Activation of a Muscle-Specific Actin Gene Promoter in Serum-Stimulated Fibroblasts

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Treatment of AKR-2B mouse fibroblasts with serum growth factors or inhibitors of protein synthesis, such as cycloheximide, results in a stimulation of cytoskeletal β -actin transcription but has no effect on transcription of muscle-specific isoforms, such as the vascular smooth muscle (VSM) α -actin gene. Deletion mapping and site-specific mutagenesis studies demonstrated that a single "CArG" element of the general form CC(A/T)₆GG was necessary and possibly sufficient to impart serum and cycloheximide-inducibility to the β -actin promoter. Although the VSM α -actin promoter exhibits at least three similar sequence elements, it remained refractory to serum and cycloheximide induction. However, deletion of a 33 base pair sequence between -191 and -224 relative to the transcription start site resulted in the transcriptional activation of this muscle-specific promoter in rapidly growing or serum-stimulated fibroblasts. Although the activity of this truncated promoter was potentiated by cycloheximide in a manner indistinguishable from that of the β -actin promoter, this was dependent on a more complex array of interacting elements. These included at least one CArG box and a putative upstream activating element closely associated with the -191 to -224 inhibitory sequences. These results demonstrate that the expression of a muscle-specific actin gene in fibroblasts is suppressed by a *cis*-acting negative control element and that in the absence of this element, the promoter is responsive to growth factor-induced signal transduction pathways.

INTRODUCTION

The genomes of higher vertebrates encode six major isoforms of the contractile protein actin. Two of these, the β - and γ -cytoskeletal actins, are expressed in all cells and are the principal structural proteins of cellular microfilaments. Interestingly, both cytoskeletal actin genes are members of a diverse class of cellular "immediate early" genes, the prototypical example of which is the *c-fos* proto-oncogene (Greenberg and Ziff, 1984; Lau and Nathans, 1987). The salient feature of these genes is that their transcriptional activation is one of the earliest catalogued responses of cells in culture to mitogenic stimulation by serum or purified peptide growth factors.

Although expression of the two cytoskeletal actin genes is strongly correlated with cell growth and division, the opposite is true for the muscle-specific actin genes. These genes, which encode two striated and two smooth muscle isoforms, are expressed mainly on terminal differentiation of cells of myogenic lineage. In cell culture model systems, this generally occurs spontaneously at high cell density and is coordinated with the cessation of cell proliferation (Cox *et al.*, 1990, and references therein). Thus, although the cytoskeletal and muscle-specific actins are closely related proteins, they are differentially expressed in response to what are generally considered to be mutually exclusive events, cell growth and cell differentiation.

Two general types of *cis*-acting sequence elements have been shown to be required for cell- and developmental stage-specific transcription of muscle-specific actin genes. One of these, termed an E-box, has been shown to bind helix-loop-helix proteins of the MyoD family of myogenic regulators (reviewed by Olson, 1990). The other, termed a CArG box (Minty and Kedes, 1986), has the general form CC(A/T)₆GG and has been shown to bind *trans*-acting factors closely related to, and in some cases identical to, the mammalian serum response factor (SRF) (Boxer *et al.*, 1989; Walsh, 1989; Lee *et al.*, 1991; Mohun *et al.*, 1991; Pollock and Treisman, 1991). The SRF was initially identified as the cognate binding protein of the *c-fos* serum response element (SRE), a 22 base pair (bp) dyad symmetry sequence required for serum-dependent activation of *c-fos* transcription (Treisman, 1986, 1987). Interestingly, the central 10 bp core of the *c-fos* SRE is a CArG box, mutation of which eliminates SRF binding and serum-dependent enhancer activity (Greenberg *et al.*, 1987; Leung and Miyamoto, 1989). Thus, a common sequence motif, the CArG box, is required for both differentiation-specific and growth-specific transcription of certain genes.

In the present report, we show that the mouse cytoskeletal β -actin gene promoter contains a single CArG motif that specifically binds *in vitro* synthesized SRF and that is required for serum-dependent activation of transcription. Moreover, this sequence also mediates the well-established ability of cycloheximide to potentiate this effect (Elder *et al.*, 1984; Greenberg *et al.*, 1986) and, indeed, to induce transcription in the absence of serum growth factors (Subramaniam *et al.*, 1989). On the basis of these and other data, we suggest that the β -actin CArG box is an integral component of a negatively regulated serum-responsive enhancer element. In contrast, we demonstrate that expression of the muscle-specific vascular smooth muscle (VSM) α -actin gene in fibroblasts is negatively regulated by an additional and fundamentally different mechanism that appears to involve a strong transcriptional silencing element. Most importantly, a truncated promoter lacking this element can be induced by both serum and cycloheximide, suggesting that the element is a dominant negative regulator of functional serum-responsive transcriptional enhancer elements contained within the VSM α -actin promoter.

MATERIALS AND METHODS

Cell Culture and Transfection

Mouse embryo-derived AKR-2B cells were cultured to 80–90% confluency in T-175 flasks containing McCoy's 5A medium (GIBCO, Grand Island, NY) and 5% fetal calf serum (FCS) (Hyclone, Logan, UT) at 37°C and 5% CO₂ (temperature and CO₂ concentration are the same for all following incubations). Cells were trypsinized and plated on 100 mm dishes (Corning Glass, Corning, NY) at a concentration of 5×10^5 to 1×10^6 cells per dish in McCoy's 5A and 5% FCS. Cells were grown for 24 h and then transfected with 15 μ g of the indicated plasmids using a DEAE-dextran (Pharmacia, Piscataway, NJ) technique

(Lopata *et al.*, 1984). Transfected cells were grown for 24 h in McCoy's 5A and 10% FCS. Rapidly growing cells were harvested at this point, and equal amounts of total protein from each were assayed for chloramphenicol acetyltransferase (CAT) activity by the method of Gorman *et al.* (1982). Alternatively, transfected cells were rendered quiescent by washing twice with serum-free medium MCDB 402 (Hazleton, Lenexa, KS) and incubating an additional 36 h in MCDB 402. Quiescent cells were then stimulated for 6 h by the addition of fresh MCDB 402 containing 20% FCS or for 4 h with the same medium additionally containing 10 μ g/ml cycloheximide (CHX) (Sigma, St. Louis, MO). The latter was followed by a 2-h washout with medium lacking CHX as previously described (Elder *et al.*, 1988). Cells were harvested and assayed for CAT activity as described above.

Construction of β -Actin Plasmids

Recombinant plasmid p99 β Ac-CAT was constructed by making a 99-base synthetic copy of the mouse β -actin proximal promoter region +7 to -92 and its complement on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. Gel-purified oligonucleotides were phosphorylated, annealed, and inserted between the *Sal*I and *Bam*HI sites of the promoterless reporter plasmid pBLCAT3 (Luckow and Schutz, 1987). The plasmids p57 β Actk-CAT and p26 β Actk-CAT contain subdivisions of the β -actin promoter similarly inserted upstream of the herpes simplex virus (HSV) thymidine kinase (tk) promoter in pBLCAT2.

Run-Off Transcription

Nuclear run-off transcription assays were performed as previously described (Elder *et al.*, 1984).

Primer Extension

Total RNA from cells stimulated with 20% FCS and 10 μ g/ml CHX as described was harvested by the method of Chomczynski and Sacchi (1987). Primer extension analysis was performed as described by Elsholtz *et al.* (1986). For each reaction, 40 μ g of RNA was annealed to 3 ng of ³²P γ -ATP (Amersham, Arlington Heights, IL) labeled CAT33 oligonucleotide primer. The primer was then extended to the initiation site using AMV reverse transcriptase (Life Sciences, St. Petersburg, FL). Extension products were analyzed on a 10% polyacrylamide 7 M urea gel.

Construction of VSM α -Actin Deletion Mutants

Deletion mutants of the VSM α -actin 5' regulatory region were constructed using polymerase chain reaction (PCR) amplification. Oligonucleotide primers designed with 5' *Sal*I and 3' *Bam*HI restriction sites were made on an Applied Biosystems model 394 DNA/RNA synthesizer and desalted over a Sephadex G-25 NAP column (Pharmacia) in distilled water. Primers were annealed to 1 μ g of template DNA (pSB18a) (Min *et al.*, 1990) and amplified 30 cycles in a Perkin-Elmer-Cetus (Norwalk, CT) DNA thermalcycler using standard PCR kit (Perkin-Elmer-Cetus) reaction mixes and times (94°C $\times$ 1 min; 50°C $\times$ 2 min; 72°C $\times$ 3 min). PCR products were gel purified from a 1% agarose gel using Costar (Cambridge, MA) spin-X columns. VSM fragments were then *Sal*I/*Bam*HI digested and ligated into pBLCAT3 that was similarly digested and calf intestinal phosphatase treated. Transformation was done into *Escherichia coli* HB101 cells using standard CaCl₂ techniques.

Construction of *Stu*I Mutations

Site-directed *Stu*I mutations were made using the splicing by overlap extension method (Horton *et al.*, 1989). The following oligos were synthesized and purified as described earlier. Small-case letters denote a clamping sequence for the restriction enzymes. Underlined letters

denote *Sal* I or *Bam*HI sites, whereas italicized letters indicate a *Stu* I site. Bold capitals denote VSM sequence.

- a) VSMP4*Sal*-gatcat**GTCGACG**CAGAACAGAGGAATG;
- b) VSMP α Stu-I-CTTCtaggccttt**AGCAAAGACGGG**CTGA;
- c) VSMPStuI-GCTaaaggcctaa**GAAGCGAGTGGG**AGG;
- d) VSMPBam-catgat**GGATCCTGACAGCG**ACTGGCT;
- e) VSMP α Stu2-**ACAA**ttaggccttt**ACCTCAGCACAAA**ACTC;
- f) VSMPStu2-**AGGT**aaaggcctaa**TTGTGTTAGAGTGAACGG**;

For the first PCR reaction, the following primer sets were annealed to 500 ng of template DNA (pC3VSMP2) and amplified 35 cycles at conditions described previously: 1) a + b; 2) c + d; 3) a + e; and 4) f + d. PCR products were gel purified from a 2% agarose gel using Costar spin-X columns. For the second PCR reaction, 10 μ l of eluant from the columns (total volume of eluants was 100–200 μ l) was used as template for the reaction. Primers a + d were annealed to eluants from 1) + 2) and 3) + 4) and amplified as before. PCR products were gel purified as described, and fragments were then *Sal* I/*Bam*HI digested, ligated into pBLCAT3, and transformed as before.

All cloned sequences described in this paper were confirmed by double-stranded dideoxy sequencing using a sequenase kit (United States Biochemical, Cleveland, OH).

In vitro Transcription and Translation of SRF

Approximately 2 μ g of SRF vector pG3.15 hSRF (a derivative of the SRF cDNA plasmid pG3.5) containing 8 bp 5' to the start of transcription and linearized by *Bst* EII at bp 2879 was transcribed and translated using the TnT T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's recommendation.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed similar to the method of Fried and Crothers (1981), except rabbit reticulocyte lysate with *in vitro* translated hSRF (SRF) or unprogrammed lysate (UL) was used rather than nuclear extract. Each reaction contained a 0.5 μ g of poly[dI-dC], 0.9 mM EDTA, pH 8.0, 1 mM dithiothreitol (DTT), 20 000 cpm (~0.4 ng) of 32 P end-labeled probe and 15 μ l of rabbit reticulocyte lysate in a reaction volume of 20 μ l. Whole cell extract buffer (15 μ l; 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) replaced lysate in the free probe mixture. All mixtures were incubated for 30 min at room temperature and then loaded onto a 4% non-denaturing polyacrylamide gel. The samples were electrophoresed at 150 V in 0.5 $\times$ TBE (45 mM tris(hydroxymethyl)aminomethane, 45 mM boric acid, 1 mM EDTA, pH 8.3) for 1.5 h at room temperature and then the gel was dried and autoradiographed.

RESULTS

Isotype-Specific Stimulation of Actin Gene Transcription by CHX

We have previously shown that cytoskeletal, but not muscle-specific, actin gene transcription is stimulated by epidermal growth factor and other serum growth factors in AKR-2B mouse fibroblasts (Elder *et al.*, 1984; Blatti *et al.*, 1988). Additionally, we have shown that cytoskeletal actin transcription can also be stimulated in the absence of growth factors by CHX and have suggested that this property reflects the action of a labile repressor of serum-responsive enhancer elements

(Subramaniam *et al.*, 1989). In the experiment shown in Figure 1, we compared the transcriptional activity of the cytoskeletal β -actin gene with that of the muscle-specific VSM α -actin gene after CHX treatment of AKR-2B mouse fibroblasts growth arrested under serum-free conditions. In agreement with our previous studies, β -actin transcription increased severalfold within 2 h of the addition of CHX. However, transcription of the VSM α -actin gene was refractory to CHX induction, indicating that repression of this muscle-specific gene must be mediated by mechanisms fundamentally different from those that regulate β -actin transcription. We obtained identical results using probes specific for the α -cardiac and α -skeletal muscle isotypes.

CHX-Induction of β -Actin Transcription is Mediated by a Promoter-Associated *Ca*RG Box

We have previously demonstrated that sequences associated with a 4.5-kb fragment containing the 5' end of the mouse β -actin gene are capable of conferring serum- and CHX-dependent transcription to a linked CAT gene as determined by measurements of both CAT enzymatic activity and the abundance of correctly initiated RNA transcripts (Elder *et al.*, 1988). Subsequent deletion mapping experiments localized the sequence elements involved to a 99-bp fragment encompassing the sequences from +7 to -92 relative to the start site of transcription. To further define these elements, synthetic double-stranded oligonucleotides corresponding to this region, or to subfragments thereof, were prepared and inserted into either the promoterless reporter plasmid pBLCAT3 or into a derivative containing the HSV tk promoter (pBLCAT2). These constructs were then transfected into AKR-2B fibroblasts, and their ability to direct serum and CHX-dependent transcription was examined. Figure 2 illustrates a representative series of experiments. Although the ability of these constructs to direct serum-inducible transcription varied somewhat from experiment to experiment (generally ranging from

Figure 1. Isotype-specific stimulation of actin gene transcription by CHX. AKR-2B cells were grown to near confluency in medium containing 10% FCS and then rendered quiescent by placement in serum-free medium for 48 h. CHX was added to a final concentration of 10 μ g/ml for the indicated times. Nuclei were isolated and used for nuclear run-off transcription assays as described (Elder *et al.*, 1984). 32 P-labeled transcripts were then hybridized to actin isotype-specific cDNA probes (Ponte *et al.*, 1983; Min *et al.*, 1988) immobilized on nitrocellulose in the form of duplicate slots.

Figure 2. Serum and CHX-dependent stimulation of β -actin promoter activity. Double-stranded synthetic oligonucleotides corresponding to the indicated regions of the mouse β -actin promoter were linked to the promoterless reporter plasmid pBLCAT3 or to the tk promoter in the reporter plasmid pBLCAT2. The indicated constructs were then transfected into AKR-2B cells that were grown for 24 h in medium containing 10% FCS and then rendered quiescent by placement in serum-free medium for 36 h. They were then stimulated for 6 h by the addition of fresh medium containing 20% FCS or the same containing 10 μ g/ml CHX. The numbers above the lanes denote independent transfection experiments.

2- to 8-fold), the addition of CHX consistently resulted in up to a 50-fold stimulation over basal levels. Similar results were obtained by treatment with CHX in the absence of serum factors. We attribute the more consistent results obtained using CHX to the fact that CHX inhibits normal transcriptional attenuation that occurs after serum-stimulation (Greenberg *et al.*, 1986). Because

the time course of attenuation can vary considerably, particularly in transient expression experiments, the use of CHX to inhibit attenuation allows a more accurate measurement of the inherent strength of serum-responsive enhancer elements (Subramaniam *et al.*, 1989). Our results were essentially the same using the intact wild-type β -actin promoter (p99 β Ac-CAT) or two in-

A β -Actin

B VSM α -Actin

Figure 3. Nucleotide sequence of mouse actin gene promoters. (A) Nucleotide sequence of the mouse β -actin promoter fragment contained in p99 β Ac-CAT (Figure 2). (B) Nucleotide sequence of a 270-bp fragment of the mouse VSM α -actin promoter (Min *et al.*, 1990). Boxed sequences correspond to CArG or CArG-like motifs, whereas underlining denotes consensus CAAT or TATA boxes.

ternal subfragments linked to the heterologous tk promoter (p57 β Actk-CAT, p26 β Actk-CAT). As shown in Figure 2, this latter 26-bp fragment contains a single CArG box (CCTTTTATGG) but exhibits little similarity outside of this motif to what we have previously shown to be a functionally similar *c-fos* SRE (Subramanian *et al.*, 1989). The complete nucleotide sequence of the 99-bp β -actin promoter is shown in Figure 3A.

To verify the essential requirement for the CCTTTTATGG sequence, a mutant was prepared in which an unrelated 10-bp sequence containing a *Stu*I restriction site was substituted for the CCTTTTATGG sequence within an otherwise wild-type +7 to -92 β -actin promoter fragment. Figure 4 illustrates that this mutation reduced the basal promoter activity severalfold and completely eliminated serum and CHX inducibility. It is important to note that the CCTTTTATGG sequence is conserved in place within the human β -actin promoter and is similarly required for serum-dependent transcription (Ng *et al.*, 1989). Although we cannot yet discount a requirement for additional flanking sequence specificity, the lack of flanking sequence similarity with the functionally similar *c-fos* SRE suggests that any such requirement cannot be too stringent. Moreover, differences between the two CArG box motifs themselves suggest that some flexibility is permitted here as well.

A Strong Negative Control Element Suppresses VSM α -Actin Promoter Activity in Fibroblasts

The transcriptional inactivity of the VSM α -actin gene in fibroblasts (Figure 1) could be due to differences in *trans*-acting regulatory factors, a nonpermissive chromosomal organization, DNA methylation, or some combination thereof. To study this aspect further, we examined the activity of cloned VSM α -actin promoters transfected under conditions that do not permit chromosomal integration (Lopata *et al.*, 1984). To facilitate these experiments, a series of sequential deletion mutants of an 1109-bp mouse VSM α -actin promoter (Min *et al.*, 1990) was constructed and inserted into the promoterless reporter plasmid pBLCAT3. These are also described elsewhere (Foster *et al.*, 1992) and are illustrated diagrammatically in Figure 5. As shown in Figure 3B, this promoter contains three CArG elements (nos. 1, 2, and 4) and one CArG-like element lying in an opposite orientation (no. 3). Each construct was transfected in duplicate into parallel cultures of subconfluent AKR-2B cells and cellular extracts analyzed for CAT activity 24 h after refeeding with fresh serum-containing medium. Figure 6 illustrates that constructs that retained sequences upstream of position -191 were inefficiently expressed. Because the frequency of stable chromosomal integration is very low under these conditions (10^{-3} to 10^{-4}), the lack of endogenous VSM α -actin transcription in fibroblasts (Figure 1) cannot be explained solely on

Figure 4. Serum and CHX-stimulated activity of wild-type and mutant β -actin promoters. The indicated wild-type (p99 β Ac) and mutant (p99 β AcStu) promoters were inserted into pBLCAT3 and assayed for their ability to direct serum and CHX-dependent transcription in AKR-2B cells as described in Figure 2. The numbers above the lanes denote independent transfection experiments.

the basis of a nonpermissive chromosomal organization or DNA methylation pattern. Notably, deletion of a 33-bp fragment from -191 to -224 resulted in a dramatic activation of promoter activity that approximated that observed in parallel cultures transfected with a CAT gene under control of the β -actin promoter. However, deletion of an additional 41 bp to position -150 resulted in an equally dramatic loss of promoter activity despite the retention of the two most promoter-proximal CArG motifs and a TATA box. This latter 41-bp fragment spans the "inverted" CArG-like sequence GGAAGA-GACC, but the functional significance of this sequence has not yet been determined. Irrespective, the results of these experiments suggest that the expression of the VSM α -actin gene in fibroblasts is regulated by a closely associated group of positive and negative elements mapping, in part, between positions -224 and -150. A similar organization of positive and negative elements has been noted previously in studies of the chicken VSM, and skeletal muscle α -actin gene promoters (Carroll *et al.*, 1988; Chow and Schwartz, 1990). Carroll *et al.* (1988) have termed this region a "governor" element and similarly localized it to position -257 to -123 of the chicken VSM α -actin promoter.

The -191 to -224 Sequence Specifically Represses Serum and CHX-Inducibility of the VSM α -Actin Promoter

Because the experiments shown in Figure 6 used mid-log phase cultures, they provided no information as to whether deletion of any of the indicated sequences may have altered the inducibility properties of the VSM α -actin promoter. In particular, we wondered whether sequences within the -191 to -224 region may specifically repress the ability of any of the CArG motifs to mediate serum and CHX-dependent expression. To examine this

Figure 5. Deletion mutants of the mouse VSM α -actin promoter and associated 5' flanking sequences. The uppermost construct (pC3VSMP1) corresponds to a previously described 1109 bp segment of the mouse VSM α -actin gene and associated 5' flanking sequences (Min *et al.*, 1990). Progressive deletions of this sequence were constructed by PCR amplification techniques (MATERIALS AND METHODS) and cloned into pBLCAT3. The lined box corresponds to the TATA box, whereas the solid boxes correspond to the CArG motifs illustrated in Fig. 3B.

issue, the series of VSM α -actin promoter constructs shown in Figure 5 were transfected into AKR-2B fibroblasts that were subsequently rendered quiescent by placement in serum-free medium. As a control, parallel cultures were again transfected with p99 β Ac-CAT. Cells were then stimulated for 6 h by the addition of fresh medium containing 20% FCS or the same plus 10 μ g/ml CHX. As shown in Figure 7, cells transfected with VSM α -actin promoter constructs that retained sequences upstream of position -191 (pC3VSMP1-3) exhibited no detectable serum-inducible CAT activity. A

Figure 6. Activity of VSM α -actin promoter deletion mutants in rapidly growing fibroblasts. Subconfluent cultures of AKR-2B cells were transfected in duplicate with the deletion constructs shown in Figure 5. Cellular extracts were prepared after 24 h of additional growth in fresh medium containing 10% FCS and assayed for CAT activity. The β -actin promoter construct p99 β Ac was transfected into parallel cultures as a control.

small and variable degree of potentiation was observed in cells stimulated in the presence of CHX. However, a similar effect has been observed previously in control cultures transfected with pBLCAT2 (Subramaniam *et al.*, 1989) and is known to reflect a nonspecific stabilization of total cellular mRNA (Meister *et al.*, 1979). In contrast, the truncated promoter lacking sequences upstream of position -191 (pC3VSMP4) exhibited a high degree of serum inducibility (~ 10 -fold over basal levels) that was strongly potentiated by CHX. For simplicity, we now refer to this construct as the "unrestricted" VSM α -actin promoter. In addition, this unrestricted VSM α -actin promoter exhibited an approximately twofold increase in basal promoter activity relative to the less truncated constructs, a property also associated with functional serum response elements (Treisman, 1986). Notably, further deletion to position -150 resulted in the loss of inducibility, again indicating an essential stimulatory function for sequences between -150 and -191.

To more rigorously establish a governor role for the -224 to -150 sequences, cells were cotransfected with p99 β Ac-CAT and either PC3VSMP3, PC3VSMP4, or PC3VSMP5. After stimulation with serum and CHX, total RNA was isolated and used for primer extension analysis as previously described (Elder *et al.*, 1988). Correctly initiated transcripts from the β -actin promoter yield a 129 nucleotide long primer extension product (using a CAT sequence-specific primer), whereas correctly initiated transcripts from the VSM α -actin pro-

Figure 7. Serum and CHX-stimulated promoter activity of VSM α -actin deletion mutants. The seven deletion mutants of the VSM α -actin promoter shown in Figure 5, along with the β -actin promoter construct p99 β Ac, were transfected into AKR-2B cells that were subsequently treated as described in Figure 2. Cellular extracts were prepared and analyzed for CAT activity. (A) Quiescent cells. (B) Quiescent cells stimulated for 6 h with fresh medium containing 20% FCS. (C) Quiescent cells stimulated as in B in the presence of 10 μ g/ml CHX. The percentage conversion of chloramphenicol to its acetylated derivatives (% Con) was determined using an AMBIS Radioanalytic Imaging Scanner (Ambis Systems, San Diego, CA). The 1,3 diacetate derivatives are not shown in the figure but were included (when present) in the quantitative analysis.

moter yield a 168 nucleotide long primer extension product. As shown in Figure 8, transcripts from the β -actin promoter were readily detectable in cells cotransfected with either the VSMP3, P4, or P5 constructs, whereas VSM α -actin transcripts were only evident in cells cotransfected with the VSMP4 construct. These results reinforce the conclusions drawn from measurements of CAT enzymatic activity and further indicate that the dramatic differences observed between the VSMP3, P4, and P5 constructs cannot be accounted for by variations in transfection efficiency.

Individual VSM α -Actin CarG Boxes are Weak SREs

The ability of the unrestricted VSM α -actin promoter to direct serum and CHX-inducible expression strongly suggested that this promoter contained at least one functional SRE whose effect is normally suppressed by a linked negative control element. Obvious candidate SREs included VSM CarG boxes 1 and 2, both of which are highly conserved among vertebrate species (Min *et al.*, 1990). To compare the activity of these elements with the β -actin SRE within the context of a common promoter, synthetic copies were linked to the tk promoter in pBLCAT2. The VSM sequences also included

Figure 8. Primer extension analysis of RNA transcripts derived from cotransfected β -actin and VSM α -actin promoters. AKR-2B cells were cotransfected (7.5 μ g each) with CAT constructs driven by the indicated β -actin and truncated VSM α -actin promoters. Cells were treated with serum and CHX as described in Figure 2 and total RNA was isolated. Primer extension analysis was performed as described in MATERIALS AND METHODS using a primer complementary to nucleotides 29–61 of the CAT coding sequence. M, *Msp* I digest of pBR322 DNA; arrows denote the position of predicted primer extension products derived from the indicated promoter-specific transcripts.

8 bp on each side of the respective CarG box and were thus structurally analogous to the construct p26 β Actk-CAT (Figure 2). However, the data shown in Figure 9 demonstrated that their ability to confer serum and CHX-inducible expression on the tk promoter was greatly reduced relative to the β -actin SRE sequence. This experiment has been repeated numerous times and

Figure 9. Serum and CHX-dependent enhancer activity of VSM α -actin and β -actin CarG boxes. Synthetic copies of VSM α -actin CarG box 1 (p α 2SM1) or CarG box 2 (pC2 α 2SM2) along with 8 bp of their immediate flanking sequence were linked to the tk promoter in pBLCAT2. These were transfected into AKR-2B cells, and their ability to direct serum and CHX-dependent expression was determined as described in Figures 2 and 7. For comparative purposes, parallel cultures were transfected with the additional indicated constructs. Solid bars indicate CAT activity in quiescent cells, cross-hatched bars indicate CAT activity in 6-h serum-stimulated cells, whereas shaded bars indicate CAT activity in cells stimulated for 6 h with serum plus CHX.

indicates that the VSM CArG motifs and/or their immediate flanking sequence environment differ from the β -actin (and *c-fos*) SREs in some functionally important way. One obvious structural distinction is that both VSM α -actin CArG boxes differ from the canonical CC(A/T)₆GG sequence by virtue of a single G/C substitution within the central 6-bp spacer.

CArG Box 2 is an Essential Component of a Complex VSM α -Actin SRE and is Required for SRF Binding

The data of Figure 9 demonstrated that neither CArG box 1 or CArG box 2 was sufficient to confer serum and CHX-inducible transcription on a linked tk promoter. Moreover, a VSM α -actin promoter truncated upstream of position -150 (pC3VSMP5), but which retained both CArG boxes in their normal position, also lacked appreciable inducibility (Figures 7 and 8). However, neither experiment eliminated the possibility that these highly conserved motifs are an essential part of a more complex serum response element that includes additional sequences, such as those within the -150 to -191 region. To investigate this possibility, two site-directed mutants of a construct containing the -150 to -191 sequences (pC3VSMP4) were prepared in which either box 1 (pC3VSMP4Stu1) or box 2 (pC3VSMP4Stu2) was replaced by an unrelated 10-bp sequence incorporating a *Stu1* site. We demonstrated earlier (Figure 4) that an analogous replacement of the CArG box within the β -actin promoter abolishes the ability of this promoter to respond to both serum and CHX. Figure 10 illustrates that mutation of CArG box 2 had a similar effect on the unrestricted VSM α -actin promoter construct pC3VSMP4. However, mutation of CArG box 1 was far less deleterious, indicating that CArG box 2 is the more critical of the two elements and is indeed essential to serum and CHX-stimulated transcription. We hasten to point out, however, that additional data would be required to rule out the possibility of a modest synergistic effect resulting from the two elements acting in combination.

Mutations in the central CArG box core of the *c-fos* SRE have been shown to impair both serum-inducible enhancer activity and SRF binding (Greenberg *et al.*, 1987; Leung and Miyamoto, 1989). To determine whether a similar correlation exists for the β -actin and VSM α -actin promoters, we analyzed the ability of the wild-type promoters and their respective CArG box mutants to bind in vitro translated SRF. A coupled transcription/reticulocyte lysate translation system was programmed with a plasmid encoding full-length human SRF (Norman *et al.*, 1988). Aliquots were then mixed with the appropriate radiolabeled probes, and the resulting protein-DNA complexes were resolved by electrophoresis on low ionic strength polyacrylamide gels (Fried and Crothers, 1981).

The data presented in Figure 11 illustrate that both the β -actin promoter (p99 β Ac) and the truncated VSM

Figure 10. Effect of CArG box mutations on VSM α -actin promoter activity. PCR-mediated site-specific mutagenesis techniques (MATERIALS AND METHODS) were used to derive the indicated mutants of the VSM α -actin deletion construct pC3VSMP4. The solid boxes represent an alteration of the indicated CArG box, whereas the resulting sequence is shown above the box. These constructs, along with the indicated controls (pC3VSMP3, pC3VSMP4), were transfected into AKR-2B cells, and their ability to direct serum and CHX-inducible expression was determined as described in Figures 2 and 7. (A) Quiescent cells. (B) Cells stimulated for 6 h with fresh medium containing 20% FCS. (C) Quiescent cells stimulated as in B in the presence of 10 μ g/ml CHX.

α -actin promoter PC3VSMP4 yielded a prominent protein-DNA complex of low mobility that was not formed when the transcription/translation step was omitted (UL). Thus, these complexes most likely reflect the binding of in vitro translated SRF to sites in the actin promoter fragments. Interestingly, the SRF-specific complex formed using the β -actin promoter comigrated with an SRF-specific complex formed using a control oligonucleotide containing the *c-fos* SRE (left), whereas the VSMP4 complex migrated somewhat slower (right). Both SRF-specific complexes were abolished, however, when the CArG box mutants β Ac-*Stu* and VSMP4 *Stu*2 were used as probes. Although these results do not prove that SRF binding is required for serum and CHX-inducible promoter activity in transfected cells, they do show that CArG box mutations that eliminate such promoter activity also abolish the ability of the promoters to specifically bind SRF. Moreover, the greatly diminished ability of the VSMP4 *Stu*2 mutant to bind SRF, despite the presence of an intact CArG box 1, further suggests that CArG box 2 is the more important of the two elements.

DISCUSSION

The experiments presented in this paper demonstrate that deletion of VSM α -actin promoter sequences be-

Figure 11. Effect of CArG box mutations on SRF binding to β -actin and VSM α -actin promoters. Electrophoretic mobility shift assays were used to resolve protein-DNA complexes formed between the indicated DNA probes and SRF synthesized in a coupled transcription/reticulocyte lysate translation system (Promega TnT). Details are described in MATERIALS AND METHODS. FP, free probe; UL, unprogrammed lysate; SRF(2), lysate programmed with 2 μ g SRF plasmid; SRF(4) lysate programmed with 4 μ g SRF plasmid. Arrows indicate positions of SRF-specific complexes as judged by a comparison of complexes formed using programmed and unprogrammed lysates.

tween -191 and -224 permit the growth-dependent expression of this muscle-specific actin gene in fibroblasts. This was evidenced by the activation of promoter activity in rapidly dividing cells and by specific induction by serum factors and inhibitors of protein synthesis in G_0 -arrested cells. Within the limits of the assay system used, these properties are indistinguishable from those displayed by the cytoskeletal β - and γ -actin genes (Greenberg and Ziff, 1984; Elder *et al.*, 1984, 1988; Greenberg *et al.*, 1986). This conservation of regulatory properties is a novel finding that has interesting implications for both the evolution and for the developmental regulation of the actin multigene family in vertebrates.

Because invertebrate organisms such as *Drosophila* contain only cytoskeletal-type actins, the emergence of muscle-specific isoforms is presumed to have occurred by gene duplication followed by chromosomal dispersion and divergence (reviewed by Buckingham, 1985). Our data suggest that at least one muscle-specific gene, the VSM α -actin gene, retains molecular determinants that presumably coupled the expression of the ancestral cytoskeletal-type genes to cell proliferation. However, these determinants appear to be suppressed in proliferating fibroblasts by a negative element not found in the cytoskeletal genes. To accommodate these facts into a coherent model for developmental regulation, it seems necessary to postulate that at least some of the molecular determinants required for growth-dependent expression are also required for muscle-specific expression. Clearly, CArG box 2 is an attractive candidate for such a determinant given its high degree of evolutionary conservation. Alternatively, serum and CHX-dependent expression of the cytoskeletal β -actin and VSM α -actin genes may be mediated by completely independent mechanisms. We do not favor this hypothesis, however, because mutation of a similar sequence motif, the CArG box, eliminates serum and CHX-dependent transcription directed by both promoters and results in a similar loss of SRF binding capacity. Moreover, in a previous study

of the *c-fos* and cytoskeletal γ -actin SRE sequences, we found that mutations that eliminated CHX-inducibility were identical to those known to eliminate SRF binding and serum-inducibility (Subramaniam *et al.*, 1989). Based on these considerations, we favor a model in which the serum and CHX-inducible properties of the β -actin and VSM α -actin promoters require binding of SRF or an SRF-related protein.

Although the transcriptional activation of both the β -actin and VSM α -actin promoters in serum and CHX-stimulated fibroblasts may require the participation of SRF, the mechanism(s) by which SRF is recruited to the promoter and/or its subsequent interaction with other factors may well be promoter specific. In clear contrast to the β -actin SRE, neither of the tested VSM α -actin CArG elements were capable of conferring appreciable serum and CHX inducibility to a heterologous promoter or to their own promoter in the absence of additional upstream sequences (-150 to -191). Based on these findings, we suggest that under the conditions encountered in the living cell, the inherent affinity of SRF or SRF-related proteins for VSM CArG boxes 1 and 2 may be quite low. In the context of the unrestricted VSM α -actin promoter, however, SRF binding to CArG box 2 may be stimulated because CArG box 2 was shown to be absolutely required for both inducibility (Figure 10) and for the binding of *in vitro* synthesized SRF (Figure 11). We propose a model in which cooperative interactions between a -150 to -191 cognate binding protein(s) and SRF result in the occupation of CArG box 2 by SRF and the activation of growth-dependent expression in fibroblasts. Additional cooperative effects could also result in the further recruitment of SRF to the CArG box 1 site and could account, in principle, for the reduced mobility of the VSMP4-SRF complex relative to that formed with the *c-fos* SRE probe observed in Figure 11. However, gel shift experiments using the VSMP4 *Stu* I mutant are not consistent with this interpretation, suggesting that other mechanisms may

Figure 12. Model for growth-dependent expression of the VSM α -actin gene in fibroblasts. The essential features of this model are described in the text (see DISCUSSION). A and B represent two possible ways in which a negative factor could inhibit the transcriptional activating function of a closely associated positive factor. The solid arrows (C and D) depict a cooperative stimulation of SRF binding to CarG box 2, whereas the broken arrows represent additional cooperative effects not excluded by the data presented in the article. Binding of SRF may be precluded by mutation of CarG box 2 (E) or by deletion of the positive element upstream of -150 (F).

account for the observed mobility difference. Irrespective, cooperative stimulation of SRF binding to CarG box 2 could be impaired by a negative element that is closely associated with the -150 to -191 positive element. This could occur either by exclusion of a cognant binding protein(s) by a repressor or by direct protein-protein interaction. We have illustrated these possibilities in Figure 12 and are currently testing key aspects of this model.

The structural similarity between CarG boxes found in the promoters of muscle-specific actin genes and those of serum-responsive transcriptional enhancer elements, such as the *c-fos* SRE, have prompted several recent studies of their functional similarity. Conflicting results have been reported with some investigations, suggesting functional interchangeability (Taylor *et al.*, 1989; Tuil *et al.*, 1990), whereas others have indicated distinct differences (Walsh, 1989; Santoro and Walsh, 1991; Taylor, 1991). Although our own studies do not allow broad

generalizations, they clearly suggest that CarG elements derived from the promoter of the mouse VSM α -actin gene are not fully equivalent to the β -actin SRE. In particular, neither CarG box was capable of conferring appreciable serum- or CHX-inducible expression on a linked tk promoter. Rather, they required complementation by additional positive elements within the VSM α -actin promoter itself. Thus, in this particular case, the function of individual CarG boxes seems highly dependent on the sequence context of the specific promoter in which they are embedded. CarG boxes may represent a family of elements that can use combinatorial interactions between their cognant binding proteins and other promoter-specific accessory proteins to mediate widely diverse patterns of gene expression.

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