Expression of the *SM22α* Promoter in Transgenic Mice Provides Evidence for Distinct Transcriptional Regulatory Programs in Vascular and Visceral Smooth Muscle Cells

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Abstract. SM22α is a putative calcium-binding protein that is expressed in cardiac, smooth, and skeletal muscle lineages during mouse embryogenesis and in adult smooth muscle cells (SMC). To define the mechanisms that regulate smooth muscle–specific gene transcription, we isolated the *SM22α* gene and analyzed its 5'-flanking region for elements that direct smooth muscle expression in transgenic mice. Using a series of promoter deletions, a region of the *SM22α* promoter containing 445 base pairs of 5'-flanking sequence was found to be sufficient to direct the specific expression of a *lacZ* transgene in mouse embryos in the vascular smooth, cardiac, and skeletal muscle lineages in a temporospatial pattern similar to that of the endogenous *SM22α* gene. However, in contrast to the endogenous gene, transgene expression was not detected in venous, nor visceral SMCs. This *SM22α-lacZ* transgene was therefore able to distinguish between the transcriptional regulatory programs that control gene expression in vascular and visceral SMCs and revealed heretofore unrecognized differences between these SMC types. These results suggest that distinct transcriptional regulatory programs control muscle gene expression in vascular and visceral SMCs.

T HE three major muscle cell types, skeletal, cardiac, and smooth, express many of the same muscle structural genes. However, each muscle type is unique in its contractile properties and morphology and each appears to use distinct regulatory mechanisms to control muscle gene expression. Whereas the molecular mechanisms that control differentiation of skeletal and cardiac muscles have begun to be elucidated (Weintraub et al., 1991; Buckingham, 1994; Olson and Klein, 1994; Bodmer, 1995), little is known of the myogenic program that controls smooth muscle myogenesis and no transcription factors that regulate smooth muscle cell (SMC)[1]-specific gene expression have yet been identified. The developmental pathways that lead to the formation of different SMC types are also unknown.

Unlike skeletal and cardiac muscle cells, which differentiate irreversibly, SMCs exhibit phenotypic plasticity and can readily undergo transitions between the proliferative and differentiated states (Glukhova et al., 1991; Frid et al., 1992; Schwartz et al., 1995). This modulation is apparent in progressively cultured SMCs in which expression of several contractile protein genes is extinguished as the cells acquire a synthetic, proliferative phenotype (Chambley-Campbell, 1981; Owens, 1986; Thyberg, 1987). Because of this plasticity, the properties of SMCs in tissue culture may not reflect those in vivo.

To begin to investigate the molecular mechanisms that regulate SMC gene expression in vivo, we have analyzed the expression of SMC-specific genes during mouse embryogenesis (Miano et al., 1994; Li et al., 1996). We are interested in the regulation of *SM22α* expression because *SM22α* is highly expressed in all adult smooth muscle–containing tissues (Lees-Miller et al., 1987; Shanahan et al., 1993; Duband et al., 1993). Previous studies indicated that *SM22α* encodes a 22-kD protein (Lees-Miller et al., 1987), which shares sequence homology with *Drosophila* mp20 (Ayme-Southgate et al., 1989) and NP25 (Ren, 1994), as well as calponin, a troponin T–like protein that interacts with F-actin, tropomyosin, and calmodulin (Duband et al., 1993). Recently, we reported that *SM22α* is expressed in cardiac, smooth, and skeletal muscle cells during early em-

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1. *Abbreviations used in this paper:* bHLH, basic helix-loop-helix; MHC, myosin heavy chain; MLC, myosin light chain; SMC, smooth muscle cell.

bryogenesis but becomes restricted to smooth muscle lineages at late embryonic stages and throughout adulthood (Li et al., 1996).

Here we isolated the mouse $SM22\alpha$ gene and analyzed the regulation of the $SM22\alpha$ promoter in transgenic mice as well as in tissue culture cells. Our results demonstrated that the proximal promoter of $SM22\alpha$ was sufficient to direct expression of a reporter gene in cultured SMCs. In transgenic mice, this promoter exhibited a temporospatial expression pattern similar to that of endogenous $SM22\alpha$ transcripts except that transgene expression was not detected in visceral or venous SMCs during embryogenesis. These results reveal previously unrecognized heterogeneity among arterial, venous, and visceral SMCs and suggest that distinct transcriptional regulatory mechanisms exist for the control of $SM22\alpha$ gene expression in different SMC lineages.

Materials and Methods

Isolation and Characterization of Mouse SM22α Genomic Clones

An $SM22\alpha$ genomic clone was isolated by screening an SV129 mouse genomic library (Stratagene Corp., La Jolla, CA) using the 1,078-bp mouse $SM22\alpha$ cDNA as a probe (Li et al., 1996). Southern blot analysis of mouse genomic DNA and the $SM22\alpha$ genomic clone showed the same restriction maps, indicating that the genomic clone was not rearranged. DNA was hybridized on duplicate filter lifts overnight at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.05 M Na₂HPO₄, pH 7.0, 0.1% SDS, 100 µg/ml of salmon sperm DNA. After washing in 1× SSC, 0.1% SDS, positive plaques were carried through three successive rounds of screening. One genomic clone with a 15-kb insert that contained the entire $SM22\alpha$ gene and 5' flanking sequences was chosen for further characterization.

The intron/exon organization of the $SM22\alpha$ gene was determined by sequence comparison between genomic DNA and cDNA, and by PCR using primers from the cDNA sequence. The intron-exon boundaries were consistent with the AG/GT splicing consensus sequence.

RNase Protection Analysis

Total RNA from adult mouse tissues and cell lines was isolated by the acid phenol protocol (Chomczynski and Sacchi, 1987). $SM22\alpha$ transcripts were detected by RNase protection using the Maxi-Script and RPA kits (Ambion, Austin, TX). Approximately 15 µg of total RNA was hybridized to an in vitro transcribed $SM22\alpha$ riboprobe ($\sim$1 × 10⁵ cpm) corresponding to a 275-bp PCR product described previously (Li et al., 1996).

Generation of SM22α-Luciferase and SM22α-lacZ Reporters

The 15-kb $SM22\alpha$ genomic phage clone was excised by digestion with NotI and subcloned into pBluescript SKII+ (Stratagene Corp.) for further analysis. Initially, a 3,893-bp BalI-fragment was subcloned into the pBasic-luc (Nordeen, 1988) and pBS-lacZ (Cheng et al., 1992) vectors, yielding the plasmids, pSM2735/1093-luc and pSM2735/1093-lacZ, respectively. This BalI fragment contained 2,735-bp of 5' flanking region, the 64-bp first exon and 1,093-bp of the first intron. To eliminate the intronic sequence, a 2,798-bp PCR fragment containing 2,735-bp of 5'-flanking sequence and the first 62-bp of exon 1 of the $SM22\alpha$ gene was generated using synthetic oligonucleotides A and B as primers (the 3' end of primer A was located at −2,735; primer B was reversed and complementary to sequence +41 to +62 with a SalI site engineered at the 3' end). This PCR fragment was cloned into the TA vector (Invitrogen, San Diego, CA), yielding the plasmid pSM2735-TA. The insert was then excised by digestion with BamHI and SalI and the resulting BamHI/SalI fragment, corresponding to sequences from −2,735 to +62, was cloned into pBasic-luc and pBS-lacZ to yield the plasmids pSM2735-luc and pSM2735-lacZ. A 1,406-bp EcoRI/SalI fragment and a 508-bp PstI/SalI fragment were excised from the pSM2735-TA construct and subcloned into pBasic-luc and

pBS-lacZ, resulting in constructs pSM1343-luc, pSM445-luc, pSM1343-lacZ, and pSM445-lacZ. Finally, a fragment containing 144-bp of 5'-flanking sequence and 62 bp of exon 1 was generated by PCR and subcloned into pBasic-luc, resulting in the construct pSM144-luc. The orientations and identities of the inserted fragments in all constructs were confirmed by sequencing.

Transfection and Luciferase Assays

The primary rat aortic SMC cells (gift from Dr. M. Majesky, Baylor College of Medicine, Houston, TX), 10T1/2 and F9 cells were split and seeded in 6 cm dishes. After 24 h, cells were $\sim$70% confluent before transfection. F9 cells were transfected at $\sim$50% confluence. 5 µg of each luciferase reporter was transfected. Transient transfections were performed by calcium phosphate precipitation as described previously (Li et al., 1992). 48 h later, cells were harvested in 300 µl 0.1 M Tris-HCl (pH 7.8) containing 1 mM DTT. After three cycles of freeze/thaw, cells were spun for 5 min at 4°C. Aliquots ($\sim$20 µl) of supernatant containing equal quantities of protein were mixed with 330 µl of reaction buffer (0.1 M Tris-HCl, pH 7.8, 5 mM ATP, 15 mM MgSO₄, and 1 mM DTT) and 100 µl 1 mM luciferin (Analytic Luminescence Laboratories, San Diego, CA). Transfection efficiency of different cell lines was determined by comparing the expression of pSV₂-luc, which contains the SV-40 enhancer and promoter. All transfections were repeated two to six times.

Transgenic Mice

Plasmids pSM-2735/1093-lacZ, pSM-2735-lacZ, pSM-1343-lacZ and pSM445-lacZ were tested for $SM22\alpha$ promoter activity in transgenic mice. Methods for production and analysis of transgenic mice were described previously (Cheng et al., 1992). The same temporospatial expression pattern was observed in multiple independent transgenic lines. For histological analysis, samples were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS at 4°C for 30 min to 2 h (depending on sample size). After rinsing in PBS and staining with X-gal, samples were dehydrated with ethanol and cleared in xylene, and embedded in paraffin. For larger samples, 0.1% sodium deoxycholate and 0.2% NP-40 were added to PBS for rinsing and staining. Embryos were sectioned on a microtome at a thickness of 5–10 µm, and counterstained with hematoxylin and eosin.

To better visualize the vasculature of the embryo, stained embryos were dehydrated in 100% methanol for 2 d and cleared in a solution of 2 vol of benzyl benzoate per volume of benzyl alcohol for 1–3 h before photography.

Results

Characterization of the SM22α Gene

To begin to study the regulatory mechanisms that control $SM22\alpha$ expression, we isolated the mouse $SM22\alpha$ gene and characterized its structure. As reported previously, the gene spans 5.9 kb and contains 5 exons (Solway et al., 1995). The transcriptional initiation site for $SM22\alpha$ was determined by primer extension and RNase protection and agreed with the results reported by Solway et al. (1995).

The sequence, extending from −2,735 through the first exon to +1,093 bp in the first intron was determined (GenBank EMBL/DDBJ accession number U36589). An A/T-rich sequence, TTTAAA, which could serve as a TATA box, was located at −28 bp. Sequence analysis revealed a number of sequence motifs in the 5'-flanking region of the gene that have been implicated in transcriptional regulation of smooth, skeletal, and cardiac muscle genes (Solway et al., 1995; and data not shown). In particular, two CArG boxes, CC(A/T)₆GG, (Gustafson et al., 1988), are present 150- and 274-bp upstream of the transcription initiation site.

Identification of the SM22α 5' Flanking Region That Confers the Transcriptional Activity In Vitro

To choose a cell line suitable for analysis of the SM22α promoter activity, the SM22α expression profile in different cell lines was determined by RNase protection assays. Although SM22α expression was restricted to SMCs in adult mouse tissues (Li et al., 1996), SM22α transcripts were detected in cell lines derived from different origins, including the 10T1/2 and 3T3 fibroblast cell lines, the BC3H1 muscle cell line, which has properties of smooth and skeletal muscle (Schubert et al., 1974; Edmondson and Olson, 1989), the C2 skeletal muscle cell line, and the P19 embryonal carcinoma cell line (Fig. 1). Expression of SM22α transcripts was also detected in primary rat aortic SMCs (not shown). However, no expression was detected in the F9 teratocarcinoma cell line. In the BC3H1, C2, and P19 cell lines, we observed no difference in SM22α expression in differentiated or undifferentiated cells.

As a first step in identifying cis-regulatory elements that may be responsible for the transcriptional regulation of SM22α, we created a series of SM22α-luciferase reporter genes using DNA fragments that extended varying distances upstream from the first exon. These reporters were tested for expression by transient transfection in primary rat aortic SMCs. The reporter pSM2735-luc, which extends from the first exon to −2,735 bp, was expressed at a level similar to pSM445-luc, indicating that sequences between the first exon and −445 bp are sufficient to confer transcriptional activity in cultured SMCs (Fig. 2 A). The reporter pSM1343-luc was expressed at a slightly higher level than pSM2735-luc, suggesting the possible existence of a negative element between −2,735 and −1,343 bp. Deletion from −445 to −144 resulted in a dramatic decrease in promoter activity, indicating that sequences in this region are important for SM22α transcription.

To determine whether the isolated promoter was active in other cell types, pSM2735-luc was also tested in 10T1/2 fibroblasts and F9 teratocarcinoma cells (Fig. 2 B). This reporter was expressed at a threefold higher level in SMCs

A.

B.

Figure 2. Expression of SM22α-luciferase constructs in cultured cells. (A) DNA fragments extending from nucleotide +62 to the indicated distances upstream of SM22α were tested for transcriptional activity in the luciferase vector. The promoter activity of each construct was tested by transfecting into passage 25 primary rat aortic SMCs. Activities are expressed relative to that of pSM2735-luc. (B) Specificity of isolated SM22α promoter in different cell lines. The construct pSM2735-luc, or pSM445-luc or pBasic-luc was transfected into F9 cells, 10T1/2 cells, and rat aortic SMCs. Activities were determined using equal amounts of protein and were normalized for transfection efficiency in different cell lines by comparing the activities of pSV2-luc in different cell lines. The results represent the averages of two to six transfection experiments.

than in 10T1/2 cells and was inactive in F9 cells, in agreement with the expression of SM22α transcripts in these cell types (Fig. 2 B). The construct pSM445-luc was expressed similarly to pSM2735-luc in the different cell types (not shown). Both of the above SM22α-luciferase were also active in COS cells (not shown). These results suggest that sequences in the 445-bp proximal promoter of SM22α are sufficient to provide transcriptional activity in tissue culture cells. In a previous study, SM22α was reported to be active only in SMCs in vitro and not in 3T3, COS, or HepG2 (Solway et al., 1995).

Generation of Transgenic Mice Harboring SM22α-lacZ Transgenes

To determine whether the SM22α regulatiory sequences tested in tissue culture cells were sufficient to direct appropriate expression in vivo, we generated two lacZ reporter genes: one that extended from −2,735 to +62 bp

Figure 1. SM22α mRNA expression in a variety of cell lines detected by RNase protection. Approximately 15 μg of total cellular RNA from the indicated cell lines and adult mouse tissues was analyzed for the expression of SM22α mRNA by RNase protection. The protected fragment for SM22α is 275-bp in length. RNA from stomach was included for positive control. U and D indicate undifferentiated and differentiated cells, respectively. The conditions for differentiation of each cell line were described previously (Miano et al., 1994).

(pSM2735-lacZ), the other from −2,735 to 1,093 bp in the first intron (pSM2735/1093-lacZ). These reporter genes were used to generate transgenic mice that were examined for lacZ expression as founders and stable transgenic lines. Both constructs showed comparable expression patterns.

The expression pattern of lacZ in one of the representative transgenic lines harboring pSM2735/1093-lacZ is shown in Fig. 3. Whole mount-staining for lacZ expression revealed that transgene expression was first detected in the primitive heart tube at E8.75 (Fig. 3 A), consistent with the initial expression of SM22α transcripts detected by in situ hybridization (Li et al., 1996). At E9.0, the expression of lacZ in the bulbus cordis and outflow tract increased and expression began to be detected in the dorsal aorta (Fig. 3 B). At E10.0 and 11.5, lacZ expression increased in the dorsal aorta, aortic arches and structures of the heart including the bulbus cordis and the truncus arteriosus (Fig. 3, C and D). Expression in the rostral somites was detectable at E9.5 at a low level (not shown), but became apparent at E10.0 to 12.5 in a rostral-to-caudal fashion (Fig. 3, C and D). However, expression in the somites was transient and only lasted for 2–3 d in each somite. Beginning at E13.5, expression in the heart and somites diminished (Fig. 3 E). LacZ staining marked all major vessels in the head and trunk region at this stage (Fig. 3 E). Transgene expression in the vasculature increased continuously through E14.5 (Fig. 3 F) and E15.5 (not shown). During these stages, expression in intercostal vessels could be seen clearly. At all stages examined, the expression of the SM22α-lacZ transgene overlapped with the expression of SM22α transcripts in the vascular smooth, cardiac, and skeletal muscle lineages, with no apparent delay between expression of SM22α transcripts and the transgene.

The primitive heart tube was the earliest site of transgene expression (Fig. 3 A). However, in contrast to the endogenous SM22α gene which was expressed at comparable levels in the bulbus cordis (future right ventricle) and the ventricle of the primitive heart tube (Li et al., 1996),
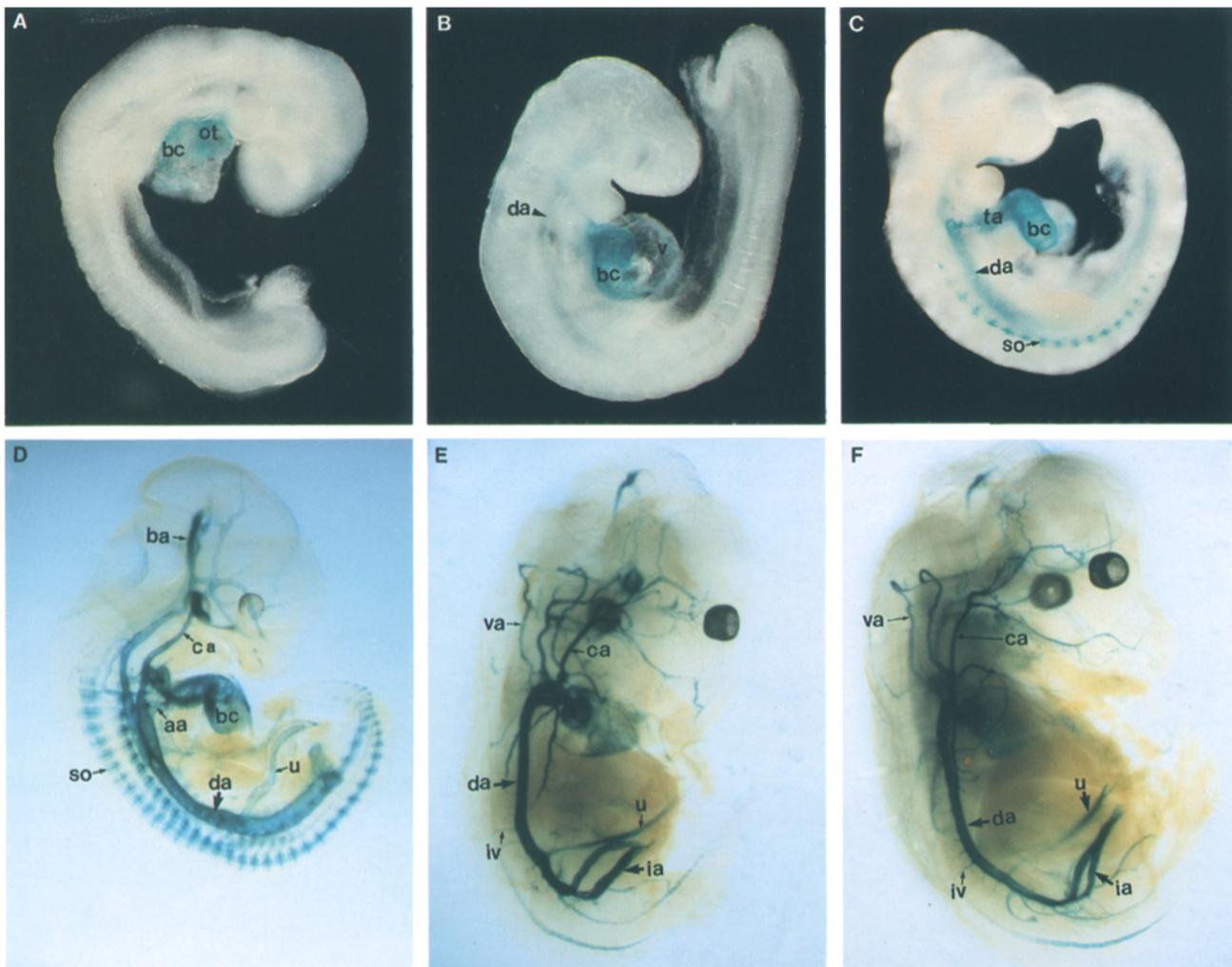
Figure 3. Expression of the *SM22α* promoter in transgenic mice. Transgenic mice harboring pSM2735/1093-lacZ were generated and the resulting embryos were stained for lacZ activity. (*A*) LacZ activity was detected in the bulbus cordis (*bc*) and outflow tract (*ot*) of the primitive heart at E8.75. (*B*) Beginning at E9.0, lacZ activity was detected in the dorsal aorta (*da*). (*C*) Expression of lacZ in the somites (*so*) became apparent at E10.0. To better visualize the vasculature, embryos from E11.5 (*D*), 13.5 (*E*), and 14.5 (*F*) were cleared as described in Materials and Methods. *aa*, aortic arches; *ba*, basilar artery; *bc*, bulbus cordis; *ca*, carotid artery; *da*, dorsal aorta; *ia*, iliac artery; *iv*, intercostal vessel; *ot*, outflow tract; *so*, somite; *ta*, truncus arteriosus; *u*, umbilical vessel; *v*, ventricle; *va*, vertebral artery.

the transgene was expressed only in the bulbus cordis (Fig. 3, A–D). No lacZ expression was detected in the left ventricle in more than five independent transgenic mice analyzed, suggesting that different cis-regulatory elements are required to control gene expression in different regions of the developing heart.

While expression of the transgene was restricted to the bulbus cordis, we observed sporadic expression in cells of the ventricle and atria (not shown). Whether this represents migrating cells or a heterogenous population of muscle cells in the heart region remains to be determined.

Histological analysis of transverse and frontal sections of E11.5 embryos revealed the expression of the transgene in the myocardium, myotomes, and aorta (Fig. 4, A–C). At E11.5, lacZ was clearly expressed in the trabeculated wall of the bulbus cordis and in the outflow tract (Fig. 4, A and B). However, the bulbar ridges within the outflow tract of the heart, which form the aortico–pulmonary spiral septum, did not show lacZ expression (Fig. 4 B). At this stage, lacZ expression was also detected in the common carotid arteries and dorsal aorta, demarcating the muscle layer of the vessels (Fig. 4, A and C). LacZ expression in the somites was restricted to the myotomal region (Fig. 4 C), where the endogenous gene was expressed (Li et al., 1996).

Specific Expression of the SM22α-lacZ Transgene in Arterial SMCs during Embryogenesis

By E13.5, lacZ activity became restricted to the major vessels throughout the embryo (Fig. 5 A). LacZ expression also demarcated the newly formed pulmonary trunk, which becomes the outlet of the right ventricle. The junction where the ascending aorta and the left ventricle meet was also marked by transgene expression (Fig. 5, B and C). No expression was detected in the bulbar ridges of the outflow tract in the heart or in the trabeculated myocardium of the right ventricle (Fig. 5 B). A high level of transgene expression was observed in the branches of the pulmonary arteries and the descending aorta (Fig. 5, B and C). Under high magnification, transgene expression was seen to specifically demarcate the medial layer of the vessel wall; the transgene was not expressed in endothelial cells which form the single cell layer lining the lumen of the vessel (Fig. 5, D and E). We conclude, therefore, that the SM22α promoter is transcriptionally active in SMCs, but not in endothelial cells of the vessel wall.

In contrast to endogenous SM22α, which is expressed at high levels in visceral SMCs beginning at E13.5, no transgene expression was detected in these cells at this stage of development. Sagittal sections of the abdominal region at 13.5 dpc revealed transgene expression in the umbilical arteries (Fig. 5 A). However, no expression was detected in the hindgut or stomach (Fig. 5 A). At the thoracic level, no expression was observed in the large bronchi or branches of the bronchi in the lung (Fig. 5, B and C; Fig. 6 C).

The absence of transgene expression in visceral SMCs was apparent in transverse sections of embryos at E14.5 (Fig. 6). At this stage, the lumen of the stomach is large, and the structures of the hindgut, bladder, kidney, and liver are easily recognized in the peritoneal cavity (Fig. 6 A). However, lacZ expression was observed only in the descending aorta, umbilical arteries, and femoral arteries, and no expression was detected in the well-defined muscle layers of the stomach, gut or bladder (Fig. 6, A and B). Together, these results show that the regions of the SM22α
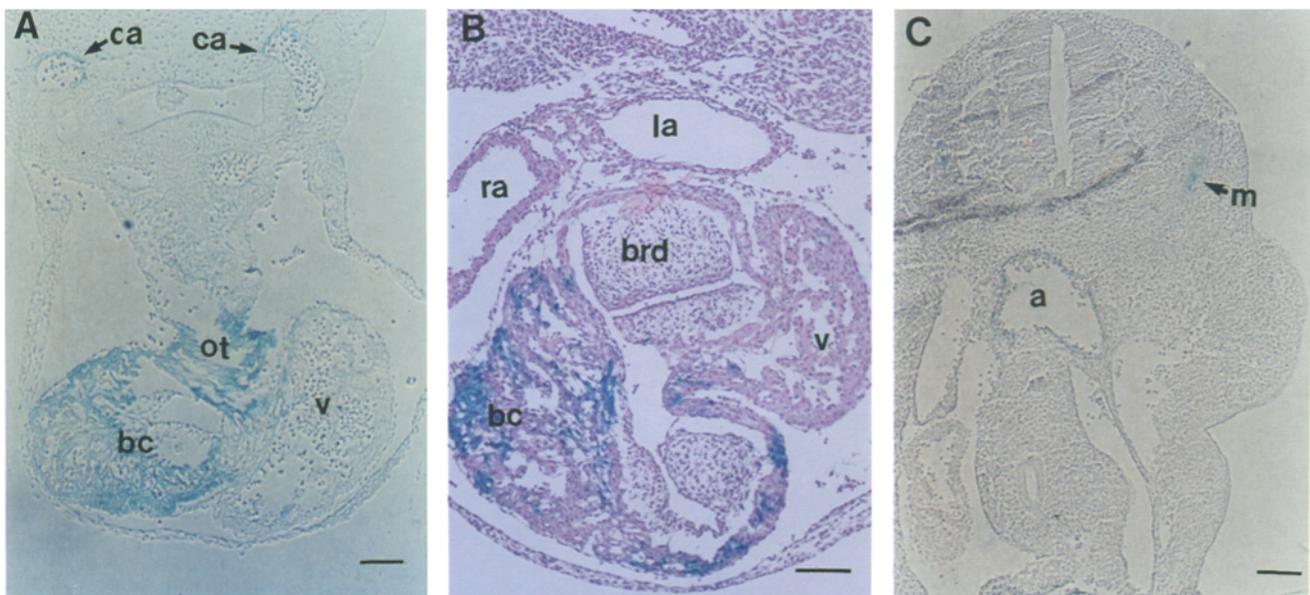
Figure 4. Expression of SM22α-lacZ transgene in the bulbus cordis, outflow tract, aorta, and somites of 11.5 d embryos. Transgenic mice harboring pSM2735/1093-lacZ were generated and the resulting embryos were stained for lacZ activity. (A) Frontal section through the ventricle of the heart of an 11.5 d transgenic embryo shows transgene expression in bulbus cordis (bc), outflow tract (ot), and the carotid arteries (ca). (B) Transverse section through the heart region of an 11.5 d embryo revealed lacZ expression in the trabeculated wall of the bulbus cordis (bc), not in the left or right atria (la and ra), ventricle (v), or bulbar ridges (brd) of the heart. (C) Frontal section through the caudal somites of an 11.5 d embryo shows transgene expression in myotome (m) and dorsal aorta (a). Bar, 100 μm.
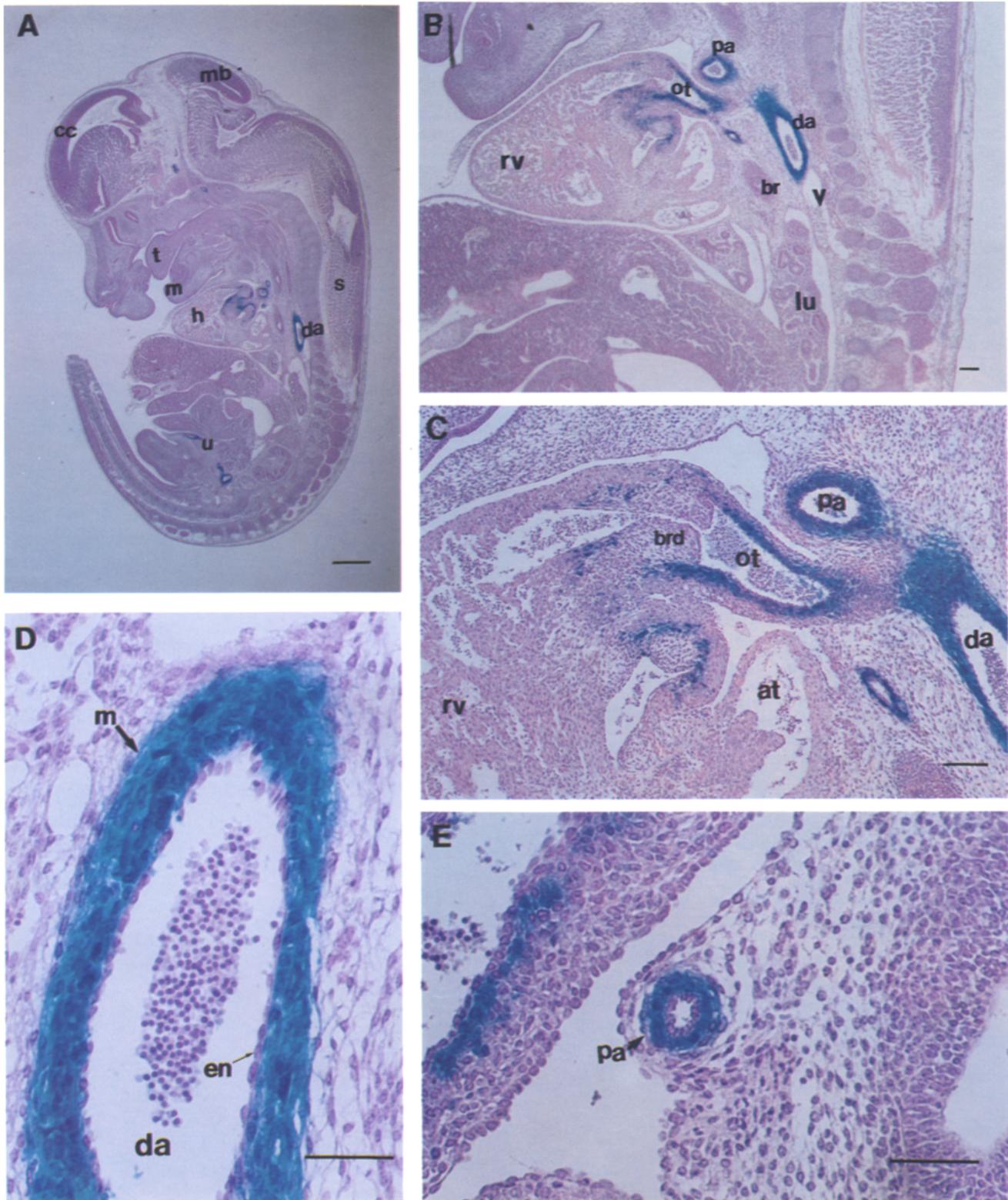
Figure 5. Expression of *SM22α*-lacZ transgene in the arterial SMCs of a 13.5 d embryo. Transgenic mice harboring pSM2735/1093-lacZ were generated and the resulting embryos were stained for lacZ activity. (*A*) A 13.5 d transgenic embryo was stained for *LacZ* and sagittal sections were stained with hematoxylin and eosin. Transgene expression is seen in major vessels in the head and trunk region. (*B*) High magnification of *A* showing the right ventricle of the heart. Transgene expression can be seen in the outflow tract in the heart (*ot*), branches of the pulmonary arteries (*pa*), and descending aorta (*da*). However, no expression was observed in the myocardium of the right ventricle (*rv*), the bronchus (*br*), or the vena cava (*v*). (*C*) A higher magnification of *B* shows that the bulbar ridge (*brd*) in the outflow tract of the heart does not express the transgene. (*D* and *E*) High magnification of *A* in the region of the descending aorta (*da*) and branches of the pulmonary artery (*pa*) shows the specific expression of the transgene in the muscle layer (*m*) around the arteries, not in the endothelial cells (*en*) lining the lumen. Bars: (*A*) 500 μm; (*B–E*) 100 μm.
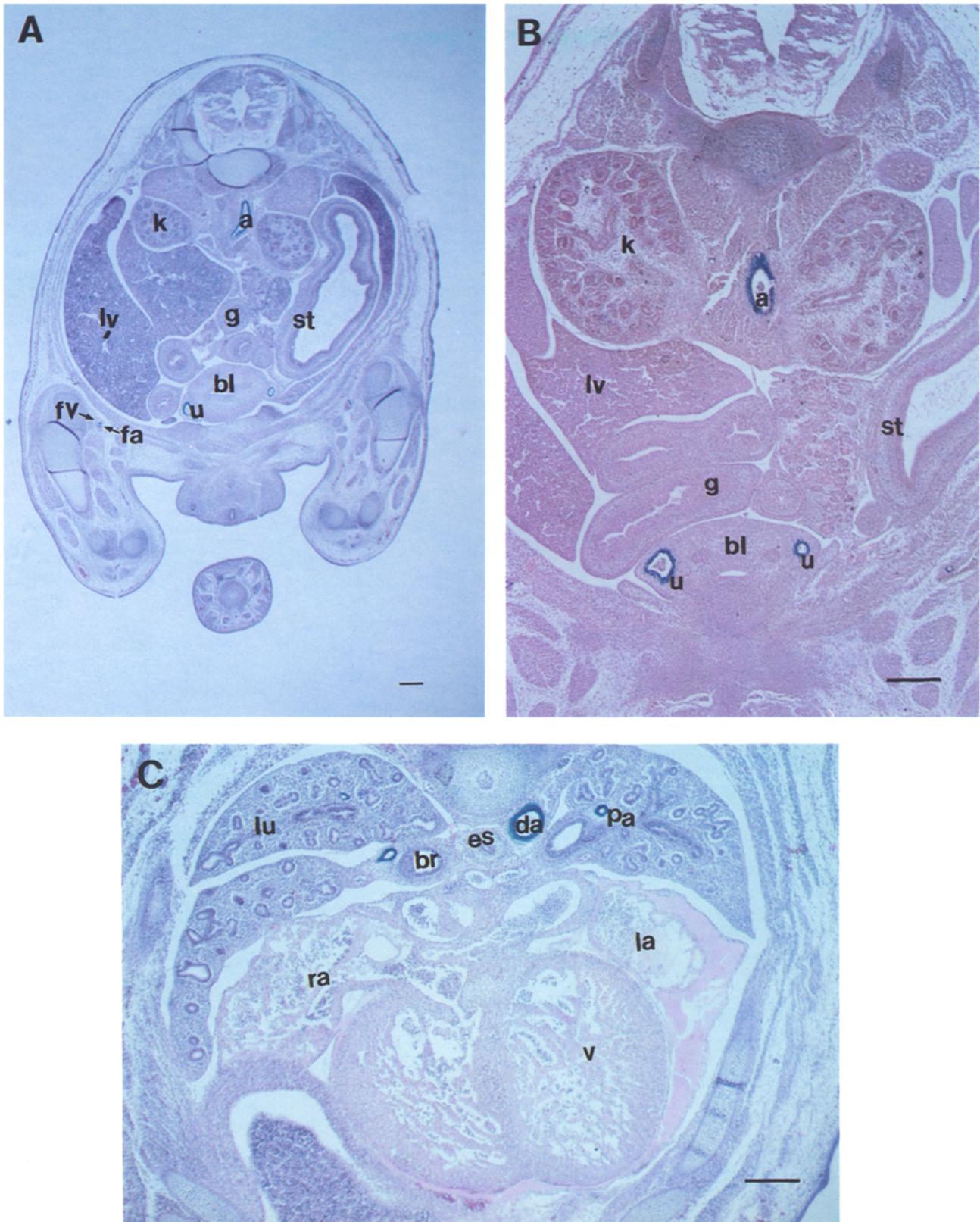
Figure 6. Absence of expression of the *SM22α*-lacZ transgene in visceral SMCs of a 14.5 d embryo. Transgenic mice harboring pSM2735/1093-lacZ were generated and the resulting embryos were stained for lacZ activity. A 14.5 d transgenic embryo was stained for lacZ and transverse sections were cut and stained with hematoxylin and eosin. (*A*) Transverse section at the abdominal level reveals specific expression in abdominal aorta (*a*) and umbilical arteries (*u*), and the absence of expression in myometrial layers of the gut (*g*), stomach (*st*), and bladder (*bl*). Specific expression of the transgene in femoral artery (*fa*) but not the femoral vein (*fv*) is also observed. (*B*) Higher magnification of the transverse section at a similar level as *A* in the 14.5 d embryo. (*C*) Transverse section at the thoracic level of the same 14.5 d transgenic embryo as in *A* shows the expression in descending aorta (*da*) and branches of pulmonary arteries (*pa*), but not in bronchus (*br*) or branches of the bronchus in the lung. *k*, kidney; *la*, left atrium; *lv*, liver; *ra*, right atrium; *v*, ventricle. Bar, 100 μm.

promoter we tested were active in vascular, but not visceral SMCs, suggesting that *SM22α* expression in visceral SMCs is controlled by regulatory elements separable from those for vascular SMC expression.

Specific Expression of the SM22α Promoter in Conductive Arterial, But Not Venous SMCs

It is well established that SMCs are heterogenous with respect to embryonic origin. Even within the vasculature, SMCs are further specified into conductive arterial, nonconductive, and venous SMCs. When examining transgene expression in the venous system, we observed that the transgene was not expressed in the inferior vena cava at 13.5 dpc (Fig. 5 *B*). Similarly, no transgene expression was seen in the cardinal vein, nor the femoral vein at E14.5 (Fig. 6). This contrasts with the expression of the endogenous *SM22α* gene, which is expressed in SMCs in all of these structures. Although the femoral vein is very small in size, *SM22α* transcripts are clearly detectable (Li et al., 1996). The absence of transgene expression in venous SMCs was apparent at adult stages. Analysis of adult heart from *SM22α*-lacZ transgenics revealed lacZ expression in the descending aorta, but not in the inferior vena cava or esophagus (Fig. 7 *A*). This observation was confirmed by histological analysis of transverse sections of the same heart (Fig. 7 *B*).

In a frontal view of the heart, transgene expression was clearly present in the ascending aorta, aortic arch, left and right common carotid arteries, and right subclavian artery (Fig. 7 *C*). No expression was detected in the veins penetrating the lung. We also did not detect expression in the coronary arteries passing through the heart. The failure to detect LacZ expression in coronary arteries, veins and visceral organs suggests that distinct regulatory mechanisms direct *SM22α* gene expression in different smooth muscle lineages.

The 445 bp of 5'-Flanking Sequence Is Sufficient to Provide Temporospatial Expression Specificity of the SM22α Promoter

To begin to define the minimal sequences required for expression of *SM22α* in vivo, we also examined the expression of lacZ reporter constructs containing 1,343 and 445 bp of 5' flanking sequence. Both constructs showed the same temporospatial expression pattern as the constructs that extended to −2,735 bp (not shown). These results suggest that the expression patterns described above are dependent on proximal promoter sequences and that sequences between −2,735 and −445 bp do not contain essential muscle regulatory elements.

Discussion

To begin to define the molecular mechanisms that control smooth muscle gene expression, we examined the regulation of the *SM22α* promoter in transgenic mice. Our results demonstrate that the 445-bp sequence preceding the transcription initiation site of *SM22α* is sufficient to direct the expression of a lacZ transgene in cardiac, smooth and skeletal muscle lineages. However, in contrast to the endogenous *SM22α* gene, which is expressed in all SMC

types throughout embryogenesis and adulthood, this promoter was selectively activated in conductive arterial SMCs, but was inactive in venous and visceral SMCs. These results suggest that distinct regulatory mechanisms control *SM22α* expression in different smooth muscle lineages during embryogenesis.

Distinct Mechanisms for Regulation of Smooth Muscle Gene Expression in Different Smooth Muscle Lineages

It is well established that SMCs have at least two distinct embryonic origins, the mesoderm and the neural crest (Schwartz et al., 1990). Embryological studies have demonstrated that SMCs within the dorsal aorta are derived from local mesenchymal cells and that SMC determination occurs before cell condensation around the endothelial channel (Manasek, 1971; Nakamura, 1988), which may signal the differentiation of SMCs. Chick–quail chimera techniques for cell lineage mapping have shown that the regions of the large arteries derived from the branchial arches (systemic aorta, pulmonary arteries, and common carotid arteries) arise from neural crest–ectomesenchymal cells, while the more distal segments of these large arteries may contain a mixture of both ectomesenchymal and mesoderm-derived SMCs (Le Leivre et al., 1975).

Coronary arteries are mesodermal in origin (Le Lievre et al., 1975). However, disruption of the neural crest in birds results in abnormal development of the coronary arteries (Hood and Rosenquist, 1992), suggesting that the neural crest plays a role in normal coronary artery development. The origins of venous SMCs are not well characterized, but these SMCs are likely to be derived from mesodermal mesenchymal cells. In contrast, visceral SMCs, which are distinct from vascular SMCs in contractile properties and pharmacologic responses (Akerlund, 1994; Zingg et al., 1995), arise from mesoderm-derived local mesenchymal cells, whose proper differentiation appears to depend on epithelial cell–mediated signals (Cunha et al., 1989).

The observation that SMCs are derived from distinct embryonic origins raises several interesting questions regarding the mechanisms that control SMC lineage specification and the molecular basis for the heterogeneity and functional diversity of SMCs. As an initial step towards addressing these issues, we have analyzed SMC gene expression in different muscle types. We observed that the onset of expression of *SM22α* (Li et al., 1996), SM myosin heavy chain (MHC) (Miano et al., 1994) and calponin (Miano and Olson, 1996) in vascular SMCs preceded that in visceral SMCs. Similarly, SM α-actin is expressed significantly earlier in SMCs at the origin of the coronary arteries than in SMCs of the cardiac outflow arteries (Hood and Rosenquist, 1992). In SMCs derived from different physiological or pathological conditions, gene expression was observed to be regulated differentially (Cremona et al., 1995; Schwartz et al., 1995). The expression of *calponin*, *SM22α*, and SM *α-actin*, for example, appears not to be coordinately regulated in human atherosclerotic plaques (Shanahan et al., 1994). These results reveal a previously unrecognized molecular heterogeneity of gene expression in different SMC types and may account for distinct functions of SMCs. The *SM22α* promoter provides the first molecular marker for dissecting the regulatory mecha-
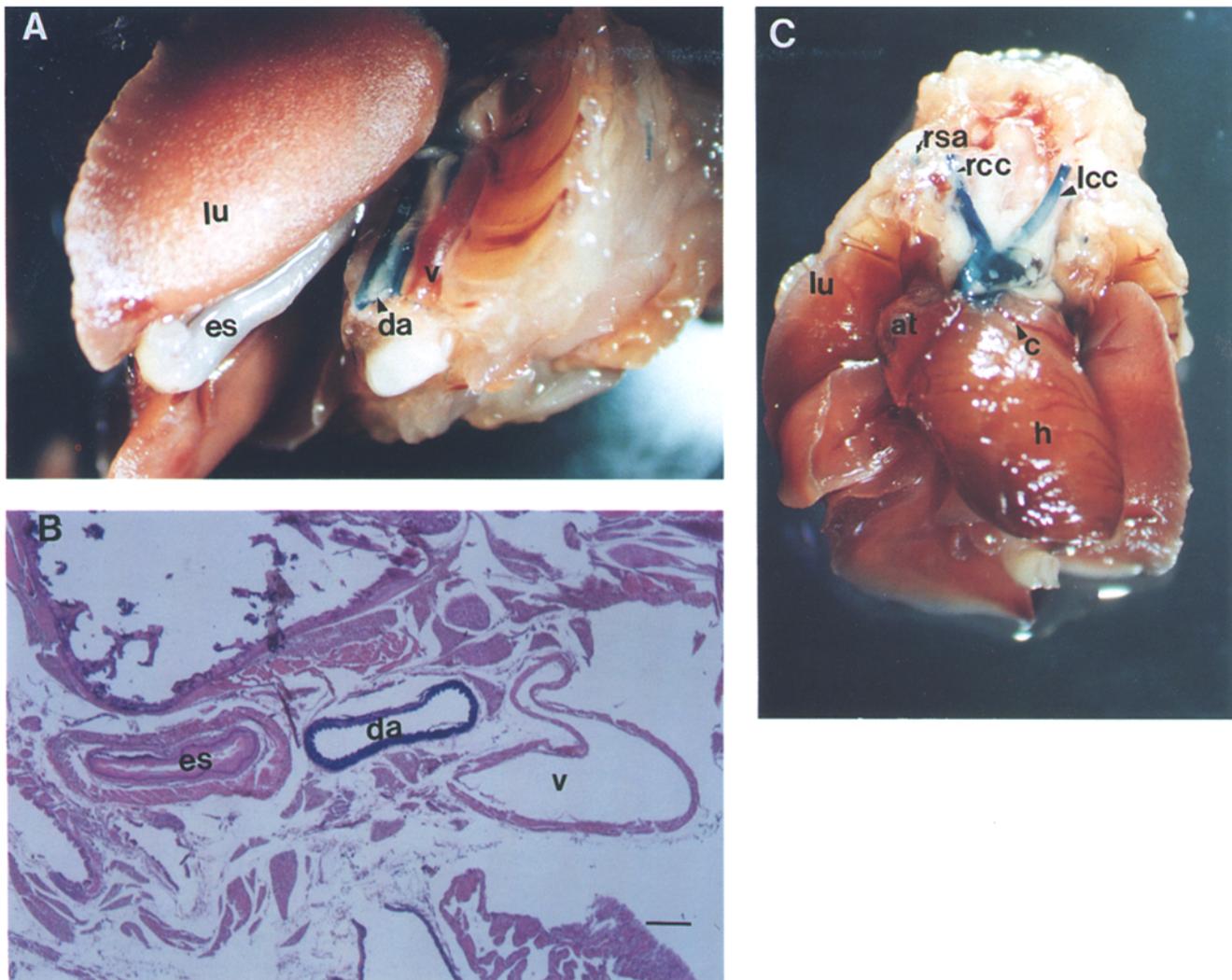
Figure 7. Expression of the *SM22α*-lacZ transgene in the descending aorta of an adult transgenic mouse. Transgenic mice harboring pSM2735/1093-lacZ were generated and the resulting embryos were stained for lacZ activity. (*A*) Gross anatomy of the heart region shown in a dorsal view reveals transgene expression in the descending aorta (*da*), but not in the vein (*v*), the esophagus (*es*) or the lung (*lu*). (*B*) Transverse section of the heart in *A* shows transgene expression in the muscle wall of the descending aorta (*da*), but not in the vein (*v*), nor in the esophagus (*es*). (*C*) A frontal view of the heart demonstrates transgene expression in ascending aorta, aortic arch, and left common carotid artery (*lcc*), right common carotid artery (*rcc*), and the right subclavian artery (*rsa*). No transgene expression was detected in veins entering the lung or in the coronary arteries (*c*). Bar, 200 μm.

nisms that control SMC-specific transcription in these different SMC types in vivo.

The SM22α-LacZ Transgene Serves as an Early Marker for the Right Ventricle during Heart Morphogenesis

Endogenous *SM22α* is expressed in the left and right ventricles of the heart prior to E10.5 and thereafter its expression becomes restricted to the right ventricle at E12.5 and disappears from the heart by E13.5 (Li et al., 1996). Our *SM22α-lacZ* transgenes were also expressed in the bulbus cordis (the future right ventricle) and in the outflow tract, but expression was never observed in the left ventricle. This spatial restriction in expression of these transgenes occurred by the onset of cardiac looping, suggesting that the left and right polarity of the ventricles is established at the early stages of heart development. The differential expression of the *SM22α-lacZ* transgenes in the left and right ventricles also suggests that *cis*-acting elements that direct transcription in the left ventricle are missing from the 5′-flanking regions we tested and demonstrates that a single gene can be subject to different regulatory mechanisms in different regions of the heart.

The simplest explanation for the more restricted expression of *SM22α-lacZ* transgenes than of the endogenous *SM22α* gene is that the 5′-flanking sequence we tested contains only a subset of the regulatory elements for the gene. Although seemingly unlikely, we cannot formally exclude the possibility that the observed differential expression of the promoter in left and right ventricles is due to differences in processing of the exogenous lac-Z transcripts. This disparity could also be explained by the exist-

ence of another *SM22α* gene that may be expressed in those *SM22α*-expressing cell types in which the transgene is not expressed. This seems less likely because recent studies indicate that *SM22α* is encoded by a single copy gene (Solway et al., 1995).

Previous studies on the diversification of cardiomyogenic cell lineages focused on the atria and ventricles. Several atrial and ventricle lineage-specific markers, such as atrial-specific MHC (Yutzey et al., 1994), atrial-specific myosin light chain (MLC)-2 (Kubalak et al., 1994), and ventricle-specific MLC-2 (O'Brien et al., 1993), have been identified. Those studies suggest that the differentiation and diversification of atrial and ventricular cells occurs during the earliest stages of cardiogenesis (Yutzey and Bader, 1995). Less is known about possible molecular heterogeneity between the left and right ventricles. Recently, *MLC-3F* gene regulatory sequences were shown to be active in the left ventricle and atria, but not in the outflow tract, nor in the bulbus cordis during embryogenesis (Kelly et al., 1995). This contrasts with the expression of our *SM22α* transgenes, which exclusively mark the bulbus cordis and the outflow tract. That the *SM22α* and *MLC-3F* promoters exhibit mutually exclusive expression patterns in the left and right ventricles suggests that muscle gene expression is controlled by different regulatory mechanisms in these two regions of the developing heart. Our results also demonstrate that the diversification of myocardial cells to left and right ventricular lineages occurs at an early stage of cardiogenesis.

The SM22α Promoter Reveals Different Expression Specificity In Vitro and In Vivo

Whereas endogenous *SM22α* is expressed specifically in myogenic lineages in vivo, we detected *SM22α* transcripts in a variety of muscle and nonmuscle cell types in culture. The *SM22α* promoter was also less tissue restricted in its expression in cultured cells than might be expected from the strict cell-type specificity of *SM22α* expression in vivo. Deletion analysis indicated that the 445-bp proximal promoter of *SM22α* was sufficient to direct transcription in these different tissue culture cell types. This finding contrasts with a recent study in which the proximal *SM22α* was shown to be active only in SMCs in vitro (Solway et al., 1995).

Studies of SMC gene promoters in tissue culture cells have provided useful information about the *cis*-acting elements that control SMC gene expression in vitro (Kahari et al., 1990; Katoh et al., 1994; Shimizu et al., 1995). However, whether these regulatory elements are also sufficient to confer temporospatial expression specificity in vivo, where higher orders of transcription control exist (Felsenfeld, 1992), remains unknown because there have been no previous studies demonstrating SMC-specific gene expression in transgenic mice.

Transcriptional Control of Muscle Gene Expression

Significant progress has been made toward identifying the transcription factors that control skeletal and cardiac muscle gene expression (Olson, 1993; Buckingham, 1994). However, the mechanisms that regulate SMC-specific gene expression remain largely unknown. Skeletal muscle

gene expression is controlled by a family of skeletal muscle-specific basic-helix-loop-helix (bHLH) proteins, MyoD, Myogenin, Myf5, and MRF4, which activate muscle gene expression by binding to the E box consensus sequence (CANNTG) in the control regions of muscle structural genes (Olson, 1990; Weintraub et al., 1991;). There are no E boxes in the 445-bp *SM22α* promoter that directs cell type–restricted expression, suggesting that bHLH factors are not essential for *SM22α* gene expression.

CArG boxes have been shown to play an important role in the control of skeletal, cardiac, and smooth muscle genes (Gustafson et al., 1988; Sartorelli et al., 1990; Treisman, 1990; Blank et al., 1992). Deletion of the region of the *SM22α* promoter containing the two CArG boxes resulted in about a sixfold reduction in promoter activity in vitro, suggesting that these CArG boxes may be important for *SM22α* gene expression in SMCs. Interestingly, the promoter of the *SM α*-actin gene, which is expressed in a similar pattern to *SM22α* in the SMC lineage, contains two CArG elements in nearly the same spatial orientation as those in the *SM22α* promoter and optimal activity of the *SM α-actin* promoter in cultured SMCs requires these elements (Shimizu et al., 1995).

Members of the MEF2 family of MADS box transcription factors have also been shown to be expressed in cardiac, skeletal, and smooth muscle cells (reviewed in Olson et al., 1995). Loss of function mutations of the single *mef2* gene in *Drosophila* result in a loss of muscle differentiation in cardiac, skeletal, and visceral muscle cells (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). There is an A/T-rich element in the *SM22α* promoter that resembles a MEF2 site, but it does not perfectly fit the MEF2 consensus binding site. Whether MEF2 acts through this element or indirectly through another element in the *SM22α* promoter remains to be determined.

Further dissecting the *SM22α* promoter in transgenic mice is likely to provide insights into the molecular mechanisms that control early cardiovascular development and SMC lineage determination and differentiation. The isolated *SM22α* promoter also offers an opportunity to genetically manipulate the physiology and pathology of the cardiovascular system by directing genes involved in cell growth and differentiation into the cardiovascular system.

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