried out using the reaction mixture of a first-strand cDNA synthesis kit (Pharmacia Biotech) in the presence of 40 pmol of the antisense primer. After 60 min incubation at 37°C, 25 pmol of each of the primers and 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech) were added. Combinations of forward (5′-ACC GGT TGT TGC AGC GGA GG-3′) and reverse (5′-GCT TGC TCA GAA TCA CGC C-3′ for human SM22α cDNA nt No. 240 to 222) primers were selected for amplification with 30 cycles of denaturation (94°C, 40 s), annealing (58°C, 30 s), and polymerization (72°C, 1 min 30 s).

Human Chromosomal Assignment—Chromosomal assignment of the human SM22α gene was carried out by fluorescence *in situ* hybridization (FISH). Lymphocytes isolated from human blood were cultured in α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal calf serum and phytohemagglutinin at 37°C for 68-72 h. The lymphocyte cultures were treated with 5-bromo-2′-deoxyuridine (0.18 mg/ml, Sigma Chemicals, USA) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and re-cultured at 37°C for 6 h in α-MEM with thymidine (2.5 μg/ml, Sigma Chemicals). Cells were harvested and slides were made by using standard procedures, including hypotonic treatment, fixation, and air-drying.

A 6.5 kb *Bam*HI-*Eco*RI fragment spanning from exon 1 to a part of exon 5 of the SM22α gene was subcloned into the pUC18 vector and then labeled with dATP using BioNick labeling kit (BRL, Gaitherburg, USA) at 15°C for 1 h. The procedure for FISH detection was performed according to the method described by Heng *et al.* (*19*). Briefly, slides were baked at 55°C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 2×SSC for 2 min at 70°C followed by dehydration with ethanol. Probes were denatured at 75°C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulfate. Probes were loaded and hybridized on the denatured chromosomal slides overnight. FISH signals and the 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) banding pattern was recorded separately, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes.

Plasmid Preparation and Cytosine DNA Methylation—A 6.5-kb *Bam*HI/*Eco*RI SM22α genomic subfragment, spanning from a 480-bp 5′-flanking segment to a part of exon 5, was first subcloned into *Bam*HI/*Eco*RI-digested pUC18 vector. The *Xho*I linker was ligated into the *Bam*HI site of the pUC18 vector containing the 6.5-kb SM22α subfragment. The *Xho*I/*Dra*I fragment (containing the promoter sequence from bp −26 to −480) was subcloned into the *Xho*I/blunt-ended *Hin*dIII sites of the pGL2-Basic vector (Promega, Madison, USA), and its correct orientation (5′ to 3′ relative to the luciferase reporter gene) was confirmed by DNA sequence analysis. The construct was designated as pGL2-455SM22. The SM22α promoter sequence from bp −26 to −480 was excised from the luciferase reporter plasmid by the *Xho*I/*Xba*I digestion and purified by 0.8% agarose gel electrophoresis. The fragment containing the promoter sequence was methylated by *Hpa*II methylase (2 U/1 μg DNA) (New England Biolabs, Beverly, USA) in the presence of 80 μM *S*-adenosylmethionine for 1 h at 37°C. Complete methylation of the *Hpa*II sites was confirmed by the protection against the *Hpa*II digestion. The specifically methylated DNA fragment (2 μg) and control unmethylated fragment (2 μg) were each ligated to 10 μg of the *Xho*I/*Xba*I-digested pGL2-Basic luciferase reporter plasmid for 5 h at 37°C. After confirming ligation by agarose gel analysis, the ligation mixture was phenol/chloroform-extracted, chloroform-extracted, and ethanol-precipitated. Ten micrograms of the closed circular plasmid was used for each transfection assay.

Cell Culture—The rat SMC cell line PASMC was derived from pulmonary arteries of adult Sprague-Dawley rats (*20*). The PASMC cells maintain differentiated properties of SMC through multiple subcultures (*20*). Primary-cultured human aortic smooth muscle cells (HS4001) were purchased from Kurabo Biomedical Business (Osaka). The cells were cultured in the manufacturer's recommended medium. Murine skeletal muscle myoblasts C2C12 (CRL-1772) and BC3H1 (CRL-1443), murine fibroblast cell lines C3H/10T1/2 Clone 8 (CCL 226) and NIH3T3 (CRL-1658) were purchased from American Type Culture Collection (Rockville, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, USA) supplemented with 10% (v/v) (growing condition) or 0.5% (v/v) (differentiation condition) fetal bovine serum (Upstate Biotechnologies, Waltham, USA) and 1% penicillin/streptomycin at 37°C under a humidified atmosphere containing 5% (v/v) CO_2.

Transfections, Luciferase, and β-Galactosidase Assays—PASMC cells and C2C12 cells were cultured in DMEM containing 10% fetal bovine serum, split and plated 24 h prior to transfection. Cells (2×10^4) at ~70% confluence in a 3-cm-diameter dish were transfected with 10 μg of the methylated or unmethylated luciferase reporter plasmid, 3 μg of the pSVβ-gal reference plasmid (Promega), and 30 μg of DOTAP reagent (Boehringer Mannheim) according to the manufacturer's protocol. Forty-eight hours following transfection, cells in a 3-cm-diameter dish were harvested in 100 μl of the cell lysis buffer (PicaGene Luciferase Assay System, Toyo Ink, Tokyo). After centrifugation at 12,000×*g* for 10 min at 4°C, aliquots (20 and 30 μl) of the supernatants were used for the luciferase and β-galactosidase assays, respectively. Luciferase activity was measured by a BLR-201 luminescence reader (Aloka, Tokyo). β-Galactosidase activity was measured using the β-galactosidase Enzyme Assay System (Promega). Briefly, 30 μl of the supernatant was mixed with 30 μl of Assay 2X Buffer [200 mM sodium phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside] in a 96-well plate. After incubation at 37°C for 2 h, the reaction was stopped by addition of 90 μl of 1 M Tris solution and then the absorbance of the samples at 415 nm was mea-

sured with an MTP-120 plate reader (Corona Electric, Ibaraki). All experiments were repeated at least three times to check reproducibility. Luciferase activities (light unit) were corrected for variations in transfection efficiences as determined by assaying cell extracts for β-galactosidase activity. Transfection efficiency of different cell lines was estimated by comparing expression of the pSV2-luc gene containing the SV40 enhancer and promoter. Data are expressed as % normalized light units ± SE as compared with control experiments.

RESULTS

cDNA Cloning and Expression of Human SM22α—A full length clone encoding human SM22α was isolated by screening of a λgt11 fetal aorta cDNA library. There were no isoform variants generated through alternative mRNA splicing. In the longest cDNA isolated, containing a 1,064-bp sequence, a single open reading frame (ORF) was found. Starting at the first in-frame ATG triplet, 48 base pairs from its 5′ end, the ORF sequence encodes a 201 amino-acid peptide with a predicted molecular mass of 22,611, and an isoelectric point of 9.54 (net charge +4.5 at neutral pH). The in-frame nonsense codon (TAG) is followed by 403 nt of a 3′-untranslated sequence. A polyadenylation signal (AATA) is present 20 bp upstream of the poly(A) tail. The sequence of the synthesized oligonucleotide probe was found in the human SM22α sequence from nt 471 to 506. The full length nucleotide sequence was identical to the reported cDNA sequence (WS3-10) (*21*) from cultured fibroblasts of a Werner's syndrome patient, except that two missense mutations (nt 193 of the human SM22α sequence; G to C, Arg-49 to Pro-49, and nt 358; A to C, Ser-104 to Tyr-104), a total of four neutral replacements (nt 209; C to G, nt 682; C to G, nt 881; T to G, nt 957; C to G) and one insertion (A at nt 1056 of the WS3-10 cDNA sequence) were found. The human SM22α sequence is 86% identical to that of the originally identified chicken SM22α (*15*). Of the 27 different residues, 25 are conservative substitutions distributed throughout the 201 amino acids of the human SM22α peptide.

RNA blot analysis from various human fetal tissues and cell lines probed with the cloned full length human SM22α cDNA revealed a 1.2 kb transcript in widely varying amounts (Fig. 1). The SM22α mRNA appears to be relatively abundant in smooth, cardiac and skeletal muscle-lineage cells or tissues containing significant amount of SMC: aorta, small intestine, placenta, heart (more abundant in atrium than in ventricle), spleen, lung, and kidney. No hybridization signal could be detected in adrenal cortex, thymus, Caco-2 (intestinal carcinoma), Hep3B (hepatocellular carcinoma), or HeLa cells. High-level expression of the SM22α transcript was found in HUSK-1, a human skeletal muscle myoblast line which was cloned from the thigh muscle of a 22 week abortus at autopsy (established by Kohtz, S. at The Mount Sinai Medical Center, and kindly provided by Nadal-Ginard B.).

Structure and Chromosome Location of the Human SM22α Gene—Using the random-primed, DIG-labeled, full length cDNA (1,064-bp) as a probe, we screened 5×10^5 plaques of a human genomic library constructed in the EMBL3 vector. Two positive EMBL3 phage clones were isolated and sequenced, representing two distinct classes of overlapping clones which contain the 5′-flanking region and entire coding sequence of the human SM22α gene. The human SM22α gene is composed of five exons and four introns spanning approximately 9.5 kb of genomic DNA (Fig. 2, a and b). As shown in Fig. 3, the oligocapping assay involving RNAs from aortic smooth muscle tissues and primary cultured aortic SMC revealed a major transcriptional initiation site located 75-bp upstream of the ATG translational initiation codon, which hereafter will be designated as +1. Two minor sites were identified 56-bp and 118-bp upstream of the translation initiation codon. The human SM22α gene does not contain either TATAA canonical sequences or a consensus initiator sequence (*22*) around its transcription start site. However, the nucleotide sequence TTTAAA, which might function as a TATA box, was present 28-bp upstream of the start site. Comparison of the nucleotide sequences of human, mouse, and rat SM22α gene promoters revealed a highly conserved region spanning from bp −1 to −270 (Fig. 4). Within this region, there are two CArG/SRF-boxes [$CC(A/T)_6GG$] located at bp −147 and −274, and one GC-box/Sp1-binding site (CCCGCC/GGCGGG) (*23*) located at bp −233, which were 100% conserved with the mouse and rat sequences. A search for consensus *cis*-acting sequences in the 5′-flanking 2,160-bp region revealed one additional GC-box/Sp1-binding site located at −1635 and one MEF-2 like sequence [$(C/T)TAAAAATAAC(C/T)_3$] (*24*) located at bp −573.

Using a 6.5 kb *Bam*HI-*Eco*RI digested genomic fragment as a probe, we mapped the localization of the SM22α gene in the human genome by chromosomal *in situ* hybridization. A total of 100 metaphase cells were examined. Of these, 89 cells showed signals on one pair of the chromo-

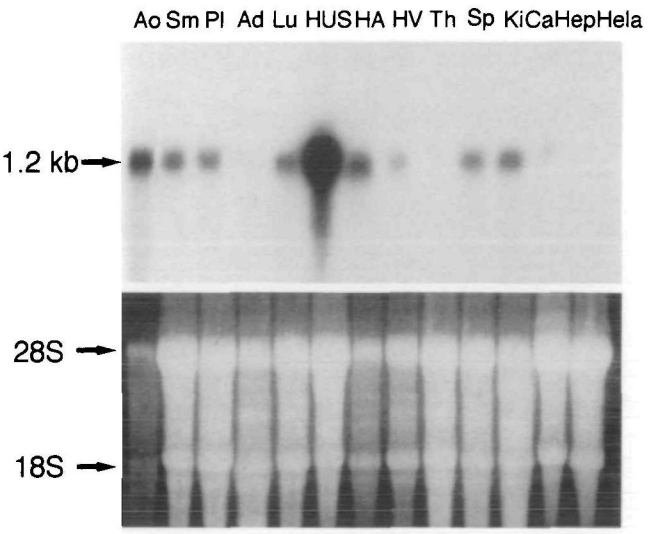

Fig. 1. **Nothern blot analysis of SM22α mRNA in human fetal tissues and the cell lines.** Each lane contains 10 μg of total RNA isolated from the indicated tissues and the cells. Ao: aorta, Sm: small intestine, Pl: placenta, Ad: adrenal cortex, Lu: lung, HUS: HUSK-1 (skeletal myoblast), HA: heart atrium, HV: heart ventricle, Th: thymus, Sp: spleen, Ki: kidney, Ca: Caco-2 (intestinal carcinoma), Hep: Hep3B (hepatocellular carcinoma), Hela: Hela (cervical carcinoma). The bottom panel shows the ethidium-stained, formaldehyde-containing gel prior to membrane transfer of RNA. The locations of the 28 S and 18 S ribosomal RNA are indicated to the left of the gel.

a

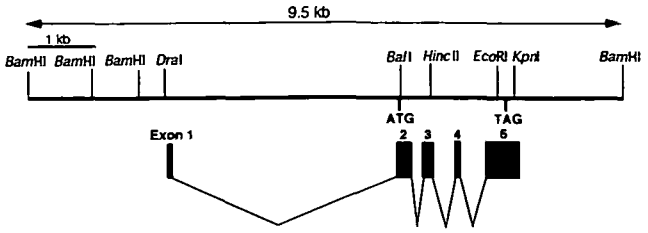

Fig. 2. **Structure of the human SM22α gene.** (a) A schematic representation of the exon-intron organization and partial restriction endonuclease map of the human SM22α gene. Exons are shown as shaded boxes. (b) The nucleotide sequence of the exons (upper-case letters) and introns (lower-case letters) and 2,159 bp of 5′-flanking sequence are shown. Some of the known regulatory elements described in the text are boxed and indicated in bold. The consensus splice donor-acceptor junctions (ag/gt) are underlined. The arrowhead indicates the 5′-terminus of the cloned cDNA sequence. Each predicted amino acid is represented by a single letter code.

b

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gatccccttcgtcccacctggtccaccccagatgctgaggatgggggagctcaggcggggcctctgctttggggatggga
atgtgtttttctcccaaacttgtttttatagctctgcttgaagggctgggagatgaggtgggtctggatcttttctcaga
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EXON 1                          ▼
ATCCTGTCTGTCCGAACCCAGACACAAGTCTTCACTCCTTCCTGCGAGCCCTGAGGAAGCCTTgtgagtgcattggctgg
ggcttggagggaagttgggct ..... ~ 4kb ..... ccgggtgaaagcagagtgctccctgaccctctgccctc
                        EXON 2
cctcctccaccctggcctgctttag CTTTCCCCAGAC ATG GCC AAC AAG GGT CCT TCC TAT GGC ATG
                                       M   A   N   K   G   P   S   Y   G   M
AGC CGC GAA GTG CAG TCC AAA ATC GAG AAG AAG TAT GAC GAG GAG CTG GAG GAG CGG CTG
 S   R   E   V   Q   S   K   I   E   K   K   Y   D   E   E   L   E   E   R   L
GTG GAG TGG ATC ATA GTG CAG TGT GGC CCT GAT GTG GGC CGC CCA GAC CGT GGG CGC TTG
 V   E   W   I   I   V   Q   C   G   P   D   V   G   R   P   D   R   G   R   L
GGC TTC CAG GTC TGG CTG AAG AAT GGC GTG gtgagtggcaccctgggctagggcgctgggggggctggggt
 G   F   Q   V   W   L   K   N   G   V
                                                                    EXON 3
gtgacccccctgtgagtcctgggccaatccctgaggactgctaagctgcgtcctatgccctatgcctggtag ATT CTG
                                                                        I   L
AGC AAG CTG GTG AAC AGC CTG TAC CCT GAT GGC TCC AAG CCG GTG AAG GTG CCC GAG AAC
 S   K   L   V   N   S   L   Y   P   D   G   S   K   P   V   K   V   P   E   N
CCA CCC TCC ATG GTC TTC AAG CAG ATG GAG CAG GTG GCT CAG TTC CTG AAG GCG GCT GAG
 P   P   S   M   V   F   K   Q   M   E   Q   V   A   Q   F   L   K   A   A   E
GAC TAT GGG GTC ATC AAG ACT GAC ATG TTC CAG ACT GTT GAC CTC TTT GAA G gtagagagg
 D   Y   G   V   I   K   T   D   M   F   Q   T   V   D   L   F   E
agaatgctgggggaggaggtgggcaggaggacagggtgctgggacagggagagggtatgaccaaatatgccacaactag
gggtgtgctcgcccgcacacagcagggatgggatatgccgagaataacacgccacgctcacagggcccactgagaggcc
tcccttgaattggggacaactcttggccctggtttggccatttttttgtgagagacgggggcaggccctggcttggagt
                                                EXON 4
cttgtttatacgttcttgatgttcatctcctctctcctgtcttctcacag  GC AAA GAC ATG GCA GCA GTG
                                                     G   K   D   M   A   A   V
CAG AGG ACC CTG ATG GCT TTG GGC AGC TTG GCA GTG ACC AAG AAT GAT GGG CAC TAC CGT
 Q   R   T   L   M   A   L   G   S   L   A   V   T   K   N   D   G   H   Y   R
GGA GAT CCC AAC TGG TTT ATG AA gtatgtggccccagggagcttgagtctccgcatggggtgggaggtgg
 G   D   P   N   W   F   M   K
cttgttctaaggagcttgcgggaaggattaggggaagcagatagccaagaaaggataaagtgagggtctgggatgggga
ataatgggtccttaatactccttgacccctcccttccacccctcctgcgctcagtctccctagcctatgaggcaagcta
gattagggaaaaaaagtgcaacaggaaggcaatgggattgggctaggacgtaacagagggatcagaaaacgggtggaaa
                                                EXON 5
acacacagttctaccaagtctttatcctgcttcctcctcttctag G AAA GCG CAG GAG CAT AAG AGG GAA
                                                  K   A   Q   E   H   K   R   E
TTC ACA GAG AGC CAG CTG CAG GAG GGA AAG CAT GTC ATT GGC CTT CAG ATG GGC AGC AAC
 F   T   E   S   Q   L   Q   E   G   K   H   V   I   G   L   Q   M   G   S   N
AGA GGG GCC TCC CAG GCC GGC ATG ACA GGC TAC GGA CGA CCT CGG CAG ATC ATC AGT TAG
 R   G   A   S   Q   A   G   M   T   G   Y   G   R   P   R   Q   I   I   S   *
AGCGGAGAGGGCTAGCCCTGAGCCCGGCCCTCCCCCAGCTCCTTGGCTGCAGCCATCCCGCTTAGCCTGCCTCACCCAC
ACCCGTGTGGTACCTTCAGCCCTGGCCAAGCTTTGAGGCTCTGTCACTGAGCAATGGTAACTGCACCTGGGCAGCTCCT
CCCTGTGCCCCCAGCCTCAGCCCAACTTCTTACCCGAAAGCATCACTGCCTTGGCCCCTCCCTCCCGGCTGCCCCCATC
ACCTCTACTGTCTCCTCCCTGGGCTAAGCAGGGGAGAAGCGGGCTGGGGGTAGCCTGGATGTGGGCCAAGTCCACTGTC
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GAACTCTCAAAAAAAA
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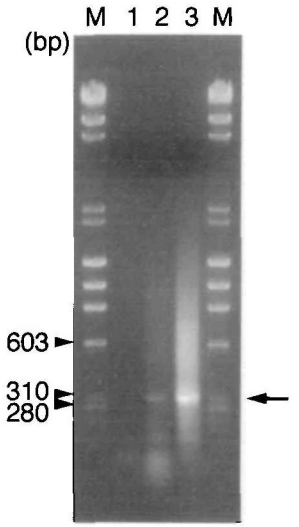

Fig. 3. **Agarose gel electrophoresis of PCR products of oligo-capped human SM22α mRNA.** Lane M: molecular weight markers (the sizes of marker bands are indicated in bp to the left of the gel). Lane 1, negative control PCR amplification in which the template cDNA was omitted, but forward and reverse primers (see text) were included. Poly(A)$^+$ RNA was prepared from primary cultures of human aortic SMC (lane 2) and adult human aortic SMC tissues (lane 3). Both RNA preparations yielded a major PCR product of approximately 300 nt that is indicated by the arrow to the right of the gel.

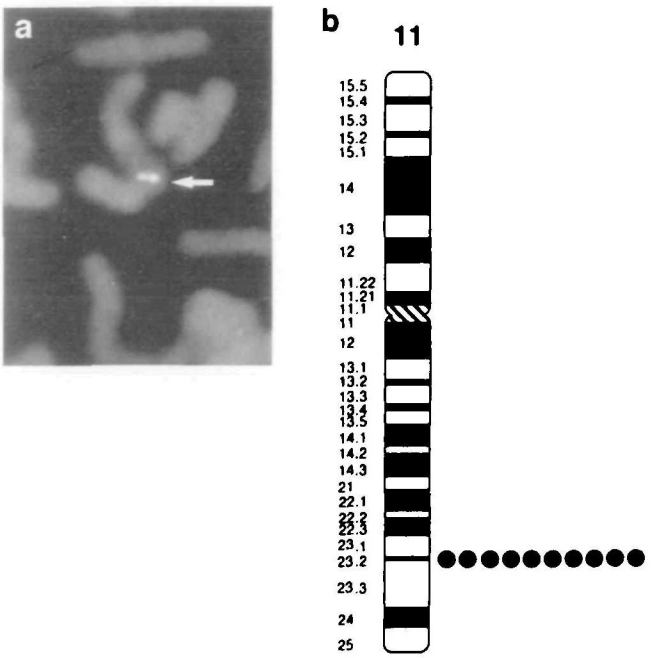

Fig. 5. **Localization of the SM22α gene at human chromosome 11q23.2 by fluorescence *in situ* hybridization.** (a) Metaphase chromosomes stained with propidium iodide showing the FITC signals at the long arm of chromosome 11 (arrow). (b) Diagram of the banding patterns in human chromosome 11, indicating the distribution of labeled sites determined based on 10 photographs by FISH analysis to chromosome region 11q23.2.

```
                                                    MEF-2-like box
human  ggaagacgcactcggggcctcagtttcctcatctataaaatggggatgtaattacaccctcacactgtagctgtgagtattcaatg.agagc.actgcaa  -514
rat    -ttct--.-----t-aa---------t-a--g--g-c---g---ctg----ctc-ta----tt-..ac----c-----g-ga--g-a--gc-.tt---tt  -487
mouse  -a---g-.----tt-aa-----a---t----a--g-t--------tg----c-g-tat----t-a-aa--..-g--acg-ga--g-.-ag--gtt----t  -467

human  agggcctgg.tgtggagtaggtcctcaggaaaggttggatcccatgtcccatcagagctaaaagccccaggaggagagggtggctggtttgtccccacaa  -415
rat    --t------.----c--cca-g-tg---tgg--ac-a-t-t---...--a-ctg--tt---------...tttca-a-a-----t-t---------...  -398
mouse  --t------t----c--cca-g-tg---tc---ac-a-t-----...--a-ctcgattttaaa.--tt-ca..---a--------t---------....  -378
                                                    MEF-2-like box

human  acccctgggattcccggctccccagcccttgcccctctctccagccagactctattgaactccccctcttctcaaactcggggccagagaacagtgaag  -315
rat    ..........-g-aa-----ta--a-gt--...----g--gg----a-t-----a--g-a--t----c-t--ttt---att-.a-a---c-g-ccc---  -314
mouse  ..........--g-a--.....---...---t-g--gg----a-----..----tg--t--c-c-t--ttt---att-.a---c-g-ccc---  -306
                                CArG/SRF-box                                    GC-box/Sp1-binding site
human  taggagcagc.cgtaagtccgggcagggtcctgtccataaaaggctttcccgggccggct.cccgccggcagcgtgccccgccccg..gcccgctccat  -219
rat    -c--g-----aa-g---.gtttt-gt-------ccataaaaggt--------.....cca--ct-a---c-..---ccgcccc.aac-----ag---  -225
mouse  -cc-g-t-a-aa-g---.ggttt----------ccataaaaggt-------.-----....--ct-a---c-..---ccgcccc-acc-----ag---  -215
                                                                CArG/SRF-box
human  ctccaaagcatgcagagaatgtctc.ggcag.ccccggtagactgctccaacttggtgtctttccccaaatatggagcctgtgtggagtcactgggggag  -121
rat    -------------------------g---t-c-----ac-------------------------ccaaatatgg-------------g-g-----c--  -125
mouse  -------------------------c---t-c-----ac-------------------------ccaaatatgg-------------g-g-----cg-  -115

human  ccgggg.............gtggggagcggagccggcttcctctagcagggaggggg.ccgaggagcgagccagtgggggaggctgacatcaccacggcg  -35
rat    -t----cgggtggggtggt-----a---ca---a-a-----atgg-----ag--c-...-....--t-.-g---gca-a----.---------tg-ct--  -34
mouse  --c---.............----t----ca---a-a-----atgg---g-------c---...----.-a-g-c-a---g-..---------tg-cta-  -35
          TATA-like box                    transcription start site                                exon 1
human  gcagccctttaaaccctcacccagccagcgcccgATCCTGTCTGTCCGAACCCAGACA.CAAGTCTTCACTCCTTCCTGCG.AGCCCTGAGGAAGCCTT  +63
rat    -.--g--tttaaa-------------g-------|G--C-----CA-C-G---------G---CG--A-T---------A---CC-CAAAC--CC-------|  +65
mouse  --.-g--tttaaa-------------g-------|G--C-----C--C-G---------CCGAAG--A-T---------A-TCC-CAAAC--CC-------|  +64
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Fig. 4. **Comparison of the 5′-flanking regulatory and exon 1 regions of human, rat, and mouse SM22α genes.** Hyphens indicate conserved nucleotides between SM22α sequences from human and rat (*6*), or human and mouse (*5*). Gaps (.) were introduced for optimal alignment. The TATA-like box, CArG/SRF-box, GC-box/Sp1-binding site, and MEF-2-like sequence are boxed and shown in bold. The start of transcription (+1) and the entire sequence of exon 1 that constitutes a portion of the 5′-untranslated region is boxed and denoted by shadowing.

somes. Based on DAPI banding patterns, the SM22α gene was assigned to the long arm of chromosome 11 (Fig. 5a). Its precise position at human chromosome 11q23.2 was further determined based on a summary of 10 photographs (Fig. 5b). Such specific accumulation of the signals could not be detected on any other chromosome.

Expression of SM22α mRNA in Cultured SMC and Non-SMC Lines—We next examined the expression of SM22α mRNA in various murine-derived cultured cells including PASMC (*20*), undifferentiated skeletal muscle-lineage cells (C2C12 and BC3H1), and fibroblasts (NIH3T3 and C3H10T1/2) in several growth conditions (Fig. 6). All of these cells expressed one predominant SM22α mRNA transcript of approximately 1.2 kb in the serum-stimulated and subconfluent culture condition. Following growth arrest by serum-deprivation for 3 days, the amount of SM22α mRNA is increased up to 4-fold in SMC, but decreased in the skeletal muscle-lineage cells and fibroblasts. As shown in Fig. 6, BC3H1 cultures in the growth condition (10% FCS), harvested at 3 days postconfluence, had SM22α mRNA levels 8-10-fold lower than those of exponentially proliferating cultures, suggesting that down-regulation of the SM22α transcript in these cells may be related to withdrawal from the cell cycle.

Downregulation of SM22α Expression in Diseased Human Arteries—Directional cloning of the human SM22α coding region into the pGEX 3X expression plasmid provided a system for synthesizing the GST-SM22α fusion protein. Human SM22α polypeptide with an apparent molecular weight of 22,000 was purified from supernatant fraction of the cell lysates on a glutathione-Sepharose affinity column, followed by digestion with Factor Xa (Fig. 7). Cross-reactivity of a polyclonal anti-gizzard SM22α antibody to the purified human SM22α polypeptide was verified by immunoblot analysis (Fig. 7). Using this antibody as a probe, immunoreactive forms of SM22α were detected in medial SMC layers of human coronary arteries at 6 days after balloon angioplasty. The SM22α protein was expressed in some but not all of the α-SMC actin-positive cells in the inner half of the medial SMC layer where SMC underwent undifferentiation (Fig. 8). This heterogeneous pattern of SM22α expression in α-SMC actin positive cells was also found at the mRNA level in both the media and the intima of atheromatous human coronary arteries (*25*).

Repression of The Promoter Activity by Specific CpG Methylation—In order to identify the underlying mechanisms that regulate transcription of the SM22α gene in SMC- and undifferentiated skeletal muscle-lineage cells, a series of transient transfections were performed. The SM22α promoter-luciferase reporter gene constructs (pGL2-455SM22) were transfected into a SMC-lineage cell PASMC and an undifferentiated skeletal muscle-lineage cell C2C12, both of which express high levels of the SM22α mRNA (Fig. 6). We used a 5′-flanking 455-bp fragment,

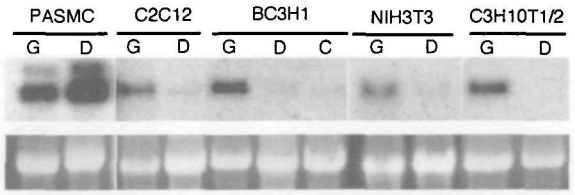

Fig. 6. **SM22α mRNA expression in a variety of cell lines detected by Northern blot analysis.** The top panel shows a Northern blot analysis of total cellular RNA (15 μg) isolated from rat pulmonary artery SMC (PASMC), mouse skeletal muscle myoblasts (C2C12 and BC3H1), and mouse fibroblast (NIH3T3 and C3H10T1/2). G and D indicate serum-stimulated (undifferentiated) and differentiated cells, respectively. The conditions for differentiation are described under "EXPERIMENTAL PROCEDURES." The bottom panel shows 18 S ribosomal RNA on the ethidium-stained, formaldehyde-containing gel prior to membrane transfer of RNA.

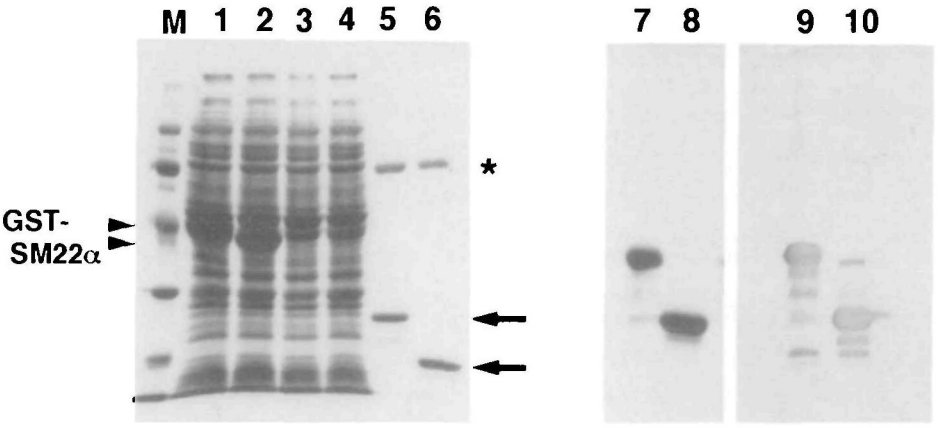

Fig. 7. **Bacterial protein expression and antibody characterization.** Coomassie Blue-stained SDS, 12.5% polyacrylamide gel showing purification of the human SM22α polypeptide (*lanes 1 to 8*). GST-SM22α fusion proteins from the full length cDNA clone (nt. 52 to 1,064) (lanes 1, 3, 5, and 7) or the truncated cDNA clone (nt. 52 to 531 at the *Eco*RI site) (lanes 2, 4, 6, and 8) were induced by addition of 0.1 mM IPTG for 4 h in 2 liter cultures of transformed *E. coli*. All subsequent procedures were performed at 4°C. The bacteria was collected by centrifugation at 4,000×*g* for 10 min. The pellet was dissolved in 30 ml of PBS containing 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM sodium tetrathionate, and then sonicated for 30 s. After addition of Triton X-100 to a final concentration of 1%, the homogenate was centrifuged at 20,000×*g* for 30 min. The supernatant fraction (lanes 1 and 2) was applied to a glutathione-Sepharose 4B (10 ml) affinity column. Following complete removal of the flow-through fraction (lanes 3 and 4), human placenta Factor Xa [20 U in 1 ml of Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM $CaCl_2$] was applied to the column, and the solution was circulated for 10 h at 4°C. The eluted fraction (lanes 5 and 6; arrows indicate full length and truncated SM22α polypeptides) was applied to the DE52 (Pharmacia Biotech.) ion exchange column (1×4 cm) to remove BSA derived from Factor Xa suspension (indicated by an asterisk [*]). The human SM22α polypeptides in the flow-through fraction from the DE52 column were dialyzed against 20 mM sodium acetate buffer (pH 5.6) containing 0.5 mM DTT and 0.1 mM EGTA, and further purified by Mono S FPLC column chromatography (Pharmacia Biotech.) (lanes 7 and 8). In the immunoblot analysis, the polyclonal antibody raised against chicken gizzard SM22α cross-reacted with both the full length (lane 9) and the truncated (lane 10) human SM22α polypeptides. Lane M, molecular weight markers; phosphorylase *b* (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100).

spanning from bp −26 to −480, which corresponds to a region of the mouse and rat SM22α promoters required to direct the maximum transcriptional activity (*5, 6, 10, 11*). Transfection of both PASMC and C2C12 myoblasts with the plasmid pGL2-455SM22 resulted in 200- to 300-fold induction in luciferase activity compared to the promoterless control plasmid, pGL2-Basic. This level of transcriptional activity was approximately 50% of that obtained following transfection of PASMC and C2C12 myoblasts with the Rous sarcoma virus promoter-containing luciferase reporter plasmid, pRSV-luc (data not shown). The proximal 455-bp segment of the human SM22α gene contains two CArG/SRF-boxes located at bp −147 and −274, and one GC-box/Sp1-binding site located at bp −233, which are perfectly conserved with the mouse and rat SM22α genes (Fig. 4). Interestingly, DNA sequence analysis revealed that a cluster of the CpG dinucleotides, a *cis*-acting element for specific DNA methylation, is present around the two CArG/SRF boxes and the proximal GC-box/Sp1-binding site (Fig. 9). The nucleotide sequences of the 5′-flanking 1,200-bp region of the human, rat and mouse SM22α genes are characterized by low density of the CpG dinucleotides with a CpG/GpC ratio of 0.39, 0.29, and 0.39, respectively. These values are close to the density in the genome as a whole (CpG/GpC ~0.2) that characterizes tissue-specific genes (*26*). A number of previous studies have established that DNA methylation in the promoter region can reduce the rate of transcription of genes (*26-28*). Recent studies suggest that repression of the genes by promoter methylation may be mediated *via* a

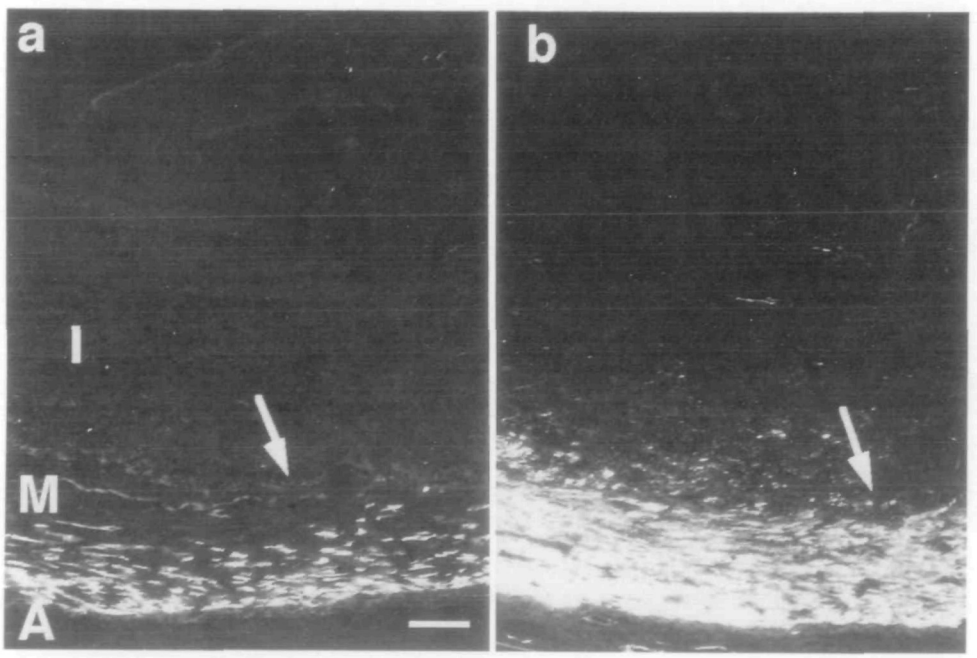

Fig. 8. **Expression of SM22α protein in the diseased human artery.** Serial sections of frozen human coronary artery at 6 days after balloon angioplasty were labeled with a polyclonal antibody raised agaist chicken gizzard SM22α (a) or with a monoclonal antibody to SM α-actin (b). Note that the SM22α expression in media appears to be patchy with only some of the SM α-actin positive cells expressing high levels of SM22α protein adjacent to SMC expressing no detectable SM22α protein. The arrows indicate the location of the elastic lamina. The scale bar represents 100 μm. I: intima, M: media, and A: adventitia.

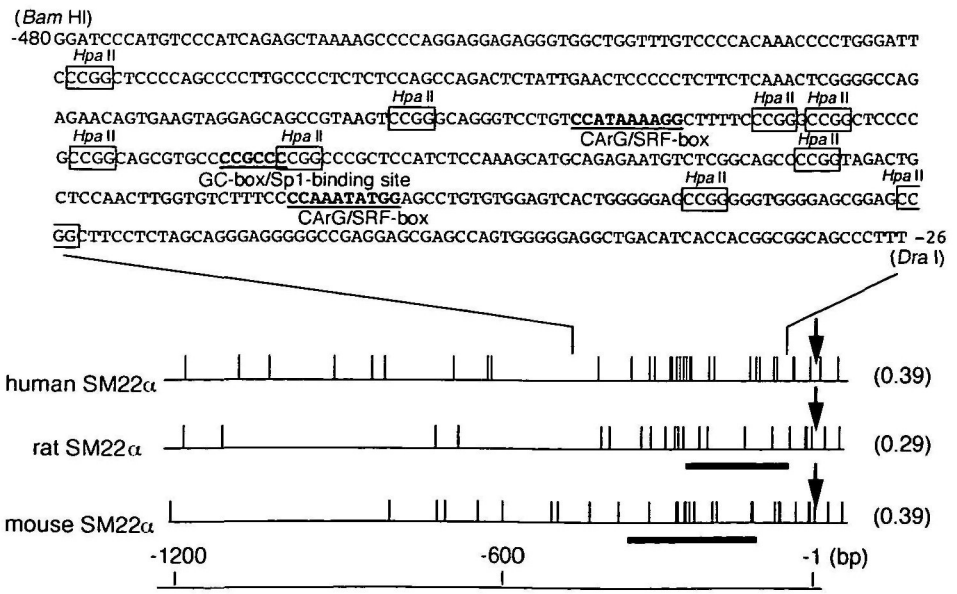

Fig. 9. **Distribution of the CpG dinucleotides within the 1,200-bp 5′-flanking region of the human, rat, and mouse SM22α genes.** The nucleotide sequence of the 455-bp segment of the human SM22α promoter is shown. *Hpa*II methylation sites are boxed and indicated. Two CArG/SRF-boxes and the proximal GC-box/Sp1-binding site are underlined and denoted in bold. The start of transcription is denoted by arrows. The underlined segment of the rat and mouse SM22α genes indicates the minimum promoter region required for optimal expression in SMC cultures (*5, 6*). The numbers in parenthesis represent CpG/GpC ratio within the 1,200-bp 5′-flanking sequence of each gene.

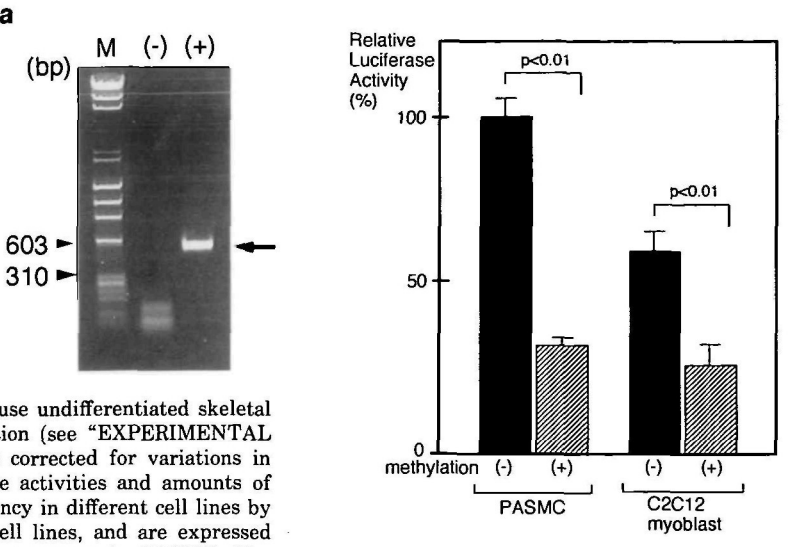

Fig. 10. **Repression of SM22α promoter activity by cytosine DNA methylation.** (a) The SM22α promoter sequence from bp −26 to −480 was excised from the luciferase reporter plasmid (pGL2-455SM22) by *Xho*I/*Xba*I digestion. The purified fragment containing the 455-bp promoter sequence was incubated for 1 h at 37°C in the absence (minus) and the presence (plus) of *Hpa*II methylase. Complete methylation of all of the nine *Hpa*II sites was confirmed by the protection against the *Hpa*II digestion (arrow), and then the fragment was ligated to the *Xho*I/*Xba*I-digested pGL2-Basic luciferase reporter plasmid. Lane M: molecular weight markers (the sizes of marker bands are indicated in bp to the left of the gel). (b) The methylated (hatched bar) or unmethylated (black bar) SM22α-luciferase construct was transfected into rat pulmonary artery SMC (PASMC) and mouse undifferentiated skeletal muscle myoblasts (C2C12) in the growing culture condition (see "EXPERIMENTAL PROCEDURES"). Luciferase activities (light unit) were corrected for variations in transfection efficiencies as determined by β-galactosidase activities and amounts of protein. Activities were normalized for transfection efficiency in different cell lines by comparing the activities of pSV2-luc gene in different cell lines, and are expressed relative to that of the unmethylated SM22α-luciferase construct in PASMC. The experiment was repeated four times, and data are means±SE ($n=6$) from a representative experiment. Statistical analysis was performed using the unpaired Student's *t* test.

methyl-CpG binding protein that binds to 5′-methylcytosines by direct inhibition of the binding of transcription factors (*29, 30*). We therefore examined the effects of DNA methylation at the specific CpG dinucleotides in the 455-bp promoter sequence by treatment with *Hpa*II DNA methylase. Within the 455-bp segments, there are nine *Hpa*II methylation sites (CCGG) located at bp −99, −118, −186, −230, −248, −254, −264, −296, and −400 (Fig. 9). When the SM22α promoter was methylated, the promoter activity was significantly reduced to 32.8±1.3% of the control value in SMC ($p<0.01$) and 25.5±7.4% of the control value in C2C12 myoblast ($p<0.01$) (Fig. 10, a and b).

DISCUSSION

In this study, we report the isolation and structural characterization of the human SM22α gene, which is mapped to the chromosome region 11q23.2. The exon-intron organization and the 5′-flanking promoter sequence of the human SM22α gene exhibit a high degree of evolutionary conservation. The SM22α mRNA is expressed in SMC and non-SMC cultures in a cell growth-regulated manner. We have shown that the level of expression of SM22α in SMC was reduced when SMC underwent undifferentiation and proliferation in both cultured SMC lines and arterial tissues. In contrast, in non-SMC mesenchymal cells, the SM22α gene was expressed in proliferating cells but was down-regulated in quiescent cultures. An important question regarding control of the SM22α promoter is what mechanisms repress the promoter activity in extra-SMC tissues *in vivo* as well as in growth-arrested non-SMC lines *in vitro*, and activate the promoter during differentiation of SMC. Our present experiments demonstrate that methylation of the specific CpG sites within the 455-bp 5′-flanking sequence of the human SM22α gene mediates transcriptional repression in SMC and undifferentiated skeletal muscle-lineage cells.

The SM22α gene promoter contains two 10-bp sequence motifs, $CC(A/T)_6GG$ (CArG/SRF-box) which are 100% conserved between mouse (*5*), rat (*6*), and human genes. The CArG/SRF motif is a *cis*-acting element which regulates skeletal or cardiac muscle-specific expression of the actin gene, basal expression of the non-muscle actin gene, and serum-induced transient expression of the *c-fos* gene (*31, 32*). Recent studies indicate that two highly conserved CArG/SRF-boxes of the SM α-actin promoter mediate serum-induced activation of this gene in fibroblasts (*33*), vascular SMC and mesangial cells (*34*). In addition, transcriptional activation of the chicken caldesmon gene in cultured SMC and chicken embryonic fibroblasts has been shown to be mediated by a single CArG box-like motif within the promoter (*35*). These studies with cultured cells suggest that the CArG/SRF motif is an essential *cis*-element for optimal expression of the SMC lineage-specific gene promoters. However, candidate CArG box-binding nuclear factors or regulatory factors capable of specifically inducing the SMC promoter activity have not yet been characterized. Also, relatively little is known about transcriptional mechanisms underlying repression of the SMC differentiation-specific genes. The results of this study showed that methylation of the CpG sites clustered around conserved elements of the CArG/SRF-boxes and GC-box/Sp1-binding site of the human SM22α promoter reduces its transcriptional activity. Methylation of cytosine in the CpG dinucleotides is often associated with repression of the gene expression (*26-28*). In such cases, critical methylation sites typically have been located in or around functional promoter sequences at the 5′ end of the genes (*28, 29*). In the case of the mouse SM22α gene, two CArG/SRF-boxes in the promoter region have been reported to be essential for transcriptional activity (*11*). It has also been shown that a GC-box/Sp1-binding site in the mouse adenine phosphoribosyltransferase gene promoter is required to protect a CpG island from *de novo* methylation (*36, 37*). Site-specific DNA methylation may therefore reduce the SM22α promoter activity by blocking binding to the two CArG/SRF-boxes or the proximal GC-box/Sp1-

binding site of nuclear factors required for optimal transcription. The promoter activity of the SM22α gene was not fully inactivated by methylation of the *Hpa*II sites (Fig. 10b), raising the possibility that methylation of the CpG dinucleotides other than the *Hpa*II sites (CCGG) may have additional suppressive effects. This seems unlikely because the activity of the SM22α promoter fully methylated by CpG (Sss) methylase was reduced to comparable levels to that of the promoter methylated by the *Hpa*II methylase (unpublished observation). It is possible that additional regulatory mechanisms for silencing the SM22α promoter may exist.

Previous studies demonstrated that SM22α mRNA is expressed in a variety of cultured non-SMC lines including fibroblasts, skeletal muscle myoblasts, and embryonal carcinoma cell line (*4-6, 10*). These results are consistent with our observation that the SM22α mRNA is expressed in C2C12, BC3H1, NIH 3T3 cells, and C3H10T1/2 cells by Northern blot analysis using the cloned human SM22α full length cDNA as a probe. However, in contrast to the previous report (*10*), we showed that expression of the SM22α transcript in these non-SMC cultures was regulated by cell growth and proliferation (Fig. 6). When exponentially growing cells were made quiescent by serum deprivation or confluency, the SM22α expression was decreased in terms of steady-state mRNA levels (Fig. 6). Analogous to serum-induced upregulation of the SM α-actin gene in cultured non-SMC lines (*33, 34*), induction of the SM22α mRNA expression during G_0/G_1 transition of NIH3T3 fibroblast was originally described by Almendral *et al.* (*4*). They showed that transcription of the SM22α gene was dramatically increased within 15 min of serum stimulation, reaching maximal levels at around 1 h and slowly decreasing until 8 h, suggesting that induction of the SM22α gene is a direct consequence of a signaling mechanism triggered by a ligand-receptor interaction (*4*). It seems likely that the cell cycle-dependent induction of the SMC gene expression in non-SMC mesenchymal cells is not restricted to the SM α-actin gene, but rather is a commom feature among the members of the SMC differentiation-specific gene family.

Although the SM22α transcript is detected in cultured cell lines derived from different origins, SM22α expression *in vivo* has been shown to be restricted to SMC in adult mouse tissues (*5, 8*) and chicken embryos (*9*). The discrepancy between the *in vitro* and the *in vivo* patterns of expression of this gene was also noted in the analysis of the promoter activity of the murine SM22α gene. The mouse *SM22α promoter-lacZ* transgene, containing a 5'-flanking 2,735-bp segment plus a 1,093-bp segment of the first intron, that is active in a variety of cultured cell lines (*10*), has been shown to be expressed exclusively in SMC during embryogenesis of transgenic mice (*10*). Moreover, in contrast to the endogenous gene, expression of this *SM22α-lacZ* transgene was restricted to the arterial SMC of large vessels, and was not detected in venous or visceral SMCs (*10*). Similarly, the *SM22α promoter-lacZ* transgene containing 441 bp of the mouse SM22α 5'-flanking sequence was active solely in vascular, but not visceral SMCs of transgenic mice (*11*). Results in other systems show that an all-or-none behavior of the transcriptional control can arise at the level of chromatin structure (*37-39*). DNA methylation which affects chromatin structure (*27, 28, 40*) is a candidate control mechanism that could confer higher orders of regulation upon the SM22α gene promoters. Because we recently identified a *cis*-acting domain for interaction with a methyl-cytosine binding protein in the 5'-flanking promoter region of the human calponin gene (*41*), a member of the SMC differentiation-specific gene family (*7, 9, 42-44*), DNA methylation, independent of sequence context of each promoter, may also explain the coordinate expressional regulation of the SMC differentiation-specific genes. In fact, we have evidence that regional DNA methylation of the human SMC calponin gene had a strong inhibitory effect on gene expression (unpublished observation). While these results are suggestive, further studies demonstrating a distinct difference in the methylation levels at the specific CpG sites within the SM22α promoter between expressing and non-expressing cells, will be required to uncover the physiological role of DNA methylation in the regulation of SMC-specific gene promoters. The cloned human SM22α gene reported here will be a useful tool for such experiments.

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REFERENCES

1. Sappino, A.P., Schurch, W., and Gabbiani, G. (1990) Differentiation repertoire of fibroblastic cells: Expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.* **63**, 144-161
2. Owens, G.K. (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75**, 487-517
3. Shah, N.M., Groves, A.K., and Anderson, D.J. (1996) Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85**, 331-343
4. Almendral, J.M., Santaren, J.F., Perera, J., Zerial, M., and Bravo, R. (1989) Expression, cloning and cDNA sequence of a fibroblast serum-regulated gene encoding a putative actin-associated protein (p27). *Exp. Cell Res.* **181**, 518-530
5. Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morriesey, E.E., Ip, H.S., and Parmacek, M.S. (1995) Structure and expression of a smooth muscle cell-specific gene, SM22α. *J. Biol. Chem.* **270**, 13460-13469
6. Kemp, P.R., Osbourn, J.K., Grainger, D.J., and Metcalf, C. (1995) Cloning and analysis of the promoter region of the rat SM22α gene. *Biochem. J.* **310**, 1037-1043
7. Takahashi, K. and Nadal-Ginard, B. (1991) Molecular cloning and sequence analysis of smooth muscle calponin. *J. Biol. Chem.* **266**, 13284-13288
8. Li, L., Miano, J.M., Cserjesi, P., and Olson, E.N. (1996) SM22α, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ. Res.* **78**, 188-195
9. Duband, J.-L., Gimona, M., Scatena, S., Sartore, S., and Small, J.V. (1993) Calponin and SM22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* **55**, 1-11
10. Li, L., Miano, J.M., Mercer, B., and Olson, E.N. (1996) Expression of the SM22α promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol.* **132**, 849-859
11. Kim, S., Ip, H.S., Lu, M.M., Clendenin, C., and Parmacek, M.S. (1997) A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages.

Mol. Cell Biol. **17**, 2266-2278
12. Lees-Miller, J.P., Heeley, D.H., Smillie, L.B., and Kay, C.M. (1987) Isolation and characterization of an abundant and novel 22-kDa protein (SM22) from chicken gizzard smooth muscle. *J. Biol. Chem.* **262**, 2988-2993
13. Takahashi, K., Hiwada, K., and Kokubu, T. (1987) Occurrence of anti-gizzard p34K antibody cross-reactive components in bovine smooth muscles and non-smooth muscle tissues. *Life Sci.* **41**, 291-296
14. Towbin, H., Staehlin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
15. Pearlstone, J.R., Weber, M., Lees-Miller, J.P., Carpenter, M.R., and Smillie, L.B. (1987) Amino acid sequence of chicken gizzard smooth muscle SM22α. *J. Biol. Chem.* **262**, 5985-5991
16. Stallcup, M.R. and Washington, L.D. (1983) Region-specific initiation of mouse mammary tumor virus RNA synthesis by endogenous RNA polymerase II in preparations of cell nuclei. *J. Biol. Chem.* **258**, 2802-2807
17. Tomatsu, S. and Sasaki, Y. (1989) The organization and the complete nucleotide sequence of the human NADH-cytochrome b5 reductase gene. *Gene* **80**, 353-361
18. Maruyama, K. and Sugano, S. (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138**, 171-174
19. Heng, H.H.Q., Squire, J., and Tsui, L.-C. (1992) High resolution mapping of mammalian genes by in situ hybridization to free chromatin. *Proc. Natl. Acad. Sci. USA* **89**, 9509-9513
20. Rothman, A., Kulik, T.J., Taubman, M.B., Berk, B.C., Smith, C.W.J., and Nadal-Ginard, B. (1992) Development and characterization of a cloned rat pulmonary arterial smooth muscle cell line that maintains differentiated properties through multiple subcultures. *Circulation* **86**, 1977-1986
21. Thweatt, R., Lumpkin, C.K., Jr., and Goldstein, S. (1992) A novel gene encoding a smooth muscle protein is overexpressed in senescent human fibroblasts. *Biochem. Biophys. Res. Commun.* **187**, 1-7
22. Smale, S.T. and Baltimore, D. (1989) The "initiator" as a transcription control element. *Cell* **57**, 103-113
23. Briggs, M.R., Kadonaga, J.T., Bell, S.P., and Tjian, R. (1986) Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234**, 47-52
24. Gossett, L.A., Kelvin, D.J., Sternberg, E.A., and Olson, E.N. (1989) A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell. Biol.* **9**, 5022-5033
25. Shanahan, C.M., Cary, N.R.B., Metcalf, J.C., and Weissberg, P.L. (1994) High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J. Clin. Invest.* **93**, 2393-2402
26. Razin, A. and Kafri, T. (1994) DNA methylation from embryo to adult in *Progress in Nucleic Acid Research and Molecular Biology* (Cohn, W.E. and Moldave, K., eds.) Vol. 48, pp. 53-81, Academic Press, San Diego
27. Doerfler, W. (1983) DNA methylation and gene activity. *Annu. Rev. Biochem.* **52**, 93-124
28. Bird, A. (1992) The essentials of DNA methylation. *Cell* **70**, 5-8
29. Watt, F. and Molly, P.L. (1988) Cytosine methylation prevents binding to DNA of a Hela cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev.* **2**, 1136-1143
30. Boyes, J. and Bird, A. (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: Evidence for involvement of a methyl-CpG binding protein. *EMBO J.* **11**, 327-333
31. Muscat, G.O., Gustafson, T.A., and Treisman, R. (1988) A common factor regulated skeletal and cardiac α-actin gene transcription in muscle. *Mol. Cell Biol.* **8**, 4120-4133
32. Treisman, R. (1985) Structure and function of serum response factor in *Transcriptional Regulation* (McKnight, S.L. and Yamamoto, K.R., eds.) pp. 881-905, Cold Spring Harbor Laboratory Press, New York
33. Kim, J.-H., Bushel, P.R., and Kumar, C.C. (1993) Smooth muscle α-actin promoter activity is induced by serum stimulation of fibroblast cells. *Biochem. Biophys. Res. Commun.* **190**, 1115-1121
34. Simonson, M.S., Walsh, K., Kumar, C.C., Bushel, P., and Herman, W.H. (1995) Two proximal CArG elements regulate SM α-actin promoter, a genetic marker of activated phenotype of mesangial cells. *Am. J. Physiol.* **268**, F760-F769
35. Yano, H., Hayashi, K., Momiyama, T., Saga, H., Haruna, M., and Sobue, K. (1995) Transcriptional regulation of the chicken caldesmon gene. *J. Biol. Chem.* **270**, 23661-23666
36. Brandeis, M., Frank, D., Keshet, L., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994) Sp1 elements protect a CpG island from de novo methylation. *Nature* **371**, 435-438
37. Macleod, D., Charlton, J., Mullins, J., and Bird, A.P. (1994) Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. *Genes Dev.* **8**, 2282-2292
38. Boyes, J. and Felsenfeld, G. (1996) Tissue-specific factors additively increase the probability of the all-or-none formation of a hypersensitive site. *EMBO J.* **15**, 2496-2507
39. Felsenfeld, G. (1996) Chromatin unfolds. *Cell* **86**, 13-19
40. Keshet, I., Lieman-Hurwitz, J., and Cedar, H. (1986) DNA methylation affects the formation of active chromatin. *Cell* **44**, 535-543
41. Takahashi, K., Tazunoki, T., Okada, T., Ohgami, K., Miwa, T., Miki, A., and Shibata, N. (1996) The 5′-flanking region of the human smooth muscle cell calponin gene contains a *cis*-acting domain for interaction with a methylated DNA-binding transcription repressor. *J. Biochem.* **120**, 18-21
42. Takahashi, K., Masuda, H., Takagi, M., Eshita, Y., and Shibata, N. (1995) Structure and expression of vascular smooth muscle calponin in *Regulation of the Contractile Cycle in Smooth Muscle* (Nakano, T. and Hartshorne, D.J., eds.) pp. 201-209, Springer-Verlag, Tokyo
43. Winder, S.J. and Walsh, M.P. (1996) Calponin. *Curr. Topics Cell. Regul.* **34**, 33-61
44. Gimona, M. and Small, J.V. (1995) Calponin in *Biochemistry of Smooth Muscle Contraction* (Barany, M., ed.) pp. 91-103, Academic Press, New York