MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 5090–5100
0270-7306/91/105090-11$02.00/0

Vol. 11, No. 10

Activation of Skeletal α-Actin Gene Transcription: The Cooperative Formation of Serum Response Factor-Binding Complexes over Positive *cis*-Acting Promoter Serum Response Elements Displaces a Negative-Acting Nuclear Factor Enriched in Replicating Myoblasts and Nonmyogenic Cells

TE-CHUNG LEE,[1] KING-LAU CHOW,[2] PING FANG,[1] AND ROBERT J. SCHWARTZ[1]*

Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030,[1] and Department of Molecular Genetics, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Avenue, Bronx, New York 10461[2]

Received 17 January 1991/Accepted 5 July 1991

Three upstream CBAR *cis*-acting promoter elements, containing the inner core CC(A/T)$_6$GG of the serum response element (SRE), are required for myogenic cell type-restricted expression of the avian skeletal α-actin gene (K. L. Chow and R. J. Schwartz, Mol. Cell. Biol. 10:528–538, 1990). These actin SRE elements display differential binding properties with two distinct nuclear proteins, serum response factor (SRF) and another factor described here as F-ACT1. SRF is able to bind to all actin SREs with various affinities. This multisite interaction is marked by cooperative binding events in that the two high-affinity proximal and distal SREs facilitate the weak central-site interaction with SRF, leading to the formation of a higher-order SRF-promoter complex. Functional analyses reveal that undisrupted multiple SRF-DNA interactions are absolutely essential for promoter activity in myogenic cells. F-ACT1, present at higher levels in nonmyogenic cells and replicating myoblasts than in myotubes, binds solely to the proximal SRE, and its binding is mutually exclusive with that of SRF owing to their overlapping base contacts. The cooperative promoter binding by SRF, however, can effectively displace prebound F-ACT1. In addition, an intact F-ACT1 binding site acts as a negative promoter element by restricting developmentally timed expression in myoblasts. F-ACT1 may therefore act as a repressor of skeletal α-actin gene transcription. This interplay between F-ACT1 and SRF may constitute a developmental as well as a physiologically regulated mechanism which modulates sarcomeric actin gene expression.

Differentiation of muscle cells involves the developmental progression of mesodermal cells into committed proliferating myoblasts, which subsequently withdraw from the cell cycle and fuse to form myotubes (41). Myoblast fusion is accompanied by the repression of a subset of nonmuscle genes and the broad activation of a family of muscle-specific genes (10, 40). The skeletal and cardiac actin genes are among the best studied of these muscle-specific genes. Gene transfer experiments in muscle cell culture and transgenic animals indicate that the 5′-flanking promoter regions of the two genes appear to be sufficient, to a large degree, for the development- and tissue-specific expression of the sarcomeric α-actin genes (14, 26, 31).

Previously, we showed that the capacity for selective expression of the chicken skeletal α-actin gene resides within approximately 200 bp upstream from the transcription initiation site (1, 14). Using site-directed linker-scanning mutagenesis, we have recently shown that four positive *cis*-acting promoter elements are absolutely required for cell type-restricted expression of the skeletal α-actin gene in myogenic cells (8). These regulatory sites include a TATA box-like domain and three upstream elements described as CBARs (5, 6), which also resemble the decanucleotide motif CC(A/T)$_6$GG of CArG boxes and the inner core of the c-*fos* serum response element (SRE). In comparison, a region from −107 to the transcriptional start, which contains the −24 TATA box and the −85 proximal SRE, provided basal promoter activity in the *Xenopus* oocyte nuclear transcription assay system (1). This core promoter region, which provides low constitutive levels of expression in prefusion myoblasts, is also capable of being stimulated during myogenic differentiation, but at levels considerably lower than the whole promoter (1, 51). In addition, mutagenesis of the −130 central SRE revealed a vital role for potentiating transcription in myogenic cells, while in passaged fibroblasts, the intact central SRE minimally affected basal promoter activity (8). Thus, interactions shared between multiple SREs appear to be required in part for myogenic cell-specified skeletal α-actin gene activity.

The three SRE elements required for the skeletal α-actin promoter function exhibit an interesting spatial alignment: they are centered at −85, −130, and −175. We note that the center-to-center distance between two adjacent SREs is exactly 45 bp, and as a result, the middle SRE is predicted to be positioned on the helix face opposite that of its two neighboring elements. Although the significance of this stereospecific SRE phasing remains to be defined, we have recently suggested that the undeformed actin promoter is not optimized with respect to interaction between adjacent SRE elements and that a half-turn of torsional deformation within the inter-SRE region may be essential for driving promoter activity (7) in myogenic cells. Clearly, identification of protein factors interacting with these SRE elements will help

* Corresponding author.

uncover how these complex protein-promoter interactions may be achieved.

Recently, the serum response factor (SRF), a phosphoprotein of 62 to 67 kDa that binds to the c-*fos* SRE, has been purified to homogeneity by several groups (35, 39, 47). SRF binds as a dimer and symmetrically to various SRE elements with a broad range of affinities. The sarcomeric SRE family is evolutionarily conserved among striated α-actin genes from amphibians to humans, and their presence can often be correlated with promoter activity (15). However, the contribution of these elements to promoter function during muscle differentiation remains largely undefined and raises several important issues. For instance, in the c-*fos* gene, serum-inducible transcriptional activation of the gene is dependent on the SRE element (47). If the SRE elements are required for α-striated actin gene activity, what prevents the skeletal and cardiac actin genes from being expressed in replicating myoblasts? Furthermore, consensus SRE/CArG elements appear to have different contextual sequences surrounding the inner core. A recent study by Taylor et al. (45) showed that the most proximal cardiac actin CArG box and the SRE are functionally interchangeable. In addition, cross-binding of nuclear factors to this core motif in the c-*fos* and α-actin gene promoters has been reported (29, 51). Does the sequences flanking the core motif play a role in modulating affinity and/or specificity for factor binding? Finally, mutagenesis studies carried out on several striated α-actin promoters have suggested either a functional redundancy (27, 28) or an absolute requirement for each of these repeated SRE elements (8). Therefore, what is the regulatory role for multiple SRE elements in the α-actin 5′ promoter region?

These issues were dealt with by examining the interactions of the avian skeletal α-actin promoter with embryonic muscle nuclear proteins and pure SRF for SRE-binding activity. Here we show that the three SREs are not equivalent in binding to SRF and to another distinct nuclear factor described as F-ACT1. SRF cooperative binding to the proximal and distal actin SREs was observed to facilitate SRF binding to the weaker central site, forming a higher-order tertiary SRF-promoter complex. Since mutagenesis at any one of the SREs prevents promoter activity and inhibits the formation of the tertiary SRF-binding complex, this study indicates that each intact SRF-binding site is not functionally redundant but is indispensable for potentiating α-actin gene transcription. Thus, this study suggests an important positive regulatory role for SRF. We observed that avian F-ACT1 binding activity, which appears to be similar to that of the mammalian factors MAPF1, CBF2, and CF-1 (16, 37a, 45), is preferentially enriched in replicating myoblasts and in nonmuscle cells and gradually decreases during myogenesis. F-ACT1 has mutually exclusive overlapping base contacts with SRF only to the proximal SRE and is effectively displaced by cooperative SRF-promoter interactions. Reduced F-ACT1 binding by site-directed mutagenesis stimulated α-actin promoter activity severalfold during early myogenesis. Therefore, F-ACT1 might serve as a repressor by competing with SRF for the proximal SRE, thereby attenuating higher-order SRF complexes during early muscle development.

MATERIALS AND METHODS

Preparation of embryonic muscle cell cultures and crude nuclear extracts. White Leghorn eggs from the Department of Poultry Science, Texas A&M University, College Station, Tex., were incubated at 38°C. Whole muscle tissues of day 17 embryos were removed by dissection, rinsed with H_2O, frozen in liquid nitrogen, and stored at −80°C. Muscle nuclear extracts were prepared according to the procedure described by Dignam et al. (9) except that Mg^{2+} was omitted and 0.05% Nonidet P-40 was included in the buffer. Protein concentrations were measured by the method of Bradford (3) with bovine serum albumin as the standard. All nuclear extracts were stored in aliquots at −80°C. Primary myoblast and fibroblast cultures were prepared as described by Hayward and Schwartz (18).

Fractionation of muscle nuclear extracts. Muscle nuclear extracts were fractionated in the cold room to enrich for F-ACT1 and SRF activities. For partial purification of F-ACT1, 20 ml of the crude extract (200 mg) was loaded directly onto a 10-ml heparin-agarose column equilibrated in column buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4], 60 mM KCl, 1 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol). The column was first washed extensively with 0.2 M KCl, and F-ACT1 was eluted with 0.35 M KCl. Fractions containing F-ACT1-binding activity were pooled and dialyzed for 3 h in column buffer supplemented with 0.05% Nonidet P-40. The dialysate was aliquoted for storage at −80°C. For partial purification of the muscle SRF, 20 ml of crude nuclear extracts was passed through a 10-ml DEAE Sephadex A-25 column, and the flowthrough fractions were applied directly to a 10-ml heparin-agarose column. The column was washed with 0.35 M KCl, and SRF was eluted with 0.5 M KCl. This 0.35 to 0.5 M KCl step fraction was dialyzed and stored in aliquots at −80°C.

Preparation of bacterially expressed SRF. Plasmid pARSRF-Nde, which is a T7 polymerase expression vector (43) capable of producing full-length SRF protein upon IPTG (isopropyl-β-D-thiogalactopyranoside) induction, was generously provided by J. R. Manak and R. Prywes and was described by Manak et al. (22). *Escherichia coli* BL21 harboring the plasmid was grown at 37°C to an OD_{600} of 0.4 in TYP medium supplemented with ampicillin (50 μg/ml). Synthesis of SRF was then induced with 1 mM IPTG for 1.5 to 2 h, after which cells were spun down, washed once in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0), and resuspended in a 40× packed-cell volume of the dialysis buffer described above. Cells were disrupted on ice by the Branson Sonifier microtip with six 15-s bursts at a power setting of 4. The lysate was clarified in a microfuge for 20 min, and the supernatant, which was found to contain a large amount of overexpressed SRF, was aliquoted for storage at −80°C. Partial purification of the recombinant SRF was done as follows. A 10-ml amount of the lysate was applied to a 10-ml phosphocellulose column equilibrated with column buffer (pH 7.4). The flowthrough fractions were collected and applied to a 5-ml heparin-agarose column. The column was washed with 0.35 M KCl, and SRF was eluted with 0.5 M KCl. SRF was then dialyzed and stored as described above.

Plasmid DNA and oligonucleotide probes. The wild-type and mutant skeletal α-actin-CAT gene fusion constructs were described previously (8). The M19 DNA was cut with *Bgl*II and *Eag*I to release the 34-bp wild-type SRE1 fragment; the vector DNA was isolated and religated with a synthetic 34-bp *Bgl*II-*Eag*I fragment, which changed the SRE1 sequence 5′-CCAAATATGGCGAC-3′ to 5′-CCAAA TATGGATCC-3′. This promoter mutant (M14.5) was then subcloned into the vector pTZ-CAT as described previously (8). Transfection studies were carried out as described previously (8). The 225-bp wild-type and mutant chicken

skeletal α-actin promoter probes were generated by *Sma*I and *Hin*dIII digestion (−201 to +24) of the MCAT constructs, end labeled with [^{32}P]dCTP and Klenow, and gel purified before use. Double-stranded oligonucleotides representing each of the four positive *cis*-acting promoter elements were synthesized and designated as follows: ATAA oligo, −37 to −15; SRE1 oligo, −98 to −76; SRE2 oligo, −143 to −123; SRE3 oligo, −190 to −168. The human c-*fos* SRE was synthesized as 5′-GGATGTCCATATTAGGACA TCTG-3′. The herpes simplex virus (HSV) thymidine kinase promoter fragment from −33 to +11 (containing the CCAAT box) was generated from pTK-CAT (kindly provided by Ming-Jer Tsai) by digestion with *Bgl*II and *Hin*dIII.

Band shift, DNase I footprinting, and dimethyl sulfate methylation interference assays. Band shift assays (11) were typically performed in a 15-μl reaction volume at room temperature. Each reaction mix contained 3 μg of double-stranded poly(dI-dC), 0.5 to 1 ng of end-labeled DNA probe, and 2 to 10 μg of protein of crude nuclear or bacterial extracts in 10 mM Tris (pH 8.0)–0.1 mM EDTA–2 mM dithiothreitol–5% glycerol plus 15 mM KCl (low-salt condition) or 100 mM KCl (high-salt condition). The assembled mixture was incubated for 15 min, after which 2 μl of loading buffer (6× type III) (23) was added, and the sample was loaded onto a 5% polyacrylamide gel which had been prerun in 1× Tris-glycine buffer (23) at room temperature for 20 min. Electrophoresis was carried out for 2 h at 180 V.

In the methylation interference experiments, the probe DNA was partially methylated with dimethyl sulfate (24) before band shift assays were done. The bound and free species were separated on preparative gels, electroeluted, extracted with phenol-chloroform, and ethanol precipitated. Subsequent cleavage by piperidine was done as described by Maxam and Gilbert (24). After being heated to 90°C for 2 min in 95% formamide, samples were electrophoresed at 1,600 V on an 8% acrylamide sequencing gel in 1× TBE (23).

DNase I footprinting binding conditions were the same as those described for the band shift assay except that 2 mM $MgCl_2$ was included in the reaction mix. DNase I digestion was initiated by addition of 1 to 3 μl of various concentrations of DNase I (Pharmacia FPLC grade; 10 U/μl) diluted with H_2O just before use. Digestion was done for 1 to 3 min at room temperature; the reaction was stopped by addition of 80 μl of carrier solution (10 mM EDTA, 0.3 M sodium acetate, 0.5 mg of tRNA per ml) and 80 μl of phenol-chloroform (1:1). The samples were vortexed vigorously, spun, extracted again with chloroform, and then ethanol precipitated. The DNA pellet was suspended in formamide-dye solution and run on sequencing gels essentially as described above.

RESULTS

We have shown previously that promoter activity and developmental regulation of the chicken skeletal α-actin gene are confined to a 200-bp segment immediately upstream from the start of transcription and that four essential positive *cis*-acting elements are present within this region (8, 15). Among these essential promoter sequences are three elements containing the inner core, $CC(A/T)_6GG$, of the SRE, and they are hereafter referred to as SRE1 (−85), SRE2 (−130), and SRE3 (−175). In this study, we focused on two nuclear factors which interact differentially with the three SRE elements. We wanted to determine whether these nuclear factors might be involved in sarcomeric α-actin gene expression and whether they were related to each other. One

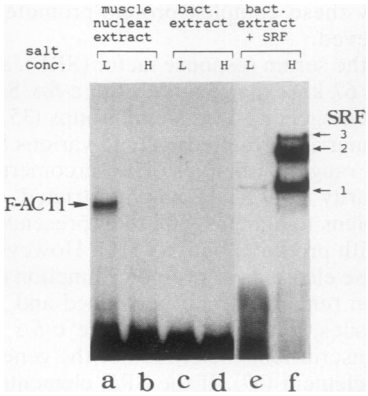

FIG. 1. Salt concentration in DNA band shift assays affects the formation of F-ACT1 and SRF complexes with the α-actin promoter. The 225-bp skeletal α-actin promoter fragment (−201 to +24) was used as a labeled probe. Proteins were day 17 embryonic muscle nuclear extracts (a and b, 5 μg), bacterial crude extracts prepared from host cells harboring the parental SRF expression vector without the SRF cDNA insert (c and d, 4 μg), and bacterial crude extracts from host cells overexpressing SRF (e and f, 4 μg). The KCl concentration in each binding reaction mix is indicated on the top by L (15 mM) or H (100 mM). Three SRF-promoter complexes were observed and are marked by 1, 2, and 3.

of the factors, which we designated F-ACT1, can be easily detected in crude nuclear extracts (Fig. 1). The other is the SRF, which is difficult to detect in crude nuclear extracts prepared from muscle tissue, as observed by Walsh (51). We therefore used a bacterially expressed SRF synthesized from a construct capable of making full-length SRF protein (22) to facilitate these analyses.

Salt concentrations differentially affect F-ACT1 and SRF binding to the α-actin promoter. As a first step toward identifying nuclear factors that might interact with the actin promoter, we used the 225-bp promoter fragment (−201 to +24) as a probe for band shift assays under a variety of gel buffer conditions (Tris-glycine, Tris-borate, and Tris-acetate). Figure 1 shows a band shift assay in which F-ACT1, the major binding activity in the crude muscle nuclear extract, was found to exhibit a profound salt sensitivity in the binding reaction. High salt (100 mM KCl, lane b) substantially weakened complex formation in comparison to that with low salt (15 mM KCl, lane a) regardless of the gel buffer system. The exquisite salt sensitivity displayed by the F-ACT1 complex may well account for its eluding detection in several previous studies (2, 28, 32). Surprisingly, high-salt conditions dramatically enhanced formation of the SRF-promoter complex (compare lanes e and f in Fig. 1), which migrated more slowly than the F-ACT1 complex, and was absent in lanes c and d, where the bacterial soluble extract was prepared from host cells harboring the parental expression vector without the SRF insert. This stabilization effect of high salt on the SRF-DNA complex has also been documented previously (35). Because of the striking signal enhancement, two more slowly migrating complexes were also observed. The presence of three SRF complexes is informative, since the 225-bp promoter fragment used here contains three SRE elements. The observed binding conditions for F-ACT1 and SRF were therefore adopted for the following studies.

F-ACT1 binds only to the proximal SRE, while SRF interacts differentially with the three elements. The binding spec-

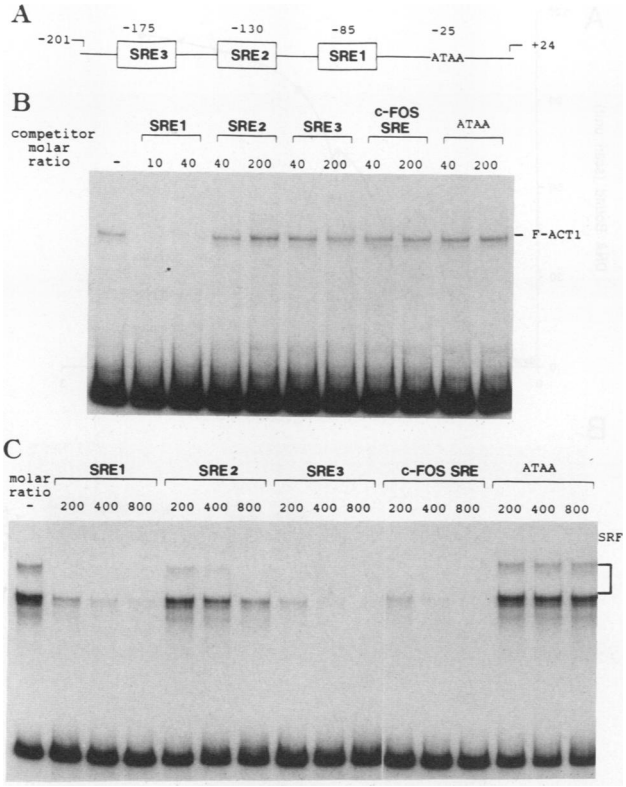

FIG. 2. Band shift assays with competitive oligonucleotides show specific binding of F-ACT1 and SRF to the skeletal α-actin promoter. The five duplex oligonucleotide competitors, each containing SRE1, SRE2, SRE3, ATAA, or the human c-*fos* SRE, were constructed as described in the text. (A) Positions of the four *cis*-acting elements carried on the promoter probe. (B) Competition assay at two competitor/probe molar ratios, with 5 μg of muscle nuclear extract as shown above each lane. (C) Competition assay at three competitor/probe molar ratios, with 2 μg of crude bacterial SRF in each lane.

ificities of F-ACT1 and SRF were first determined by band shift competition assays with double-stranded oligonucleotides corresponding to each of the four skeletal α-actin promoter *cis*-acting elements (Fig. 2A) and the human c-*fos* SRE. Figure 2B indicates that binding competition against the F-ACT1 complex with the SRE1 element occurred even at a competitor-probe molar ratio of 10. Increasing the molar ratio for other competitors from 40 to 200 did not appreciably reduce the F-ACT1 complex. Interestingly, the c-*fos* SRE also failed to compete against the F-ACT1 complex. Figure 2C, on the other hand, indicates that all three skeletal α-actin SRE elements competed against the SRF-promoter complexes. The SRE2 sequence appears to be the least efficient competitor, based on the competition efficiency. The reliability of these results was demonstrated by a positive control (c-*fos* SRE) and a negative control (ATAA) in our band shift assays. The observation that all three SREs compete with the α-actin promoter for SRF binding is consistent with the presence of three SRF-promoter complexes. A band shift assay with the SRE oligonucleotides as probes was performed to directly assess their affinities for SRF (Fig. 3). As expected, the SRE2 element displays poorer SRF binding capability than SRE1, SRE3, or the c-*fos* SRE.

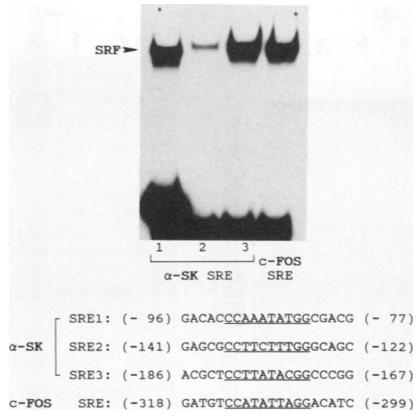

FIG. 3. Duplex SRE oligonucleotides display differential affinities for SRF. The duplex oligonucleotide competitors used in Fig. 2 were end labeled and used as probes for the binding assay. A 4-μg amount of the crude bacterial SRF was used for each lane. Oligonucleotide sequences are shown below, with the decanucleotide core motif underlined. Their positions in the skeletal α-actin (α-SK) or the human c-*fos* promoter are also indicated.

SRF forms a multicomplex through ordered occupation of the three SRE sites. The observed differential SRF-promoter interaction can be better visualized by examining the pattern of DNase I footprints on both DNA strands. To achieve this, we first developed a simple method to obtain large quantities of relatively pure bacterial SRF by using phosphocellulose and heparin-agarose chromatography. DNase I footprinting analyses with purified SRF (Fig. 4) showed that the proximal SRE1 and the distal SRE3 were occupied first by SRF. The central SRE2 site was eventually protected at higher SRF concentrations, confirming the observed differential affinities of SRF for the three actin SRE elements. Unexpectedly, protection of the SRE2 was stronger on the minus strand than on the plus strand. The stronger protection on the minus strand was also accompanied by DNase-enhanced bands in the inter-SRE regions, suggestive of DNA structural changes induced in the regions upon formation of the SRF-promoter multicomplex. Although the cause for the differential DNase protection patterns of the SRE2 is not evident, it may be related to the spatial relationship between the three actin SRE elements, since the SRE2 site is predicted to be positioned on the helix face opposite its two neighboring SRE elements. Notably, the SRE1 and SRE3 elements were invariably found to be protected simultaneously against DNase I digestion, even at the minimal SRF concentration required for protection. The possibility that SRE1 and SRE3 may be bound cooperatively by SRF was addressed in more detail.

Multisite interaction of SRF is marked by cooperative DNA-binding events. Although multiple SRE elements are found in the upstream promoter region of the sarcomeric actin and several growth factor-inducible genes (13, 32), it has not been assessed how these repeated DNA elements contribute to promoter activity. This question can be resolved in part by determining whether SRF bound at one site helps increase the affinity for SRF of an adjacent site, as protein-protein cooperation has been found to play a decisive role in promoter function (20). This recognition prompted us to determine whether the SRF binding over the multiple skeletal α-actin SRE elements of various affinities involves cooperative protein-protein interactions. We were

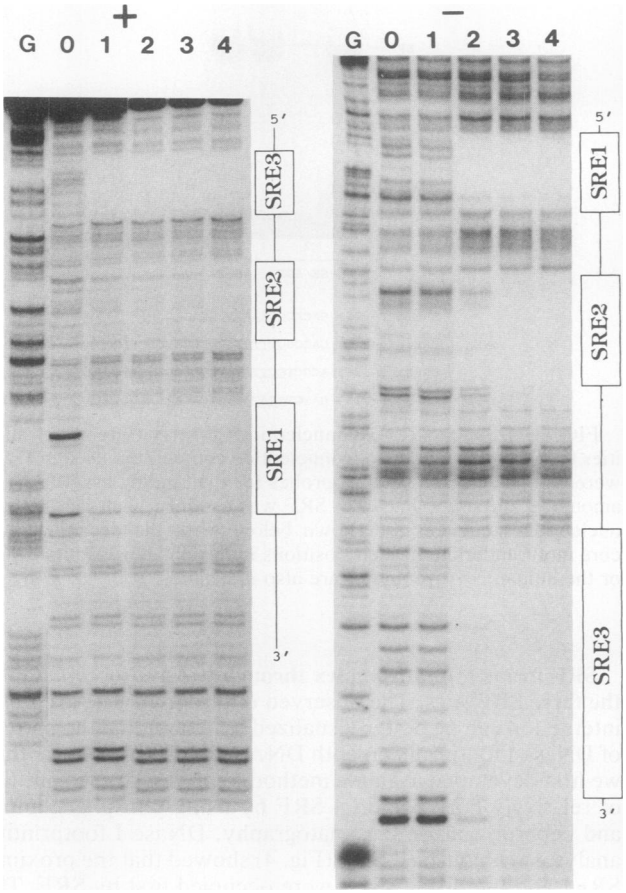

FIG. 4. DNase I footprints show that SRF forms a multicomplex through an ordered occupation of the three SRE sites. The 225-bp actin promoter fragment was inserted into the *Sma*I site of plasmid pTZ19R, and the promoter probe was then generated by double digestion with *Hin*dIII and *Eco*RI. The plus and minus strands were labeled by filling in with Klenow in the presence of [^{32}P]dCTP (*Hin*dIII site) and [^{32}P]dATP (*Eco*RI site). Lane G represents G-specific cleavage by dimethyl sulfate; lane 0 is free DNA cleaved by DNase I. In lanes 1 to 4, 0.5, 1.0, 1.5, and 2.0 μg, respectively, of purified bacterial SRF was incubated with DNA in the presence of 100 ng of poly(dI-dC).

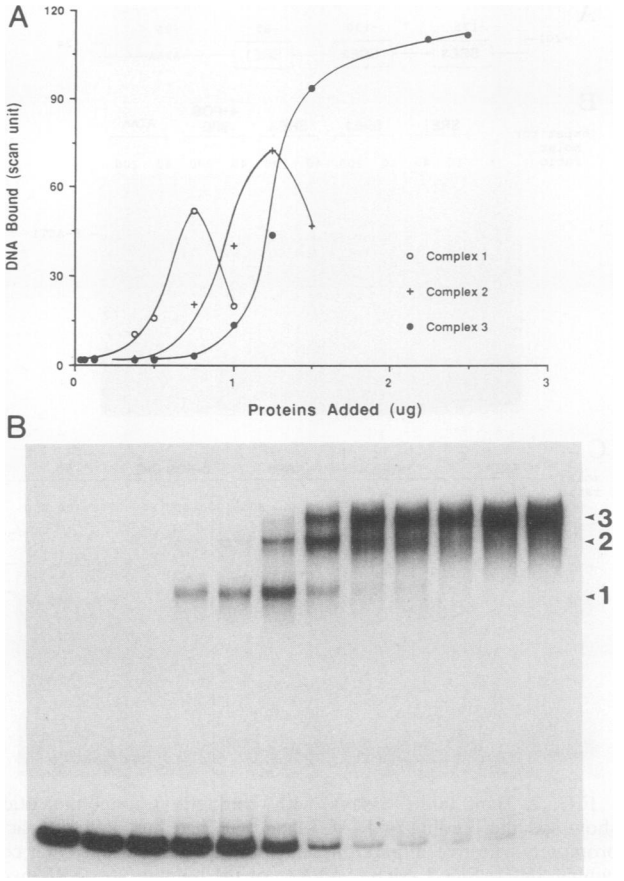

FIG. 5. SRF binds cooperatively to the three actin SRE elements. The 225-bp promoter probe and partially purified bacterially expressed SRF were used in the band shift assay. Data shown in panel A were derived from the experiment presented in panel B, which was a band shift assay with increasing amounts of the partially purified SRF in the presence of 100 ng of poly(dI-dC). The amount of protein in each lane, from left to right, was 0.06, 0.12, 0.25, 0.38, 0.50, 0.75, 1.0, 1.25, 1.5, 2.0, 2.25, and 2.5 μg, respectively. DNA probes retained in each of the three SRF complexes (1, 2, and 3) were quantitated by densitometry and are presented in arbitrary scan units.

able to explore this possibility by using purified bacterial SRF.

A band shift assay with a series of SRF doses, shown in Fig. 5B, clearly illustrates a cooperative SRF binding event. Figure 5A depicts a sigmoid curve for the appearance of all three SRF-promoter complexes. Since the first complex is solely contributed by SRF binding over a single SRE1 or SRE3 site, its sigmoid appearance most likely results from SRF binding as a dimer, as reported previously by Norman et al. (30). Similar behavior has been observed for other DNA-binding proteins that bind as dimers but which can dissociate into monomers at low concentration (20, 33). Formation of the SRF secondary and tertiary complexes must, however, involve cooperative protein-protein interaction between SRFs bound to adjacent SRE sites, based on the following observations: first, the second complex emerges while fully unoccupied promoter probes are still abundant, suggesting cooperation between SRE1 and SRE3; second, the third complex appears precociously, prior to complete saturation of the two high-affinity sites, indicating that occupancy at the high-affinity sites can facilitate SRF binding over the weaker-affinity site, since the independent affinity of the SRE2 for SRF is about 10-fold lower than that of the SRE1-SRE3 site (Fig. 2 and 3); finally, this protein cooperation results in the stabilization of the higher-order SRF-promoter complex, as judged by the increased stoichiometry of the promoter probe retained on the secondary and tertiary complexes. It is important to note that the observed cooperativity occurred over less than a fivefold change of protein concentrations and therefore is likely to be physiologically significant.

SRF-mediated multicomplex is essential for promoter activity. Having demonstrated the SRE-binding characteristics of F-ACT1 and SRF, we sought to examine the correlation between factor binding in vitro and promoter activity in vivo. We took advantage of the available site-directed α-actin promoter mutants whose activities have been examined previously by transfection studies (8) and are summarized in Fig. 6A for a convenient reference. As anticipated, muta-

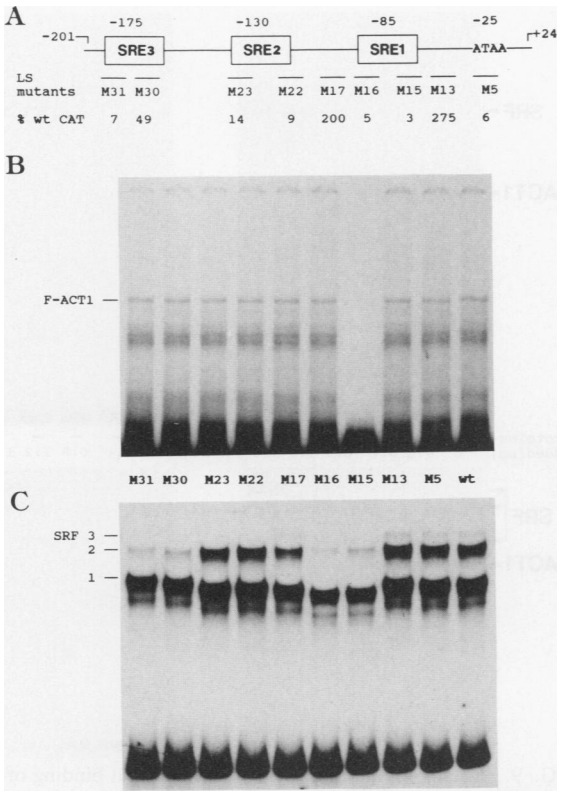

FIG. 6. Mutations of the positively acting SREs eliminate SRF binding and transcription activity. (A) Linker-scanning (LS) mutations across the promoter region are indicated by horizontal lines. Chloramphenicol acetyltransferase (CAT) activities, reported previously (8), are shown below for convenient reference as percent wild-type (wt) CAT activity. (B and C) Band shift assays with the 225-bp promoter probes carrying each of the LS mutations. Either 5 μg of muscle nuclear extract (B) or 4 μg of crude bacterial SRF (C) was used in each lane.

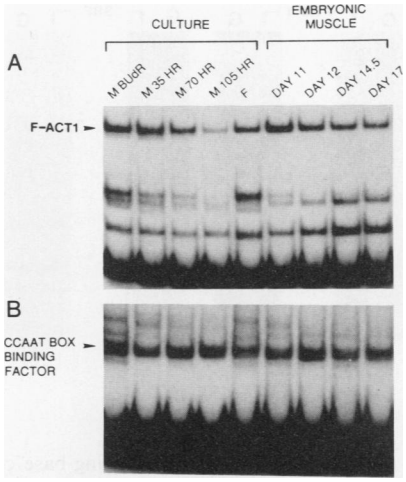

FIG. 7. F-ACT1 binding activity is decreased during embryonic myogenesis. Nuclear extracts were prepared either from primary myoblast (M) and fibroblast (F) cultures established from the breast muscle of day 11 chicken embryos or from day 11 to 17 embryonic muscle tissues. The myoblast culture treated with 30 μM 5′-bromo-2′-deoxyuridine (M BUdR) provides a source of replicating myoblasts. (A) Cloned SRE1 fragment (−105 to −70) as probe. (B) CCAAT box element of the HSV thymidine kinase promoter (−33 to +11) as probe.

tions changing nucleotide sequences in the ATAA, SRE2, or SRE3 element did not affect promoter binding by F-ACT1 (Fig. 6B). The mutation M15, which alters the right side of the SRE1 core from CCAAATATGG to CCAAGATCTG (−91 to −82), clearly disrupted the binding of F-ACT1. On the other hand, M16, which changed the left side of the core from CACCCAAATATGG to AGATCTAATATGG (−94 to −82), allowed normal F-ACT1 binding but dramatically inhibited promoter activity. Likewise, a deleterious effect on promoter activity was found for mutations affecting either the SRE2 (M22 and M23) or the SRE3 (M30 and M31), neither of which is a binding site for F-ACT1. These observations indicate that F-ACT1 selectively binds to the 3′ portion of the proximal SRE1.

By contrast, the SRE1 (M15 and M16) and the SRE3 (M30 and M31) mutations each diminished overall SRF binding by severely blocking formation of the secondary and tertiary complexes (Fig. 6C). Here, the first complex is only partially affected, since a strong SRF-binding site is always present in either of the mutant promoters. Thus, in M15 and M16, the first complex is contributed solely by SRF bound to the SRE3 site, and that in M30 and M31 results exclusively from the SRF-SRE1 interaction. Notably, the gel mobility of the first complex in M15 and M16 is faster than that in M30 and M31. This appears to be due to differential SRF-induced promoter bending, as SRF has been shown to cause DNA bending upon binding to the c-*fos* SRE (17). The SRE2 mutations (M22 and M23), as predicted, eliminated only the third complex. An analysis of binding versus function revealed a positive correlation between SRF binding in vitro and promoter activity in vivo, as evidenced by the observation that undisrupted SRF-DNA multisite interactions are absolutely essential for promoter function, regardless of their affinities for SRF. These results strongly suggest a positive regulatory role for SRF in skeletal α-actin gene expression.

F-ACT1 activity is diminished during myogenesis and enriched in replicating myoblasts and nonmuscle tissues. Developmental upregulation of the skeletal α-actin mRNA occurs following the fusion of mononucleated myoblasts and increases over 50-fold through terminal differentiation (18). Nuclear extracts taken from different stages of myogenic as well as nonmyogenic cell cultures and tissues were used in band shift assays to assess the relative levels of F-ACT1. In Fig. 7A, F-ACT1 was found to be greatest in replicating undifferentiated myoblasts grown in medium with 30 μM 5′-bromo-2′-deoxyuridine, a condition which inhibits the accumulation of detectable skeletal α-actin transcripts. F-ACT1 was subsequently reduced as myoblasts (35 h) progressed through fusion (70 h) and became differentiated myotubes (105 h), while skeletal α-actin mRNA levels peaked. Similarly, the level of F-ACT1 was greater in day 11 embryonic muscle tissue, which contains primarily myoblasts, and was reduced in day 17 embryonic muscle, which contains predominantly myotubes. An independent assessment of the quality of the extracts is also provided in Fig. 7B, which shows that the HSV thymidine kinase CCAAT box-binding factor remains relatively constant throughout the avian embryonic development. The quantitation of the F-ACT1 binding activity during muscle differentiation has been independently and repeatedly confirmed in the laboratory. Furthermore, nuclear extracts from chicken brain,

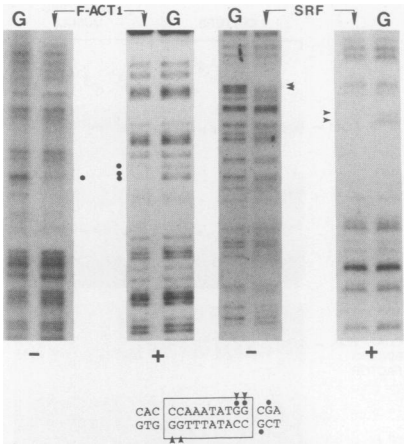

FIG. 8. F-ACT1 and SRF have overlapping base contacts over the right side of the SRE1 inner core. The 225-bp promoter used in the assay was either 5′-end labeled with kinase (for F-ACT1) or 3′-end labeled with Klenow (for SRF). Lanes G represent free probes cleaved with the guanine-specific reaction. Plus and minus strands are indicated on the bottom. Protected G residues are differentially marked on each strand (dots mark F-ACT1 contacts, and arrowheads mark SRF contacts) and are summarized below along with the SRE1 sequence.

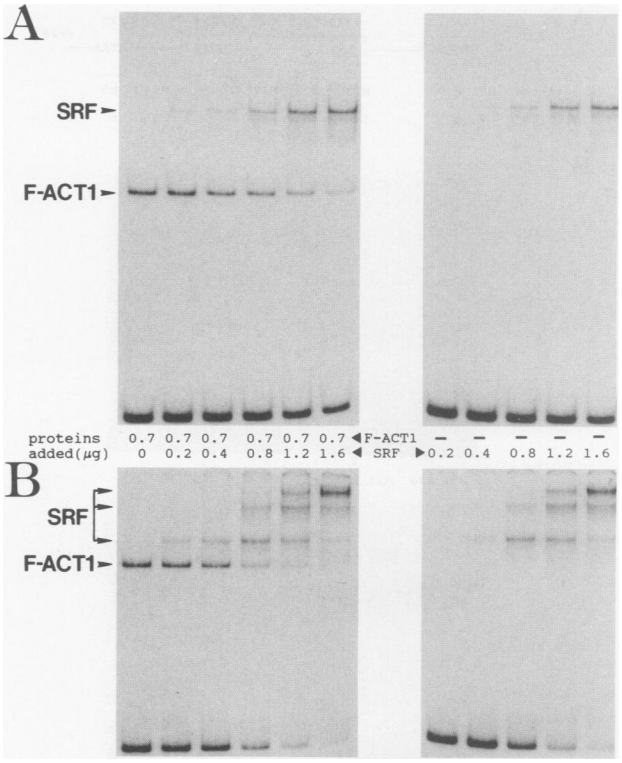

FIG. 9. Protein competition assays indicate that binding of SRF and F-ACT1 to the SRE1 site is mutually exclusive. Band shift assays shown in this figure were performed with six times less DNA probe than ordinarily used in order to observe the competition effect between F-ACT1 and SRF. Band shift assays were done at 100 mM KCl; 0.7 μg of the HeLa cell crude nuclear extract was used as a source for high levels of F-ACT1 activity. As a control for SRF binding activity alone, the HeLa extract was omitted in the right panels. The partially purified recombinant SRF, with increasing amounts of protein as indicated, was added together with the HeLa extract. DNA probes were added last. (A) Cloned SRE1 fragment as probe. (B) The 225-bp promoter fragment as probe. Conditions in panels A and B were exactly the same except for the probe used.

retina, and HeLa cells were also compared for tissue distribution of F-ACT1, and the levels of F-ACT1 among these tissues appear to be HeLa >> retina > brain > muscle (data not shown).

Our band shift analyses invariably revealed several faster-migrating complexes with all nuclear extracts. We had carried out a series of limited proteolytic digestion on F-ACT1 with proteinase K prior to the band shift assay. These experiments lend support to the possibility that the faster-migrating complexes may originate from interactions of the F-ACT1 proteolytic fragments with the DNA probe (data not shown).

The developmental downregulation and preferential enrichment in nonmuscle tissues of the F-ACT1 binding activity, together with a positive regulatory role for the SRF binding sites, led us to propose that F-ACT1 may function as a repressor in skeletal α-actin gene expression. This repressor action of F-ACT1 can be achieved by a direct competitive binding event, since both F-ACT1 and SRF bind specifically to the SRE1 site. Experiments presented below provide support for this proposed molecular mechanism.

F-ACT1 and SRF have overlapping base contacts over the SRE1 site. A mutually exclusive DNA-binding event between two competitive factors can be achieved by their binding with overlapping nucleotide contacts. Indeed, the promoter mutation M15, which blocked SRE1 binding by both F-ACT1 and SRF (Fig. 6), indicates that they may have overlapping base contacts over the right half of the SRE1. The $CC(A/T)_6GG$ core motif, which is contained within the SRE family, has an imperfect inverted repeat, the CC and GG doublets. Previously, several SRE/CArG-binding factors have been shown to contact the GG doublets either symmetrically (both strands) or asymmetrically (only one strand) (16, 38, 51). We therefore performed a methylation interference assay to determine whether F-ACT1 and SRF have mutually exclusive nucleotide contacts. Figure 8 shows that F-ACT1 asymmetrically contacts the GG doublet on the plus strand at positions −82 and −83. In comparison, SRF makes contact with both pairs of the GG doublets, as also reported by others (16, 38). Thus, F-ACT1 and SRF have distinct but overlapping nucleotide contacts on the SRE1 element, suggesting that their binding to the SRE1 site is mutually exclusive.

SRF DNA binding results in displacement of F-ACT1 from the SRE1 site. Having demonstrated the overlapping DNA contacts of F-ACT1 and SRF, we went on to determine whether their SRE binding is indeed mutually exclusive. Since F-ACT1 and SRF have distinct gel mobilities, this can be accomplished by performing a band shift assay under a limiting DNA probe condition, in which one of the two protein factors competing for the same target DNA can be titrated out by gradually changing the protein ratio in favor of the other factor. We illustrate such a competitive DNA-binding event between F-ACT1 and SRF, both on the proximal SRE site (Fig. 9A) and over three adjacent SRE elements (Fig. 9B). Figure 9A shows that even on the proximal SRE, increasing SRF-binding activity (by increasing the SRF dosage) was able to displace F-ACT1 accordingly from the SRE1. Furthermore, when this protein competition assay was performed under the same conditions except with the 225-bp promoter probe containing all three

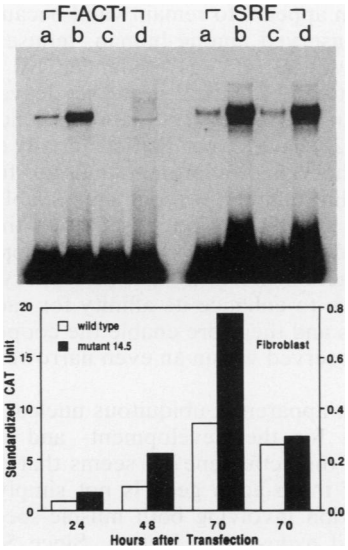

FIG. 10. Weakened F-ACT1–SRE1 interaction stimulates α-actin promoter activity in primary muscle cultures. (Top) Band shift assay with both the 225-bp wild-type (a and b) and M14.5 (c and d) promoter fragments. Heparin-agarose-purified F-ACT1 was used for the left panel, and crude bacterial SRF was used for the right panel. Lanes a and c, 0.1 μg of F-ACT1 and 2 μg of crude SRF; lanes b and d, 0.2 μg of F-ACT1 and 10 μg of crude SRF. (Bottom) Transient-transfection assay for the wild-type and M14.5 promoter-CAT fusion constructs. The data are representative of three transfection experiments and are the averages of two or more separate determinations. The CAT activity unit is standardized as percent conversion of chloramphenicol per milligram of protein per minute. The scale for myoblasts is on the left and that for fibroblasts is on the right. Myoblasts were transfected 24 h after plating and harvested 24, 48, and 70 h after transfection. Fibroblasts were harvested 70 h after transfection.

SRE elements, a more pronounced DNA-binding competition by SRF against F-ACT1 became evident (Fig. 9B). This appears to be due to the cooperative DNA-binding interaction of SRF, leading to a more efficient promoter-binding mechanism by SRF and subsequently a more effective F-ACT1-displacing mechanism. This finding is quite novel and may account for the presence of multiple SRE elements found in the promoter region of several sarcomeric α-actin genes.

Weakened F-ACT1-DNA interaction stimulates promoter activity in myoblasts. To provide a functional test for the proposal that F-ACT1 acts as a repressor in restricting actin promoter activity, it is essential to differentially eliminate F-ACT1 binding without affecting SRF binding. Since we have shown in Fig. 8 that F-ACT1 but not SRF makes additional base contacts 3′ to the SRE1 decanucleotide core, we further constructed a site-directed promoter mutant, M14.5, which changes the SRE1 sequence 5′-CCAAAT ATGGCGAC-3′ to CCAAATATGGATCC. A band shift assay (Fig. 10, top) confirms that this mutation selectively weakens F-ACT1 binding without appreciably disrupting SRF-DNA interaction. Notably, the mutant promoter DNA retains fractional F-ACT1-binding interaction, in contrast to the M15 mutant, in which the GG dinucleotide common to both F-ACT1 and SRF is altered and F-ACT1 binding is totally eliminated (Fig. 6B). This observation indicates that the SRE core is indeed the major contact site between F-ACT1 and DNA.

The effect of the M14.5 mutation on promoter activity was then examined by a transient-transfection assay in both primary myoblast and fibroblast cultures (8), and the results are depicted in Fig. 10 (bottom). Expression of the wild-type and mutant M14.5 promoter-CAT fusion constructs is induced during terminal myogenic differentiation. An increase in M14.5-CAT activity could be seen as early as 24 h after transfection, and the stimulation remained evident after myoblast fusion. However, from the band shift analysis presented above, we believe that the M14.5 promoter activity is still partially restricted by F-ACT1 due to residual interaction between F-ACT1 and the mutant SRE1 element. This may explain why transcriptional activity was only stimulated by a factor of 2 to 3 over the wild-type level. In contrast, the M14.5 mutation had little effect on promoter activity in fibroblasts, suggesting that myogenic activators deficient in these cells are required to promote α-actin gene transcription. In summary, these results are consistent with our proposal that SRF (activator) and F-ACT1 (repressor) compete for the same promoter element (SRE1).

DISCUSSION

Two distinct actin SRE-binding factors. We showed that the three skeletal α-actin SRE elements can be specifically and differentially recognized by two protein factors, F-ACT1 and SRF, which display several distinguishing properties. First, F-ACT1 binds exclusively to the SRE1, whereas SRF exhibits a relaxed interaction specificity for the broad SRE family. Second, F-ACT1 and SRF contact the SRE element in an asymmetrical and symmetrical manner, respectively. Third, high salt concentrations block complex formation of F-ACT1 but enhance that of SRF. Fourth, the two complexes have disparate gel mobilities. Fifth, F-ACT1 and SRF do not have any common antigenic determinants (data not shown). These biochemical and immunological criteria strongly suggest that they are not closely related polypeptides. Similar to our findings here, Ryan et al. (38) have reported two distinct c-*fos* SRE-binding factors, the 62-kDa protein and SRF, in a lymphoblast cell line, and Walsh (51) has also shown two SRE-binding factors, MAPF1 and SRF, in a human T-lymphocyte nuclear extract. Thus, like several eukaryotic transcriptional *cis*-acting elements, the SRE elements bind at least two distinct nuclear factors and are likely to play a complex regulatory role in muscle-specific actin gene expression.

F-ACT1, the 62-kDa protein, and MAPF1 appear to resemble each other in a number of aspects: they are abundant in crude extracts, contact the GG doublet of the core motif asymmetrically, and are ubiquitous. F-ACT1 and the 62-kDa protein were further found to have a similar molecular mass (10a), and their binding activities are both sensitive to high salt and weakened by the use of plasmid DNA as a nonspecific competitor. F-ACT1 does differ, however, from the other two proteins in that it fails to appreciably recognize the c-*fos* SRE element. Although the cause of this discrepancy is not understood, it may lie in our use of embryonic muscle tissue instead of transformed cell lines in preparing the nuclear extract. In addition, variations in preparing duplex oligonucleotides for band shift analyses could also contribute to the disparity. Interestingly, Taylor et al. (45) and Boxer et al. (2) could only demonstrate a single CArG box-binding activity attributable to SRF. Higher salt concentrations or the use of plasmid DNA as nonspecific competitors in their band shift assays may well account for their inability to detect a F-ACT1-like binding activity.

Three nonequivalent actin SRE elements. Multiple SRE elements are present in the promoter region of several sarcomeric actin- and growth factor-inducible genes. Our results explicitly demonstrate that the skeletal α-actin SRE elements display nonequivalent factor-binding interactions. The proximal one, SRE1, differs from the central (SRE2) and the distal (SRE3) elements in its unique dual interactions with F-ACT1 and SRE. SRE2 is the weakest of the three SRE binding sites. Furthermore, given the unknown importance of the contextual nucleotide sequences surrounding the core motif and the potential of unidentified protein interactions at these sites, we believe that the three SRE elements are not functionally equivalent. Taylor et al. (45) and Tuil et al. (49) concluded that the CArG box can be functionally replaced by the c-*fos* SRE for muscle-specific gene expression. It seems to us that the outcome of this CArG box substitution experiment may well depend on the factor-binding properties of the particular elements examined. For instance, the SRE3 described here, the cardiac α-actin CArG1, and the c-*fos* SRE may be quite similar because they all have a strong affinity for SRF and do not interact appreciably with factors showing asymmetrical footprints, such as F-ACT1, MAPF1, CF1, and CBF2 (16, 37a, 45). By contrast, the skeletal SRE1 and SRE2 may behave differently owing to the dual factor-binding specificity of the former and the weak SRE affinity of the latter. This view of ours, although it must be tested, is strengthened by the observation that replacement of the SRE1 by the c-*fos* SRE relaxes tissue-specific expression of the skeletal α-actin promoter (51). Thus, the presence of the common motif $CC(A/T)_6GG$ in the SRE/CArG family does not guarantee their functional equivalence.

A cooperative SRE-binding mode by SRF. Why are there multiple copies of the SRF binding site in the skeletal α-actin promoter? The answer may lie in the cooperative effect of the multiple binding sites, as with GAL4-mediated transcriptional activation (12). Indeed, we showed that the two high-affinity proximal and distal SREs are first bound cooperatively by SRF with concurrent DNA bending, which then facilitates the weak central-site interaction with SRF, leading to the formation of a higher-order SRF-promoter complex. In this respect, we have recently proposed that the undeformed wild-type actin promoter is not optimized with respect to interactions among neighboring SRE sites and may require some torsional DNA deformation in the inter-SRE regions to generate a stable transcription complex (7). Since SRF is capable of inducing DNA bending (Fig. 7) (17), the proposed essential DNA torsional deformation may be accomplished by SRF-promoter interactions. This SRF-induced DNA bending may act to spatially reorient adjacent promoter elements so as to form an energetically favorable multiprotein-DNA complex in which the promoter is then held in a transcriptionally active conformation. Indeed, our DNase I footprint analyses suggest some DNA conformational changes in the inter-SRE region upon formation of the higher-order SRF-promoter complex, although this could only be seen on the minus promoter DNA strand.

SRF and positive regulation of the actin promoter. We have shown that the three SRF binding sites are concordant with the positive *cis*-acting promoter elements, suggesting that SRF may serve as a positive regulator in activation of the skeletal α-actin promoter. This view is supported, though not proven, by the reports that SRF binding sites present in several promoters function as activating elements in transfection studies (26, 36, 51). Although our results are mainly derived from the use of a bacterially expressed human SRF, the conclusion appears to remain valid because, first, SRFs are highly conserved among human, mouse, chicken, and *Xenopus laevis* (30, 45), as evidenced by immunological cross-reactivity and identical protease cleavage patterns of the human and avian SRFs (data not shown). Second, Manak et al. (22) have shown that bacterially expressed SRF can be phosphorylated by protein kinase to further increase its DNA-binding affinity without appreciably changing its DNA-binding specificity. Our observation that the bacterially expressed SRF is still capable of cooperative DNA-binding interaction indicates that phosphorylation of SRF may also serve to enhance its affinity for each of the actin SRE elements and therefore enable the cooperative binding mode to be observed within an even narrower range of SRF dosages.

How can an apparently ubiquitous nuclear factor such as SRF account for the development- and muscle-specific expression of the actin gene? It seems that muscle-specific expression of the α-actin gene is not simply the outcome of an interaction involving both muscle-specific *cis*-acting sequences and *trans*-acting factors. Since SRF is a phosphoprotein (34, 38), muscle excitation and contractile coupling may provide a signalling event (50) leading to differential SRF protein phosphorylation, which may then subtly alter protein-protein or protein-DNA interaction and therefore specifically activate the actin promoter. Other post-translational modifications may be involved as well. Another possibility is that some myogenic factor may associate with SRF to form a ternary complex over the SRE element and subsequently modify its interaction with the actin promoter. In this respect, we have recently identified an E-box element (21a) situated between the skeletal α-actin SRE2 and SRE3. This actin E-box has been shown to interact specifically with MyoD and another nonmyogenic factor (21). Thus, the E-box may function to provide yet another level of muscle-specified gene expression. It can also be envisioned that a negative regulator preferentially enriched in nonmuscle tissue may interfere with SRF binding through mutual exclusion over the SRE. Alternatively, this negative regulator can associate with SRF to attenuate its ability to bind to the SRE. Various combinations of these modes may operate at different stages during myogenesis.

F-ACT1 functions as a repressor by precluding SRF binding over the SRE1 site. Negative regulation of transcription has been recognized as a key control element of differential gene expression, as evidenced in many prokaryotic and eukaryotic systems (4, 37, 42). In bacterial and phage systems, binding of repressors and RNA polymerase to the promoter usually constitutes a mutually exclusive event, so that the repressor protein blocks transcription initiation. Such competitive interactions might explain why muscle-restricted genes are transcriptionally suppressed in replicating myoblasts and activated in postreplicative myoblasts (25, 40). In particular, skeletal α-actin transcripts appear following the end of myoblast replication in fused myotubes (19). Our DNA-binding and footprinting analyses firmly establish that F-ACT1 and SRF have overlapping base contacts over the SRE1 site and that the two proteins bind to the SRE1 element in a mutually exclusive manner. We further show that the M14.5 SRE1 mutation, which dramatically reduces F-ACT1 but not SRF binding, also stimulates promoter activity in myogenic cells. Since F-ACT1 is preferentially enriched in replicating myoblasts and nonmuscle cells, this competitive factor might serve to inhibit skeletal α-actin gene activity even in the presence of SRF. We found that F-ACT1 levels were substantially reduced during myogenic

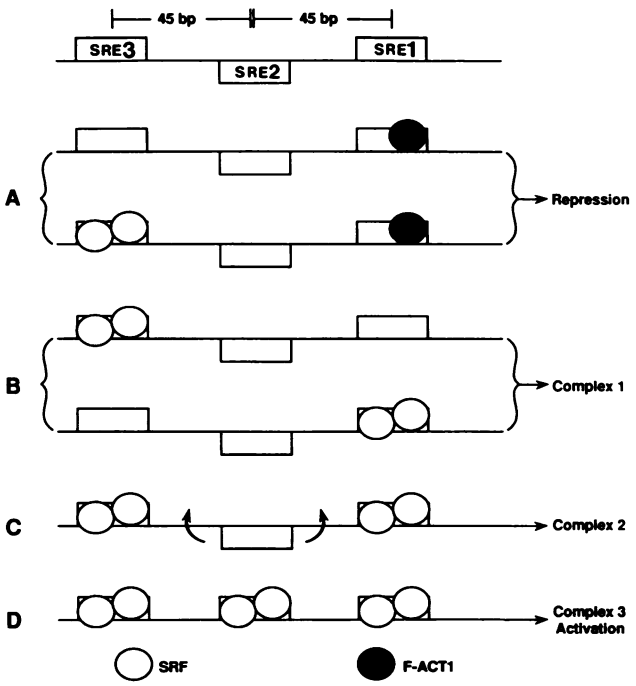

FIG. 11. Model depicting differential regulation of the skeletal α-actin promoter through a competitive interplay between SRF and F-ACT1. Each pair of actin SRE elements exhibit a center-to-center distance of exactly 45 bp, and therefore the SRE2 site is expected to be positioned on an opposite DNA helix face relative to SRE1 and SRE3. Four sequential stages of promoter occupancy (A through D) are proposed to account for our data. Upon formation of the SRF complex 2, DNA twisting may be induced in the inter-SRE regions (indicated by arrows), which may be important for stereospecific alignment of the participating transcription factors. Not shown in the model is the protein-protein contact made between pairs of the DNA-bound SRF dimers, which contributes to the cooperative SRF-DNA interaction.

terminal differentiation, correlating well with upregulation of the skeletal α-actin gene. Although F-ACT1 is still detectable in late-stage embryonic muscle tissue, as shown by our band shift assay under the low-salt condition, its DNA-binding ability in vivo may be greatly reduced owing to the high intracellular salt concentration (140 mM K^+ [44]), which would favor SRF binding, based on our observation in Fig. 1. Thus, in differentiated muscle, the cooperative DNA-binding mode of SRF provides an effective mechanism to overcome the binding competition from F-ACT1. F-ACT1 thus resembles bacterial repressors in that it appears to interfere with the DNA binding of a positive transcription factor, SRF. Since the formation of the SRF-mediated multiprotein complex is essential for promoter function, F-ACT1 may exert its inhibitory effect by blocking its formation.

To summarize our conclusions, a proposed model is depicted in Fig. 11. In replicating myoblasts and nonmyogenic cells (stage A), the skeletal α-actin promoter is repressed by the abundant presence of F-ACT1, which precludes SRF from binding to the SRE1 site via mutual exclusion and presumably disrupts its interaction with the transcription machinery. During muscle differentiation (stages B to D), the ratio of SRF/F-ACT1 binding activities is changed in favor of SRF, so that the SRE1 site now becomes accessible to SRF. This can be achieved by the developmental downregulation of F-ACT1 and/or by increasing SRF binding activity. The latter can be easily accomplished by increased SRF synthesis or by phosphorylation of SRF, which has been shown to promote SRF binding activity (22). Formation of the higher-order SRF-promoter complex is then facilitated through cooperative SRF-SRF interaction, with the presumptive involvement of DNA twisting in the inter-SRE region (7, 17) (Fig. 4), which may ultimately bring about a precise stereospecific alignment of the participating transcription factors.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants HL38401 and 87-HL-26-H from the National Institutes of Health.

We acknowledge J. R. Manak and R. Prywes for the kind gift of the SRF expression vector and Jennifer Moss for her helpful suggestions in this study.

REFERENCES

1. **Bergsma, D. J., J. M. Grichnik, L. M. A. Gosset, and R. J. Schwartz.** 1986. Delimitation and characterization of *cis*-acting DNA sequence required for the regulated expression and transcriptional control of the chicken skeletal α-actin gene. Mol. Cell. Biol. **6**:2462–2475.
2. **Boxer, L. M., R. Prywes, R. G. Roeder, and L. Kedes.** 1989. The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response factor. Mol. Cell. Biol. **9**:515–522.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brent, R.** 1985. Repression of transcription in yeast. Cell **42**:3–4.
5. **Carroll, S. L., D. J. Bergsma, and R. J. Schwartz.** 1986. Structure and complete nucleotide sequence of the chicken α-smooth muscle (aortic) actin gene: an actin gene which produces multiple mRNAs. J. Biol. Chem. **261**:8965–8976.
6. **Carroll, S. L., D. J. Bergsma, and R. J. Schwartz.** 1988. A 29-nucleotide DNA segment containing an evolutionarily conserved motif is required in *cis* for cell-type-restricted repression of chicken α-smooth muscle actin gene core promoter. Mol. Cell. Biol. **8**:241–250.
7. **Chow, K.-L., M. E. Hogan, and R. J. Schwartz.** 1991. Phased *cis*-acting promoter elements interact at short distances to direct avian skeletal α-actin gene transcription. Proc. Natl. Acad. Sci. USA **88**:1301–1305.
8. **Chow, K.-L., and R. J. Schwartz.** 1990. A combination of closely associated positive and negative *cis*-acting promoter elements regulates transcription of the skeletal α-actin gene. Mol. Cell. Biol. **10**:528–538.
9. **Dignam, J. D., P. Martin, B. S. Shastry, and R. G. Roeder.** 1983. Eukaryotic gene transcription initiation with purified components. Methods Enzymol. **101**:582–598.
10. **Emerson, C., D. Fischman, B. Nadal-Ginard, and M. A. Q. Siddiqui (ed.).** 1986. Molecular biology of muscle development. Alan R. Liss, Inc., New York.

10a. **Fang et al.** Unpublished data.

11. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of *lac* repressor-operator interaction by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
12. **Giniger, E., and M. Ptashne.** 1988. Cooperative DNA binding of the yeast transcriptional activator GAL4. Proc. Natl. Acad. Sci. USA **85**:382–386.
13. **Gius, D., X. Cao, F. J. Rauscher III, D. R. Cohen, T. Curran, and V. P. Sukhatme.** 1990. Transcriptional activation and repression by Fos are independent functions: the C terminus represses immediate-early gene expression via CArG elements. Mol. Cell. Biol. **10**:4243–4255.
14. **Grichnik, J. M., D. J. Bergsma, and R. J. Schwartz.** 1986. Tissue restricted and stage specific transcription is maintained within 411 nucleotides flanking the 5′ end of the chicken α-actin gene. Nucleic Acids Res. **14**:1683–1701.
15. **Grichnik, J. M., B. A. French, and R. J. Schwartz.** 1988. The

chicken skeletal α-actin gene promoter region exhibits partial dyad symmetry and a capacity to drive bidirectional transcription. Mol. Cell. Biol. **8**:4587–4597.
16. **Gustafson, T. A., and L. Kedes.** 1989. Identification of multiple proteins that interact with functional regions of the human cardiac α-actin promoter. Mol. Cell. Biol. **9**:3269–3283.
17. **Gustafson, T. A., A. Taylor, and L. Kedes.** 1989. DNA bending is induced by a transcription factor that interacts with the human c-*fos* and α-actin promoters. Proc. Natl. Acad. Sci. USA **86**:2162–2166.
18. **Hayward, L. J., and R. J. Schwartz.** 1986. Sequential expression of chicken actin genes during myogenesis. J. Cell Biol. **102**: 1485–1493.
19. **Hayward, L. J., Y. Y. Zhu, and R. J. Schwartz.** 1988. Cellular localization of muscle and nonmuscle actin mRNAs in chicken primary myogenic cultures: the induction of α-skeletal actin mRNA is regulated independently of α-cardiac actin gene expression. J. Cell Biol. **106**:2077–2086.
20. **Hochschild, A., and M. Ptashne.** 1986. Cooperative binding of λ repressors to sites separated by integral turns of the DNA helix. Cell **44**:681–687.
21. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
21a. **Lee, T.-C.** Unpublished data.
22. **Manak, J. R., N. de Bischop, R. M. Kris, and R. Prywes.** 1990. Casein kinase II enhances the DNA binding activity of serum response factor. Genes Dev. **4**:955–967.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
25. **Medford, R. M., H. T. Nguyen, and B. Nadal-Ginard.** 1983. Transcriptional and cell cycle mediated regulation of myosin heavy chain gene expression during muscle cell differentiation. J. Biol. Chem. **258**:11063–11073.
26. **Minty, A., and L. Kedes.** 1986. Upstream region of the human cardiac actin gene that modulates its transcription in muscle cells: presence of evolutionarily conserved repeated motif. Mol. Cell. Biol. **6**:2125–2136.
27. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory role in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7**:2803–2813.
28. **Mohun, T. J., M. V. Taylor, N. Garrett, and J. B. Gurdon.** 1989. The CArG promoter sequence is necessary for muscle-specific transcription of the cardiac actin gene in *Xenopus* embryos. EMBO J. **8**:1153–1161.
29. **Muscat, G. O., T. A. Gustafson, and L. Kedes.** 1988. A common factor regulates skeletal and cardiac α-actin gene transcription in muscle. Mol. Cell. Biol. **8**:4120–4133.
30. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-*fos* serum response element. Cell **55**:989–1003.
31. **Petropoulous, C. J., M. P. Rosenberg, N. A. Jenkins, N. G. Copeland, and S. H. Hughes.** 1989. The chicken skeletal muscle α-actin promoter is tissue specific in transgenic mice. Mol. Cell. Biol. **9**:3785–3792.
32. **Phan-Dinh-Tuy, F., D. Tuil, F. Schweighoffer, C. Pinset, A. Kahn, and A. Minty.** 1988. The "CC.Ar.GG." box. A protein-binding site common to transcription-regulatory regions of the cardiac actin, c-*fos* and interleukin-2 receptor genes. Eur. J. Biochem. **173**:507–515.
33. **Phillips, S. E. V., I. Manfield, I. Parsons, B. E. Davidson, J. B. Rafferty, W. S. Somers, D. Margarita, G. N. Cohen, I. Saint-Girons, and P. G. Stockley.** 1989. Cooperative tandem binding of *met* repressor of *Escherichia coli*. Nature (London) **341**:711–715.
34. **Prywes, R., A. Dutta, J. A. Cromlish, and R. G. Roeder.** 1988. Phosphorylation of serum response factor, a factor that binds to the serum response elements of the c-*fos* enhancer. Proc. Natl. Acad. Sci. USA **85**:7206–7210.
35. **Prywes, R., and R. G. Roeder.** 1987. Purification of the c-*fos* enhancer binding protein. Mol. Cell. Biol. **7**:3482–3489.
36. **Quitschke, W. W., L. DePonti-Zilli, Z.-Y. Lin, and B. M. Paterson.** 1989. Identification of two nuclear factor-binding domains on the chicken cardiac actin promoter: implications for regulation of the gene. Mol. Cell. Biol. **9**:3218–3230.
37. **Reznikoff, W. S., D. A. Siegele, D. W. Cowing, and C. A. Gross.** 1985. The regulation of transcription initiation in bacteria. Annu. Rev. Genet. **19**:355–387.
37a. **Riggs, K. C., K. T. Merrell, G. Wilson, and K. Calame.** 1991. Common factor 1 is a transcriptional activator which binds in the c-*myc* promoter, the skeletal α-actin promoter, and the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **11**: 1765–1769.
38. **Ryan, W. A., Jr., B. R. Franza, Jr., and M. Z. Gilman.** 1989. Two distinct cellular phosphoproteins bind to the c-*fos* serum response element. EMBO J. **8**:1785–1792.
39. **Schroter, H., P. Shaw, and A. Nordheim.** 1987. Purification of intercalator-released p67, a polypeptide that interacts specifically with the c-*fos* serum response element. Nucleic Acids Res. **15**:10145–10158.
40. **Schwartz, R. J., and K. N. Rothblum.** 1981. Gene switching in myogenesis: differential expression of the actin multigene family. Biochemistry **20**:4122–4129.
41. **Schwartz, R. J., and E. M. Stone.** 1983. Cloning of contractile protein genes. Cell Muscle Motil. **3**:195–257.
42. **Struhl, K.** 1985. Negative control at a distance mediates catabolite repression in yeast. Nature (London) **317**:822–824.
43. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
44. **Sweadner, K. J., and S. M. Goldin.** 1980. Active transport of sodium and potassium ions: mechanism, function and regulation. N. Engl. J. Med. **302**:777–783.
45. **Taylor, M., R. Treisman, N. Garrett, and T. Mohun.** 1989. Muscle-specific (CArG) and serum-responsive (SRE) promoter elements are functionally interchangeable in *Xenopus* embryos and mouse fibroblasts. Development **106**:67–78.
46. **Treisman, R.** 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5′ element and c-*fos* 3′ sequences. Cell **42**:889–902.
47. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcription response of the c-*fos* gene to serum factors. Cell **46**:567–574.
48. **Treisman, R.** 1987. Identification and purification of a polypeptide that binds the c-*fos* serum response element. EMBO J. **6**:2711–2717.
49. **Tuil, D., N. Clergue, D. Montarras, C. Pinset, A. Kahn, and F. Phan-Dinh-Tuy.** 1990. CCArGG boxes, *cis*-acting elements with a dual specificity. Muscle-specific transcriptional activation and serum responsiveness. J. Mol. Biol. **213**:677–686.
50. **Vergara, J., R. Y. Tsien, and M. Delay.** 1985. Inositol 1,4,5-triphosphate: a possible chemical link in excitation-contraction coupling in muscle. Proc. Natl. Acad. Sci. USA **82**:6352–6356.
51. **Walsh, K.** 1989. Cross-binding of factors to functionally different promoter elements in the c-*fos* and skeletal actin genes. Mol. Cell. Biol. **9**:2191–2201.
52. **Walsh, K., and P. Schimmel.** 1987. Two nuclear factors compete for the skeletal muscle actin promoter. J. Biol. Chem. **262**:9429–9432.