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Identification of Multiple Proteins That Interact with Functional Regions of the Human Cardiac α-Actin Promoter

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5′ Sequences of the human cardiac α-actin gene are involved in the tissue-specific and developmental regulation of the gene. Deletion analyses combined with transient expression experiments in muscle cells have demonstrated three primary regions of functional importance (A. Minty and L. Kedes, Mol. Cell. Biol. 6:2125–2136, 1986; T. Miwa and L. Kedes, Mol. Cell. Biol. 7:2803–2813, 1987), and we have previously demonstrated binding of a protein indistinguishable from serum response factor (SRF) to the most proximal region (T. A. Gustafson, T. Miwa, L. M. Boxer, and L. Kedes, Mol. Cell. Biol. 8:4110–4119, 1988). In this report, we examine protein interaction with the remainder of the promoter. Gel shift and footprinting assays revealed that at least seven distinct nuclear proteins interacted with known and putative regulatory regions of the promoter. The transcription factor Sp1 bound to eight sites, as demonstrated by footprinting assays and gel shift analysis with purified Sp1. Purified CCAAT box-binding transcription factor CTF/NF-I and Sp1 were shown to interact with the far-upstream regulatory element at −410, and footprint analysis showed extensive overlap of these two sites. Two unidentified proteins with similar but distinct footprints interacted with the second region of functional importance at −140, which contains the second CArG motif [CC(A+T rich)$_6$GG], and these proteins were shown to be distinct from SRF. SRF was found to bind to the remaining three CArG boxes, two of which were closely interdigitated with Sp1 sites. In addition, CArG box 4 was found to interact with SRF and another distinct protein whose footprint was contained within the SRF-binding site. Sequences surrounding the TATA box were also shown to bind proteins. Sp1 was shown to bind to a site immediately downstream from the TATA box and to a site within the first exon. Thus, each of the three functional upstream regions, as defined by transfection assays, was shown to interact with five factors: Sp1 and CTF/NF-I at the upstream site, two unidentified proteins at the central site, and SRF at the most proximal site. These results suggest that expression of the cardiac actin gene in muscle cells is controlled by complex interactions among multiple upstream and intragenic elements.

Promoters of eucaryotic genes that are transcribed by RNA polymerase II require *cis*-acting regulatory elements for both constitutive and regulated gene expression. These elements include sequences near the start of transcription such as the TATA box, a variety of upstream regulatory sequences generally located within 50 to 400 base pairs of the start site, and enhancer elements that may be located at great distances upstream or downstream of the start site (for review, see reference 37). In all cases thus far examined, these *cis*-acting regulatory sequences interact with one or more sequence-specific DNA-binding proteins that can activate or repress transcription. Although it is believed that regulation of the amount, cellular localization, or activity of regulatory proteins is important for transcriptional regulation, little is known about the mechanisms by which specific protein-DNA interactions regulate gene expression or how these interactions are integrated into the overall pattern of tissue-specific gene expression during development.

Cellular differentiation involves the induction of genes required for the specialized function of the cell as well as repression of genes associated with the undifferentiated state. Differentiation of muscle cells involves an ordered sequence of morphological and molecular events which can be reproduced in vitro by using established myogenic cell lines (16, 60). Myogenesis involves the fusion of proliferating undifferentiated myoblasts to form highly organized, multinucleated myotubes. Fusion is accompanied by the rapid accumulation of muscle-specific gene products, including the cardiac and skeletal actins (2, 8, 28), and numerous other gene products, including myosin heavy and light chains, tropomyosin, troponin, and muscle creatine kinase (3, 9, 12, 13).

The cardiac and skeletal actin genes are among the best studied of these tissue-specific genes. The sarcomeric α-actin gene family consists of two evolutionarily conserved genes, which are expressed predominantly in either cardiac or skeletal muscle (25, 28, 39, 57). The 5′-flanking regions of the human cardiac and skeletal actin genes appear to be sufficient to account for the strict developmental and tissue-specific regulation of these genes (40, 41, 43, 48). Preliminary footprint analysis of these two promoters has revealed that at least one common factor interacts with regulatory sequences of both actin genes, located at nucleotides −89 and −100 of the skeletal and cardiac promoters, respectively (26, 47). This factor is biochemically and immunologically indistinguishable from the serum response factor (SRF) that binds to the c-*fos* serum response element (10, 56). In addition, the

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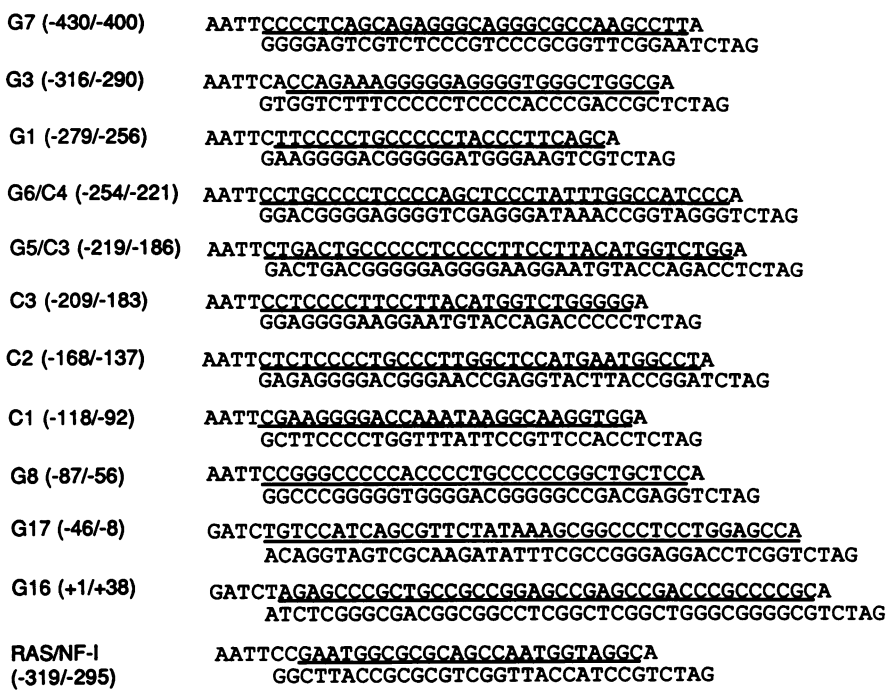

FIG. 1. Oligonucleotide sequences. Sequences refer to nucleotides in relation to the cap site and are underlined (see reference 41 for actin sequences and reference 31 for *ras* NF-I sequences).

human cardiac α-actin promoter contains two distal regulatory elements (−443/−395) and (−177/−142), which account for approximately two-thirds of optimal expression in muscle cells (41, 43).

The variety and number of protein-binding sites on most gene promoters suggests that the regulation of gene transcription is a result of complex interactions between multiple DNA-binding proteins. It is clear from a number of studies, for example, that the simultaneous binding of multiple proteins at neighboring sites is required for optimal promoter activation (32, 35, 46, 53). Other studies suggest that genes can be regulated in negative and positive manners by the binding of multiple factors to overlapping binding sites (4–7, 15, 24, 34, 36, 55, 59). These studies suggest the possibility that different cell types regulate expression of a gene in a positive or negative manner through the utilization of sets of DNA-binding proteins capable of interacting with overlapping or interdigitated binding sites. Up or down regulation of gene expression might therefore be a result of subtle alterations in the relative concentrations or structures of these interacting sets of binding proteins.

To begin to understand the nature of the protein-DNA interactions that regulate expression of the human cardiac actin gene, we have used gel shift and footprinting assays to show that a number of proteins interact with functional regions of the gene. We show that at least seven distinct proteins interact with 13 binding sites located between nucleotides −443 and +68 on the human cardiac actin gene. These proteins include SRF, the CCAAT box-binding transcription factor CTF/NF-I, Sp1, and three unidentified factors. In addition, a protein-binding site was identified just upstream of the TATA box, and Sp1 was shown to bind immediately downstream of the TATA box. In vitro binding assays showed that expression of the proteins examined was not limited to differentiated muscle cells, although subtle differences in gel shift patterns or of the relative binding activities of the proteins were evident between muscle and nonmuscle cells and between undifferentiated and differentiated muscle cells. Thus, regulation of the cardiac actin gene appears to be a result of a complex interaction of multiple distinct protein factors with their cognate binding sites and possibly with one another.

MATERIALS AND METHODS

Cell culture and extract preparation. Cells were cultured and nuclear extracts were prepared as previously described (26). C2 (60) and HeLa cells were grown in Dulbecco modified Eagle medium with 20 and 10% fetal calf serum, respectively. C2 cells were harvested at 40 to 60% confluency for myoblasts. Differentiation was induced by changing confluent myoblast cultures to Dulbecco modified Eagle medium containing 2% horse serum, and extracts were prepared 2 to 3 days later. Nuclear extracts were prepared as described previously (17) except that the protease inhibitors leupeptin and aprotinin (2 μg/ml; Calbiochem-Behring) were added to all solutions. Nuclear proteins were extracted with 0.4 M NaCl. Protein concentrations were measured by the method of Bradford (11); extracts were divided into equal portions and stored at −80°C.

Plasmid DNAs and synthetic oligonucleotides. The SH and SE probes were excised from pHCA443CAT plasmid derivatives (41) by cleavage with *Hin*dIII and with *Sty*I or *Sty*I-*Eco*RI, respectively. The ET probe has been described elsewhere (26). The oligonucleotide probes (Fig. 1) were synthesized on an Applied Biosystems DNA synthesizer and gel purified. Probes were end labeled and gel purified before use.

Gel mobility shift and footprinting assays. Binding reactions for gel mobility shift assays have been previously described (26); in this study, poly(dI-dC) was used as a nonspecific competitor instead of pUC19 DNA, and 5 μg of bovine serum albumin was included in each reaction, since it was found to increase the signal. pUC19 DNA was not used

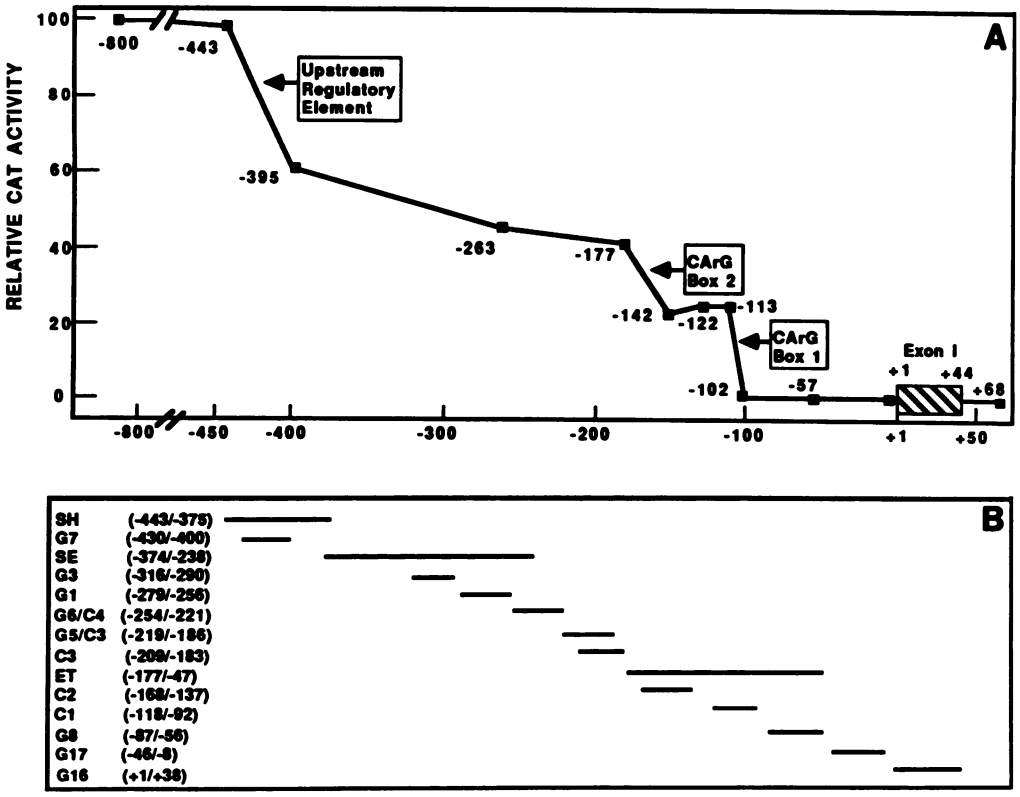

FIG. 2. Summary of transient transfection experiments in C2 muscle cells, delineating functional regions of the human cardiac actin promoter and promoter fragments used in gel shift and footprinting assays. (A) Results (summarized from references 41 and 43) representing relative CAT activities of a series of unidirectional 5′ deletion mutants after transient transfection into C2 myoblasts. (B) Promoter fragments used in this study and their locations relative to the transcriptional start site.

as a nonspecific competitor, since it was found to compete for CArG-binding factor 2 (CBF-2), CBF-3, Sp1, and CTF/NF-I binding. The gel shift assays with the purified Sp1 and CTF/NF-I proteins were carried out in an identical manner except that no nonspecific competitor DNAs were used. Approximately 2 to 4 ng of purified Sp1 or CTF/NF-I protein was used per binding reaction. In the methylation interference experiments, the probe DNAs were partially methylated with dimethyl sulfate (38) before complex formation. The complexed and free DNA was localized on gel mobility shift assays, eluted, treated with piperidine, and analyzed on sequencing gels as previously described (26) except that the synthetic oligonucleotides were used rather than restriction fragments. In one case (the G17 probe), the DNA was suspended in 90 μl of 20 mM ammonium acetate (pH 7)–1 mM EDTA for 30 min at 90°C before addition of 10 μl of piperidine and was incubated for an additional 30 min at 90°C to cleave at methylated adenine and guanine residues (38). Each oligonucleotide strand was individually 5′ end labeled and subsequently annealed with a slight excess of the opposite unlabeled strand before methylation, gel shift analysis, and complex elution. After piperidine cleavage, electrophoresis was carried out on 15% polyacrylamide–45% urea sequencing gels. DNase I footprinting was performed as previously described (20).

RESULTS

Gel shift analysis of cardiac actin promoter fragments. The 5′-flanking DNA of the human cardiac actin gene has been examined extensively, using deletional mutagenesis and transfection assays of promoter fragments linked to the chloramphenicol acetyltransferase (CAT) gene (41, 43). These functional assays have demonstrated at least three DNA regions whose removal results in markedly reduced CAT activities of the transfected constructs in muscle cells (Fig. 2A). These are located between nucleotides −443 and −395, −177 and −142, and −113 and −102. We and others have previously demonstrated protein interaction with the most proximal site, which contains a CArG motif [CC(A+T rich)$_6$GG] (26, 49). The protein that binds to this element is indistinguishable immunologically from the SRF which binds to the dyad symmetry element of the c-*fos* promoter (10, 26, 49). We have referred to this protein previously as CBF, for CArG-binding factor (26), but in this report it will be referred to as SRF.

To examine protein interaction with the remainder of the promoter, a number of DNA fragments (summarized in Fig. 2B) were used in gel shift assays. These probes were named as follows. First, the SH, SE, and ET probes are the *Sty*I-*Hin*dIII, *Sty*I-*Eco*RI, and *Eco*RI-*Tth*111I promoter fragments described in Materials and Methods. Second, some oligonucleotide probes were designated by the letter G because they contained G-rich protein-binding sites which, as we show below, interact with Sp1. The four probes containing CArG boxes (41) are designated by the letter C. The two probes containing both CArG elements and guanine-rich sites are indicated by both C and G. The sequences of the synthetic oligonucleotide probes are shown in Fig. 1. As shown in the following figures, most of these DNA

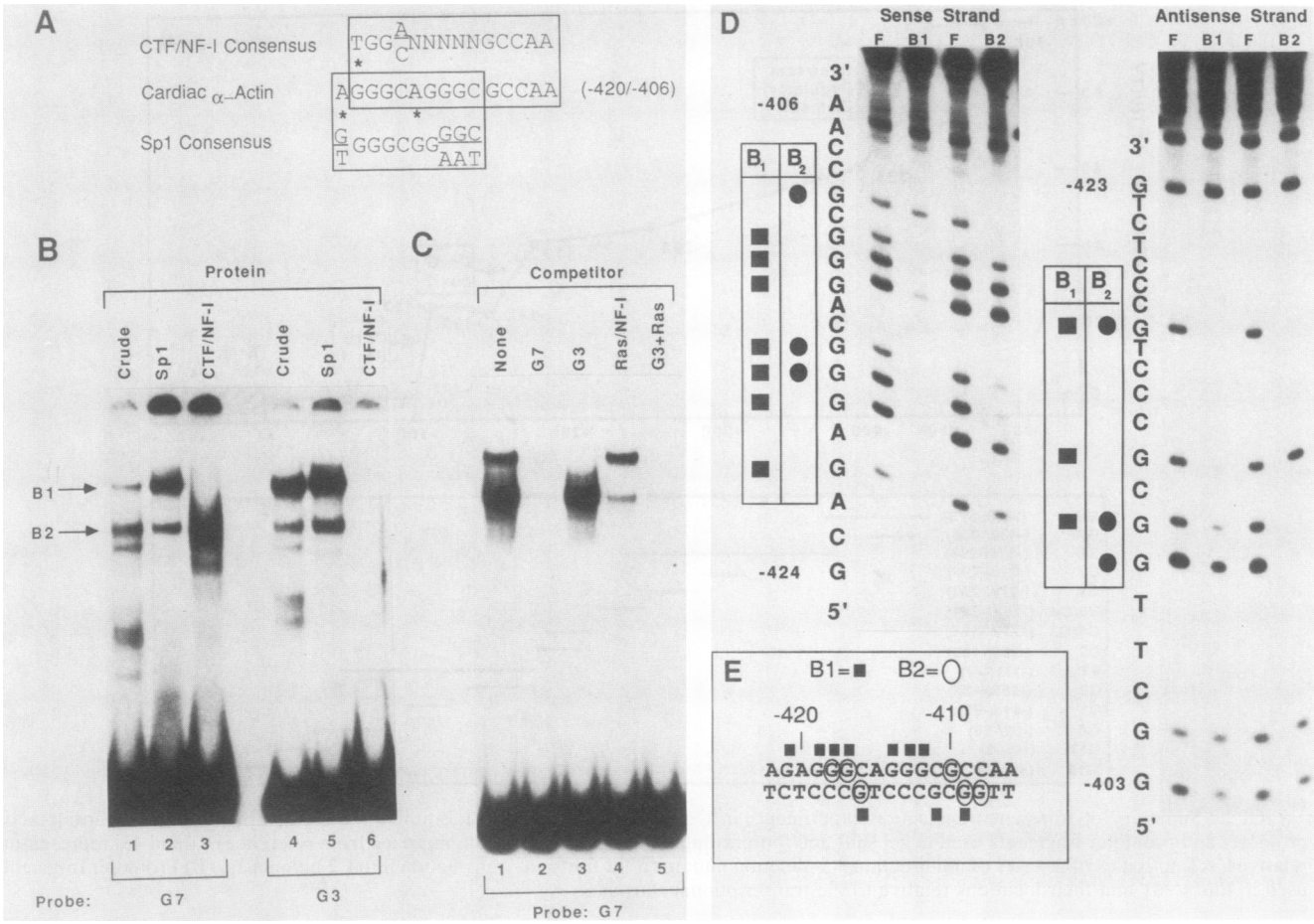

FIG. 3. Binding of Sp1 and CTF/NF-I to overlapping sequences between −430 and −400. (A) Sequence of the cardiac actin promoter (−420 to −406), showing consensus binding sites for CTF/NF-I and Sp1. *, Nucleotide differing from the consensus sequence. (B) Gel shift analysis using crude C2 nuclear extracts and purified (>95%) preparations of Sp1 and CTF/NF-I. The G7 probe was incubated with either crude C2 nuclear extract (lane 1) or purified preparations of Sp1 (lane 2) and CTF/NF-I (lane 3) and electrophoresed. Lanes 4 to 6 represent an identical experiment except that the G3 probe, containing an Sp1 site, was used. (C) Gel shift competition experiments. Lane 1 shows the gel shift pattern of crude C2 nuclear extract after incubation with the G7 probe; 200-fold-excess amounts of G7 (lane 2), G3 (lane 3), *ras* NF-I, an oligonucleotide derived from c-*ras* containing a high-affinity CTF/NF-I site (lane 4), and G3 and *ras* NF-I together (lane 5) were added as competitors to the binding reactions. (D) Methylation interference footprinting of B1 and B2 protein complexes with the G7 probe. Sequences representing top (sense)- and bottom (antisense)-strand interference analyses are shown on the left. F, Free probe; B1 and B2, upper and lower complexes, respectively. Squares and circles represent guanine nucleotides whose methylation interfered with B1 (Sp1) and B2 (CTF/NF-I) protein interaction, respectively. (E) Summary of methylation interference footprinting results. The circled nucleotides are those whose methylation interfered with CTF/NF-I binding (B2); squares indicate those that interfered with Sp1 binding (B1).

fragments were found to interact specifically with nuclear proteins prepared from the mouse myogenic cell line C2 (60).

Interaction of Sp1 and CTF/NF-I with the distal regulatory region of the human cardiac actin promoter. Deletion studies of the cardiac actin promoter have delineated a transcriptionally important region localized between −443 and −395 (41; Fig. 2A). When a DNA fragment encompassing these regulatory sequences (designated SH) was analyzed for protein binding in gel shift assays (19, 22), two major shifted sequence-specific complexes of differing mobilities were observed by using nuclear extracts from C2 muscle cells. A double-stranded oligonucleotide (designated G7) corresponding to nucleotides −430 to −400 was synthesized and found to retain specific binding activity identical to that of the parent SH fragment (data not shown).

In analyzing this 30-base-pair region contained within the G7 probe, we noticed overlapping consensus binding sites for CTF/NF-I (13 of 14 nucleotides) (31) and Sp1 (8 of 10) (33) (Fig. 3A). To determine whether either of these transcription factors might bind to the G7 probe, we used pure preparations of Sp1 (provided by J. Kadonaga and R. Tjian) and CTF/NF-I (provided by K. Jones) in gel shift assays. When the G7 probe was incubated with pure Sp1 (Fig. 3B, lane 2), we observed two shifted complexes, designated B1 and B2, that comigrated with the two specific complexes detected after incubation with crude nuclear extracts, although B1 was always more intense than B2 when pure Sp1 was used. The explanation for an Sp1 doublet in gel shift assays is unclear, although it has been observed by others (15) and may be due in part to posttranslational processing (29).

When the G7 probe was incubated with pure CTF/NF-I (Fig. 3B, lane 3), a diffuse complex that comigrated with B2 was observed. The heterogeneity of this complex was similar to that observed by Jones et al. (31) and appeared to be due to multiple forms of CTF/NF-I present in HeLa cells, which

may be a result of differential mRNA splicing (52) or multiple subunits (14). The faint complexes of faster mobility observed after incubation with crude nuclear extract (lanes 1 and 4) appeared to represent binding to partially degraded proteins, since they have been routinely observed with crude nuclear extracts after prolonged storage.

To further define Sp1 binding, a second oligonucleotide (designated G3) was synthesized that corresponded to nucleotides −316 to −290 of the cardiac actin promoter, since this region contains a putative Sp1-binding site. This fragment did indeed bind purified Sp1 (Fig. 3B, lane 5) but did not interact with CTF/NF-I (lane 6). This result confirmed that the G3 probe contained an Sp1 recognition site and, in addition, demonstrated the sequence specificity of CTF/NF-I interaction with the G7 probe. These data suggested that in the case of the G7 probe, the B1 complex represented Sp1 binding, whereas the B2 complex may have represented a combination of CTF/NF-I and Sp1 binding.

This idea was confirmed by gel shift competition experiments (Fig. 3C) in which the G7 probe was incubated with crude C2 nuclear extracts in the absence or presence of a variety of competitor DNAs containing either an Sp1 site (G3) or a high-affinity CTF/NF-I site derived from the human c-*ras* promoter (31). Whereas self-competition with G7 DNA abolished binding of both complexes (lane 2), the G3 competitor eliminated the B1 complex only (lane 3). In contrast, the c-*ras* NF-I DNA reduced B2 complex binding only (lane 4). The signal remaining at B2 after c-*ras* competition appeared to represent the Sp1 component of the B2 complex, since it could be eliminated by addition of excess unlabeled G3 (lane 5). These experiments clearly demonstrated that the B1 complex represented Sp1 binding, whereas the B2 complex was made up primarily of CTF/NF-I binding activity in addition to a small amount of Sp1.

Methylation interference footprinting showing extensive overlap of Sp1 and CTF/NF-I binding to the upstream regulatory element. To more critically analyze the specific nucleotides that interact with these two proteins, a methylation interference assay was used (54). Since the B2 complex was shown to represent a mixture of Sp1 and CTF/NF-I, this complex was footprinted after competition with unlabeled G3 oligonucleotide to remove the Sp1 component. Interference footprinting of both complexes revealed 12 guanine nucleotides on the two strands between −421 and −408 whose methylation interfered markedly with protein binding (Fig. 4D). Furthermore, numerous clear differences in the interference patterns of the two shifted complexes were observed. Specifically, methylation of guanines at positions −408 (antisense strand) and −410 (sense strand) interfered with B2 (CTF/NF-I) binding but had negligible effect on B1 (Sp1) binding. Conversely, methylation of guanines at positions −411 (antisense strand) and −412, −413, −414, −419, and −421 (sense strand) blocked Sp1 binding to a greater degree than it blocked CTF/NF-I binding. In addition, methylation of guanines at positions −409 and −416 (antisense), as well as at −417 and −418 (sense), affected binding of both factors equally. These methylation interference patterns (Fig. 4E) correspond well with previously characterized Sp1 (23) and CTF/NF-I (50) interference footprints, and they clearly demonstrate that both Sp1 and CTF/NF-I bind to the same DNA segment and that they each make close contact with overlapping but distinct sets of nucleotides.

Sp1 interacts with a number of promoter sites. Preliminary footprinting studies (data not shown) revealed a number of binding sites that were extremely G+C rich and therefore

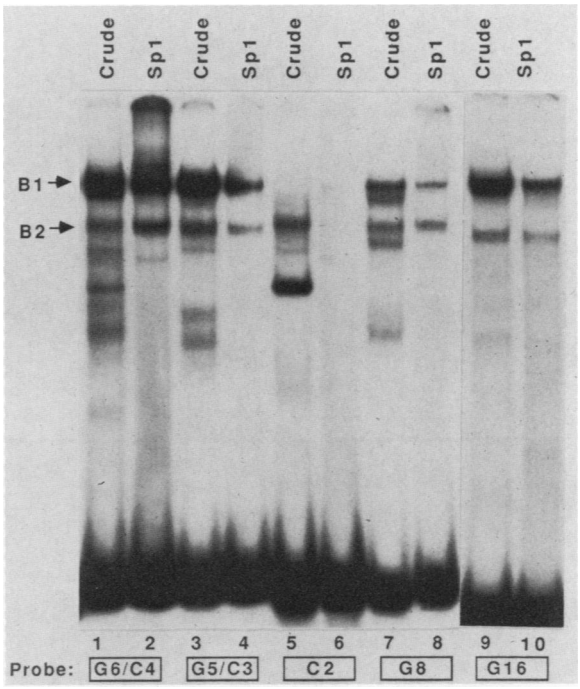

FIG. 4. Interaction of purified Sp1 with four additional regions of the cardiac actin promoter. The indicated labeled probes were incubated with crude C2 nuclear extracts (odd-numbered lanes) or purified preparations of Sp1 (even-numbered lanes) and analyzed by gel shift assay as described in Materials and Methods.

reminiscent of Sp1 sites. Oligonucleotide probes G6/C4, G5/C3, C2, G8, and G16, corresponding to these regions, were synthesized (Fig. 1) and used in gel shift assays with either crude nuclear extracts or the highly purified preparation (>95%) of Sp1 purified from HeLa cells described above. When incubated with Sp1, all probes except C2 showed two shifted complexes that comigrated with the complexes seen with crude extracts (Fig. 4). The probe containing the CArG box 2 (CArG 2) sequences (C2) showed little binding to Sp1 despite the existence of a G+C-rich element. To further examine Sp1 interaction with these sites, gel shift competition studies were performed; the results suggested that G3, G6/C4, and G5/C3 showed high affinity for Sp1, whereas G7 and G1 interacted more weakly and C1 and C2 interacted not at all (data not shown). Thus, Sp1 interacted with various degrees of binding affinity with at least six sites in the 5′-flanking DNA and at one site (G16) within the first exon of the cardiac actin promoter.

To begin to examine Sp1 interaction with cardiac actin promoter elements, methylation interference footprinting was carried out with the G3, G8, and G16 probes, since they had been shown to represent medium- to high-affinity sites for Sp1 interaction. Only the results obtained from the more prominent B1 complex are presented (Fig. 5). Footprint analysis of the B2 complex was identical to that for the B1 complex with all probes (data not shown). Methylation of a number of guanine nucleotides in each probe interfered markedly with Sp1 binding (see Fig. 5). It should be noted that only the G16 site contains a perfect Sp1 consensus in which the core sequence is GGCGGG (33). The footprint of the G8 probe is also unusual in that three rather than two consecutive runs of guanines appear to be involved with Sp1 binding. This finding suggests that the G8 probe may contain two overlapping Sp1-binding sites. The region of the cardiac

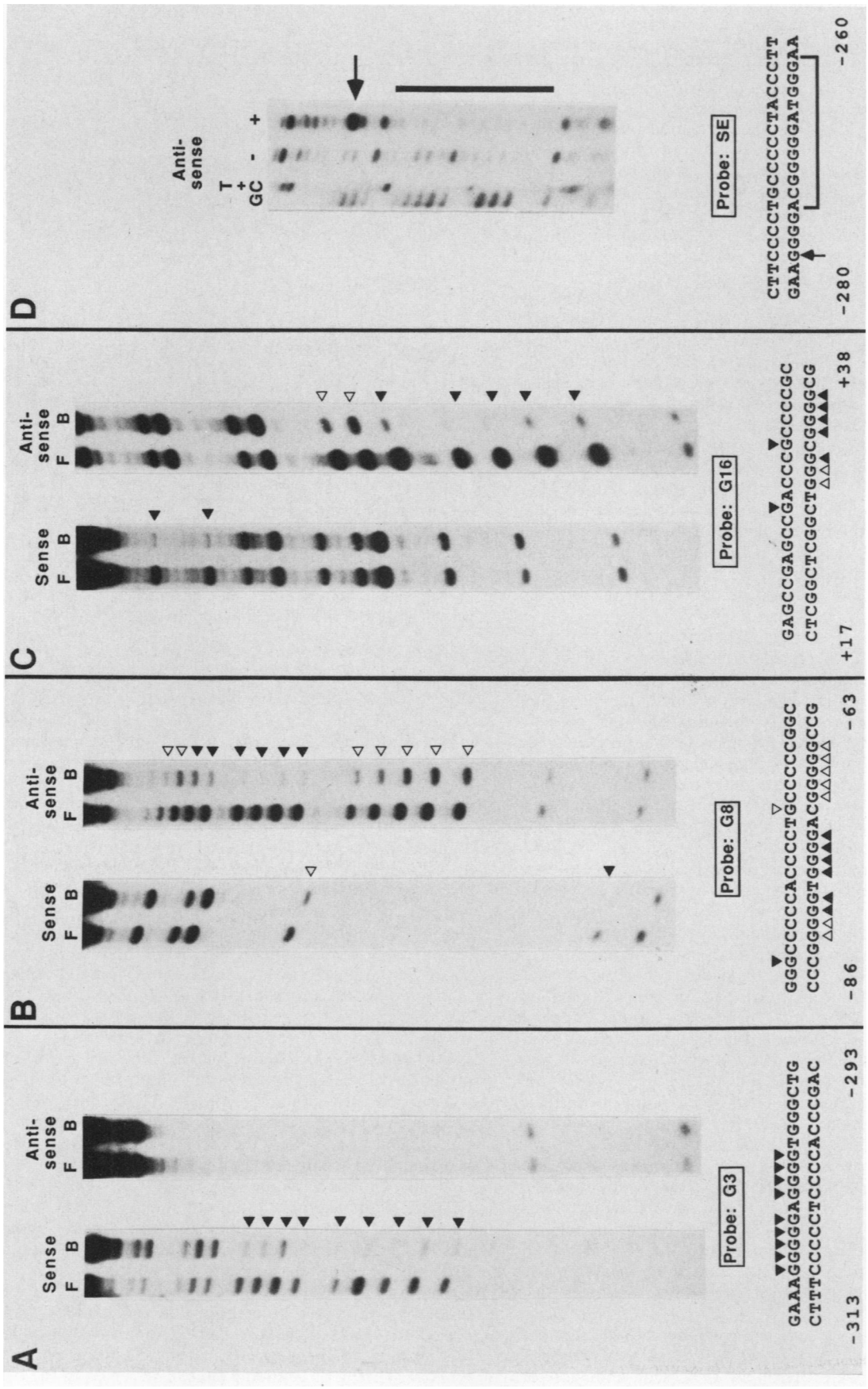

FIG. 5. Footprint analysis of Sp1 interaction with the G3 (A), G8 (B), G16 (C), and SE (D) probes. The G3, G8, and G16 probes were end labeled, incubated with C2 crude nuclear extract, and loaded on gel shift gels, and the resulting complexes were analyzed by methylation interference footprinting as described in Materials and Methods. The sense and antisense strands are indicated. F, Free probe; B, bound probe; ◀ and ◁, nucleotides whose methylation interfered with protein binding strongly and weakly, respectively. The SE probe was end labeled and used in a DNase I protection assay as described in Materials and Methods. The guanine and C+T reactions are shown on the left. −, No added protein; +, reactions to which C2 extract was added.

actin promoter corresponding to the G1 probe competed weakly for Sp1 binding but did not give a gel shift when used as a probe (data not shown). Since methylation interference footprinting could not be done in this case, we used the SE probe, containing the G1 region, in a DNase I protection assay. A clear footprint corresponding to the G-rich core of the putative Sp1 site as well as a hypersensitive site at approximately −277 could be observed. Despite differences from the Sp1 consensus as noted, these four footprints are nevertheless characteristic of Sp1 binding (33), and functional Sp1 sites that do not match the classical consensus have been demonstrated (18, 30).

Interaction of distinct proteins with CArG 1 and 2. It has been previously demonstrated that the CArG box [CC(A+T rich)$_6$ GG] located at −100 interacts with a protein in muscle cell nuclei that is biochemically and immunologically indistinguishable from the nonmuscle SRF, which interacts with the c-*fos* dyad symmetry element (10, 26, 49). To determine whether the other three CArG boxes previously noted by sequence similarity (41) also interacted with SRF, the experiments shown in Fig. 6 were carried out. The probe corresponding to the proximal CArG box (designated C1) was incubated with C2 myotube nuclear extract in the absence or presence of excess specific competitors (Fig. 6A). We first wanted to determine whether any of the other CArG boxes, located at −140 (CArG 2), −191 (CArG 3), and −228 (CArG 4), could compete for SRF binding to CArG 1; therefore, oligonucleotides corresponding to these regions, designated C2, C3, and G6/C4, were used as competitors. When added to the binding reactions at a 200- to 250-fold molar excess, all of the CArG box-containing probes were able to compete for SRF binding to CArG 1 but with markedly differing degrees of efficiency (Fig. 6A). The order of affinity for SRF appeared to be CArG 1 = CArG 3 > CArG 4 >> CArG 2. Conversely, the G3 probe, corresponding to an Sp1 site (Fig. 3), did not compete for SRF binding. The complex of faster mobility seen in lane 2 after self-competition was seen occasionally and appeared to represent a nonspecific interaction, since its binding was not inhibited by excess unlabeled DNA.

The conclusion that CArG 1, 3, and 4 interact with SRF was strengthened by the demonstration that each of these probes showed a complex of indistinguishable mobility when electrophoresed side by side, although the G6/C4 probe showed additional complexes of similar mobilities (data not shown). Surprisingly, the C2 probe, containing CArG 2, showed a gel shift pattern different from those of the other CArG elements (Fig. 6B). This CArG 2-containing probe showed two specific complexes of shifted mobility whose migration was faster than that of the complex observed with CArG 1, 3, or 4. When the CArG 2-containing probe was used in competition experiments, the only competitor that showed any diminution of binding was C2 DNA (Fig. 6B). Thus, CArG 2 could compete weakly for SRF binding to CArG 1 but appeared to interact much more strongly with two other protein complexes that had little or no affinity for the other three CArG elements. It should be noted that we had previously failed to detect protein binding to fragments containing CArG 2 (26). This result was due to the use of pUC19 DNA as a nonspecific competitor rather than poly(dI-dC), as was used in this study. Apparently, pUC19 DNA contains sites with relatively high affinity for the protein(s) that binds CArG 2. In any event, the data shown in Fig. 6 clearly show that under identical binding conditions, CArG 1 and 2 interacted with distinct proteins.

Methylation interference footprinting of CArG 2-binding factors. When the C2 probe containing CArG 2 was used for methylation interference footprinting a distinctive pattern was seen that differed markedly from the pattern observed previously with SRF binding to CArG 1 (26). Methylation of either of two guanines on the sense strand at −152 or −153 completely eliminated protein binding, whereas methylation of the guanine at −145 interfered weakly with binding (Fig. 6C). On the antisense strand, methylation of three guanines was found to interfere with factor binding, although one clear difference was observed between the B1 and B2 complexes. Whereas binding of the factors represented by both complexes was blocked by methylation of guanines at −149 or −151, methylation of the G at −148 interfered only with binding of the B2 complex. Whether this difference was due to interaction with a different protein or to modification of a single factor is unclear. We will refer to these proteins as CBF-2 and CBF-3, as represented by the B1 and B2 complexes, respectively.

The nucleotides that interact closely with these proteins are located within a region of the promoter which has been examined extensively by using deletion and linker scanner mutagenesis techniques combined with transient-transfection assays (43). These data are summarized in Fig. 6D. As shown by 5′ deletion analysis of sequences upstream from −153 and −142, there was a 40 to 60% drop in activity as compared with the parent construct containing nucleotides up to −177 (43). Similarly, a number of linker scanner mutants were analyzed functionally, and the two that showed dramatic reductions in expression (LS3 and LS4) corresponded well to the binding site. Conversely, the two linker scanner mutations that abutted the binding site on either side had little effect (LS1 and LS2). Finally, an internal deletion mutant (ID7) that deleted the entire binding site also showed a marked drop in function. Thus, one or both of the proteins that interacted with this site appeared to be functionally important in the expression of the cardiac actin gene in transient-expression assays. We noted that this region contains on the antisense strand a CTF/NF-I-like site consisting of the sequence 5′-GCCAA-3′. Thus, it is possible that the CBF-2 and CBF-3 proteins belong to the CCAAT-binding protein family. It should be noted that the CArG 2-binding protein factors are clearly different from the CTF/NF-I peptides that bind at −410, since the shifted complexes of the C2 probe showed mobilities much different from that of the G7 probe; also, no competition of the CTF/NF-I band was observed with C2 DNA (data not shown).

Binding of SRF and Sp1 to closely spaced sites near CArG 3 and 4. When the G5/C3 probe was used in gel shift experiments, two protein-DNA complexes of distinguishable mobilities were observed, both of which were eliminated by excess unlabeled G5/C3 DNA in the binding reaction (Fig. 7A). When an excess of unlabeled G3 DNA (corresponding to an Sp1 site) was included as competitor, binding of the faster-migrating complex B2 was eliminated, but the slower-migrating complex B1 was unaffected. The B2 complex therefore represents Sp1 binding. When excess of unlabeled C1 DNA, containing the CArG 1 element (which binds SRF; 26), was included in the binding reaction, only the slower-migrating B1 complex was efficiently competed against, showing that this complex represents SRF binding.

To determine the precise sites of interaction of these proteins, methylation interference footprinting was performed with this probe. To distinguish the methylation interference footprints of the individual proteins, interference assays were performed after competition with DNA fragments containing either Sp1 (G3)- or SRF (C1)-binding

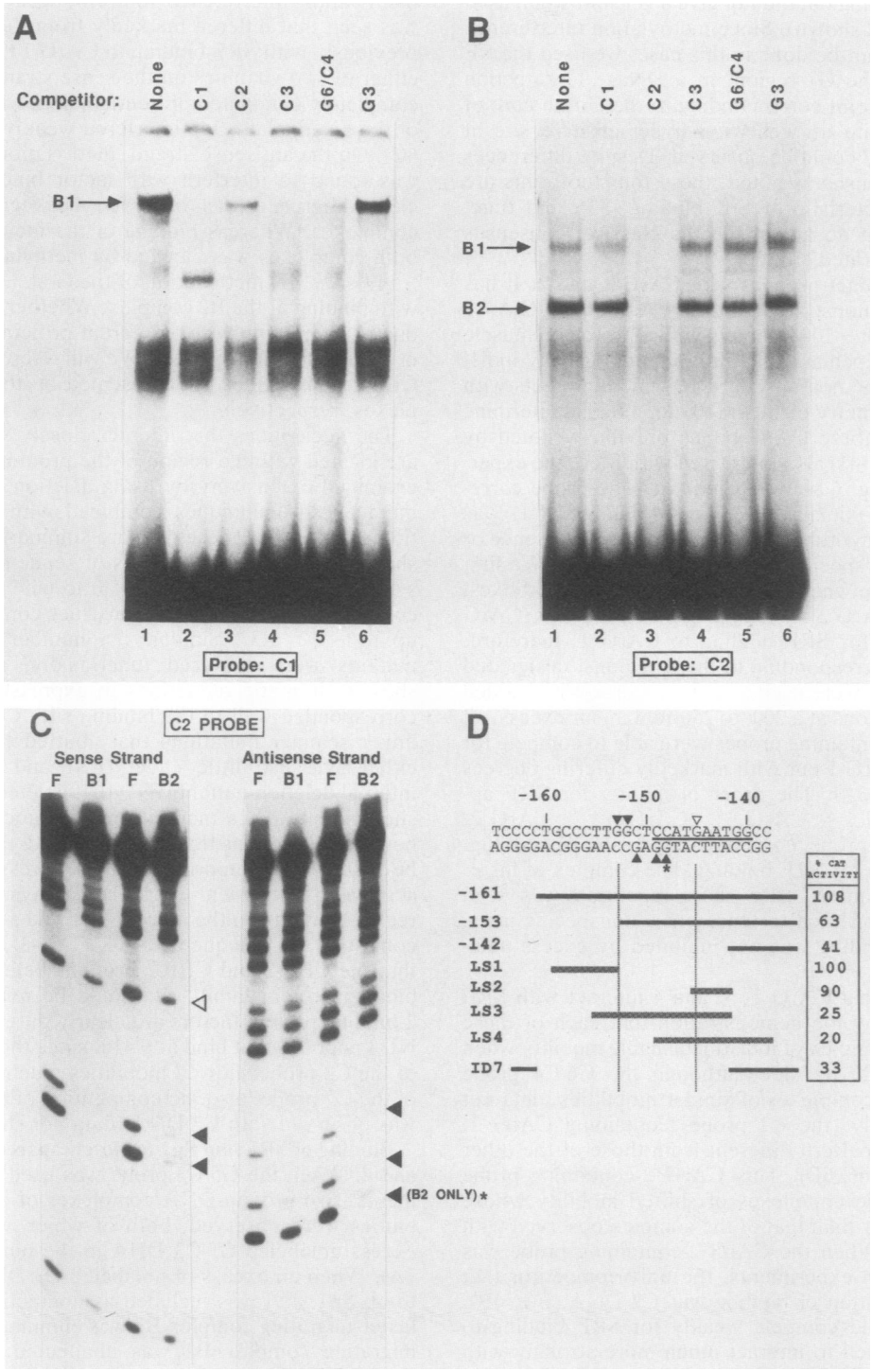

FIG. 6. Gel shift competition analysis of probes containing CArG boxes and methylation interference footprinting of CArG 2. (A) The CArG 1-containing probe (C1) was labeled and analyzed by gel shift assay in the absence or presence of a 200-fold molar excess of each indicated unlabeled DNA. The shifted complex corresponding to SRF binding is shown as B1. (B) A probe containing CArG 2 (C2) was labeled and analyzed by gel shift assay in the absence or presence of a 200-fold molar excess of each indicated unlabeled DNA. B1 and B2 refer to the two specifically competed complexes. See Fig. 2 for locations of the DNAs and Fig. 1 for the sequences of the oligonucleotides. (C) Analysis of the C2 probe by methylation interference footprinting as described in Materials and Methods. F, Free probe; B1 and B2, retarded complexes shown in Fig. 9B. (D) Summary of methylation interference and functional data. *, Nucleotide whose methylation interfered with binding of the B2 complex only. A summary of previous unidirectional 5′ deletion, linker scanner mutation (LS), and internal deletion (ID) data is presented with the representative CAT activities in relation to the wild-type construct (43). Bars represent positions of the deletions and mutations introduced into the promoter.

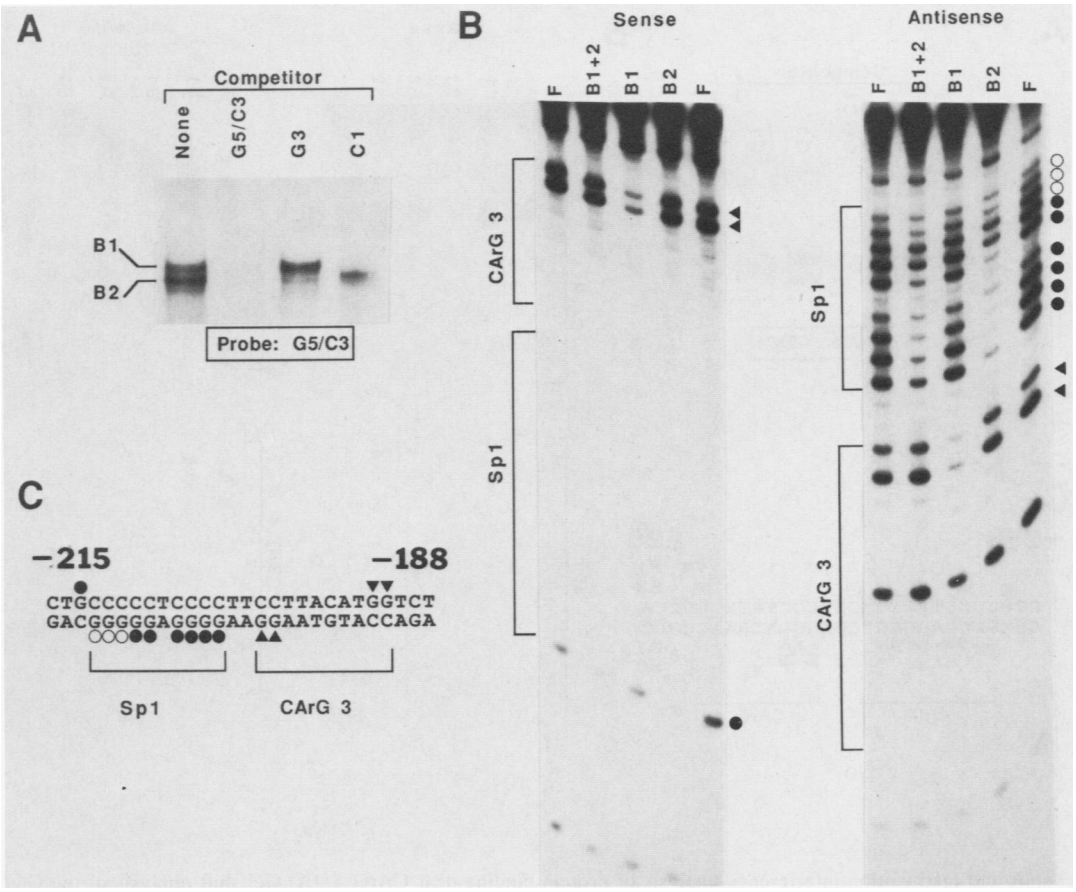

FIG. 7. Gel shift and methylation interference analysis of protein binding near CArG 3. (A) Gel shift analysis of the G5/C3 probe after competition with self, G3 (Sp1 site), and C1 (SRF site) DNAs. Only the shifted complexes are shown. (B) Methylation interference analysis of the B1 and B2 complexes. B1+2, Footprinting of both complexes together. B1 and B2 were footprinted separately after G3 or C1 competition to remove the Sp1- or SRF-binding component, respectively. Symbols: ● and ○, bases whose methylation interfered strongly and weakly, respectively, with Sp1 binding; ◀, bases that interfered with SRF binding. (C) Summary of methylation interference data.

activity. In this manner, each complex could be isolated from the gel with little or no cross-contamination of the other complex. When the G5/C3 probe was used, the footprint pattern of complexes B1 and B2 together showed an Sp1-like pattern in which methylation of consecutive runs of guanines interfered with protein binding (Fig. 7B and C). When the B1 complex was footprinted after competition of Sp1 binding with the G3 oligonucleotide, a very different pattern was revealed in which methylation of each pair of guanine nucleotides at either end of the CArG box interfered with binding. This pattern was identical to that observed with SRF binding to CArG 1 (26). Conversely, when the B2 complex was footprinted, the Sp1-like footprint was revealed. Thus, Sp1 and SRF can interact with this small region of DNA and interact closely with nucleotides that are separated by at most 2 base pairs. (Extracts varied as to the relative levels of Sp1 and SRF [see below]; also, the extract used for the footprinting pattern seen in Fig. 7B had more Sp1 than SRF, and the footprint of the B1-plus-B2 complexes therefore showed primarily the B2 or Sp1 footprint.)

As in the case of the G5/C3 probe, the G6/C4 probe, containing CArG 4, was also shown to bind Sp1 (Fig. 4). In the case of G6/C4, three protein-DNA complexes were evident. The B1 and B2 complexes could be competed against by DNAs containing SRF- and Sp1-binding sites, respectively, in a manner identical to that observed with the G5/C3 probe (Fig. 8A). Therefore, these complexes represent SRF and Sp1 binding, respectively. The B3 complex, although slightly diminished by addition of C1 competitor, was competed against efficiently only by self DNA and therefore represents a third and distinct protein-DNA complex. Methylation interference footprinting was again performed after competition with the C1 and G3 DNAs in order to footprint the three individual complexes. The B1 footprint was characteristic of SRF binding in that each set of GG nucleotides at both ends of the CArG box interacted closely with the protein (−228 and −229 sense; −236 and −237 antisense) (Fig. 8B). In this case, though, two additional G's, located at −235 antisense and at −240 sense, were also involved in protein binding. As expected, B2 gave a footprint characteristic of Sp1 in which two consecutive runs of guanines separated by an adenine interacted closely with Sp1. The footprint of B3 was somewhat surprising in that it was contained within the SRF footprint. The B3 footprint was nevertheless clearly distinct from that of B1. Whereas methylation of the guanine (−229 sense) interfered with binding of both the B1 and B3 complexes, methylation of the neighboring guanine (−228 sense) interfered only with B1 complex binding and, in fact, enhanced binding to the B3 complex, as shown by the increased intensity of this base in the interference assay. Analysis of the antisense strand showed that methylation of the three guanines also inter-

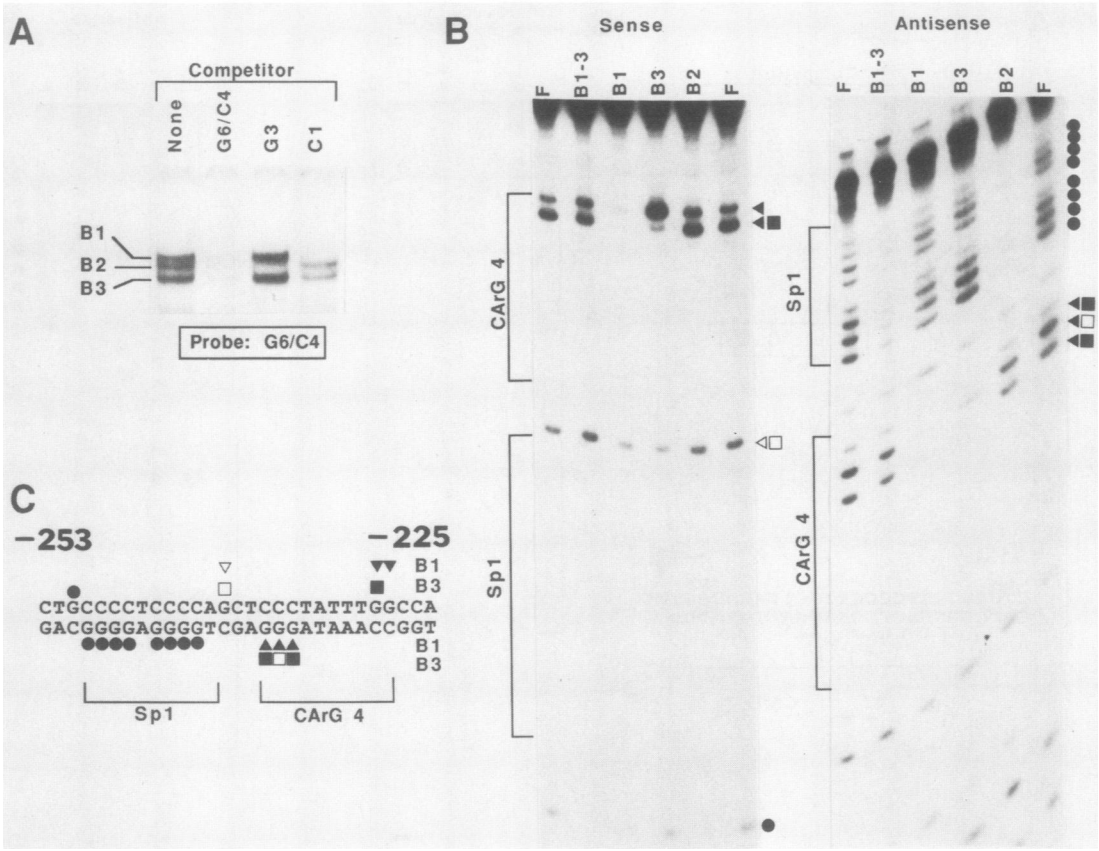

FIG. 8. Gel shift and methylation interference analysis of protein binding near CArG 4. (A) Gel shift analysis of the G6/C4 probe after competition with self, G3, and C1 DNAs. Only the shifted complexes are shown. (B) Methylation interference analysis of the B1, B2, and B3 complexes. See the legend to Fig. 7 for explanation. Squares indicate the B3 protein complex. (C) Summary of methylation interference data.

fered with binding of both complexes, although methylation of the central G consistently interfered less with B3 binding than with B1 binding. The differences between the B1 and B3 footprints and the lack of competition of B3 by C1 DNA showed that a protein distinct from SRF interacted with an overlapping site centered at CArG 4. In addition, the complex represented by B3 was not affected by competition with probes containing CArG 2 or 3 (data not shown). Thus, CArG 4 interacts with SRF and a second unidentified protein that we designate CBF-4. In addition, an Sp1 site is located 2 to 4 base pairs upstream of CArG 4, reminiscent of the juxtaposition of an Sp1 site immediately upstream of CArG 3.

Interaction of the TATA box region with an unidentified protein and Sp1. When the G17 probe, corresponding to the TATA box region, was used in gel shift assays, three specific complexes of retarded mobility were observed (Fig. 9A). Unexpectedly, the G3 DNA, which contains an Sp1 site, was found to compete for binding to the shifted complex of slowest mobility (B1). To confirm this finding, the G17 probe was incubated with purified Sp1 and found to bind, giving rise to the two characteristic Sp1 complexes, the fastest of which comigrated with the B3 complex (Fig. 9A). These results were particularly surprising because sequences contained within this DNA region showed little similarity to consensus Sp1-binding sites.

To define the sequences involved in Sp1 binding as well as the proteins that gave rise to the B2 and B3 complexes (designated TUBF, for TATA upstream binding factor), methylation interference footprinting was carried out (Fig. 9B and C). Each complex was footprinted independently, and the B2 and B3 complexes were footprinted after removal of the Sp1 component by addition of excess unlabeled G3 DNA. The B1 complex representing Sp1 was found to footprint immediately downstream of the TATA box and made close contact with 10 purine nucleotides, located between nucleotides −25 and −16. This is an unusual site, since it does not contain two extended runs of guanines, as is typical for Sp1, but does contain an extended run of purines whose methylation clearly interfered with Sp1 binding. The footprints for complexes B2 and B3 were identical, and both complexes were found to footprint immediately upstream of the TATA box. Specifically, methylation of 6 nucleotides between −43 and −35 interfered with protein binding (Fig. 9C).

Protein-binding activities are not muscle specific, although Sp1-binding activity is reduced during myogenic differentiation. To determine whether any of the proteins identified in this study were expressed only in muscle cells, extracts were prepared from HeLa cells, and the gel shift patterns were compared with those obtained from C2 cells. We have previously shown that indistinguishable forms of SRF are expressed in C2 and HeLa cells (26). All of the probes shown to bind proteins in this study were found to bind nuclear proteins prepared from both cell types (Fig. 10A). Binding to the G7 probe was examined in the absence and presence of

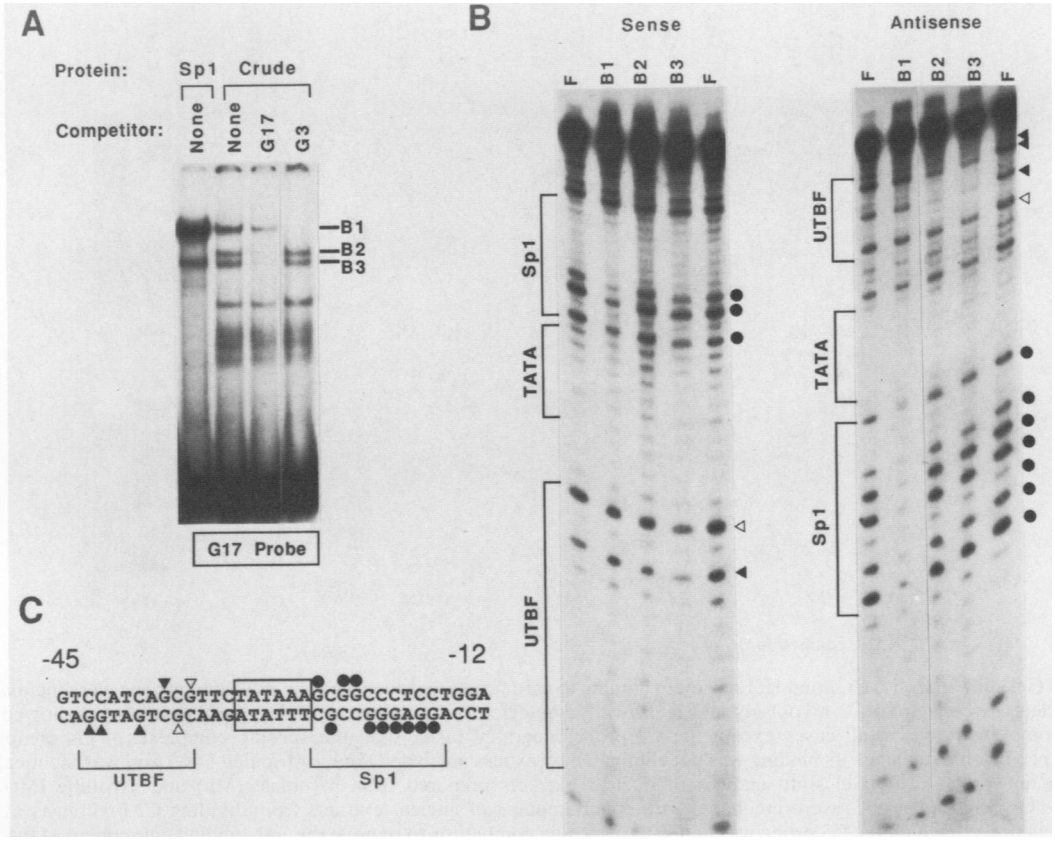

FIG. 9. Gel shift and methylation interference analysis of protein binding near the TATA box. (A) Gel shift analysis with purified Sp1 and C2 crude nuclear extracts, using the G17 probe. Competition experiments were performed with self and G3 DNAs at a 100-fold molar excess. (B) Methylation interference footprinting of Sp1 and TUBF interaction. B2 and B3 were footprinted after G3 competition to remove the Sp1 component from B3. Shown are bases whose methylation interfered with Sp1 binding (●) and with TUBF binding (◁, ◀), as well as the location of the TATA box. (C) Summary of methylation interference data.

excess unlabeled G3 DNA in order to examine Sp1 and CTF/NF-I binding independently (see legend to Fig. 3 for further explanation). It is clear that C2 and HeLa cells showed different patterns of CTF/NF-I binding to the G7 probe. The gel-shifted complex from HeLa cells was much broader, and the majority ran slower than did the CTF/NF-I species present in C2 nuclei. Sp1 was expressed in both cell types, as shown by indistinguishable patterns of protein interaction with the G3 probe. To examine CBF-4, the G6/C4 probe was used in gel shift assays after addition of excess G3 DNA. This was done to remove the Sp1 component (B2) such that the B1 and B3 complexes, representing SRF and CBF-4, respectively, would be more clearly seen (Fig. 8). HeLa extracts contained similar levels of SRF and CBF-4. HeLa cells also showed similar levels of CBF-3, as measured by binding of complex B2 to the C2 probe. Upon longer exposure of the autoradiograms, HeLa cells were also found to express low levels of CBF-2, corresponding to the complex of slowest mobility, with the C2 probe (data not shown). Use of the G17 probe showed that both cell types contained similar levels of TUBF and Sp1. HeLa extracts routinely showed an additional complex of retarded mobility that was evident as a faster-migrating complex in all HeLa lanes. This complex was identical for all probes examined and appeared to represent a sequence-independent interaction, since it was not efficiently competed against by excess unlabeled probe, nor did it show a footprint in methylation interference assays (data not shown).

To determine whether changes in cardiac actin gene expression during myogenic differentiation might be due to alterations in transcription factor-binding activity, nuclear extracts were prepared from C2 cells at early and late stages of in vitro differentiation and compared in the gel shift assay. The binding activities of CTF/NF-I (faster-migrating G7 complex), SRF and CBF-4 (slower- and faster-migrating G6/C4 complexes), CBF-2 and CBF-3 (C2 complexes), and TUBF (G17 doublet) remained relatively constant during differentiation (Fig. 10B). The only consistent change appeared to be a drop in Sp1-binding activity as the cells fused and became differentiated myotubes. This was particularly apparent with the G7 and G17 probes, which showed similar levels of the CTF/NF-I and TUBF, respectively, but little or no Sp1 binding in the myotube extract. It should be noted that the results of these assays may not reflect a drop in Sp1-binding activity; it is equally possible that binding of the other proteins may have increased with respect to Sp1. Whatever the explanation, it is clear that in vitro binding activities of these proteins changed with respect to one another as myogenic differentiation proceeded.

DISCUSSION

In this paper, we report the identification of multiple sites of protein interaction with the human cardiac actin promoter. Further, we have identified three of these proteins, CTF/NF-I, Sp1, and SRF, the latter two of which were

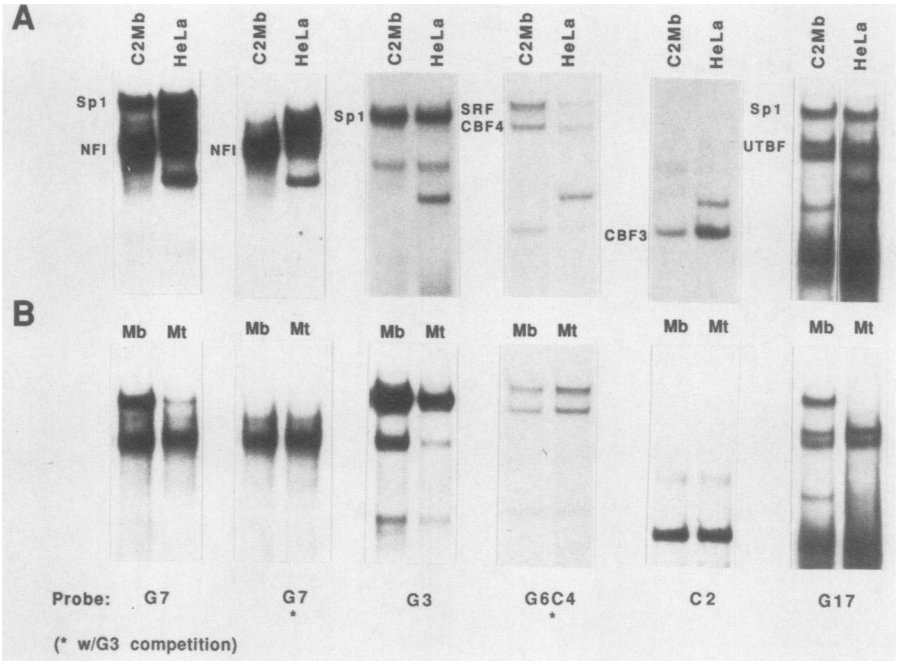

FIG. 10. (A) Gel shift analysis of C2 and HeLa protein binding to cardiac actin sequences. The indicated probes were incubated with equal amounts of nuclear extracts from C2 myoblasts and HeLa cells. As discussed in the text, HeLa extracts routinely showed an additional protein-DNA complex which, in all cases except the C2 probe, migrated faster than the specific complexes. This complex represents non-sequence-specific binding, since its binding was not eliminated by excess unlabeled double-stranded DNA and was identical for all probes examined (data not shown). (B) Gel shift analysis of nuclear extracts prepared from myoblast (Mb) and myotube (Mt) stages of C2 differentiation. The indicated probes were incubated with equal amounts of nuclear extracts from dividing C2 myoblasts or differentiated myotubes. *, Reactions to which the G3 probe was added as a specific competitor to remove the Sp1-binding component of the G7 and G6/C4 probes that interacted with more than one protein in order to more easily visualize the other binding factors.

found to interact with multiple sites on the promoter. A diagrammatic model of the interaction of these proteins is shown in Fig. 11, in which the sites of protein interaction are superimposed on a representation of the relative CAT activities of a series of unidirectional 5′ deletions (41, 43). It is clear from the footprinting data that a number of different proteins interact with regions of the promoter whose deletion results in markedly decreased function in transient-transfection assays. These proteins include CTF/NF-I and Sp1 at −410, CBF-2 and CBF-3 at −140, and SRF at −100. The relationship between footprinting and functional data is particularly clear for CBF-2 and CBF-3 (Fig. 6D), although the relative contribution of these two proteins is unclear. The functional significance of the Sp1-, SRF-, and CBF-4-binding sites located between −191 and −313 is not as obvious because of the relatively small loss of CAT activity

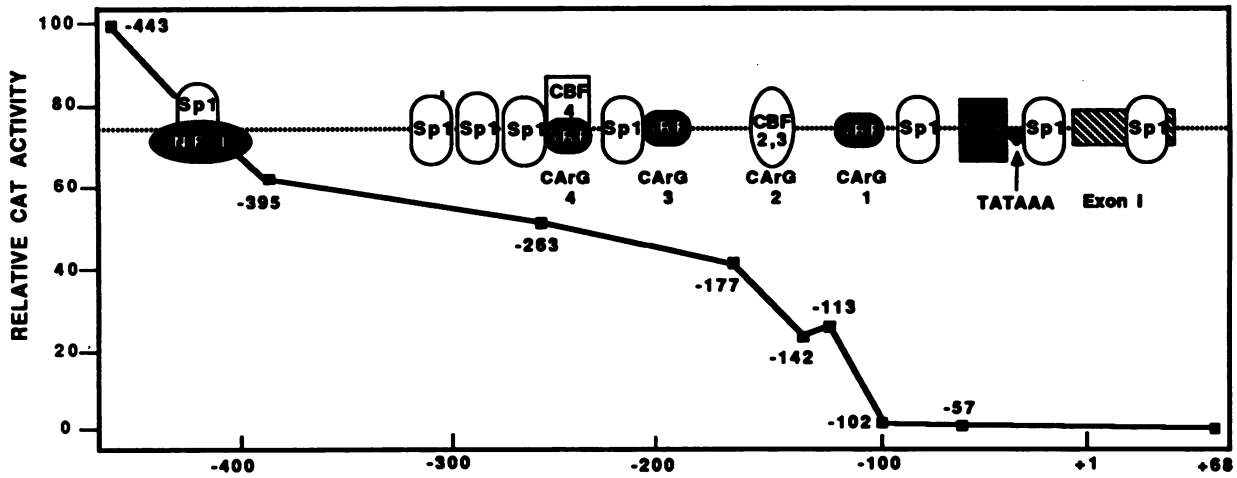

FIG. 11. Comparison of protein-binding and functional data. The protein-binding sites demonstrated in this study are superimposed on the functional activities of a series of 5′ deletion mutants (41, 43).

as this region is deleted. It is possible that these proteins serve to form a more stable transcription complex and might, for example, act to promote interaction of the upstream regulatory element (−443 to −395) with basal promoter elements. In this event, deletion of these binding sites would not be expected to show further decreases in CAT activity, since the upstream functional element has already been deleted.

It is clear that the majority of the Sp1 sites are not located in regions of the promoter whose removal results in dramatic losses of activity in transient-expression assays (Fig. 11). This suggests a number of possibilities. First, Sp1 may not act as a positive modulator of cardiac actin gene regulation. Although to our knowledge negative regulation by Sp1 has not been reported, our demonstration that Sp1 binds at interdigitating or overlapping sites, including those which bind SRF (at CArG 3 and 4), CBF-4 (at CArG 4), CTF/NF-I (at −410), and near the TATA box, suggests that steric constraints are placed on DNA binding by these proteins by virtue of their proximity with Sp1 sites. The demonstration that Sp1 binding appears to drop during myogenic differentiation (Fig. 10) might suggest a model by which Sp1 acts as a negative modulator of actin transcription in the undifferentiated state by exclusion of the factors mentioned above. This may be an oversimplistic view, and Sp1 may in fact act in a positive manner but require interaction with additional factors to be active.

The unusual sequences with which Sp1 interacts on the cardiac actin promoter also deserve comment. The generally accepted Sp1 consensus as proposed by Kadonaga et al. (33) is

$$\frac{G}{T}GGGCGG\frac{GGC}{AAT},$$

whereas the cardiac actin Sp1 sites show the slightly different consensus,

$$\begin{array}{ccccccccccc} G_7 & G_7 & G_7 & G_5 & A_5 & G_7 & G_7 & G_7 & G_5 & G_4 \\ & & C_1 & C_1 & & & & C_1 & C_2 & \\ & & T_1 & T_1 & & & & T_1 & T_1 & \end{array}$$

when the Sp1 sites (excluding the G17 site) are aligned. Thus, for the sites outlined in this report, two consecutive runs of three guanines separated by an adenine appear to be preferred. In fact, only one site contains the core consensus GGGCGG (in exon I). Similar differences in recognition sequences for Sp1 that lack the core consensus and retain function have been demonstrated (18, 31).

The general phenomenon of overlapping protein-binding sites, which appears to be a recurring theme on the cardiac actin promoter, is reminiscent of numerous procaryotic regulatory mechanisms (for a review, see reference 51). One of the more unusual of the overlapping sites examined in this report involves Sp1 and CTF/NF-I binding at −410. Footprint analysis showed that four nucleotides interacted closely with both proteins, suggesting that binding must be mutually exclusive. If both of these proteins are important for gene regulation, each of their binding domains is likely to be conserved in evolution. Although limited upstream sequence information is available, it is clear that these sequences have been conserved between humans and mice. The sequences are as follows, with the binding site underlined (21, 41):

Thus, species as divergent as humans and mice show extensive conservation, and the nucleotides critical for Sp1 and CTF/NF-I binding have been conserved despite considerable divergence of the surrounding sequence.

The demonstration that at least three proteins besides SRF could interact with the CArG elements of the cardiac actin promoter in vitro suggests another possible point of regulation of this gene. The CBF-2 and CBF-3 proteins, which interact with the second CArG element, show a footprint pattern similar to that of the MAPF proteins, identified by Walsh and Schimmel (58), which interact with the most proximal CArG box of the chicken skeletal actin gene. Although the footprint and gel shift patterns are similar, these appear to be different proteins since in our hands HeLa cells showed the same gel shift pattern as did muscle cells (Fig. 10A), whereas the MAPF proteins reported by Walsh and Schimmel (58) showed distinct gel shift patterns between muscle and nonmuscle cells. In addition, the proximity of the CArG 2 footprints to the CCAAT box binding consensus GCCAA on the antisense strand suggests that the proteins may be members of the CTF/NF-I family of proteins that are distinct from the CTF/NF-I proteins which bind at −410. Further experiments will be required to answer this question. It is also interesting that despite the demonstration that CArG 1, 3, and 4 interact with SRF, only CArG 1 appears to be functional in CAT assays using deletion constructs after transient transfection into C2 myoblasts (41). This result could be due to the close interaction of Sp1 with CArG 3 and 4, which may sterically hinder SRF binding; alternatively, it is possible that the SRF-binding sites are functionally redundant and that only a single CArG element which binds SRF is required for promoter activation.

We have previously suggested that CArG 1 and 2 interact with the same factor (42, 43). This notion was based on in vivo competition experiments showing that cotransfection with a large molar excess of DNA fragments that contained CArG 2 could compete against activity of a CAT construct containing only CArG 1 (42). Therefore, it was somewhat surprising that these elements bound different factors in gel shift assays under identical binding conditions. Nevertheless, the affinity differences noted in the in vivo competition experiments in which CArG 2 was a weaker competitor than CArG 1 are compatible with the results presented in Fig. 6A, which show that CArG 2 can compete weakly for SRF binding to CArG 1.

Despite the clear mobility differences noted between C2 and HeLa CTF/NF-I proteins, the finding that the remaining functional DNA regions all appear to interact with ubiquitous transcription factors that are present in most if not all cell types does not explain why the cardiac actin promoter is expressed at high levels only in cells of muscle origin (41, 43). It is likely that combinations of regulatory mechanisms play roles in determining the cell specificity of mammalian gene expression. A number of recent studies (1, 27, 29, 44, 52) suggest that tissue specificity may be a result of subtle differences in transcription factors that may not always be evident by in vitro DNA binding assays, and it remains possible that extremely diverse patterns of differential gene regulation are regulated by ubiquitous factors that are in fact much different from cell type to cell type.

```
Human  CTCAGCAGAGGGCAGGGCGCCAAGCCTTCCCACTGCA (−428 to −392)
        X  XX  X              X      XX XXXX
Mouse  TTCTCCA-AGGGCAGGGCGCCAAGTCTTCCGGCATTG (−462 to −427)
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