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The Dystrophin Promoter Is Negatively Regulated by YY1 in Undifferentiated Muscle Cells*

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The dystrophin gene transcription is up-regulated during muscle cell differentiation. Its expression in muscle cells is induced by the binding of the positive regulators serum response factor and dystrophin promoter bending factor (DPBF) on a regulatory CArG element present on the promoter. Here we show that the dystrophin CArG box is also recognized by the zinc finger nuclear factor YY1. Transient transfection experiments show that YY1 negatively regulates dystrophin transcription in C2C12 muscle cells. On the dystrophin CArG element YY1 competes with the structural factor DPBF. We further show that YY1 and DPBF binding to the CArG element induce opposite DNA bends suggesting that their binding induces alternative promoter structures. Along with C2C12 myotube formation YY1 is reduced and we observed that YY1, but not DPBF, is a substrate of *m*-calpain, a protease that is up-regulated in muscle cell differentiation. Thus, high levels of YY1 in non-differentiated muscle cells down-regulate the dystrophin promoter, at least in part, by interfering with the spatial organization of the promoter.

The dystrophin gene, which is altered in Duchenne and Becker muscular dystrophies, is transcribed in skeletal and cardiac muscle cells from a muscle-specific promoter (1–3). The minimal dystrophin promoter (−96 to +30) drives the transcription of the CAT reporter gene preferentially in muscle cells, and it is induced by muscle cell differentiation from myoblasts to myotubes (4, 5). The main regulatory element of the dystrophin promoter is a CArG box that is recognized by the serum response factor (SRF),[1] which requires the activity of the dystrophin promoter bending factor (DPBF). The latter acts as an architectural component that alters the promoter structure and enhances dystrophin transcription probably facilitating interactions between SRF and the other components of the transcriptional complex (5).

A CArG element was first found in the *c-fos* promoter where it has been named serum response element (SRE) because of its ability to respond to serum stimulation signaling (6). CArG elements are also present in several muscle-specific promoters (7–10). Both SRE and muscle-specific CArG elements are recognized by SRF (11–15). Indication of SRF involvement in muscle-specific transcription is also suggested by its pattern of expression since SRF is mainly expressed in myogenic tissues (16, 17). Moreover, SRF interacts with muscle-specific factors including myogenin, MyoD, and Nkx-2.5 (18–20).

CArG elements can also be recognized by the nuclear factor YY1. YY1 is a zinc finger protein that binds the DNA with a high degree of flexibility in its DNA recognition (21). It can act as a transcriptional repressor or activator and, when binding at the initiator element, it becomes a component of the basal transcription complex (22–25). YY1 interacts with several transcription factors including Sp1, c-Myc, p300, TAFII55, ATF/CREB, and TFIIB and with the histone deacetylase HDA2/mRPD3, suggesting that YY1 activation or repression might be mediated by these interactions (26–33). Moreover, YY1 has been found associated with the matrix suggesting that it may mediate gene-matrix interactions by linking the promoter with nuclear matrix-associated proteins (34). On the c-*fos* promoter YY1 binds to three different sites (31, 35, 36). Because the activity of at least one of these sites was dependent on the orientation of its binding site, it was proposed that the different effects of YY1 on transcription could be due to its ability to bend DNA (36). On the muscle-specific CArG element YY1 acts as a repressor and it was proposed that YY1-dependent repression was due to its competitive binding with SRF (35, 37, 38).

Here we analyzed the developmentally regulated muscle-specific dystrophin promoter. We present evidence that the dystrophin CArG box is recognized by YY1, which acts as a negative regulator of the dystrophin promoter. We further show that YY1 competes for binding with the structural factor DPBF. Moreover, YY1 and DPBF bend DNA in opposite orientations with respect to a fixed bend. These results suggest that YY1 and DPBF regulate the dystrophin promoter transcription negatively or positively by competing with each other, at least in part, by alternatively organizing the DNA structure.

EXPERIMENTAL PROCEDURES

Plasmid Construction—To generate the DMD-CAT constructs the dystrophin core promoter was amplified as described previously (5), using the oligonucleotide primers (1) CAGGTCTAGAACACTGAGTGAGTCAACAC and (2) GGATAAGCTTACTCATGTCCTATTATGGGAAACCAACTTGAG for the −93 DMD-CAT and (1) and (3) GGATAAGCTTGAGAGAGAAGGCGGGTC for −72 DMD-CAT. The polymerase chain reaction product was cloned between *Hin*dIII and *Xba*I sites of the plasmid pUC-CAT (39).

The YY1 expression vectors (CMV-YY1 and CMV-ReY) were obtained by cloning the full-length coding sequence of the human YY1 in the pcDNA3 vector (Invitrogen) under control of the cytomegalovirus (CMV) promoter. The GST vector for YY1 expression in *Escherichia coli* was made by cloning the full-length coding sequence of the human protein downstream from the coding region for the thrombin cleavage site in the pGEX-2T vector (Amersham Pharmacia Biotech).

The set of six phasing vectors (FG 277–282) was made by cloning the annealed oligonucleotides (4) AATTCTCATCTCCTATTATGGGAAACCGAGCT and (5) CGGTTTCCCATAATAGGAGATGAG between the *Sac*I and the *Eco*RI sites of the plasmids pSB-10, -12, -14, -16, -18, and -20 (40), kindly provided by A. D. Sharrocks. All the plasmid structures were verified by sequencing.

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[1] The abbreviations used are: SRF, serum response factor; SRE, serum response element; DPBF, dystrophin promoter bending factor; CMV, cytomegalovirus; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

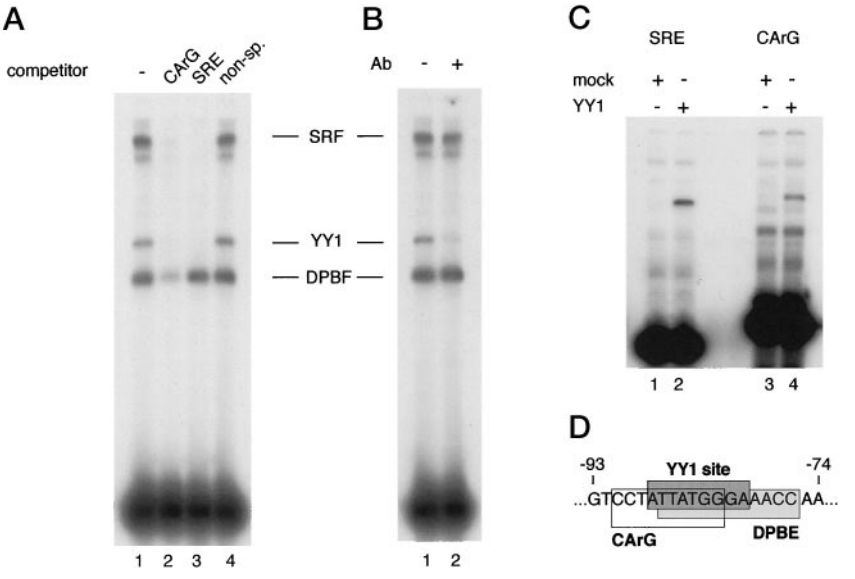

FIG. 1. **YY1 recognizes the dystrophin CArG box.** *A,* nuclear extracts of C2C12 muscle cells were incubated with the probe CArG. Competition experiments were performed with 100-fold molar excess of the indicated oligonucleotides containing the dystrophin CArG box (*CArG*), the *c-fos* SRE element (*SRE*), or an unrelated sequence (*non-sp*.). *B,* nuclear extracts were preincubated with anti-YY1 polyclonal antibody (*Ab*). In the presence of anti-YY1 antibodies in the binding reaction (+), the formation of the putative DNA-YY1 complex was inhibited. *C, in vitro* synthesized YY1 binds to both SRE and the dystrophin CArG element. *D*, graphical representation of the dystrophin CArG box. SRF, YY1, and DPBF binding sites are indicated.

Cell Culture, Transfection, and CAT Assays—C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection of the promoter CAT constructs was performed by the standard calcium phosphate method. Typically, a transfection experiment included 2 μg of reporter plasmid and 0.75 μg of RSV-LacZ plasmid as transfection efficiency control (41). Where indicated increasing amounts of expression plasmids (1, 2, and 4 μg) were added. The amount of transfected plasmids was held constant by the addition of pcDNA3. After transfection, the differentiation of C2C12 cells into myotubes was allowed by changing growth medium to Dulbecco's modified Eagle's medium containing 2% horse serum. CAT activity was determined as described previously (42). Acetylated forms of chloramphenicol were quantified by scanning the thin-layer chromatography plates using the Molecular Dynamics Image Quant radioanalytic system. A minimum of four independent transfection experiments were performed with independent DNA preparations.

The activity of the wild type dystrophin promoter (−93 DMD-CAT) was about 120% in C2C12 cells with respect to the pSV40-CAT used as positive control.

Protein Preparation—Preparation of nuclear extracts was performed as described previously (43). Cold *in vitro* translated proteins were prepared using the TNT™ Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's description. The construct FG236 (5) was used for the synthesis of YY1 and the plasmid pCITE-2a (Novagen) for the mock reaction.

YY1 was expressed in *E. coli* as glutathione *S*-transferase (GST) fusion protein, purified by glutathione affinity chromatography and eluted from the column by adding 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0), and the GST domain was removed by thrombin digestion (0.01 unit/μl for 45 min at room temperature). The amount of protein recovered was determined by SDS-PAGE followed by staining with Coomassie Blue and comparison with protein standards. DPBF was obtained from C2C12 nuclear extracts and purified as described previously (5).

Gel Mobility Shift Assay—The probe and competitors for gel mobility assays were obtained by annealing of the following oligonucleotides: (2) and (6) ATGGATCCTCAAGTTGGTTTCCCATAATAGGAGATGAGTAAGCTT; for the probe (CArG), (7) GGATAAGCTTACACAGGATGTCCATATTAGGACAT and (8) ATGGATCATGTCCTAATATGGACATCCATGTGTAGCTT; for SRE, (9) AGCTGGAGGAAAAACTGTTTCATATACAGAAGGCGT and (10) GATCACGCCTTCTGTATGAAACAGTTTTTCCTCC for nonspecific competitor (non-sp.). The probe utilized was obtained by terminal labeling of annealed oligonucleotides.

Binding reactions (20 μl) contained 10 μg of nuclear extracts and 2 μg of poly(dI-dC) in 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol. 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 DNA. Complexes were allowed to form for 10 min on ice, and 5 μl of Ficoll 20% were added before being resolved on 6% 39:1 acrylamide-bisacrylamide gels in 0.5% Tris borate-EDTA. Where indicated, 1 μl of anti-YY1 polyclonal antibody (Santa Cruz) was added in the reaction mixture.

The gel mobility shift reactions of Fig. 4 contained 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 20% glycerol, 0.05% Nonidet P-40, 1 mg/ml bovine serum albumin and the amount of recombinant proteins indicated in the legend. The calpain cleavage assay was performed by adding 1 μl of purified *m*-calpain (Calbiochem) and incubating the reaction at 37 °C for 1 h in the presence of $CaCl_2$ (2 mM final).

Phasing Analysis—DNA probes for phasing analysis were prepared by polymerase chain reaction amplifications, using the external primers (11) TATGTATCATACACATAC and (12) GAAATTAATACGACTCAC labeled with [γ-^{32}P]dATP and purified by acrylamide gel electrophoresis. The binding reactions were performed as described for the gel mobility shift assays, and the complexes were resolved on 8% polyacrylamide gels in 0.5% Tris borate-EDTA. The mobilities of free DNA and protein-DNA complexes were determined by measuring the distances traveled from the origin of the gel, and the ratios were plotted as a function of the spacer length. Curve fitting was carried out using the program CA-Cricket Graph III.

RESULTS

YY1 Binds to the CArG Element of the Dystrophin Promoter—The minimal dystrophin promoter transcriptional activation in muscle cells depends on a CArG box, which is in part similar to the c-*fos* SRE. Both SRE and dystrophin CArG boxes are recognized by SRF while the ternary complex factor TCF and the bending factor DPBF are specific for the SRE and for the dystrophin CArG element, respectively (5). To test whether in addition to SRF other proteins bind to both elements we performed competition experiments with the c-*fos* SRE on the dystrophin CArG. Using electrophoretic mobility retardation assays with nuclear extracts of C2C12 cells and a probe spanning positions −93 to −68 of the dystrophin promoter (CArG) we identified three distinct retarded complexes. The slower and the faster migrating complexes contained SRF and DPBF nuclear factors, respectively (5). Competition with 100-fold molar excess of a cold double-stranded oligonucleotide carrying the c-*fos* SRE, which is recognized by both SRF and YY1 (35), abolished both the slower and intermediate migrating bands (Fig. 1*A*, *lane 3*) suggesting that the band with intermediate mobility contained YY1. The addition of polyclonal anti-YY1 antibodies specifically inhibited the formation of the intermediate complex (Fig. 1*B*, *lane 2*). To verify further that YY1 binds to the dystrophin CArG box we performed band shift experiments with *in vitro* synthesized YY1. YY1 synthesized *in vitro* recognized both the SRE and the dystrophin CArG element (Fig. 1*C*). Taken together, these data demonstrate that YY1 specifically recognizes the dystrophin CArG element.

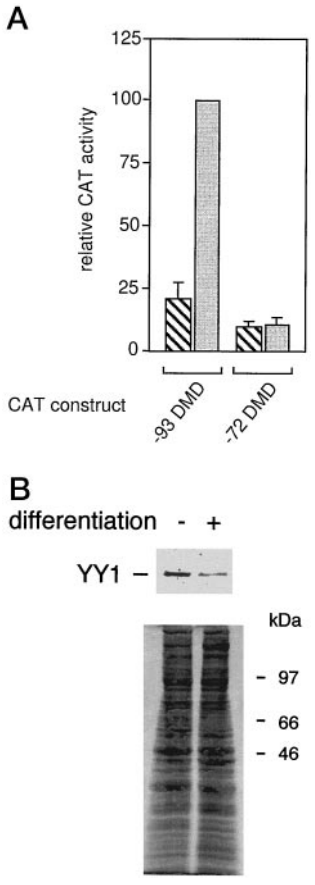

FIG. 2. **The minimal muscle-specific dystrophin promoter containing a CArG element is up-regulated in differentiated cells in culture.** *A*, transient transfection in C2C12 muscle cells of the minimal promoter (−93 DMD CAT) or a promoter deletion construct that does not contain the CArG element (−72 DMD) before (*dashed box*) or after (*shaded box*) muscle differentiation obtained by cell growth in horse serum. *B,* Western blotting with polyclonal anti-YY1 antibodies in nuclear extracts from undifferentiated and differentiated C2C12 cells. Coomassie Blue staining as a control for equal loading was used as shown in the *lower panel*.

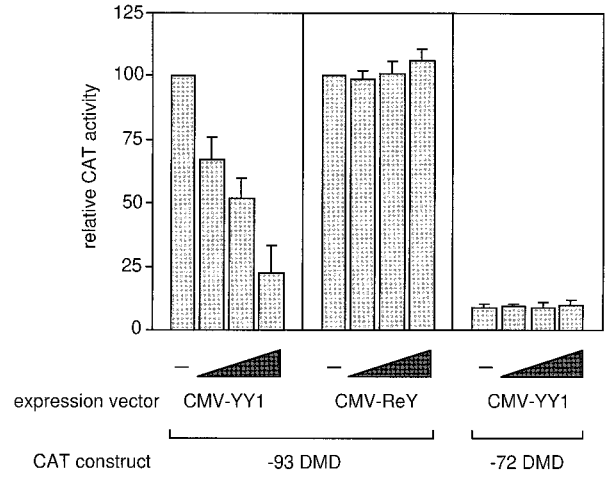

FIG. 3. **Exogenous YY1 expression down-regulates the minimal dystrophin promoter carrying the dystrophin CArG box in muscle cells.** The relative CAT activities of the reporter plasmids transfected alone or together with increasing amounts of the plamids carrying the human YY1 cDNA in the sense (CMV-YY1) or in the antisense orientation CMV-ReY are shown. The data shown are the means with the standard error derived from four independent experiments.

YY1 Binding to the CArG Box Represses Dystrophin Promoter Transcription—In C2C12 muscle cells the dystrophin promoter containing the CArG element is induced by cell differentiation *in vitro* by about 4-fold. This induction is mediated by the CArG element since a deleted promoter lacking the CArG box shows a reduced transcriptional activity and is no longer induced by cell differentiation (Fig. 2*A*). The promoter activation correlates with a down modulation of YY1 in cells treated in the same way (Fig. 2*B*).

We therefore tested whether the overexpression of YY1 in differentiated cells would play a negative role on promoter transcription. We cotransfected, in C2C12 muscle cells, an expression vector in which the YY1 cDNA was cloned under the control of the viral CMV promoter (CMV-YY1) with the minimal dystrophin promoter fused to the chloramphenicol acetyltransferase gene (−93 DMD-CAT). Increasing amounts of CMV-YY1 negatively regulated the expression of the DMD-CAT construct, but not the promoter lacking the CArG element −72 DMD-CAT (Fig. 3). Moreover, increasing amounts of the plasmid carrying the YY1 in the antisense orientation (CMV-ReY) did not affect the activity of the −93 DMD-CAT construct thereby excluding the possibility that cryptical plasmid sequences affected dystrophin promoter activity. Thus, YY1 has a negative effect on the dystrophin transcription, and its repression is mediated by the CArG element.

YY1 Competes with DPBF for Binding to the Dystrophin CArG Box Changing the Architectural Conformation of the Promoter—The partial overlap of the proteins binding to the dystrophin CArG element suggested that these proteins might influence their binding. Competitive binding between YY1 and SRF was described previously for other muscle-specific CArG elements (35, 37, 38). As on the dystrophin promoter, the YY1 binding site partially overlaps with the DPBF binding site, we investigated whether YY1 could interfere with the binding of DPBF. In band shift experiments the binding of DPBF to the dystrophin promoter was negatively influenced by the addition of YY1 to the binding mixture (Fig. 4, compare *lanes 4-6* with *1–3*). Thus, YY1 competes with DPBF for the binding on the dystrophin CArG box suggesting that their binding on the promoter is mutually exclusive.

Since both YY1 and DPBF induce bending of the DNA we investigated the structural changes induced by these proteins. YY1 bends the DNA approximately 80°, and it was suggested that its binding could influence the interactions between proteins bound to the two flanking elements (36). The binding of DPBF to the dystrophin promoter induces a bend of approximately 60° in the double helix axis with a bend center in the stretch of three A nucleotides between positions −79 and −78 just downstream of the CArG box, and its binding acts positively on dystrophin transcription (Fig. 1*D*) (5). As the YY1 center of bending is between the C and A nucleotides of the CCAT core on the dystrophin promoter this position would be between −84 and −83 at about 4 bases upstream with respect to the DPBF-induced bending (Fig. 1*D*). To test the hypothesis that these proteins induce a definite bending and to measure the angle induced by each protein with respect to a fixed bend, we performed phasing analysis with each protein on the same DNA probe. The probes used for the phasing analysis contain an intrinsic bend induced by an AT-rich track at different distances from the binding site of interest and thereby vary the helical phasing of the two sequences. If the DNA-protein complex under investigation contains a bend, its relative mobility will vary such that it is lowest when the two bends have the same orientation (in-phase) and highest when the two bends have an opposite orientation (out-of-phase) (40, 44). We prepared six probes carrying the AT-rich sequence separated by a set of spacers (10 to 20 base pairs) from the CArG box. The

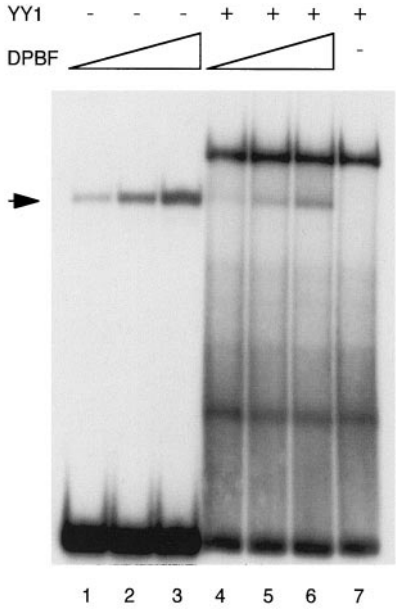

FIG. 4. **YY1 and DPBF compete for binding to the dystrophin CArG.** Results of mobility shift assays of YY1 and DPBF proteins either alone or in combination are shown. YY1 inhibits DPBF binding. *Lanes 1–3* and *4–6* contain the same increasing amounts of DPBF. *Lanes 4–7* contain 50 ng of YY1. The *arrow* indicates the DPBF complex.

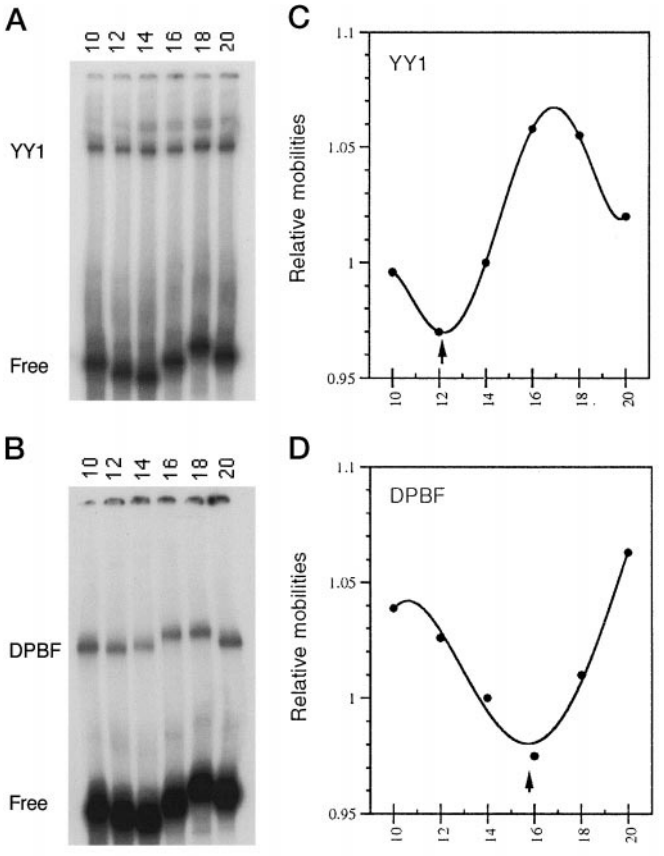

FIG. 5. **YY1 and DPBF change the architectural conformation of the promoter differently.** Results of phasing analyses of YY1-DNA complexes (*A*) and DPBF-DNA complexes (*B*) are shown. An intrinsic bend is separated by a linker from the dystrophin CArG box. The length of the linkers (10–20 bp) is indicated. *C*, phasing plot of the relative mobilities of complexes obtained with YY1 and with DPBF (*D*). The minima of the curves are shown by an *arrow*.

complexes formed independently with YY1 or DPBF were resolved by polyacrylamide gel electrophoresis. Fig. 5, *A* and *B,* shows the results of the phasing analysis of YY1 and DPBF, respectively. To determine the relative orientations of the protein-induced DNA bend, the relative mobilities of the complexes were plotted as a function of the spacer length (Fig. 5, *C* and *D*). The minima of the curves, corresponding to the minimal relative mobilities of the complexes, were obtained with a spacer of 12 bp and of 16 bp for the complexes containing YY1 and DPBF, respectively. Since the difference between these two spacers is 4 bp and the turn of the DNA helix is on average 10.5 bp, YY1 and DPBF proved to bend the promoter DNA in almost opposite orientations.

Unlike YY1, DPBF Is Not Proteolytically Degraded by m-Calpain in Vitro—Previous studies demonstrated that YY1, but not SRF, decreases during muscle-cell development and argue that YY1 reduction is due to its proteolytic degradation in differentiated muscle cells by the calcium-activated protease *m*-calpain, which is supported by the fact that *m*-calpain selectively cleaved YY1 but not SRF (37, 45). Moreover, the activity of *m*-calpain is up-regulated during myogenic differentiation and correlates with myoblast fusion (46–48). We reasoned that if the degradation of YY1 is involved in the mechanisms associated with the release of transcriptional repression of muscle-specific genes during myogenic differentiation, and that DPBF plays an antagonistic role with respect to YY1, DPBF, like SRF, should be insensitive to proteolytical degradation by *m*-calpain. In band shift experiments the incubation of YY1 with *m*-calpain in the reaction mixture reduced the intensity of the retarded complex (Fig. 6, *lane 2*). Under the same conditions, the complex containing DPBF was not affected (*lane 5*). To establish that the effect observed was due to the proteolytic activity of *m*-calpain and not, for example, due to a nonspecific inhibition of the binding capacity, we reproduced the reaction in the presence of leupeptin, an inhibitor of *m*-calpain activity (49). The addition of leupeptin in the reaction is sufficient to prevent the loss of YY1 binding. Thus, unlike YY1, DPBF is not proteolytically degraded by *m*-calpain.

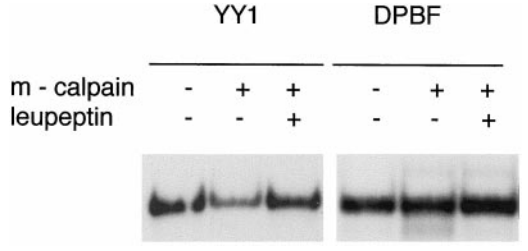

FIG. 6. **Calpain digests YY1 but not DPBF.** Purified proteins were incubated with *m*-calpain prior to incubation with the specific probes. Retarded bands containing either YY1 or DPBF are indicated.

DISCUSSION

The dystrophin muscle-specific promoter drives transcription in a developmental specific manner. Up-regulation of the promoter correlates with down-regulation of the nuclear factor YY1. We provide evidence that increased transcription of dystrophin is mediated by the release of YY1 repression during muscle cell differentiation *in vitro*. We show that the structural factors YY1 and DPBF bind competitively to the promoter and induce different bends of the DNA. Thus, the substitution of YY1 with DPBF, during muscle cell development, induces a change in the DNA structure. We propose that this structural change contributes to transcriptional activation of the dystrophin promoter.

The nuclear factor YY1 is developmentally down-regulated during muscle differentiation, and it was proposed that YY1

reduction in differentiated myotubes is due to its proteolytic degradation (37, 45). This is consistent with the idea that high levels of YY1 in non-differentiated myoblasts keep several muscle-specific genes at a low level of transcription, and upon differentiation, YY1-specific degradation allows the expression of genes at late stages. We observed that YY1 binds directly to the dystrophin CArG element. This binding was shown both with nuclear extracts and also with *in vitro* synthesized YY1 suggesting that recognition of the dystrophin CArG box by YY1 does not need accessory factors. Binding of YY1 to the dystrophin promoter may have a functional role since promoter activation correlates with a reduction in YY1 levels in C2C12 differentiated to form myotubes. In fact, cotransfection experiments demonstrated a negative effect of YY1 on the dystrophin promoter suggesting that the binding of YY1 to the CArG element is responsible for promoter repression. This was corroborated by the observation that deletion of the CArG element abolished both promoter activation during myogenic differentiation and YY1 repression. CArG elements are present in the promoters of many genes including serum-responsive genes and several muscle-specific genes. The muscle-specific dystrophin CArG element is recognized by SRF and by the structural factor DPBF, which works as a SRF accessory factor. Thus, unlike the c-*fos* SRE, in which full activation of the promoter is obtained following binding of the TCF accessory factor, transcriptional activation of the dystrophin promoter requires DPBF (5). We observed that, on the dystrophin promoter, YY1 competes with DPBF. In support of the hypothesis that during myogenic differentiation calpain-dependent reduction of YY1 may release the promoter repression we observed that YY1, but not DPBF, is selectively degraded by calcium-activated *m*-calpain.

Both YY1 and DPBF are bending factors and exert an opposite effect on promoter transcription. This led us to explore the possibility that bending could be relevant for promoter activity. Phasing analysis revealed that both these proteins induce a detectable bend when they recognize the dystrophin CArG element. However, when binding on the dystrophin CArG element, these factors bend the DNA in opposite directions suggesting that the alternative bending induced by YY1 or DPBF has opposite effects on promoter structure. Thus, it is likely that the alternative binding of YY1 and DPBF to the promoter contributes to promoter regulation by the induction of structural changes. Indirect evidence that the promoter structure is crucial for its proper activity was also observed by base insertions of different lengths between the CArG element and the TATA box: all insertions tested resulted in promoter down-regulation,[2] suggesting that the distance between elements of the promoter is crucial for the proper assembly of the various components of the transcription machinery.

It is possible that, as cellular DNA is organized in chromatin, the differential bending induced by these factors plays a role in promoter accessibility. Thus, the alternative binding of YY1 or DPBF as structural factors may lead to the formation of highly specific alternative complexes by recruiting specific proteins on the promoter. Several nuclear factors as well as histone deacetylase and matrix components are known to interact with YY1 (22, 26–34). From this perspective, only a subset of these factors would be selected on the dystrophin promoter by YY1 because of structural constraints. The reduction of YY1 during myogenic differentiation would lead to DPBF binding to the dystrophin CArG element. The alternative bending induced by DPBF on the promoter and its specific protein-protein interactions would lead to the formation of a specific transcriptionally active assembly complex. It is conceivable that some components are in common between these alternative complexes on the CArG element, and a simple change in the helical phasing is sufficient to determine the proper stereochemical organization for either repression or activation. Future experiments will address this model. For example it will be interesting to test whether both YY1 and DPBF are able to recognize the DNA organized in nucleosomal structures and facilitate the access of different regulatory factors by exposing the DNA on the nucleosome.

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