A Novel Smooth Muscle-specific Enhancer Regulates Transcription of the Smooth Muscle Myosin Heavy Chain Gene in Vascular Smooth Muscle Cells*

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Transient DNA transfection analysis of 5' end deletion mutants of the rabbit smooth muscle myosin heavy chain (SMHC) gene promoter was performed in primary cultures of rabbit vascular smooth muscle cells (VSMC). A positive element located at position −1,332 upstream of the transcription start site consistently gave the highest relative chloramphenicol acetyltransferase (CAT) activity (6.3 ± 1.5-fold over the minimal SMHC promoter), suggesting that inclusion of the extra 107-base pair (bp) DNA fragment between −1,332 and −1,225 could significantly enhance CAT activity in VSMC. Transfection of mutants into several muscle and nonmuscle cell lines did not show any significant CAT activity above control, showing that factors unique to smooth muscle cells were required for SMHC expression. Gel shift analysis indicated that multiple factors interacted with the 107-bp element, two of which appeared to show smooth muscle specificity. Tests of enhancer function in transfected VSMC indicated that the 107-bp fragment behaved as a classical enhancer, i.e. independently of position and orientation. These results indicate that a novel DNA element may regulate the tissue-restricted expression of the SMHC gene and provides the first example of a role for a smooth muscle-specific enhancer in VSMC.

Little is known about the control of myogenesis in smooth muscle cells of either vascular or visceral origin. Like skeletal and cardiac muscle types, smooth muscle cells are derived from mesodermal precursors, but the factors regulating the selection of this particular developmental pathway are unknown. Growth and terminal differentiation in skeletal muscle is under the complex control of the MyoD family of transcriptional regulators (1), and skeletal muscle-specific gene expression is also dependent on a growing number of auxiliary transcription factors (2). In cardiac muscle less is known about lineage determination, but a large number of transcriptional regulators have been identified that appear to regulate the events associated with terminal differentiation and expression of cardiac-specific genes (3).

In smooth muscle cells, growth and differentiation appear to be regulated by mechanisms that distinguish them from the mutually exclusive events associated with striated muscle types. During late fetal development at a point just prior to birth, smooth muscle cells activate a program of gene expression associated with the differentiated phenotype (4). Smooth muscle myosin heavy chain and α-actin are expressed at this time in a manner analogous to the synthesis of proteins required for the formation of the contractile apparatus in striated muscle. No myogenic factors have been identified that may participate in the regulation of these early events in smooth muscle cells. In fact very few genes have been characterized to date that may provide clues about the activation of smooth muscle-specific gene expression. The smooth muscle α-actin gene has been studied for some time (5–7), but its expression is not tissue-restricted and can be detected in other cell types.

Despite the transition from growth to quiescence during development, smooth muscle cells can be stimulated by growth factors to re-enter the cell cycle and undergo new rounds of cell division (8). A new wave of vascular smooth muscle cell (VSMC) proliferation appears to be one of the hallmarks associated with the development of vascular lesions, and these cycling cells undergo a major restructuring of the cell phenotype. This modulation of the phenotype includes a reduction or loss of some of the major features that are characteristic of differentiated cells including smooth muscle myosin. The mechanism of this inhibition of myogenesis in VSMC is unknown.

To study the regulation of myogenesis in smooth muscle cells, we previously isolated the smooth muscle myosin heavy chain (SMHC) gene (9), which is known to encode a number of splicing variants (10–13). The expression of the SMHC isoforms was found to be highly tissue-restricted to smooth muscle tissues, although a low level of expression could also be detected in lung connective tissue cells (14). SMHC expression was also developmentally regulated (15), suggesting that unique factors may be important for activation of SMHC expression in smooth muscle cells. In this report we present the first evidence for the existence of a putative smooth muscle-specific transcriptional enhancer that regulates expression of the SMHC gene.

EXPERIMENTAL PROCEDURES

Creation of SMHC Deletion Mutants—The SMHC promoter was previously isolated as a genomic clone (9), which extended approximately 2.3 kb upstream of the transcription start site. A 3.0-kb XbaI restriction fragment containing all the upstream DNA sequence, the first untranslated exon, and part of the first intron was used to create a nested set of 5′ end deletion mutants (Promega, Erase-a-base), and the inserts were isolated with SstI and BssHII. The BssHII site is located −4 bp upstream of the transcription start site and conveniently allowed

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‡The abbreviations used are: SMHC, smooth muscle myosin heavy chain; VSMC, vascular smooth muscle cell(s); CAT, chloramphenicol acetyltransferase; kb, kilobase(s); FCS, fetal calf serum; BES, 2-(bis[2-hydroxyethyl]amino)ethanesulfonic acid; bp, base pair(s).
construction of mutants that all included the TATA box but excluded the untranslated exon and first intron sequences. XbaI linkers were added to the ends of the 5' end-deleted fragments, and inserts were subcloned into the promoterless reporter gene pCAT Basic (Promega). Mutants were sequenced in both orientations using the Sequenase construction of mutants that all included the TATA box but excluded the enhancer fragment was isolated with HindIII linkers for subcloning in the 5’ – 3’ direction, the enhancer fragment was isolated with HindIII and PstI and ligated upstream of the pRSMHC-112. Orientations and copy number were verified by restriction mapping. The effect of the putative 107 bp enhancer was tested in the enhancerless vector pCAT Promoter (Promega) driven by the viral SV40 promoter. The enhancer fragment was isolated with PstI and subcloned downstream of the CAT gene in both orientations. The vector pBLCAT2 (20), which contains the Herpes simplex virus thymidine kinase promoter, was also used to test for an effect of the 107 bp enhancer fragment on the heterologous vector pCAT Promoter. The 107 bp fragment was isolated from the pRSMHC-112 vector constructed above using HindIII and subcloned in both orientations in pBLCAT2. Transient DNA transfections into primary VSMC were performed as above.

In additional experiments to dissect the functional domains of the SMHC enhancer, a series of five DNA restriction fragments from the enhancer (see Fig. 5) were cloned in a 3’ – 5’ direction into the enhancerless vector pCAT Promoter. Transient DNA transfections into primary VSMC were performed with these vectors to determine which sequences were responsible for the enhancer activity.

Animal Tissues—Intact adult rabbit thoracic aorta for isolation of purified nuclear proteins was quickly and carefully isolated, and the endothelial cell layer was removed by gentle scraping of the luminal surface. Strips of intact tenia coli were carefully dissected away from the underlying intestinal tissue and processed for use in both primary tissue culture and preparation of nuclear protein extracts. Segments (~4 cm) of large intestine were also isolated, and the luminal surface was gently scraped to remove smooth muscle cell layers. The smooth muscle cell layer was then used for cell culture and preparation of nuclear protein extracts. All tissues were quickly and thoroughly washed in ice-cold sterile 0.9% saline during preparation; tissue for nuclear protein extraction was rapidly frozen in liquid nitrogen.

Gel Shift Analysis—Preparation of purified nuclear protein extracts from cell lines, acrylamide gel electrophoresis of DNA-protein complexes, and labeling of DNA restriction fragments and oligonucleotides were based on the protocols described in Ref. 21. Frozen tissue from aorta, tenia coli, and intestine was powdered in liquid nitrogen and resuspended in buffer for Dounce homogenization.

Briefly for analysis of complexes, 5 μg of purified nuclear protein extract was preincubated for 10 min at 4°C with 2 μg of poly(d-I-c) in binding buffer containing 10 mM Hepes, pH 7.9, 10% glycerol, 0.5 mM EDTA, pH 8.0, 1 mM MgCl₂, and 0.5 mM dithiothreitol. Final salt concentration was 50–100 mM, and the final reaction volume was 20 μL. The reaction mixture was then incubated with 1 μL of 32P-labeled probe (100 fmol/μL) for 20 min at 4°C. Samples were run on 4–5% acrylamide gels (401) at 150 V, and gels were dried and exposed to film.

Oligonucleotides for use in gel shift analysis were obtained in high purity liquid chromatography purified form (Oswel DNA Service), and complementary sequences were annealed to form double-stranded probes (see Fig. 5). The sequences were: oligo A (30-mer), 5’-CCGGCCGGACGGGACGGCCGAGCCA-3’; CARG (18-mer), 5’-CAGCTATTATAGTACGG-3’; oligo B (30-mer), 5’-TATTTAGTACGG-3’; oligo C (43-mer), 5’-GGCGAGCCGGCGGTCTGATGACGGCTGCATGGC-3’; oligo D (40-mer), 5’-GCTGATAGTCGTCGGGCGGCTGATGACGGCTGCATGGC-3’; and oligo E (25-mer), 5’-GCTGATAGTCGTCGGGCGGCTGATGACGGCTGCATGGC-3’.

Other Methods—Total RNA was isolated using guanidinium thiocyanate, and RNase protection assays were performed as described (11). The 219-bp PstI-EcoRI DNA fragment used for RNAse protection analysis of SMHC mRNAs was derived from the 3’ end of SMHC C2 (10). This rabbit-derived probe simultaneously detects SM1 and SM2 mRNAs in a manner analogous to the rat probe (11).

RESULTS

DNA Sequence Analysis of Rabbit SMHC Promoter—The transcription start site was previously identified (9), and the data in Fig. 1 show the position of the 79-bp 5’-untranslated exon. Included in this exon is a single consensus E box (1 motif). A consensus TATA box motif lies –24 bp upstream of the start site. (Other binding sites for general transcription factors are present but are not shown for clarity). Highlighted in Fig. 1 are several consensus DNA sequences present in the SMHC gene that are known to be required for the binding of skeletal and cardiac muscle-specific transcription factors. Present in the 2.3-kb of upstream sequence are six E box motifs, one perfect and one slightly imperfect CARG-box (22) motif, one MEF2-like (2) motif, and one MCAT-like (3) motif. We have presently no

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information to decide whether these defined sequences are also functionally important for SMHC gene transcription. The position of the putative enhancer sequence (see below) is marked by brackets in Fig. 1.

SMHC Promoter Is Strongly Active in VSMC and Is Regulated by Both Positive and Negative Elements—When a series of 11 SMHC 5' end deletion mutants were transfected into primary VSMC, several regions of positive and negative CAT activity could be identified (Fig. 2). The shortest mutant used in this study, pRSMHC-112, gave an activity 9-62-fold above the promoterless control pCAT Basic, suggesting that this basal level of activity might correspond to the SMHC minimal promoter. Therefore this level of CAT activity from the minimal promoter was subsequently used as the baseline to calculate CAT activity from the longer mutants (Fig. 2). Compared with the minimal promoter, CAT activity increased 2-fold by the addition of a further 67-bp (mutant pRSMHC-179), suggesting the presence of a positive regulatory sequence in this region. This degree of CAT activity was maintained at approximately the same level for a further ;1 kb of upstream sequence with the possibility of small degrees of negative regulation occurring at pRSMHC-424 and pRSMHC-942. However, a surprisingly large increase in CAT activity (6-fold over basal activity) was observed with mutant pRSMHC-1,332, which gave the largest measured CAT activity of all the SMHC promoter mutants studied. This observation suggested the existence of a strong positive regulatory element in this region of the gene. This activity was slightly reduced to 5-fold by the addition of a further 285 bp of sequence (pRSMHC-1,617) and was maintained at this lower level for the longest mutants up to pRSMHC-2,305.

The possible involvement of the 5' untranslated region (Fig. 1) and first intron sequences in regulating SMHC expression was also tested by their ability to drive CAT expression in VSMC. When an ;12-kb fragment containing the promoter sequences (Fig. 2) plus ;1.0 kb of 5' sequence from the first intron was used in transfected VSMC, we could not detect any CAT activity from the reporter construct (data not shown). Further tests are required to determine if the remainder of the ~12-kb first intron sequences possess any regulatory activity.

Identification of a Vascular Smooth Muscle-specific Positive DNA Element Required for SMHC Transcription—To determine whether the relatively large increase in CAT activity obtained from mutant pRSMHC-1,332 (Fig. 2) might be smooth muscle-specific, mutants pRSMHC-1,332, -2,305, and -1,225 were transfected into several different muscle and nonmuscle cell lines. The minimal promoter mutant pRSMHC-112 was also transfected to allow expression of CAT data relative to the minimal promoter. The results in Fig. 3 show CAT activity from the three mutants in primary VSMC, primary visceral smooth muscle cells (tenia coli and large intestine), and four different cell lines. Compared with primary VSMC, only a relatively low level of CAT activity was measurable from the mutants in both types of primary visceral cells. Although surprising at first, we subsequently found using sensitive RNase protection assays that the level of SM1 and SM2 mRNAs in both types of visceral cells was extremely low compared with primary VSMC (data not shown). Specific antibodies to SM1 and SM2 protein isoforms also gave a barely detectable signal using immunofluorescence of cultured tenia coli cells (data not shown). Thus, the data suggest that the putative positive regulatory element of SMHC transcription, which is evident in primary VSMC, was either present in low amounts and/or nonfunctional in primary visceral cells.

The Rb1 cell line derived from rabbit aortic VSMC (17) was used because of the large degree of smooth a-actin expressed in these cells. However, when we tested Rb1 cells for endogenous SMHC expression, we found no evidence at the mRNA or protein level for SMHC expression in these cells (data not shown). The same was true for the rat aorta VSMC cell lines A7r5 and SV40LTSMC (data not shown). When the two most active SMHC mutants, ~2,305 and ~1,332, were transfected into Rb1 and A7r5 cells, we detected a very low level of CAT activity (Fig. 3). These results together suggested that the Rb1 and
A7r5 cells, although both derived from aortic VSMC, were lacking the positive regulator required for SMHC expression evident in primary VSMC. Therefore, in two smooth muscle cell lines tested, neither of which express the endogenous SMHC gene, we could not detect significant CAT activity from the transfected SMHC mutants.

No CAT activity from the SMHC mutants above the level of the basal promoter could be detected in rabbit skin fibroblast RAB9 and mouse skeletal C2C12 cell lines (Fig. 3), nor in rat skeletal L6, rabbit kidney epithelial RK13, or mouse Swiss 3T3 fibroblasts (data not shown). These data thus provide supporting evidence for the role of a smooth muscle-specific factor in regulating SMHC transcription. The SMHC DNA sequence between positions -1,332 and -1,225 may contain a recognition element(s) that functions to enhance SMHC gene expression in smooth muscle cells. An alternative explanation is that a negative regulator of SMHC expression exists in nonsmooth muscle cell types.

**SMHC Gene Expression in VSMC Is Regulated by a Novel Enhancer DNA Element in the SMHC Promoter**

The marked transition to a region of very high CAT activity observed from mutant pRSMHC-1,332 (Fig. 2) suggested that this region of the SMHC promoter contains a novel enhancer DNA element.
the gene contained DNA sequences vital for high levels of SMHC gene expression in VSMC. To test whether the extra 107-bp fragment from pRSMHC-1,332 was important for regulation of SMHC transcription, its ability to augment the level of CAT activity from the SMHC minimal promoter was tested in VSMC. When a single copy of the 107-bp fragment was placed in either orientation immediately upstream of pRSMHC-112, a 3–6-fold increase in CAT activity was observed compared with the basal level (Fig. 4). The effect was most apparent when the 107-bp fragment was placed in the 3' → 5' orientation, which increased the activity of the basal promoter to the 6-fold level observed for the most active mutant pRSMHC-1,332 (Fig. 2). This result therefore provided the first clue that the fragment was able to enhance transcription of its own promoter.

To determine whether the 107-bp fragment could enhance the activity of a heterologous promoter, the DNA was placed downstream and in both orientations of the CAT gene driven by the SV40 promoter in the enhancerless vector pCAT Promoter. The results in Fig. 4 show that the 107-bp fragment was capable of restoring an enhancer-like function to the SV40 driven CAT gene. The effect was greatest with the fragment in the 5' → 3' orientation and was remarkable in that the activity of the positive control vector in VSMC (~8-fold) was identical to the level obtained when the vector contained the wild-type SV40 enhancer sequence (Fig. 2).

The putative 107-bp putative enhancer was also placed upstream and in both orientations of the eukaryotic Herpes simplex virus thymidine kinase promoter driving CAT expression in the vector pBLCAT2. The data in Fig. 4 show that the 107-bp fragment caused a striking increase in CAT activity from the heterologous tk promoter when transfected into primary VSMC. The levels of CAT activity (~7–11-fold above the minimal promoter) were even greater than those obtained from the SMHC promoter (Fig. 2), thus raising the possibility that the 107-bp fragment was not maximally active in driving SMHC expression.

Dissection of the 107-bp Enhancer Element Reveals Multiple Positively Acting Regions—To further dissect the putative SMHC enhancer sequence and test for activation of transcription, a series of five DNA restriction fragments were generated from the enhancer sequence (Fig. 5). When these fragments were subcloned into pCAT Promoter and transfected into primary VSMC, each showed that it could enhance transcription of the CAT gene (Fig. 6). Surprisingly, RF V (57 bp) on its own was able to enhance CAT activity to a level (~6-fold) that was similar to the level induced by the intact 5' end deletion mutant pRSMHC-1,332. This observation suggested that RF V may possess most of the information required for smooth muscle-specific SMHC expression. However, the fact that the shorter RF III (21 bp) and RF IV (29 bp) fragments were also capable of significantly enhancing CAT activity (~4–5-fold) from pCAT Promoter suggests that these sequences may also be important for SMHC transcription. Interestingly when oligo C (43-mer) was ligated to pRSMHC-1,225 to produce pRSMHC-1,256* (Fig. 6), it did not increase CAT activity above the 2-fold level observed for pRSMHC-1,225 on its own, thus suggesting two possibilities. First, when RF III is present on its own promoter, its enhancing activity is attenuated by downstream sequences. Second, additional distal sequences in the 107-bp enhancer

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**Fig. 4.** Putative enhancer driving SMHC expression in VSMC. A single copy of the 107-bp enhancer DNA element located between positions -1,332 and -1,225 was placed in several orientations: upstream of the basal SMHC promoter pRSMHC-112, downstream of the SV40 promoter-driven CAT gene in the enhancerless vector pCAT promoter, and upstream of the eukaryotic heterologous herpes simplex virus thymidine kinase promoter in pBLCAT2. Constructs were transfected into primary VSMC and assayed for CAT activity (mean ± S.D.). The data expressed are relative to the minimal SMHC promoter.

**Fig. 5.** Analysis of SMHC enhancer. Transfection analysis localized SMHC enhancer activity to positions -1,332 to -1,225 upstream of the transcription start site. A series of five DNA restriction fragments (RF I–RF V) were isolated and subcloned into pCAT promoter and also used as probes in gel shift analysis. Additional probes used in gel shift analysis included oligos A–E and the CAArg oligo.
Fragment are required for full enhancer activity. Furthermore, the observation that the activity of RF V is attenuated when incorporated into RF I and RF II suggests that these longer DNA sequences may contain regions that suppress the activity of RF V. These data point to multiple control regions in the SMHC enhancer and together provide new evidence for the existence of a putative DNA enhancer element in the SMHC gene that interacts with factors unique to vascular smooth muscle cells.

Multiple Factors Bind to the 107 bp SMHC Enhancer Sequence—To determine whether the increased SMHC transcriptional activity generated by the putative 107-bp enhancer fragment resulted from a unique set of protein-DNA interactions, we performed a series of gel shift assays using restriction fragments and specific oligonucleotide DNA probes from the enhancer sequence (Fig. 5). First, to obtain an overall picture of the number of putative nuclear proteins binding to the enhancer, the entire 107-bp fragment was used as a probe. The results in Fig. 7 show that at least two factors from intact aorta interacted with the enhancer, and both appeared to be present in nuclear extracts from nonsmooth muscle cell types. When all cell types were tested, at least four factors apparently bound to the enhancer, all of which were effectively competed out by 100-fold molar excess of the cold probe. When oligos A, B, D, and E were used individually as competitors (Fig. 7), each showed only a low level of competition for different factors in the extracts tested. These data therefore indicate that multiple sequences in the 107-bp enhancer fragment are recognized by factors from different cell types.

Proximal SMHC Enhancer Sequence May Bind a Putative Smooth Muscle Cell-specific Factor—To begin a more detailed analysis of factors binding to the SMHC enhancer, specific probes from the enhancer sequence (Fig. 5) were used for multiple gel shift assays. When RF III (21 bp) from the proximal enhancer was used as probe, a pattern of DNA-protein interactions was observed (Fig. 8) that suggested that nuclear extracts from smooth muscle cells may contain a unique binding activity. A single, strong binding activity designated VFI (Fig. 8, lane 2) was most evident in extracts from native aorta and was competed out by cold competitor (and oligo E, not shown) but not by cold nonspecific competitor. This activity was not evident in the other nonsmooth muscle cell lines tested (Fig. 8, lanes 9–14). However, a low level of VFI activity was present in extracts from rabbit VSMC at passage one (Fig. 8, lane 5), VSMC at passage two in SFM (Fig. 8, lane 7) and in vascular A7r5 cells (Fig. 8, lane 10). This activity was not detectable in passage two cells grown in 10% FCS or in primary visceral smooth muscle cells from tenia coli. (RNase protection analysis of RNA from cultured tenia coli cells showed a very low level of VFI activity.)

Fig. 6. Multicomponent nature of SMHC enhancer. A single copy of each of the restriction fragments RF I–RF V was subcloned into the enhancerless vector pCAT promoter, and the constructs were transfected into primary VSMC. The construct pSMHC-1,256° was derived by ligating oligo C (43-mer) to pSMHC-1,225. The data for pSMHC-112 and -1,332 were taken from Fig. 2; the data for pCAT promoter containing the entire 107-bp fragment were taken from Fig. 4. The data (mean ± S.D.) expressed are relative to the minimal SMHC promoter.

Fig. 7. Gel shift analysis of purified nuclear protein extracts binding to the complete 107-bp SMHC enhancer. Extracts from intact aorta, 3T3 (fibroblasts), Rb1 (aorta), C2C12 (skeletal), RK13 (epithelial), and RAB9 (fibroblast) cells were reacted with and without competitors. All competitors were added at 100-fold molar excess. Lanes 1 and 2 show probe alone; a minus sign indicates no competitor; a plus sign indicates with competitor; S, cold specific competitor; A, oligo A; B, oligo B; D, oligo D; E, oligo E. The arrowheads point to bands that were not competed out by cold nonspecific competitor.
SM1 and SM2 mRNA expression; data not shown). When extracts were prepared from native tenia coli and large intestine, it was also possible to detect VFI activity (data not shown). These data therefore suggest that smooth muscle cells express levels of VFI activity that resemble the pattern of endogenous SMHC mRNA and protein expression that occurs in intact tissue and cultured VSMC (11, 13).

A second weak DNA binding activity designated VFII was observed in primary VSMC extracts and also in the Rb1 and A7r5 vascular cell lines but not in any of the non-smooth muscle cell lines tested. Because VFII activity could not be detected in extracts from native aorta, it suggests that the activity may result from growth of vascular cells in culture. The data in Fig. 8 also show that the RF III probe detected a single binding activity in non-smooth muscle cells that could be partially competed out by cold specific competitor in C2C12 cells (Fig. 8, lanes 11 and 12). This activity migrated with a mobility between VFI and VFII and was reproducible (data not shown).

When oligo E was used as probe (which differs from RF III by eight nucleotides), a faint band resembling the pattern of VFI expression was evident in primary VSMC, and this activity was competed out by cold specific competitor (data not shown). This band was not detectable in non-smooth muscle cell extracts. A second major activity was present in primary VSMC and all other cell lines tested. This activity was almost totally competed out in VSMC but was only partially competed out by cold specific competitor in non-smooth muscle cell extracts, analogous to the intermediate activity shown in Fig. 8. (A faint band was also evident only in extracts from native aorta and A7r5 cells when oligo C was used as probe and was effectively competed out by cold specific probe and oligo E, data not shown). Taken together these data provide preliminary evidence for the existence of a smooth muscle-specific activity in the proximal region of the SMHC enhancer.

Central (57 bp) Portion of SMHC Enhancer Binds Multiple Factors—Gel shift analyses with RF V as probe showed that several factors recognized this sequence, one of which was
Effect (Fig. 9, strongly competed out by oligo B with the CA rG oligo having no competitor, but the smooth muscle cell-related activity was apparently present in all cell types examined (Fig. 9). At least four factors were observed for all cell types put together, but the data in Fig. 9 suggest that one of these factors may be unique to smooth muscle cells. A reproducible band migrating just above the single, common band was observed in extracts from intact aorta (Fig. 9A), cultured VSMC, and tenia coli cells (Fig. 9B) but could not be seen in extracts from large intestine. All visible bands were effectively competed out by cold specific competitor, but the smooth muscle cell-related activity was strongly competed out by oligo B with the CA rG oligo having no effect (Fig. 9, A and B). Interestingly, in intact aorta, cultured VSMC, and tenia coli extracts, the common band was most effectively competed out by the CA rG oligo, whereas CA rG had little or no effect on this activity in the other nonsmooth muscle cell lines tested. These results therefore suggest that the RF V probe has exposed a further smooth muscle-related binding activity that does not apparently possess a CA rG-like activity.

Also, the SMHC CA rG-like sequence apparently binds a factor that distinguishes it from any CA rG activity present in nonsmooth muscle cell extracts.

Rabbit Primary VSMC Express a CA rG-like Activity—The data in Fig. 9 indicated that when RF V was used as probe, the CA rG sequence was able to compete out an activity present in extracts from smooth muscle cells. To confirm that a CA rG-like activity was present in smooth muscle cells, gel shift assays were performed with the CA rG oligo as probe. The results in Fig. 10 show that a single major factor from VSMC recognized the CA rG sequence, and this activity was competed out by cold specific competitor. (This band was not competed by any non-specific competitor tested except calf thymus DNA, data not shown). This factor was also present in the Rb1 and RAB9 cell lines tested but not in others (Fig. 10), although a more slowly migrating, specific faint band was also evident in A7r5, C2C12, and RK13 cells. This observation suggested that SMHC transcription was regulated by a CA rG-like activity that was not unique to VSMC. When oligo B was used as probe, a single band was also evident in all cell lines studied, although its mobility in C2C12 and A7r5 extracts was slower as above (data not shown). This activity was partially competed by the CA rG sequence (data not shown), thus suggesting a role for this sequence in the regulation of SMHC transcription.

Additional Factors Bind to the Distal SMHC Enhancer Element—When RF IV was used as probe in gel shift analysis, at least two factors could be identified in all smooth muscle cells studied except intact aorta (data not shown). These bands were also evident in other cell types that included the presence of additional factors. Thus the distal region of the SMHC enhancer shows DNA binding activity but was not unique to smooth muscle cells.

The results taken together therefore suggest that the enhanced transcriptional activity obtained from the intact SMHC promoter in VSMC (Fig. 2) is associated with unique DNA-protein interactions that appear to reside in a 78-bp region of the proximal SMHC enhancer sequence. These factors may be responsible for the dramatic increase in CAT reporter activity shown in VSMC when RF V was positioned immediately upstream of the SMHC minimal promoter (Figs. 4 and 6).

DISCUSSION

We present new data revealing the existence of a novel eukaryotic enhancer DNA element that functions to regulate SMHC gene expression in primary VSMC. This is the first example of a putative enhancer whose activity appears to be tissue restricted to VSMC.

Transient DNA transfection analysis of the rabbit SMHC promoter in primary VSMC revealed regions of positive and negative activity. However, a 107-bp element between positions −1,332 and −1,225 was found to function in a classical enhancer-like manner, i.e. independently of position and orientation. An inspection of the DNA sequences in this region revealed the presence of a slightly imperfect CA rG box consensus, but otherwise data base searches did not reveal any other known muscle-related sequences. The CA rG box is known to be an essential cis regulatory element for skeletal muscle-specific gene expression (22), but we presently have no information to implicate a function for this slightly imperfect element (ending AG instead of GG) located in the 107-bp enhancer. It is possible that the CA rG-like sequence performs an essential role in VSMC, and preliminary gel shift analysis (Fig. 10) showed that primary VSMC possessed an activity capable of specifically binding to the CA rG-like sequence. However, the data also showed that a similar activity was present in other cell types, implying that the activity was not unique to smooth muscle. The cardiac α-MHC gene is the only other myosin gene known to date (23) that is regulated by an enhancer but in this case is unresponsive to transactivation by the MyoD family of regulators (1).

The absence of known muscle-related sequences in the 107-bp enhancer fragment raises the possibility that this new smooth muscle-specific enhancer interacts with factors unique to smooth muscle cells. There is considerable evidence to support this idea because analysis of SMHC expression in vivo demonstrates a very high degree of tissue-restricted regulation (10, 11). Furthermore, SMHC promoter-driven CAT activity is not detectable in nonsmooth muscle cells (Fig. 3), and the absence of endogenous SMHC expression in VSMC cell lines, such as Rb1 and A7r5, strengthens the view that only primary VSMC possess the factors required for SMHC expression. The observation that primary visceral smooth muscle cells do not support SMHC-driven CAT activity is most likely related to the fact that these cultured cells express extremely low levels of endogenous SMHC mRNA and protein. This may be the result of a low level of transcription factor activity required for binding to the SMHC enhancer.

Our results showing that the 107-bp enhancer appears to be multicomponent (Fig. 6) and that multiple factors appear to bind to the sequence (Figs. 7–10) suggest that this region of the SMHC gene is under complex control. However, our gel shift analysis identified a putative smooth muscle-specific binding factor (Fig. 8) whose apparent abundance in purified nuclear extracts from smooth muscle cells, gel shift analysis of purified nuclear protein extracts binding to the enhancer CA rG-like sequence (18-mer). Extracts used were primary VSMC at passage two (P2), Rb1, RAB9, A7r5, C2C12, and RK13. Plus and minus signs indicate with cold specific competitor and without competitor, respectively. Lane 1 shows probe alone. The closed arrowhead points to the major band visible in VSMC.
extracts largely resembles the pattern of endogenous SMHC expression. Thus the abundance of VFI was greatest in intact aorta, decreased when VSMC were grown in culture, decreased further during passaging of VSMC but whose level could be partially restored when confluent VSMC were maintained in serum-free medium. This matches perfectly the expression of the endogenous SMHC gene (11–14) and argues in favor of a link between VFI and SMHC expression. It remains a puzzle, however, why the DNA sequence in RF III that is bound by the factor VFI does not apparently augment transcription of the SMHC promoter (Fig. 6). Further work is required to complete the characterization of this region. Similarly, it is presently unclear whether the augmented CAT activity induced by RF V (Fig. 6) is linked to the presence of a second putative smooth muscle related factor shown in Fig. 9. In extracts from intact aorta, for example, at least three factors bound to the RF V probe, two of which appeared to be present in other cell types. These results imply that the interaction between a number of factors in this region of the SMHC gene determine the extent to which the gene is transcribed in smooth muscle cells.

Compared with the advances made in our understanding of striated muscle development (1), relatively few studies so far have addressed the question of myogenesis in smooth muscle cells. Previous reports on the smooth \(\alpha\)-actin promoter (5–7) have shown that CArG box sequences are essential for activity and that a number of positive and negative cis elements regulate expression. However, unlike the data in the present study, the smooth \(\alpha\)-actin promoter is active in fibroblast and skeletal myoblast cells. Recently it was shown that SMHC exclusively marks the smooth muscle lineage during mouse embryogenesis (24) and suggests that this gene may be regulated by factors unique to smooth muscle cells. Here we present strong evidence to show that the SMHC promoter is only active in primary VSMC and that maximal expression is dependent on an enhancer DNA element that appears to be functional only in primary VSMC. We have now created transgenic mouse cell lines where the SMHC promoter was used to drive lacZ expression to determine the role of the enhancer during smooth muscle development. These data pave the way for further analysis of smooth muscle-specific gene expression and may eventually provide clues on the inhibition of myogenesis that is associated with proliferation of VSMC in vascular disease.3

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REFERENCES

1. Edmondson, D. G., and Olson, E. N. (1993) J. Biol. Chem. 268, 755–758
2. Yu, Y.-T., Brettbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Gene & Dev. 6, 1783–1788
3. Mar, J. H., and Ordahl, C. P. (1990) Mol. Cell. Biol. 10, 4271–4283
4. Schwartz, S. M., Campbell, G. R., and Campbell, J. H. (1989) Circ. Res. 58, 487–484
5. Carrell, D. L., Bergma, D. J., and Schwartz, R. J. (1988) Mol. Cell. Biol. 8, 241–250
6. Blank, R. S., McQuinn, T. C., Yin, K. C., Thompson, M. M., Takeyasu, K., Schwartz, R. J., and Owens, G. K. (1992) J. Biol. Chem. 267, 984–989
7. Foster, D. N., Min, B., Foster, L. K., Stoflet, E. S., Sun, S., Getz, M. J., and Strauch, A. R. (1992) J. Biol. Chem. 267, 11955–12003
8. Ross, R. (1993) Nature 362, 801–809
9. Babij, P., Kelly, C., and Periasamy, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10676–10680
10. Nagai, R., Kuroo, M., Babij, P., and Periasamy, M. (1989) J. Biol. Chem. 264, 9734–9737
11. Babij, P., and Periasamy, M. (1989) J. Mol. Biol. 210, 673–679
12. Babij, P. (1993) Nucleic Acids Res. 21, 1467–1471
13. Babij, P., Kawamoto, S., White, S., Adelstein, R. S., and Periasamy, M. (1992) Am. J. Physiol. 262, C607–C613
14. Babij, P., Zhao, J., White, S., Woodcock-Mitchell, J., Mitchell, J., Abscher, M., Baldor, L., Periasamy, M., and Low, R. B. (1993) Am. J. Physiol. 265, L127–L132
15. Kuroo, M., Nagai, R., Tsuchimochi, H., Katoh, H., Yazaki, Y., Ohkubo, A., and Takaku, F. (1989) J. Biol. Chem. 264, 18272–18275
16. Chamley-Campbell, J. H., Campbell, G. R., and Ross, R. (1979) Physiol. Rev. 59, 1–59
17. Nachtigal, M., Nagai, M. L., Greenspan, P., Nachtigal, S. A., and Legrand, A. (1989) In Vitro 25, 683–688
18. Reilly, C. (1990) J. Cell. Physiol. 142, 342–351
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Luckow, B., and Schutz, G. (1987) Nucleic Acids Res. 15, 5490
21. Latchman, D. S. (1993) Transcription Factors, pp. 1–26, IRL Press, Oxford University Press, Oxford
22. Minty, A., and Kedes, L. (1986) Nucleic Acids Res. 14, 892–898
23. Thompson, W. R., Nadal-Ginard, B., and Mahdavi, V. (1992) J. Biol. Chem. 267, 22678–22688
24. Miano, J. M., Cserjesi, P., Ligon, K. L., Periasamy, M., and Olson, E. N. (1994) Circ. Res. 75, 803–812
25. Kuroo, M., Koukianov, E., Kopras, E., Zilberman, A., and Periasamy, M. (1994) J. Biol. Chem. 269, 30538–30545

2 P. Babij, unpublished observations.

3 During revision of this manuscript a paper was published by Katoh et al. (25) describing the rabbit SMHC promoter but did not make reference to any enhancer-like activity.
A Novel Smooth Muscle-specific Enhancer Regulates Transcription of the Smooth Muscle Myosin Heavy Chain Gene in Vascular Smooth Muscle Cells
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