J. Biochem. **120**, 18-21 (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[1]

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Received for publication, May 7, 1996

The human smooth muscle cell (SMC) calponin gene, which is composed of seven exons and six introns, spanning an approximately 11.2 kilobase (kb) genomic DNA, has been isolated and characterized by sequence analysis. As determined by primer extension mapping and rapid amplification of mRNA transcripts, a major transcription start site of the calponin gene is located at 101 base-pairs (bp) upstream of the ATG start codon. A striking feature of the 5′-flanking, 5′-untranslated, and amino-terminal protein coding regions is the presence of an *Alu* repetitive sequence and a stretch of DNA sequence identified using a methyl CpG-binding protein affinity column [Cross, S.H., Charlton, J.A., Nan, X., and Bird, A.P. (1994) *Nature Genet.* 6, 236-244]. The results of this study provide a new insight into the molecular mechanism underlying regulation of SMC-lineage specific gene expression.

Key words: actin-binding protein, *Alu* repetitive sequence, calponin, CpG island, DNA methylation, smooth muscle-specific gene.

Calponin was originally isolated as an actin-associated protein from chicken gizzard and bovine aortic SMC (*1-3*). Structural analysis of cDNAs encoding calponin isoforms has revealed the presence of three types of genes with distinct expressional regulation (*4-9*). Each of the three calponin genes encodes distinct classes of isoforms categorized into acidic (pI 5-6), neutral (pI 7-8), and basic (pI 8-10) calponins on the basis of their isoelectric points. The basic calponin gene [also called the calponin-h1 gene (*5*)] encodes an originally isolated calponin isoform, which is predominantly SMC-specific (*4, 7, 9-12*). In addition, the α (high molecular)- and β (low molecular)-isoforms encoded by the SMC calponin gene have been found as products of alternative mRNA splicing in chicken smooth muscle tissues (*4, 8*). More recently, cDNA clones encoding a novel acidic calponin isoform with a deduced 330-amino-acid polypeptide have been isolated, and shown to be expressed in both smooth muscle and extra-smooth muscle tissues of adult rats (*6, 13*). The neutral calponin is the equivalent of calponin-h2 (*5*), isolated from mouse uterus and porcine stomach smooth muscle tissues. In recent reports, we showed that a human equivalent of mouse calponin-h2 is also expressed in both smooth muscle and extra-smooth muscle tissues (*14, 15*).

Previous studies have suggested that a SMC-specific isoform of calponin may play an auxilliary regulatory role in smooth muscle contraction (*16-19*), but, when costitutively expressed, it may also have cell proliferation-inhibitory activity independent of contractility (*20, 21*). Regulated expression of the SMC calponin gene has been reported during the development and differentiation of SMC (*7, 9-12*). Furthermore, down regulation of calponin expression has been found in hyperplastic and de-differentiated SMC in diseased human arteries, such as in atherosclerosis and restenosis following balloon angioplasty (*22*). It is likely that elucidation of the *cis*-acting elements and *trans*-acting factors that regulate expression of the human SMC calponin gene may provide a great deal of information concerning the physiology of SMC differentiation and the pathogenesis of vascular disorders at the molecular level.

A full length cDNA clone encoding human SMC calponin (basic calponin) was isolated from aortic cDNA libraries (Stratagene, La Jolla, USA) constructed in the λZAP II vector, using the random primed ^{32}P-labeled *Sac*I-*Sma*I fragment of chicken gizzard calponin β cDNA (*4*) as a probe. The nucleotide sequence of the cDNA was determined on both strands with an Applied Biosystem Model 373A DNA sequencer according to the manufacturer's instructions, and was deposited in the DDBJ/GenBank™/EMBL database (accession No. D17408). Using the random primed ^{32}P-labeled full length cDNA (1,536 bp) as a probe, we screened 5×10^5 plaques of a human genomic library (Stratagene) constructed in the λDASH vector prepared from male Caucasian leucocytes. Prehybridization and hybridization were performed in a solution comprizing 5× SSC, 5× Denhardt's solution, and 1% SDS at 65°C for 12 h.

[1] This work was supported in part by Research Grants for Cardiovascular Diseases (5-2, 6-2, and 6-6) from the Ministry of Health and Welfare of Japan. The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EMBL Data Bank with accession number D85611.

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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. Two hybridized clones were isolated, representing two distinct classes of overlapping clones on the basis of the insert size. The purified phage DNA was subcloned into the pBluescript plasmid vector (Stratagene) for restriction endonuclease mapping and sequencing. A 13.7 kb DNA fragment containing the 5′ flanking region and the entire coding sequence of the human SMC calponin gene was sequenced through the construction of restriction endonuclease fragment subclones and ExoIII nuclease-digested subclones using an Erase-A-Base kit (Promega, Madison, USA), as well as synthetic oligonucleotide primers. DNA sequencing was carried out on both strands using an Applied BioSystem model 373A DNA sequencer.

Two independent assays, primer extension and the 5′-RACE protocol (*23*), were used to identify the transcription initiation site of SMC calponin. Total RNA was prepared from liquid nitrogen-frozen adult human aortic smooth muscle tissue using an ISOGEN RNA purification kit (Wako Pure Chemicals, Osaka). A 42-nt oligonucleotide primer (5′-AGAGGCAGC GGCTGAAGTTCCGTCTGCA-CACTCTTCCCTCCT-3′) was synthesized as the antisense sequence of exon 1 (nt Nos. 12 to 53) and 5′-end-labeled with [γ-^{32}P]ATP. Reverse transcription of 20 μg of the total RNA was carried out using the reaction mixture of a first-strand cDNA synthesis kit (Pharmacia Biotech Inc., Uppsala, Sweden) in the presence of 10 pmol of the ^{32}P-labeled primer. After 60 min incubation at 37°C, the reaction product was extracted with phenol/chloroform, precipitated with ethanol, and then run on a 6% acrylamide sequencing gel. Mapping of the 5′ end of the human SMC calponin mRNA was also carried out by 5′-specific primer extension and cloning using 1 μg of total RNA and a 5′ RACE System (Life Technologies, Gaithersburg, USA).

Two positive λDASH phage clones were isolated and sequenced, representing two distinct classes of overlapping clones which contain the entire human SMC calponin gene. It is composed of seven exons and six introns, spanning approximately 11.2-kb of genomic DNA. The nucleotide sequences and precise exon-intron organization of the gene will be reported in an accompanying paper (in preparation). As shown in Fig. 1, the primer extension assay involving RNA from aortic smooth muscle tissue revealed a major transcriptional initiation site located at 101-bp upstream of the ATG translational initiation codon, which hereafter will be designated as +1. A weak band was observed for 103-bp upstream of the translation initiation codon. Similarly, two major 5′-RACE products extended to the same sites as those obtained by the primer extension method (data not shown).

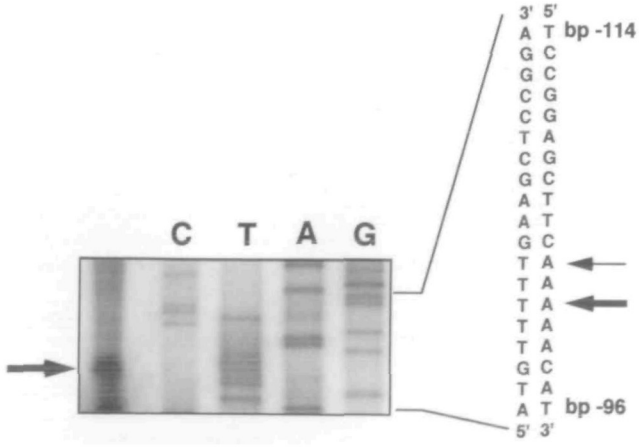

Fig. 1. **Mapping of transcriptional start sites by primer extension.** Two major extended products (left lane) were obtained using RNA prepared from adult human aortic SMC tissue. They are located at the nucleotides indicated by arrows in the genomic sequence on the right.

The human SMC calponin gene does not contain either TATAA canonical sequences or a consensus initiator sequence (*24*) around its transcriptional start site. A search for consensus *cis*-acting sequences revealed three GC-boxes/Sp1-binding sites (CCCGCC/GGCGGG) (*25*) located at bp +79, −67, and −804, six E-boxes (CANNTG) (*26*) located at bp +4, −93, −428, −637, −964, and −975, three CCAAT boxes (CCAAT) located at bp −44, −133, and −244, three half palindromic motifs of estrogen responsive elements (ERE) (TGACC/GGTCA) (*27*) located at bp −198, −283, and −623, two GATA binding sites (AGATAA) (*28*) located at bp −297 and −360, and one TPA-responsive element/AP1-binding site (TGACTCA) located at bp −842 (Fig. 2a). Unlike the previously characterized SMC-lineage specific genes, including those of SMC α-actin (*29*), SMC myosin heavy chain (*30*), and SM22α (*31, 32*), no consensus CArG/rSRF and MEF-2 binding sites are present in the 5′ flanking 2500-nt region of the human SMC calponin gene. The 5′ flanking 1221 nt sequence was used to carry out a BLAST search (*33*) of the DDBJ/GenBank™/EMBL databases. The searches revealed an *Alu* repetitive sequence located from bp −552 to −860, and a previously identified DNA sequence that is capable of binding to the methyl CpG-binding protein affinity column (*34*), located from bp +154 to −557 (Fig. 2, a and b). The nucleotide sequence of this DNA stretch spanning from bp +164 to −1057 of the human SMC calponin gene is characterized by a relatively high GC content (56%) and a low density of CpG dinucleotides (27 CpGs over 1221 bp), with a CpG/GpC ratio of 0.30. This value is close to the density of CpGs in the genome as a whole (CpG/GpC ratio, ~0.2), which is characteristic for tissue-specific genes (*35*). However, fifteen of the 27 CpGs are concentrated in the immediately 5′ region of the gene, spanning from bp +164 to −152. The 5′-flanking 1200 nt sequence surrounding the transcriptional start site of the mouse SMC calponin (calponin-h1) gene (*7-9*) contains only seven CpGs, having an average CpG/GpC ratio of 0.11.

A number of previous studies have established that DNA methylation in the promoter region can reduce the rate of transcription of genes (*35, 36*). Recent studies suggested that the repression of genes by promoter methylation is mediated *via* a methyl-CpG binding protein that binds to 5′-methylcytosines, preventing the formation of the transcription initiation complex by direct inhibition of the binding of transcription factors (*37, 38*). In addition, CpG methylation in the human *Alu* elements associated with the 5′-end of genes has been shown to contribute to the long-term silencing of the transcription *in vitro* (*39*). The identification of a *cis*-acting domain for interaction with the methyl-cytosine binding protein in the 5′-flanking promoter region of the human SMC calponin gene suggests that

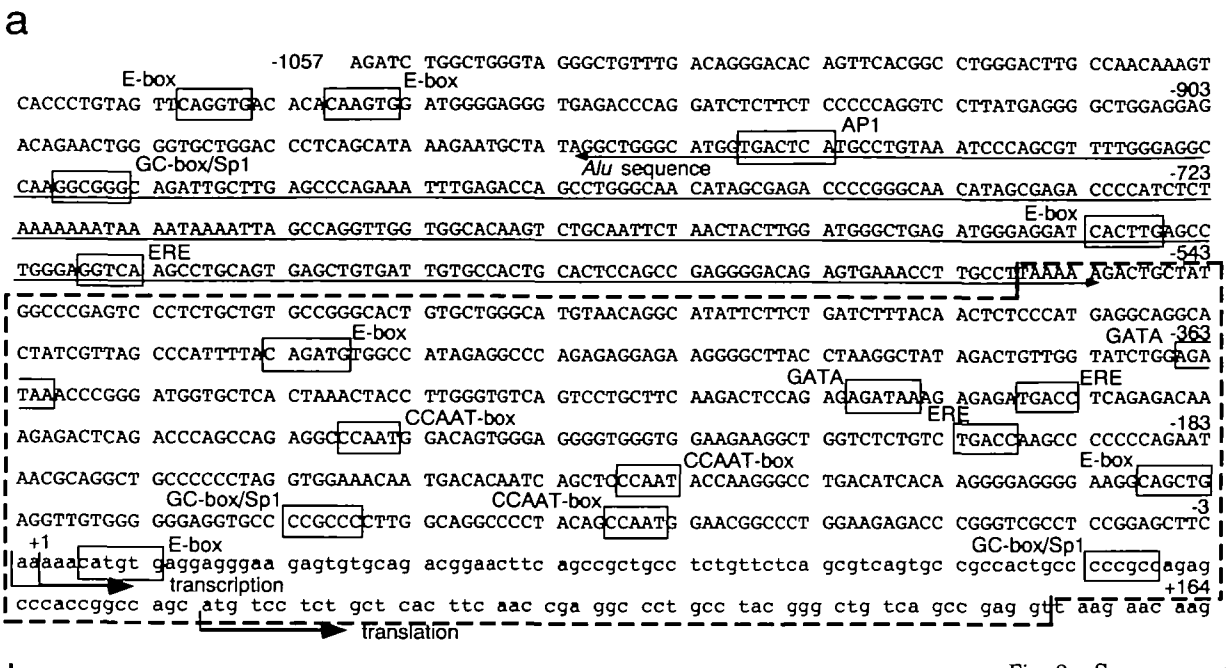

Fig. 2. **Sequence analysis of the 5′-flanking, 5′-untranslated, and amino-terminal protein coding regions of the human SMC calponin gene.** (a) Known regulatory elements are boxed. The *Alu* repetitive sequence is underlined. A DNA segment identified with a methyl CpG-binding protein affinity column is boxed with a broken line. (b) CpG and GpC plots covering the region of genomic DNA described in (a). The arrow indicates the translation start site (ATG initiation codon).

methylation of the DNA may be a possible mechanism for repression of the calponin-gene expression in extra-smooth muscle tissues in adult as well as in phenotypically de-differentiated SMC. Repression of the calponin expression persists stably throughout DNA replication and cell division in cultured de-differentiated SMC (*7, 10, 40*). Furthermore, the cloned SMC calponin promoter is active in a wider range of cells, including both SMC and non-SMC, than the endogenous SMC calponin gene (for human SMC calponin gene, manuscript in preparation; for mouse SMC calponin gene, see Ref. *9*). The characteristics of repression of the promoter activity by cytosine methylation (*35-38*) are in accord with the results of experiments on the cellular calponin expression described above. In order to confirm, it is necessary to demonstrate the distinct difference in the levels of cytosine methylation in the promoter region of the SMC calponin gene between expressing and non-expressing cells as well as the inverse correlation between methylation level and transcriptional activity of this gene. The cloned 5′-promoter region of the human SMC calponin gene reported here will be a useful tool for such experiments.

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