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Cross-Binding of Factors to Functionally Different Promoter Elements in c-*fos* and Skeletal Actin Genes

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A conserved 28-base-pair element in the skeletal actin promoter was sufficient to activate muscle-specific expression when placed upstream of a TATA element. This muscle regulatory element (MRE) is similar in structure to the serum response element (SRE), which is present in the promoters of the c-*fos* proto-oncogene and the nonmuscle actin genes. The SRE can function as a constitutive promoter element. Though the MRE and SRE differed in their tissue-specific expression properties, both elements bound to the same protein factors in vitro. These proteins are the serum response factor (SRF) and the muscle actin promoter factors 1 and 2 (MAPF1 and MAPF2). The SRF and MAPF proteins were resolved by chromatographic procedures, and they differed in their relative affinities for each element. The factors were further distinguished by their distinct, but overlapping, methylation interference footprint patterns on each element. These data indicate that the differences in tissue-specific expression may be due to a complex interaction of protein factors with these sequences.

To study aspects of cellular differentiation, the protein factors that bind to cell-type-specific promoter elements are being analyzed (2–5, 25, 44). The rationale for these experiments is that development is controlled, at least in part, by changes in gene transcription. To understand the processes that regulate transcription during muscle development, factors that interact with an essential element in the promoter of the chicken α-skeletal actin gene have been identified (51, 52a). This gene is activated when myoblasts withdraw from the cell cycle and fuse to form multinucleated myotubes in animal tissues, primary cultures, and some, but not all, myogenic cell lines (6, 19, 32, 39, 41, 52).

Muscle actin promoter factors 1 and 2 (MAPF1 and MAPF2) are the two major chicken skeletal actin promoter-binding activities in nuclear extracts (51, 52a). MAPF1 is the predominant binding activity in extracts from nonmyocyte cell lines and from embryonic chicken skeletal muscle cells. Myogenic cell lines, such as L6 and C2, and clonally derived cultures of human myocytes contain the factor MAPF2. MAPF1 and MAPF2 can be separated by a number of chromatographic procedures, but they produce the same methylation interference footprint between 78 and 83 nucleotides upstream from the start of transcription (Fig. 1). The molecular basis for the difference between the two muscle promoter factors is unknown; however, mixing experiments with different cell types suggest that one factor does not arise from the other during the preparation of nuclear extract (52a). Regulatory elements in other tissue-specific genes also bind to heterogeneous proteins that produce identical footprints (4, 44). In some cases, these proteins are encoded by different transcripts.

Base substitutions and internal deletions in the skeletal actin promoter show that the MAPF-binding site (52) and the TATA element (19) are essential for expression in myocytes. A single transversion mutation in the MAPF footprint can disrupt binding of these proteins in vitro and eliminate expression in muscle cells (without any observable activation of expression in nonmuscle cells). The MAPF footprint occurs within a dyad symmetry element that is conserved in the actin genes (22, 32, 33, 47). These sequences are found in muscle and nonmuscle genes, and they have been collectively referred to as "CArG boxes" (29). In the α-smooth actin promoter, this sequence motif has been implicated in the repression of expression (7), and in the human β-actin gene, this sequence is a factor-binding site that functions as an enhancer of transcription in NIH 3T3 cells (24). Recently, it was shown that the analogous sequence in the human cardiac actin promoter is also an essential factor-binding site; however, it is not known whether this sequence functions as a tissue-specific regulatory element or as a basal promoter element (20).

Here it is shown that the chicken skeletal α-actin dyad symmetry element that encompasses the MAPF-binding site is sufficient for muscle-specific expression when it is placed upstream of a TATA element. This muscle regulatory element (MRE) is similar in sequence to the serum response elements (SREs) identified in the promoters of the c-*fos* proto-oncogene and a nonmuscle actin gene (Fig. 1). The SRE mediates the rapid and transient induction of expression by growth factors in serum and other signals (12, 15, 42, 46, 48). Unlike the MRE, the SRE can function as a constitutive promoter element. The serum response factor (SRF) specifically binds to the c-*fos* SRE (16, 18, 34, 35, 38, 49, 50). Here data are presented that MAPF1 (and MAPF2) can also bind to the c-*fos* SRE but with a lower apparent affinity than to the skeletal actin MRE. Conversely, the SRF binds to the muscle element. These data indicate that the differences in tissue specificity of expression from the c-*fos* SRE and the skeletal actin MRE may result from a complex interaction of protein factors with these DNA elements.

MATERIALS AND METHODS

Cell culture and factor preparations. Primary cultures of breast skeletal muscle, skin fibroblasts, liver, and brain were prepared from 10- to 12-day-old chicken embryos (13, 19). Cells were plated 24 h before they were transfected. Primary cells were grown in minimal essential medium with 10%

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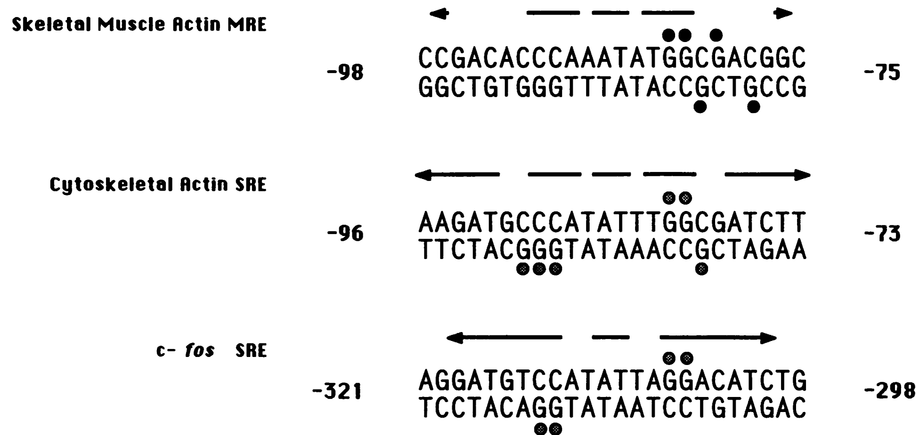


FIG. 1. Comparison of the sequences of the chicken skeletal actin MRE and the c-*fos* and *Xenopis laevis* (type 5) actin SREs. Dots above or below the corresponding nucleotides indicate the points of methylation interference for the MAPF proteins (solid) or the SRFs (stippled) on these sequences (16,30,38,49,51). MAPF1 and MAPF2 have the same methylation interference footprint on the skeletal actin MRE. The arrows indicate the symmetry of the elements.

horse serum–2 or 4% chicken embryo extract. BALB/c 3T3 fibroblasts were grown in Dulbecco modified Eagle medium (DME) with 10% calf serum or DME with 1.25% fetal bovine serum–3.75% calf serum. Nuclear extracts were prepared by the method of Dignam et al. (9).

Extracts for subsequent fractionation experiments were prepared from day 12 embryonic chicken breast muscle by mixing 1 part tissue with 1 part buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 10 mM KCl, 1.5 mM EDTA, and 10 μg/ml each of leupeptin, pepstatin, chymostatin, and soybean trypsin inhibitor). The tissue was homogenized by ultrasonic vibration, using the microtip of Branson cell disruptor (Sonifier 200) at setting 3 (six 30-s bursts). The particulate material was removed by centrifugation at 100,000 × *g* for 30 min. The supernatant fraction from the centrifugation was immediately loaded onto a heparin-Sepharose column that was equilibrated in 25 mM Tris (pH 7.5)–100 mM KCl–1 mM EDTA–10% glycerol–1 μg of leupeptin per ml–1 μg of pepstatin per ml. Proteins were eluted from the column with a steep, linear gradient to 1 M KCl. All procedures were performed at 4°C.

Affinity-column-purified fractions of the SRF were a gift from T. Hayes. BALB/c 3T3 nuclear extracts were precipitated with ammonium sulfate and suspended, and soluble proteins were chromatographed on a Sephacryl S300 column. Fractions with SRE-binding activity were pooled and chromatographed on a non-SRE DNA-affinity column. The flowthrough fractions were then chromatographed on an SRE DNA-affinity column (50). The SRF eluted between 1.0 and 1.2 M KCl.

Plasmid constructions. The construction of the plasmids 155SK CAT, 155SK *Mlu*I CAT, and 155SK T-83 CAT was described previously (52). The plasmid 155SK CAT contains the chicken skeletal actin promoter sequences from positions −12 to −155 fused, with a *Hin*dIII linker, to the chloramphenicol acetyltransferase (CAT) reporter gene that is upstream from simian virus 40 (SV40) sequences (17). The FOS-SK CAT element substitution and the transversion mutation at position −90 were prepared by removing the sequences between the *Apa*I and *Eag*I sites in 155SK CAT and replacing them with the appropriate sequences (Fig. 2). Cassettes with compatible restriction endonuclease ends for ligation were prepared on a Systec Microsyn-1450, purified by polyacrylamide gel electrophoresis, and annealed prior to use (1). DNA manipulations were done by the method of Maniatis et al. (26). The structures of FOS-SK CAT and the mutant promoters were confirmed by dideoxy-chain termination sequencing after they were subcloned into an M13 vector (36).

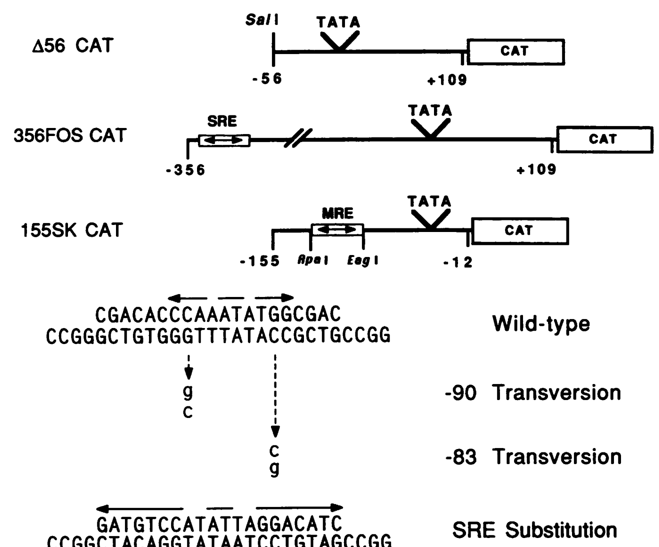


FIG. 2. Summary of plasmid constructions. (Top) DNA elements were inserted in the *Sal*I site of the test plasmid, Δ56 CAT, that has the mouse c-*fos* gene sequences between positions −56 and +109 upstream from the CAT reporter gene. The plasmid 356FOS CAT contains the c-*fos* sequences from positions −356 to +109 and includes the SRE. The plasmid 155SK CAT has the chicken skeletal α-actin sequences from positions −12 to −155 and includes the MRE. (Bottom) The sequences between positions −78 and −97 of 155SK CAT were removed with the restriction endonucleases *Apa*I and *Eag*I and replaced by the indicated fragments. The replacement sequences contain transversion mutations at positions −83 (155SK T-83) or −90 (155SK T-90) or a 20-nucleotide segment of the c-*fos* SRE (FOS-SK). The SRE substitution places the center of the symmetry of the element 1 nucleotide upstream from that of the MRE. The symmetry of the element is indicated by arrows above the sequence.

The plasmid MRE/Δ56 CAT was constructed by cloning the chicken skeletal α-actin sequences (positions −73 to −100) into the *Sal*I site of the test plasmid Δ56 CAT. This fragment was synthesized on an Applied Biosystems 380B DNA synthesizer. The sequences are: 5′-TCGAGCCCGA CACCCAAATATGGCGACGGCCG-3′ and 5′-TCGACGG CCGTCGCCATATTTGGGTGTCGGGC-3′. The structure of MRE/Δ56 CAT was confirmed by chemical cleavage sequencing (28). The plasmid Δ56 CAT contains the mouse c-*fos* gene sequences from positions −56 to +109, and the plasmid 356FOS CAT contains sequences from −356 to +109 upstream from the CAT reporter gene (Fig. 2 top) (16). SRE/Δ56 CAT contains the c-*fos* SRE from positions −320 to −298 cloned into the *Sal*I site of Δ56 CAT. The plasmids 356FOS CAT, Δ56 CAT, and SRE/Δ56 CAT were gifts from M. Z. Gilman.

The template for the synthesis of actin-CAT cRNA probes (pACAT.BS) was prepared by subcloning an *Eco*RI-*Sma*I fragment of the 155SK CAT plasmid into the Bluescribe M13+ vector (Stratagene). The subcloned fragment contains the skeletal actin promoter sequences and a portion of the CAT gene.

DNA-binding assay. Electrophoretic mobility shift assays were performed with 0.3 to 0.5 ng of ^{32}P-labeled promoter fragments (14). Fragments containing the chicken skeletal muscle regulatory element (positions −73 to −100) or the c-*fos* SRE (positions −298 to −320), cloned in the *Sal*I site of pBS M13+ (Vector Cloning Systems), were excised by digestions with *Eco*RI and *Hin*dIII. Other binding experiments utilized fragments prepared by digesting CAT plasmid 155SK, FOS-SK, 155SK T-83, or 155SK T-90 with *Bst*NI to produce 83-base-pair (bp) fragments that contain the region of interest. Probes were prepared by treating fragments with calf intestinal alkaline phosphatase followed by polynucleotide kinase and [γ-^{32}P]ATP (>6,000 Ci/mmol; New England BioLabs) (26). All probes were purified by polyacrylamide gel electrophoresis before use. Binding reaction mixtures contained approximately 5 μg of extract protein and 10 mM Tris (pH 7.5), 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 8% glycerol, and 1 μg of poly(dI-dC) · poly(dI-dC) in 10 μl. After a brief incubation at room temperature (approximately 10 min), binding mixes were loaded onto a 4% polyacrylamide gel and electrophoresis was performed at 10 V/cm in 0.25 × TBE buffer (26). Gels were dried and exposed to film at −70°C with an intensifying screen.

Methylation interference footprints were determined with end-labeled fragments that were partially methylated with dimethyl sulfate (43). Complexed and free DNAs were fractionated by polyacrylamide gel electrophoresis, excised, and eluted into 50 mM Tris (pH 7.5)–0.2 M KCl–1 mM EDTA. The eluted DNA was purified with Elutip-d columns (Schleicher & Schuell, Inc.), ethanol precipitated, and cleaved with 1.0 M piperidine for 30 min at 90°C. The cleaved DNA was loaded onto an 8% acrylamide–50% urea sequencing gel. After electrophoresis, the gels were exposed to film at −70°C with an intensifying screen.

Transfections and analysis of gene expression. Cells were transfected with 9 μg of test plasmid DNA–1 μg of the plasmid pXGH5 by the calcium phosphate method (53). The plasmid pXGH5 has the metallothionine promoter fused to the human growth hormone gene (40). Cells were harvested 2 to 3 days after transfection, and protein or RNA extracts were prepared. To account for differences in transfection efficiencies, the level of human growth hormone in the medium was determined by radioimmunoassay (Nichols Institute Diagnostics). CAT activities were determined by the method of Gorman et al. (17). Alternatively, RNA was analyzed by ribonuclease protection (15), using a cRNA probe prepared from a subclone of 155SK CAT. The radiolabeled cRNA probes were prepared by using *Sma*I-cut pACAT.BS and T7 polymerase. All cRNA probes were purified by polyacrylamide gel electrophoresis before use. The probe for the muscle promoter constructs extends from the *Eco*RI site in the CAT gene to position −155 in the promoter of 155SK CAT and is approximately 390 nucleotides in length.

For tests of serum-inducible expression, BALB/c 3T3 cells were cotransfected with 9 μg of test plasmid and 1 μg of the plasmid πSVHPα2, which contains human α globin under the control of the SV40 promoter. At 15 h after transfection, the medium containing the precipitated DNA was replaced with fresh DME medium–0.5% calf serum. At 2 days later, the medium was changed to fresh DME (minus serum) or DME with 10% calf serum (plus serum). RNA was rapidly prepared 40 min after the addition of the fresh medium. For each condition, 5 μg of RNA was analyzed by ribonuclease protection with cRNA probes specific for the actin-CAT transcripts (155SK and FOS-SK), for the endogenous and transfected c-*fos* transcripts, and for α globin. Details on some of these probes and plasmids were previously published (15).

RESULTS

Identification of an MRE. The skeletal actin promoter contains a dyad symmetry element that specifically binds to MAPF1 and MAPF2. The dyad symmetry element was tested for its ability to activate the expression of a test promoter. The chicken skeletal actin sequence (from positions −73 to −100) was inserted upstream of a c-*fos* promoter construction that is truncated at position −56. This test promoter supplies a TATA element, and it is fused to the CAT reporter gene (Fig. 2 top) (16). The test promoter with no inserted sequences (Δ56 CAT) produced relatively low levels of CAT activity when it was transfected into cultures of embryonic chicken breast muscle or nonmuscle cells (Table 1). When the 28-bp muscle actin element was inserted into the *Sal*I site (MRE/Δ56 CAT), CAT expression was elevated 11-fold in muscle cultures but not in nonmuscle cultures. The 28-bp element activated muscle-specific expression in an orientation-independent manner. Separate experiments showed that expression from MRE/Δ56 CAT is within a factor of four of the expression from a larger skeletal actin promoter construct that contains 2 kilobase pairs of upstream sequences. These results indicate that the dyad symmetry element is an important component of the promoter. This 28-bp MRE is the smallest sequence identified to contain information that is sufficient for muscle-specific expression. Though the MRE is only active in muscle, nuclear extracts from muscle and nonmuscle cell types contain sequence-specific MRE-binding activities (51, 52a).

The tissue specificity of c-*fos* SRE expression was also tested in the Δ56 CAT construct. The SRE activated expression of the test promoter in primary cultures of muscle and nonmuscle cells (Table 1). Expression was activated 15-fold in myocytes, 16-fold in hepatocytes, and 8-fold in fibroblasts. These results are in agreement with reports that the SRE, from the c-*fos* or *Xenopus* cytoskeletal actin genes, will activate basal expression in fibroblasts (30, 50). The expression from a larger c-*fos* promoter construct, 356FOS CAT, was also not tissue restricted (Table 2). This larger construct contains mouse c-*fos* sequences from positions

TABLE 1. Tissue-specific expression properties of the MRE and the SRE

Construct[a]	Relative CAT activity in[b]:			
	Muscle	Liver	Fibroblast	Brain
Δ56 CAT	1.0	1.0	1.0	1.0
MRE/Δ56 CAT	10.8	0.4	1.7	0.9
Invert.MRE/Δ56 CAT	14.0	2.0*		0.3*
SRE/Δ56 CAT	15.4	16.2	7.9	

[a] Embryonic chicken cells were cultured and transfected with the test plasmid (Δ56 CAT) with no inserted sequences, the c-*fos* SRE (positions −298 to −320) (SRE/Δ56 CAT), or the MRE (positions −73 to −100) in the wild-type orientation (MRE/Δ56 CAT) or in the reverse orientation (Invert. MRE/Δ56CAT). DNA elements were inserted in the *Sal*I site of Δ56 CAT. Test plasmids were cotransfected with the internal control plasmid, pXGH5, which has the human growth hormone gene under the control of the metallothionine promoter (40).

[b] With each cell type, CAT activity levels are expressed relative to the activity found with Δ56 CAT which is assigned a value of 1.0. The quantity of extract used in the CAT assays was adjusted so that the level of human growth hormone was constant for a particular cell type. Unless indicated by the asterisk, the values reported are the averages of two or more individual determinations.

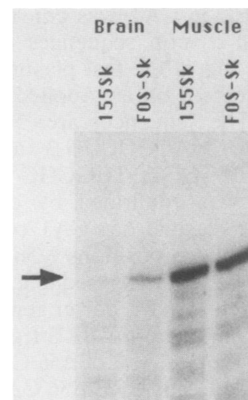


FIG. 3. Ribonuclease protection analysis of transcripts from the wild-type skeletal actin promoter and the chimera. Three plates of primary muscle or brain cultures were transfected with 155SK CAT or with FOS-SK CAT. At 2 days after transfection, RNA was prepared, pooled, and analyzed. The full-length probe was 390 nucleotides, and the protected fragments (arrow) are approximately 240 nucleotides.

−356 to +109 and includes the SRE. These experiments demonstrate that the c-*fos* SRE can function as a constitutive promoter element, but the skeletal actin MRE only activates expression in muscle cultures.

To extend these observations, the c-*fos* SRE was placed in the context of flanking muscle sequences to test whether this would alter the expression properties of the muscle promoter. A chimeric promoter of 144 nucleotides was constructed in which 20 nucleotides of the MRE (positions −78 to −97) were replaced by 20 nucleotides of the c-*fos* SRE (Fig. 2 bottom). This chimera is referred to as FOS-SK. Primary cultures were transfected with CAT gene constructs under the control of the nonsubstituted skeletal actin promoter (155SK) or the chimeric promoter (FOS-SK). In skeletal muscle cultures, high levels of CAT expression were found with both promoters (Table 2). This level of CAT expression is three- to fourfold greater than that obtained from the SV40 early-region transcription unit (pSV2CAT) (17). Ribonuclease protection assays of the RNA isolated from breast muscle cultures showed that the 155SK and FOS-SK promoters also utilize the same start of transcription (Fig. 3). This site maps to a position that is similar, if not identical, to the start site that was determined for a similar chicken skeletal actin-CAT construct (19). Mutated muscle promoters (155SK T-83 and 155SK T-90) were also tested as controls to show that the substituted muscle sequence is important for expression (Fig. 2). Both point mutations reduced expression in skeletal muscle cultures by a factor of 30 or more (Table 2). Collectively, these experiments demonstrate that the MRE is an essential element in the skeletal actin promoter, but it can be replaced by the c-*fos* SRE to produce a functional heterologous promoter.

In nonmuscle cultures, the expression from the chimeric promoter was considerably higher than that from the nonsubstituted promoter (Table 2). In cultures of embryonic brain, the FOS-SK construct produced fourfold more CAT activity than did 155SK (Table 2). In these cultures, the chimera was almost as active as the SV40 enhancer/promoter. RNase protection analysis showed that the start of transcription for the FOS-SK promoter in brain is the same as that in muscle (Fig. 3). Similar results were also obtained with transfected cultures of embryonic liver and fibroblasts (Table 2). These experiments showed that the replacement of the MRE by the SRE will relax the tissue-restricted expression of the muscle promoter and indicated further that these elements are functionally different.

TABLE 2. Tissue-specific promoter expression[a]

Promoter	Relative CAT activity in[b]:			
	Muscle	Brain	Fibroblast	Liver
SV40	100	100	100	100
356FOS	79		93	53*
FOS-SK	360	91	93	80
155SK	310	23	23	21
155SK T-83	11			
155SK T-90	6			
None	<1	6	8	4

[a] Embryonic chicken cultures were cotransfected with plasmids containing the CAT reporter gene downstream from a test promoter (or no promoter) and the internal control plasmid pXGH5.

[b] The amount of extract used in the CAT assays was adjusted so the level of growth hormone was constant for a particular cell type. In each set of cultures, CAT activities are expressed relative to the activity of the SV40 promoter (pSV2CAT), which is assigned the value of 100. These data are representative of multiple transfection experiments and, unless specified by the asterisk, are the averages of two or more separate determinations.

Skeletal actin promoter can be induced by serum. The ability of the SRE to mediate inducible expression in response to serum and other agents has been extensively characterized (12, 15, 42, 46, 48). The skeletal muscle actin and c-*fos* promoters were examined for their abilities to be induced by serum to determine whether they differed with respect to this property. Mouse fibroblasts were transfected and deprived of serum for 48 h. RNA was rapidly isolated from parallel cultures 40 min after the addition of serum-free medium or medium containing 10% serum. The RNA was analyzed by ribonuclease protection analysis (Fig. 4). The transfected (356FOS CAT) and endogenous c-*fos* genes, the muscle promoter (155SK CAT), and the chimeric promoter (FOS-SK CAT) constructs are all induced by serum. The 5′ ends of the mRNA for the FOS-SK CAT and 155SK CAT constructs map to the same site that was found in muscle cultures. The inducible expression from the transfected muscle actin promoter construct is dependent upon a functional MRE because a hexanucleotide substitution, from

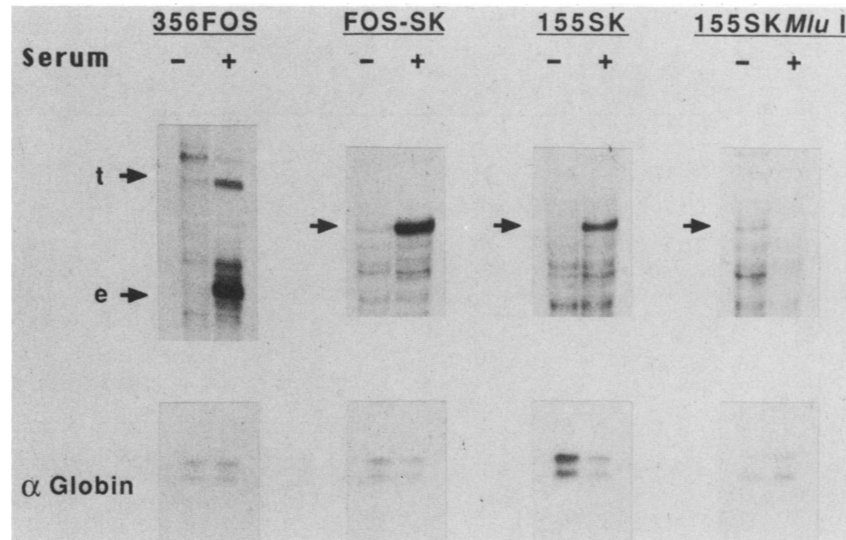


FIG. 4. Serum-inducible expression of the c-*fos* and skeletal actin promoters. BALB/c 3T3 fibroblasts were cotransfected with the test plasmids and the internal control plasmid (πSVHPα2) that encodes the human α-globin gene. The plasmid 155SK *Mlu*I has a hexanucleotide substitution mutation in the MRE. After 2 days of serum deprivation, parallel cultures were treated with medium plus 10% calf serum (+) or with serum-free medium (−). RNA was rapidly harvested 40 minutes after the medium change and was analyzed by ribonuclease protection. The protected cRNA fragments are 240 nucleotides for the actin-CAT constructs, 128 nucleotides for the transfected c-*fos* gene (t), 110 nucleotides for the endogenous c-*fos* gene (e), and 95 nucleotides for the upper α-globin band.

positions −78 to −83 (155SK*Mlu*I CAT), blocks the serum effect. This hexanucleotide substitution also decreases expression in transfected muscle cultures by a factor of 50 (52).

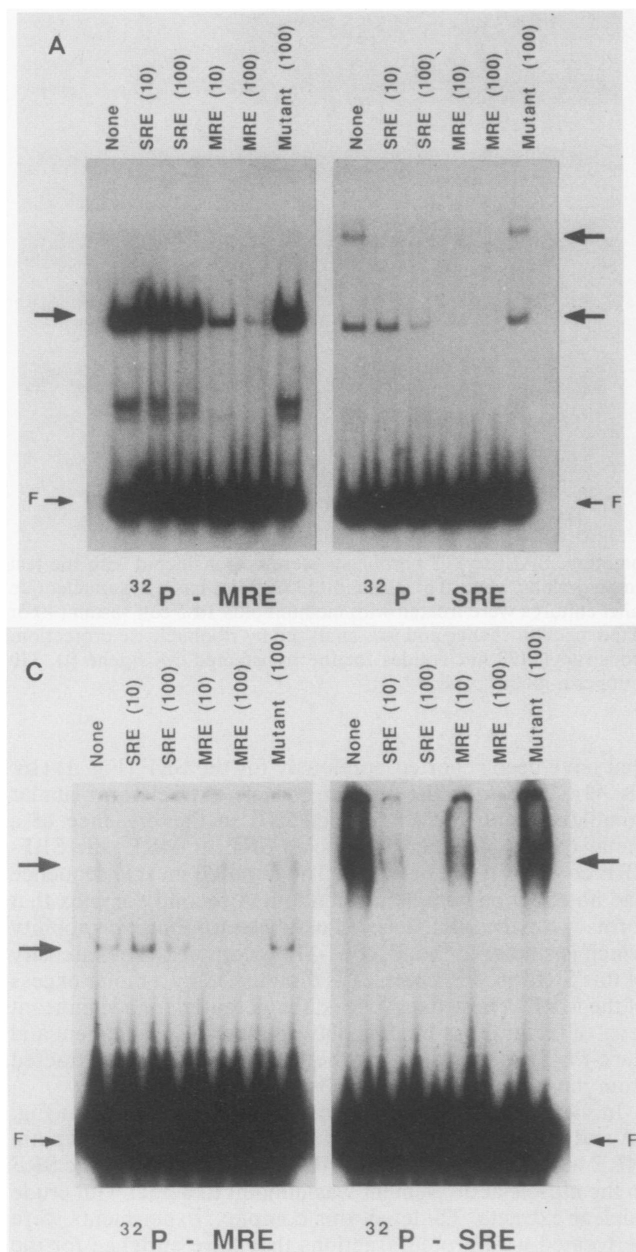
Factor cross-binding between the MRE and the SRE. Figure 1 compares the sequences of the SREs from the c-*fos* and *Xenopus* cytoskeletal actin genes to the analogous sequence in the chicken muscle actin promoter. On the basis of binding competition experiments, it was shown that the mammalian SRF binds both of the nonmuscle elements (30). The methylation interference footprints of the SRF on both SREs are similar (Fig. 1). Also indicated is the methylation interference footprint of MAPF1 and MAPF2 on the skeletal actin MRE. The MAPF footprint differs from that of the SRF in that the interference pattern is only seen on one side of the palindrome and that it extends further down that arm.

To investigate similarities between the SRE and the MRE, the protein factors that interact with these sequences were compared. The protein-binding properties of the c-*fos* SRE and the skeletal actin MRE were tested with an embryonic chicken pectoralis muscle extract, a nonmuscle (T-lymphocyte) nuclear extract, and an affinity column-purified fraction of the SRF (Fig. 5). When a radiolabeled probe of the skeletal actin MRE is used for binding, MAPF1 forms the predominant complex with factors in T lymphocytes or chicken breast muscle extracts (Fig. 5A and B). In the presence of a molar excess of nonlabeled muscle actin sequence, the intensity of the MAPF1-DNA complex is diminished. This competition is sequence-specific because a single transversion mutation in the muscle sequence, corresponding to position −83 (see Fig. 2 bottom), eliminates the competition. Under these conditions, the c-*fos* SRE is a poor competitor.

The binding of factors to the c-*fos* SRE was tested with a human T-lymphocyte extract (Fig. 5A). SRF binding gives rise to the complex with the slowest electrophoretic mobility. The methylation interference footprint of this complex (not shown) revealed the same protein-DNA interactions that have been reported previously for the SRF (Fig. 1) (16, 38, 49). Factors in the chicken muscle extract form similar complexes with the SRE (Fig. 5B). In the presence of a molar excess of either nonlabeled SRE or MRE, the SRF-SRE complex is not detected. The mutated muscle sequence had no effect on complex formation. A second complex that forms with the SRE has a faster electrophoretic mobility which is similar to the MAPF1-MRE complex. The intensity of this SRE-protein complex is diminished by a molar excess of the MRE. These data suggest the potential for a significant level of factor cross-binding between the muscle element and the c-*fos* SRE regardless of whether the factors are extracted from muscle or nonmuscle cells.

In view of the competition experiments presented in Fig. 5A and B, the possibility of SRF binding to the skeletal actin MRE was examined in greater detail. The binding of the SRF to the muscle actin element was difficult to detect with crude nuclear extracts. To detect this complex, experiments were performed with protein fractions that were enriched for the SRF by chromatography on an affinity resin containing the c-*fos* SRE. As with the crude extracts, a molar excess of nonlabeled SRE or MRE blocked complex formation between the SRF in this fraction and the ^{32}P-SRE. (Fig. 5C). The affinity column-purified SRF-DNA complex formed a diffuse band on the autoradiogram. The molecular basis for the diffuse band is unknown, but this behavior was found with all affinity column-purified fractions of the SRF. Methylation interference analysis of this SRE complex (not shown) revealed the same footprint pattern that has been reported for the SRF.

When this enriched SRF fraction was used for binding to a radiolabeled fragment of the skeletal actin MRE, a low-intensity band with a similar electrophoretic mobility as that of the SRF-SRE complex was observed (Fig. 5C). This MRE-protein complex is not detected when a molar excess of nonlabeled MRE or SRE is included in the binding reaction. These data are consistent with the hypothesis that the SRF can bind to the muscle actin sequence, but this



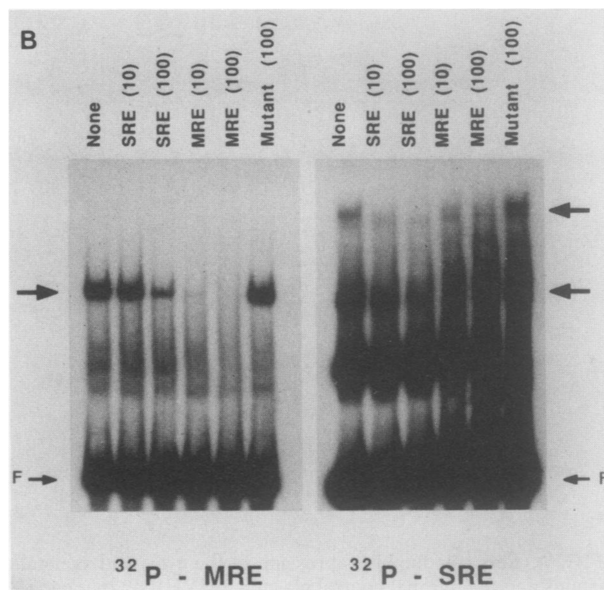


FIG. 5. Competition for complex formation between nuclear proteins and the c-*fos* SRE or the skeletal actin MRE. Binding was examined with extracts that were prepared from H9 T lymphocytes (A), chicken embryo pectoralis muscle (B), or an affinity column-purified fraction of the SRF (C). Binding was tested with ^{32}P-labeled probes of the c-*fos* SRE (positions −298 to −320) or the chicken skeletal actin MRE (positions −73 to −100). Complexes were competed by the addition of 10 or 100 ng of the nonlabeled oligonucleotide duplex of the c-*fos* SRE, the skeletal actin MRE, or the MRE with a transversion mutation at the position that corresponds to −83 (Mutant). Approximately 5,000 cpm of probe was used in each binding reaction. The autoradiograms in panels A, B, and C were exposed for different lengths of time. The arrows indicate the positions of the free fragment (F) and the putative MAPF1-DNA complexes (lower arrows) and SRF-DNA complexes (upper arrows).

interaction is weaker than SRF binding to the SRE. A complex with a fast electrophoretic mobility was also detected when the affinity-purified SRF fraction was used with the radiolabeled muscle actin sequence (Fig. 5C). The competition of this complex by DNA fragments is similar to what is seen with the MAPF1-DNA complex. Thus, it appears that the affinity column-purified material also contains a small quantity of MAPF1. The level of this putative MAPF1 activity varied among different SRF preparations.

To investigate the identities of these DNA-binding activities further, heparin-Sepharose column profiles of embryonic muscle extracts were analyzed (Fig. 6). The column fractions were assayed for skeletal actin MRE and the c-*fos* SRE-binding activities. The SRF eluted later than MAPF1 in the salt gradient, and fractions were obtained that are highly enriched for one binding activity or the other. When the probe was the skeletal actin MRE, a second peak of binding activity was detected that has a slow electrophoretic mobility and has the same elution profile as the SRF. This MRE-binding activity was not detected in muscle extracts before chromatography (Fig. 5B). In addition, a second peak of SRE-binding activity cochromatographed with MAPF1. These SRE- and MRE-binding activities also cochromatographed on mono Q resin, and the same behavior was observed with heparin-Sepharose-fractionated HeLa or T-lymphocyte nuclear extracts (not shown). Collectively, the cochromatography and sequence-specific competition data indicate that MAPF1 binds the c-*fos* SRE and that the SRF binds the skeletal actin MRE. The MAPF1-SRE complex was detected in crude extracts, but the SRF-MRE complex was only seen with fractions that were enriched for SRF by heparin-Sepharose or DNA-affinity chromatography.

The relative intensities of the autoradiogram bands suggested that MAPF1 preferentially binds to the skeletal actin MRE and that the SRF preferentially binds to the c-*fos* SRE. Quantitative competition experiments with nonlabeled oligonucleotide duplexes showed that the MRE is a 20-fold-better competitor than the SRE for the MAPF1-DNA complex. Conversely, the c-*fos* SRE is a threefold-better competitor than the MRE for the SRF-DNA complex. The apparent affinities of these factors for the DNA elements depends on the conditions of the electrophoretic mobility shift assay and on the factor levels in the nuclear extract. MAPF1 binding to

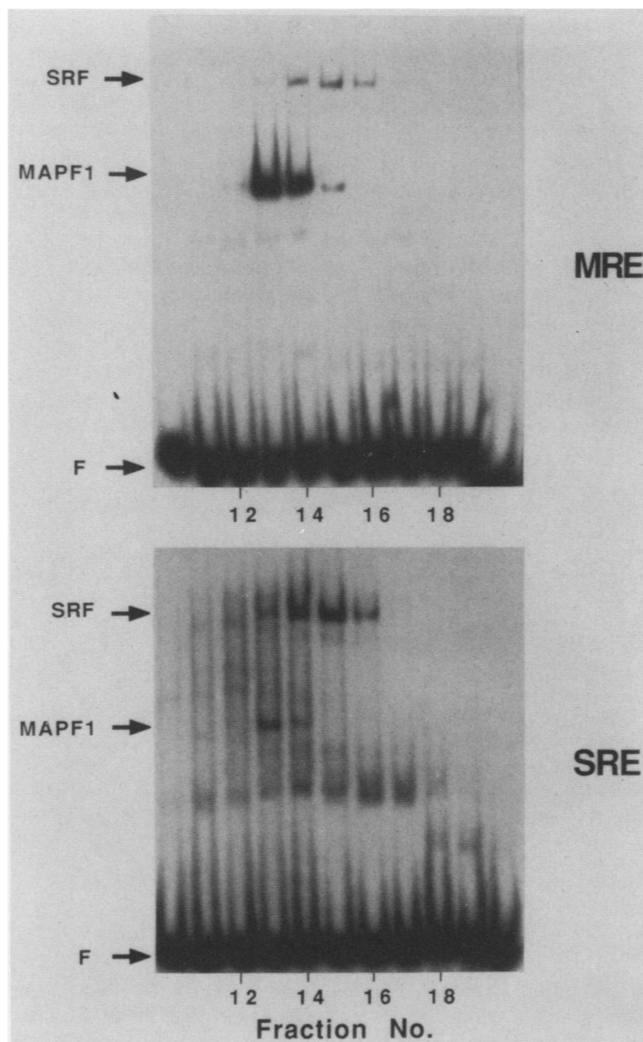


FIG. 6. Heparin-Sepharose profile of c-*fos* ^{32}P-labeled SRE- and skeletal actin ^{32}P-labeled MRE-binding activities in extracts of embryonic chicken pectoralis muscle. Proteins were eluted with a linear gradient from 100 mM to 1 M KCl. MAPF1 began to elute at 420 mM KCl, and the SRF began to elute at 480 mM KCl. The arrows indicate the positions of the MAPF1-DNA and SRF-DNA complexes. F, Free fragment.

either element was favored by a Tris-borate-EDTA gel buffer, while the SRF interaction was favored by a Tris-glycine-EDTA gel buffer. Furthermore, the MAPF1-DNA interaction was diminished relative to the SRF-DNA interaction by higher salt concentrations (100 mM versus 30 mM KCl) or by guanidine hydrochloride (at 600 mM) in the binding mixes. These experiments indicate that direct correlations between factor binding in vitro and in vivo may be inappropriate due to uncertainties about the conditions in the nucleus.

MAPF2, the muscle actin promoter-binding activity found in nuclear extracts from the L6 and C2 myogenic cell lines and in clonally derived human myocytes, can be resolved from MAPF1 and the SRF by chromatography (51, 52a). The MAPF2-DNA complex can be distinguished further by its electrophoretic mobility (which is greater than that of MAPF1 or the SRF). The specificity of MAPF2 binding appears to be identical to that of MAPF1 (51, 52, 52a). Although MAPF2 can also bind to the c-*fos* SRE, the experiments presented in this report focus on MAPF1 for reasons of convenience. However, critical observations made with MAPF1 have also been confirmed with MAPF2.

SRF and MAPF1 have overlapping footprints. Methylation interference analysis was performed on the MAPF1-SRE and on the SRF-MRE complexes to examine these interactions in greater detail. MAPF1 makes contacts with the major groove on the upstream portion of the c-*fos* SRE at positions −313, −314, and −316. The binding is slightly diminished by methylation at position −319 (Fig. 7A). The same footprint was obtained with factors extracted from HeLa or T cells. The MAPF2-SRE complex obtained with rat L6 myocyte extracts has the same methylation interference footprint as the MAPF1-SRE complex (not shown). As with the skeletal actin MRE, the methylation interference footprint of the MAPF proteins occurs on one side of the interrupted inverted repeat. However, the MAPF footprint is on the 3′ half of the muscle actin dyad (Fig. 1) (51) and on the 5′ half of the c-*fos* dyad (Fig. 7A).

The methylation interference footprint of the SRF on the muscle promoter sequence is shown in Fig. 7B. The N-7 methylation of guanine residues at position −91, −90, −83, or −82 interferes with SRF binding to the muscle promoter. The methylation of position −81 weakly interferes with binding. This pattern of interference is similar to the footprint of the SRF on the *Xenopus* cytoskeletal actin promoter but differs from the MAPF footprint on the muscle element. The MAPF1 and SRF footprints overlap at positions −83, −82, and −81. Thus, the SRF and the MAPF proteins have distinct but overlapping methylation interference footprints on the skeletal actin MRE and on the c-*fos* SRE.

SRF contacts in the MRE are important for expression. Mutations were introduced into the skeletal actin MRE to test whether the integrity of the factor-binding site is important for expression in myocytes. These binding assays used heparin-Sepharose fractions of embryonic muscle extracts that were enriched for SRF or MAPF1. To test the importance of the upstream sequences, a transversion mutation was introduced at position −90 (Fig. 2 bottom). This mutation blocks detectable SRF binding, but has little or no effect on MAPF1 binding (Fig. 8), and decreases expression in myocyte cultures by a factor of 50 (Table 2). The G-C base pair at −83 is at the position that is symmetrical to −90 in the palindrome. A transversion mutation at position −83 decreases the muscle-specific expression by a factor of 30 (Table 2). This mutation blocks detectable binding of the SRF and MAPF1 in vitro (Fig. 8). (The identity of the lower complex obtained with the SRF-enriched fraction is not known; however, it is probably not MAPF1 because binding occurs with the −83 mutation.) These experiments demonstrate that the integrity of the SRF/MAPF-binding site is essential for muscle-specific expression. Furthermore, these data show that a sole MAPF1 in vitro binding site is not sufficient for expression in muscle and indicate that the SRF may be involved in the activation of the muscle gene. Also shown in Fig. 8 is the binding of the SRF and MAPF1 to a segment of the FOS-SK promoter. This chimera was made by replacing a segment of the MRE with the SRE (Fig. 2 bottom). As expected, this element substitution results in an enhancement of the SRF interaction, while MAPF1 binding is diminished.

DISCUSSION

The data presented in this report show that the skeletal actin MRE and the c-*fos* SRE differ in their tissue-specific

FIG. 7. Methylation interference footprints of (A) MAPF1 on the c-*fos* SRE and of (B) the SRF on the skeletal actin MRE. End-labeled probes were partially methylated with dimethyl sulfate and then were incubated with T-cell nuclear extracts (for MAPF1) or with the affinity column-purified fraction of the SRF. After electrophoresis, the protein-DNA complexes (lane C) and the free fragments (lane F) were excised, purified, and cleaved with piperidine. The unused methylated probe was also cleaved with piperidine to provide a G ladder. The positions of binding interference are indicated with arrows. The methylation interference footprints of the complexes are summarized below the panels. The dots indicate the guanidine residues whose methylation completely or partially interferes with factor binding.

expression properties. The MRE is only active in transfected muscle cultures, but the SRE will activate expression in many cell types. Despite this difference in function, both elements interact with the same protein factors in vitro. Here it is shown that the SRF can bind to the skeletal actin regulatory element in addition to the c-*fos* SRE. Furthermore, MAPF1 and MAPF2 have been identified as new c-*fos* SRE-binding activities. The interaction of the MAPF proteins with the SRE can be detected with crude nuclear extracts and with purified fractions. The SRF-MRE complex is difficult to detect in crude extracts, but binding is apparent when purified fractions are used.

The SRF has a number of properties that allow it to be distinguished from the MAPF proteins. First, the MAPF1 (and MAPF2) complex has a faster electrophoretic mobility than the SRF complex. Second, these factors can be separated by chromatographic procedures. Third, the factors differ in their relative affinities for the SRE and the MRE. On the basis of the competition data and of the intensities of the autoradiograms of the DNA-protein complexes, the SRF has a higher affinity in vitro for the c-*fos* element than for the skeletal actin MRE. Conversely, MAPF1 has a higher affinity for the muscle actin element. Finally, the SRF and MAPF1 complexes have distinct but overlapping methylation interference footprints on both elements. On the basis of methylation interference footprint data, it appears that the SRF also binds to similar elements in the human skeletal and cardiac actin promoters (20, 31).

The binding of multiple nuclear factors with overlapping sites has been reported for other promoter elements (3). For example, the factors NF-κB and H2TF1 interact with sequences in the promoter of a major histocompatibility gene and the κ immunoglobulin gene enhancer (2). NF-κB contacts a subset of the sequences contacted by H2TF1, and the factors differ in their affinities for each element. The CTF transcription factors appear to be a family of proteins that bind differentially to the CCAAT elements of a number of genes (8, 37). These factors share characteristics with the SRF and MAPF proteins in that they have different methylation footprints and vary in their affinities for DNA elements that are similar in sequence. In addition, there are reports of proteins that bind DNA in a cooperative manner though they

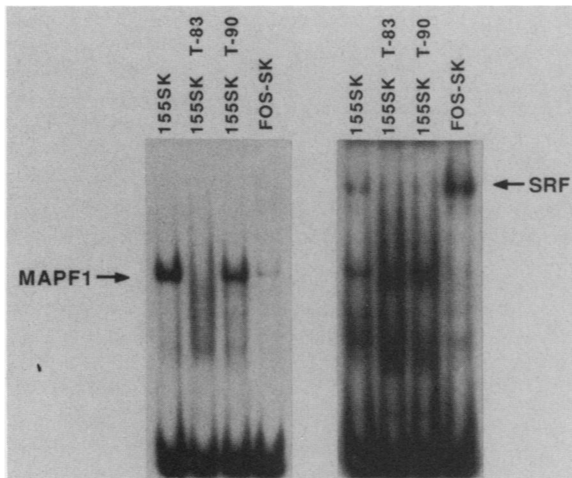


FIG. 8. Effects of mutations and substitutions on MAPF1 and SRF binding to the skeletal actin promoter. Segments of the wild-type skeletal actin promoter (155SK), the chimeric promoter (FOS-SK), and the skeletal actin promoter with transversion mutations at positions −90 (155SK T-90) or −83 (155SK T-83) were tested for binding to the SRF or MAPF1. The promoter fragments used for probes were obtained by *Bst*NI digestions. The same amount of radiolabeled probe was used in each reaction experiment. Binding was tested with fractions that were enriched for MAPF1 (left panel) or SRF (right panel) by heparin-Sepharose chromatography of embryonic chicken pectoralis muscle extracts.

individually have overlapping footprints. This behavior has been found with the *Escherichia coli lac* promoter in which the binding of the CAP protein enhances the binding of RNA polymerase even though the individual proteins have overlapping binding sites (14). The element that controls the cell-type-specific switching genes in yeasts is also similar in this regard. In this system, a cell-type-specific repressor interacts cooperatively with a ubiquitous protein (GRM/PRTF) in binding to an operator sequence (5, 25). These observations may be particularly relevant to the skeletal actin MRE system because this sequence is similar to that of the yeast element, and a yeast factor that is identical or closely related to the factor GRM/PRTF can bind to the human c-*fos* SRE (21).

The chicken skeletal actin gene is activated upon myocyte differentiation, and it is inactive in nonmuscle cell types (19, 32, 52). These regulatory properties are also displayed by a 28-bp element, which encompasses the SRF/MAPF binding site, when it is placed upstream of a nonmuscle sequence that supplies a TATA element (MRE/Δ56 CAT in Table 1). Though a number of muscle promoters have been characterized, this element is the smallest sequence identified to contain sufficient information for muscle-specific expression. Other *cis*-acting regions that have been shown to confer muscle-specific expression in heterologous promoter constructs include the creatine kinase (M) gene enhancer (257 to 352 bp) (23, 45), the myosin light chain 1/3 gene enhancer (0.9 kilobase-pair) (10), and a 67-bp segment of the cardiac troponin T promoter (27). The troponin gene segment contains a sequence motif that is present in other muscle genes but appears to be different from the skeletal actin MRE.

In contrast to the skeletal actin MRE, the c-*fos* SRE activates expression in muscle and nonmuscle cells. Similarly, constitutive basal expression is also obtained from a larger c-*fos* promoter construct (positions −356 to +109). A feature that contributes to differences in *cis*-element properties is the context of the element relative to other regulatory sequences. The spatial arrangement of DNA elements can lead to differences in protein factor interactions on the promoter and this, in turn, results in differences in regulation. To test the effect of flanking muscle actin promoter sequences on expression, a chimeric promoter was constructed in which the MRE was replaced by the c-*fos* SRE. The chimeric promoter (FOS-SK) was as active as the nonsubstituted skeletal actin promoter in transfected muscle cultures; however, the chimera was more active in nonmuscle cultures (Table 2). These data indicate further that the expression from the c-*fos* SRE is not tissue restricted and that the MRE regulates, at least in part, the tissue-specific expression of the muscle promoter.

The SRE is required for the induction of c-*fos* expression by factors in serum (16, 18, 34, 48). Here it is shown that the chicken skeletal actin promoter is similar to the c-*fos* promoter in that it can also be induced by serum (Fig. 4). Furthermore, this induction is prevented by a mutation in the MRE. It has been reported that the endogenous muscle actin gene is not induced by a serum growth factor (11). The discrepancy between this previous report and these data may result from differences in expression that result from the episomal rather than chromosomal location of the muscle gene, from species differences, or because a sensitive RNase protection assay was employed in these experiments to measure transcript levels. The experiments presented here show that the c-*fos* and chicken skeletal actin promoters differ considerably in the tissue specificity of expression but apparently not in their induction by serum growth factors.

The mutation of the factor-binding site in the skeletal actin promoter indicates that the integrity of this sequence is critical for the activation of expression in myocytes. Particularly striking is the finding that a mutation that disrupts SRF binding in vitro, while leaving the MAPF-binding site intact, is sufficient to block expression. This implicates the SRF in the activation of muscle gene expression and complicates models that account for the differences in tissue-specific expression between the SRE and the MRE. One hypothesis is that both the factors are capable of co-occupying the same DNA element, but differences in the relative affinities of SRF and MAPF may control the tissue specificity of expression. An alternative hypothesis is that the regulation is achieved by a mechanism that involves a competition between these proteins for the common binding sites. Finally, it is possible that other, yet unidentified proteins, may be required for regulation by interacting with these elements or by controlling the accessibility of SRF and MAPF to the DNA. Work is currently in progress to test these models by characterizing the expression properties of mutant DNA elements that differ in their affinities for these proteins.

The data presented in this report reveal that the SRF and MAPF proteins have overlapping specificities for DNA elements which have different functions. It is possible that the protein factors that bind these sequences are closely related. For example, the genes that encode the SRF and MAPF factors may have evolved from a common ancestral gene. Alternatively, these factors may be encoded by the same gene, but posttranscriptional modifications give rise to proteins with different properties. This factor diversity may contribute to differences in the tissue-specific expression properties of the SRE and MRE.

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