Expression of the Smooth Muscle Cell Calponin Gene Marks the Early Cardiac and Smooth Muscle Cell Lineages during Mouse Embryogenesis*

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Although several genes are considered markers for vascular smooth muscle cell (SMC) differentiation, few have been rigorously tested for SMC specificity in mammals, particularly during development where considerable overlap exists between different muscle gene programs. Here we describe the temporospatial expression pattern of the SMC calponin gene (formerly h1 or basic calponin) during mouse embryogenesis and in adult mouse tissues and cell lines. Whereas SMC calponin mRNA expression is restricted exclusively to SMCs in adult tissues, during early embryogenesis, SMC calponin transcripts are expressed throughout the developing cardiac tube as well as in differentiating SMCs. Transcription of the SMC calponin gene initiates at two closely juxtaposed sites in the absence of a consensus TATAA or initiator element. Transient transfection assays in cultured SMC demonstrated that high level SMC calponin promoter activity required no more than 549 nucleotides of 5’ sequence. In contrast to the strict cell type-specificity of SMC calponin mRNA expression, the SMC calponin promoter showed activity in several cell lines that do not express the endogenous SMC calponin gene. These results demonstrate that SMC calponin responds to cardiac and smooth muscle gene regulatory programs and suggest that the cardiac and smooth muscle cell lineages may share a common gene regulatory program early in embryogenesis, which diverges as the heart matures. The finding that the isolated SMC calponin promoter is active in a wider range of cells than the endogenous SMC calponin gene also suggests that long-range repression or higher order regulatory mechanism(s) are involved in cell-specific regulation of SMC calponin expression.

The discovery of cell-specific transcription factors that trigger differentiation in skeletal and cardiac muscle has led to a search for similar regulatory factors in smooth muscle cells (SMCs), whose differentiation program is impaired during the course of intimal disease (1). Although several transcription factors have been documented in SMCs (2–8), none display the specificity commonly associated with factors that control cell identity by activating batteries of cell-specific genes (9). Given the similarities between skeletal, cardiac, and smooth muscle cells, it is reasonable to anticipate that these different muscle cell types may share certain aspects of a myogenic gene regulatory program.

In contrast to skeletal and cardiac muscle, which are derived from distinct populations of mesodermal precursor cells, SMCs arise throughout the embryo from diverse precursor cell types. The mechanisms that specify the SMC phenotype and the embryonic origins of the many different types of SMCs remain unclear. There have been relatively few studies that have examined the temporospatial patterns of expression of SMC-specific genes during embryogenesis. However, the few SMC genes that have been examined have been found to exhibit different expression patterns. Smooth muscle myosin heavy chain (SMMHC), for example, is expressed only in the SMC lineage, appearing initially in the dorsal aorta at 10.5 days postcoitum (10). In contrast, smooth muscle α-actin (SM α-actin) is expressed in the cardiac, skeletal, and smooth muscle cell lineages during embryogenesis and in the adult (11–14). SM22α is also expressed in cardiac, skeletal, and smooth muscle cells in the embryo before becoming restricted to SMCs late in embryogenesis (14, 15). Dissection of the cis-acting regulatory elements associated with these and other SMC genes will be an important step toward understanding the similarities and differences in the myogenic regulatory programs in the three major muscle cell types.

One approach to identify SMC-specific regulatory factors is to analyze promoters of genes that are unique to SMC lineages. The best studied SMC promoter is that of SM α-actin (16, 17). Defining SMC-specific transcription factors that activate the SM α-actin promoter, however, is complicated by its expression in multiple cell types during embryogenesis and in the adult (11–13). In addition to SM α-actin, other SMC gene promoters have been cloned and partially characterized including elastin (18), SMMHC (19), and SM22α (15, 20). As yet, no cis elements have been shown to confer SMC-specific expression of these promoters.

The abbreviations used are: SMC, smooth muscle cell(s); SM α-actin, smooth muscle α-actin; SMMHC, smooth muscle myosin heavy chain; RASMC, rat aortic smooth muscle cell(s); RACE, rapid amplification of cDNA end; PCR, polymerase chain reaction; nt, nucleotide(s); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Calponin is a thin filament-associated protein that apparently functions as a negative regulatory element for SMC contraction (21), but may also have more broad cellular activities independent of contractility (22). Three distinct mammalian calponin genes have been described based on their expression and nucleotide sequence differences (23–25). Whereas much effort has been directed toward understanding the function of different calponin proteins, relatively little is known about their specificity of mRNA expression. Based on studies conducted with antisera and cDNA probes, calponin was shown to predominate in SMCs (23, 26, 27), but was also present in other cell types (28, 29). These studies, however, could not adequately distinguish between the three calponin genes. A similar problem was recently approached with respect to SMMHC mRNA expression using stringent assays for the unambiguous assignment of this marker to only SMC lineages (10).

In this study, we examined the temporospatial expression pattern of the basic or h1 calponin (hereafter referred as SMC calponin) during mouse embryogenesis and in adult mouse tissues. Our results demonstrate that SMC calponin is strictly specific for adult SMCs, but that during embryogenesis it is expressed throughout the early cardiac tube. While the SMC calponin gene is expressed exclusively in SMC lineages and embryonic heart, its promoter, which lacks core sequence elements typical of other muscle genes, displays activity in cell lines that do not express the endogenous transcript. These results reveal similarities between the cardiac and smooth muscle gene regulatory programs during early embryogenesis and suggest that complex mechanisms govern the cell type-specific expression of SMC calponin.

MATERIALS AND METHODS

Cell Culture—The culture conditions for many of the cell lines analyzed here have been described previously (10). Primary rat aortic SMCs (RASMC) were obtained by a modified explant protocol (30). Briefly, rat aortae were rinsed in phosphate-buffered saline, carefully stripped of adherent periaortic fat and endothelium, and subjected to a 20-min digestion in 1% collagenase, 0.25% type I elastase, and 1% soybean trypsin inhibitor as described (31). Vessels were then rinsed in Dulbecco's modified Eagle's medium and aseptically cut into small rings. The rings were placed under sterile coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/l-glutamine for 5–7 days. The cells were routinely split 1:5 and maintained in the same medium. The identity of our RASMC cultures was verified by assaying for the presence of SMC calponin protein in a number of cell types. Cells were washed 3 times in phosphate-buffered saline, scraped into extraction buffer (35), sheared 10 times through a 22-gauge needle, and boiled for 10 min. After spinning 10 min in a microcentrifuge, equivalent volumes of supernatant were loaded on a 10% polyacrylamide gel and stained with Coomassie Blue to verify protein integrity and equal loading. In a separate gel, resolved proteins were electroblotted to a nylon membrane (Zeta Probe; Bio-Rad, Hercules, CA) and blocked in buffer 5% non-fat milk (35) for 1 h at room temperature. Western Blotting—A monoclonal antibody to SMC calponin (clone hCP; Product No. C-2687) was purchased from Sigma and used to test for the presence of SMC calponin protein in a number of cell types. Positive immunostaining was observed only in RASMC. Proteins were electrophoresed in a 5% polyacrylamide, 7 μM urea gel and visualized following autoradiographic exposure (Kodak X-Omat AR; Rochester, NY).

In Situ Hybridization—Adult heart, skeletal muscle, small intestine, and uterus as well as staged mouse embryos were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraplast. Sections (8–10 μm) were mounted on triple-washed silane-coated glass slides (HCS Inc., Glen Head, NY) and processed for in situ hybridization as described previously (10). Adjunct sections of 9.5- and 13.5-day embryos were hybridized with either a riboprobe corresponding to mouse SMMHC (10) or mouse SMC calponin (see above). Slides were dipped in emulsion (Kodak NBT-2; Rochester, NY) and exposed for 9 days at 4°C before development. Sections were subjected to digital photography and microscopy for analysis and photography (Nikon SMZ-U Zoom 1:10).

Genomic Screening and Sequencing—The XL 1 Blue MRA (P2) strain of bacteria (Stratagene) was used to generate ~4 × 10^6 recombinants/plate of the SV129 mouse genomic library (Stratagene). About 500,000 recombinants (~1.5 genomic equivalents) were lifted in duplicate onto nitrocellulose filters (Schleicher & Schuell), denatured, and neutralized as described (32) and then hybridized for at least 6 h in 50% formamide, 5 × SSC, 5 × Denhardt’s, 0.5% SDS, and 200 μg/ml salmon sperm DNA at 42°C. The 195-nt mouse SMC calponin probe above was used as a preimmune labeled (Boehringer Mannheim) in the presence of (α-32P) dCTP (3000 Ci/mmol; Amersham) and added to the prehybridization mixture for an additional 16 h. Filters were washed at high stringency (final wash was 0.1% SSC, 0.1% SDS, 157-nt 3′-end-labeled with [32P]dCTP). Three positive clones (14 kilobase pairs) selected for the SMC calponin gene. A total of 16 positive clones were detected after primary screening. Two clones of ~18 (CALP-5) and 14 kilobase pairs (CALP-8) were plaque purified by multiple rounds of screening using freshly labeled probe. Restriction digest analysis of these clones revealed they were independent, but overlapping (see Fig. 4). Genomic Southern blotting of the CALP-5 and CALP-8 clones with DNA derived from the liver of an SV129 mouse verified the authenticity of both clones.

In order to clone the SMC calponin gene, three independent assays were performed to map the SMC calponin transcription initiation sites. Firstly, the nucleotide sequences present in the 5′ ends were determined by reverse transcription using oligo(dT) primer and T7 reverse transcriptase (Ambion, Austin, TX) in the presence of [α-32P]dUTP (800 Ci/mmol; Amersham). Approximately 15 μg of total RNA was hybridized to each radiolabeled riboprobe according to the manufacturer's instructions (Ambion RPA II). In some experiments, a 157-nt 3′-translated region riboprobe corresponding to the murine Sm α-actin cDNA (34) or an 18 S riboprobe (Ambion) was co-hybridized with the calponin riboprobe. Protected SMC calponin riboprobes were shown to be hybridized in a 5% polyacrylamide, 7 μM urea gel and visualized following autoradiographic exposure (Kodak X-Omat AR; Rochester, NY).

Western Blotting—A monoclonal antibody to SMC calponin (clone hCP; Product No. C-2687) was purchased from Sigma and used to test for the presence of SMC calponin protein in a number of cell types. Positive immunostaining was observed only in RASMC. Proteins were electrophoresed in a 5% polyacrylamide, 7 μM urea gel and stained with Coomassie Blue to verify protein integrity and equal loading. In a separate gel, resolved proteins were electroblotted to a nylon membrane (Zeta Probe; Bio-Rad, Hercules, CA) and blocked in buffer 5% non-fat milk (35) for 1 h at room temperature. The blot was then incubated with the monoclonal calponin antibody at a dilution of 1:2500 in buffered 1% non-fat milk and then incubated with a horseradish peroxidase-linked secondary anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1 h at room temperature. After washing in buffered 1% non-fat milk, the blot was washed in buffered 1% non-fat milk and then incubated with a horseradish peroxidase-linked secondary anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1 h at room temperature. The blot was then incubated in 1% non-fat milk and then incubated with a horseradish peroxidase-linked secondary anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1 h at room temperature. The blot was then incubated in buffered 1% non-fat milk and then incubated with a horseradish peroxidase-linked secondary anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1 h at room temperature. The blot was then incubated in buffered 1% non-fat milk and then incubated with a horseradish peroxidase-linked secondary anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1 h at room temperature.

SMC Calponin Gene Expression and Promoter Analysis

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Restriction enzyme-clamped PCR primersto

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reverse transcriptase (Bethesda Research Laboratories) in a final volume of 20 μl. Reactions were stopped by adding 60 μl of dH₂O, 70 μl of 5 M HClO₄, and 2 μl of 0.5 M EDTA and precipitated in 500 μl of ethanol. Dried samples were resuspended in 10 μl of Sequenase stop solution (U. S. Biochemical), denatured at 75°C, and resolved in a 6% polyacrylamide, 8 M urea gel. A sequencing ladder using a calponin cDNA clone with the same primer as that used to anneal the RNA was loaded adjacently to the primer extension reactions for fine mapping of the start sites.

RNase protection analysis and 5' RACE were used in conjunction with primer extension for mapping the start sites. For RNase protection, total RNA was hybridized to one of two independent riboprobes (see Fig. 5A) and the protected fragments resolved in a 5% polyacrylamide, 7 M urea gel. Sequencing reactions of a calponin cDNA were carried out using primers to the 3' most end of each riboprobe. Total RNA from uterus or liver was also subjected to 5' RACE as described in Ref. 10. Cells were either serum-deprived for 3 days (Q) or serum-stimulated (P) for 24 h following quiescence. Exposure time was for 8 h. The PC12 cell line was induced to differentiate by treating subconfluent cells with 10 ng/ml nerve growth factor. D, protein extracts (~50 μg) from the indicated cell lines were processed for Western blotting and incubated with a monoclonal antibody to human SMC calponin as described under “Materials and Methods.” Cells were either serum-deprived for 3 days (Q) or serum-stimulated and then stimulated with 10% fetal bovine serum for 24 h. The growth state of each SMC line did not have any discernable effect on SMC calponin protein levels. The absence of any immunoreactive signal in 10T1/2 cells verifies the specificity of the antisera for only SMC calponin.
50% confluence with 10 \( \mu g \) of plasmid DNA. Primary RASMC were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and used between passage 15 and 30. Although the cell lines grow at different rates, every effort was made to transfect approximately equivalent cell numbers between lines. In some experiments, a plasmid carrying the \( \beta \)-galactosidase gene was cotransfected to correct for varying transfection efficiencies between cell lines.

Transfections were typically carried out for 12-16 h followed by 48 h of recovery and growth. Cells were harvested in cold phosphate-buffered saline, spun down, and resuspended in 200 \( \mu l \) of 0.25M Tris-HCl, pH 7.8. Cell lysates were then briefly sonicated, spun down and stored at \(-80\) °C before use. Neither mild sonication nor multiple freeze thawing influenced luciferase activity. Total protein was measured by the Bradford assay (Bio-Rad). Luciferase activity was assayed according to the manufacturer's specifications (Promega). A Turner Model 20 luminometer was used to measure the light reactivity of firefly luciferase. The relative light units were then normalized to total protein and expressed as a percent of the normalized luciferase activity obtained with the pGL3 control vector, which contains the SV40 promoter/enhancer (Promega). The data reflect the means (±S.E.) of at least four independent experiments done in duplicate.

RESULTS

SMC Calponin Transcripts in Adult Mouse Tissues and Cell Lines—RNase protection assays were performed to accurately assess the specificity of SMC calponin mRNA expression in adult mouse tissues and a variety of cell lines. Consistent with previous reports (21, 24), SMC calponin transcripts were restricted to adult mouse tissues with a SMC component (Fig. 1). Upon overexposure, however, transcripts were detected in most tissues due to the presence of blood vessels (see below). In cultured cells, SMC calponin was expressed in proliferating BC3H1 cells, which have been reported to exhibit properties of smooth and skeletal muscle, and only weakly in the differentiated P19 embryonal carcinoma cell line, which also has the potential to differentiate into smooth muscle (38) (Fig. 1B). Interestingly, SMC calponin mRNA expression was extinguished in differentiated BC3H1 cells (Fig. 1B). No other mouse lines examined expressed SMC calponin mRNA including 3T3, 10T1/2, C2, F9, and embryonic stem cells (Fig. 1B). These findings contrast with the expression of SM \( \alpha \)-actin, which was expressed in most cell lines analyzed (Fig. 1B).

No SMC calponin transcripts were detected in the rat L6 skeletal myoblast line, PC12 cells, or the HepG2 liver cell line (Fig. 1C). On the other hand, a prominent signal was observed in the A7r5 fetal rat aortic SMC line as well as primary

![Fig. 2. Localization of SMC calponin transcripts in adult mouse tissues. Sections of adult mouse heart (A), skeletal muscle (B), small intestine (C), and uterus (D) were processed for in situ hybridization as described under “Materials and Methods” and photographed under darkfield microscopy. SMC calponin was detected in the SMC-containing tissues of small intestine (C) and uterus (D), but was only detected in blood vessels (arrows) of the heart (A) and skeletal muscle (B). Abbreviations are: en, endometrium; ve, villous epithelium.](http://www.jbc.org/)

![Fig. 3. mRNA expression of SMC markers in staged mouse embryos. Adjacent sagittal sections of 9.5 days postcoitum (A and B) or 13.5 days postcoitum (C and D) mouse embryos were hybridized to a mouse riboprobe corresponding either to SMMHC (A and C) or SMC calponin (B and D). Note the intense SMC calponin hybridization signal in the heart of both 9.5- and 13.5-day postcoitum embryos. Consistent with a previous report (10), no SMMHC signal was ever observed in tissues without a SMC component. Abbreviations are: br, brain; fb, forebrain; gu, gut; he, heart; and li, liver.](http://www.jbc.org/)
RASMC (Fig. 1C). These latter cells also expressed SMC calponin protein as determined by Western blotting (Fig. 1D). Importantly, SMC calponin mRNA was expressed at high levels in rat SMC irrespective of passage number or growth state (Fig. 1C). Together, these results show SMC calponin to be a highly restricted marker for SMC lineages. They also demonstrate the utility of both the A7r5 fetal rat aortic SMC line and multiply passaged primary RASMC for analyzing SMC calponin promoter activity (see below).

Expression of SMC Calponin mRNA in Adult and Developing Mouse Tissues—Several adult mouse tissues were analyzed by in situ hybridization as an additional measure of SMC calponin's specificity for SMC lineages. In adult heart and skeletal muscle, for example, SMC calponin mRNA was only observed in the smooth muscle tissue enveloping these organs (Fig. 2, A and B). In small intestine and uterus, a strong hybridization signal was present in the smooth muscle tissue enveloping these organs (Fig. 2, C and D). Thus, SMC calponin mRNA is only expressed in vascular or visceral SMCs, and the RNase protection signal observed in tissues without a SMC component is solely attributable to blood vessels.

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SMC Calponin Gene Expression and Promoter Analysis

Fig. 6

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SMC calponin mRNA first appeared at low levels in the heart at 8.5 days postcoitum. As shown in Fig. 3B, 9.5-day postcoitum embryos displayed a strong hybridization signal throughout all chambers of the heart. This embryonic cardiac expression of SMC calponin persisted up to 13.5 days postcoitum at which time the mRNA could be detected in several SMC-containing tissues including lung, gut, and blood vessels (Fig. 3D). This expression pattern differs from that of SMMHC mRNA which, as shown previously (10), is only expressed in SMC lineages during development (Fig. 3C). The expression of SMC calponin mRNA in the heart subsided by 15.5 days postcoitum. At no time during development did we observe SMC calponin mRNA in the heart or elsewhere. Together our findings show clearly that SMC calponin, with the exception of early heart expression, SMC calponin is restricted to SMC lineages.

Characterization of the Mouse SMC Calponin Gene—The mouse SMC calponin gene was isolated and characterized by sequence analysis and PCR. The gene contains 7 exons spanning 9451 nucleotides of DNA (Fig. 4; accession number U28932). The first exon of SMC calponin comprises both untranslated and translated sequences (Fig. 4), a feature uncommon among SMC genes which typically have an untranslated first exon (16, 17, 19, 20, 39). The putative calmodulin- and actin-binding domains of SMC calponin (40) are split by introns. All intronic sequences at each splice site (except intron 3) conform to the “gt/ag rule” (Ref. 41, Table I).

Interestingly, the initial cloning of SMC calponin in chickens revealed two transcripts (designated α and β) that apparently arose due to alternative splicing (23). The putative splice site in the chicken SMC calponin cDNA maps precisely to the boundary of the sixth intron and seventh exon of the mouse SMC calponin gene. RT-PCR analysis of several mouse tissues with primers flanking this region, however, failed to reveal any splice variants of the mouse SMC calponin gene. Thus, the splicing phenomenon of SMC calponin described by Takahashi and Nadal-Ginard (23) appears to be restricted to chickens.

SMC Calponin Transcription Initiates at Two Sites in the Absence of a Consensus TATAAA Box or Initiator—Three independent assays were used to identify the transcription initiation site of SMC calponin: primer extension, RNase protection, and rapid amplification of the 5′ cDNA end (5′ RACE). Fig. 5A is a schematic of the SMC calponin gene showing the position of the probes and primers utilized for these analyses. Primer extension of uterus and stomach RNA revealed two bands of equivalent intensity indicating that the SMC calponin gene has two major sites of transcription initiation (Fig. 5B). These bands were not present in RNA from liver (Fig. 5B). The position of these bands places the 5′ end of the SMC calponin mRNA 77 and 80 nt from the initiating methionine (Fig. 5A). Similarly, RNase protection using two probes (Fig. 5A) showed two major protected fragments in stomach and uterus (but not liver) that coincided with the primer extended products (Fig. 5C). Finally, two major 5′ RACE products extended to the same site as that achieved by primer extension and RNase protection (Fig. 5D). These results establish the presence of two closely juxtaposed transcription initiation sites for the SMC calponin gene. The position of the two start sites, designated S2 and S2′, is illustrated in Fig. 5E.

Most SMC structural or cytosolic genes contain a TATA box in their 5′ promoter (16, 17, 19, 20, 39). SMC calponin, however, has no consensus TATAAA box (Fig. 5E). Moreover, no consensus initiator sequence (42) is present around its transcription start sites. There is, however, a sequence (TTCAAAAA) that may serve as a weak binding site for TATA binding protein (Fig. 5E and Fig. 6). Immediately 5′ of S2 is a stretch of 14 purines (underlined sequence in Fig. 6). Further upstream, a consensus CCAAT box is present as is a GC box (Fig. 6). Sequence analysis of the 5′ 3000 nt of SMC calponin promoter revealed several consensus binding sites for regulatory factors involved with muscle transcription including E-boxes (43) and GATA binding sites (44) (Fig. 6; accession number U37071). No consensus MEF-2 binding sites (45) are present in the 5′ 3000 nt of SMC calponin. Finally, several stretches of alternating purine/pyrimidine dinucleotides are present (double underlined sequences in Fig. 6).

SMC Calponin Promoter Activity in Cell Lines—PCR and restriction digestion-mediated deletions of the SMC calponin promoter (Fig. 7A) were generated to study its activity in cultured cell lines. The results of five independent transfections in passaged RASMC are summarized in Fig. 7B. The 3000 nt promoter construct, –3000 CALPLuc, yielded high activity in cultured RASMC. Progressive deletions of this construct revealed that as little as 549 nt retained full promoter activity (Fig. 7B). The –115 CALPLuc promoter construct displayed no activity despite the presence of both transcription start sites and upstream GC and CCAAT boxes (see Fig. 6). In fact, –115 CALPLuc was expressed at a level 2 orders of magnitude less than the promoterless pG3 basic reporter (Fig. 7B). The –1342 CALPLuc promoter construct, which displayed high activity in primary RASM (Fig. 7B), exhibited no luciferase activity if the 3′ 115 nt were removed. This finding further supports our transcription initiation site mapping data (see Fig. 5).

To ascertain the specificity of SMC calponin promoter activity in vitro, several cell lines were transfected with either the promoterless pG3 basic vector, the –3000 CALPLuc promoter, or the –549 CALPLuc promoter. Although these promoters showed higher relative activity in two cell lines, several cell lines that do not express the endogenous SMC calponin transcript displayed some SMC calponin promoter activity (Fig. 8). Only the F9 teratocarcinoma cell line exhibited relatively low luciferase activity with both SMC calponin promoter constructs (Fig. 8). In general, the –549 CALPLuc promoter construct displayed higher activity than the –3000 CALPLuc construct, particularly in the 10T1/2 cell line (Fig. 8). This suggests the presence of negative regulatory elements between –3000 and –549. These results demonstrate functional SMC calponin promoter activity in vascular SMC and, to a lesser extent, several cell lines that do not express the SMC calponin transcript. This activity is largely imparted by sequences between –549 and –115 of the SMC calponin promoter.

**DISCUSSION**

SMC Calponin: A Highly Restricted SMC Marker Expressed in Embryonic Heart—All three adult muscle types, skeletal, cardiac, and smooth, are distinguished by their different structural organization of contractile elements and functionally distinct modes of contraction. During embryogenesis, however, they express many of the same contractile protein genes. Both skeletal and cardiac muscle, for example, express SM α-actin, a...
gene that has been used extensively as a marker for SMC lineages (11, 46). We recently documented the expression of a SMC marker of unknown function, SM22α, in embryonic heart and skeletal muscle of the mouse (14). Here we show that SMC calponin first appears in the heart during mouse embryogenesis. Recently, Duband and co-workers (27) showed that SMC calponin and SM22α protein were expressed exclusively in SMC during chicken embryogenesis, but they did not measure the transcripts to these genes. Similarly, we did not assay the corresponding proteins in embryonic mice so it is possible that these markers are transcribed, but untranslated in embryonic mouse cardiac tissue.

The mRNA expression of SM α-actin, SM22α, and SMC calponin in the embryonic mouse heart should be contrasted with the notable absence of SMMHC transcripts in developing cardiac tissue (Fig. 3 and Ref. 10). These unique patterns of SMC gene expression during mammalian development suggest that distinct regulatory factors control each gene. Identifying these regulatory factors should provide insight into the mechanisms for SMC transcription and may contribute to an understanding of the complexity of SMC phenotypes that characterize many vascular lesions.

The functional significance of a shared genetic program between developing cardiac and smooth muscle has yet to be elucidated. One intriguing possibility is that cardiac muscle traverses a SMC-like phenotype during its ontogeny, a concept that is supported by the distinct differences in embryonic versus postnatal cardiac contractility (47). In this regard, it will be of interest to determine whether the decompensated adult heart, which expresses a fetal cardiac phenotype (48), expresses SMC calponin. Support for such expression is provided by studies in hypertrophied rat hearts, which express the SM α-actin gene (13). If SMC proteins participate in some aspect of cardiac contractility, then they would do so in the absence of their native thick filament, SMMHC.

In addition to embryonic heart expression, SMC calponin mRNA was noted in proliferating BC3H1 cells. This cell line, originally thought to be of SMC origin (49), is defective for terminal differentiation into sarcomeric muscle (50). When stimulated to exit the cell cycle and differentiate, BC3H1 cells acquire a number of sarcomeric markers and, at the same time, lose several SMC markers (50). The reversible expression of SMC calponin mRNA in BC3H1 cells is consistent with these findings and suggests that proliferating BC3H1 cells more closely resemble a SMC-like phenotype. Moreover, as with other SMC markers (50), SMC calponin mRNA can be reinduced by serum-stimulating differentiated BC3H1, which leads to their re-entry into the cell cycle. Thus, this cell line may be useful as a tool to uncover regulatory factors that activate SMC calponin gene expression in vitro.

With the exception of embryonic heart and proliferating BC3H1 cells, SMC calponin mRNA was only expressed in SMC lineages. No evidence of expression was noted in skeletal muscle or cell lines derived from skeletal muscle. Furthermore, no transcripts were detected in embryonic cell lines (ES, F9), fibroblasts (3T3 or 10T1/2), or endothelial (human umbilical vein) cells. Thus, early reports of calponin protein expression in such tissues as the adrenal gland (51) and such cells as platelets, fibroblasts, and endothelial cells (28,29), probably reflected the presence of a recently cloned non-muscle (acidic) calponin (25).

The SMC Calponin Promoter Displays Unusual Features not Commonly Found in Other SMC Gene Promoters—Neither a TATAA nor an initiator consensus element was found in the SMC calponin promoter. The absence of such elements is often associated with multiple transcription initiation sites (42).

**Fig. 7.** SMC calponin promoter activity in cultured RASMC. A, schematic of progressive S′ SMC calponin promoter deletions. Shown is a partial restriction map of the sites used to construct each deletion construct into the pGL3 basic luciferase vector. Note that the two BamHI sites were artificially engineered by PCR (see "Materials and Methods"). The two transcription start sites are indicated by arrows. The black box corresponds to the S′ 60 nt of untranslated SMC calponin cDNA sequence, the 3′ end of which is 19 nt upstream of the initiating methionine. The numbers represent the distance of the 3′ end of each promoter construct from the S′ start site. Abbreviations are: B, BamHI; H, HindIII; and N, Ncol. B, five independent transfections of RASMC (passage number 15–30) with each indicated SMC calponin promoter construct were performed as described under "Materials and Methods." The relative light units of luciferase were normalized to total protein (passage number 15–30) with each indicated SMC calponin promoter construct. The mean percent of pGL3 control + S.E. of mean was computed as described in the legend to Fig. 7B. Values represent the mean percent of pGL3 control ± S.E. of mean.

**Fig. 8.** SMC calponin promoter activity in different cell lines. Four independent transfections were carried out in the indicated cell lines with the promoterless pGL3 basic vector, −549 CALPLuc and −3000 CALPLuc as described under "Materials and Methods." Activity was computed as described in the legend to Fig. 7B. Values represent the mean percent of pGL3 control ± S.E. of mean.
Thus, it is not surprising that transcription initiation of the SMC calponin gene occurs at two closely juxtaposed guanine nucleotides. Similar characteristics are found in the human elastin gene (52), but not SM MHC (19), SM actin (16, 17), SM γ-actin (39), or SM22α (20), all of which contain sequences closely related to the consensus TATAAA box upstream of a single major initiation site. Although no obvious TATAAA element is present in the SMC calponin promoter, there is the sequence TTTCAAAA 28 nt upstream of the 5' most start site that could conceivably serve as a weak binding element for TATA binding protein, since there is considerable heterogeneity in sequences that can bind TATA binding protein (53).

The proximal promoter of SMC calponin shares many features with another TATAA/initiator-less promoter, thymidylate synthase (54). Both genes have a stretch of purines immediately 5' of an initiation site. In addition, the SMC calponin promoter has an upstream polyuridine tract that immediately follows a purine/pyrimidine dinucleotide repeat (see Fig. 6). These unique DNA sequences have been shown to play a role in transcription control. For example, polyuridine tracts form DNA triple helices that serve as directional attenuators of transcription (55). Purine/pyrimidine dinucleotide repeats promote the formation of Z DNA (56), which has recently been shown to regulate the transcription of the c-myc proto-oncogene (57). Such mechanisms of control could exclude transcription of SMC calponin in non-SMC-containing tissues.

Another shared feature between SMC calponin and thymidylate synthase is the presence of GC-rich sequences in their 5'-flanking region. Both genes, for example, have a GC box whose binding factors may interact with the basal transcriptional machinery to facilitate preinitiation complex formation and transcription (54). Despite the presence of a GC box, as well as a CCAAT box and sequences around both initiation sites, the -115 CALPLuc promoter construct was totally inactive. This result indicates that upstream activators between -115 and -549 play a crucial role in the initiation of SMC calponin transcription. Sequence analysis of this region reveals two E-boxes and one GATA site.

Based on its highly restricted pattern of mRNA expression, we predicted that the SMC calponin promoter would be similarly restricted in activity. Although promoter activity was very high in SMC, we found it to be active in several cell lines, including L6, C2 myoblasts, and 10T1/2 fibroblasts, none of which express the endogenous gene. Only the F9 teratocarcinoma cell line showed low promoter activity. These findings suggest either a distal inhibition element involved in long range repression of SMC calponin expression in non-SMCs is missing from our promoter constructs or some other mode of transcriptional regulation confers SMC calponin’s specificity for SMC lineages.

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Additions and Corrections

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Expression of the smooth muscle cell calponin gene marks the early cardiac and smooth muscle cell lineages during mouse embryogenesis.

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The luciferase activity reported for the construct −115 CALPLuc in Fig. 7B is incorrect due to a probable mutation in the coding region of the pGL3 basic luciferase gene. A newly cloned −115 CALPLuc reporter construct gave normalized luciferase activity approximating 20% of the pGL3 control. Investigators are encouraged to take care in the interpretation of seemingly unusual luciferase activity as spontaneous mutations in the pGL3 basic luciferase gene may occur during amplification in bacteria (Yang, N. N., Venugopalan, M., Hardikar, S., and Glasebrook, A. (1997) Science 275, 1249.

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Two-dimensional crystals of photosystem I in higher plant grana margins.

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One of Dr. Holzenburg's affiliations was omitted. He is also affiliated with the Department of Biology, University of Leeds, LS29JT, United Kingdom.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.