MOLECULAR AND CELLULAR BIOLOGY, Apr. 1991, p. 1944–1953
0270-7306/91/041944-10$02.00/0

Vol. 11, No. 4

Identification of Single-Stranded-DNA-Binding Proteins That Interact with Muscle Gene Elements

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Received 24 September 1990/Accepted 4 January 1991

A sequence-specific DNA-binding protein from skeletal-muscle extracts that binds to probes of three muscle gene DNA elements is identified. This protein, referred to as muscle factor 3, forms the predominant nucleoprotein complex with the MCAT gene sequence motif in an electrophoretic mobility shift assay. This protein also binds to the skeletal actin muscle regulatory element, which contains the conserved CArG motif, and to a creatine kinase enhancer probe, which contains the E-box motif, a MyoD-binding site. Muscle factor 3 has a potent sequence-specific, single-stranded-DNA-binding activity. The specificity of this interaction was demonstrated by sequence-specific competition and by mutations that diminished or eliminated detectable complex formation. MyoD, a myogenic determination factor that is distinct from muscle factor 3, also bound to single-stranded-DNA probes in a sequence-specific manner, but other transcription factors did not. Multiple copies of the MCAT motif activated the expression of a heterologous promoter, and a mutation that eliminated expression was correlated with diminished factor binding. Muscle factor 3 and MyoD may be members of a class of DNA-binding proteins that modulate gene expression by their abilities to recognize DNA with unusual secondary structure in addition to specific sequence.

Myocyte development involves the coordinated activation of many genes. Aspects of this regulation have been elucidated by the identification of nuclear proteins that specifically bind to muscle gene DNA regulatory elements and by the isolation of myogenic determination factors. A number of conserved factor-binding sites have been identified in the promoters and enhancers of muscle-specific genes. The sequence motif CANNTG, or E-box, occurs in the muscle-specific creatine kinase and myosin light-chain 1/3 genes (8, 18). This motif is the binding site for myogenic determination factors and other proteins that share a conserved helix-loop-helix motif (3, 24, 29, 30). Another conserved sequence motif, CATTCCT, or MCAT, occurs in a variety of muscle gene promoters and is essential for expression from the cardiac troponin T promoter (25). A ubiquitous protein has been identified that specifically binds to MCAT motifs from the chicken cardiac troponin T and the skeletal actin promoters (26). The $CC(A/T)_6GG$ motif, or CArG, occurs in single or multiple copies in the promoters of the sarcomeric actin genes as well as in the nonmuscle actins, interleukin receptor, and c-*fos* genes (27, 32, 42). The proximal CArG motif in the chicken skeletal actin promoter forms the core of a muscle regulatory element (MRE) that is sufficient for muscle-specific expression upstream from a TATA element (44). In contrast, the CArG motif in the c-*fos* promoter forms the core of the serum response element (SRE), which confers rapid and transient activation of expression in response to serum growth factors and is required for basal, constitutive expression in the nonmuscle actin genes (28, 43). DNA elements that contain the CArG motif interact with multiple nuclear factors in vitro. These factors include the serum response factor (SRF; a 67-kDa phosphoprotein), muscle actin promoter factor 1 (MAPF1; a 62-kDa phosphoprotein), and MAPF2 (11, 28, 32, 35, 43, 44). MAPF1 and MAPF2 are distinct proteins that differ in their cell-type distributions but have similar DNA-binding properties (46). Another 62-kDa phosphoprotein, referred to as p62, cannot bind directly to DNA but it is capable of forming a ternary complex with the SRF and the SRE (37).

Changes in DNA conformation may also influence transcription. Larsen and Weintraub (23) originally proposed the existence of a class of regulatory proteins that are opaque to B-DNA but that specifically recognize altered structures of DNA. Alterations in DNA structure can result from bending, transitions to Z- or H-DNA conformations, the action of helicases, or the action of proteins that separate DNA strands upon binding. Studies of chromatin sensitivity to S1 nuclease and bromoacetaldehyde provide evidence for the existence of altered DNA conformations (21); however, few S1 nuclease-sensitive sites map to known DNA regulatory elements, and their role in transcription is generally not clear. One approach to understanding the function of altered DNA conformations is to identify and characterize nuclear factors that potentially bind to or stabilize these structures. In this study we compared the nuclear factor-binding properties of three DNA elements from muscle-specific genes. We identified a DNA-binding activity in skeletal muscle extracts, referred to as muscle factor 3 (MF3), that is site specific but has a relatively broad specificity for DNA. Upon further investigation, we determined that this factor binds to single-stranded probes in a sequence-specific manner. MyoD, a myogenic determination protein, also binds to single-stranded DNA probes. The sequence-specific, single-stranded-DNA-binding activities of MF3 and MyoD may allow these proteins to recognize altered conformations of DNA that arise in chromatin.

MATERIALS AND METHODS

Cell culture and factor preparations. Primary cultures of breast skeletal muscle or liver were prepared from 12-day-old chicken embryos (9). Cells were plated on 60-mm Primaria dishes (Falcon) 24 h prior to transfection. Cells were

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grown in minimal essential medium with 10% horse serum and chicken embryo extract (GIBCO). The level of chicken embryo extract was 4% for the muscle cultures, but the liver cultures were grown with 1% extract. The lower levels of embryo extract are critical for high transfection efficiencies in liver. Extracts for DNA-binding and fractionation experiments were prepared from day 12 to 14 embryonic-chicken skeletal-muscle tissue by mixing 1 part tissue with 1 part buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 100 mM KCl, 1.5 mM EDTA, and 10 μg of each of leupeptin, pepstatin, chymostatin, and trypsin inhibitor per ml or 25 mM Tris, 400 mM NaCl, 1 mM EDTA, and 10 μg of leupeptin per ml). Between 20 and 50 g of tissue was homogenized at 4°C by ultrasonic vibration using a Branson cell disruptor, and the particulate material was removed by centrifugation at 100,000 × *g* for 30 min. Nuclear extracts prepared by the method of Dignam et al. (7) have identical DNA-binding activities; however, the tissue homogenization method was used for these studies because the large volumes required for fractionation could be processed more rapidly. The supernatant fraction from the centrifugation was applied to a heparin-Sepharose column that was equilibrated at 4°C in 25 mM Tris (pH 7.5)–100 mM NaCl–1 mM EDTA–10% glycerol–1 μg of leupeptin per ml–1 μg of pepstatin per ml. Unless indicated otherwise, proteins were eluted from the column with a linear gradient to 1 M NaCl. Recombinant MyoD and the anti-MyoD antibodies were gifts from A. Lassar and H. Weintraub. *Escherichia coli* single-stranded-DNA-binding protein was from United States Biochemicals.

Transfections and analysis of gene expression. Cells were transfected with 9 μg of test plasmid DNA and 0.5 μg of the plasmid pXGH5 by the calcium phosphate method (48). The plasmid pXGH5, which has the metallothionine promoter fused to the human growth hormone gene, is used as an internal control to account for differences in transfection efficiencies (36). The medium on the cells was replaced 1 h before the DNA-calcium phosphate mixture was added. The medium-DNA-calcium phosphate mix remained on the cells overnight before it was removed and replaced by fresh medium after the plates were washed. All cells were harvested 2 days after transfection, and protein extracts were prepared as described by Gorman et al. (12). The level of human growth hormone in the medium was determined by radioimmunoassay (Nichols Institute Diagnostics). Chloramphenicol acetyltransferase (CAT) activities were determined by the method of Gorman et al. (12).

Plasmid constructions and preparation. The plasmid Δ56 CAT contains the truncated mouse c-*fos* gene from positions −56 to +109 fused to the CAT reporter gene (11). Test sequences were cloned into a unique *Sal*I site that is immediately upstream from the truncated c-*fos* promoter. Oligonucleotide cassettes of wild-type and mutated MCAT elements, with compatible *Sal*I ends for ligation, were prepared on an Applied Biosystems 380B DNA synthesizer, purified by denaturing polyacrylamide gel electrophoresis and SEP-PAK C18 cartridges (Waters Associates), and annealed prior to use. The MCAT element includes the chicken skeletal actin promoter sequences from positions −51 to −74. The MCAT mutation changes the core sequence from CATTCCT to GGGCCCT. The structures of the inserted sequences were confirmed by dideoxy chain termination sequencing of the double-stranded plasmid using Sequenase (United States Biochemical) according to the directions of the manufacturer.

DNA-binding assay. Electrophoretic mobility shift assays

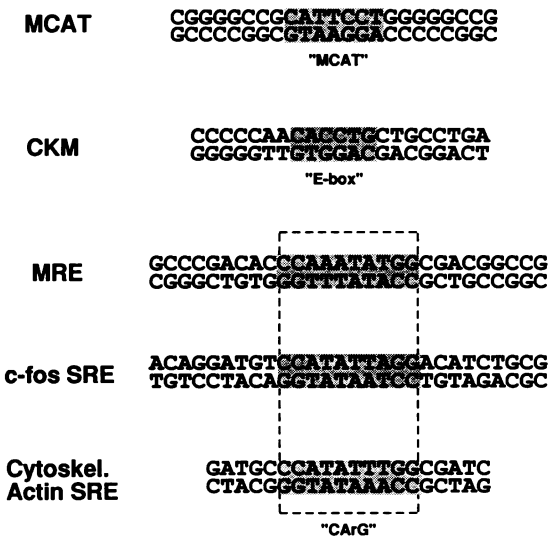


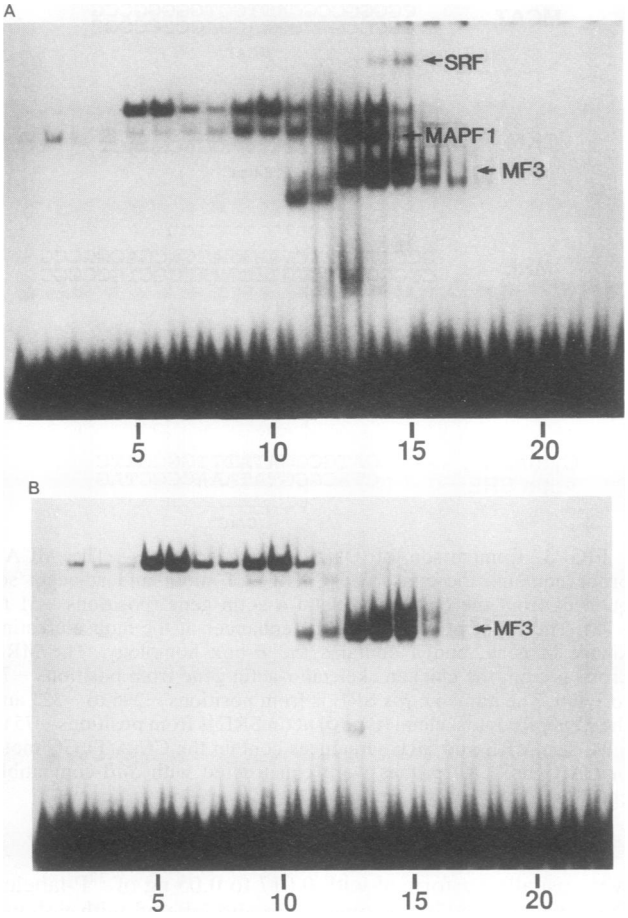
FIG. 1. Comparison of DNA probe sequences. The MCAT probe contains the conserved CATTCCT motif and adjacent sequences from the chicken skeletal α-actin gene (positions −51 to −74). The CKM probe is from the enhancer of the mouse creatine kinase M gene, and it contains the E-box homology. The MRE probe is from the chicken skeletal α-actin gene from positions −73 to −100. The human c-*fos* SRE is from positions −296 to −323 and the *Xenopus* cytoskeletal (type 5) actin SRE is from positions −75 to −94. The MRE and SRE sequences contain the $CC(A/T)_6GG$ motif or CArG box. All probes were constructed with *Sal*I-compatible ends.

were usually performed with 0.017 to 0.05 ng of ^{32}P-labeled DNA fragments (10). Probes were end labeled with polynucleotide kinase and [γ-^{32}P]ATP (>4,500 Ci/mmol; Amersham). Binding reactions contained between 0.25 and 1.0 μl of column fraction and 10 mM Tris (pH 7.5), 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 8% glycerol, and 0.1 to 1.0 μg of poly(dI-dC) · poly(dI-dC) in 10 μl (total volume). Changes in these binding reaction components are specified in the figure legends. Following a brief incubation at room temperature, binding mixes were loaded onto a 5% polyacrylamide gel and electrophoresis was performed at 10 V cm^{-1} in 22 mM Tris borate–0.5 mM EDTA buffer. Gels were dried and exposed to film for 6 to 12 h at −70°C with an intensifying screen.

Interference footprints were determined with end-labeled fragments that were modified with diethyl pyrocarbonate (39). Complexed and free DNAs were fractionated by native polyacrylamide gel electrophoresis, excised, and eluted into 50 mM Tris (pH 7.5)–0.2 M KCl–1 mM EDTA. The eluted DNA was purified with Elutip-d columns (Schleicher & Schuell), ethanol precipitated, and cleaved with 1.0 M piperidine for 30 min at 90°C. The cleaved DNA was loaded on an 20% acrylamide–50% urea sequencing gel. Following electrophoresis, the gels were exposed to film at −70°C with an intensifying screen. Autoradiographs were scanned with a Pharmacia LKB Ultroscan XL laser densitometer.

RESULTS

MF3 binds to multiple DNA probes but not to the SRE. The factor-binding properties of three muscle gene DNA regulatory elements were compared by using nuclear proteins from embryonic chicken muscle. The sequences of the MRE,



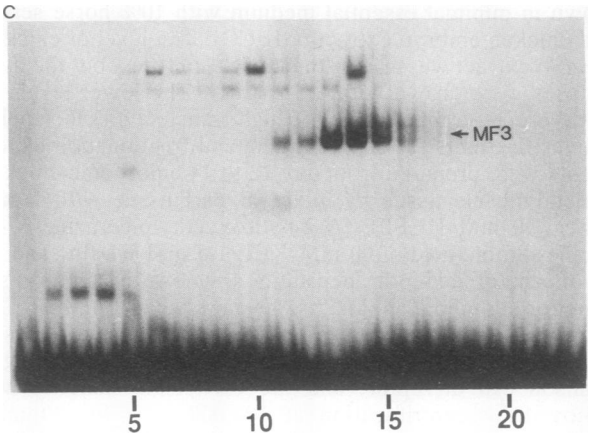


FIG. 2. Separation of chicken pectoralis muscle DNA-binding activities by heparin-Sepharose chromatography. The extract was applied to the column at 100 mM NaCl and eluted with a linear salt gradient that began at fraction number 8 and increased by approximately 66 mM per fraction. Individual fractions were assayed for their abilities to form nucleoprotein complexes with the MRE (A), MCAT (B), and CKM (C), double-stranded probes by using the electrophoretic mobility shift assay. The MAPF1-MRE, SRF-MRE, and MF3-DNA complexes are indicated by arrows. Each 10 μl of reaction mixture contained 2 μl of fraction, 50 pg of probe, and 1 μg of poly(dI-dC) · poly(dI-dC).

MCAT, and creatine kinase M (CKM) E-box probes are shown in Fig. 1. An extract of embryonic muscle was fractionated by heparin-Sepharose chromatography, and the individual fractions were analyzed for DNA-binding activities by using the electrophoretic mobility shift assay (Fig. 2). The MRE probe formed the greatest number of nucleoprotein complexes across the column profile. Two previously characterized factors, MAPF1 and SRF, bound only to the MRE probe. MAPF1 eluted at approximately 450 mM NaCl, and the SRF eluted at approximately 600 mM NaCl. The identities of MAPF1 and SRF are known from the proteins' positions in the column profile and by their electrophoretic mobilities as determined in previous studies (44, 46, 47). The position of MEF1 in the column profile is not known. MEF1 is reported to bind to the CKM probe, and it shares antigenicity with MyoD (4, 24).

A new protein that bound to all three probes eluted between 500 and 600 mM NaCl. This new binding activity was usually detected as a doublet in the gel shift assay, with the lower, intenser band eluting slightly before the upper band in the salt gradient. Further investigations revealed that this protein, detected by its binding to the three probes, also copurified by DNA affinity chromatography. The protein, or proteins, that forms these complexes is generally referred to as factor 3. This activity appears to be ubiquitous (data not shown); however, in this study we have focused on skeletal-muscle fractions and thus we refer to this activity as MF3 in subsequent discussions. DNA-binding activities other than MAPF1, SRF, and MF3 were not analyzed further in this study.

Competition experiments with nonlabeled DNA sequences were performed to test the specificity of the MF3-DNA interaction (Fig. 3A). These experiments utilized a heparin-Sepharose column fraction that contained MAPF1, SRF, and MF3. The three DNA probes bound to MF3, giving rise to doublet nucleoprotein complexes with identical electrophoretic mobilities. The MAPF1 and SRF complexes with the MRE were also detected. The MF3-DNA complexes were sensitive to competition by a molar excess of the MCAT fragment but not by the same level of a control oligonucleotide duplex made to a different nucleotide sequence. The relative intensities of the ^{32}P-labeled complexes indicated that MF3 preferentially bound to the MCAT probe. Quantitative competition experiments showed that the preference of binding to these duplex probes is MCAT > CKM > MRE (not shown). Consistent with these findings, the MRE is a weak competitor of the MF3-MRE complex; however, the SRF and the MAPF1 complexes are effectively eliminated (Fig. 3A). The complex labeled ns that appears slightly above the MAPF1-DNA complex in Fig. 3A apparently results from the nonspecific interaction of an abundant protein with the DNA, because it bound equally well to all probes and was not sensitive to competition by a molar excess of nonlabeled fragments.

MF3 was tested for its ability to form complexes with the SRE sequences from the c-*fos* proto-oncogene and *Xenopus* type 5 cytoskeletal actin gene. The skeletal actin MRE is structurally similar to the SREs on the basis of their core CC(A/T)$_6$GG motifs. The overall sequence similarities between the MRE and the SREs are 50% for the c-*fos* element and 80% for the nonmuscle actin element when the central 20 nucleotides are compared (Fig. 1). MF3 did not form a detectable complex with either SRE probe, which suggests that the factor recognizes MRE sequences that occur outside

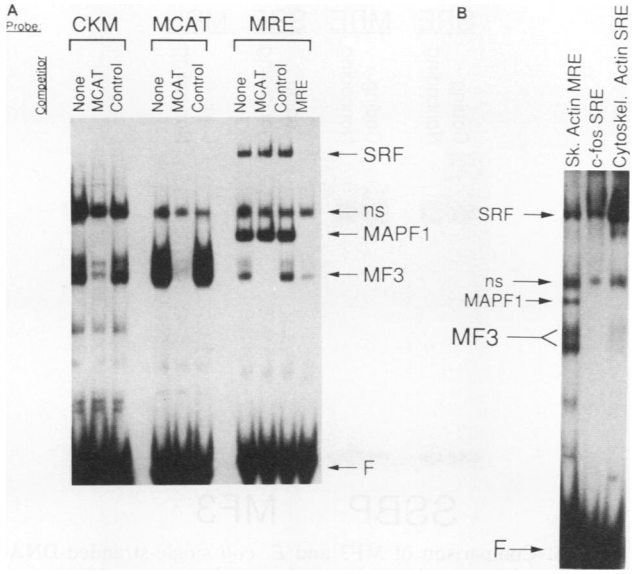


FIG. 3. Specificity of the MF3-DNA interaction. (A) Complexes between nuclear factors and the MRE, MCAT, and CKM double-stranded probes were formed in the absence or presence of a molar excess of nonlabeled DNA probe. The nonlabeled competitor DNA was MCAT, MRE, or a 20-bp control oligonucleotide duplex that was synthesized to unrelated sequences from the protein-coding region of the rabbit CKM gene. Binding mixtures contained 1.4 μg of proteins from fraction number 14 of the heparin-Sepharose profile of embryonic-chicken skeletal muscle shown in Fig. 2. The level of DNA probe was 50 pg except for CKM, which was at 200 pg in the binding mixture. The level of poly(dI-dC) · poly(dI-dC) was 250 ng, and the level of competitor was 20 ng. The positions of the SRF-, MAPF1-, and MF3-DNA complexes and the free fragments (F) are indicated by arrows. The position of a putative nonspecific protein-DNA complex (ns) is also indicated. (B) Complex formation between nuclear factors and the skeletal actin MRE, the human c-*fos* SRE (positions −298 to −321), and the cytoskeletal (type 5) actin SRE from *Xenopus laevis* (positions −75 to −94). Each 10 μl of reaction mixture contained 17 pg of probe, 250 ng of poly(dI-dC) · poly(dI-dC), and 2 μl of fraction number 15 from the heparin-Sepharose profile shown in Fig. 2. This protein fraction is enriched for SRF and MF3 but contains little MAPF1.

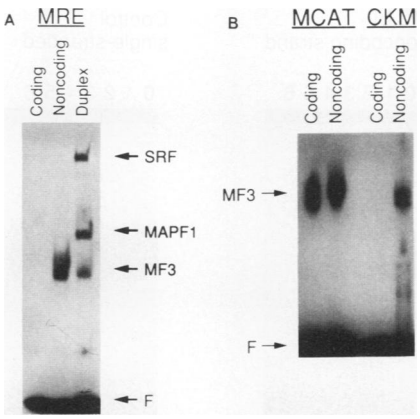


FIG. 4. MF3 binds to single-stranded DNA probes. (A) MF3, MAPF1, and SRF binding to single-stranded and duplex probes of the skeletal actin MRE in the electrophoretic mobility shift assay. The source of factors was a pool of fractions 13, 14, and 15 from the heparin-Sepharose profile shown in Fig. 2. The 10-μl binding-reaction mixtures contained 1.5 μl of pooled column fraction (1.2 μg of protein), 100 ng of poly(dI-dC) · poly(dI-dC), and 50 pg of probe. (B) MF3 binding to single-stranded probes of the MCAT and CKM elements. Individual coding and noncoding strands were incubated with 500 ng of poly(dI-dC) · poly(dI-dC), 25 pg of probe, and 0.3 μl of an embryonic skeletal-muscle fraction. The protein fraction was from a heparin-Sepharose column that was eluted between 0.3 and 0.6 M NaCl.

of the highly conserved $CC(A/T)_6GG$ motif (Fig. 3B). Taken together, the binding and copurification data indicate that the MF3-DNA complex results from the specific interaction of the same factor, or group of factors, with the MRE, MCAT, and CKM probes. The specificity of the MF3-DNA interaction is demonstrated by the sequence-specific competition and by a preference for probe sequence in complex formation. However, MF3 appears to have a broader specificity than SRF or MAPF1 for binding to DNA.

MF3 recognizes altered DNA structures. Because the MRE, MCAT, and CKM probes lack obvious sequence similarities, we reasoned that other structural features of the probe may be involved in the specific recognition of DNA by MF3. To test this possibility, we examined factor binding to the duplex and to the individual coding (upper) and noncoding (lower) strands of the DNA elements. These experiments revealed that MF3 was capable of forming a complex with the noncoding strand of the MRE, but no detectable complex was obtained with the coding strand of this element (Fig. 4A). The identity of this single-stranded-DNA-binding activity as MF3 was confirmed by copurification and by sequence-specific competition experiments. The previously characterized factors, SRF and MAPF1, differed from MF3 in that they formed detectable complexes only with the double-stranded MRE probe (Fig. 4A). The individual coding and noncoding strands of the MCAT and CKM probes were also tested for binding to MF3 in the electrophoretic mobility shift assay (Fig. 4B). MF3 formed complexes with the CKM noncoding strand and with the coding and noncoding strands of the MCAT element.

The sequence specificity of the single-stranded-DNA-binding activity was tested by competition with single-stranded and double-stranded DNA probes (Fig. 5). These experiments demonstrated that the binding was site specific and that MF3 had at least a 100-fold-higher affinity for the noncoding strand of the MCAT element than for the duplex probe. Similar results were obtained with the noncoding strands of the MRE and CKM elements (not shown).

To investigate the specificity of the MF3–single-stranded-DNA interaction further, the binding properties of MF3 were compared with that of the *E. coli* single-stranded binding protein (SSBP). SSBP is a helix-destabilizing protein that binds nonspecifically to single-stranded DNA (5). In an electrophoretic mobility shift assay, SSBP formed complexes with the individual strands of the c-*fos* SRE and the MRE (Fig. 6). In contrast, MF3 bound only to the noncoding strand of the MRE, and no complexes were formed with either strand of the SRE. Additional direct binding and competition experiments with 14 different single-stranded DNA probes failed to identify another site of MF3 interaction (not shown). Taken together, these results demonstrate that MF3 can be distinguished from MAPF1, SRF, and *E. coli* SSBP because it possesses a sequence-specific, single-stranded-DNA-binding activity.

MyoD recognizes an altered DNA structure. Because MF3 binds to the creatine kinase probe that contains the E-box

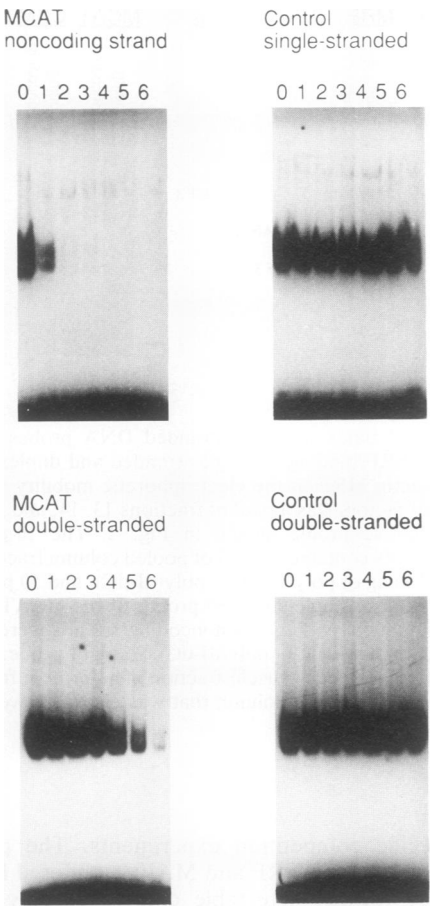


FIG. 5. Sequence-specific competition of the MF3 nucleoprotein complex by single- and double-stranded DNAs. Complexes were formed between the noncoding strand of the MRE and MF3 in the presence or absence of nonlabeled competitor DNAs. Competitors were the noncoding strand of the MCAT element, the double-stranded MCAT element, a control single-stranded sequence that was synthesized to a 20-nucleotide stretch of unrelated sequences from the protein-coding region of the rabbit CKM gene, and the human c-*fos* SRE from positions −298 to −321 (double-stranded control). Lanes: 0, no competitor; 1, 1 ng of competitor; 2, 3 ng; 3, 10 ng; 4, 30 ng; 5, 100 ng; and 6, 300 ng. The source of MF3 was 1 μl of a heparin-Sepharose fraction (0.3 to 0.6 M NaCl gradient) from embryonic-chicken skeletal muscle. Binding reaction mixtures contained 25 pg of probe and 100 ng of poly(dI-dC) · poly(dI-dC).

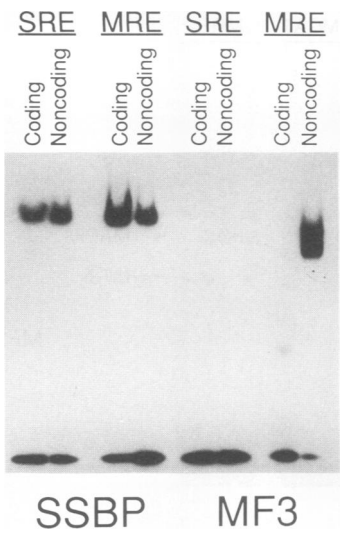


FIG. 6. Comparison of MF3 and *E. coli* single-stranded-DNA-binding protein interactions with single-stranded probes. The source of MF3 was a heparin-Sepharose fraction (0.3 to 0.6 M NaCl gradient) of embryonic-chicken muscle (1 μl), and the SSBP was present at 9.4 ng in the binding mix. Binding mixes also contained 40 pg of probe and 100 ng of poly(dI-dC) · poly(dI-dC). The SRE probes were sequences −296 to −323 of the human c-*fos* gene.

motif, we tested whether MyoD also has a single-stranded-DNA-binding activity. A recombinant glutathione *S*-transferase–MyoD fusion protein (24) was used in the electrophoretic mobility shift assay with single- and double-stranded CKM probes (Fig. 7A). MyoD formed a single predominant complex with the double-stranded CKM probe, and two predominant complexes were detected with the noncoding strand of the creatine kinase enhancer fragment. These complexes were not obtained when a glutathione *S*-transferase control extract that lacks MyoD was used in the binding assay (not shown). Quantitative competition experiments were performed with single- and double-stranded-DNA sequences to further characterize the specificity of the single-stranded-DNA-binding activity of MyoD (Fig. 7B). The CKM duplex and the CKM noncoding strand were equally effective at competing for the binding of MyoD to the CKM duplex probe. In contrast, the noncoding strand of the MCAT element, a high-affinity MF3 binding site, was not effective as a competitor (compare Fig. 5 and 7B). These data indicate that the binding of MyoD to single-stranded DNA is a high-affinity, sequence-specific interaction. The inability of the MCAT sequence to compete the MyoD nucleoprotein complex suggested that MF3 and MyoD are distinct factors. Additional direct binding experiments also failed to detect complexes between MyoD and the MRE or MCAT probes even with long exposures of the gel to the autoradiograph, indicating further that MF3 and MyoD have different sequence specificities (not shown). These results are consistent with a previous report that MyoD does not bind to the skeletal actin promoter (from positions −12 to −148), which contains the MRE and MCAT sequences (24). Furthermore, judged by their inability to alter the mobility or the appearance of the MF3-DNA complex in a gel shift assay (not shown), antibodies directed against the entire MyoD protein or the carboxyl-terminal half did not cross-react with MF3. Taken together, these data strongly indicate that MyoD and MF3 are different proteins despite a similarity in their abilities to bind to single-stranded probes in a sequence-specific manner.

Identification of sequences required for MF3 binding to the MRE. The binding of MF3 to the MRE but not to the structurally related SRE suggested that it should be possible to localize the subset of sequences within the MRE that are required for MF3 binding. Synthetic oligonucleotides were constructed that fused portions of the noncoding strands of the skeletal actin MRE and the c-*fos* SRE. Since the SRE does not bind to MF3, sequences required for factor binding could be identified by making relatively small changes in the overall structure of the element. The interaction of MF3 with these sequences was evaluated by direct binding to the radiolabeled fragment and by the ability of the nonlabeled fragment to compete for complex formation. The data from

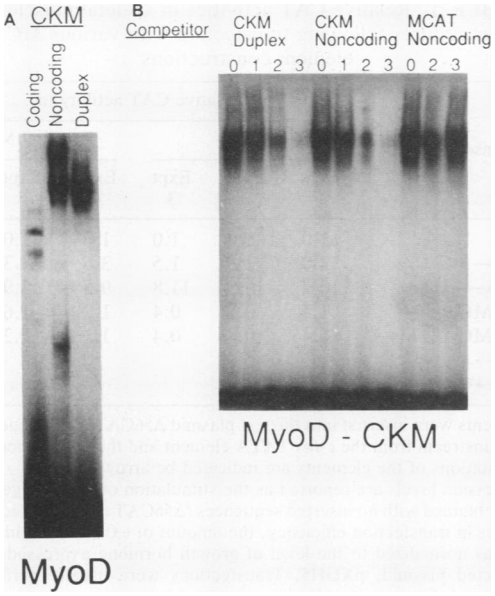


FIG. 7. MyoD binds to single-stranded and double-stranded DNA probes. (A) Binding of the bacterially produced MyoD–glutathione *S*-transferase fusion protein (MyoD) to the single-stranded and double-stranded CKM probes in an electrophoretic mobility shift assay. DNA-binding mixes contained 25 pg of probe, no poly(dI-dC) · poly(dI-dC), and 0.3 μg of the MyoD fusion protein. (B) Competition of the MyoD–duplex-CKM complex by single- and double-stranded DNAs. Lanes: 0, no competitor; 1, 3 ng of nonlabeled competitor; 2, 10 ng; and 3, 30 ng. Binding mixes contained MyoD fusion protein, 100 ng of poly(dI-dC) · poly(dI-dC), and 25 pg of the double-stranded CKM E-box probe. The binding buffer also included 1 mM dithiothreitol and 0.5% Nonidet P-40. Differences in the composition of the binding buffer or the presence or absence of poly(dI-dC) · poly(dI-dC) did not have qualitative effects on the binding of the recombinant MyoD protein to the single-stranded or duplex probes.

		Binding
a.	5'-CGGCCGTCGCCATATTTGGGTGTCGGGC-3'	+
b.	CGGCCGTCGCCtaATaTGGGTGTCGGGC	+
c.	CGcagaTgtCCtaATTTGGGTGTCGGGC	+
d.	acaggaTgtCCATATTaGGGTGTCGGGC	+
e.	CGGCCGTCGCCATATaTGGacaTCctGt	-
f.	CGcagaTgtCCATATTTGGacaTCctGt	-
g.	CGGCCGTCGCCATATTTGGacaTCtGcg	-
h.	CGGCCGTCGCCATATTTGGGT	-

FIG. 8. Identification of nucleotides within the noncoding strand of the MRE that are required for MF3 binding. Summary of MF3-binding characteristics of wild-type (a), mutated (b through g), and truncated (h) oligonucleotides constructed to the noncoding strand of the MRE. Binding to the individual ^{32}P-labeled probes was measured directly and by the ability of a molar excess of nonlabeled sequence to compete for the formation of the MF3-DNA complex in the electrophoretic mobility shift assay. Mutations inside the boxed region eliminated MF3 binding, while mutations outside of the box had no detectable effect on binding. The sequences required for MF3 binding occur in the left-hand flanking sequences of the MRE when the duplex element is viewed in the wild-type orientation.

these experiments are summarized in Fig. 8. Sequences required for MF3 binding are located in the highly conserved 3′ portion of the MRE noncoding strand, which flanks the CC(A/T)$_6$GG core. Sequence changes in the A-T-rich region of the CArG motif or the 5′ portion of the MRE had no effect on factor binding. These results are supported by the finding that a noncoding-strand MRE probe with a 3′ truncation also failed to bind to MF3 (h in Fig. 8).

Identification and function of the MF3-binding site in the MCAT element. The MF3-binding site in the MCAT element was examined in greater detail because this sequence binds to the fewest known nuclear factors and we reasoned that it would provide the clearest indication about the role of the MF3-binding site in expression. Diethyl pyrocarbonate interference footprinting was performed to determine more precisely the nucleotides required for MF3 binding. This reagent reacts with the N-7 positions of adenine and guanine residues in single-stranded DNA, making it suitable for the analysis of MF3 binding to single-stranded probes (14). If the DNA carbethoxylation interferes with factor binding in the electrophoretic mobility shift assay, a gap is formed in the sequence ladder of the excised protein-DNA complex compared with the sequence ladder of the noncomplexed DNA. High levels of interference occurred at four positions (band intensity reduction by a factor of 5 or more) in the noncoding MCAT probe (Fig. 9A). Three of these four positions occurred within the complementary sequence of the CATT CCT motif. The modification of other positions by diethyl pyrocarbonate resulted in lower or no interference in factor binding. Interference footprints using the dimethyl sulfate modification (38) revealed a smaller footprint and less interference with the noncoding strand of the MCAT element (not shown). This weaker footprint pattern may be due to decreased steric hindrance from the smaller modifying group. Diethyl pyrocarbonate and methylation interference assays using the coding strand of the MCAT element had little or no detectable effect on complex formation. These results indicate that the N-7 position of purine bases is not of key importance in all MF3-nucleic acid interactions.

The MCAT element was mutated to directly test the role of the CATTCCT motif in MF3 binding and expression. The core CATTCCT sequence was mutated to GGGCCCT in the double-stranded element. This mutation significantly diminished but did not eliminate MF3 binding (Fig. 9B). According to quantitative competition experiments, MF3's affinity for the mutated element was lower by a factor of 10 or more. The decrease in factor affinity resulting from this mutation was due to the loss of factor binding to the noncoding strand while binding to the coding strand appeared to be unaffected (not shown). The functional significance of this MF3-binding site was tested with a transient expression assay in muscle and nonmuscle cultures. In this test system, the wild-type or mutated DNA elements are inserted upstream from the truncated c-*fos* promoter that is fused to the CAT reporter gene. The truncated promoter supplies a TATA element and is weakly active; however, the insertion of functional DNA regulatory elements upstream from this sequence will result in elevated and regulated expression of the reporter gene (28, 33, 47, 50). Primary cultures were used because we have found that high levels of muscle-specific gene expression in this system are not obtained with continuous muscle cell lines. Expression from the wild-type and the mutated skeletal actin MCAT element was compared (Table 1). A single 23-bp MCAT element functioned weakly as a promoter

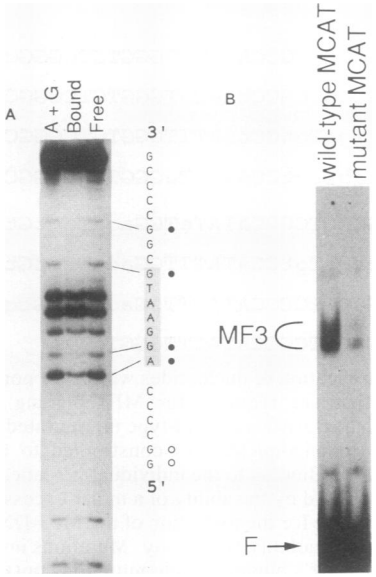


FIG. 9. Carbethoxylation interference footprint of the MF3 nucleoprotein complex and factor interaction with a mutated MCAT probe. (A) Interference footprint of the complex between MF3 and the noncoding strand of the MCAT probe. A 193-μl binding mixture contained 1 ng of diethyl pyrocarbonate-treated probe, 3 μg of poly(dI-dC) · poly(dI-dC), and 4 μl of a 0.3 to 0.6 M NaCl heparin-Sepharose fraction of embryonic chicken muscle as a source of MF3. The free fragment (Free) and MF3-DNA complex (Bound) were excised from the native gel following electrophoresis and autoradiography. The DNA was eluted, cleaved with piperidine, and run on a sequencing gel. An A+G ladder was created by treating an aliquot of unused diethyl pyrocarbonate-treated probe with piperidine. The individual lanes in the autoradiograph of the sequencing gel were scanned with a densitometer to determine the reduction in band intensity resulting from the diethyl pyrocarbonate modification. A reduction in band intensity by a factor of 5 or more is indicated by a solid dot to the right of the sequence. An open dot indicates a reduction in band intensity by a factor of 2 to 4. (B) MF3 binding to wild-type and mutated MCAT duplex probes. The mutated, double-stranded MCAT probe had the core CATTCCT sequence changed to GGGCCCT. The electrophoretic mobility shift assay was performed with 17 pg of probe, 250 ng of poly(dI-dC) · poly(dI-dC), and 0.1 μg of protein from fraction number 15 from the heparin-Sepharose profile shown in Fig. 2. F, Free fragments.

element, providing levels of reporter gene expression that were only slightly above the levels found with the TATA element alone. Significantly higher levels of expression were obtained when two MCAT elements were inserted upstream from the truncated promoter. Similar elevations in reporter gene activity were observed in the muscle and liver cultures, and a mutation of the core CATTCCT sequence eliminated expression in both cell types. The orientations of the single or multiple elements did not have a significant effect on expression in this system. Collectively, these data indicate that the CATTCCT motif contributes to the binding of MF3 and that this conserved motif can function as a basal, constitutive promoter element.

DISCUSSION

Three DNA regulatory elements are capable of forming specific nucleoprotein complexes with a binding activity from muscle that is referred to as MF3. These elements are

TABLE 1. Relative CAT activities in skeletal muscle and liver cultures following transfection with various MCAT element constructions

Insert[a]	Relative CAT activity in[b]:					
	Liver			Skeletal Muscle		
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
None	1.0	1.0	1.0	1.0	1.0	1.0
MCAT ←	2.0	1.8	1.5	3.6	4.3	
MCAT ←←	10.7	16.3	11.8	9.5	15.9	
Mutant MCAT ←	1.5	0.7	0.4	1.2	1.6	
Mutant MCAT →←	0.4	0.4	0.4	1.1	1.2	0.8
MCAT →						3.6
MCAT →←						6.4

[a] Elements were inserted into the test plasmid Δ56CAT at a unique *SalI* site which is upstream from the *c-fos* TATA element and the CAT reporter gene. The orientations of the elements are indicated be arrows.

[b] Expression levels are reported as the stimulation of reporter gene above the level obtained with no inserted sequences (Δ56CAT alone). To account for differences in transfection efficiency, the amount of extract used in the CAT assays was normalized to the level of growth hormone expressed from the cotransfected plasmid, pXGH5. Transfections were usually performed in duplicate, and the average values are reported.

skeletal actin MRE, which contains the core $CC(A/T)_6GG$ motif, a segment of the creatine kinase enhancer that contains the E-box homology, and an element that contains the CATTCCT motif that occurs in a number of muscle gene promoters. Multiple lines of evidence indicate that the three protein-DNA complexes arise from the same binding activity. The activity copurifies on heparin-Sepharose (Fig. 2) and on a DNA-affinity column (45). The three nucleoprotein complexes have the same electrophoretic mobilities and usually appear as a doublet in the autoradiograph. Finally, each of the three nucleoprotein complexes can be cross-competed by a molar excess of each DNA element but not by control DNA. The specificity of protein-DNA interaction is demonstrated by the sequence-specific competition and by mutations that eliminate detectable binding. Furthermore, MF3 does not form detectable complexes with two SREs from nonmuscle genes. Collectively, these data demonstrate that MF3 specifically interacts with the MRE, MCAT, and CKM probes despite the absence of obvious regions of extensive sequence similarity between these three probes. Thus it appears that MF3 is a site-specific protein with limited specificity.

Multiple copies of the skeletal actin MCAT element activated reporter gene expression in the heterologous promoter assay, but a single copy of the motif had little effect. A 4-bp mutation in the conserved sequence motif eliminated expression and significantly diminished MF3 binding in vitro. Similar effects on expression have been reported by Mar and Ordahl (25), who found that a 129-bp segment of the chicken cardiac troponin T promoter, containing two identical MCAT motifs, was sufficient to confer muscle-specific expression. Expression from the 129-bp segment was eliminated by a mutation in a single MCAT motif. In contrast to results with the troponin T gene, we found that two MCAT elements activated expression from the heterologous promoter in a nonmuscle cell type. This result was surprising in view of the fact that MCAT motifs occur in many muscle-specific promoters and suggests a role for this element in tissue-specific regulation. A possible explanation for these findings is that the sequences that flank the core MCAT motif contribute to the regulation of expression. It should be

noted that the single MCAT element in the chicken skeletal actin promoter contributes minimally to the overall regulation of the promoter (6). In comparison, the skeletal actin MRE is sufficient for muscle-specific expression when it is placed upstream from the c-*fos* TATA element (44). The sequences within the MRE that are required for MF3 binding, 5′-GGTGTCGGG-3′, occur upstream from the core $CC(A/T)_6GG$ motif on the noncoding strand (Fig. 8). This MF3-binding site occurs entirely within a 20-bp segment of the skeletal actin promoter that is conserved between rodent and chicken (1). Until this report, no factors have been identified that interact with the conserved sequences upstream from the CArG motif.

An unusual feature of MF3 is its ability to specifically bind to non-B conformations of DNA. This is indicated by the formation of nucleoprotein complexes with single-stranded probes in an electrophoretic mobility shift assay. MyoD is similar to MF3 in that it can also bind to a single-stranded DNA probe in a sequence-specific manner. Previous to this study, MyoD has been reported to possess only a double-stranded, major-groove DNA-binding activity (24). The precise conformation of the single-stranded DNA that is recognized by MF3 and MyoD is not known. Possibilities include randomly coiled single strands, intrastrand hairpin structures, or higher-order complexes that form from the interaction of two or more single-stranded molecules. We favor the hypothesis that MF3 recognizes specific sequences in randomly coiled single strands, because the MRE, MCAT, and CKM probes contain little or no symmetry and because it is unlikely that similar hairpins or higher-order DNA complexes can form with all three probes. However, in the case of MyoD, the structure of the non-B DNA-binding site has been identified (45). Since this structure is in low abundance in the single-stranded pool, it follows from the quantitative competition experiments shown in Fig. 7B that the affinity of MyoD for this non-B site is significantly higher than it is for the double stranded E-box.

MF3 has a higher affinity for single-stranded DNA than for double-stranded DNA (Fig. 5). On the basis of these observations, it is reasonable to assume that the factor will have a higher apparent affinity for sites in shorter duplex fragments because they are more likely to have a single-stranded character. MF3 does bind to large DNA fragments that are excised from plasmids, but with larger probes, the interaction with MF3 is greatly enhanced by heating the DNA prior to use. Presumably, heating allows the formation of alternative DNA conformations which bind to MF3 with higher affinities. Similar DNA-binding properties have been described for nuclear proteins that bind to rat satellite DNA (40) and for the estrogen receptor, which has a 60-fold-greater affinity for the noncoding strand of the estrogen regulatory element than for the duplex element (22). Svaren et al. (40) have found that duplex DNA probes as long as 400 bp can denature and form relatively stable hairpin structures following ethanol precipitation and drying. These data indicate that energetically favorable, altered conformations of DNA can arise under the conditions of routine manipulation. These data also illustrate the difficulty of knowing with certainty the conformation of the DNA in the binding assay even when presumably double-stranded probes are used. Though we can detect MF3 binding to the larger double-stranded probes, we cannot rule out the possibility that the factor is binding to small single-stranded regions within the duplex. Experiments are in progress to determine the DNA conformations that are recognized by MF3 and to more precisely map the regions that are critical for binding.

Many double-stranded DNA-binding proteins have been identified, purified, and cloned. However, only a relatively small number of sequence-specific, single-stranded-DNA-binding proteins have been described. TFIID, which binds to the TATA element, has a proposed single-stranded-DNA-binding activity that may be required to maintain the factor's position when DNA melts during the initiation of transcription (15). Single-stranded DNA-binding activities also appear to be involved in steroid hormone control of gene expression (19, 22). A regulatory element in the adipsin gene is similar to the skeletal actin MRE in that it specifically binds to multiple protein factors, of which two preferentially bind to single-stranded probes (49). Recently, the molecular clone for a sequence-specific, single-stranded-DNA-binding protein was isolated (34). This 19-kDa protein, termed CNBP, recognizes the noncoding strand of the sterol regulatory element found in the HMG-coenzyme A reductase promoter. The consensus sequence for CNBP, 5′-GTG[G/C][G/C]G[T/C]G-3′, is similar to that of the MF3-binding site in the noncoding strand of the MRE (5′-GGTGTCGGG-3′). However, it appears that MF3 and CNBP are different proteins, because they can be separated by heparin-Sepharose chromatography, and the HMG-coenzyme A reductase element does not bind to MF3 or compete for the formation of the MF3-nucleoprotein complex (35a). Though MF3 and CNBP are distinct proteins, they may be structurally similar and belong to a unique family of DNA-binding factors that specifically recognize altered DNA structures. Finally, it is possible that MF3 is the MCAT-binding factor that has recently been described by Mar and Ordahl (26). We note that MF3 and the MCAT-binding factor interact with similar MCAT probes and that both activities are present at variable levels in extracts from different cell types. We have found that the variability between cell types results, at least in part, from an inhibitor in the crude extract that can be removed by fractionation (45).

Altered DNA conformations have long been thought to be of importance in the regulation of gene expression. The presence of altered DNA conformations in chromatin is indicated by sites that are sensitive to bromoacetaldehyde, which selectively reacts with unpaired bases (21), and to S1 nuclease, which cleaves at unpaired bases in H-, or hinged, DNA and cruciforms and at the junctions of B- and Z-DNA (16, 51). S1 nuclease-sensitive regions occur in the 5′-flanking regions of genes, and in some cases their occurrence is cell type-specific and developmentally regulated (23). We note that the skeletal actin promoter contains extensive inverted repeats that are capable of forming remarkably stable hairpin structures. These inverted repeats have been conserved between the chicken and rodent genes, and it has been proposed that they are important in the regulation of skeletal actin transcription (17). It is also of interest that CArG elements are the sites of DNA bending when they are bound by SRF (13, 20) or MAPF1 (45). DNA bending produces a torsional strain that can be released by a localized melting of the duplex (41). In the creatine kinase enhancer, adjacent low-affinity and high-affinity MyoD-binding sites are separated by a stretch of C · G that extends for up to 13 nucleotides. In other promoters these tracts have been identified as the sites of S1 nuclease cleavage (2, 31). From these considerations it is tempting to speculate that altered conformations of DNA arise in the muscle gene regulatory regions during myocyte development. The nucleic acid-binding properties of MF3 and MyoD suggest that these factors recognize and stabilize these structures.

ACKNOWLEDGMENTS

This work was supported by grants from the American Heart Association, the Mathers Foundation, and the Life and Health Insurance Medical Research Fund.

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