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Serum Response Factor and Protein-Mediated DNA Bending Contribute to Transcription of the Dystrophin Muscle-Specific Promoter

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The minimal muscle-specific dystrophin promoter contains the consensus sequence CC(A/T)$_6$GG, or the CArG element, which can be found in serum-inducible or muscle-specific promoters. The serum response factor (SRF), which mediates the transcriptional activation of the c-*fos* gene in response to serum stimulation, can bind to different CArG box elements, suggesting that it could be involved in muscle-constitutive transcription. Here we show that SRF binds to the dystrophin promoter and regulates its muscle-specific transcription. In transient transfections, an altered-binding-specificity SRF mutant restores the muscle-constitutive transcription of a dystrophin promoter with a mutation in its CArG box element. The muscle-constitutive transcription of the dystrophin promoter also requires the sequence GAAACC immediately downstream of the CArG box. This sequence is recognized by a novel DNA bending factor which was named dystrophin promoter-bending factor (DPBF). Mutations of the CArG flanking sequence abolish both DPBF binding and the promoter activity in muscle cells. Its replacement with a p62/ternary complex factor binding site changes the promoter specificity from muscle constitutive to serum responsive. These results show that, on the dystrophin promoter, the transcriptional activation induced by SRF requires the DNA bending induced by DPBF. The bending, next to the CArG box, could promote interactions between SRF and other proteins in the transcriptional complex.

The dystrophin gene, which is altered in Duchenne and Becker muscular dystrophies, is expressed primarily in skeletal and cardiac muscle and to a lesser extent in smooth muscle, kidney, and brain (12, 21, 27, 28). Dystrophin-specific expression in different tissues is controlled by independent promoters (2, 5, 6). Previous analysis showed that the muscle-specific dystrophin promoter drives the expression of a reporter gene in muscle cells and, to a lesser extent, also in non-muscle cells. Furthermore, it demonstrated that a minimal segment between −96 and +30 of the promoter with respect to the transcription start site retained the muscle-preferential expression (12, 21). This minimal dystrophin promoter contains both a CArG box consensus sequence and an E box element. Of these elements, only the former was shown to be functionally essential. Binding assays showed that the dystrophin CArG box is recognized by a ubiquitous factor. This suggested that the serum response factor (SRF) could be involved in the muscle-preferential activation of the dystrophin promoter (12).

SRF was initially isolated as the transcription factor binding to the serum response element (SRE) of the c-*fos* promoter (46). Later it was shown that SRF could also bind several muscle-specific promoters (30, 46, 47, 49), and some SRF-related molecules with muscle-specific activity have been identified in frog (3, 4, 23). Indications of SRF involvement in muscle-specific transcription are suggested by several experiments: CArG box present in muscle-specific promoters competed with SRE for SRF binding (1, 15, 24, 30, 33, 37, 47, 49), and anti-p67/SRF antibodies prevent the myoblast-to-myotube transition as well as the expression of muscle-specific troponin T in muscle cell lines (48). The mechanism by which SRF could contribute to muscle-specific transcriptional regulation is not clear. This could be due to protein-protein interactions with muscle-specific factors, as suggested by the α-actin promoter which requires the binding of SRF, SP-1, and the muscle-specific MyoD1 factor (37).

The dystrophin muscle-specific promoter examined herein provides an excellent model for studying CArG box-dependent muscle-specific transcription. We report biochemical and functional evidence that SRF binds to the dystrophin CArG box and is necessary for the muscle-constitutive transcription of the promoter. Analysis of the *cis*-acting elements revealed the existence of two more sequences necessary for the promoter activity. One of them, located immediately downstream of the CArG box, is involved in tissue-specific transcription. This DNA sequence is recognized by a nuclear factor which is able to bend the DNA and was named dystrophin promoter-bending factor (DPBF). Our data suggest that DPBF functions as an architectural component of the promoter, allowing interactions between SRF and other factors crucial for the formation of a higher-order complex in muscle cells.

MATERIALS AND METHODS

Plasmids. All DNA manipulations were carried out by standard techniques, and the plasmids' structures were verified by sequencing. The dystrophin promoter was obtained from a healthy individual as previously described (50). To generate the promoter mutants, DNA amplified by standard PCRs using the oligonucleotide primers as indicated were cloned between the *Hin*dIII and *Xba*I sites of the plasmid pUC-CAT (29). Oligonucleotides used for the PCRs introduced *Hin*dIII and *Xba*I sites, respectively, upstream of position −92 and downstream of position +37 with respect to the transcription start site. The following oligonucleotides were used for each construct: 1 (CAGGTCTAGAACACTGA GTGAGTCAACAC) and 2 (GGATAAGCTTACTCATCTCCTATTATGGG AAACCAACTTGAG) for the wild-type (wt) promoter (ML145); 1 and 6 (GG ATAAGCTTACTCATCTCCCAATCGGGGAAACCAACTTGAG) for the m1 mutant (ML151); 1 and 8 (GGATAAGCTTACTCATCTCCTATTATGGG AGTATAACTTGAGAG) for the m2 mutant (ML159); 1 and 10 (GGATAAG

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CTTACTCATCTCCTATTATGGACATCCTGCTTGAGAG) for the TCF mutant (ML156); 1, 12 (GGGGAAGCTTCATGATCTCCTATTATGGGAAACC GTCGACAGAGAGAAGGC), 13 (GCAGAAGCTTACCGTCGACAGAGA GAAGGCGGGTCACTTG), and 14 (GCCTTCTCTCTGTCGACGG) for the m3 mutant (FG182); 1, 15 (GGGGAAGCTTCATGATCTCCTATTATGGGA AACCAACTTGTGGACGAAGGC), 16 (GCAGAAGCTTACCGTCGACGA AGGCGGGTCACTTGCTTGT), and 17 (GCCTTCGTCGACAAGTTGG) for the m4 mutant (FG183); 1, 18 (GCAGAAGCTTACCGTCGACGTCACTTGC TTGTGCGCAGGT), and 19 (TGACGTCGACTCTCTCTCAAGTTGGTT) for the m5 mutant (FG184); 1 and 20 (TTCCAGGACCTGCGCACAAGCTC TGTACCCGCCTTCTCTCTCAAGT) for the m6 mutant (FG187); 1, 2, 21 (GCAGAAGCTTGCACTCGAGCGCAGGTCCTGGAATTTG), and 22 (AG TACCTCGAGCAAGTTACCCGCCTTCTCTCT) for the m7 mutant (FG186); 1 and 23 (TTCCAGGACCGTATGACAAGCAAGTGACCCGCCTTCTCTCT CAAGT) for the m8 mutant (FG188); 1, 2, 24 (CATACTCGAGGGAATTTG AAATATCCGGGGG), and 25 (CATACTCGAGTGCGCACAAGCAAGTG ACCC) for the m9 mutant (FG215); 1, 2, 26 (CATACTCGAGAATTTGAAA TATCCGGGGGC), and 27 (CATACTCGAGCCTGCGCACAAGTGAC) for the m10 mutant (FG216).

The following plasmids for expression in mammalian cells were as described previously: MLV-SRF, MLV-SRF-M2 (17), and MLV-Elk (18, 19, 34). For in vitro transcription, plasmids carrying SRF, SRF-M2, Elk-1, and Sap-1a coding sequences downstream of a T3 promoter were used.

Plasmid pBend-DPBE (FG169) was prepared by insertion of the annealed synthetic oligonucleotides 28 (CTAGACTTATGGGAAACCAACTTGAG) and 29 (TCGACTCAAGTTGGTTTCCCATAAGT) between the *Xba*I and *Sal*I restriction sites in the plasmid pBend2 (20). pBend2 was kindly provided by Marco E. Bianchi.

For the in vitro translation of YY1, the human cDNA was obtained by PCR amplification with the following oligonucleotides: 42 (AGCCCTCAGCCATGG CCTCGGGCGA) and 43 (GGATCGAATTCGAGAAGGGTCTTCTCTCTTC TTTTCAC). The amplified DNA was then cloned in the plasmid pCITE-2a (Novagen) under control of the T7 promoter (FG236) between an *Nco*I site and an *Eco*RI site. The resulting construct was checked by sequencing.

Cell culture, transfection, and CAT assays. C2C12 myoblasts and NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (FCS). Transfection of the promoter chloramphenicol acetyltransferase (CAT) constructs were performed by the standard calcium phosphate method as previously reported (29). The dystrophin promoter activity was about 120% in C2C12 cells with respect to the simian virus 40-CAT construct used as the positive control. Typically, a transfection experiment included 3 μg of reporter plasmid, and 0.6 to 1 μg of cytomegalovirus-LacZ plasmid was used for the transfection efficiency control (7). In cotransfection experiments, expression plasmids were added as indicated in the figure legends. The amount of transfected plasmids was held constant by addition of pBluescript as a DNA carrier. In serum induction experiments, after transfection, cells were incubated in Dulbecco modified Eagle's medium containing 0.5% FCS for 40 h before stimulation with 15% FCS for 6 h. Cell extracts were made by three or four cycles of freezing and thawing in 0.25 M Tris-HCl (pH 7.8) followed by centrifugation. CAT activity was determined as described by Gorman et al. (13). Acetylated forms of chloramphenicol were quantified by scanning the thin-layer chromatography plates with the Image Quant radio analytic system from Molecular Dynamics. A minimum of four independent transfection experiments were performed with independent DNA preparations. The average variation of CAT activity observed for each construct was less than 10% in different transfections. The data of cotransfection experiments with recombinant SRF and SRF-M2 were not corrected for the β-galactosidase activity, because we observed a systematic reduction of the β-galactosidase levels as previously observed by Hill and coworkers (17). In this case, six or seven independent experiments were performed.

Gel mobility shift assay. Probes and competitors for gel mobility assays were obtained by annealing of the following oligonucleotides: 2 and 3 (ATGGATCC TCAAGTTGGTTTCCCATAATAGGAGATGAGTAAGCTT) for D-CArG-wt; 4 (GGATAAGCTTACACAGGATGTCCATATTAGGACAT) and 5 (AT GGATCATGTCCTAATATGGACATCCATGTGTAGCTT) for SRE; 6 and 7 (ATGGATCCTCAAGTTGGTTTCCCCGATTGGGAGATGAGTAAGCTT) for D-CArG-M; 8 and 9 (ATGGATCCTCTCAAGTTATACTCCCATAATAG GAGATGAGTAAGCTT) for D-CArG-m2; 10 and 11 (ATGGATCCTCAAG CAGGATGTCCATAATAGGAGATGAGTAAGCTT) for TCF; 30 (AGTTC CTATTATGGGA) and 31 (TGATCCCATAATAGGA) for D-CArG; 32 (AG TTTATGGGAAACC) and 33 (TGAGGTTTCCCATAA) for DPBE; 34 (AGTTCCTATCICGGGAAACC) and 35 (TGAGGTTTCCCICIATAAGGA) for IC-1; 36 (AGTTCCTATTATIIGAAACC) and 37 (TGAGGTTTCCCATA ATAGGA) for IC-2; 38 (AGTTCCTATTATGGGIIICC) and 39 (TGAGGCC CCCCATAATAGGA) for IC-3.

Cold in vitro-translated proteins were prepared with the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's description. The synthesis of proteins was monitored by determination of [^{35}S]methionine incorporation and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Preparation of nuclear extracts was performed as previously described (38). Binding reaction mixtures (20 μl) typically contained 15 μg of nuclear extracts, 4× reaction buffer (4 mM $MgCl_2$, 240 mM KCl, 16 mM Tris [pH 7.9], 48 mM HEPES [pH 7.9], 4 mM dithiothreitol [DTT]), 2 μg of poly(dI-dC), 3 mM spermidine, and 1 mg of bovine serum albumin per ml. For assays containing proteins produced by in vitro translation, 1 μl of reticulocyte lysate was used for a standard binding reaction with 200 ng of calf thymus. Complexes were allowed to form for 30 min at room temperature, and 5 μl of 20% Ficoll was added before loading on 6% acrylamide-bisacrylamide (40:1) gels in 0.5% Tris-borate-EDTA. The probes utilized were terminally labelled oligonucleotides. Anti-SRF polyclonal antibodies, described by Hipskind et al. (19), were kindly provided by Robert Hipskind.

Circular permutation analysis. DPBF was obtained from C2C12 nuclear extracts. The extracts were concentrated with a Centricon-10 filter (Amicon), 250 μl was loaded on a Superose 12 fast protein liquid chromatography column (Pharmacia), and fractions were eluted in buffer C (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, 4 μM leupeptin, 10% glycerol). The fractions were tested by band shift. The positive fractions were purified by chromatography on a DNA affinity column consisting of Sepharose 4B (Pharmacia) coupled to the annealed and polymerized oligonucleotides 40 (GGATAAGCTTATGGGAAA CCA) and 41 (ATCCTGGTTTCCCATAAGTT). After four washes with 2 ml of buffer Z (25 mM HEPES [pH 7.6], 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% Nonidet P-40, 0 to 1 M KCl) and 0.1 M KCl, bound proteins were eluted by successive washes with 1 ml of buffer Z at increasing concentrations of KCl. Each fraction (5 μl) was tested by band shift, and the positive and clearest fractions were used for the circular permutation analysis. DNA probes for circular permutation analysis were prepared by restriction enzyme cleavage of pBend-DPBE, dephosphorylated with calf intestinal phosphatases, and purified by agarose gel electrophoresis. The probes were then labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP and purified by acrylamide gel electrophoresis. Circular permutation analysis assay was performed as described for the gel mobility shift assays. The mobilities of free DNA and DPBF-DNA complexes were determined by measuring the distances travelled from the origin of the gel, and the ratios were plotted as described in the legend to Fig. 7. The induced bending angle (α) was estimated by using the empirical equation $\mu M/\mu E = \cos \alpha/2$ (45), where μM and μE are the relative mobilities of DPBF-DNA complexes corresponding to the middle and the end of the DPBE sequence, respectively.

RESULTS

SRF binds to the muscular dystrophin promoter CArG box element. Progressive deletion analysis revealed that the region of the dystrophin promoter between −96 and −73 contains the main regulatory element of the minimal promoter in muscle cells. Moreover, site-specific mutagenesis showed that the CArG box, present within this sequence, is necessary for the muscle preference expression of the promoter (12). These studies suggested that the ubiquitous SRF, which recognizes the CArG box in a different promoter, could be involved in the muscle-specific expression of the dystrophin promoter. To investigate in more detail the transcriptional regulation of the minimal dystrophin promoter and the role of SRF in muscle-specific transcription, we first tested whether in vitro-synthesized SRF binds to the dystrophin CArG box element. SRF binds with high affinity to the c-*fos* SRE. The dystrophin CArG box differs from c-*fos* SRE both in the AT-rich core element and in the flanking sequences outside the conserved CC and GG nucleotides (the probes used are shown in Fig. 1C). As shown in Fig. 1A, in band shift experiments the dystrophin CArG box probe generated a retarded band which migrates at the same apparent molecular weight of the retarded band obtained with the c-*fos* SRE probe (compare lane 1 with lane 5 in Fig. 1A). Competition experiments showed that both oligonucleotides containing the c-*fos* SRE binding site and the dystrophin CArG box binding site cross-competed for the binding to SRF. Figure 1A, lanes 2 to 4, shows a competition experiment in which the c-*fos* SRE was used as a probe with a 100-fold molar excess of each cold oligonucleotide. Competition is obtained with either the c-*fos* SRE oligonucleotide or the dystrophin CArG box element. In contrast, an oligonucleotide carrying a nonspecific sequence, under the same conditions, did not compete for the binding to SRF (Fig. 1A, lane 4). In lanes 6 to 8 (Fig. 1A) are shown the results of the converse experiment in which the dystrophin CArG box binding was competed with the c-*fos* SRE (lane 6) or the dystrophin CArG

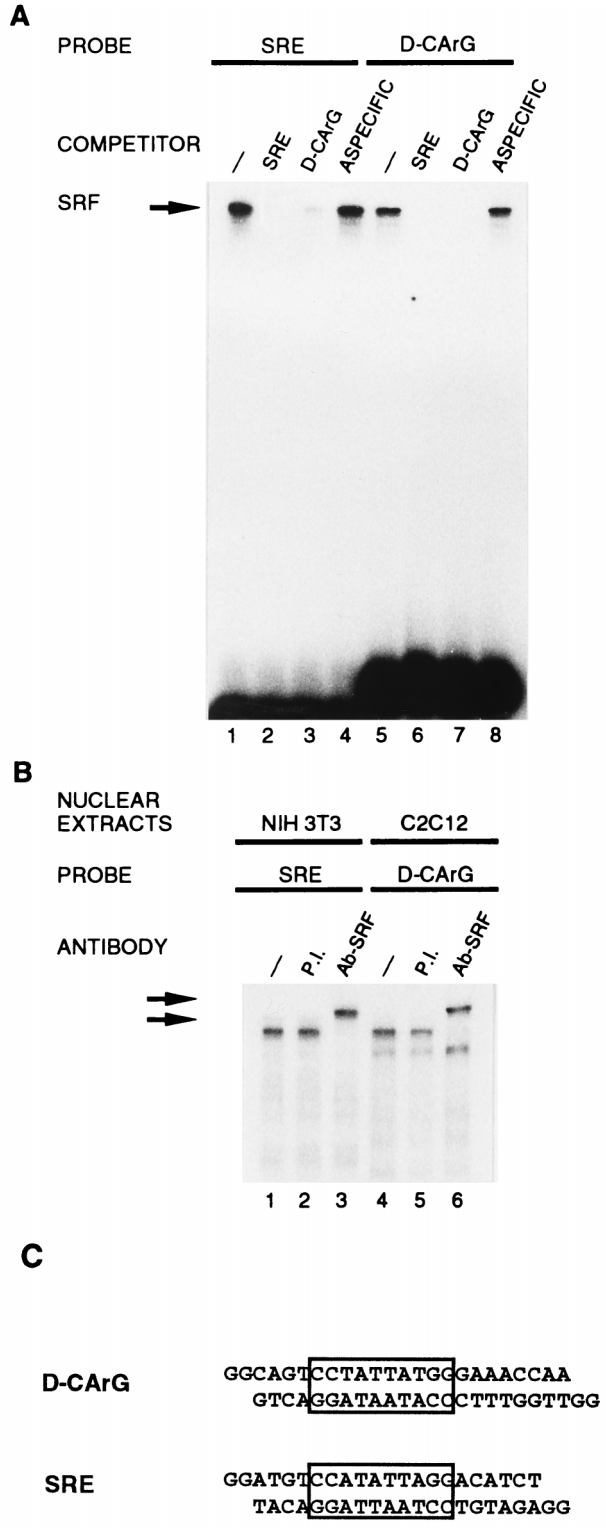


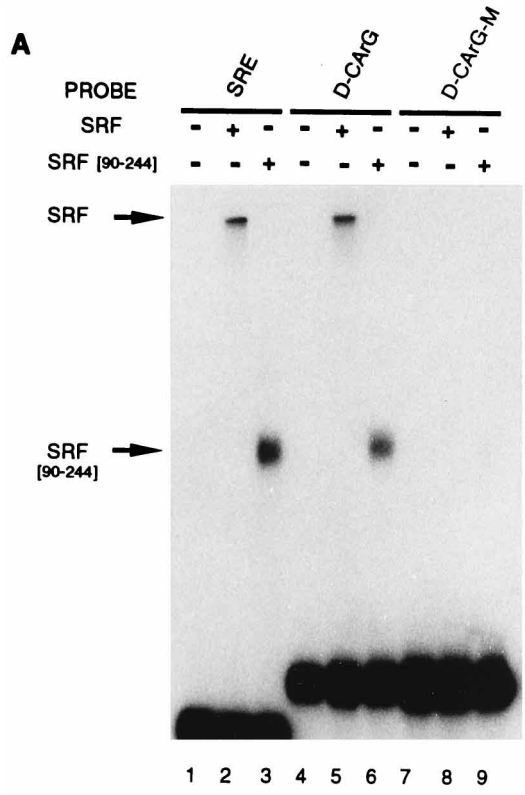
FIG. 1. The dystrophin CArG element is recognized by SRF. (A) Bindings were carried out with in vitro-synthesized SRF under standard reaction conditions. Competition was performed with a 100-fold molar excess of the indicated oligonucleotides containing the c-*fos* SRE (lanes SRE), the dystrophin CArG sequence (lanes D-CArG), and an unrelated sequence (lanes ASPECIFIC). (B) Retarded bands obtained with nuclear extracts as indicated were preincubated with either anti-SRF polyclonal antibody (lanes Ab-SRF) or preimmune serum (lanes P.I.). The nuclear extracts from NIH 3T3 cells were incubated with the c-*fos* SRE oligonucleotide, and the nuclear extracts obtained from C2C12 mouse muscle cells were incubated with an oligonucleotide containing the dystrophin CArG box sequence (D-CArG). (C) Oligonucleotides used for the experiments shown in panels A and B. SRE and the dystrophin CArG box are outlined.

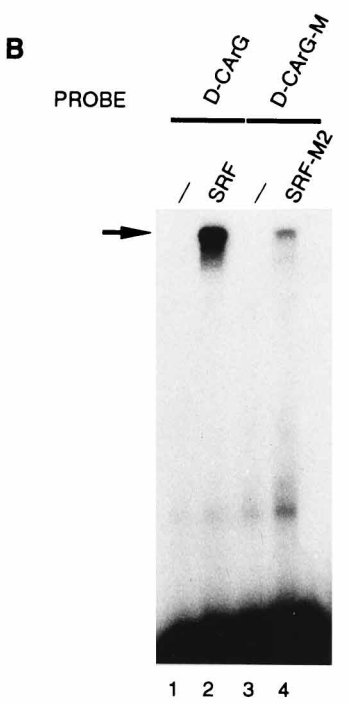
box (lane 7), but not with an oligonucleotide containing a nonspecific sequence (lane 8). To further verify that the retarded band contained SRF, we incubated the band shift reaction mixtures obtained with nuclear extracts from C2C12 muscle cells with antibodies that specifically recognize SRF. As a control, we incubated the anti-SRF antibodies with the retarded bands obtained with c-*fos* SRE and NIH 3T3 nuclear extracts. Incubation of the reaction mixture containing the dystrophin probe with anti-SRF antibodies generated a supershift similar to that obtained with the c-*fos* SRE (Fig. 1B). Thus, nuclear SRF binds to the dystrophin CArG box element.

We also tested whether MEF-2 was able to bind to the dystrophin CArG box, as this factor is muscle specific and belongs, like SRF, to the MADS box proteins (40). in vitro recombinant MEF-2 (10) did not recognize the dystrophin CArG box element (data not shown). Since on this segment of the dystrophin promoter a putative E box is also present, we tested whether MyoD1 could bind. In our experiments, the in vitro-synthesized MyoD1 was unable to bind to the dystrophin promoter (data not shown). This is in agreement with the mutagenesis experiments performed with the promoter (see below) and with previous results showing that MyoD1 is not able to transactivate the dystrophin promoter (12).

SRF altered-specificity mutant restores muscle-constitutive transcription of dystrophin promoter mutants. The binding of SRF to the dystrophin CArG box element suggests its involvement in the muscle-specific transcription of the gene. To investigate whether binding of SRF to the dystrophin CArG box element plays a functional role in the muscle-constitutive transcription, we performed transient-transfection experiments with a dystrophin promoter mutant whose expression would depend on transfected SRF. We adopted the strategy of Hill and coworkers, in which a promoter mutant could be rescued by the cotransfection of an altered-specificity SRF mutant which recognizes the mutated promoter (17). This strategy allows the effect on the promoter transcription mediated by the cellular SRF to be ignored.

Figure 2A shows the binding obtained with in vitro-synthesized SRF with the c-*fos* SRE, the wt dystrophin CArG box element, and one oligonucleotide mutant in which the AT-rich sequence of the dystrophin CArG box TATTAT was substituted with the sequence CAATCG (D-CArG-M). Both the recombinant full-length SRF and the core molecule, containing the dimerization and DNA binding domain (amino acids 90 to 244), bind to the dystrophin CArG element (Fig. 2A, lanes 5 and 6) but failed to recognize the oligonucleotide mutant D-CArG-M (lanes 8 and 9 in Fig. 2A). The CArG-M sequence is a recognition site for the yeast SRF homolog MCM1 and could also be recognized by the chimeric molecule SRF-M2 obtained by Hill and coworkers by substitution of residues 133 to 166 of the SRF DNA binding domain with the corresponding residues of the yeast MCM1 molecule (17). As shown in Fig. 2B, SRF-M2 was able to bind to the dystrophin CArG-M sequence, although less efficiently when compared with the binding obtained with an equal amount of wt SRF on the dystrophin CArG box (compare lanes 2 and 4). By transient transfections, we verified that a dystrophin mutant carrying the CArG-M site (m1) affected the promoter transcription. The introduction of the MCM1 recognition sequence in place of the wt dystrophin CArG box strongly reduced the promoter activity in C2C12 cells (Fig. 2C). As this promoter mutant was





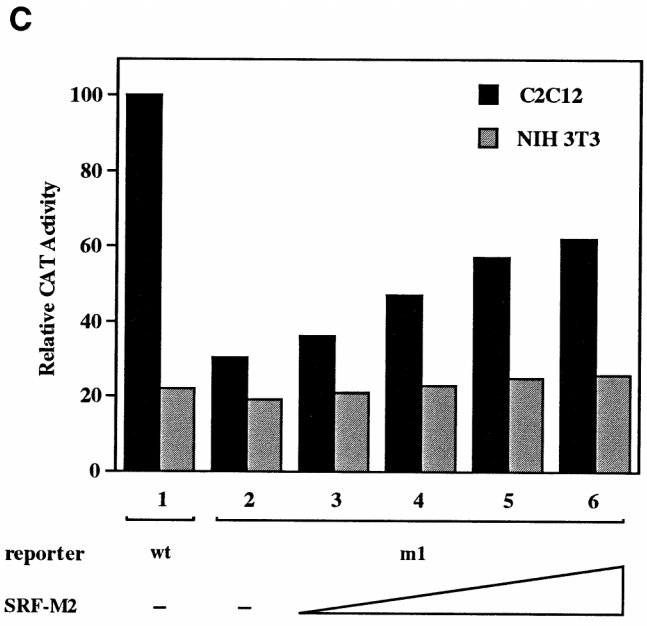


FIG. 2. SRF is necessary for the dystrophin muscle promoter transcription. (A) The full-length SRF and the core molecule containing amino acids 90 to 244 were synthesized in vitro and incubated with one oligonucleotide containing either the c-*fos* SRE sequence, the dystrophin CArG box (D-CArG), or a mutated dystrophin CArG box sequence in which the core AT-rich sequence was substituted with the MCM1 recognition sequence (D-CArG-M). (B) Binding of the in vitro-synthesized SRF to the wt dystrophin CArG box sequence (D-CArG) and binding of the SRF altered-specificity mutant SRF-M2 to the mutated dystrophin CArG box (D-CArG-M). (C) Transcriptional activation mediated by the altered-specificity mutant SRF-M2. For comparison, bars in group 1 represent the wt construct transcriptional level in arbitrary units as has been reported (the dystrophin promoter activity corresponds to about 120% with respect to the positive control simian virus 40-CAT in C2C12 cells). Bars in groups 3 to 6 show the transcriptional effect obtained by the cotransfection of the m1 construct (for the nomenclature of the mutant, see Fig. 3) with increasing amounts (50, 100, 200, and 400 ng) of SRF-M2. CAT activity in the C2C12 muscle cells and NIH 3T3 cells is indicated by black and shaded bars, respectively. The data are the averages from six independent experiments.

not recognized by SRF, this result suggests that SRF is necessary for the promoter activity in muscle cells. In order to observe whether the SRF-M2 mutant could rescue this mutant, C2C12 muscle cells or NIH 3T3 fibroblasts were cotransfected with SRF-M2 together with the m1 construct in which the dystrophin promoter contains the CArG-M mutation (a scheme of the m1 construct is shown in Fig. 3). As shown in Fig. 2C, the cotransfection of the altered-specificity mutant SRF-M2 restored the muscle-constitutive expression of the mutant promoter. Transfection of increasing amounts of SRF-M2 showed that approximately 400 ng of transfected plasmid gave the highest promoter activity, and 200 ng was sufficient to restore almost 60% of the promoter activity in the same cells. In contrast, the cotransfection of SRF-M2 did not induce expression of the dystrophin mutant promoter in NIH 3T3 cells (Fig. 2C). These results indicate that binding of SRF to the dystrophin promoter is necessary for the promoter activity but that its activity on this promoter is restricted to muscle cells.

DNA sequence flanking the dystrophin CArG element is necessary for muscle-constitutive transcription. The experiments discussed above establish that SRF contributes to muscle-constitutive transcription of the dystrophin gene. SRF is a ubiquitous molecule which activates transcription in a nontissue-specific manner in other promoter contexts. One way in which SRF regulates transcription is the interaction with other transcription factors. It has been shown that SRF interacts with Ets accessory proteins at the c-*fos* SRE element in response to serum induction and that it can cooperate with muscle-specific factors such as MyoD1 in the muscle-specific α-actin gene (9, 19, 22, 37, 39, 42, 46). To investigate whether the muscle-constitutive activation mediated by SRF on the dystrophin promoter requires additional *cis*-acting elements, we mutated

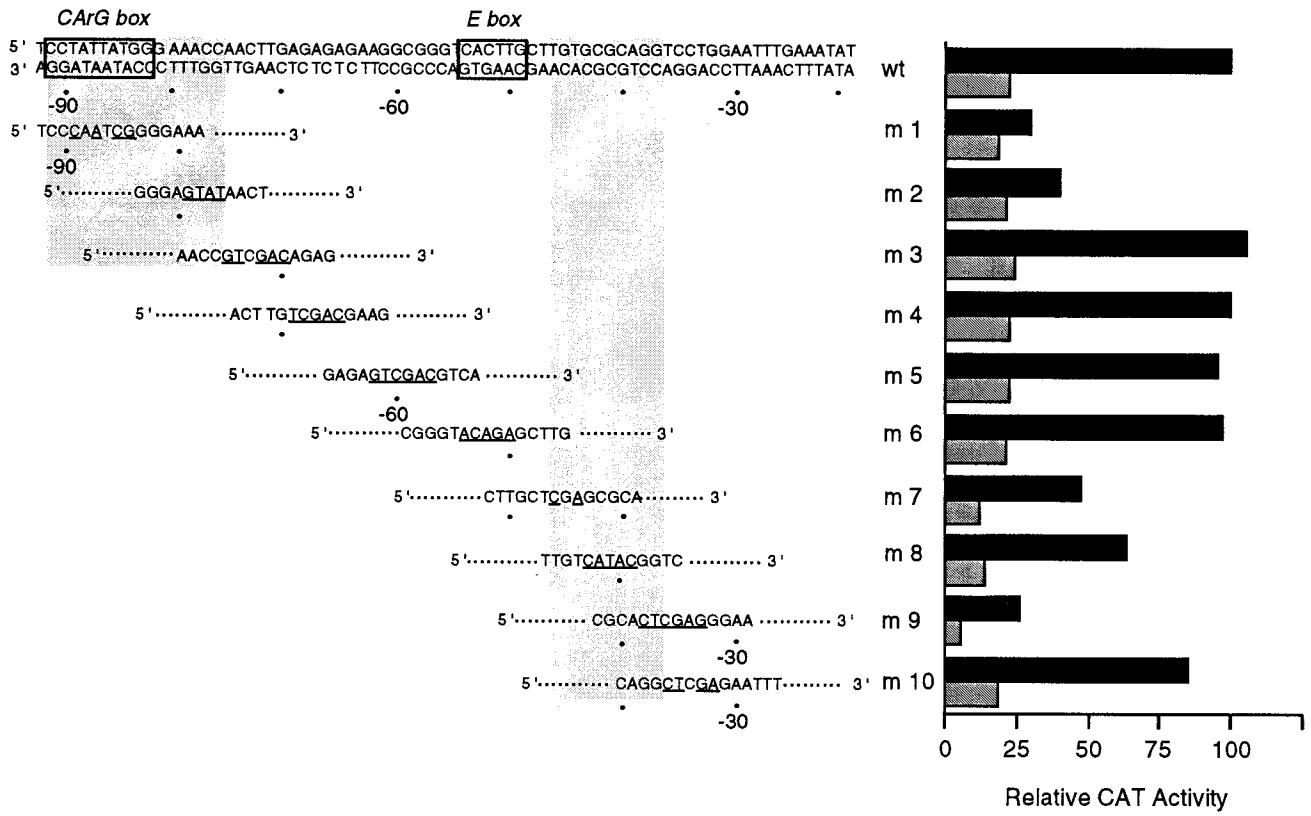


FIG. 3. Diagrammatic representation of the dystrophin promoter (−92 to −19) and the different mutants with their relative CAT activities. For each mutant, only a short region around the mutation is represented, and the bases which were changed are underlined. The shaded areas indicate the tracts of DNA in which mutations significantly decrease the transcription activity. The transfection was done in C2C12 cells (black bars) and NIH 3T3 cells (grey bars). The CArG box and a putative E box are outlined. The m1 mutant contains the same bases mutated as in the band shift probe D-CArG-M (shown in Fig. 1 and 3). The data are the averages from three independent experiments.

by linker scanning the promoter sequences downstream from the CArG box element and analyzed the transcriptional activity of mutants, spanning the region from −92 to −32 (m1 to m10 in Fig. 3) with respect to the transcription start site, both in C2C12 muscle cells and in NIH 3T3 cells. Five mutations showed a significant reduction in the transcriptional activity. Three mutations (m7, m8, and m9 [Fig. 3]) between positions −46 and −37 reduced the promoter activity both in C2C12 and in NIH 3T3 cells. These mutations define a proximal region which is apparently involved in the basal transcription of the promoter. Band shift experiments showed that this element is recognized by a ubiquitous nuclear factor (data not shown).

Mutations in the distal region defined by the mutants m1 and m2 affected the promoter transcription in the C2C12 muscle cells specifically (Fig. 3). The m1 mutation falls in the dystrophin CArG box element and inhibits SRF binding (Fig. 2A). m2 carries mutations at the bases just downstream from the CArG box. The lower transcriptional activity of the m2 mutant in C2C12 cells suggested that the 3′ flanking sequence of the CArG box is important for the promoter activity in muscle cells and that the dystrophin muscle-specific element is not merely restricted to the CArG box sequence but extends to the sequences adjacent to it. Interestingly, the linker-scanning mutagenesis showed that the putative E box is not involved in the dystrophin muscle-specific transcription, since mutations within the putative E box present in m6 did not affect the promoter transcription.

Sequence downstream from the CArG element confers specificity on the dystrophin promoter. The data presented above defined the dystrophin CArG box together with the sequence just downstream as the element responsible for the muscle-specific transcription of the minimal dystrophin promoter. Since the dystrophin CArG box is recognized by SRF, we firstly verified whether SRF could form a ternary complex with the known p62/ternary complex factor (TCF). Figure 4A shows an experiment of ternary complex formation either at the c-*fos* SRE sequence (lanes 2 and 3) or at the dystrophin CArG box sequence (lanes 5 and 6). Both probes where incubated with SRF synthesized in vitro together with the p62/TCF molecule Elk-1 or Sap-1a. At the dystrophin CArG element, the in vitro-synthesized SRF did not form a ternary complex with either Sap-1a or Elk-1, while under the same conditions, a ternary complex was formed on the c-*fos* SRE (Fig. 4A, compare lanes 2 and 3 with lanes 5 and 6). One oligonucleotide carrying the m2 mutation, which affects the promoter activity in muscle cells, still binds SRF in vitro and did not form a ternary complex with either Elk-1 or Sap-1a (Fig. 4A, lanes 8 and 9). The formation of the ternary complex with both Elk-1 and Sap-1a could be obtained with an oligonucleotide in which the dystrophin 3′ flanking sequence of the CArG box was substituted with a TCF consensus site (Fig. 4A, lanes 11 and 12). The retarded bands shown were obtained with the core SRF, because it allowed a better resolution of the supershift, but identical results were observed with the full-length SRF molecule (data not shown).

If the muscle-specific transcription induced by the dystrophin CArG box is due to its flanking sequence, the substitution of the 3′ flanking sequence of the CArG box with the consen-

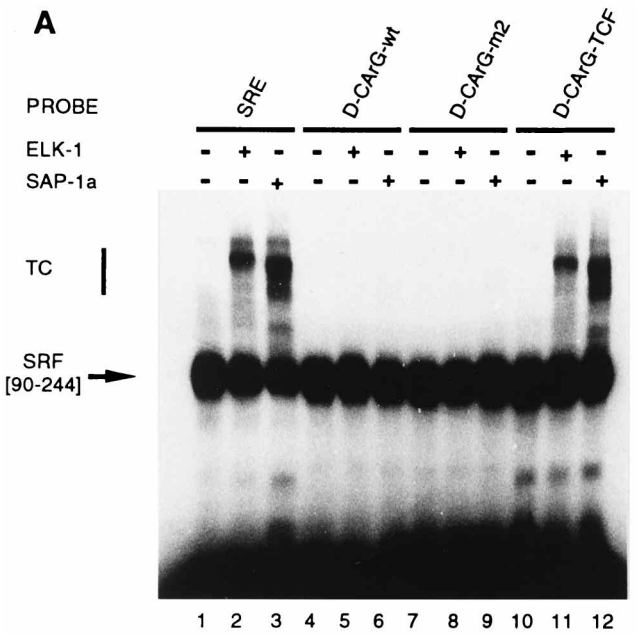
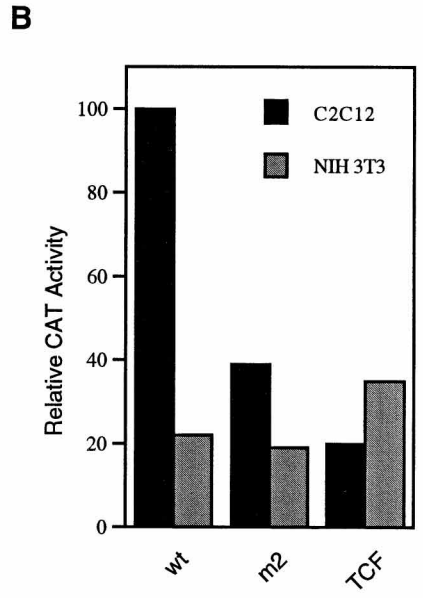
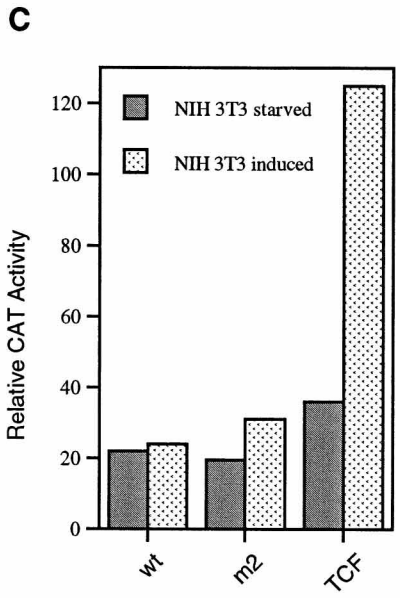


FIG. 4. The 3′ flanking sequence of the dystrophin CArG box is important for muscle-specific transcription. (A) Binding and ternary complex formation by SRF core protein, the p62/TCF molecule Elk-1 or Sap-1a, and dystrophin derivative mutants. TC, ternary complex formation. Above the lanes are indicated the probes used: c-*fos* SRE, dystrophin wt (D-CArG-wt); dystrophin 3′ flanking sequence mutant (D-CArG-m2), and dystrophin carrying next to the CArG box a p62/TCF binding site (D-CArG-TCF). +, presence of SRF cofactor in reaction mixture. −, absence of SRF cofactor in reaction mixture. (B) Cell-specific transcription of the dystrophin promoter CAT reporter genes. For the reporter CAT constructs wt and m2, see Fig. 3. The CAT construct TCF presents a TCF binding site just downstream of the CArG box (see Materials and Methods). (C) CAT activity of the same constructs transfected in NIH 3T3 cells either starved or serum induced as indicated. The data are the averages from three independent experiments.





sus TCF site could alter the dystrophin promoter specificity. To test this hypothesis, we constructed a new dystrophin mutant (TCF) in which the 3′ flanking region of the CArG sequence was mutated in order to generate a TCF consensus site in front of the reporter CAT gene and tested it in transfection experiments. This new mutant proved to be inactive in muscle cells (Fig. 4B). The serum inducibility of the TCF mutant construct was tested in NIH 3T3 cells. The cells were starved in 0.5% serum for 48 h and then treated with 15% serum. In starved cells, the dystrophin promoter carrying the TCF site, like the wt promoter and the m2 construct, is poorly transcribed. Contrary to these constructs, the TCF mutant was induced by serum treatment (Fig. 4C). These data show that the dystrophin CArG box specificity is determined by its 3′ flanking sequence and that point mutations that allow the binding of p62/TCF next to SRF could change the promoter specificity from muscle constitutive to serum inducible.

Dystrophin CArG flanking sequence is recognized by a nuclear factor that binds to the DNA major groove. To identify proteins interacting with the CArG flanking sequences and/or with SRF, we performed mobility shift experiments using C2C12 and NIH 3T3 nuclear extracts with an oligonucleotide spanning from −92 to −68 (D-CArG-wt) with respect to the transcription start site, containing both the CArG box and its 3′ flanking region. Band shift experiments allowed us to observe, besides a slowly migrating band corresponding to SRF, a specific band migrating with faster mobility with respect to SRF. Incubation of the nuclear extracts with an oligonucleotide containing the m2 (D-CArG-m2) mutation did not alter SRF binding but strongly reduced the faster-migrating band

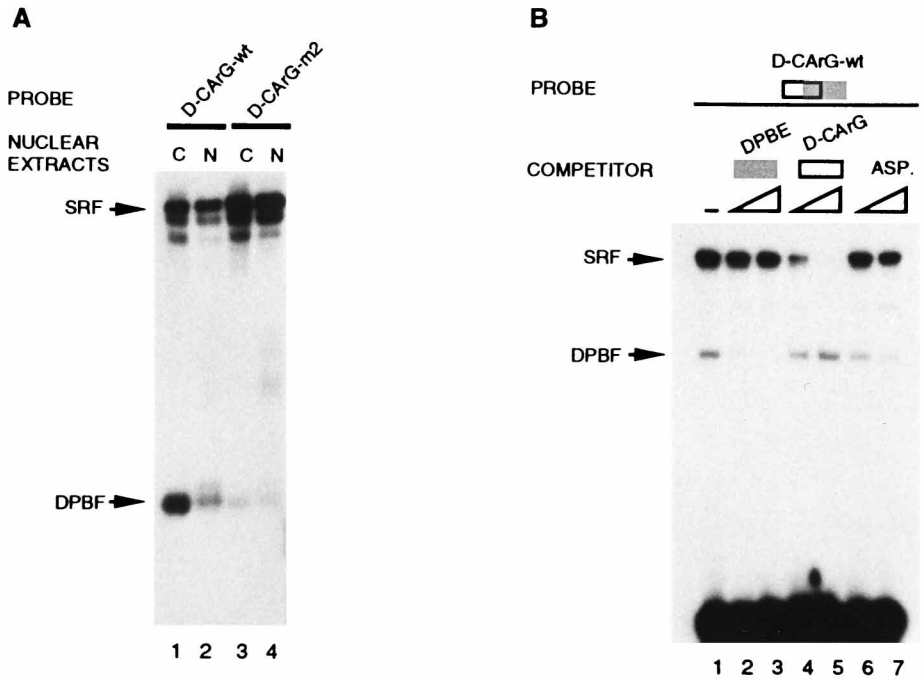


FIG. 5. Nuclear factor DPBF binds specifically to a site on the dystrophin promoter partially overlapping the CArG box. (A) Results of experiments on binding of nuclear extracts obtained from C2C12 (C) or NIH 3T3 (N) cells to the wt probe (lanes 1 and 2) and the mutated probe (lanes 3 and 4) are shown. SRF and the factor with faster mobility (DPBF) are indicated. DPBF binds the wt probe (lane 1) but does not recognize efficiently the probe containing the mutation as in m2, while SRF binding is not affected (lane 3). (B) Competition experiment. Nuclear extracts of C2C12 cells were incubated with the D-CArG-wt probe and without (lane 1) or with (lanes 2 to 7) unlabelled oligonucleotide competitors as indicated. Competition was performed with 100- and 1,000-fold molar excesses of the oligonucleotide containing the DPBE spanning from −87 to −76 of the promoter sequence (lanes 2 and 3) or the D-CArG box spanning from −92 to −81 of the dystrophin promoter sequence (lanes 4 and 5) and an unrelated sequence (lanes 6 and 7). DPBE is represented as a grey box, and D-CArG is represented as a black-bordered box.

(Fig. 5A, compare lanes 1 and 3). Thus, this binding activity correlates with the transcriptional activity of the dystrophin promoter. We named this activity DPBF (for dystrophin promoter-bending factor) (see below). DPBF activity is not restricted to C2C12 cells, since a corresponding retarded band could also be detected in NIH 3T3 nuclear extracts, although with lower intensity (Fig. 5A, compare lanes 1 and 2).

Competition experiments carried out with an oligonucleotide containing the DPBF binding site (DPBE), spanning bases from −87 to −76 of the dystrophin promoter, inhibited DPBF binding specifically without affecting SRF binding (Fig. 5B, lanes 2 and 3). Oligonucleotides containing shorter sequences failed to compete (data not shown). The competition with an oligonucleotide containing the SRF binding site alone, spanning bases −92 to −81, inhibited SRF binding specifically without affecting DPBF binding (Fig. 5B, lanes 4 and 5). A nonspecific competitor did not influence either binding within a 100-fold molar concentration and partially reduced both bindings at a 1,000-fold molar concentration. These results suggested that DPBF binding is specific for a site that extends for about 10 bases. DPBE is partially overlapping with the CArG box between bases −87 and −76 of the dystrophin promoter. SRF and DPBF can bind independently, since the specific competition of each protein did not influence the other binding. We failed to observe a ternary complex containing both proteins. This could be due either to instability of the complex in the running conditions or to the low concentration of DPBF in our assays. It could also be that their binding sites are overlapping in a way that SRF and DPBF could not bind at the same time on the same DNA molecule.

SRF binding to the DNA has been clarified by the analysis of the SRF-DNA cocrystal structure (32). SRF makes extensive contacts in the minor groove of the DNA within the AT-rich sequence of the CArG box. Its interactions with the major groove are limited to the phosphate backbone and to the G residue at the extremity of the CArG box. We checked the binding characteristic of SRF and DPBF contained in C2C12 nuclear extracts. Distamycin A is a drug with high affinity to AT-rich sequences in the minor groove of DNA, strongly interfering with TATA-box-binding protein (TBP) for DNA binding (8). As shown in Fig. 6A (lanes 3 and 4), distamycin A at concentrations of 2.5 and 25 μM severely interfered with the binding of SRF to the CArG box. On the contrary, the same concentrations of distamycin A did not affect DPBF binding to its cognate DNA sequence, suggesting that DPBF interacts with the DNA through the major groove (Fig. 6A, lanes 5 to 8).

A direct test for protein interactions with either the minor or the major groove is provided by the observation that IC base pairs resemble, in terms of hydrogen donors and acceptors, AT in the minor groove but differ from AT base pairs in the major groove. IC base pairs also resemble GC in the major groove, but differ from them in the minor groove (11, 43). We synthesized double-stranded oligonucleotides IC-1, IC-2, and IC-3 in which AT or GC base pairs were substituted with IC within the DPBF binding site (Fig. 6B). The double-stranded oligonucleotides IC-1 and IC-3 are identical to the wt probe in the minor groove, while differing in the major groove. IC-1 is mutated within the AT-rich region of the CArG box, while IC-3 differs from the wt in the major groove of the CArG box downstream sequence. Inosine substitutions present in IC-1 affected the binding of DPBF and also had a small effect on SRF binding, whereas the inosine substitutions present in IC-3 completely inhibited the binding of DPBF alone (Fig. 6C). The reduction of SRF binding to IC-1 could be due to alterations of the

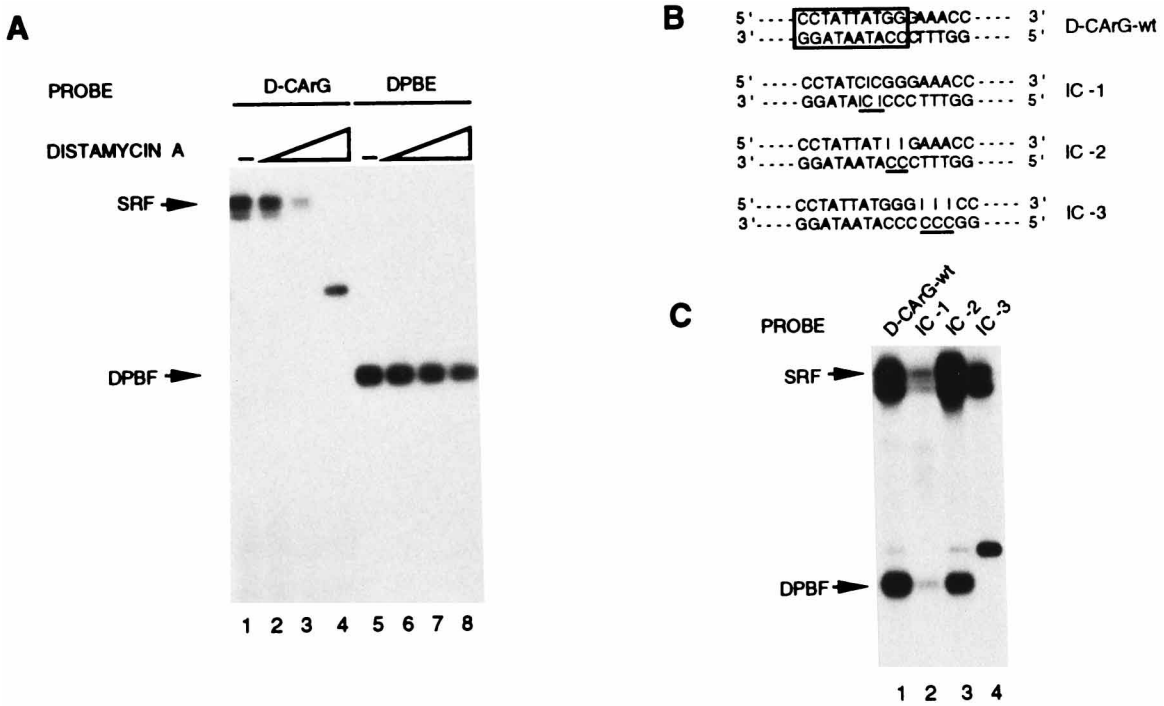


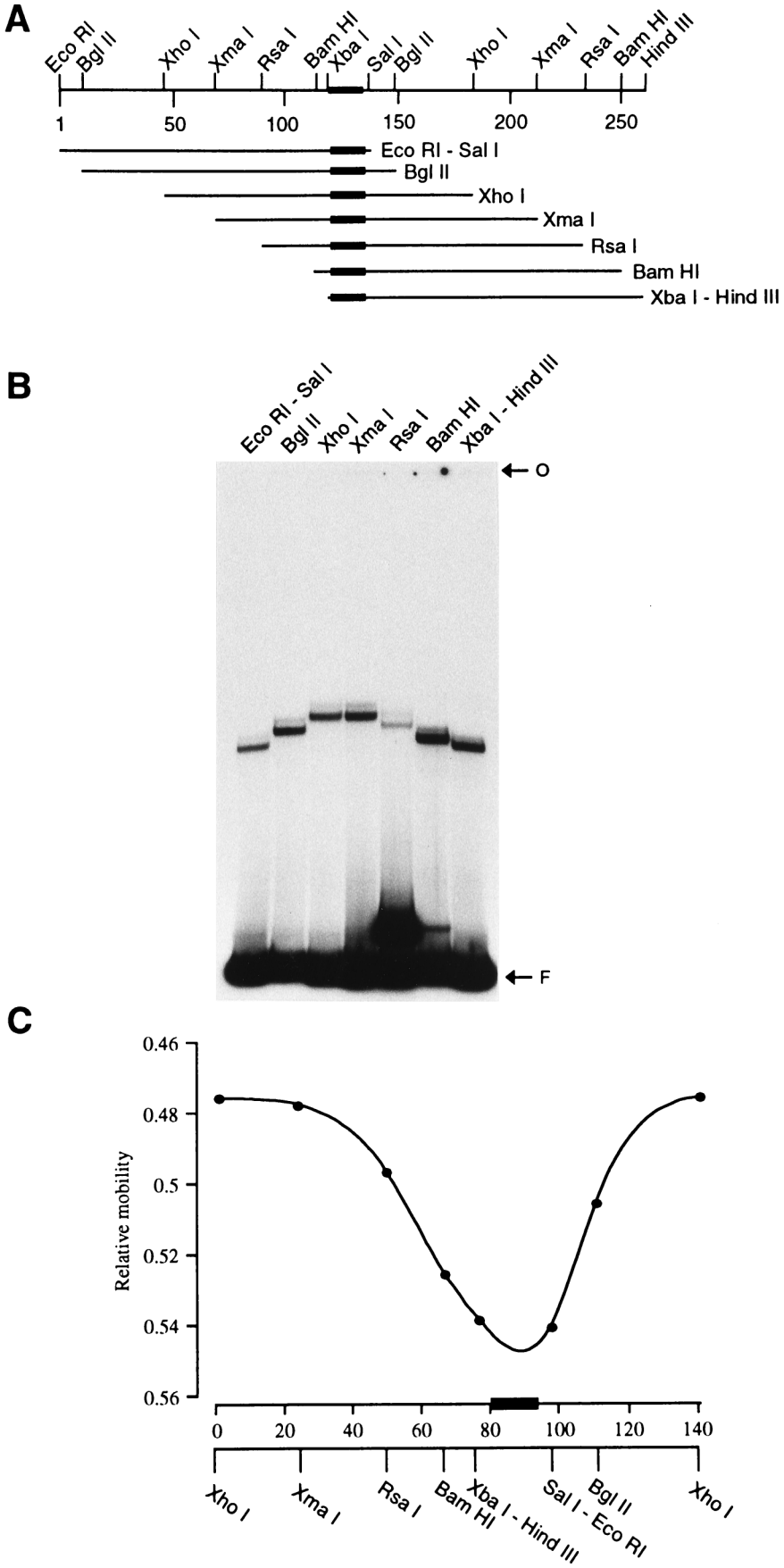
FIG. 6. DPBF binds to DPBE through contacts in the major groove. (A) Gel mobility shift assay determining the effect of distamycin A on the binding of SRF and DPBF to the DNA. C2C12 nuclear extract (5 μl) was incubated with a probe containing only the CArG box (lanes 1 to 4) or only the DPBE (lanes 5 to 8) and increasing concentrations of distamycin A: 0.25 μM (lanes 2 and 6), 2.5 μM (lanes 3 and 7), and 25 μM (lanes 4 and 8). Distamycin A competition of SRF binding allowed nonspecific binding to the probe (lane 4). (B) Schematic representation of probes used for the gel mobility shift assay shown in panel C. The CArG box is outlined in black, DPBE is represented as a grey box, and the base substitutions with inosines are underlined. The complete sequences of the probes are listed in Materials and Methods. (C) Gel mobility shift assay of C2C12 nuclear extracts incubated separately with the different probes indicated in panel B. DPBF binding is strongly reduced or abolished with inosine mutants that affect the major groove. The extra band that appears in lane 4 is nonspecific and recognizes the mutated sequence of IC-3 only.

phosphate backbone induced by the inosine residues. Inosine mutant IC-2 is identical to the wt probe in the major groove, while it differs in the minor groove at one extremity of the CArG box (Fig. 6B). IC-2 was recognized by both proteins. These observations are compatible with the crystallography data which showed that SRF does not contact the minor groove in this region (32) and suggested that DPBF does not interact with these bases of the CArG box. Taken together, these results indicated that DPBF, contrary to SRF, interacts with the DNA mainly in the major groove. Thus, the two proteins contact the DNA from opposite sites: SRF contacts DNA from the minor groove and DPBF contacts DNA from the major groove.

DPBF induces bending at the dystrophin promoter just downstream of the CArG box. DPBF is a nuclear factor which binds to its cognate site on the dystrophin promoter next to the SRF site in the major groove. Transient transfection of the multimerized dystrophin CArG box with its 3′ flanking sequence in front of the thymidine kinase promoter showed transcriptional activity in all cell types, while the multimerization of the DPBE alone did not induce transcriptional activation (not shown). These experiments suggested that the dystrophin CArG box element activates muscle-specific transcription only within the context of the dystrophin promoter and allows one to postulate that DPBF plays a structural role within the promoter. Moreover, in band shift experiments using different probes containing DPBE, we noted variations in the migration of the DPBF retarded band, suggesting that this factor could induce DNA bending.

To test the hypothesis that DPBF plays a structural role bending the DNA of the dystrophin promoter, we performed a circular permutation assay to detect possible distortion induced on linear DNA fragments by the nuclear factor DPBF. DNA fragments with a distortion in the middle of the molecule show slower migration in electrophoresis compared with DNA fragments identical in length but with a distortion near the end (51). The DPBE binding site (−87 to −76 of the dystrophin promoter) was cloned between directly repeated sequences in the plasmid pBend2 (20). Cleavage with several restriction endonucleases yielded a series of identical fragments and a circularly permuted sequence (Fig. 7A). We incubated these fragments with partially purified DPBF from C2C12 cells and

FIG. 7. Circular permutation analysis of DNA bending induced by DPBF. (A) Diagrammatic representation of the binding site of DPBF (black boxes) between a direct repeat of restriction sites. The probes obtained by the indicated restriction endonucleases and used for the assay are shown. (B) Electrophoretic mobility shift assay of the DPBF-DNA complex with the different probes shown in panel A. For this experiment, DPBF has been partially purified by gel filtration and DNA affinity chromatography of nuclear extracts. The complexes were resolved on a 6% nondenaturing polyacrylamide gel. The free DNA is indicated as F, and the origin of the gel is indicated as O. (C) Mapping of the region from the bending center to the DPBF site. The ratio of mobilities of the DPBF-DNA complex to those of unbound DNA fragments is plotted against the position of the DPBF site relative to the probe ends. The DPBF site is shown as a black box. The numbers indicate the distances in base pairs from the 5′ *Xho*I site of the DNA fragment. The extrapolation of the sides of the curve matches the bending center with the DPBF binding site. The curve fitting was performed by using Cricket Graph III.


A
Eco RI
Bgl II
Xho I
Xma I
Rsa I
Bam HI
Xba I
Sal I
Bgl II
Xho I
Xma I
Rsa I
Bam HI
Hind III
1
50
100
150
200
250
Eco RI - Sal I
Bgl II
Xho I
Xma I
Rsa I
Bam HI
Xba I - Hind III
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Eco RI - Sal I
Bgl II
Xho I
Xma I
Rsa I
Bam HI
Xba I - Hind III
O
F
C
Relative mobility
0.46
0.48
0.5
0.52
0.54
0.56
0
20
40
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80
100
120
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Xho I
Xma I
Rsa I
Bam HI
Xba I - Hind III
Sal I - Eco RI
Bgl II
Xho I

analyzed the electrophoretic mobility of the resulting complexes (Fig. 7B). No significant difference in the mobilities of the free DNA probes was observed, indicating that DPBE did not show intrinsic bending, while the complexes with DPBF bound near the middle migrated more slowly than complexes with the site near the ends. By analyzing the data in terms of a simple geometric model, we localized the site of the flexure within the DPBE fragment with a bending centered within the A tract located downstream of the CArG box and estimated a deviation from linearity of about 56° to 58°.

It was still possible that the DPBF activity corresponded to YY1, because this nuclear factor binds the major groove, and its recognition binding site can overlap CArG boxes and bend the DNA (16, 25, 26, 31). As the c-*fos* SRE oligonucleotide binds to both SRF and YY1, we used the c-*fos* SRE probe as a competitor to challenge the binding of SRF and DPBF to the dystrophin CArG box containing the DPBF binding site. As can be observed in Fig. 8A, the oligonucleotide containing SRE specifically competed for SRF binding but failed to compete for the DPBF binding activity. We also tested whether the in vitro-synthesized YY1 could bind to the oligonucleotide containing the DPBF site. As can be observed in Fig. 8B, the in vitro-synthesized YY1 recognized the c-*fos* SRE but failed to bind to the dystrophin probe. These results allow exclusion of the possibility that DPBF nuclear factor corresponds to YY1.

DISCUSSION

Here we have analyzed the dystrophin muscle-specific minimal promoter. We showed with both biochemical and functional data that for the tissue-specific activation of the promoter, SRF is required. However, SRF alone is not sufficient to induce muscle-specific transcription, but the activity of a DNA bending factor, which was named DPBF, is also required. This nuclear factor, like the known SRF cofactors, recognizes a sequence partially overlapping with the SRF binding site on the opposite side on the DNA molecule. DPBF could function as an architectural component, allowing the interaction of SRF with a muscle-specific cofactor on the promoter.

Our initial hypothesis was that the ubiquitous SRF is directly involved in the muscle-specific transcription of the dystrophin promoter. We first verified whether the dystrophin CArG box element is recognized by SRF. We observed by band shift

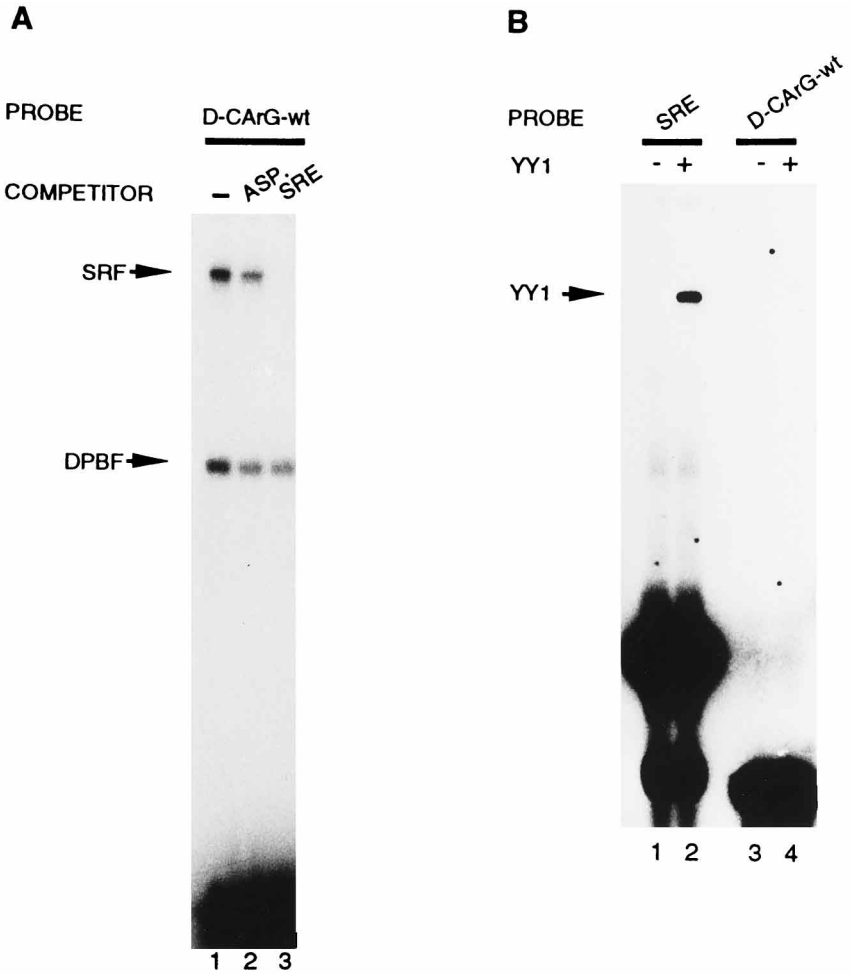

FIG. 8. DPBF binding to the dystrophin DPBE is not competed by a YY1 consensus sequence and is not recognized by in vitro-synthesized YY1. (A) The probe D-CArG-wt containing the DPBE was incubated with C2C12 nuclear extracts. The competition was performed with a 100-fold molar excess of a nonspecific oligonucleotide (ASP.) in lane 2 or with an oligonucleotide containing the SRF and YY1 binding sites of the c-*fos* promoter (SRE) in lane 3. SRE competed with D-CArG-wt for SRF binding but not for DPBF binding. (B) The SRE and D-CArG-wt probes were incubated with the products of in vitro translation, using as templates the pCITE plasmid alone (lanes 1 and 3) and the pCITE plasmid carrying the cDNA of the human YY1 (lanes 2 and 4), respectively. The retarded band containing YY1 is indicated. The dystrophin CArG box oligonucleotide containing the DPBE site is not recognized by in vitro-synthesized YY1.

experiments that the in vitro-synthesized SRF binds the dystrophin CArG element. SRF binding to the dystrophin CArG box was confirmed by binding competition experiments with SRE and the dystrophin CArG box and with anti-SRF antibodies which recognized the main retarded band obtained with the dystrophin CArG box. A possible candidate for dystrophin regulation was the related muscle-specific Mef-2 (10). However, band shift experiments with in vitro-synthesized Mef-2 did not detect binding to the dystrophin CArG box (data not shown). This result could be predicted, as Mef-2 recognizes the consensus sequence $CTA(A/T)_4TAG$, which differs from the dystrophin CArG box (35).

The binding of SRF to the dystrophin promoter suggests that SRF could be responsible for the promoter activity in muscle cells. To show that binding of SRF to the dystrophin CArG element is necessary for muscle-constitutive transcription of the dystrophin promoter, we performed transient-transfection experiments. We generated a dystrophin promoter that depends on the transfected altered-specificity mutant SRF-M2. The transient transfection of SRF-M2, together with dystrophin promoter mutant m1, recognized specifically by SRF-M2, restores the expression of the dystrophin promoter only in muscle cells. In NIH 3T3 cells, the SRF-M2 molecule is not sufficient to activate the m1 mutant construct, indicating that although SRF is present in these cells, a muscle-specific molecule involved in the promoter activation is missing.

In order to reveal other regulatory elements responsible, together with SRF, for the dystrophin transcription in muscle cells, we performed linker-scanning mutagenesis of the dystrophin promoter. These experiments identified two main *cis*-acting elements. The proximal one appears to act as a basal activator of the dystrophin promoter, as mutations in this region reduced the promoter activity in both C2C12 and NIH 3T3 cells. The distal element contains the CArG element recognized by SRF and further extends at its 3′ end. Thus, the distal element responsible for the promoter activity in muscle cells is not limited to SRF binding.

Band shift experiments with nuclear extracts allowed us to identify a nuclear factor that was named DPBF, which specifically recognized a sequence partially overlapping the dystrophin CArG box. Mutants within the DPBF recognition sequence abolished both the promoter activity in muscle cells and the binding of DPBF, thus showing that besides SRF, DPBF binding also is necessary for the promoter activity in muscle cells. However, neither SRF nor DPBF binding activity was shown to be strictly tissue specific, and we did not observe other muscle-specific binding activities on the promoter fragment. It was previously shown that the muscle-specific activation of the α-actin promoter is due to the cooperation of SRF, SP1, and a member of the MyoD family (36, 37). In this case, the tissue-specific MyoD1 factor requires the activity of the CArG box element to determine tissue-specific transcription. The dystrophin muscle-specific promoter differs from this example, because point mutations within the putative E box present on the promoter did not reduce the promoter activity, confirming that MyoD1 is not involved in the regulation of the minimal dystrophin promoter.

Synthetic promoters containing multiple copies of the dystrophin CArG box activate transcription in all cell types, while multiple copies of the DPBF binding site alone did not activate the promoter transcription (unpublished results). These results indicate that the dystrophin CArG box is not muscle specific per se. A similar behavior has been observed for virus inducibility of the beta interferon enhancer (44). Moreover, the DPBF factor does not activate transcription on its own. Taken together, these observations led to the hypothesis that DPBF acts as an architectural component in the assembly of the dystrophin promoter complex. Therefore, we analyzed a possible structural role for DPBF. Using the circular permutation assay, we observed that DPBF induces a bend of 56° to 58° in the DNA. Thus, it is possible that the role of DPBF in the muscle-specific activation of the dystrophin promoter is connected with its ability to bend the DNA. On the dystrophin promoter, SRF binds at a site centered at about nucleotide −87 with respect to the transcription start site. This is a critical distance on the DNA molecule which tends to be too rigid to allow proper interactions between molecules bound to it. DPBF binding could be necessary to allow SRF to contact other factors bound to the promoter at a critical distance. Thus, DPBF plays a role in the precise positioning of SRF in a way that it can interact specifically with a higher-order muscle-specific complex. This hypothesis is reinforced by the fact that the substitution of the dystrophin wt CArG flanking region with an optimal TCF results in the change of the promoter specificity. This result suggests that the ternary complex containing the TCF does not allow the formation of a muscle-specific complex. SRF induces a DNA bend of 72° toward the protein, while the Ets domain of the Elk-1 cofactor does not induce DNA bending or increase the DNA bending of the ternary complex (32, 41). The TCF differs considerably from DPBF, since its main function is to enhance SRF binding to the DNA. We did not observe cooperative binding between SRF and DPBF; instead, DPBF bends the DNA significantly, inducing a conformational change in the promoter. Thus, DPBF bends the DNA in a way that allows SRF to be oriented towards the general transcription complex at the right distance to allow the interaction with a muscle-specific component of the complex. Taken together, our results suggest that the binding of SRF and DPBF is necessary to recruit a muscle-specific factor, present in C2C12 cells, which does not bind directly to the promoter. In the absence of either SRF or DPBF binding, the putative muscle-specific factor cannot participate in the formation of a muscle-specific complex on the promoter.

It remains to be clarified whether DPBF and SRF bind together to the same DNA molecule or these two factors are mutually exclusive. In our assay, the mobility of the CArG-SRF complex was not altered in the presence of DPBF, and no DNase I footprint alterations due to DPBF were observed (data not published). These results suggested either that DPBF does not form a ternary complex with SRF or that the ternary complex is not stable under the conditions of analysis used. In this respect, DPBF binding resembles the homeotic Phox1 factor which is believed to form an unstable ternary complex with SRF in vitro (14). Crystallographic studies have shown that the binding of Phox1 together with SRF is possible, since this SRF contacts mainly the phosphate backbone and the minor groove, leaving the major groove accessible (32). YY1 recognizes the DNA on the major groove, and its binding site overlaps the SRE. Nevertheless, it can co-occupy the SRE with SRF at least transiently (25). DPBF could behave similarly to YY1. The DPBF binding site partially overlaps the dystrophin CArG box on the major groove. As SRF binds mainly on the minor groove, it is possible that, like YY1 or Phox1, DPBF could bind together with SRF to its cognate site. In any case, whether SRF and DPBF bind stably to the same DNA molecule is not relevant with respect to the promoter activity, since the DPBF function is not to bind cooperatively with SRF to the DNA. Thus, it is very likely that the binding of DPBF induces the right spatial positioning between the CArG box and the other component(s) of the transcription machinery by the bending induced just downstream from the CArG box. Once SRF is bound to its cognate site, DPBF could be released.

Our experiments showed that SRF can mediate both types of transcription on the dystrophin promoter: muscle constitutive or serum induced. These two activations mediated by SRF could be determined by the formation of specific complexes which bind to the sequences flanking the SRF binding site. We have provided evidence that the architectural factor DPBF is required for the muscle-specific transcription. DPBF could coordinate the assembly of a muscle-specific complex by facilitating the interactions between SRF, a muscle-specific cofactor, and the general transcription complex. Thus, multiple protein interactions promoted by DPBF binding are likely to be essential for the specificity of the dystrophin promoter. It remains to be determined whether the architectural function exerted by DPBF requires the stable binding of this bending factor within the higher-order complex or whether the transient binding of DPBF downstream from the CArG box is sufficient to position SRF within the higher-order complex.

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