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The human skeletal α-actin gene is regulated by a muscle-specific enhancer that binds three nuclear factors

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The tissue-specific distal promoter of the human skeletal α-actin gene (−1282 to −708) induces transcription in myogenic cells approximately 10-fold and, with the most proximal promoter domain (−153 to −87), it synergistically increases transcription 100-fold (Muscat and Kedes 1987). We report here that it is a short fragment of the distal promoter, the distal regulatory element (DRE) from −1282 to −1177 that functions as a muscle-specific, composite enhancer. An internal deletion in the DRE (Δ−1282/−1151) in the context of the full-length 2000 bp promoter, resulted in a 10-fold reduction in transcription. Three distinct nuclear proteins, DRF-1, DRF-2 and DRF-3, interact specifically with the DRE between positions −1260 and −1193. A site specific mutation that abolishes DRF-2 binding also results in a 10-fold reduction in transcriptional activity. The DRF-2 nuclear protein has characteristics similar to those of the muscle-specific regulatory factor, MEF-2 (Buskin and Hauschka 1989; Gossett et al., 1989). Like the MEF-2 binding site in the muscle creatine kinase enhancer, the critical DRF-2 binding site is also an A/T-rich sequence element. The DRF-2 nuclear protein binds equally well to the MCK MEF-2 binding site and to the A/T-rich regulatory element of the skeletal muscle fast-twitch troponin C gene (Gahlmann and Kedes 1990). Furthermore, this troponin C site competes in vivo for DRF-2 driven expression of the skeletal α-actin gene in C2 cells. The DRF-2 site alone, however, does not activate transcription in muscle cells when linked to the SV40 promoter. We conclude that the DRF-2 binding element is a MEF-2 binding site that is required but insufficient for regulation of muscle-specific skeletal α-actin gene expression by the DRE. Thus, muscle-specific regulation of the human skeletal α-actin gene appears to require interactions between the other elements of the composite DRE enhancer with the protein:DNA complex formed by DRF-2.

Tissue-specific gene expression, the hallmark of cellular differentiation and organismal development, often is mediated through the interaction of sequence-specific DNA-binding proteins and their cis-acting target sequences (Poellinger and Roeder, 1989; Tanaka and Herr, 1990). Myogenesis is an excellent paradigm for investigation of mechanisms governing differentiation via tissue- and developmental stage-specific gene regulation. The fusion of proliferating myoblasts to form myofibers is accompanied by the temporal regulation of genes encoding a structurally diverse group of proteins that form the muscle phenotype. These events are charac-

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terized by a sequence of isoform transitions typified by the actin multigene family. During myogenic differentiation the cytoskeletal β- and γ-actins are down regulated. In contrast the sarcomeric cardiac and skeletal α-actins are vigorously co-expressed but with distinct tissue specific and developmental patterns in heart and skeletal muscle (Hayward and Schwartz, 1982; Gunning et al., 1983; Bains et al., 1984; Vandekerckhove et al., 1986; Gunning et al., 1987). During development, cardiac α-actin appears to be the equivalent of a fetal isoform in both skeletal muscle and heart (Minty et al., 1982). In adult human skeletal muscle the skeletal isoform prevails, accounting for 95% of the α-actin proteins (Gunning et al., 1983). Conversely, in adult cardiac muscle the cardiac isotype predominates, although the level of the skeletal isoform (which can account for as much as 50% of the α-actin transcripts) seems related to the physiological state and the size of the mammal studied (Gunning et al., 1983; Vandekerckhove et al., 1986).

Our previous work (Muscat and Kedes, 1987; Muscat et al., 1988) demonstrated that 1300 base pairs of the 5′ flanking sequences of the human skeletal α-actin gene are sufficient and necessary for both optimal muscle specific expression and regulation during differentiation of myogenic cells in culture. There are at least two dominant transcriptional domains within this 1300 bp promoter: a *proximal*, cis-acting element located between position −153 and −87 relative to the start of transcription at +1, and a *distal* element located between positions −1282 and −708. These regulatory elements each induce transcription about 10-fold and synergistically increase expression 100-fold over levels achieved by the sequences 3′ of −87 (Muscat and Kedes, 1987). Furthermore, the two cis-acting elements independently and synergistically can modulate an enhancerless heterologous SV40 promoter in a tissue specific manner (Muscat and Kedes, 1987). The distal DNA region appears to be differentially utilized for maximal expression in different myogenic cell lines (Gunning et al., 1983; Bains et al., 1984; Hickey et al., 1986; Minty et al., 1986) and primary cells (Hayward and Schwartz, 1982; Gunning et al., 1987). Its deletion, for example, has minimal effect on promoter expression in myogenic L8 cells. One conclusion of these studies was that the particular combination of domains used may be dependent on the qualitative and quantitative availability of trans-acting transcription factors present in each cell type. This could account for the different and complex modulatory programs of actin gene expression observed during muscle differentiation both in vitro and in vivo.

We have now further defined the nature of the muscle-specific distal regulatory element: a 105 base pair upstream region of the skeletal α-actin gene from −1282 to −1177 appears to be responsible for high level muscle cell-specific expression. Furthermore, the element functions as a classical tissue-specific enhancer. Three distinct proteins, or protein complexes—DRF-1, DRF-2 and DRF-3—interact specifically with defined DNA segments in this region. Mutations in the A/T-rich DRF-2 binding site and in vivo competition studies establish this site as a necessary muscle-specific transcription element.

Materials and methods

Cell culture and transfection

Myogenic mouse C2 and rat L8 cells (Yaffe and Saxel, 1977a,b) and HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with fetal calf serum in 10% CO_2 as described previously (Muscat and Kedes, 1987; Muscat et al., 1988). Myogenic and HeLa cells were grown with 20% and 10% fetal calf serum respectively. The C2 and L8 cells were harvested at 50% confluency for myoblasts. Myogenesis was induced by transfer of proliferating myoblasts into serum depleted medium (DMEM containing 2% horse serum). Myotube formation was essentially complete for C2 and L8 cells 2.5 and 5.0 days after serum depletion, respectively. For transfections each 100 mm dish of ~60% confluent C2 myoblasts in 20% fetal calf serum was treated with 15 μg of DNA using calcium phosphate mediated gene transfer; 16 hours after transfection the medium was changed to 2% horse serum in DMEM medium and the cells were allowed to differentiate for an additional 48 hours before harvesting for chloramphenicol acetyl transferase (CAT) analysis (see Muscat and Kedes, 1987; Muscat et al., 1988 for details). At least two independent preparations of each DNA construct were assayed in duplicate or more often. CAT assays were performed according to the method of Gorman et al. (1982), and the percentage of acetylation was quantitated by scintillation count of thin layer chroma-

tography plates or with the AMBIS Dual Radioanalytic Imaging System.

Plasmid DNAs and oligonucleotides

The distal regulatory region of the human skeletal α-actin gene from −1284 to −708 was excised from pHSA2000CAT by cleavage with Xba I and Pvu II restriction endonucleases. This fragment was cloned into pUC19 cleaved with Xba I and Sma I. This recombinant clone is denoted as pHSA−1282/−708. The distal regulatory region contained Xba I, Fnu 4HI, Pst I and Sty I recognition sites at positions −1282, −1228, −1226 and −1177 respectively. These restriction sites were utilized to isolate shorter fragments and to end-label DNA fragments at either end.

The plasmids pHSA2000CAT Δ−1282/−1150 and pHSA2000CAT Δ−1282/−1089 were constructed by the digestion of pHSA−1282/−708 with the restriction enzyme Xba I, treatment with Bal 31 nuclease and T4 DNA polymerase, followed by the ligation of Xba I linkers. The subsequent products were cleaved with the restriction enzyme Sac I; the fragments released by enzyme digestion were isolated after electrophoresis and cloned into pHSA2000CAT digested with Xba I and Sac I which cleaved at unique sites at nucleotide positions −1282 and −626 respectively. The extent of the deletions was determined by nucleotide sequencing. These plasmids also carried the deletion Δ−708/−626; however, 5′ unidirectional deletion of these nucleotides previously has been shown to have little or no effect on transcription (Muscat and Kedes, 1987). The plasmid denoted as pHSA2000CAT LC was constructed by cloning of a DNA fragment that was not digested by Bal 31 and that went through the construction protocol unaffected. This fragment was cloned into the vector and acted as a ligation control during transfection; it carried a single deletion Δ−708/−626. This construct served as a control for the lack of functional importance of the sequences between −708 and −626.

The primers pΔ−1282/−1261, pΔ−1282/−1261 M1, and pM2 were synthesized and used in polymerase chain reactions (PCR) with a primer 3′ of the Sac 1 restriction site at nucleotide position −626. The subsequent PCR products were then cleaved with Xba I and Sac I and cloned into the wild-type plasmid cleaved with the same restriction endonucleases. Table 1 shows the primers used (deleted or mutated bases are indicated by underlining). The DNA sequences of these clones were confirmed using double-stranded sequencing and the T7 DNA polymerase system (Pharmacia).

Enhancer containing constructs were created as follows: The Xba I/Sty I fragment from −1282 to −1177 was blunt-ended and inserted with appropriate linkers into the Bgl II site of pCAT promoter (Promega) upstream of the CAT gene or into the Sph I site downstream of the CAT gene in both sense and antisense orientations. The DRE-2 site was tested for enhancer activity by cloning a double stranded 25 bp oligonucleotide (corresponding to bp −1250 to −1231 of the enhancer) with Bgl II overhanging ends into the Bgl II site of the pCAT promoter test construct upstream of the CAT gene.

Nuclear extracts and gel electrophoresis mobility shift (GEMS) assays

Nuclear extracts were prepared by the method of Dignam et al. (1983), with the addition to all solutions of 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 2 μg/ml of Aprotinin and Leupeptin (Calbiochem). Nuclear proteins were extracted with 0.4 M NaCl. Extracts were finally dialyzed against 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid, pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF and 2 μg/ml Aprotinin and Leupeptin. Protein concentrations were measured by the method of Bradford (1976). Each binding mixture (25 μl of dialysis buffer) contained 1–2 ng of a T4 polynucleotide kinase-labeled DNA fragment, 5–10 μg of protein, 5 μg of BSA, and either 2 or 3 μg of pUC18 plasmid DNA digested with MspI or poly(dI-dC)·poly(dI-dC) as a non-specific DNA

Table 1. Primers for construction of DRE mutant plasmids.

pΔ−1282/−1261	5′ GCTCTAG_____AGCCCCATATATCAGTGATATAAATAGAACCTGC 3′
pΔ−1282/−1261 M1	5′ GCTCTAG_____AGCCCCATATATCACCCATACCAATAGAACCTGC 3′
pM2	5′ GCTCTAGAAAATCTGAAAGGCATAGCCCCATATATCAGTGATCCCCATAGAACC 3′

competitor (Gustafson and Kedes 1989). The mixtures were incubated at room temperature for 20 minutes and electrophoresed through a 4% or 6% (20:1 poly-acrylamide:bis-acrylamide) gel in 80 mM Tris borate and 2 mM EDTA. Gels were then soaked in 10% acetic acid, dried, and autoradiographed (Fried and Crothers, 1981).

Methylation interference footprinting

The DNA probes were partially methylated with DMS (dimethylsulphate) for 3 to 5 minutes. Binding reactions and electrophoresis were as described for the gel mobility shift assay, except 10–15 reactions were pooled in order to augment the signal. After electrophoresis, the DNA was located by autoradiography of the wet gel for 4–10 hours, and the bound and free bands were excised from the gel. The DNA was eluted by electrophoresis and purified over DEAE elutip minicolumns (Schleicher and Schuell) as described by the manufacturer. The DNA was further treated with piperidine prior to denaturation and electrophoresis on 8–10% polyacrylamide–45% urea gels.

Results

Location of the skeletal α-actin gene distal regulatory element

In the C2 cell line, expression of the skeletal α-actin gene relies on the distal regulatory region (−1282 to −708) for almost 90% of its activity (Muscat and Kedes, 1987). We evaluated the role of the sequences between −1282 and −708 using a series of constructs with increasingly large internal deletions extending from −1282. Two of these mutant plasmids were designated pHSA2000CAT Δ−1282/−1150 and pHSA2000CAT Δ−1282/−1089 (see Materials and Methods for construction details). Three independent isolates of these internal deletions, plus the ligation control plasmid pHSA2000CAT LC and the wild-type promoter pHSA2000CAT, were transfected into C2 myoblasts. The CAT activities were measured after differentiation. The deletion of either segment from the wild-type promoter resulted in an approximately 6- to 10-fold reduction in transcription compared to the control constructs (Fig. 1, lanes 3 to 8). Reporter gene constructs carrying longer extensions of the internal deletion to include nucleotides from position −1282 to −708 all transcribed at levels identical to pHSA2000CAT Δ−1282/−1150 (data not shown). Thus the DNA sequences between −1282 and −1150 appear to be required for the transcriptional activity of the distal element.

The distal regulatory element functions as a muscle-specific enhancer.

We further delineated the size of the DRE by determining that the region between −1282 and −1177 had enhancer activity when linked to a heterologous SV40 basal promoter-CAT construct. The element was cloned in both orientations upstream and downstream of the pCAT-promoter test gene (Promega). In C2 myotubes the enhancer induced activity between 3.5- and 10-fold above background, independent of location or orientation (Fig. 2A and B, lanes 1 to 4), but in non-muscle CV1 cells no enhancer-like activity was detected (Fig. 2A and B, lanes 5 to 8; see Table 2). We refer to the sequences between −1282 and −1177 as the distal regulatory element (DRE) and conclude that this relatively small segment is sufficient to account for essentially all of the activity of the distal regulatory region.

Nuclear proteins specifically interact with the DRE

We analyzed the interaction of segments of the DRE with DNA-binding proteins prepared from nuclei of differentiated C2 myotubes. As seen in Figure 3A and B, when the DRE or the DNA segments that make up its distal and proximal halves (from −1282 to −1228 [DRE-L] and from −1226 to −1177 [DRE-R]), were used in gel electrophoresis mobility shift (GEMS) assays, they formed three different nuclear protein complexes. These three binding activities were initially designated as distal regulatory factors (DRF)-1, -2, and -3. The factors had a high degree of sequence specificity since, as shown below, the binding of DRF-2 and DRF-3 were competed only by the distal and proximal half-fragments respectively, and by intact HSA −1282/−1177. Furthermore, DRF-1 binding requires the presence of sequences spanning the L and R halves of the DRE.

We incubated nuclear extracts from either myoblast or myotube nuclei with overlapping oligonucleotides representing the left and right halves of the DRE (Fig. 4A) or with the intact DRE in order to form the DRF-1, -2, and -3 complexes. Neither synthetic oligonucleotide

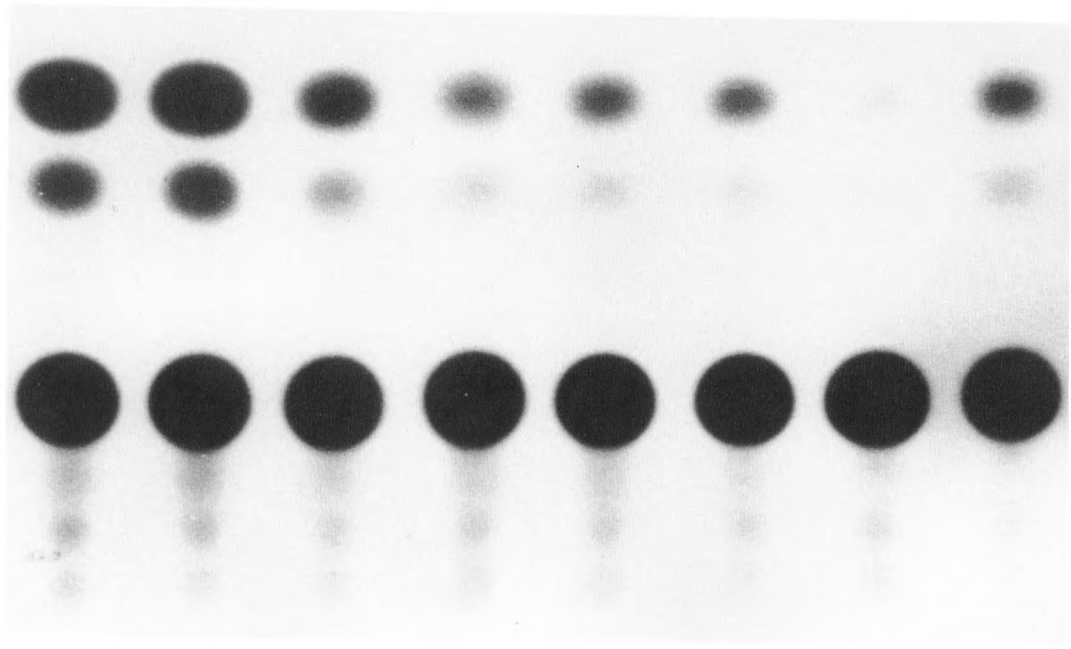


Figure 1. CAT assays demonstrating the effect of internal deletions within the wild-type skeletal α-actin promoter on expression in C2 myotubes. Cells were transfected and harvested for CAT assays as described in Materials and Methods. Percentage of acetylation is shown in parenthesis. Lane 1: pHSA2000CAT (12.8%); lane 2: pHSA2000CAT LC (18.3%); lanes 3 (6.4%), 4 (2.2%), and 5 (2.6%): pHSA2000CAT Δ−1282/−1151; lanes 6 (2.8%), 7 (0.9%), and 8 (3.2%): pHSA2000CAT Δ−1282/−1089.

efficiently competed for DRF-1 binding to the intact DRE (Fig. 4B), although the left half-element, HSA−1274/−1226, did compete weakly at molar excess of >45. In contrast, and very efficiently and specifically, the left half-element competed DRF-2 (Fig. 4C), while the right half-element, HSA−1234/−1194, competed DRF-3 binding (Fig. 4D). These results confirm the existence of three different proteins, or protein complexes, that interact with the DRE. Furthermore, these data establish that the integrity of the DRF-1 binding site is disrupted by bisection of the DRE. This splitting bisects two separated regions of DRF-1:DNA contact (see below).

The DRE plays markedly different roles in achieving maximal expression in different myogenic cell lines and during myogenesis. Whereas in C2 cells expression of the skeletal α-actin gene depends on the DRE for most of its activity, this cis-acting element functions only weakly in L8 cells. To determine whether we could distinguish the factors in C2 cells that interact with the DRE from those that bind in L8 cells, we compared the DNA-binding activities of nuclear extracts from C2 cells and from L8 cells in the myoblast and myotube stages of differentiation. The concentration of serum response factor (SRF) binding activity in each extract was constant, as assayed by its binding to the proximal CArG box of the human cardiac α-actin gene (Muscat et al., 1988; data not shown). However, the concentrations of the binding activities of DRF-1, -2, and -3 varied considerably. The results seen in Figure 3C can be summarized as follows:

- *DRF-1 binding:* DRF-1 binding was detected in both C2 and L8 myoblast extracts. After differentiation, DRF-1 activity was undetectable in L8 myotube nuclear extracts but was still active in C2 myotube extracts.
- *DRF-2 binding:* in contrast, maximal binding of DRF-2 was detected only after differentiation of either C2 or L8 myotubes.

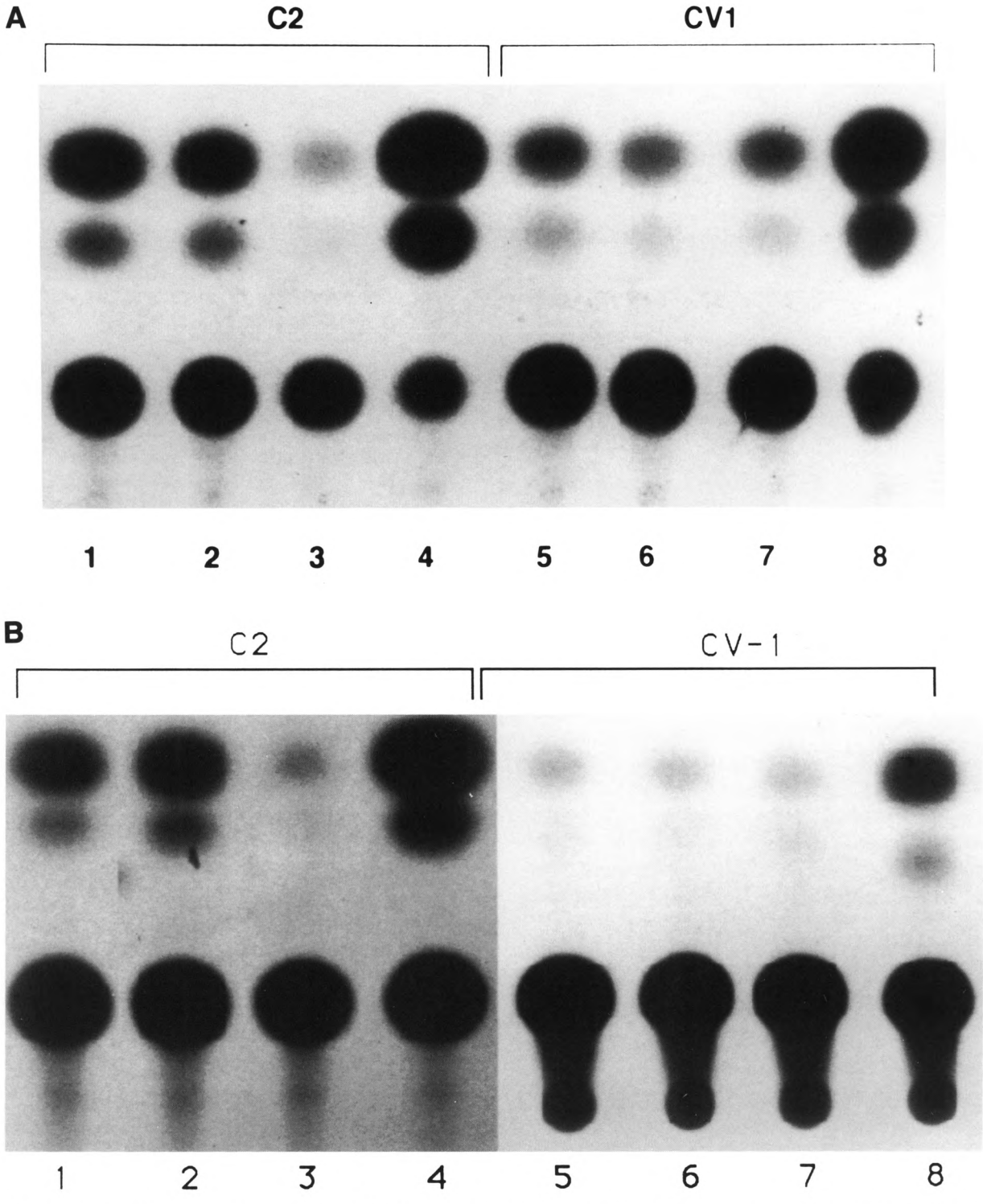


Figure 2. The distal regulatory region (DRE) is a muscle-specific enhancer. The element −1282/−1177 was cloned into the pCAT-promoter (Promega) upstream (Bgl II site: **A**, lanes 1, 2, 5, and 6) and downstream (Sph I site: **B**, lanes 1, 2, 5, and 6) of the CAT gene in sense (lanes 1 and 5) and antisense (lanes 2 and 6) orientation. CV-1 cells were transfected at 60% confluency, and expression was tested 48 hours later. In C2 myotubes the enhancer gave significant levels of activity above the pCAT plasmid (lanes 3 and 7) independent of location or orientation, but in non-muscle cells (CV-1 cells) no enhancer-like activity was detected. β-actin served as a positive control (lanes 4 and 8). Reproductions of the original autoradiograms were obtained by digital scanning and photographed with a Presentation Technologies Montage system on a Macintosh IIcx computer with 8 megabytes of RAM.

Table 2. CAT activities of DRE-SV40 constructs.

Activities are expressed as % of β-actin-CAT activity.

	UP(S)	*UP(A)*	*DS(S)*	*DS(A)*	*DRE2pCAT*	*pCAT*
C2 cells	41.1 (9.2)	29.5 (7.5)	27.6 (6.1)	15.6 (4.6)	5.2 (0.5)	6.1 (2.9)
CV-1 cells	8.1 (1.6)	10.7 (1.0)	10.0 (2.1)	11.5 (1.6)	6.6 (0.8)	5.6 (1.3)

UP = DRE cloned upstream of the SV40 promoter. DS = DRE cloned downstream of the SV40 promoter. DRE2pCAT = DRE-2 cloned upstream of the SV40 promoter. S = sense. A = antisense. Numbers in parentheses are ± S.E.M.

- *DRF-3 binding:* binding of DRF-3 can only be detected in C2 myotube extracts and is not detectable in L8 cells.

These results demonstrate that all three DRF-binding activities are present in nuclear extracts of C2 myotubes, but only DRF-2 activity can be detected in L8 myotube extracts. Use of pUC18 DNA rather than poly(dI-dC)·poly(dI-dC) as competitor DNA in these assays gave similar results (not shown).

Footprinting of the DRF-1, -2, and -3 binding sites

We used dimethylsulfate methylation interference analysis to determine the locations of nu-

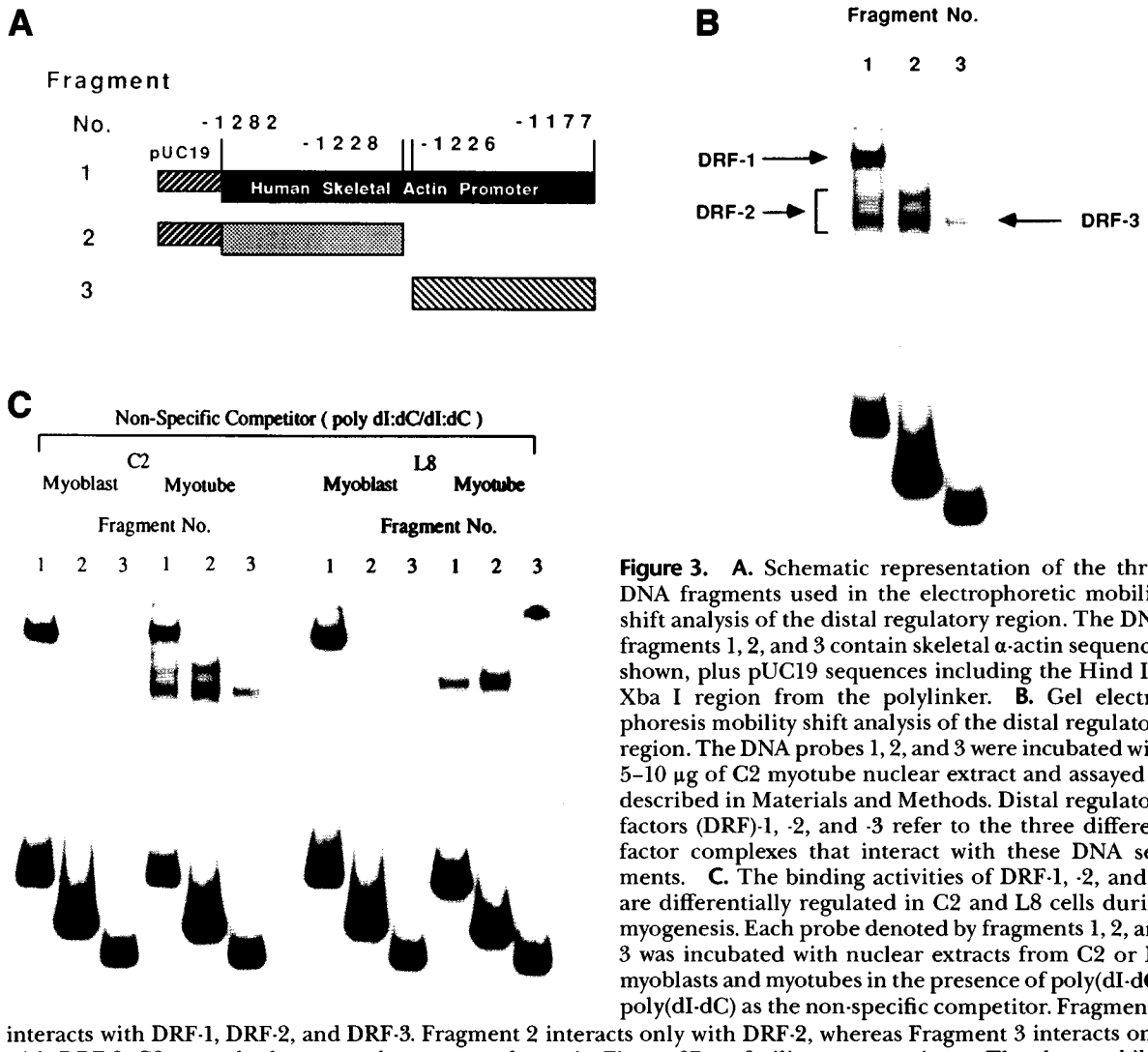
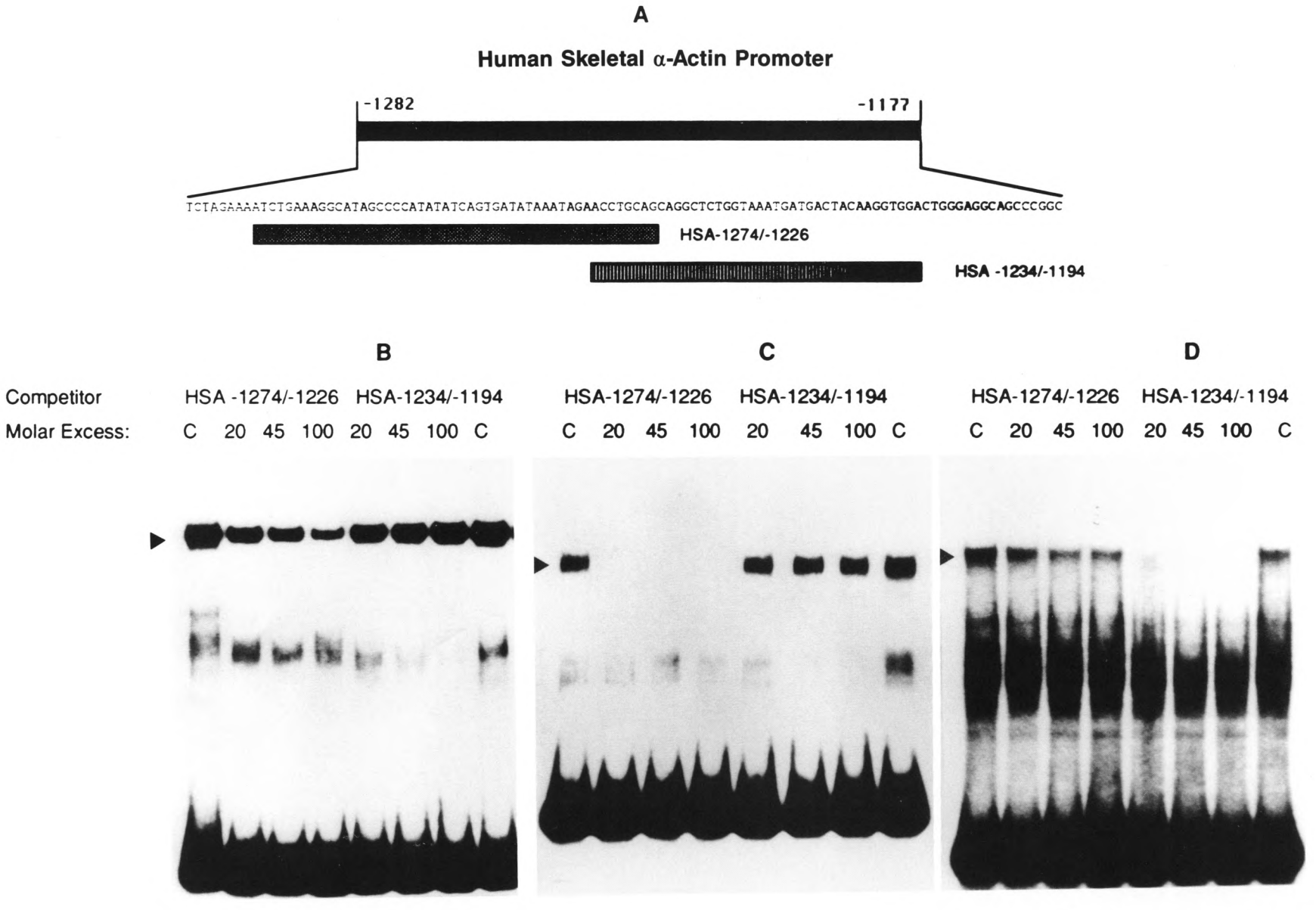


Figure 3. **A.** Schematic representation of the three DNA fragments used in the electrophoretic mobility shift analysis of the distal regulatory region. The DNA fragments 1, 2, and 3 contain skeletal α-actin sequences shown, plus pUC19 sequences including the Hind III/Xba I region from the polylinker. **B.** Gel electrophoresis mobility shift analysis of the distal regulatory region. The DNA probes 1, 2, and 3 were incubated with 5–10 μg of C2 myotube nuclear extract and assayed as described in Materials and Methods. Distal regulatory factors (DRF)-1, -2, and -3 refer to the three different factor complexes that interact with these DNA segments. **C.** The binding activities of DRF-1, -2, and -3 are differentially regulated in C2 and L8 cells during myogenesis. Each probe denoted by fragments 1, 2, and 3 was incubated with nuclear extracts from C2 or L8 myoblasts and myotubes in the presence of poly(dI-dC)·poly(dI-dC) as the non-specific competitor. Fragment 1 interacts with DRF-1, DRF-2, and DRF-3. Fragment 2 interacts only with DRF-2, whereas Fragment 3 interacts only with DRF-3. C2 myotube lanes are the same as shown in Figure 3B to facilitate comparisons. The slow mobility band in lane 3, L8 myotubes, is radiolabeled material at the top of the gel well.


A
Human Skeletal α-Actin Promoter
-1282
-1177
TCTAGAAAATCTGAAAGGCATAGCCCCATATATCAGTGATATAAATAGAACCTGCAGCAGGCTCTGGTAAATGATGACTACAAGGTGGACTGGGAGGCAGCCCGGC
HSA-1274/-1226
HSA -1234/-1194
B
C
D
Competitor
Molar Excess:
HSA -1274/-1226
HSA-1234/-1194
HSA-1274/-1226
HSA-1234/-1194
HSA-1274/-1226
HSA-1234/-1194
C 20 45 100 20 45 100 C
C 20 45 100 20 45 100 C
C 20 45 100 20 45 100 C

cleotides in the DRE that interact with DRF-1, -2, and -3. Based on the footprint data presented in Figure 5 and summarized in Figure 6, we designate the sequences that interact with DRF-1, -2, and -3 as the distal regulatory elements (DRE)-1, -2, and -3.

- *DRF-1 footprint* (Fig. 5A): methylation at either of two guanine nucleotides in the DRE interfered with binding of DRF-1. These nucleotides were located at positions −1210 and −1205 on the top and bottom strands respectively. In addition methylation of guanines at positions −1260 and −1247 on the top strand reproducibly enhanced DRF-1 binding. The locations of these contact bases on both sides of the boundary that separates the DRE-L and DRE-R sequences likely explains why neither half-contact site alone competes for binding of DRF-1 and suggests that one or both sites are required for DRF-1 binding.
- *DRF-2 footprint* (Fig. 5B): methylation of any one of three guanine nucleotides on the top strand at positions −1247, −1245, and −1235 interfered with binding of DRF-2. No contact sites were detected on the bottom strand by this method. An identical DRF-2 methylation interference pattern was obtained when L8 myotube extracts were incubated with radiolabeled HSA−1282/1228 (Fig. 5C).
- *DRF-3 footprint* (Fig. 5D): the DNA fragment DRE-R encompassing the more proximal half of the DRE was used to map the DRF-3 binding site. Contact nucleotides were located at positions −1210 and −1207 and at positions −1205, −1202, and −1193 on the top and bottom strands respectively.

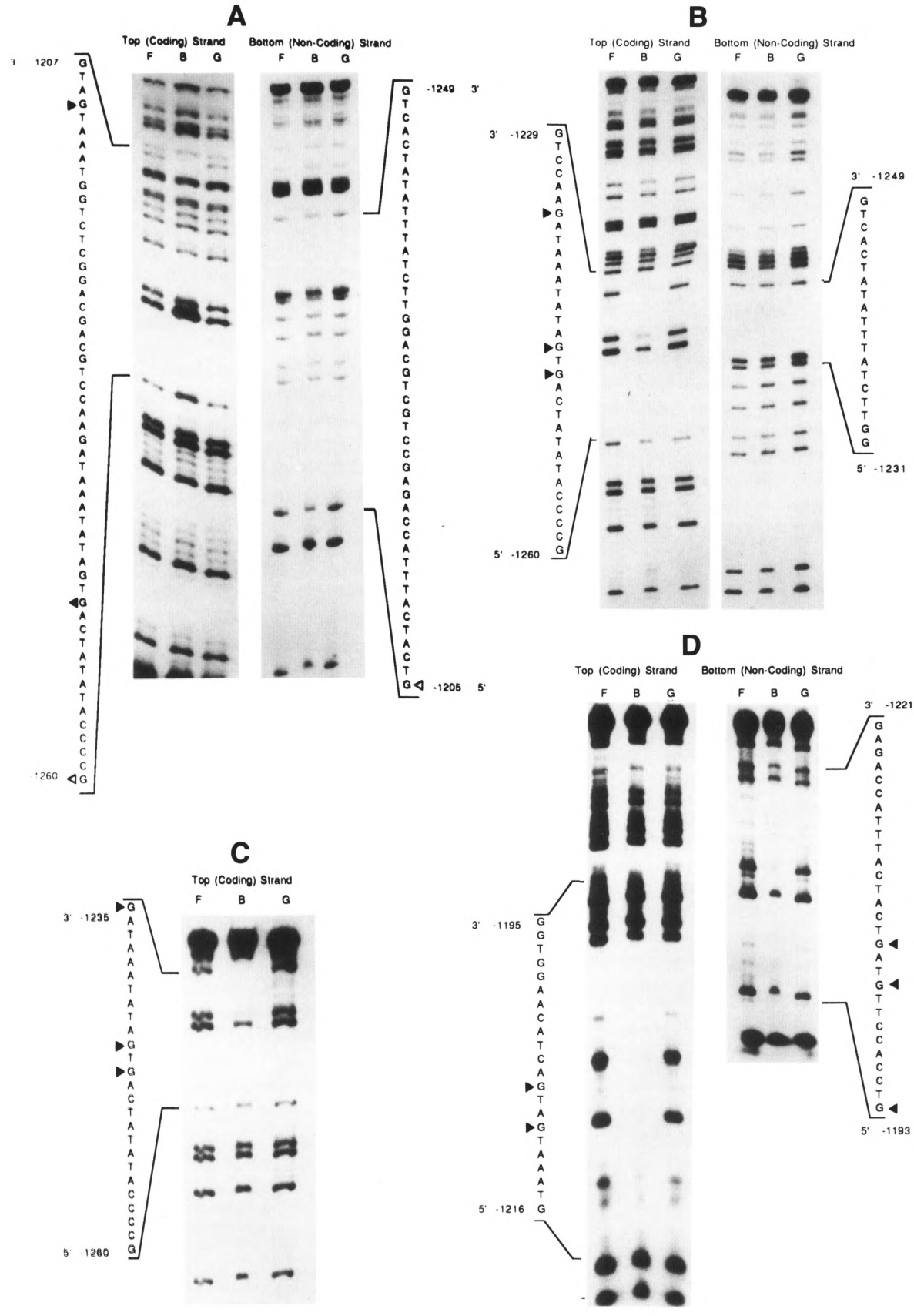
DRF-2:DRE-2 interactions appear critical for high level transcriptional activity

To identify and demonstrate which of the three distal regulatory elements and factors play key roles in the high level transcription of the skeletal α-actin gene, we constructed an additional internal mutation and two site specific mutations in DRE-1 and -2 (Fig. 6). The construct pHSA2000CAT Δ−1282/−1261 displayed wild-type levels of transcription after transfection analyses, indicating that the nucleotides between positions −1282 and −1261 were not required for transcriptional activity of the skeletal α-actin gene (Fig. 7; compare lanes 2, 4, and 6). Point mutations were then introduced into this construct within DRE-2 to create the plasmid pHSA2000CAT Δ−1282/−1261 M1, which resulted in a 3- to 5-fold reduction in transcription after transfection (Fig. 7; compare lanes 2, 4, and 7). Furthermore, and to verify the functional importance of DRE-2, we also constructed a site-specific mutation in the wild-type promoter. This plasmid pHSA2000CAT M2 contained mutations in the core of DRE-2 (see Fig. 6). This mutation resulted in approximately a 10-fold reduction in transcription after transfection (Fig. 7, lanes 3 and 5). These functional data indicate that DRE-2 is an essential sequence with respect to high level transcriptional activity of the DRE and appears to be required for most of the enhancer activity.

These mutant constructs were next tested for their ability to interfere selectively with DRF-2 binding. As seen in Figure 8A, lanes 3 and 4, the construct pHSA2000CAT Δ−1282/−1261 (which expresses CAT at native levels) competes for the binding of DRF-2 to the DRE. The two low activity constructs, M1 and M2, which contain mutations in the bases that contact DRF-2, have also lost the ability to interact with DRF-2 (Fig. 8A, lanes 5 to 8). However, as seen in Figure 8B, lanes 5, 6, and 7, all these mutated constructs can still compete for DRF-1 binding.

These data imply that the function of the DRE element requires the formation of the DRF-2:DRE-2 complex. The data also suggest that the guanine at −1247, when methylated, enhances the binding of DRF-1 and is not crucial with respect either to the binding of DRF-1

Figure 4. Gel electrophoresis mobility shift analysis of DRF-1, -2, and -3. **A.** The sequence of HSA−1282/−1177 is displayed, and the oligonucleotides used in competition (HSA−1274/−1226 and HSA−1234/−1194) are represented by the cross-hatched rectangular boxes. **B.** The effect of competition by the two oligonucleotides on the complex DRF-1 formed with the DRE probe in C2 myoblast nuclear extracts. Poly(dI-dC)·poly(dI-dC) was used as a non-specific competitor. **C.** The effect of competition by the two oligonucleotides on the complex DRF-2, formed with the DRE (HSA−1282/−1177) in C2 myotube nuclear extracts with Msp I digested pUC18 as the non-specific competitor. **D.** The effect of competition by the two oligonucleotides on the complex DRF-3, formed with the probe HSA−1226/−1177 in C2 myotube nuclear extracts with poly(dI-dC)·poly(dI-dC) as the non-specific competitor.


A
Top (Coding) Strand
Bottom (Non-Coding) Strand
F B G
F B G
B
Top (Coding) Strand
Bottom (Non-Coding) Strand
F B G
F B G
C
Top (Coding) Strand
F B G
D
Top (Coding) Strand
Bottom (Non-Coding) Strand
F B G
F B G

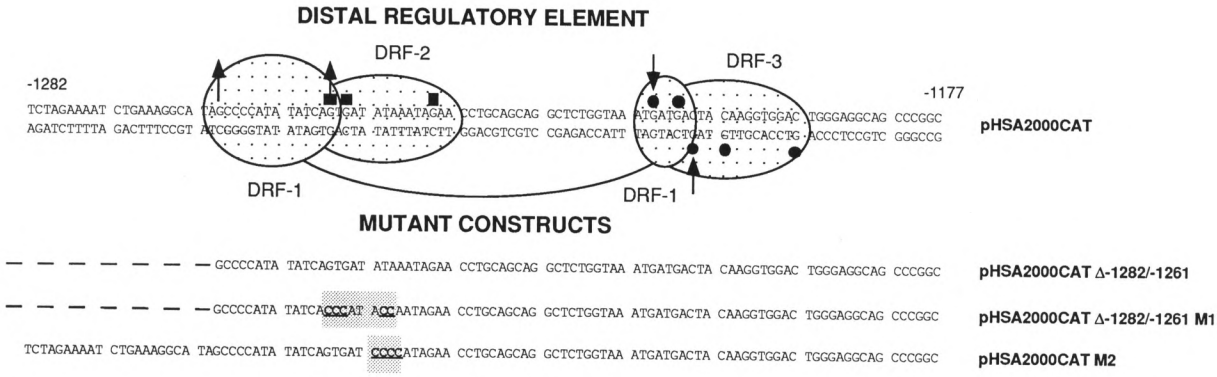


Figure 6. Summary of the DMS methylation interference footprint analysis of DRF-1, -2, and -3. Distal Regulatory Elements (DRE)-1, -2, and -3 denote the sequences that interact with DRF-1, -2, and -3. Arrows pointed away from the sequence indicate the nucleotides whose methylation enhances formation of a complex with DRF-1. Arrows directed towards the sequence denote nucleotides whose methylation interferes in complex formation with DRF-1. Nucleotides whose methylation interferes with complex formation for DRF-2 and DRF-3 are shown by squares and dots respectively. The sequences of the mutated distal regulatory elements are shown for comparison.

to the promoter or its function in the types of assays used.

The DRE-2 site has no enhancer activity as a monomer

To establish whether the DRE-2 site alone was sufficient for the muscle-specific enhancer activity of the DRE, a 25 bp double-stranded synthetic oligonucleotide was cloned into the pCAT promoter test construct in the sense orientation upstream of the CAT gene. This construct, DRE2PCAT (Table 2), gave similar levels to background in C2 cells, indicating that the DRE-2 site alone could not confer muscle specificity on a heterologous promoter but required the presence of the additional elements DRE-1 and/or DRE-3.

DRE-2 binding and function is competed by other muscle promoter elements

The A/T-rich DRE-2 binding site sequence resembles functional sites from enhancers of other muscle-specific genes, including the MEF-2 site of MCK (Gossett et al., 1989) and the PABS site of fast-twitch skeletal troponin C (TnC_f) upstream regulatory element (URE; R. Gahlmann and L. Kedes, unpublished data). The muscle-specific and strong enhancer functions of DRE-2 appear to differ from those of MEF-2 and PABS sites. The MEF-2 site of MCK plays only a modest role in its transcription in C2 cells (Gossett et al., 1989), whereas the PABS site can function as a strong enhancer in both muscle and non-muscle cells (R. Gahlmann and L. Kedes, unpublished data). We tested whether the MEF-2

Figure 5. Methylation interference footprint analysis of the DRE. **A.** Methylation interference footprint analysis of DRF-1 binding to the DRE (HSA−1282/−1177). The DNA was partially methylated with DMS prior to incubation with C2 myoblast nuclear extracts and poly(dI-dC)·poly(dI-dC). The complexed and free populations of DNA were localized on gel mobility shift assays, eluted, treated with piperidine, and analyzed on sequencing gels, as outlined in Materials and Methods. Results from the coding and non-coding strands are shown. Lane F, free probe DNA; lane B, bound probe DNA; and lane G, partial chemical degradation products of the probe cleaved at guanine nucleotides. Triangles directed *toward* the sequence denote nucleotides in the sequence whose methylation *interferes* strongly (filled triangles) or partially (open triangles) with complex formation. The nucleotides in the sequence whose methylation *enhances* complex formation are denoted by solid triangles directed *away* from the sequence. **B.** Methylation interference footprint analysis of DRF-2 binding to HSA−1282/−1177. The DNA was partially methylated with DMS prior to incubation with C2 myotube nuclear extracts and Msp I digested pUC18. Details and symbols are as in the legend to **A**. **C.** Methylation interference footprint analysis of DRF-2 binding to HSA−1282/−1228. The DNA was partially methylated with DMS prior to incubation with L8 myotube nuclear extracts and poly(dI-dC)·poly(dI-dC). Details and symbols are as in the legend to **A**. **D.** Methylation interference analysis of DRF-3 binding to HSA−1226/−1177. The DNA was partially methylated with DMS prior to incubation with C2 myotube nuclear extracts and poly(dI-dC)·poly(dI-dC). Details and symbols are as in the legend to **A**.

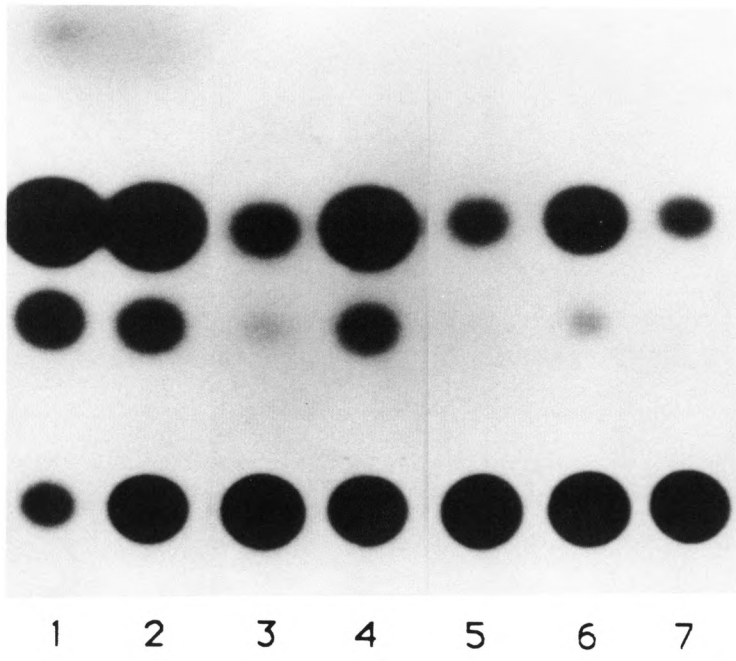


Figure 7. Mutations within the DRE effect expression in C2 cells. DNA transfection, culture conditions, and CAT assays were performed as described in the legend to Figure 1. Lane 1: pHβAPr-1-CAT; lane 2: pHSA2000CAT; lane 3: pHSA2000CAT M2; lane 4: pHSA2000CAT; lane 5: pHSA2000-CAT M2; lane 6: pHSA2000CAT Δ−1282/−1261; and lane 7: pHSA2000CAT Δ−1282/−1261M1. Multiple transfections with several independent preparations of these constructs gave the following levels of expression normalized to pHβACAT set to 100%:

Plasmid	% Activity	S.E.M.
pHSA2000CAT	56.2	±2.6
pHSA2000CAT Δ−1282/−1261	45.1	±13.1
pHSA2000CAT Δ−1282/−1261M1	13.7	±2.4
pHSA2000CAT M2	9.9	±0.4

binding sequence could compete for DRF-2 binding. The MEF-2 sequence competed for DRF-2 binding equally as well as the DRE-2 site itself over a 10- to 200-fold range of excess competitor DNA (Fig. 9a). We also found strong competition for DRF-2 binding with a 40-fold excess of troponin C fast URE PABS sequence (Fig. 9b). Thus DRE-2 shares affinities for binding to nuclear proteins with other A/T-rich enhancer elements.

To test whether such A/T-rich sequences also can compete for skeletal α-actin gene expression, we co-transfected C2 cells with HSA2000-CAT and an excess of DNA carrying either the URE PABS or non-specific sequences. The URE specifically reduced by 6-fold the activity of the −2000 skeletal α-actin wild-type promoter in C2 cells (Fig. 10). These data suggest that there is a family of A/T-rich regulatory elements in these co-expressed muscle genes which interact with the same regulatory factor(s) but function in distinguishably different manners.

Discussion

Our results show that the DNA segment from −1282 to −1177 of the human skeletal α-actin gene promoter is a classical muscle-specific enhancer. This region activates the basal promoter and drives a heterologous promoter in a muscle-specific manner. Muscle-specific enhancer sequences have been reported for a number of other genes, including the vertebrate genes for muscle creatine kinase (Gossett et al., 1989), myosin light chain 2 (Arnold et al., 1988), myosin light chain 1/3 (Donoghue et al., 1988), troponin C fast (R. Gahlmann and L. Kedes, unpublished data), troponin C cardiac (Christensen and Kedes, 1991; Parmacek et al., 1991), troponin I (Konieczny and Emerson, 1987), troponin T (Mar et al., 1988), embryonic myosin heavy chain (Bouvagnet et al., 1987), and myoglobin (Wefald et al., 1990).

Three nuclear factors, DRF-1, -2, and -3, were shown to bind specifically to this skeletal α-actin enhancer. Whereas DRF-2 and DRF-3 bind at two different single locales on the enhancer, DRF-1 binds at two separated sites that overlap with DRE-2 and DRE-3. The three binding factors were differently active during the transition from proliferating myoblasts to differentiated myotubes. Whereas DRF-1 activity is present in both myoblasts and myotubes of C2 cells, DRF-2 and DRF-3 are dramatically elevated during C2 cell differentiation. Furthermore, the levels of activity of these factors varied quite dramatically in different myogenic cell lines. In addition, we have demonstrated previously (Muscat and Kedes, 1987) that deletion of the enhancer has little effect on expression in L8 cells, whereas it is critical for expression in C2 cells. In this regard it is tempting to speculate that the ma-

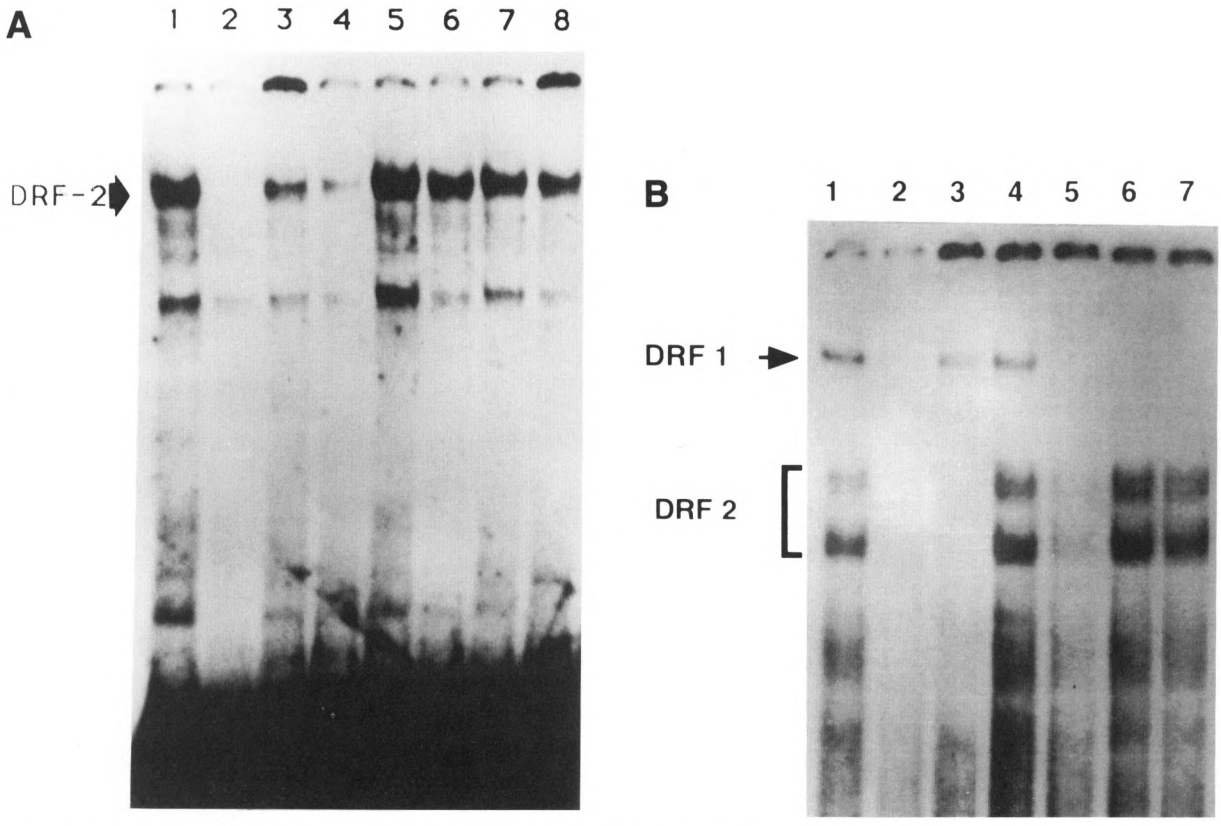


Figure 8. Gel electrophoresis mobility shift analysis of DRF-1 and DRF-2. **A.** The effect of competition by mutated DREs on the DRF-2 complex. The radiolabeled probe HSA−1282/−1222 was incubated with C2 myotube nuclear extracts and poly(dI-dC)·poly(dI-dC) as the non-specific competitor, as described in Materials and Methods. Lane 1, no competitor; lane 2, competition with 60-fold excess DNA fragment HSA−1274/−1226 (native DRE-2); lanes 3 and 4, 20- and 60-fold excess pHSA2000CAT Δ−1282/−1261 respectively; lanes 5 and 6, 20- and 60-fold excess pHSA2000CAT Δ−1282/−1261M1; lanes 7 and 8, 20- and 60-fold excess pHSA2000CAT M2. **B.** The effect of competition by the mutant DREs on the complex DRF-1 formed in vitro with the probe HSA−1282/−1177 in C2 myoblast nuclear extracts with poly(dI-dC)·poly(dI-dC) as the non-specific competitor. Lane 1, no competitor; lane 2, competed with 60-fold excess HSA−1282/−1177; lanes 3 and 4, 60-fold excess HSA−1274/−1226 (DRE-2) and HSA−1234/−1194 (DRE-3); lane 5, pHSA2000CAT Δ−1282/−1261; lane 6, 60-fold excess pHSA2000CAT Δ−1282/−1261M1; lane 7, 60-fold excess pHSA2000CAT M2.

jor differences observed in vitro with respect to DRF-3 binding activity during C2 and L8 cell differentiation, might contribute to the transcriptional differences in these two cell lines. DRF-3 is never detected during myogenic differentiation of L8 cells.

This demonstration that DRF-1, DRF-2, and DRF-3 bind to overlapping and/or closely interdigitated DNA sequences may have potential regulatory significance, since it is unlikely that all three factors can bind simultaneously. Overlapping binding sites have been reported for a number of tissue-specific genes, including albumin (Lichtsteiner et al., 1987), urokinase (von der Ahe et al., 1988), and β-fibrinogen (Baumhueter et al., 1988), and have been implicated in their cell specificity and/or developmental regulation. Preliminary in vitro analysis indicates that DNA bound by DRF-1 is refractory to interactions with DRF-2, which can be partially explained by the disparate DNA-binding affinities of these factors (G. Muscat, unpublished data). In C2 cells DRF-1 activity is present in both myoblasts and myotubes, while DRF-2 and DRF-3 are up-regulated. DRF-1 might thus have a repressive function in these cells through competitive binding (Tanaka and Herr, 1990), whereas DRF-2 and DRF-3 may prove to be cooperative in terms of gene activation. The inverse relationship between DRF-1 and DRF-2 binding is more pronounced in L8 cells, but the significance of this is unclear, as the enhancer is non-functional in this myogenic cell line.

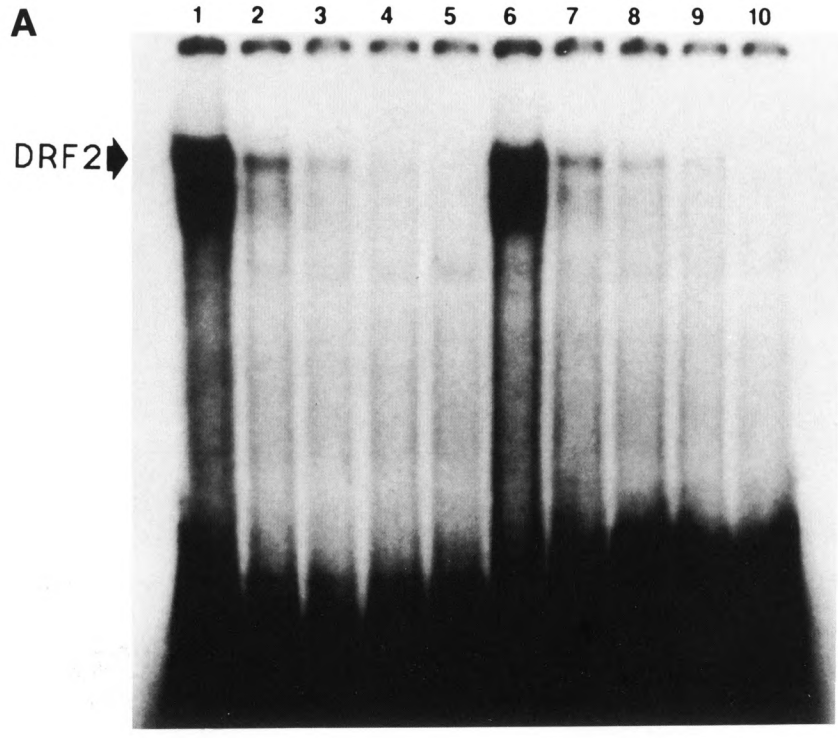


Figure 9. The effect of competition by enhancers of muscle creatine kinase and skeletal troponin C genes upon complex formation with the probe HSA−1282/−1177 in C2 myotube extracts. **A.** DRE-2 competitions were performed with 0 ng, 50 ng, 100 ng, 200 ng, and 1 μg of oligonucleotide (lanes 1 to 5). MEF-2 competitions were performed with 0 ng, 50 ng, 100 ng, 200 ng, and 1 μg of oligonucleotide (lanes 6–10). **B.** The effect of DRE-2 complex formation (control: lane 1) of competition by TnC fast PABS sequence (lane 2), self competition (lane 3), and competition with an unrelated oligonucleotide for DRE-3 (lane 4). A 40-fold molar excess of oligonucleotide competitor was used in each case.

Our mutational and competition analyses of the DRE point to the importance of DRF-2 in activating the skeletal α-actin promoter and suggest a less significant role for DRF-1 in tissue specificity. DRE-2 is an A/T-rich element with a counterpart in at least 4 other muscle-specific genes: the chicken cardiac MLC-2A gene (Arnold et al., 1988), rat MLC 1/3 gene (Donoghue et al., 1988), the mouse and rat muscle creatine kinase gene (Gossett et al., 1989; Horlick and Benfield, 1989), and the human skeletal troponin C gene (R. Gahlmann and L. Kedes, unpublished data). It is possible that the MEF-2 protein, which interacts with the MCK enhancer, is a common regulatory factor involved in the regulation of all these genes. Alternatively, the subtle differences in these A/T-rich binding sites could reflect the presence of a related family of factors with different binding specificities and functions. Different transcription factors can recognize the same consensus sequence (Jones et al., 1988), while purified factors can recognize a number of variant consensus sequences (Poellinger and Roeder, 1989 and references therein). Such degeneracy may serve to fine tune transcription during development and differentiation and account for differential gene expression in different tissues. Although we favor the likelihood that the DRF-2, which binds to the human skeletal α-actin en-

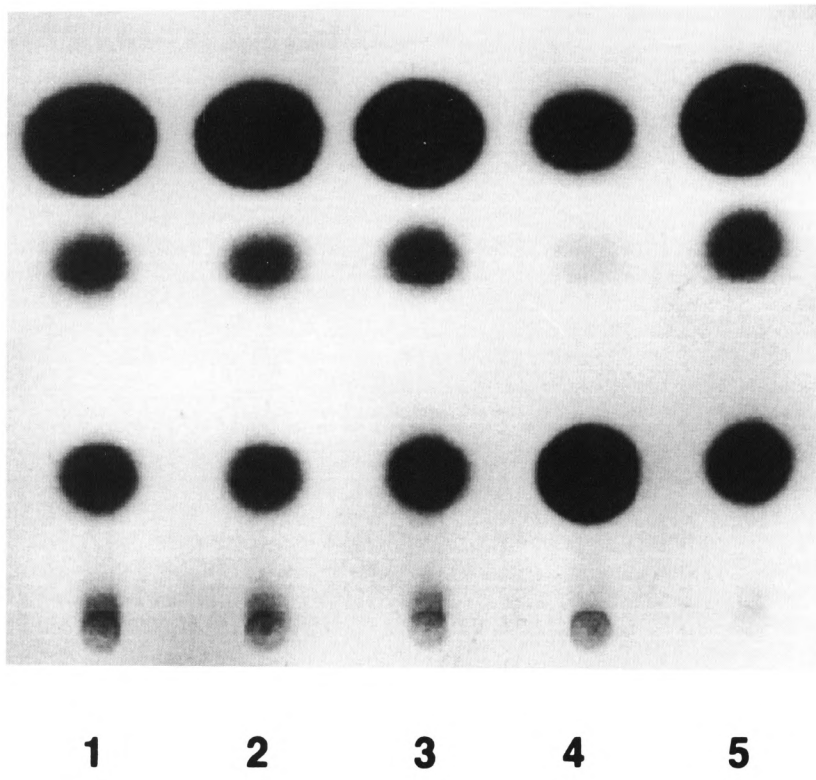


Figure 10. The TnC fast enhancer DNA (URE) inhibits HSA expression in C2 cells. C2 myoblasts were co-transfected with reporter CAT constructs and with plasmids carrying the skeletal TnC gene URE. Cells were harvested for CAT assay after differentiation. Plasmids transfected were pHSA2000-CAT (lanes 1 and 4), and pHβA-CAT (lanes 2, 3, and 5). Competitors were 17 μg pBR322 (lanes 1–3) and 17 μg pBR-URE (lanes 4–5).

hancer, and the human TnC fast PABS binding protein are the equivalent of MEF-2, formal establishment of their identity must await isolation and characterization of these proteins.

In the context of a heterologous promoter, the skeletal α-actin enhancer is muscle-specific, whereas the TnC fast enhancer expresses at high levels in all cell types (R. Gahlmann and L. Kedes, unpublished data). The discrepancy in activities of these two enhancers is more likely to be due to the loss of a repressor element in the TnC construct rather than any functional difference between the A/T-rich binding sites in the two genes. The TnC fast A/T-rich site competes the transcriptional activity of the intact skeletal α-actin promoter. This observation further supports the likelihood that the DRE-2 sequence is functionally involved in activating the skeletal α-actin promoter.

Whereas the MCK enhancer, which includes a MyoD binding site, is MyoD-responsive, the skeletal α-actin enhancer is not transactivated by MyoD in 10T1/2 cells (our unpublished data). This observation suggests that tissue specificity may be the result of one or more of the DRE binding factors and does not depend upon an interaction with MyoD or the product of another member of the MyoD myogenic determination gene family. The DRE-2 binding site alone is not sufficient to confer muscle specificity on a heterologous promoter, indicating that additional nuclear factors are necessary for tissue-restricted gene activation. Gossett et al. (1989) have observed that a MEF-2 binding site showed little or no enhancer activity as a monomer, but that multiple copies of this sequence resulted in strong activation of the MCK promoter. It appears that although the MEF-2 site is directly involved in muscle-specific gene activation, this regulation is dependent upon the presence of additional cis elements, including MyoD. In the context of the skeletal α-actin promoter, DRE-2 may depend upon heterotypic interactions with DRF-1 and/or DRF-3 for its activation properties or, alternatively, these factors may have a more modulatory role.

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