

# Regulation of Smooth Muscle $\alpha$ -Actin Expression In Vivo Is Dependent on CArG Elements Within the 5' and First Intron Promoter Regions

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**Abstract**—The aims of the present studies were to define sufficient promoter sequences required to drive endogenous expression of smooth muscle (SM)  $\alpha$ -actin and to determine whether regulation of SM  $\alpha$ -actin expression in vivo is dependent on CArG (CC(A/T)<sub>6</sub>GG) *cis* elements. Promoter deletions and site directed mutagenesis techniques were used to study gene regulation in transgenic mice as well as in smooth muscle cell (SMC) cultures. Results demonstrated that a Lac Z transgene that contained 547 bp of the 5' rat SM  $\alpha$ -actin promoter was sufficient to drive embryonic expression of SM  $\alpha$ -actin in the heart and in skeletal muscle but not in SMCs. Transient transfections into SMC cultures demonstrated that the conserved CArG element in the first intron had significant positive activity, and gel shift analyses demonstrated that the intronic CArG bound serum response factor. A transgene construct from -2600 through the first intron (p2600Int/Lac Z) was expressed in embryos and adults in a pattern that closely mimicked endogenous SM  $\alpha$ -actin expression. Expression in adult mice was completely restricted to SMCs and was detected in esophagus, stomach, intestine, lung, and nearly all blood vessels, including coronary, mesenteric, and renal vascular beds. Mutation of CArG B completely inhibited expression in all cell types, whereas mutation of the intronic CArG selectively abolished expression in SMCs, which suggests that it may act as an SMC-specific enhancer-like element. Taken together, these results provide the first in vivo evidence for the importance of multiple CArG *cis* elements in the regulation of SM  $\alpha$ -actin expression. (*Circ Res.* 1999;84:852-861.)

**Key Words:** muscle, smooth ■ transgenic mice ■ transfection ■ gene expression

Several *cis* elements and *trans*-acting factors have recently been described that regulate muscle-specific gene expression in skeletal and cardiac muscle and are required for the terminal differentiation of these muscle cell types.<sup>1,2</sup> In contrast, the mechanisms that regulate smooth muscle cell (SMC) differentiation are only poorly understood, and, to date, no transcription factors have been identified that direct SMC-specific gene expression. Because SMC maturation and differentiation are required for the full development of arteries and veins during angiogenesis and vasculogenesis, the identification of the molecular mechanisms that control SMC differentiation is important to our understanding of these processes that occur not only during development but also under pathological conditions. Furthermore, it may lead to a better understanding of SMC phenotypic modulation that has been shown to contribute to atherosclerosis and restenosis after balloon angioplasty.<sup>3,4</sup>

A major goal of our laboratory has been to elucidate control processes that regulate SMC differentiation by identifying mechanisms that control the transcription of genes such as SM  $\alpha$ -actin and SM myosin heavy chain (MHC) that are required for the contractile function of SMC.<sup>5</sup> SM  $\alpha$ -actin is a contractile protein that comprises 40% of total SMC

protein.<sup>6</sup> It is required for the contractile function of SMCs and is the first SMC differentiation marker to appear during development.<sup>7</sup> Although it is also transiently expressed in the myocardium and skeletal muscle during the development of the embryo and is expressed in myofibroblasts during wound healing, SM  $\alpha$ -actin expression in adult animals is highly restricted to SMCs or SM-like cells.<sup>8,9</sup>

Our laboratory and others have shown that the regulation of SM  $\alpha$ -actin expression involves a complex interaction of multiple positive and negative *cis* elements that act in a cell-type specific fashion.<sup>10-16</sup> For example, a 547 bp SM  $\alpha$ -actin promoter/CAT construct (p547CAT) had high activity in cultured SMCs and in L6 skeletal myotubes, which are cell types that express SM  $\alpha$ -actin in culture. However, the same construct was inactive in non-SMC-types such as endothelial cells<sup>14</sup> and AKR2B fibroblasts.<sup>17</sup> Of particular interest, we have demonstrated that two completely conserved CArG (CC(A/T)<sub>6</sub>GG) elements, CArG A at -62 and CArG B at -112, within the first 125 bps of the SM  $\alpha$ -actin 5' promoter are required for high level of expression in cultured SMCs in that mutation of either CArG abolished transcriptional activity.<sup>14</sup> Additional positive and negative *cis* elements have been described in the sequences further up-

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stream to  $-2.8$  Kb, but in cell culture systems, this region acts mainly to inhibit expression in cell types that do not normally express SM  $\alpha$ -actin.<sup>13,14</sup> The SM  $\alpha$ -actin first intron contains another completely conserved CArG element,<sup>12</sup> but the activity of the intronic CArG and its contribution to SMC-specific regulation have not been studied.

The CArG element was first described as the core sequence of the serum response element (SRE) within early response genes, such as *c-fos* (reviewed in Reference 18) but has also been shown to be required for the activity of many muscle-specific gene promoters.<sup>19–23</sup> Of interest, nearly all of the SMC differentiation marker genes characterized to date, including SM MHC, caldesmon, and telokin, contain two or more CArG elements that are required for maximal expression in cultured SMCs.<sup>14,24–28</sup> In addition, separate laboratories have reported that a conserved CArG element in the SM-22 promoter is required for the arterial expression of a Lac Z transgene in the mouse.<sup>29,30</sup> Electrophoretic mobility supershift studies demonstrated that the SM  $\alpha$ -actin CArG elements, like the SRE, bind the serum response factor (SRF).<sup>14</sup> Although recent evidence suggests that muscle-derived tissues express higher levels of SRF than non-muscle derived tissues,<sup>31</sup> SRF is thought to be ubiquitously expressed, and a critical yet presently unresolved question remains as to the mechanism of CArG-dependent regulation of SMC-specific gene expression. Evidence from this laboratory suggests that both CArGs A and B, together with highly conserved CArG flanking sequences, act cooperatively to coordinate the formation of an SRF-containing SMC-specific activation complex that may contain an SMC-selective SRF accessory protein (C.P.M., Mike M. Thompson, Susan Lawrenz-Smith, G.K.O., unpublished results, 1998).

It is well established that SMC differentiation is dependent on a large number of local environmental cues, including extracellular matrix interactions, local production of growth factors, and mechanical stresses that cannot be accurately reproduced in culture.<sup>5,32</sup> Moreover, recent studies have provided evidence that gene regulation in SMC culture systems often does not completely mimic regulation in vivo.<sup>30,33</sup> Therefore, when studying SMC differentiation, it is critical that regulatory pathways initially identified in cultured SMC are tested in vivo through the use of transgenic mice. In addition, analysis of SM-22 and SM MHC gene expression in transgenic mice has demonstrated that expression of SMC marker genes is complex and may involve “regulatory cassettes” that drive expression within some but not all SM tissues.<sup>25,29,33</sup> Therefore, transgenic studies are also critical for detecting possible heterogeneity in SMC gene regulation.

No studies to date have reported the complete characterization of the regulatory regions required to drive in vivo expression of SM  $\alpha$ -actin during development and maturation. Wang et al<sup>34</sup> recently reported that a SM  $\alpha$ -actin promoter that contains 1100 bp of 5' promoter and the entire first intron could drive expression of an IGF-1 transgene in many SM tissues. However, these studies were restricted to analysis in adult animals and focused on examination of the effects of IGF-1 overexpression in SMC rather than on the

characterization of the promoter regions required for SMC-specific expression. This is a critical issue because the SM  $\alpha$ -actin gene is known to be expressed by all three muscle types during development. In addition, although a large number of regulatory elements have been identified on the basis of studies in cultured SMCs, no studies have been reported examining whether specific *cis* elements regulate SM  $\alpha$ -actin expression in vivo. As such, the goals of the present experiments were to define the sufficient promoter sequences required to drive expression of SM  $\alpha$ -actin in vivo and whether this regulation is CArG dependent. Data presented demonstrate that a promoter construct from 2600 through the first intron is sufficient to drive high-level expression in vivo in a manner that appears to mimic the endogenous SM  $\alpha$ -actin gene. Moreover, we provide evidence that the CArG elements are required for transcriptional activation of the promoter in vivo but exhibit differential activity in SMCs versus non-SMCs.

## Materials and Methods

### Construction of Rat SM $\alpha$ -Actin Lac Z Reporters

The pUC19-Lac Z plasmid used to generate reporter gene constructs was a generous gift of Dr Eric Olson (University of Texas Southwestern, Dallas, Tex). Several deletion constructs were generated for analysis in transgenic mice. The p125/Lac Z, p547/Lac Z, and p2800/Lac Z reporters were made by subcloning the corresponding promoter regions from previously described CAT reporter constructs<sup>14</sup> into the Lac Z vector after *HindIII/Xba I* restriction digestion. Constructs that contained the first intron, p547Int/Lac Z and p2600Int/Lac Z, were subcloned from a larger genomic fragment isolated and described previously with *PmlI/XhoI* and *ScaI/XhoI* digestion, respectively.

CArG mutations in the p2600Int/Lac Z construct were made with the polymerase chain reaction (PCR)-based Excite method (Promega) as per protocol. To avoid potential PCR-induced mutations in the Lac Z reporter, the promoter was subcloned into pBluescript, and after the mutagenesis protocol was returned to the Lac Z vector. The oligonucleotides used to make these mutations contained the following sequences that have been shown to abolish SRF binding in gel shift analyses (mutated sequences are in bold): CArG A, 5'-AATTGTTTAA; CArG B, CCCTATATCA; and intronic CArG, AATAATTA AAA. Final subcloning steps and all mutations were verified by direct DNA sequencing. Before transgenic injections, all constructs were tested for Lac Z expression by transient transfection into cultured rat aortic SMC cultures to ensure functional activity of all constructs. All clones, including those that contained CArG mutations, showed at least some activity in these assays.

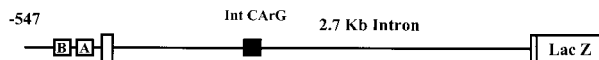
### Generation and Analysis of Transgenic Mice

All constructs were prepared for transgenic injection by removal of pUC19 backbone sequences by *NotI/EcoRI* digestion and subsequent agarose gel purification of the linearized promoter/Lac Z fragment. Transgenic mice were generated with standard methods<sup>25,35</sup> either commercially (DNX, Princeton, NJ) or within the transgenic core facility at the University of Virginia, Charlottesville. Mice (C57/B6) were analyzed transiently at several embryonic stages or by establishing founder lines that allowed more detailed analysis of transgene expression throughout development and in adult animals. Transgene presence was analyzed by PCR with genomic DNA purified from placentas (transients) or tail clips (founders) according to the method of Vemet.<sup>36</sup> Mice were euthanized by IP injection of pentobarbital (100 mg/kg), and transgene expression was analyzed as previously described.<sup>25,37</sup> All animal procedures used in these studies were reviewed and approved by the University of Virginia Animal Use and Care Committee.

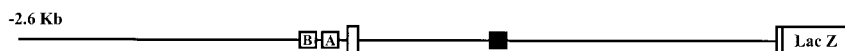
## A. (p547/Lac Z)



## B. (p547Int/Lac Z)



## C. (p2600Int/Lac Z)



**Figure 1.** Linear diagrams of Lac Z promoter constructs used to generate transgenic mice. Deletion fragments from a rat genomic clone were subcloned into the previously described pUC/AUG  $\beta$ -galactosidase vector (see Materials and Methods). A, Fragment from  $-547$  to  $+47$  bp (p547/Lac Z). B, Fragment from  $-547$  to  $+2784$  bp (p547Int/Lac Z). C, Fragment from  $-2600$  to  $+2784$  bp (p2600Int/Lac Z). CARG A at  $-72$  bp, CARG B at  $-112$  bp, the intronic CARG at  $+1001$  bp, and the 5' untranslated first exon are indicated. *NotI/EcoRI* digestion was used to remove the pUC plasmid backbone before transgenic injections.

### Cell Culture, Transient Transfections, and Reporter Gene Assays

SMCs from rat thoracic aorta were isolated and cultured as previously described.<sup>13</sup> SMCs were seeded into 6-well plates and transfected 24 hours after plating at 70% to 80% confluency. Transfections were performed with  $4 \mu\text{g}$  of plasmid DNA and the transfection reagent DOTAP (Boehringer Mannheim). Growth conditions and preparation of cell lysates for measurement of Lac Z activity were performed as previously described.<sup>14</sup> The enzyme activity of each sample was normalized to the protein concentration of each cell lysate as measured by the DC protein assay (BioRad). In each experiment, the promoterless Lac Z construct was also transfected to serve as the baseline indicator of Lac Z activity, and the activity of each promoter construct is expressed relative to promoterless activity. All activities represent at least three independent experiments, with each construct tested in triplicate per experiment. Relative Lac Z activities are expressed as the mean  $\pm$  SD computed from the results obtained from each set of transfection experiments. We did not cotransfect a viral promoter/reporter construct as a control for transfection efficiency because we have previously shown that such constructs exhibit unknown and variable squelching effects on the SM  $\alpha$ -actin promoter presumably because of competition for common transcription factors.<sup>14</sup> Moreover, we have found that inclusion of such controls are unnecessary because variations in transfection efficiency between independent experimental samples are routinely small ( $<10\%$ ).<sup>14</sup>

### Preparation of Nuclear Extracts, In Vitro Synthesis of SRF, and Electromobility Shift Assays

Nuclear extracts were prepared from confluent rat aortic SMCs by the methods of Dignam.<sup>38</sup> Culture conditions matched those used for transient transfection assays. Oligonucleotides used in electrophoretic mobility shift assays (EMSA) were purchased commercially (Operon Technologies) and include the following: CARG A, 5'-ttgctcctgtttgggaagc-3'; CARG B, 5'-gaggtccctatggtgtg-3'; and intronic CARG, 5'-ttttacctaatggaatg-3'. Probes were <sup>32</sup>P end labeled and annealed. All probes were purified on a 6% acrylamide gel, eluted in TE, and precipitated twice in ethanol.

EMSA were performed with  $20 \mu\text{L}$  of binding reaction that included  $\approx 30$  pg of labeled probe,  $5 \mu\text{g}$  of SMC nuclear extract,  $0.2$  to  $0.6 \mu\text{g}$  of poly(dI-dC) in  $1\times$  binding buffer ( $10 \text{ mmol/L}$  Tris-HCl, pH 7.5;  $100 \text{ mmol/L}$  KCl;  $50 \text{ mmol/L}$  NaCl;  $1 \text{ mmol/L}$  dithiothreitol;  $1 \text{ mmol/L}$  EDTA; and  $5\%$  glycerol). After a 30-minute incubation at room temperature, the samples were subjected to electrophoresis on a  $5\%$  polyacrylamide gel that had been prerun at  $170 \text{ V}$  for 1 hour. Electrophoresis was performed at  $170 \text{ V}$  in  $0.5\times$  TBE ( $45 \text{ mmol/L}$  Tris borate and  $1 \text{ mmol/L}$  EDTA). Gels were dried and exposed to film for 24 to 72 hours at  $-70^\circ\text{C}$ . For supershift studies,  $1 \mu\text{L}$  of SRF antibody was added after the 30-minute incubation period and the reaction was incubated for an additional 15 minutes and loaded onto the gel for electrophoresis.

### Immunohistochemical Staining of SM $\alpha$ -Actin Expression

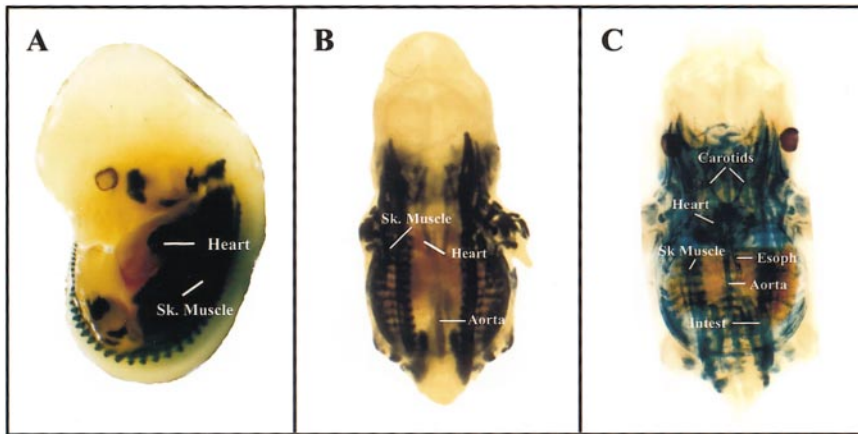
Embryos were fixed overnight in formalin. Tissues were dehydrated, incubated in 100% xylene, and embedded in paraffin. Thin sections ( $6 \mu\text{m}$ ) were placed on uncoated slides and dried on a slide warmer. Sections were cleared in 100% xylene and rehydrated through a graded ethanol series to a final incubation in PBS. Endogenous peroxidase activity was quenched by incubating the slides for 30 minutes in methanol that contained  $0.3\%$  hydrogen peroxide. Slides were subsequently rehydrated in PBS and blocked in a  $1:50$  solution of normal goat serum made in PBS. Sections were then incubated with SM  $\alpha$ -actin primary antibody for 1 hour and washed with three changes of PBS. Detection of primary antibody was performed with a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions, with 3,3'-diaminobenzidine as the chromogen.

## Results

### SM $\alpha$ -Actin Promoter Region From $-2600$ Through the First Intron Conferred In Vivo Expression of a Lac Z Reporter in a Manner Similar to That of the Endogenous Gene

Previous results from transient transfections into rat aortic SMC cultures demonstrated that reporter constructs that contained the first  $547$  bp of the SM  $\alpha$ -actin 5' promoter were expressed at high levels in only SMCs or other muscle cells that are known to express their endogenous SM  $\alpha$ -actin gene.<sup>14</sup> Therefore, we initiated our transgenic mouse studies with a construct that contained this promoter region (Figure 1A). Figure 2A shows a p547/Lac Z positive embryo at embryonic day 13.5 (E13.5), a time point when SM  $\alpha$ -actin is expressed in skeletal, cardiac, and smooth muscle.<sup>9</sup> Results showed that this promoter region was sufficient to drive transgene expression in skeletal and cardiac muscle but not in the vasculature or in any other SMC tissue. Note that in subsequent studies, a construct that contains a larger 5' region (up to  $-2800$  bps) did not result in expression in SMCs. The p2800/Lac Z construct, like p547/Lac Z, was expressed in only embryonic cardiac and skeletal muscle (data not shown).

The preceding observations indicated that additional regions of the SM  $\alpha$ -actin promoter were necessary for expression of SM  $\alpha$ -actin in SMCs in vivo. Nakano et al<sup>12</sup> previously reported that the first intron of the human gene had significant enhancer activity in cultured SMCs, an observation consistent with observations in this laboratory for the rat first intron (see Figure 4). Therefore, constructs were generated from a genomic clone that included the first intron and



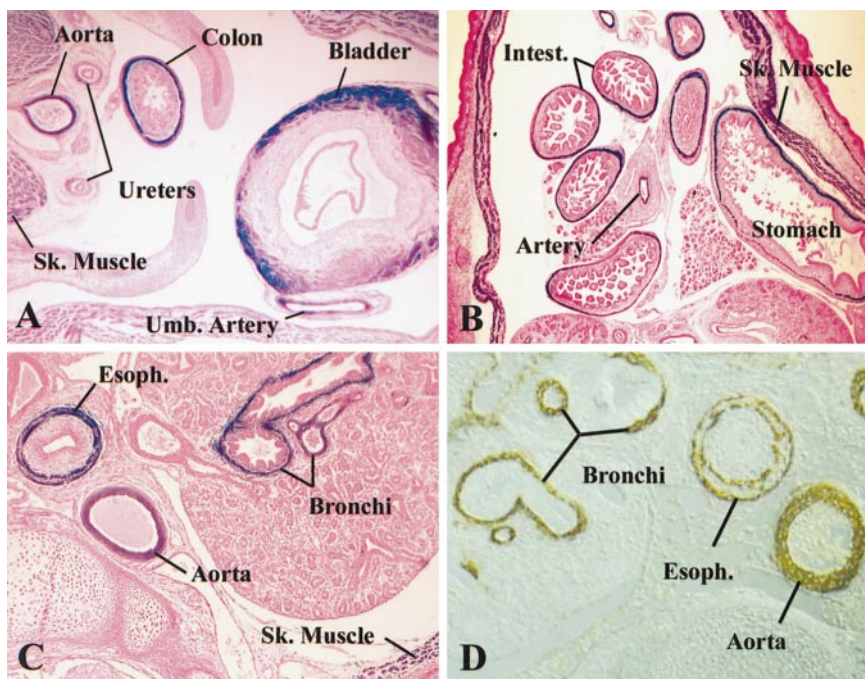
**Figure 2.** Examination of transgene expression at E13.5. The SM  $\alpha$ -actin promoter/Lac Z deletion constructs shown in Figure 1 were used to generate transgenic mice with standard transgenic procedures. Transgenic embryos at E13.5 were stained for Lac Z expression and a representative embryo from each group is shown ( $n > 4$  independent founders per group). At this embryonic stage, SM  $\alpha$ -actin is expressed in skeletal, heart, and smooth muscle. A, The p547/Lac Z construct was highly expressed in the heart and skeletal muscle but not in SMCs. B, Inclusion of the entire first intron (p547Int/Lac Z) that contains a highly conserved CARG element resulted in additional but very limited expression in the abdominal aorta and umbilical arteries. C, Promoter

sequences from -2600 bp through the first intron (p2600Int/Lac Z) were sufficient to drive expression of the Lac Z transgene that closely mimicked expression of endogenous SM  $\alpha$ -actin, with staining in heart and skeletal muscle and vascular, GI, and airway SM.

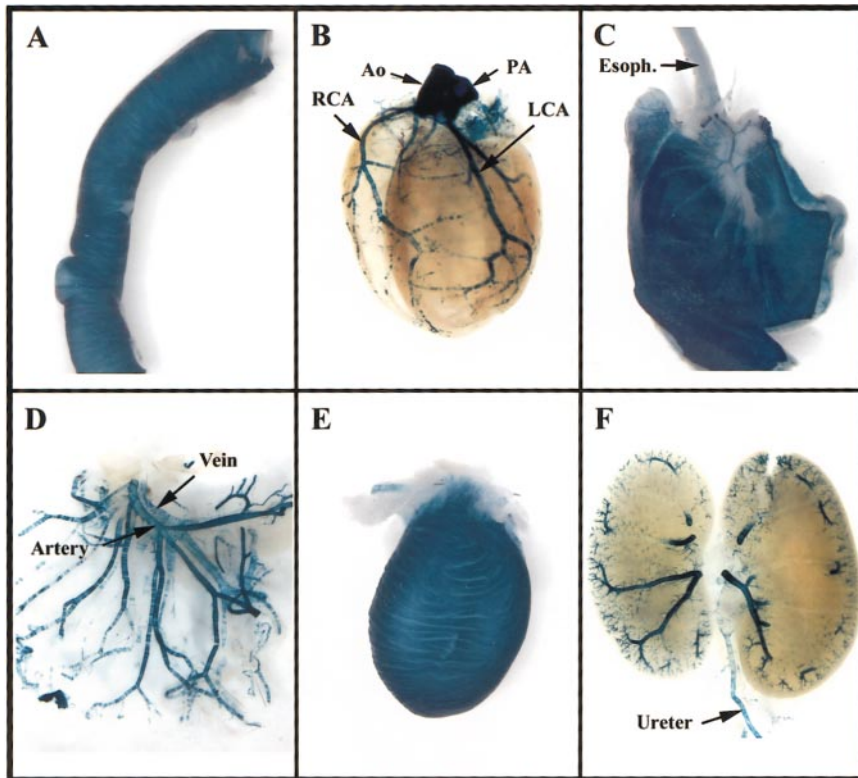
547 or 2600 bp of the 5' promoter (Figure 1B and 1C). Results shown in Figure 2B demonstrate that p547Int/Lac Z, like the p547 construct, was expressed highly in embryonic cardiac and skeletal muscle. In addition, all independent transgenic founder embryos (E13.5) generated with this construct ( $n=8$ ) expressed high levels of Lac Z in the umbilical arteries and one half showed expression in the lower portion of the abdominal aorta. These data demonstrated that the addition of the first intron to 547 bp of the 5' promoter promoted transgene expression in only a small subset of SMCs.

We next tested a transgenic construct that contained sequences from -2600 bp through the first intron (p2600Int/Lac Z). Results shown in Figure 2C demonstrated that this construct was expressed at E13.5 in a pattern that closely followed expression of the endogenous SM  $\alpha$ -actin gene, with staining in heart and skeletal muscle as well as in

multiple SM tissues, including the aorta, carotids, multiple small and large arteries, esophagus, stomach, intestine, bladder, ureter, and airway SM. Examination of histological sections from p2600Int/Lac Z animals at E10.5 to E16.5 showed that Lac Z staining was highly restricted to the vasculature or the SMC layers of SM-containing organs as well as to cardiac and skeletal muscle. Figure 3 shows representative sections at E16.5 with panel 3D showing immunohistochemical detection of SM  $\alpha$ -actin expression for comparison. Figure 4 shows p2600Int/Lac Z expression in various organs taken from adult mice 4 to 6 weeks of age. Lac Z staining was seen in nearly all adult SM tissues examined, including the esophagus, stomach, intestine, bladder, trachea, bronchi, and most blood vessels, including the coronary, mesenteric, and renal vascular beds. Histological sections taken from adult tissues are shown in Figure 5. Note that expression was completely restricted to SMCs and that the



**Figure 3.** Histological examination of p2600Int/Lac Z expression at E16.5. After Lac Z staining, embryos were fixed overnight, embedded in paraffin, sectioned at 6 to 10  $\mu$ m, and counterstained with eosin. A through C, Transverse sections were taken at multiple locations to obtain a representative sample of most SM tissues. Lac Z expression was seen in skeletal and cardiac muscle and in nearly all SM tissues including esophagus, bronchi, aorta, bladder, intestine, stomach, and most vascular beds. Note that staining in SM-containing tissues is highly restricted to SMCs. D, Immunohistochemical staining with an antibody specific for SM  $\alpha$ -actin.



**Figure 4.** Expression of the p2600Int/Lac Z transgene in adult mice. Mice that were 4 to 6 weeks old were perfusion fixed and their tissues were excised and stained overnight for Lac Z expression. Expression was observed in nearly all SMC-containing tissues examined. A, Portion of the intestines that shows uniform Lac Z staining. B, Anterior view of the heart that shows Lac Z expression in most if not all of the coronary vasculature and outflow tracts. C, View of stomach that was opened sagittally to show staining of the stomach wall and the gastric artery. Lac Z expression in the esophagus was limited to the longitudinal SMCs. D, Mesenteric vasculature removed en bloc to show SMC-specific staining of both the mesenteric arteries and veins. E, Bladder shows intense and uniform Lac Z expression. F, Splayed view of the kidney after it was cut sagittally to reveal Lac Z staining of the renal vasculature and ureter. RCA indicates right coronary artery; LCA, left coronary artery; Ao, aorta; PA, pulmonary artery.

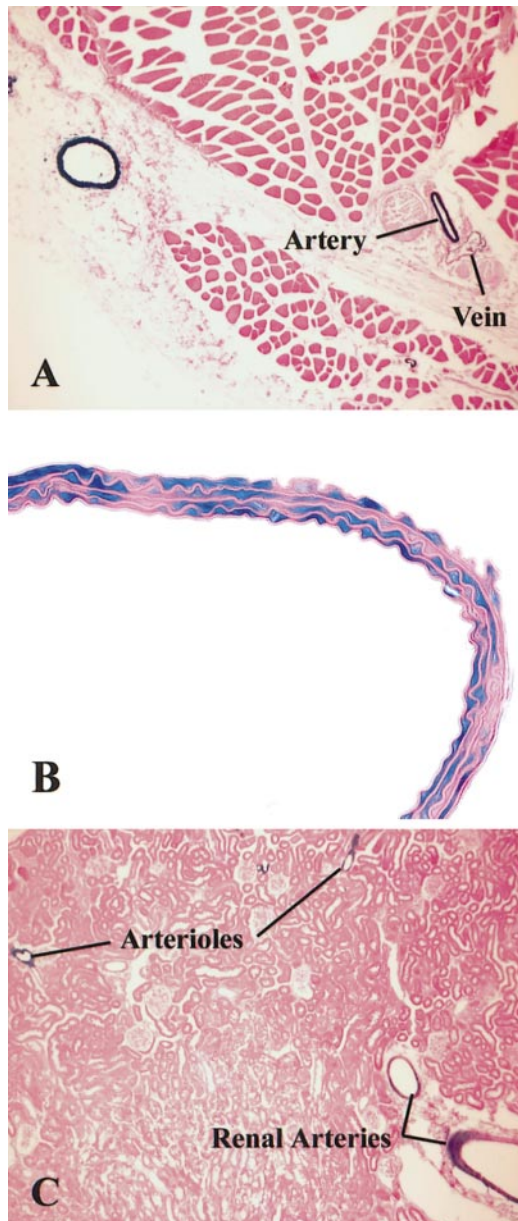
p2600Int/Lac Z transgene, which was highly expressed in skeletal and cardiac muscle during embryonic development, was no longer expressed in adult skeletal or cardiac muscle cells. The latter observation is consistent with the absence of SM  $\alpha$ -actin expression in these tissues in adult animals<sup>5</sup> and indicates that the  $-2600$  to  $+2784$  bp promoter region tested is sufficient to confer appropriate developmental regulation of this gene in multiple cell types. Expression in most structures was found to be homogeneous between individual cells with most, if not all, SMC being stained. This is in contrast to previous observations with certain SM MHC and SM-22 promoter constructs<sup>25,29,33</sup> and suggests that the p2600Int/Lac Z transgene also contains sufficient information to drive expression in SMC subtypes that have been shown to differentially express SM-22 or SM MHC transgenic constructs within a given SMC tissue.

A total of 10 independent founder lines were established with the p2600Int/Lac Z construct. Of these, six showed expression patterns during embryonic development, and as adults, that virtually mimicked expression of the endogenous SM  $\alpha$ -actin gene with two exceptions. Only one founder exhibited expression in uterine SMC, and most founders showed relatively low expression in small cranial arteries during development. However, in adult animals, we consistently detected expression in the basilar artery and other cerebral vessels (data not shown) in each of these six independent founders, which suggests that developmental signals may be important for expression of the p2600Int/Lac Z transgene in some SMC subtypes. Of the four remaining founders, two showed high expression in all vascular SMCs but only limited expression in SM-containing organs such as stomach, intestine, and bladder;

one founder showed expression only in cardiac and skeletal muscle during development; and one was expressed only in a small subset of skeletal muscle in the head and neck. Thus, although there were clearly some modest effects of insertion site on the expression pattern of the p2600Int/Lac Z transgene in a small number of founders, a high level of reproducibility of transgene expression across multiple independent founder lines existed. This provides strong evidence that the observed expression pattern was the result of sequences contained within the p2600Int/Lac Z construct and not insertional locus.

#### CArG Mutations Attenuated the Activity of p2600Int/Lac Z Activity in Cultured SMCs

Previous studies demonstrated that CArGs A and B (when contained within a construct that contained either 125 or 547 bp of the 5' promoter region) were absolutely required for expression in SMC cultures.<sup>14</sup> However, the transgenic results shown above demonstrate that additional sequences, including the CArG-containing first intron, are required for expression in vivo. Therefore, to measure the transcriptional activity of the first intron and to test the effects of mutations to CArGs A and B and the intronic CArG in the context of the promoter region shown to be sufficient for in vivo expression, we transfected cultured rat SMCs with equimolar amounts of the deletion or site-directed mutant constructs shown in Figure 6. Results demonstrated that the first intron had significant transcriptional activity in the  $-547$  and  $-2600$  context and that mutation of either CArG A or B or the intronic CArG decreased p2600Int/Lac Z activity in cultured SMCs.



**Figure 5.** Histological examination of p2600Int/Lac Z expression in various adult SM-containing tissues. Mice 4 to 6 weeks old were perfusion-fixed, and their tissues were excised and stained overnight for Lac Z expression. After Lac Z staining, tissues were further fixed overnight, embedded in paraffin, sectioned at 6 to 10  $\mu\text{m}$ , and counterstained with eosin. A, Section of thigh muscle skeletal muscle that shows Lac Z expression in a femoral artery and vein. Note that in adult animals, the p2600Int/Lac Z transgene was not expressed in skeletal muscle. B, Cross-section of the aorta that shows nearly uniform Lac Z expression in multiple SMC layers. C, Transverse section of the kidney that shows SMC-specific staining in the large renal arteries as well as smaller renal arterioles.

### SRF Bound the Intronic CARG

EMSA supershift analysis was performed to determine whether the intronic CARG, like CARGs A and B, binds SRF (Figure 7). Results demonstrated that SRF bound to the intronic CARG (lanes 3 and 6). The intronic CARG appeared to bind SRF more avidly than CARGs A and B (compare lane 3 with lanes 1 and 2), a result that is consistent with the fact that these CARGs contain a conserved G or C substitution in

their internal A/T rich nucleotide region<sup>14</sup> and that such substitutions lower SRF binding affinity.<sup>39</sup>

### CARG B Was Required for Expression of the p2600Int/Lac Z Transgene in Skeletal, Cardiac, and Smooth Muscle at E13.5, Whereas the Intronic CARG Was Required Only in SMCs

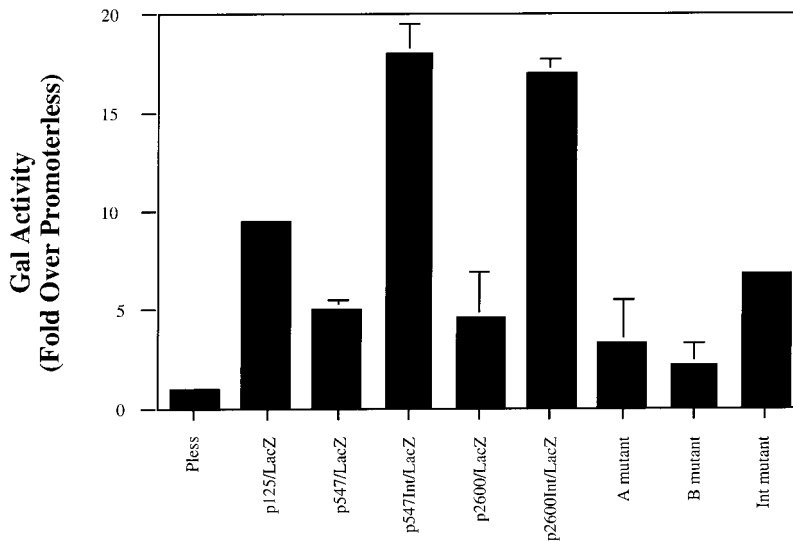
Results from our transgenic analyses of the SM  $\alpha$ -actin promoter demonstrated that the first intron was required for transgene expression in SMCs. Combined with the cell culture studies described above, these results suggest that the intronic CARG, and perhaps CARGs A and B, are required for expression of SM  $\alpha$ -actin in SMCs in vivo. Therefore, we tested whether CARG mutations affected expression of the p2600Int/Lac Z transgene in developing embryos and in adult mice. At least five independent founder lines were generated for each CARG mutant construct. Results shown in Figure 8 compare the effects of CARG mutations on Lac Z expression in mouse embryos at E13.5 when the endogenous SM  $\alpha$ -actin gene and our p2600Int/Lac Z transgene (wt) is expressed in all three muscle cell types. Mutation of CARG B (B mut) completely abolished Lac Z expression in all three muscle cell types, which indicated that it is absolutely required for SM  $\alpha$ -actin expression. Of major significance, mutation of the intronic CARG (Int mut) had no effect on cardiac or skeletal muscle expression but completely abolished expression in all SM tissues, which indicated that it is required for expression in SMCs but not in cardiac and skeletal muscle. Mutation of CARG A had no visible effect on staining in skeletal or heart muscle, but reduced or eliminated staining in some SM tissues (data not shown). However, these effects varied somewhat between founders, which suggested that the activity of this construct was somewhat sensitive to the site of transgene insertion.

### Mutations to CARG B and the Intronic CARG Abolished Expression of the p2600Int/Lac Z Transgene in SMCs in Adult Mice

To determine whether CARG elements are also required for expression in adult mice, we compared the expression of the wild-type p2600Int/Lac Z transgene construct and respective CARG mutants in 4- to 6-week-old mice (Figure 8). Results demonstrated that mutation of CARG B or the intronic CARG abolished expression in SMCs from all tissues, including trachea, lung, bladder, stomach, and intestine, and from all blood vessels, including the aorta, carotids, and coronary mesenteric, renal, and skeletal muscle arteries. Interestingly, in many founder lines, mutation of CARG A eliminated expression in SM organs and large vessels such as the aorta and carotids but only partially inhibited expression in smaller vessels such as those found in the mesenteric and skeletal muscle vascular beds.

### Discussion

Because it is well established that many of the factors that affect SMC differentiation and the transcription of SMC-specific genes cannot be reproduced accurately in cultured SMCs, the goal of the present study was to identify mechanisms that regulate SM  $\alpha$ -actin expression in vivo in trans-

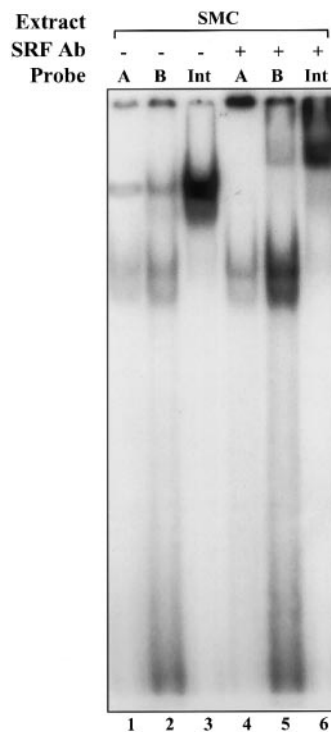


**Figure 6.** The effects of CARG mutations on p2600Int/Lac Z activity in cultured SMCs. Cultured rat SMCs (see Materials and Methods) were transfected with equimolar amounts of the indicated deletion or site-directed mutant constructs. After 48 hours, cells were lysed and galactosidase activity was measured spectrophotometrically. Galactosidase activity ( $\pm$ SD) is expressed relative to the baseline galactosidase activity of a promoterless galactosidase construct set to 1. The first intron had significant transcriptional activity in the  $-547$  and  $-2600$  context, and mutation of either CARG A or B or the intronic CARG greatly decreased p2600Int/Lac Z activity. Mutated CARG sequences were as follows: CARG A, 5'-AATTGTTAA; CARG B, CCCTATATCA; and intronic CARG, AATAATAAA.

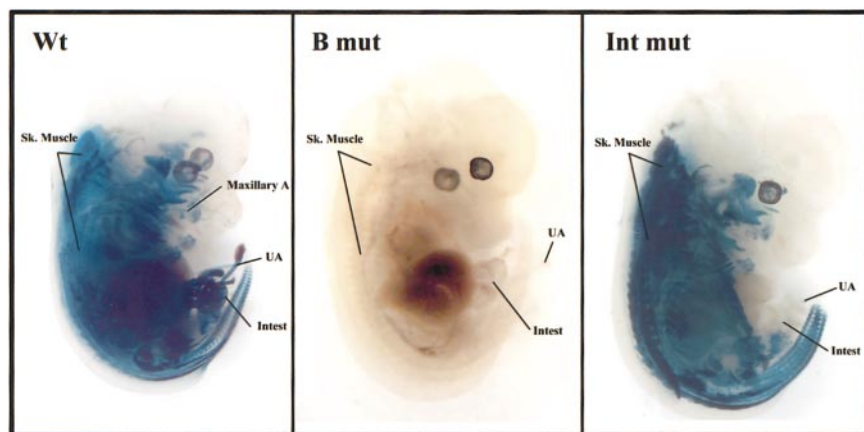
genic mice. Results demonstrated that the SM  $\alpha$ -actin first intron was required for expression of a Lac Z transgene in SMC and that the promoter regions from  $-2600$  bp through the first intron were sufficient to drive transgene expression in a pattern virtually identical to that of the endogenous gene. We also provided clear evidence that SM  $\alpha$ -actin expression is CARG-dependent and that SMC-specific regulation re-

quires unique cooperative interactions between the intronic CARG and CARGs A and B.

Results of our transgenic analyses illustrated a number of interesting features of SM  $\alpha$ -actin gene regulation that both confirm and extend previous observations in cultured SMCs but also point out some key differences. First, these results provide clear evidence that shows that SM  $\alpha$ -actin expression is differentially regulated depending on cell type. Previous studies from our laboratory demonstrated that 2800 bp of the SM  $\alpha$ -actin 5' promoter were sufficient to drive high-level expression of SM  $\alpha$ -actin only in cultured SMCs or other cell types such as L6 myotubes that are known to express their endogenous gene.<sup>13,14</sup> In contrast, this same construct was completely inactive in a variety of cell types, such as endothelial cells and AKR2B fibroblasts that do not express SM  $\alpha$ -actin.<sup>14</sup> The results presented in this study demonstrated that neither the p2800/Lac Z nor the p547/Lac Z transgenes were expressed in SMCs in vivo. However, these same constructs were expressed highly in embryonic skeletal and heart muscle, which are known to express SM  $\alpha$ -actin during embryonic development.<sup>9</sup> More extensive promoter analyses provided conclusive evidence that both the first intron and sequences from  $-547$  to  $-2600$  bp contain promoter elements required for transgenic expression in SMCs. When combined with the results of Wang et al, who used a mouse SM  $\alpha$ -actin promoter fragment from  $-1100$  bp through the first intron to overexpress IGF-1 in SMCs in vivo, our data suggest that the promoter region from  $-547$  through  $-1100$  bp contains *cis* elements necessary for expression of SM  $\alpha$ -actin in vivo. However, because of poorly defined differences between the sensitivities of the in situ methods used by Wang and the Lac Z detection methods used in our studies, it is difficult to make direct comparisons between these two model systems. Second, the differences in activity of promoter constructs in cultured SMCs versus in transgenic mice further emphasize the critical importance of studying SMC gene regulation in transgenic animals in order to reproduce complex local environmental cues that are necessary for SMC differentiation (ie, matrix interactions, neuronal and hormonal input, and mechanical stresses). Third, our



**Figure 7.** EMSA analysis of SRF binding to the intronic CARG. SMC nuclear extracts ( $5 \mu\text{g}$ ) were incubated for 20 minutes with 20 bp radiolabeled CARG A, CARG B, or intronic CARG gel shift probes. In lanes 4 to 6, SRF antibodies were added and the incubation was continued for 15 minutes. Samples were then loaded on a 5% nondenaturing polyacrylamide gel and electrophoresed at 170 V for 2 hours. Results demonstrated that SRF does bind to the intronic CARG (lanes 3 and 6) and that SRF bound the intronic CARG more avidly than CARGs A and B (compare lane 3 with lanes 1 and 2).



**Figure 8.** Effects of mutations to CARG B and the intronic CARG on the expression of the p2600Int/LacZ transgene at E13.5. Site-directed CARG mutations that have previously been shown to abolish SRF binding *in vitro* were made to CARG B and the intronic CARG in the p2600Int/Lac Z transgene construct. Transgenic mice were generated as described previously and stained for Lac Z expression at E13.5. Lac Z expression in wild-type (Wt) embryos was indicative of endogenous SM  $\alpha$ -actin expression. Mutation to CARG B (B mut) completely abolished Lac Z expression in all muscle cell types. Mutation to the intron CARG (Int mut) had no effect on skeletal muscle expression but did eliminate expression in all smooth muscle. UA indicates

umbilical artery. Mutated CARG sequences were as follows: CARG B, CCCTATATCA; intronic CARG, AATAATTA.

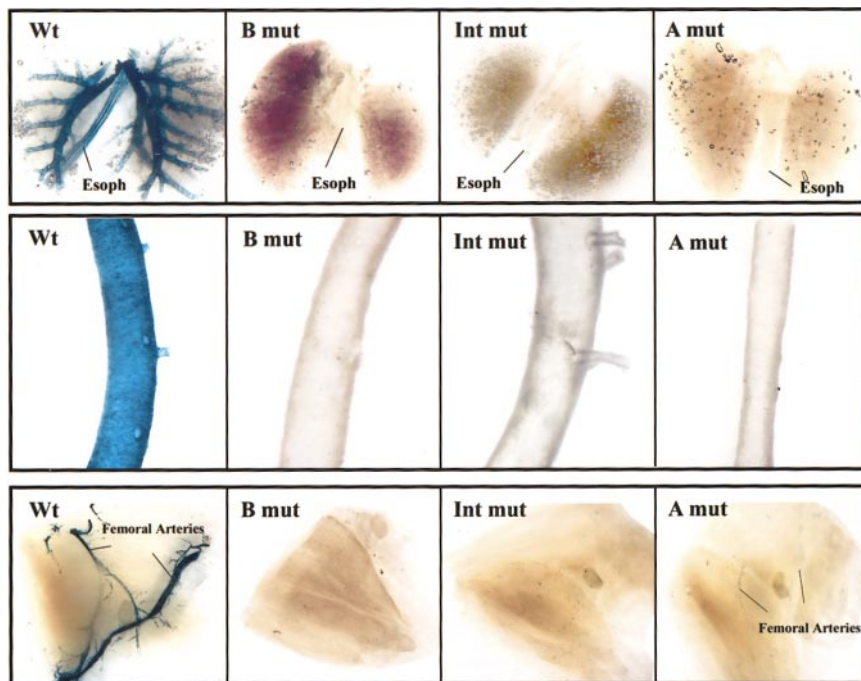
transgenic studies extend results of previous studies in cell culture<sup>10,14,16,17,40,41</sup> by providing evidence that specific *cis* elements exhibit cell-type-specific activity *in vivo*. For example, the intronic CARG was absolutely required for expression in SMCs but not in skeletal and cardiac muscle during development. A key unresolved issue will be to determine the precise combinatorial interactions of *cis* and *trans* acting factors that are required for expression in the various cell-types that express SM  $\alpha$ -actin under different physiological and pathophysiological conditions.

Because of the qualitative nature of Lac Z analysis in transgenic animals, the possibility of insertional variegation, and known SMC heterogeneity, considerable caution must be used when analyzing expression patterns among different transgenic promoters and even among independent founder lines that contain the same transgene. Nevertheless, it is interesting that expression of the p2600Int/Lac Z transgene was readily detected in nearly all SM tissues in 6 of 10 independent founder lines, and expression in those lines was remarkably homogeneous both between and within SMC populations. Recently published transgenic studies with other SMC marker gene promoters resulted in considerably different patterns of SMC expression and provided evidence for significant SMC heterogeneity. For example, a transgene driven by 441 or 1110 bp of the SM-22 5' promoter, although expressed in arterial SMCs, was not expressed in any other SM tissues.<sup>29,30</sup> In addition, a Lac Z transgene construct under the control of the SM MHC promoter region from -4299 through +11 600 bp was expressed in most SMC tissues but lacked any detectable activity in the renal and pulmonary vasculature and also showed significant cell-to-cell heterogeneity between SMCs within the same tissue.<sup>33</sup> Although the apparent homogeneity of expression observed could simply be a function of the relative strength of the SM  $\alpha$ -actin promoter, they also indicate that the p2600Int/Lac Z transgene contains sufficient information to drive expression in nearly all SMC types and that some transcriptional regulatory pathways such as those involving the CARGs may be common to all SMC subtypes. Finally, it is worth noting that the high level of SMC-specific expression observed with the p2600Int/Lac Z transgene in adult animals may make it an

attractive vector for gene therapies targeted to SM-containing tissues.

These studies are the first to report the activity of the SM  $\alpha$ -actin CARG elements *in vivo* and provide several interesting findings concerning CARG-dependent regulation of SM  $\alpha$ -actin expression. First, CARG B was absolutely required for *in vivo* expression in all three muscle cell types and may be the only CARG element required for transcriptional activity in skeletal and cardiac muscle during embryonic development. Second, CARG A, which is a much weaker CARG because it binds SRF poorly, was required for expression in nearly all SMC tissues except for the smaller resistance vessels (Figure 9). Although SMCs within large and small vessels are believed to be derived from a common mesenchymal source,<sup>42</sup> unknown differences in SMC lineage could contribute to these differences in transgene expression. Alternatively, differences may be ascribed to known differences in hemodynamic and/or other environmental stresses that could possibly regulate SM  $\alpha$ -actin expression independent of CARG A. The effects of the CARG A and B mutations on *in vivo* expression of the SM  $\alpha$ -actin transgene are somewhat analogous to the effects of mutations to the "near" (-141) and "far" (-264) CARGs described in the SM-22 promoter.<sup>29,30</sup> In those studies, mutation of the "strong" near CARG abolished expression in all cell types, whereas mutation of the much "weaker" far CARG had only limited effects on expression. Third, we showed that the intronic CARG functioned as an SMC-specific enhancer-like element that was required for expression in SMCs but not in embryonic skeletal and cardiac muscle. SRF was shown to bind intronic CARG more avidly than CARGs A and B (Figure 6), suggesting that SRF binding to the SM  $\alpha$ -actin promoter may be rate limiting in SMCs *in vivo*, thus making the presence of the strong intronic CARG required for expression. It is also possible that the intronic CARG or other elements within the first intron that interact with the intronic CARG recruit SMC-specific factors that are required for SM  $\alpha$ -actin expression *in vivo*. Although we did not detect such a factor in our gel shift analyses, this was not surprising because we only used 20 bp intronic CARG oligos as shift probes.

The requirement of multiple CARGs for p2600Int/Lac Z expression in SMCs and the fact that the CARGs have



**Figure 9.** Effects of mutations to CARGs A and B and the intronic CARG on the expression of the p2600Int/Lac Z transgene in adult mice. Adult lung, aorta, and skeletal muscle, from Wt and CARG mutant mice were processed as previously described and results of staining for Lac Z expression are shown. Mutation to CARG B (B mut) and the intronic CARG (Int mut) abolished expression in SMCs from all tissues and vascular beds. In contrast, mutation to CARG A (A mut) eliminated expression in smooth muscle organs and large vessels such as the aorta but only partially inhibited expression in smaller blood vessels.

differential effects in SMC versus non-SMC indicates that these elements act interdependently in vivo to regulate SM  $\alpha$ -actin expression. Recent evidence from studies in cultured SMCs from our laboratory demonstrated that CARG phasing and spacing are important determinants of the activity of a reporter construct that contains the first 125 bp of the 5' promoter. This suggests that CARGs A and B coordinate the formation of a transcription activation complex sufficient to drive expression at least in SMC cultures (C.P.M., Mike M. Thompson, Susan Lawrenz-Smith, G.K.O., unpublished results, 1998). The in vivo requirement for the intronic CARG, which is  $\approx 1,000$  bp 3' to CARGs A and B, suggests that this model is probably more complex. Moreover, we have shown that the highly conserved intronic region functions in only one orientation, which argues that it also has specific structural requirements important for transcription complex assembly or activation (C.P.M., unpublished results, 1998). Our results are somewhat analogous to those of Lee et al,<sup>23</sup> which demonstrated that skeletal  $\alpha$ -actin expression in cultured cells was regulated by two "strong" CARGs that facilitate binding of SRF to a third relatively weak CARG element. Given the high prevalence of multiple conserved CARG elements in many skeletal, cardiac, and SMC genes,<sup>19–23</sup> this model that involves interdependence of multiple CARG elements may be an important general mechanism for regulating muscle-specific gene expression.

In summary, these studies are the first to provide evidence that CARG elements play a critical role in transcriptional regulation of the SM  $\alpha$ -actin gene in vivo and that they exhibit differential activity in SMCs versus non-SMCs. This study and others have shown that the activity of each of these elements is regulated by binding to SRF. Therefore, a key goal for further studies is to determine the mechanisms that confer SMC-specific regulation by a ubiquitously expressed transcription factor such as SRF.

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