Developmental Pattern of Expression and Genomic Organization of the Calponin-h1 Gene

A CONTRACTILE SMOOTH MUSCLE CELL MARKER* (Received for publication, September 6, 1995)

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Calponin-h1 is a 34-kDa myofibrillar thin filament, actin-binding protein that is expressed exclusively in smooth muscle cells (SMCs) in adult animals. To examine the molecular mechanisms that regulate SMC-specific gene expression, we have examined the temporal, spatial, and cell cycle-regulated patterns of expression of calponin-h1 gene expression and isolated and structurally characterized the murine calponin-h1 gene. Calponin-h1 mRNA is expressed exclusively in SMC-containing tissues in adult animals. During murine embryonic development, calponin-h1 gene expression is (i) detectable in E9.5 embryos in the dorsal aorta, cardiac outflow tract, and tubular heart, (ii) sequentially up-regulated in SMC-containing tissues, and (iii) down-regulated to non-detectable levels in the heart during late fetal development. In addition, the gene is expressed in resting rat aortic SMCs, but its expression is rapidly down-regulated when growth-arrested cells re-enter phase G1 of the cell cycle and proliferate. Calponin-h1 is encoded by a 10.7-kilobase single copy gene composed of seven exons, which is part of a multigene family. Transient transfection analyses demonstrated that 1.5 kilobases of calponin-h1 5′-flanking sequence is sufficient to program high level transcription of a luciferase reporter gene in cultured primary rat aortic SMCs and the smooth muscle cell line, A7r5. Taken together, these data suggest that the calponin-h1 gene will serve as an excellent model system with which to examine the molecular mechanisms that regulate SMC lineage specification, differentiation, and phenotypic modulation.

The vascular smooth muscle cell (SMC)1 is responsible for maintaining both arterial tone via contraction-relaxation and vessel integrity by proliferation and synthesis of extracellular matrix (1, 2). During postnatal development, SMCs located in the arterial tunica media are maintained in the resting, or G0/G1, stage of the cell cycle and express high levels of contractile protein isoforms (3). However, in response to vascular injury and the concomitant release of growth factors, vascular SMCs re-enter the cell cycle, proliferate, and modulate their phenotype to subserve a primarily synthetic function (4–13). This phenotypic modulation has been implicated in the pathogenesis of a number of disease states, including atherosclerosis and restenosis following percutaneous transluminal coronary angioplasty (3, 14–17). Despite the central role of SMCs in vascular biology, relatively little is currently understood about the molecular mechanisms that control SMC lineage specification, differentiation, and phenotypic modulation. This is due, in part, to the fact that relatively few SMC lineage-specific markers have been identified and to the poorly understood embryological origin(s) of the SMC lineage(s) (18–27).

Previous studies have suggested that the myofibrillar thin filament protein calponin is expressed exclusively in SMCs (11, 28–31). Two species of calponin mRNA, designated h1 and h2, have been identified, each of which is encoded by a separate gene (32). Interestingly, only the h1 protein product is detectable in SMC-containing tissues (32). In addition, a non-muscle acidic isoform of calponin with distinct functional properties has recently been described (33). Several findings suggest that calponin may regulate SMC contraction: (i) calponin binds to the thin filament proteins actin, tropomyosin, calmodulin, and calsequestrin (34–38), (ii) calponin can reversibly inhibit the actin-calmodulin-dependent mobility of actin on immobilized myosin (41), (iv) calponin induces conformational changes in F-actin (42), and (v) calponin decreases the rate of cross-bridge cycling and increases maximum force production by smooth muscle myosin (43). Despite these in vitro findings, the molecular mechanism by which calponin regulates smooth muscle cell contraction in vivo remains unknown.

In the studies described in this report, we have used the murine calponin-h1 cDNA as a molecular probe to define the spatial and temporal pattern of calponin-h1 gene expression during development. In addition, we have isolated and structurally characterized a murine calponin-h1 genomic clone and performed a series of transient transfection analyses using calponin/luciferase reporter plasmids. These studies demonstrated that the calponin-h1 gene is expressed in a developmentally regulated, SMC-specific pattern. The 10.7-kb calponin-h1 gene is a single copy gene that is a member of a multigene family composed of proteins with an evolutionarily conserved actin-binding domain. Finally, transient transfection analyses demonstrate that the murine calponin-h1 promoter programs high level transcription of a luciferase reporter gene in primary cultures of rat aortic SMCs and the SMC line, A7r5. Taken together, these data suggest that the murine calponin-h1 gene should serve as an excellent model system in which to examine the transcriptional mechanisms that control SMC lineage specification and differentiation.

The abbreviations used are: SMC, smooth muscle cells; kb, kilobase(s); bp, base pair(s); Rb, retinoblastoma; FACS, fluorescence-activated cell sorting.
**MATERIALS AND METHODS**

Isolation of Murine Calponin-h1 cDNAs—The coding region (bp 98–988) of the murine calponin-h1 cDNA was isolated by performing reverse transcription-polymerase chain reaction using murine uterine RNA and synthetic 5'- and 3'-oligonucleotide primers constructed from the previously published sequence of the mouse calponin-h1 cDNA (GenBank Accession No. Z19542) as described previously (44). The 891-bp reaction product (excluding the restriction enzyme sites) was subcloned into BamHI-HindII-digested pGEM7Z (Promega) and was utilized for genomic screening, Southern blot, and Northern blot analyses. In addition, a 467-bp murine calponin-h1 cDNA fragment (bp 193–659), derived from the coding region of the cDNA, and a 746-bp cDNA fragment (bp 659–1404), derived from the coding region and 3'-untranslated region of the calponin-h1 cDNA were isolated using reverse transcription-polymerase chain reaction using murine uterine RNA and synthetic 5'- and 3'-oligonucleotide primers as described previously (44) and utilized for in situ hybridization analyses.

Isolation of Murine Calponin-h1 Genomic Clones—Approximately 1 × 10^6 recombinant phage from a murine 129Sv Lambda F II genomic library (Stratagene) were screened with the radiolabeled 891-bp murine calponin-h1 cDNA probe, and positively hybridizing clones were purified to homogeneity as described previously (44, 45). Each clone was analyzed by Southern blot analysis, and one clone spanning the length of the gene (mCalp-SA) including approximately 6 kb of 5'-flanking sequence was used for all subsequent experiments.

Cell Culture and Cell Cycle Analyses—The rat A7r5 cell line, which was derived from embryonic thoracic aorta, was grown as described previously (46). Murine NIH 3T3 cells, C3H10T1/2 cells, 5S6 myoblasts and NIH 3T3-EP352 cells, and murine EL-4 cells were grown as described previously (46, 48). Primary rat aortic SMCs were isolated from 12–16-week-old Sprague-Dawley rats as described previously (47). Cell cycle analysis was performed on propidium iodide-stained SMCs 0, 8, 12, 16-, and 24-hour post-stimulation by fluorescence-activated cell sorting using a Becton Dickinson FACSCAN and CellFIT computer software as described previously (47).

DNA and RNA Blot Analyses—High molecular weight DNA was prepared from strain 129Sv mice as described previously (44). Southern blot analyses were performed using the radiolabeled 891-bp murine calponin-h1 cDNA probe and a 706-bp genomic subfragment under the highly stringent conditions described previously (44). For Northern blot analyses, RNA was prepared from tissues isolated from 12-week-old 129Sv mice (Jackson Laboratories) as described previously (44). In addition, RNA was prepared from cultures of primary rat aortic SMCs and a variety of smooth muscle actin mononuclear antibodies (47). In all experiments, only second or third passage SMCs were utilized. For the cel cycle analyses, SMCs from the third passage were placed in serum-free medium (50% Dulbecco’s minimal essential medium, 45% Ham’s F-12, 10% glucose (292 mg/ml), insulin (5 mg/ml), transferrin (5 mg/ml), se-L-glutamine (292 mg/ml), insulin (5 mg/ml), transferrin (5 mg/ml), selenium acid (5 ng/ml)), 100 U/ml penicillin, and 100 μg/ml streptomycin) for 24 hours. The cells were then harvested, lysed, and the lysates were analyzed by gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then probed with a radioactive probe specific for calponin-h1 and visualized by autoradiography. To detect nonspecific background, hybridization was performed with the radiolabeled sense calponin-h1 riboprobe to alternate sections under identical conditions.

**RESULTS**

SMC Lineage-restricted Expression of the Calponin-h1 Gene—Previous studies have suggested that the thin filament protein calponin is expressed exclusively in SMC-containing tissues (11, 28–31). To determine the in vivo pattern of calponin-h1 gene expression in adult mice, the calponin-h1 cDNA was hybridized to Northern blots containing RNA preparations from 12-week-old mouse tissues. As shown in Fig. 1A, the murine calponin-h1 cDNA hybridized to a single mRNA species of approximately 1.5 kb (arrow). In adult mice, the calponin-h1 gene is expressed at high levels in SMC-containing tissues including aorta, small intestine, lung, and uterus. Prolonged autoradiographic exposure demonstrated a low intensity hybridization signal in both the heart and thymus. The faint hybridization signal of approximately 1.2 kb represents cross-hybridization to the structurally related SMC-specific protein SM22α (46).

To determine the cell specificity of calponin-h1 gene expression, the calponin-h1 cDNA probe was hybridized to Northern
**Structure and Expression of the Calponin-h1 Gene**

**The in vivo tissue distribution and cellular specificity of calponin-h1 gene expression.** A, the top panel shows a Northern blot analysis of RNA samples isolated from adult murine tissues hybridized to the radiolabeled calponin-h1 cDNA probe. RNA size markers are shown in kilobases to the left of the blot. The calponin-h1 cDNA hybridized to a single 1.5-kb species of mRNA, which was present in SMC-containing tissues (arrow). The bottom panel shows the ethidium bromide-stained formaldehyde-containing gel prior to membrane transfer of RNA. The locations of the 28S and 18S ribosomal RNA bands are indicated to the left of the gel. B, the top panel shows a Northern blot analysis of RNA samples isolated from primary rat aortic SMCs (VSMC), A7r5, NIH 3T3 (3T3), C3H10T1/2 (10T1/2), COS-7, SaIb myoblasts and myotubes (SaIb.2 Blasts and Tubes), Hep G2, and EL-4 cells hybridized to the radiolabeled calponin-h1 cDNA probe. The calponin-h1 cDNA probe hybridized to a 1.5-kb species of mRNA (arrow) present in primary VSMCs, A7r5 cells, and C2C12 myoblasts. The bottom panel shows an ethidium bromide-stained formaldehyde-containing gel prior to transfer of RNA.

Blots containing RNAs prepared from freshly isolated primary rat aortic SMCs, the smooth muscle cell line, A7r5, murine NIH 3T3 and C3H10T1/2 fibroblasts, the monkey SV-40 transformed kidney cell line, COS-7, murine SaIb myoblasts and myotubes, the human hepatocellular carcinoma-derived cell line, Hep G2, and the murine lymphoid cell line, EL-4. High levels of calponin-h1 mRNA were detected in primary rat aortic SMCs and the smooth muscle cell line, A7r5 (Fig. 1B, lanes 1 and 2), while the gene was not expressed in other cell lines including NIH 3T3, C3H10T1/2, COS-7, SaIb, Hep G2, and EL-4 cells even after prolonged autoradiographic exposure. Thus, calponin is expressed in a lineage-restricted fashion in primary vascular SMCs and the SMC line, A7r5.

**Developmental Regulation of Calponin-h1 Gene Expression in Vivo**—To determine the temporal and spatial patterns of calponin-h1 gene expression during mouse development, a series of in situ hybridization experiments were performed on staged murine embryos using radiolabeled antisense and sense (control) calponin-h1 cDNA probes derived from both the coding and 3′-untranslated regions of the murine calponin-h1 cDNA. In postcoital day (E) 9.5 mouse embryos, calponin-h1 mRNA was abundantly expressed in the aortic outflow tract or truncus arteriosus (open arrow), dorsal aorta (white arrow), as well as in the developing heart tube (Fig. 2A). Of note, several studies suggest that the SMCs in the aortic outflow tract are derived from the neural crest, while those in the dorsal aorta are derived from the lateral plate mesoderm (50–52). No expression could be detected in other tissues including the oropharynx, esophageal region of the foregut, midgut, hindgut, lung bud, or urogenital ridge. In E14.0 embryos, calponin mRNA is detectable in the branch arteries, foregut, midgut, hindgut, developing lung bud, and urogenital ridge (Fig. 2C). Once again, the sense probe did not hybridize to the E14.0 embryo (Fig. 2D). Higher power views reveal that calponin-h1 mRNA is expressed exclusively in the lamina propria surrounding the gut epithelium (white arrows, Fig. 3B), bronchial epithelium (white arrow, Fig. 3C), the urogenital ridge surrounding the urogenital sinus (white arrow, Fig. 3D), and the medial layer surrounding large arteries such as the dorsal aorta (Fig. 2C) as well as the smaller branch arteries (white arrow, Fig. 3A).

In E18.5 mouse embryos, calponin-h1 mRNA gene expression can no longer be detected above background levels in the heart, while the gene continues to be expressed abundantly in the aorta as well as smaller branch arteries, the esophagus, stomach, upper and lower intestine, pulmonary bronchi and bronchioles, and the bladder (Fig. 2E). At this late fetal stage, low level nonspecific hybridization to the sense probe was detected in the heart, liver, and some skeletal muscles (Fig. 2F). Taken together, these analyses demonstrate that calponin-h1 gene expression is (i) initially detectable at least as early as embryonic day 9.5 in the dorsal aorta, the aortic outflow tract, and the developing heart tube, (ii) sequentially up-regulated in SMC-containing tissues including the smaller branch arteries, primitive gut, lung bud, and urinary tract where it continues to be expressed throughout adult development, and (iii) down-regulated to non-detectable levels during late fetal development in the four-chambered heart.

Calponin-h1 is a Marker of the Contractile/Arrested SMC Phenotype—Within the arterial wall the majority of SMCs are maintained in the G0/G1 stage of the cell cycle and express contractile protein isoforms (3). However, in response to vascular injury, SMCs are stimulated to pass through the G1/S checkpoint of the cell cycle, proliferate, and modulate their phenotype by up-regulating the expression of intracellular matrix proteins (4–13). Thus, it was of interest to determine the pattern of calponin-h1 gene expression in quiescent and proliferating SMCs. To address this question, primary cultures of third passage rat aortic SMCs were synchronized in the G0/G1 stage of the cell cycle by serum starvation for 72 h. Under these conditions, 85% of SMCs are arrested in G0/G1, of the cell cycle as assayed by propidium iodide staining and fluorescence-activated cell sorting analyses (Fig. 4A, left panel). The cells were then serum-stimulated, and RNA was harvested from replicate cultures at 0, 8, 12, 16, and 24 h post-stimulation. Following serum stimulation, primary rat aortic SMCs begin to pass through the G1/S transition point at approximately 12 h (data not shown), and by 24 h post-stimulation, greater than 50% of the cells are in the S and G2 + M phases of the cell cycle (Fig. 4A, right panel). Northern blot analyses revealed that at approximately 12 h post-stimulation (coincident with the G1/S transition), calponin-h1 mRNA began to be down-regulated, and by 24 h post-stimulation, a marked reduction in calponin gene expression was noted (Fig. 4B, arrow). Quantitative phosphorimage analyses of signal intensity demonstrated a 50% decrease in signal intensity within 24 h from the time of serum stimulation (Fig. 4C). In contrast, hybridization of this membrane to a cDNA encoding the retinoblastoma gene product (Fig. 4, B and C) demonstrated a slight increase in Rb gene expression in proliferating SMCs post-serum stimulation. Similarly, we have reported previously that expression of the SMC-
specific gene, SM22α, is stable in cell cycle-arrested and proliferating SMCs (46). Thus, these data demonstrate that calponin-h1 gene expression is down-regulated when quiescent vascular SMCs re-enter the cell cycle and proliferate and suggest that calponin-h1 is a specific marker of the contractile SMC phenotype.

Calponin-h1 Is a Member of a Multigene Family—Previous investigators have reported that calponin shares structural domains in common with a number of other thin filament myofibrillar proteins including troponin T, troponin I, and caldesmon (29). In addition, we and others (18, 46, 53) have reported high level sequence identity with the SMC-specific protein SM22α. To determine whether calponin-h1 was encoded by a single copy gene in the murine genome and whether this gene was part of a multigene family, we hybridized the calponin-h1 cDNA probe to Southern blots containing murine...
genomic DNA under both high and low stringency conditions. Under high stringency conditions, the calponin-h1 cDNA probe hybridized to 1–2 BamHI, EcoRI, HindIII, PstI, and XbaI fragments, suggesting that calponin-h1 is encoded by a single copy murine gene (Fig. 5A). However, under moderate stringency conditions (2 × SSC, 0.1% SDS at 50 °C), between four and eight additional bands were detected in each lane (Fig. 5B). Taken together, these data demonstrate that calponin-h1 is a single copy gene that is a member of a multigene family.

Isolation and Structural Characterization of Calponin-h1 Genomic Clones—A full-length murine calponin-h1 genomic clone was isolated by screening a murine 129SV genomic library with a calponin-h1 cDNA probe under high stringency conditions. A partial restriction map of this 18-kb clone, designated mCalp-5A, is shown in Fig. 6A. Exons were identified by Southern hybridization with specific cDNA fragments, and their boundaries were confirmed by DNA sequence analyses. The murine calponin-h1 gene is composed of seven exons spanning approximately 10.7 kb of genomic DNA. Each of the splice junctions (Fig. 6C, underlined) conforms to the consensus splice donor-acceptor patterns described by Brentnach and Chambon (54).

The transcriptional start site was identified by RNase protection and primer extension analyses (Fig. 7). As shown in Fig. 7A, primer extension analyses utilizing an antisense synthetic oligonucleotide corresponding to bp +80 to +115 of the previously published sequence of the murine calponin-h1 cDNA (GenBank Accession No. Z19542) (32) resulted in two major extended products of 93 and 95 bp, respectively (arrows), each of which was thermostable up to a temperature of 56 °C. In addition, a less abundant (10% relative signal intensity) 118-bp primer extension product was also detected upon prolonged autoradiographic exposure (dashed arrow). Thus, primer extension analyses suggests that the 5′ most transcriptional start site is located 101 bp 5′ of the translational initiation codon (Fig. 6C, closed arrowhead) with alternative transcriptional start sites located 78 and 76 bp 5′ of the initiation codon (Fig. 6C, black dots). To complement the primer extension analyses, RNase protection analyses were also performed using an antisense cRNA probe corresponding to bp –160 to +97 of the calponin-h1 genomic sequence as deduced by DNA sequence, Southern blot, and primer extension analyses (Fig. 7B). These analyses revealed several major protected fragments of 97 (37% relative signal intensity), 73, 71, and 69 bp, respectively (Fig. 7B, arrows). Thus, RNase protection analyses suggests that the 5′ most transcriptional start site is located 101 bp 5′ of the initiation codon (Fig. 6C, closed arrowhead) with alternative transcriptional start sites located 78, 76, and 74 bp (Fig. 6C, closed arrowheads) 5′ of the initiation codon. Taken together, these analyses served to identify the 5′ most transcriptional

![Image](http://www.jbc.org/)

**Fig. 4.** Down-regulation of calponin-h1 gene expression in proliferating SMCs. A, the left panel shows the FACS cell cycle analysis of propidium iodide-stained SMCs following 72 h of serum starvation. 85% of cells were in G0/G1 phase, 6% of cells in S phase, and 9% of cells in G2 + M phase as assessed using CellFIT software program. The right panel shows the FACS analysis of propidium iodide-stained SMCs 24 h post-serum stimulation. 47% of cells were in G0/G1 phase, 6% of cells in S phase, and 9% of cells in G2 + M phase. B, the left panel shows the Northern blot analysis of RNA prepared from G0/G1 synchronized cultures of primary rat aortic SMCs at t0 and 8, 12, 16, and 24 h post-serum stimulation hybridized to the radiolabeled calponin-h1 cDNA probe. The bottom panel shows the ethidium bromide-stained formaldehyde-containing gel prior to membrane transfer of RNA. The location of the 28S and 18S ribosomal RNA bands are indicated to the left of the gel. C, quantitative image analysis of the Northern blot hybridization signals. The autoradiographic hybridization signals (dpm) for calponin-h1 mRNA were quantitated using a Molecular Dynamics PhosphorImager, and each signal was normalized to the quantitated dpm obtained at time t0 and is reported as normalized gene expression.
Structure and Expression of the Calponin-h1 Gene

A sequence homology search of the protein sequence databases demonstrated that this 29-amino acid motif is conserved across species within each of the calponin isoforms (29, 32, 33, 55) (Fig. 8). Interestingly, five direct repeats of this amino acid motif are present in the recently identified unc-87 body wall muscle protein of *Caenorhabditis elegans* (55) (Fig. 8). In addition, single copies of this motif are present in the chicken, rat, mouse, and *Drosophila* neuronal protein NP25 (58), and the rat muscle protein of (59) (Fig. 8). Comparison of the amino acid sequence of this motif across species and family members revealed a conserved 29-amino acid direct repeat (amino acids 164–192, 204–232, and 243–271) (Fig. 6). As shown in Fig. 6, the full-length calponin-h1 cDNA is composed of a 101-bp 5'-untranslated region, an 892-bp open reading frame, and a 494-bp 3'-untranslated region. The predicted protein contains three 29-amino acid direct repeats (amino acids 164–192, 204–232, and 243–271) (Fig. 6B, gray boxes). As shown in Fig. 6B, the full-length calponin-h1 cDNA is composed of a 101-bp 5'-untranslated region, an 892-bp open reading frame, and a 494-bp 3'-untranslated region. The predicted protein contains three 29-amino acid direct repeats (amino acids 164–192, 204–232, and 243–271). (Fig. 6B, gray boxes). A sequence homology search of the protein sequence data bases demonstrated that this 29-amino acid motif is conserved across species within each of the calponin isoforms (29, 32, 33, 55) (Fig. 8). Interestingly, five direct repeats of this amino acid motif are present in the recently identified unc-87 body wall muscle protein of *Caenorhabditis elegans* (55) (Fig. 8). In addition, single copies of this motif are present in the chicken, rat, and murine SM22a proteins (13, 18, 46, 53, 56, 57), the rat neuronal protein NP25 (58), and the Drosophila muscle protein mp20 (59) (Fig. 8). Comparison of the amino acid sequence of this motif across species and family members revealed a consensus sequence of (I/V)GLQMGTNK.
positive control plasmid pGL2-Control, which contains the
mately 50% of that demonstrated after transfection of the
low transfection of the p-1500cal/luc plasmid was approxi-
A
The level of luciferase activity fol-
mid pGL2-Basic (Fig. 9
increases in luciferase activity as compared to the control plas-
فلanking sequence, resulted 180- and 290-fold, respectively,
region of skeletal, cardiac, and smooth muscle genes identified
-acting sequences present in the transcriptional regulatory
DNA sequence analysis software (Eastman Kodak Co., IBI).
The immediate 5'-flanking sequence did not contain a consen-
TATA or CAAT box. A search for previously described
potential transcriptional regulatory elements using MacVector
sequence flanking the cap site was searched for
transcriptional regulatory elements using MacVector
DNA sequence analysis software (Eastman Kodak Co., IBI).
The immediate 5'-flanking sequence did not contain a consen-
TATA or CAAT box. A search for previously described
cis-acting sequences present in the transcriptional regulatory
regions of skeletal, cardiac, and smooth muscle genes identified
one consensus GATA binding site (WGATAR) (61) located at bp
-273, two consensus CACC boxes (62) located at bp −322 and
-214, and seven consensus E boxes (63) located at bp −244,
-441, −660, −916, −954, −961, −1150, and −1224. However,
unlike each of the three previously characterized smooth muscle
long-exon/unrestricted or lineage-specific genes, including the
SM22α (46), SM-myosin heavy chain (64), and SM-α-actin (65,
66), the immediate 5'-flanking sequence of the calponin-h1
gene did not contain a consensus CArG/SRF binding site (67,
68). In addition, four consensus binding sites for the ubiqui-
tously expressed transcription factor AP2 were identified at
bps −39, −214, −237, and −322 and one consensus binding
site for Sp1 was identified at bp −983.

Identification of the Calponin-h1 Transcrip-
tional Promoter—To confirm that the immediate 5'-flanking region of the
calponin-h1 gene functions as a transcriptional promoter in
SMCs, a series of transient transfections was performed using
calponin-h1/luciferase reporter constructs and the SMC line,
A7r5, as well as primary cultures of rat aortic SMCs, both of
which express the gene (see Fig. 1B). Transfection of A7r5 cells
with the plasmids p-3400cal/luc, containing 3.4 kb of 5'-flank-
ing sequence, or the p-1500cal/luc, containing 1.5 kb of 5'
flanking sequence, resulted 180- and 290-fold, respectively,
increases in luciferase activity as compared to the control plasmid
pGL2-Basic (Fig. 9A). The level of luciferase activity
following transfection of the p-1500cal/luc plasmid was approxi-
ately 50% of that demonstrated after transfection of the
positive control plasmid pGL2-Control, which contains the
SV40 promoter and transcriptional enhancer.

To test the activity of the calponin-h1 promoter in primary
SMCs, confluent cultures of low passage rat aortic SMCs
(which express abundant levels of calponin mRNA) were tran-
siently transfected with the p-1500cal/luc reporter plasmid.
Under these transfection conditions, the p-1500cal/luc plasmid
increased luciferase activity approximately 40-fold above levels
obtained following transfection with the promoterless pGL2-
Basic plasmid (Fig. 9B). Once again, this level of transcripti-
onal activity was approximately 50% of that obtained follow-
ing transfection with the positive control plasmid, pGL2-
Control (Fig. 9B). Of note, transfection of subconfluent cultures
of proliferating primary rat aortic SMCs with the p-1500cal/luc
plasmid resulted in significantly lower levels of transcriptional
activity relative to the positive control plasmid, suggesting that
the down-regulation of calponin-h1 gene expression in prolifer-
ating SMCs may be transcriptionally regulated (data not

| Protein                | aa sequence       |
|------------------------|-------------------|
| Mouse calponin h1      | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Mouse calponin h1      | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Mouse calponin h1      | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Chicken calponin α     | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Chicken calponin β     | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Pig calponin h1        | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Rat calponin, acidic    | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| C. elegans unc-87      | VIHLKQGVRQKQGGRQ |
| C. elegans unc-87      | VIHLKQGVRQKQGGRQ |
| Human SM22α            | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Human SM22α            | IGLGGMKSNRASGQGGMATCTGRHDLDFK |
| Drosophila mp20        | VLHLKQGVRQKQGGRQ |

**Fig. 8.** Evolutionary conservation of the 29-amino acid calpo-
nin repeat domain. A computer-based search of the protein data
bases revealed that the calponin 29-amino acid repeat domain is con-
served in other proteins including chicken calponin α, chicken calponin
β, pig calponin-h1, rat acidic calponin, C. elegans unc-87, mouse SM22α,
human SM22α, and Drosophila mp20. The location of this motif within
each respective protein is indicated in the column under aa. The actual
amino acid sequence of each motif is indicated. Amino acid identity or
conservation is indicated by gray shading. A consensus amino acid
motif derived from this analysis is shown below the protein sequences.
shown). Taken together, these data demonstrate that the immediate 5’-flanking region of the calponin-h1 gene contains a promoter that is active in both quiescent primary cultures of rat aortic SMCs and the smooth muscle cell line, A7r5.

**DISCUSSION**

The myofibrillar thin filament actin-binding protein, calponin, is believed to regulate SMC contraction and has been proposed as a candidate marker of the SMC lineage (11, 13, 28–31). In this report, we have examined the spatial and temporal pattern of calponin-h1 gene expression during murine development. In addition, we have defined the pattern of calponin-h1 gene expression in quiescent and proliferating cultured rat aortic SMCs. We have isolated and structurally characterized the murine calponin-h1 gene, which has provided novel insights into the evolution and function of the calponin multigene family. Finally, we have demonstrated that the immediate 5’-flanking region of the calponin-h1 gene directs high level transcription of the calponin-h1 gene in primary cultures of rat aortic SMCs. These data are relevant to several unresolved issues in SMC biology including (i) the developmental programs that control SMC lineage specification, (ii) the molecular mechanisms that regulate SMC phenotypic modulation, and (iii) the evolution and function of the calponin multigene family.

In contrast to the skeletal muscle lineage (for review, see Ref. 69), relatively little is currently understood about the molecular mechanisms that control SMC lineage specification and differentiation. This is due in part to the poorly defined embryological origin of SMCs (18–26), the lack of definitive markers of the SMC lineage (27), and the phenotypic plasticity of this muscle cell lineage (4–13). In this report, we have determined the pattern of expression of the murine calponin-h1 gene during murine embryogenesis and in adult mice to investigate its utility as a marker of the SMC lineage. As anticipated, the calponin-h1 gene is expressed solely in SMC-containing tissues in adult animals demonstrating that the pattern of expression of the protein is regulated at the level of gene expression. Calponin mRNA was detected at least as early as embryonic day 9.5 in the dorsal aorta as well as the aortic outflow tract. Thus, the calponin-h1 gene represents one of the earliest developmental markers of the SMC lineage. Second, we have shown that calponin-h1 gene expression is rapidly down-regulated when primary rat aortic SMCs begin to pass through the G2/S checkpoint of the cell cycle and proliferate. Taken together, these data suggest that the calponin-h1 gene will serve as an excellent model system to elucidate the molecular mechanisms that regulate the SMC specification and differentiation.

Our results demonstrated that the calponin-h1 gene is not only expressed in SMCs but is also transiently expressed in the early embryonic heart. Of note, several other genes that are expressed in a SMC-specific or lineage-restricted fashion during postnatal development including SM22a2 and SM-α-actin (70, 71) are also expressed in embryonic cardiac myocytes. Similarly, expression of vascular smooth muscle α-actin in avian cardiogenesis correlates with the onset of cardiomyocyte differentiation (70). Given the common embryological origin of some SMCs and cardiac progenitors from the lateral plate mesoderm, it is tempting to speculate that a common developmental program might direct early cardiac and SMC lineage specification. In this regard, it will be of interest to determine whether distinct or similar sets of cis-acting sequences and trans-acting factors control transcription of the calponin-h1 gene in each of these two early muscle cell lineages. In this regard, it is noteworthy that the calponin-h1 promoter contains a GATA and CACCC box5p1 binding sites. Similar sites have been identified previously in several cardiac-specific transcriptional regulatory elements (72–74).

Finally, structural analyses of the calponin-h1 gene revealed some new insights into the function and evolution of the calponin multigene family. The calponin-h1 protein contains three conserved direct repeats of 29 amino acids (amino acids 145–192, 204–232, and 243–271). Previous investigators have demonstrated that amino acids 145–182 encode an actin-binding domain (39, 75). In addition, each of these repeats contains a conserved threonine residue, which is phosphorylatable by protein kinase C in vitro (60). This motif is present in other putative actin-binding proteins including the non-muscle, acidic isoform of calponin, the human, murine, rat, and chicken SM22α proteins (13, 18, 46, 53, 56, 57), the Drosophila muscle protein mp20 (59), and the recently described C. elegans unc-87 body wall protein (55) (Fig. 8). Interestingly, mutation of the unc-87 protein, which contains five copies of this motif, results in variable degrees of paralysis (55). As such, these data are consistent with the hypothesis that the calponin multigene family evolved from a common ancestral protein with a conserved amino acid motif that encodes an actin-binding domain, which is regulated by phosphorylation. Future studies of this conserved domain may provide insights into the function of calponin and related proteins in modulating the contractile phenotype.

**Acknowledgments**—We thank G. Eichele for providing a protocol for performing in situ hybridization on mouse embryo sections and Eric N. Olson for helpful discussions. We thank Jeffrey M. Leiden and M. Celeste Simon for reviewing the manuscript. We thank Amy Murphy for expert secretarial assistance and Lisa R. Goldtschak for expert preparation of illustrations.

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J. Biol. Chem. 1996, 271:395-403.
doi: 10.1074/jbc.271.1.395

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