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Muscle-Specific Expression of the Troponin I Gene Requires Interactions between Helix-Loop-Helix Muscle Regulatory Factors and Ubiquitous Transcription Factors

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The quail fast skeletal troponin I (TnI) gene is a member of the contractile protein gene set and is expressed exclusively in differentiated skeletal muscle cells. TnI gene transcription is controlled by an internal regulatory element (IRE), located within the first intron, that functions as a muscle-specific enhancer. Recent studies have shown that the TnI IRE may interact directly with the muscle regulatory factors MyoD, myogenin, and Myf-5 to produce a muscle-specific expression pattern, since these factors *trans*-activate cotransfected TnI gene constructs in C3H10T1/2 fibroblasts. In this study, we have examined the protein-IRE interactions that are responsible for transcriptionally activating the TnI gene during skeletal muscle development. We demonstrate that the helix-loop-helix muscle regulatory factors MyoD, myogenin, Myf-5, and MRF4, when complexed with the immunoglobulin enhancer-binding protein E12, interact with identical nucleotides within a muscle regulatory factor-binding site (MRF site) located in the TnI IRE. The nuclear proteins that bind to the MRF site are restricted to skeletal muscle cells, since protein extracts from HeLa, L, and C3H10T1/2 fibroblasts do not contain similar binding activities. Importantly, the TnI MRF site alone is not sufficient to elicit the full enhancer activity associated with the IRE. Instead, two additional regions (site I and site II) are required. The proteins that interact with site I and site II are expressed in both muscle and nonmuscle cell types and by themselves are ineffective in activating TnI gene expression. However, when the MRF site is positioned upstream or downstream of site I and site II, full enhancer activity is restored. We conclude that helix-loop-helix muscle regulatory factors must interact with ubiquitously expressed proteins to generate the active TnI transcription complex that is present in differentiated muscle fibers.

The muscle regulatory factors MyoD (14), myogenin (17, 50), Myf-5 (7), and MRF4 (6, 31, 41) are thought to play important roles in controlling skeletal myogenesis. Forced expression of these factors in a variety of nonmuscle cell types leads to the production of stable muscle cell lineages. Expression of MyoD, myogenin, and MRF4 occurs in the early somitic regions of developing embryos (43a, 44), suggesting that these regulatory proteins serve important functions in the formation of the embryonic trunk and limb musculatures. The muscle regulatory factors also *trans*-activate expression of cotransfected contractile protein genes (7, 17, 28, 49, 52), demonstrating that MyoD, myogenin, Myf-5, and MRF4 regulate terminal differentiation events as well. The precise mechanism by which myogenic determination and differentiation is controlled by each muscle regulatory factor, however, is poorly understood.

Members of the muscle regulatory factor family share extensive amino acid sequence similarity within a central domain that includes a basic region and a helix-loop-helix motif (b/HLH) (reviewed in reference 38). Similar b/HLH domains also are present in several other regulatory proteins, including the immunoglobulin enhancer-binding proteins E12 and E47 (34, 35) and the *Drosophila* regulatory proteins daughterless (10, 12), achaete-scute (1), and twist (45). The common HLH motif shared by these proteins allows members of this family to form homo- and hetero-oligomers. These associations are believed to be necessary for the factors to interact with DNA via their respective basic regions (13, 34, 35). Although MyoD and myogenin can form homo-oligomers, it is the formation of MyoD and myogenin hetero-oligomers with the ubiquitously expressed b/HLH protein E12 that greatly enhances their ability to bind to specific DNA sequences (8, 35). The intriguing hypothesis that differential gene expression is controlled by a combinatorial mechanism involving dynamic interactions between tissue-specific and ubiquitous transcription factors is supported by these and other studies (5).

Skeletal muscle cells rely on the coordinate expression of numerous gene products, including those encoded by the α-actin; myosin heavy-chain; myosin light-chain; troponin C, I (TnI), and T; muscle creatine kinase (MCK); and acetylcholine receptor genes to form a functional contractile apparatus. Although these genes are genetically unlinked and evolutionarily unrelated, their expression is restricted to differentiated skeletal muscle. For many of these genes, the *cis*-acting DNA regulatory sequences that control their muscle-specific expression have been identified (reviewed in reference 42). For the MCK and acetylcholine receptor α-subunit genes, sequence analysis and protein-DNA-binding studies have revealed a DNA motif that resembles the κE2 site (or E-box) that is present in the immunoglobulin light-chain gene enhancer (9, 26, 34, 40). The related DNA motif within these enhancers can serve as binding sites for MyoD and myogenin (8, 25, 39). Mutations within the MCK muscle regulatory factor-binding site (MRF site) alter the ability of MyoD and myogenin to bind to this element and block the transcriptional activation of the MCK gene (8, 25). The observations that MRF sites also are found in the genes coding for myosin light chain 1/3 (MLC) (16), the δ subunit of the acetylcholine receptor (47), and TnI (37, 51) raise the possibility that all contractile protein genes are regulated

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developmentally through common MRF sites. Although this is an attractive model that could account for the coordinate regulation of this evolutionarily unrelated gene set, most eucaryotic enhancers utilize multiple protein-binding sites to produce a controlled transcription pattern (reviewed in reference 2). Therefore, it is likely that other regulatory factors, in cooperation with MyoD, myogenin, Myf-5, and MRF4, must be recruited to initiate and maintain muscle-specific expression.

To further understand how the contractile protein genes are regulated during skeletal myogenesis, we have focused our attention on the quail fast skeletal TnI gene (4). Expression of the TnI gene is controlled through an internal regulatory element (IRE) that is located within the first intron (23, 51). The TnI IRE functions as a tissue-specific enhancer, conferring muscle-specific expression on heterologous genes, independent of position or orientation. Sequence analysis of the TnI IRE has revealed several potential protein-binding sites that may be responsible for developmentally regulating this contractile protein gene.

In an attempt to identify the specific regulatory elements and protein factors that control TnI gene expression, we have examined the protein-DNA interactions that are associated with the TnI IRE. In this study, we report that the muscle regulatory factors MyoD, myogenin, Myf-5, and MRF4, in conjunction with the ubiquitous protein factor E12, bind to the TnI MRF site located in the 5′ portion of the IRE. Proteins which bind to the MRF site are restricted to skeletal muscle cells, since extracts from C3H10T1/2, L, and HeLa cells do not contain similar binding activities. Interestingly, the TnI MRF site alone is not sufficient to elicit the full enhancer activity associated with the IRE. Instead, two additional protein-binding sites (site I and site II) are required. The proteins that interact with site I and site II are expressed in both muscle and nonmuscle cell types and are incapable of fully activating TnI transcription in the absence of the MRF site. However, when site I and site II are positioned downstream or upstream of the MRF site, complete enhancer activity is restored. Our results suggest that the HLH muscle regulatory factors must interact with ubiquitous protein factors to generate a complete muscle-specific TnI transcription complex.

MATERIALS AND METHODS

Cell culture and DNA transfections. C3H10T1/2, L (TK^-), and HeLa cells were obtained from the American Type Culture Collection and maintained in growth medium containing Eagle basal medium (GIBCO) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml). The myogenic cell line 23A2 was derived from C3H10T1/2 cells as described previously (21) and maintained in growth medium supplemented with 15% fetal bovine serum. Myogenic differentiation of 23A2 myoblasts was induced by the addition of ITS medium as described previously (51).

Transient DNA transfections were performed by standard procedures (51). Confluent cultures of 23A2 myoblasts (1.5×10^6 cells per 100-mm-diameter dish) were transfected with a calcium phosphate DNA precipitate containing 5 μg of test plasmid and 5 μg of the control β-galactosidase expression vector RSVLacZ (20). Four hours after the addition of DNA, the myoblasts were subjected to a 2-min osmotic shock in serum-free medium containing 20% glycerol and were fed differentiation medium (ITS). Two days after transfection, myofiber cell extracts were prepared and β-galactosidase activity was determined as described previously (36, 51). The amount of extract used for each chloramphenicol acetyltransferase (CAT) assay was normalized to the specific activity of β-galactosidase in each protein sample. CAT assays were carried out as described by Gorman et al. (18), and the percent acetylated chloramphenicol was quantitated by liquid scintillation counting. A minimum of three independent transfections was performed for each experimental gene construct.

trans-Activation transfections of C3H10T1/2 cells were performed as described by Yutzey et al. (52). DNA precipitates consisted of 5 μg of a plasmid containing the MyoD cDNA cloned into the pEMSVscribeα2 expression vector (14) or the expression vector without an insert and 5 μg of the test CAT plasmid. Precipitates were added to 10^6 C3H10T1/2 cells per 100-mm-diameter dish. Four hours after the addition of DNA, the cultures were glycerol shocked as described above and fed growth medium containing Eagle basal medium and 15% fetal bovine serum. Twenty-four hours after transfection, cultures were induced to differentiate by the addition of ITS. After 2 days in differentiation medium, protein extracts were prepared and analyzed as described above, with the exception that 30 μg of protein was used in each assay.

Experimental gene constructions. The TnI IRE, which encompasses nucleotides +634 to +781 of the quail TnI gene, was inserted into the *Hin*dIII site of pBluescript KS^+ (Stratagene) in both orientations (51). 5′ and 3′ deletions of the IRE were generated by digesting both IRE-pBluescript constructs with *Apa*I and *Xho*I and treating them with exonuclease III (see Fig. 1). The reaction mixtures were treated with S1 nuclease, and *Bgl*II linkers were added. Each deletion mutant subsequently was excised from pBluescript and ligated into the *Bam*HI and *Hin*dIII sites of TKCAT, which contains nucleotides −109 to +51 of the herpes simplex virus thymidine kinase gene linked to the CAT reporter gene (30). Internal deletions 3′Δ4/5′Δ8, 3′Δ5/5′Δ8, and 3′Δ8/5′Δ8 were constructed by ligating a *Bgl*II-*Kpn*I 5′Δ8 fragment next to the respective 3′ deletion fragment contained within pBluescript. Each internal deletion then was cloned into the *Hin*dIII site of TKCAT. Deletion 5′/3′Δ1 was produced by digesting the IRE with *Pst*I and *Mbo*II, treating the isolated insert with S1 nuclease, and ligating *Hin*dIII linkers onto the ends to facilitate cloning into the TKCAT vector.

Site-directed mutagenesis was performed by using the Amersham oligonucleotide-directed in vitro mutagenesis system version 2. Oligonucleotides containing specific point mutations were hybridized to the single-stranded IRE-pBluescript clone, and the second strand was extended by Klenow polymerase as per the manufacturer's recommendations. The oligonucleotides used in these studies contained the following mutations (indicated by lowercase letters): mutant MRF site, AGACAGCaaaAGCTCCTTG; mutant site I, GCTCCCCgaCaATTTTTAG; mutant site II, TTC CCCCCaaAaCCCCTTG (see Fig. 3 and 7 for the wild-type MRF site, site I, and site II DNA sequences). Wild-type and mutant IRE inserts were subsequently cloned into the *Hin*dIII site of E1b TATA-CAT (27). The precise nucleotide sequence of each deleted and mutated IRE construct was verified by dideoxy sequencing (11).

In vitro transcription and translation. m^7GpppG-capped RNAs were synthesized in vitro from linearized DNA templates (1 μg) by using T3 RNA polymerase and a TransProbe T in vitro transcription kit (Pharmacia). Reaction mixtures were incubated at 37°C for 1 h, after which DNase I was

added for an additional 10 min. Newly synthesized RNA was extracted with phenol-chloroform, ethanol precipitated, and suspended in 20 μl of TE (10 mM Tris, pH 7.8, 1 mM EDTA). Linear DNA templates were generated from the mouse MyoD (14), rat myogenin (50), rat MRF4 (41), human Myf-5 (7), and human E12 (34) cDNAs cloned into the pBluescript KS$^+$ vector (Stratagene).

For in vitro translations, 2 μl (~2 ng) of RNA transcribed in vitro was added to 48 μl of a reaction mixture containing a rabbit reticulocyte lysate (Promega) and incubated at 30°C for 1 h. Aliquots of the translation products were stored at −80°C. Radioactive translation products were generated by supplementing the amino acid mixtures with L-[^{35}S]methionine (>800 Ci/mmol; Amersham). Synthesis of the ^{35}S-labeled protein products was monitored by electrophoresis through a 12% discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel (24). The gel was fixed in 50% methanol–7.5% acetic acid for 30 min and in 100% acetic acid for an additional 30 min. Fixed gels were treated with En^{3}Hance (New England Nuclear) for 1 h, vacuum dried, and exposed to Kodak XAR film.

Preparation of nuclear extracts. Nuclear extracts were prepared essentially as described by Dignam et al. (15) with 0.42 M NaCl for protein extractions. All solutions contained 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg each of leupeptin, aprotinin, and pepstatin protease inhibitors (Sigma) per ml. The protein concentration of each extract was determined by the modified Bradford assay (Bio-Rad). Aliquots of the nuclear extracts were stored at −80°C.

Gel mobility shift assays. The DNA probes used in this study were generated by standard procedures (43). Briefly, the TnI IRE, 3′Δ4, and 5′Δ8 DNA fragments were treated with alkaline phosphatase (Boehringer Mannheim), labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear), and purified by gel electrophoresis. Oligonucleotide probes were generated by labeling one DNA strand with T4 polynucleotide kinase and [γ-^{32}P]ATP. Unincorporated [γ-^{32}P]ATP was removed by passing the reaction through a G-50 (Pharmacia) spun column. The single-stranded, labeled DNA then was mixed with a 10-fold molar excess of the unlabeled complementary oligonucleotide strand, heated to 100°C for 5 min, and allowed to anneal at room temperature.

Gel mobility shift assays were conducted by incubating end-labeled probes (2 to 13 fmol, ~5,000 cpm) with either 0.5 μg of poly(dI-dC) (Sigma) and 3 μl of in vitro-translated products or 2 μg of poly(dI-dC) and 4 μg of nuclear protein extracts in a total volume of 20 μl. Binding-reaction mixtures also contained 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9), 50 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 10% glycerol. When appropriate, unlabeled DNA fragments were added as competitors. Binding-reaction mixtures were incubated on ice for 20 min, loaded onto a 5% polyacrylamide gel (30:1, acrylamide-bisacrylamide) containing 2.5% glycerol, and electrophoresed at room temperature at 8 V/cm for 3 to 3.5 h in 12.5 mM Tris (pH 8.5)–95 mM glycine–and 0.5 mM EDTA. After electrophoresis, the gels were fixed in 10% methanol–10% acetic acid, vacuum dried, and exposed to Kodak XAR film. For antibody interference, gel mobility shift assays were performed under the conditions described above with the exception that the 1-8 anti-MyoD monoclonal antibody (1 μg) (provided by S. Kohtz) or a polyclonal antimyogenin antibody (8) (2 μl) was added to the reaction mixture.

Competitor DNAs used in the gel mobility shift assays were isolated by electroelution from a 7% polyacrylamide gel, extracted with phenol-chloroform, ethanol precipitated, resuspended in TE, and quantitated by using a DNA fluorometer (Hoefer TKO100). These DNAs included the TnI IRE (nucleotides +634 to +781), deletion or point mutants of the TnI IRE, an *Eco*RI-*Pvu*II pGEM fragment (nucleotides 10 to 108) (Promega), an *Ava*I-*Nco*I MCK enhancer fragment (nucleotides −1206 to −1097) from pΔEΔ (9), and a 180-bp *Bam*HI MLC enhancer fragment from pMLC1 CAT180 (provided by N. Rosenthal). Double-stranded oligonucleotide DNA competitors were generated by mixing the coding and noncoding strands of each oligonucleotide at a 1:1 ratio. After annealing, the double-stranded DNA was isolated from a 12% polyacrylamide gel as described above. The nucleotide sequences of the wild-type and mutated (underlined) TnI MRF site oligonucleotides are given below. The nucleotides shown in lowercase letters are not present in the IRE but were added to facilitate DNA ligations.

GTTACC
gatcCGTCTGAGGAGACAGCTGCAGCTCCa
GCAGACTCCTCTGTCGACGTCGAGGttcga
CAATGG

Methylation interference assays. Methylation interference assays were performed by the modified method of Ausubel et al. (3). The pBS 3′Δ4 clone was labeled at the *Eco*RI site on the coding strand or at the *Bgl*II site on the noncoding strand with [γ-^{32}P]ATP as described above. Similarly, the pBS 5′Δ7 clone was labeled at the *Hin*dIII site on the coding strand and at the *Bgl*II site on the noncoding strand. All probes were purified by electrophoresis through a 7% polyacrylamide gel. The DNA probes were partially methylated by incubating ~0.5 μg of end-labeled DNA probes (~10^7 cpm), 200 μl of methylation buffer (New England Nuclear), and 2 μl of dimethyl sulfate (New England Nuclear) at room temperature for 5 min (3). Preparative gel mobility shift assays were performed with 0.3 to 1 pmol of methylated probes (~5 × 10^5 cpm), either with 20 μl of in vitro-translated products and 2 μg of poly(dI-dC) or with 60 μg of nuclear extracts and 6 μg of poly(dI-dC) in a total volume of 50 μl. Reaction mixtures were incubated on ice for 30 min and electrophoresed through a 5% polyacrylamide gel as described for the gel mobility shift assays. Wet gels were exposed to Kodak XAR film for 2 h, and the gel slices containing either the free probe or the shifted protein-DNA complex were excised. The DNA probes were eluted from the gel with an IBI electroelution unit (International Biotechnologies, Inc.), extracted with phenol-chloroform, and ethanol precipitated. DNA pellets were suspended in 1 M piperidine and cleaved at 90°C for 30 min. Maxam and Gilbert (29) G and A/G sequencing reactions were performed with the New England Nuclear sequencing kit. All reactions were analyzed by electrophoresis through a 12% polyacrylamide denaturing gel.

RESULTS

The muscle regulatory factors MyoD, myogenin, Myf-5, and MRF4 bind to the TnI IRE in the presence of E12. The developmental regulation of the TnI gene is controlled by an internal regulatory element (IRE) (Fig. 1) which acts as a muscle-specific enhancer (51). The TnI IRE and the enhancer region of the MCK gene (9, 19) share a similar sequence that is related to the κE2 site (or E-box) found in several immunoglobulin gene enhancers (26, 34). The iden-

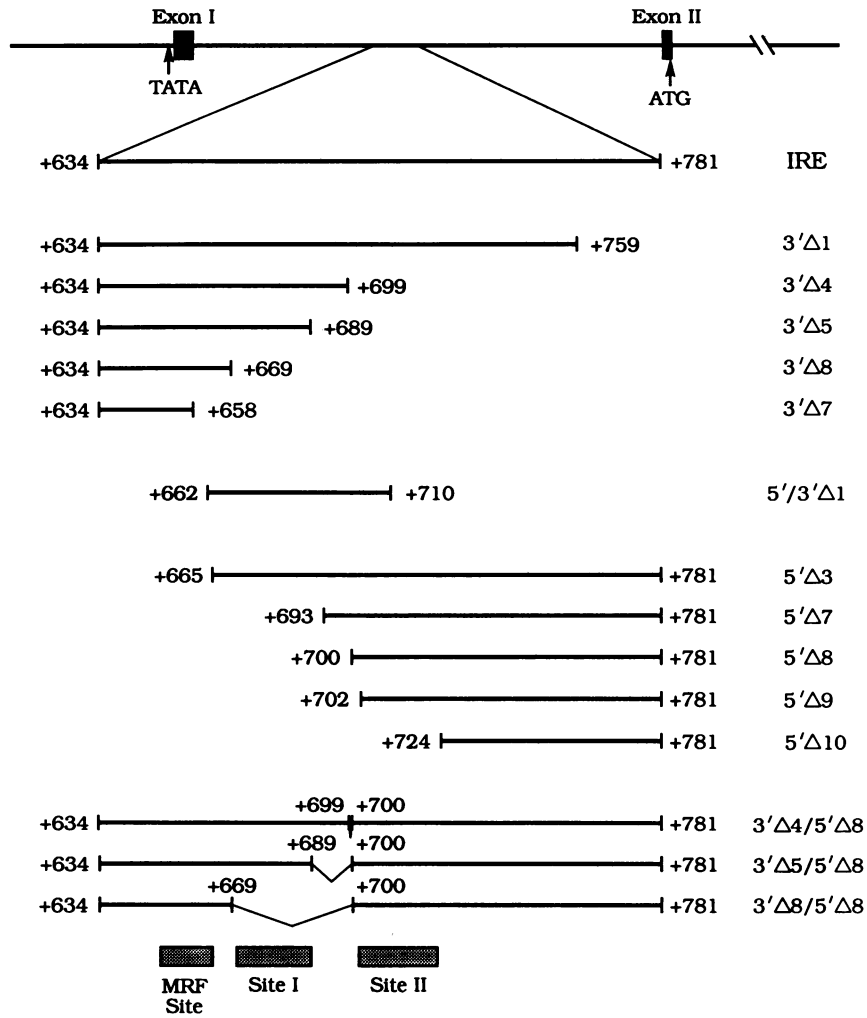

FIG. 1. Deletion mutants of the TnI IRE. The 5′ region of the quail TnI gene, including the promoter, exon I, intron I, and exon II, is represented in the top line. TATA and ATG indicate the TATA consensus sequence and translation start site, respectively. The muscle-specific IRE enhancer is located within the first intron, between positions +634 and +781. The 5′ and 3′ IRE deletion mutants and internal deletion mutants were constructed as described in Materials and Methods. The boxes indicate the locations of the MRF site, site I, and site II regulatory elements (see text for details).

tified MCK enhancer region, which we have termed the MRF site, interacts with the muscle regulatory factors MyoD (25) and myogenin (8) and is required for normal expression of the MCK gene. To investigate the importance of the IRE MRF site (nucleotides +650 to +663) in controlling TnI gene expression, we first examined the ability of the muscle regulatory factors to recognize and bind to the TnI IRE. For these studies, protein products derived from the cloned muscle regulatory factors MyoD (14), myogenin (50), and MRF4 (41) were synthesized in vitro or cosynthesized with the ubiquitous immunoglobulin transcription factor E12 (34). The protein products generated in the rabbit reticulocyte lysates corresponded to the specific regulatory factor RNAs that were incubated in the reaction mixtures, with the cotranslated RNAs producing two major proteins (Fig. 2A). Incubating the individual protein factors with a ^{32}P-labeled TnI IRE probe did not yield specific protein-DNA complexes in gel mobility shift assays (Fig. 2B). However, when the TnI IRE was incubated with cotranslated MyoD-E12, myogenin-E12, or MRF4-E12 proteins, a prominent protein-DNA complex was produced (Fig. 2B), suggesting that hetero-oligomers of E12 and each of the muscle regulatory factors recognize and bind to a specific DNA sequence located within the TnI IRE. Similar results also were obtained when the muscle regulatory factor Myf-5 (7) was cotranslated with E12 and tested for binding activity with the TnI IRE (data not shown). Interestingly, cotranslated MyoD-myogenin, MyoD-MRF4, and myogenin-MRF4 proteins failed to bind to the TnI IRE (data not shown), indicating that these muscle regulatory factors cannot substitute for E12 in forming active DNA-binding complexes.

In order to determine whether the protein-DNA complexes detected in these experiments specifically interact with the MRF site, we performed a series of competition assays with different portions of the IRE (Fig. 1). As expected, a 50-fold molar excess of unlabeled TnI IRE DNA competed efficiently for the MRF4-E12 hetero-oligomer complex (Fig. 2C). Similar competition was observed when the 3′Δ4 DNA fragment, which contains the MRF site, or an oligonucleotide that contains the TnI MRF site (see Materi-

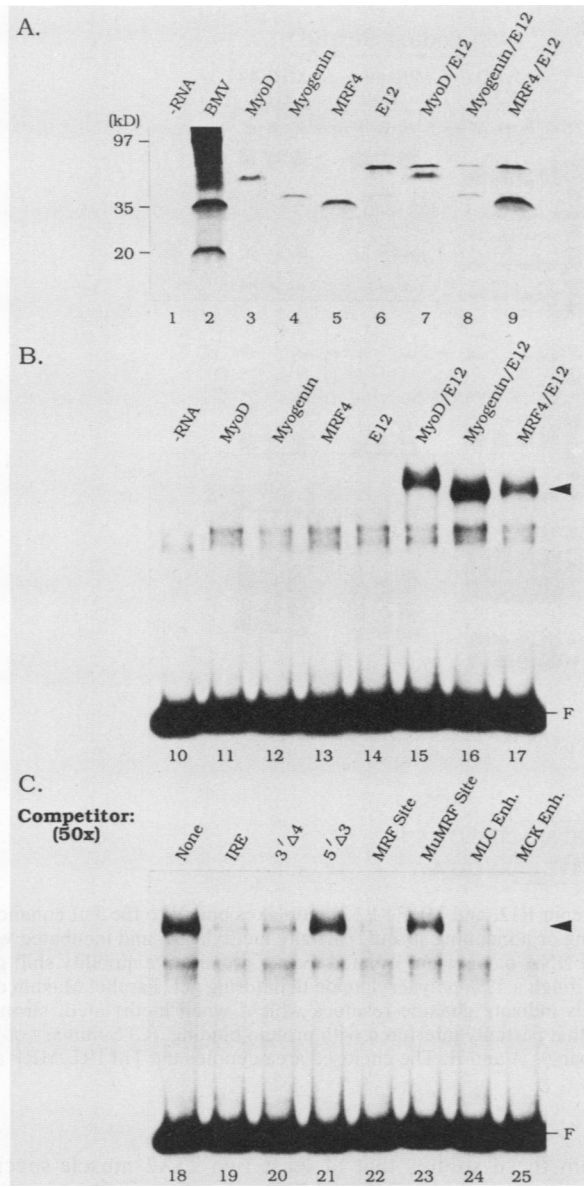

FIG. 2. MyoD-E12, myogenin-E12, and MRF4-E12 hetero-oligomers bind to the TnI IRE. (A) ^{35}S-labeled protein products were translated in vitro from MyoD, myogenin, MRF4, and E12 RNA templates (lanes 3 to 6) or cotranslated from MyoD-E12, myogenin-E12, and MRF4-E12 RNA templates (lanes 7 to 9) and analyzed by SDS-polyacrylamide gel electrophoresis. Translation reactions carried out in the absence of RNA (lane 1) or in the presence of Brome mosaic virus (BMV) RNAs (lane 2) served as negative and positive controls, respectively. The predicted sizes of the translated BMV polypeptides are indicated on the left in kilodaltons (kD). (B) Each translation product (3 μl) generated in panel A (minus ^{35}S-methionine) was incubated with a ^{32}P-labeled TnI IRE probe. The specific protein-DNA complexes (arrowhead) generated in gel mobility shift assays and the free, unbound IRE probe (F) are indicated. Note that MyoD, myogenin, MRF4, and E12 homo-oligomers do not interact with the TnI IRE. (C) The TnI IRE probe was incubated with MRF4-E12 cotranslated proteins in the presence of unlabeled competitor DNAs. The TnI IRE, 3′Δ4, and 5′Δ3 (Fig. 1) DNA fragments along with oligonucleotides containing the TnI MRF site (MRF Site) or a mutated TnI MRF site (MuMRF Site), plus DNA fragments containing the muscle-specific enhancers from the MLC (MLC Enh.) or MCK (MCK Enh.) gene were used as competitors (50-fold molar excess). DNA competitors that contain identified MRF sites efficiently competed for the MRF4-E12 protein complex (see text for details).

als and Methods for details) was included in these reactions. In contrast, the 5′Δ3 DNA fragment, which lacks the TnI MRF site, or an oligonucleotide containing a mutated TnI MRF site (see Materials and Methods for details) did not compete efficiently for the MRF4-E12 complex (Fig. 2C). Related MRF sites contained within the MCK (9) and MLC (16) enhancer regions also competed for binding (Fig. 2C), confirming that the MRF4-E12 complex recognizes the MRF sites present within the TnI, MCK, and MLC enhancers. Identical results were obtained when MyoD-E12, myogenin-E12, and Myf-5-E12 complexes were tested with these competitors (data not shown), indicating that each muscle regulatory factor, in conjunction with E12, recognizes and binds to the MRF site located within the TnI IRE.

Since the DNA-binding characteristics of the MyoD-E12, myogenin-E12, and MRF4-E12 complexes were comparable in the gel mobility shift assays, we investigated whether the precise DNA recognition sequences for the muscle regulatory factors were identical. Methylation interference assays were performed with the 3′Δ4 deletion fragment and the cotranslated products of the MyoD-E12, myogenin-E12, and MRF4-E12 RNAs. The methylation of three specific guanines in each DNA strand within the TnI MRF site (nucleotides +650 to +663) strongly interfered with the binding of the muscle regulatory factor complexes (Fig. 3). Methylation of an additional flanking guanine residue in each DNA strand also partially inhibited the interactions of the MyoD-E12, myogenin-E12, and MRF4-E12 complexes, demonstrating that the methylation interference patterns observed for the coding and noncoding DNA strands were symmetrical (Fig. 3C). Similar results were obtained when we examined the TnI IRE contact points for the Myf-5-E12 complex (data not shown), again indicating that each muscle regulatory factor-E12 oligomer recognizes and binds to identical nucleotides within the TnI MRF site.

Skeletal muscle nuclear extracts contain muscle-specific factors that recognize and bind to the TnI MRF site. Since in vitro-translated complexes containing E12 and MyoD, myogenin, Myf-5, or MRF4 proteins bind to the TnI MRF site, we predicted that similar binding activity should be present in nuclear extracts obtained from skeletal muscle cells. For these studies, nuclear extracts derived from the 23A2 myogenic cell line or from C3H10T1/2 fibroblasts were incubated with an oligonucleotide probe containing the TnI MRF site. Nuclear extracts from 23A2 myofibers interacted with the TnI MRF probe, generating at least three specific protein-DNA complexes (M1, M2, and M3) (Fig. 4A). Extracts from C3H10T1/2 fibroblasts did not contain a specific MRF site-binding activity (Fig. 4A). Nuclear extracts from HeLa and L cells also failed to produce specific protein-DNA complexes (data not shown), indicating that the nuclear factors which interact with the TnI MRF site are muscle specific.

Competition analyses revealed that the myofiber proteins interact directly with the TnI MRF site. DNA competitors, such as the complete TnI IRE, the 3′Δ4 deletion fragment (Fig. 1), or the MRF site oligonucleotide, efficiently inhibited the formation of the M1, M2, and M3 protein-DNA complexes (Fig. 4A). The MCK and MLC enhancer competitors also inhibited the formation of the M1 and M2 complexes and partially prevented formation of the M3 complex. The 5′Δ3 IRE deletion fragment (Fig. 1) and the mutant MRF site oligonucleotide did not compete for the muscle-specific proteins that bind to the TnI MRF site (Fig. 4A). Similar but less-abundant M1, M2, and M3 protein-DNA complexes were detected in 23A2 myoblasts (data not shown), suggesting that MRF site-binding activities also are present in the

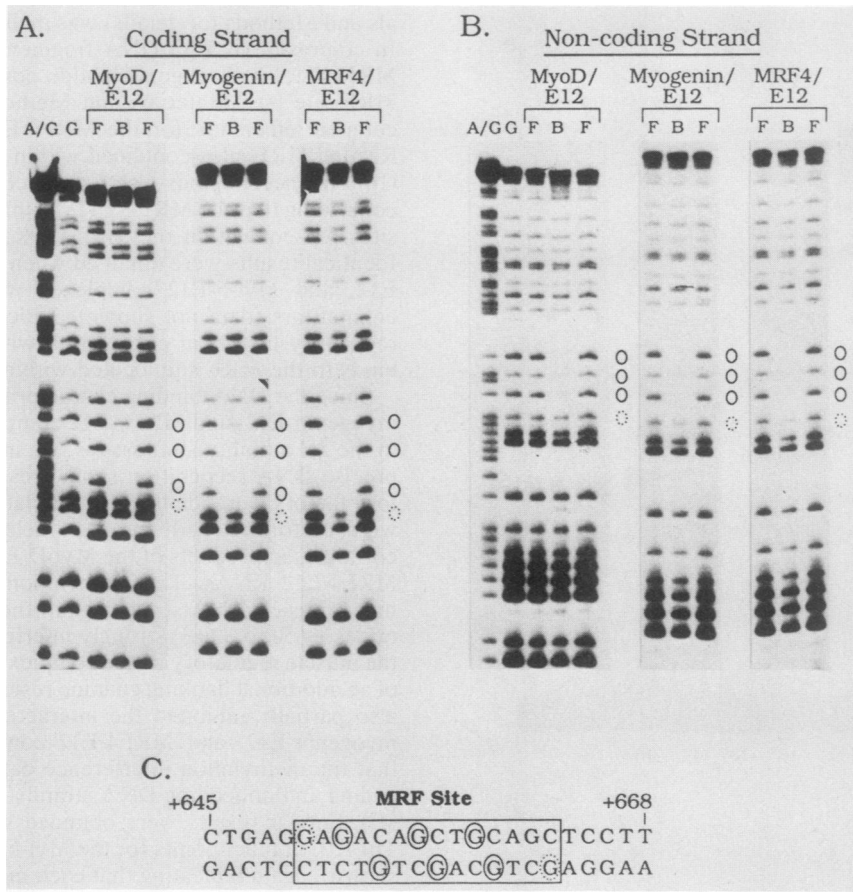

FIG. 3. DNA methylation interference analyses of the MyoD-E12, myogenin-E12, and MRF4-E12 complexes bound to the TnI enhancer. (A and B) The TnI IRE deletion fragment 3′Δ4 was end labeled on the coding or noncoding strand, partially methylated, and incubated with the indicated cotranslated protein products. Free (F) and protein-bound (B) DNA bands were isolated from a preparative mobility shift gel, cleaved at methylated guanine residues, and analyzed by electrophoresis through a 12% polyacrylamide denaturing gel. Parallel Maxam and Gilbert (29) A/G and G sequencing reactions also are shown. Whole ovals indicate guanine residues which, when methylated, strongly interfered with protein binding. Broken ovals indicate methylated guanines that partially interfered with protein binding. (C) Summary of the methylation interference analyses derived from the binding experiments in panels A and B. The enclosed area denotes the TnI IRE MRF site (nucleotides +650 to +663).

proliferating undifferentiated cell population. At this time, however, it is unclear whether these activities are present in both myoblast and myofiber cells or whether these activities result from the few (<1%) precociously differentiated cells that are found within the myoblast cultures.

The 23A2 myogenic cell line expresses the muscle regulatory factors MyoD and myogenin but not MRF4 or Myf-5 (40a, 41). In order to determine whether the DNA-binding activities present within 23A2 myofiber extracts contain MyoD or myogenin or both, gel mobility shift assays were performed in the presence or absence of MyoD-specific and myogenin-specific antibodies. Coincubation of the MRF site oligonucleotide with cotranslated MyoD-E12 proteins and MyoD antibodies abolished the DNA binding of the MyoD-E12 complex but did not prevent the myogenin-E12 complex from interacting with the MRF site (Fig. 4B). In contrast, addition of the myogenin-specific antibodies produced a supershifted complex in the myogenin-E12 group but did not affect the binding of the MyoD-E12 complex to the oligonucleotide. Interestingly, incubation of 23A2 nuclear proteins with MyoD-specific antibodies inhibited the formation of the M2 complex, while incubation with myogenin-specific antibodies supershifted the M3 complex (Fig. 4B). We conclude from these studies that at least two 23A2 muscle-specific binding complexes (M2 and M3) are antigenically related to MyoD and to myogenin, suggesting that the TnI gene partially relies on the interaction of MyoD and myogenin to produce its normal expression pattern.

The TnI MRF site is necessary but not sufficient for the complete enhancer activity associated with the TnI IRE. Since the MRF site in the 5′ region of the TnI IRE (nucleotides +650 to +663) can be occupied by each of the in vitro-translated muscle regulatory factors and by DNA-binding complexes present within muscle nuclear protein extracts, we tested whether this region alone is responsible for the enhancer activity of the TnI IRE. For these studies, a series of IRE deletion mutants was constructed (Fig. 1) and ligated 5′ to a TKCAT reporter gene (see Materials and Methods for details). Each gene construct was introduced into 23A2 myoblasts, which then were induced to differentiate into myofibers. Deletion of the MRF site in mutant 5′Δ3 (Fig. 1) reduced the enhancer activity of the IRE by 70% (Table 1). Complete IRE activity was restored, however, when the MRF site was added to the 3′ portion of mutant 5′Δ3 (data not shown), demonstrating that the MRF site is essential for full enhancer activity. Surprisingly, mutant 3′Δ8, which

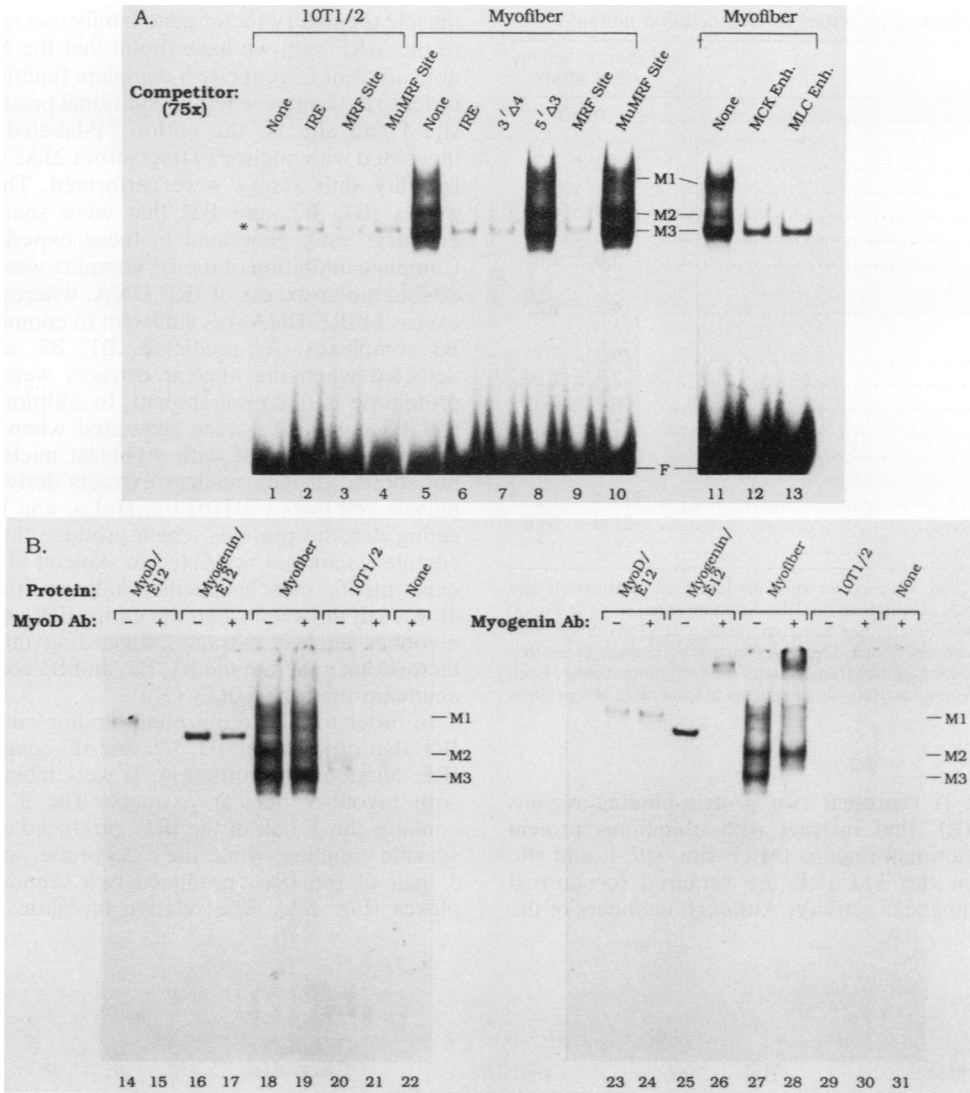

FIG. 4. Muscle-specific proteins interact with the TnI MRF site to produce three specific complexes. (A) A ^{32}P-labeled MRF oligonucleotide probe (see Materials and Methods) was incubated with 20 μg of nuclear protein extracts derived either from C3H10T1/2 fibroblasts or from 23A2 myofibers. Protein-DNA complexes were analyzed as described in Materials and Methods and in the legend to Fig. 2. M1, M2, and M3 denote three muscle-specific protein-DNA complexes that were obtained with 23A2 myofiber proteins but not with C3H10T1/2 nuclear proteins. The asterisk indicates nonspecific DNA-binding activities that migrated slightly above the M3 complex. The DNA competitors used in these assays are as described in the legend to Fig. 2. Note that in lanes 11 to 13, the gel was not electrophoresed sufficiently to separate the nonspecific band from the M3 complex. (B) Gel mobility shift assays were performed in the absence (−) or presence (+) of a MyoD-specific or myogenin-specific antibody (Ab). The MyoD antibody inhibited the formation of the MyoD-E12 and M2 complexes but did not affect significantly the formation of the myogenin-E12, M1, or M3 complexes. The myogenin antibody supershifted the myogenin-E12 and M3 complexes but did not alter the migration of the MyoD-E12, M1, or M2 complexes. In this experiment, the free DNA probe was electrophoresed off the gel to allow maximum separation of the M1, M2, and M3 complexes.

contains the entire MRF site but lacks the 3′ portion of the IRE, exhibited only 13% of the wild-type enhancer activity. These results imply that the MRF site, although necessary, is not sufficient to elicit the full muscle-specific enhancer activity associated with the complete TnI IRE.

Analysis of other mutant gene constructs revealed two additional regions of the IRE that are required to generate a complete functional enhancer. A 12-fold decrease in enhancer activity was observed when nucleotide positions +665 to +692 (site I) were deleted from the IRE (compare 5′Δ7 with 5′Δ3, Table 1). Removal of a second region (site II), located between nucleotides +700 and +759, also resulted in a decrease in enhancer activity, despite the presence of the MRF site and site I (compare 3′Δ4 with 3′Δ1, Table 1). An IRE construct that contains an intact MRF site and site II but lacks site I (3′Δ8/5′Δ8), exhibited 5% of the activity associated with the complete TnI enhancer, and constructs that contain site I (5′/3′Δ1) or site II (5′Δ7) alone showed only 4 or 2% of the wild-type IRE activity, respectively. Therefore, although three regions of the IRE (MRF site, site I, and site II) appear to be important to the generation of full enhancer activity, none of the three regions alone is sufficient to produce wild-type levels of TnI gene expression.

TABLE 1. Enhancer activities of IRE deletion mutants

IRE deletion mutant[a]	Enhancer activity (% ± SEM)[b]
IRE	100.0
3′Δ1	140.1 ± 7.7
3′Δ4	57.3 ± 8.0
3′Δ5	6.9 ± 1.6
3′Δ8	12.6 ± 3.5
3′Δ7	5.9 ± 1.0
5′/3′Δ1	4.3 ± 1.2
5′Δ3	29.2 ± 2.7
5′Δ7	2.4 ± 1.6
5′Δ8	6.4 ± 3.3
5′Δ9	7.2 ± 2.3
5′Δ10	4.7 ± 2.0
3′Δ4/5′Δ8	113.4 ± 3.2
3′Δ5/5′Δ8	21.8 ± 10.6
3′Δ8/5′Δ8	5.3 ± 0.5

[a] Ligated 5′ to the TKCAT reporter gene as described in Materials and Methods. The nucleotide positions of the IRE mutations are shown in Fig. 1.
[b] CAT expression was determined after transfecting 23A2 myofibers with the indicated gene constructs. Values are presented as a percentage of the IRE activity obtained in parallel control transfections for each mutant gene. Each value represents the average activity obtained from at least three independent transfections.

Site I and site II represent two protein-binding regions within the TnI IRE that interact with ubiquitous protein factors. Three functional regions (MRF site, site I, and site II) present within the TnI IRE are required for normal muscle-specific enhancer activity. Although members of the muscle regulatory factor gene family can recognize and bind to the MRF site, we have found that the MRF site alone is not sufficient to generate a complete functional enhancer. In order to determine whether additional protein factors bind to site I and site II, the entire ^{32}P-labeled IRE probe was incubated with nuclear extracts from 23A2 myofibers and gel mobility shift assays were performed. Three shifted complexes (B1, B2, and B3) that were specific for the IRE enhancer were generated in these experiments (Fig. 5A). Complete inhibition of the B1 complex was achieved with an 80-fold molar excess of IRE DNA, whereas a 20-fold molar excess of IRE DNA was sufficient to compete for the B2 and B3 complexes. As predicted, B1, B2, and B3 were not detected when the nuclear extracts were pretreated with proteinase K (data not shown). In addition, similar B1, B2, and B3 complexes were generated when the labeled IRE probe was incubated with myoblast nuclear extracts (data not shown) or with nuclear extracts derived from the nonmuscle cell lines C3H10T1/2, HeLa, and L (Fig. 5B), indicating that the proteins which produce the B1, B2, and B3 complexes are not restricted to skeletal myofibers. In these experiments, muscle-specific binding to the MRF site (Fig. 4) was not detected when the entire IRE was incubated with myofiber nuclear extracts, suggesting that the ubiquitous factors that generate the B1, B2, and B3 complexes are more abundant than the MRFs (27a).

In order to map the protein-binding sites within the TnI IRE that produce the B1, B2, and B3 complexes, individual IRE deletion fragments (Fig. 1) were labeled and incubated with myofiber nuclear extracts. The 3′Δ4 probe, which contains the 5′ half of the IRE, produced a single sequence-specific complex, while the 5′Δ8 probe, which contains the 3′ half of the IRE, produced two sequence-specific complexes (Fig. 6A). The relative mobilities of these shifted

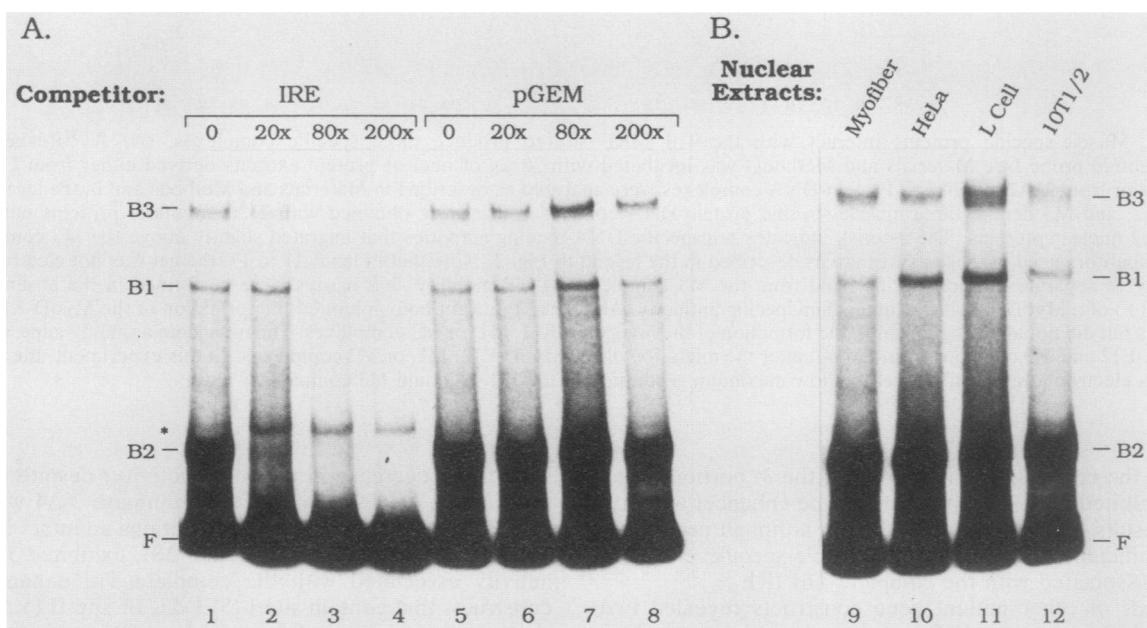

FIG. 5. Three ubiquitous protein complexes recognize and bind to the TnI IRE. (A) The ^{32}P-labeled TnI IRE, 4 μg of myofiber nuclear extracts, and increasing concentrations of IRE or nonspecific pGEM competitor DNAs were incubated as described in Materials and Methods. B1, B2, and B3 denote individual protein-DNA complexes that were generated with nuclear proteins and the TnI IRE probe (F, free, unbound IRE probe). The asterisk indicates a nonspecific protein-DNA complex. (B) The TnI IRE probe was incubated with nuclear protein extracts derived from myofiber, HeLa, L, and C3H10T1/2 cells and analyzed as in panel A. Note that each protein extract contains the B1, B2, and B3 binding activities.

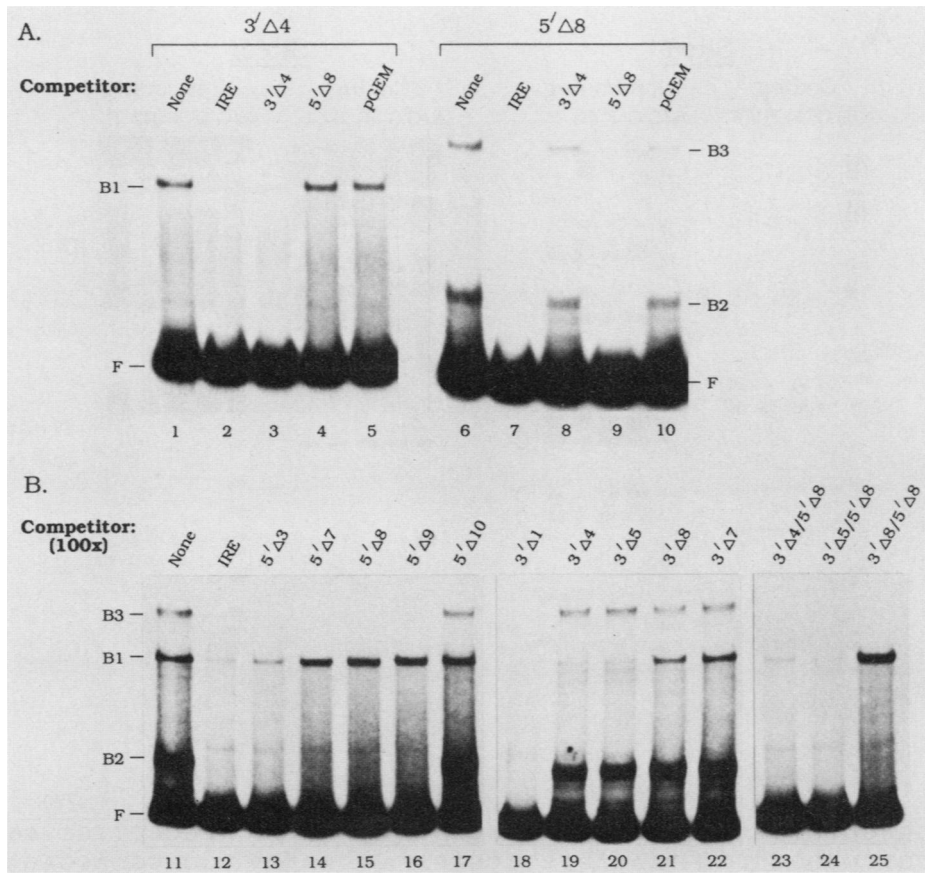

FIG. 6. Competition analyses for the B1, B2, and B3 protein-DNA complexes. (A) 3′Δ4 or 5′Δ8 DNA probes were incubated with 4 μg of myofiber nuclear extracts and either 100-fold (lanes 1 to 5) or 50-fold (lanes 6 to 10) molar excess of the indicated competitor DNAs. The B1 complex was generated with the 3′Δ4 probe, while the B2 and B3 complexes were generated with the 5′Δ8 probe. (B) The ^{32}P-labeled TnI IRE probe and 4 μg of myofiber nuclear extract were incubated with the indicated competitor DNA fragments (100-fold molar excess) (Fig. 1). The B1 complex was inhibited by excess 5′Δ3 and 3′Δ5 DNAs but not by 5′Δ7 and 3′Δ8 DNAs, while the B2 and B3 complexes were inhibited by excess 5′Δ9 and 3′Δ1 DNAs but not by 5′Δ10 and 3′Δ4 DNAs, demonstrating that the B1 complex consists of proteins that interact with nucleotides +670 to +689 (site I) while the B2 and B3 complexes consist of proteins that bind to nucleotides +702 to +723 (site II) (see Fig. 1 and text for details).

bands suggested that the protein(s) which is responsible for the B1 complex interacts with the 3′Δ4 probe while the proteins that produce the B2 and B3 complexes interact with the 5′Δ8 probe (compare Fig. 5 and 6A). Excess IRE or 3′Δ4 DNAs were efficient competitors for the B1 complex, whereas excess 5′Δ8 DNA did not compete for B1 binding (Fig. 6A). In contrast, excess IRE or 5′Δ8 DNAs, but not 3′Δ4 DNA, competed for B2 and B3 binding, confirming that the 5′ portion of the TnI IRE contains the binding site for the B1 complex while the 3′ portion of the IRE includes the binding site(s) for the B2 and B3 complexes. Identical results were obtained when HeLa nuclear extracts were tested with these DNA probes and competitors (data not shown), again suggesting that ubiquitous factors are responsible for the formation of the B1, B2, and B3 complexes.

By utilizing specific IRE deletion mutants in competition assays, we also determined the nucleotide borders associated with the B1, B2, and B3 complexes. The protein(s) which produced the B1 complex recognizes nucleotides +670 to +689 of the IRE, since competition for B1 binding was achieved with 3′Δ5 and 5′Δ3 DNAs but not with 3′Δ8 and 5′Δ7 DNAs (Fig. 1 and 6B). Similarly, the B2 and B3 complexes were produced by proteins that recognize nucleotides +702 to +723 of the IRE. Competition for these complexes occurred with 3′Δ1 and 5′Δ9 DNAs but not with 3′Δ4 and 5′Δ10 DNAs (Fig. 6B). These results, in conjunction with the enhancer activity assays in Table 1, suggest that the binding of ubiquitous proteins to the IRE corresponds to two functional regions, with the B1 complex interacting with site I and the B2 and B3 complexes interacting with site II.

To reveal the precise contact points of the ubiquitous protein factors within the IRE, 23A2 myofiber nuclear extracts were incubated with the 3′Δ4 or 5′Δ7 IRE probes and the B1 and B2 complexes were analyzed by methylation interference assays. A single methylated guanine residue in the coding strand and three methylated guanine residues in the noncoding strand of site I strongly interfered with the formation of the B1 complex. An additional guanine residue on the noncoding strand also appeared to be involved in the formation of the B1 complex (Fig. 7A). The central portion of site I (Fig. 7C) resembles a DNA consensus sequence (CCCCAGGC) to which the transcription factor AP-2 binds (32), suggesting that the B1 protein factor may be similar or identical to the AP-2 protein. For site II, nine guanine residues in the noncoding strand were crucial for B2 binding,

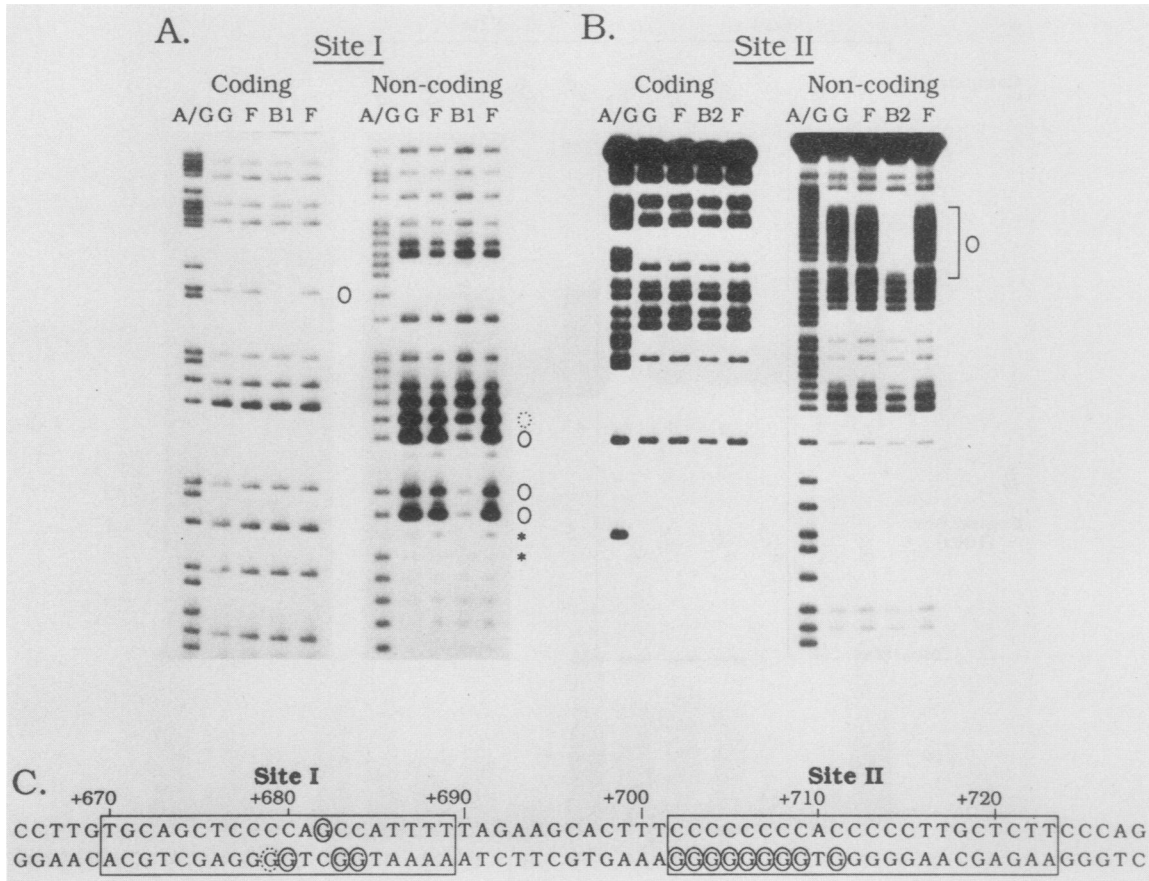

FIG. 7. Methylation interference assays of the protein-DNA interactions associated with the B1 and B2 complexes. (A) 3′Δ4 DNA probes were end labeled on the coding or noncoding strand, partially methylated, and incubated with myofiber nuclear extracts as described for Fig. 3. Gel slices containing the free DNA probe (F) and the B1 complex were excised from the preparative gel, cleaved with piperidine, and analyzed by electrophoresis through a 12% denaturing gel. Parallel Maxam and Gilbert (29) A/G and G sequencing reactions also are shown. Methylated guanine residues that strongly interfered with protein binding are indicated by whole ovals, while the broken oval indicates a single methylated guanine residue that partially interfered with protein binding. Asterisks represent nonguanine residues which also interacted with the protein(s) in the B1 complex. (B) Methylation interference assays were performed as described for panel A, with the exception that the 5′Δ7 DNA probe was used in the gel mobility shift assays and the B2 complex was isolated. (C) Nucleotide sequences of the B1 (site I) and B2 (site II) protein-binding sites present within the TnI IRE. The nucleotide borders of site I and site II and the guanine residues that interact with the B1 and B2 protein complexes are indicated.

while methylation of the single guanine residue in the coding strand did not alter the B2-binding activity (Fig. 7B). Identical methylation interference patterns for the B1 and B2 complexes were obtained with C3H10T1/2 nuclear extracts (data not shown), further supporting the hypothesis that the protein factors which interact with site I and site II in muscle cells are identical to those found in nonmuscle cells. Unfortunately, we have been unable to obtain an unambiguous methylation interference pattern for B3, probably because of the relatively low levels and weak protein-DNA interactions associated with this complex. However, in DNA competition assays, an IRE deletion mutant containing the 5′ portion of site II (nucleotides +662 to +710) efficiently competed for the B2 complex but not for the B3 complex (data not shown). This result suggests that the protein factor(s) that produces the B2 complex binds to the 5′ half of site II, while the protein(s) responsible for the B3 complex binds to the 3′ half of site II.

Complete *trans*-activation of the TnI gene by the muscle regulatory factors requires cooperation between the MRF site, site I, and site II. We have reported previously that the TnI gene is *trans*-activated efficiently when C3H10T1/2 fibroblasts are cotransfected with MyoD or myogenin but not MRF4 (52). To examine the individual roles of the MRF site, site I, and site II in *trans*-activation of TnI by these muscle regulatory factors, we tested individual mutant IRE constructs in cotransfection assays. In order to minimize potential regulatory interference from heterologous promoters, we inserted the intact TnI IRE 5′ to the E1b TATA-CAT (TATA-CAT) reporter gene (see Materials and Methods for details). TATA-CAT contains a 13-mer oligonucleotide derived from the adenovirus E1b TATA consensus sequence ligated 5′ to the CAT gene (27). Cotransfection of C3H10T1/2 fibroblasts with a MyoD expression vector and TATA-CAT did not produce a *trans*-activation response (Fig. 8). However, cotransfection of the MyoD expression vector and the IRE-TATA-CAT gene produced very high levels of CAT expression (Fig. 8), demonstrating that *trans*-activation of the reporter TATA-CAT gene requires an intact IRE. Similar results were obtained when myogenin was tested in these experiments (data not shown).

Since the muscle regulatory factors bind exclusively to the

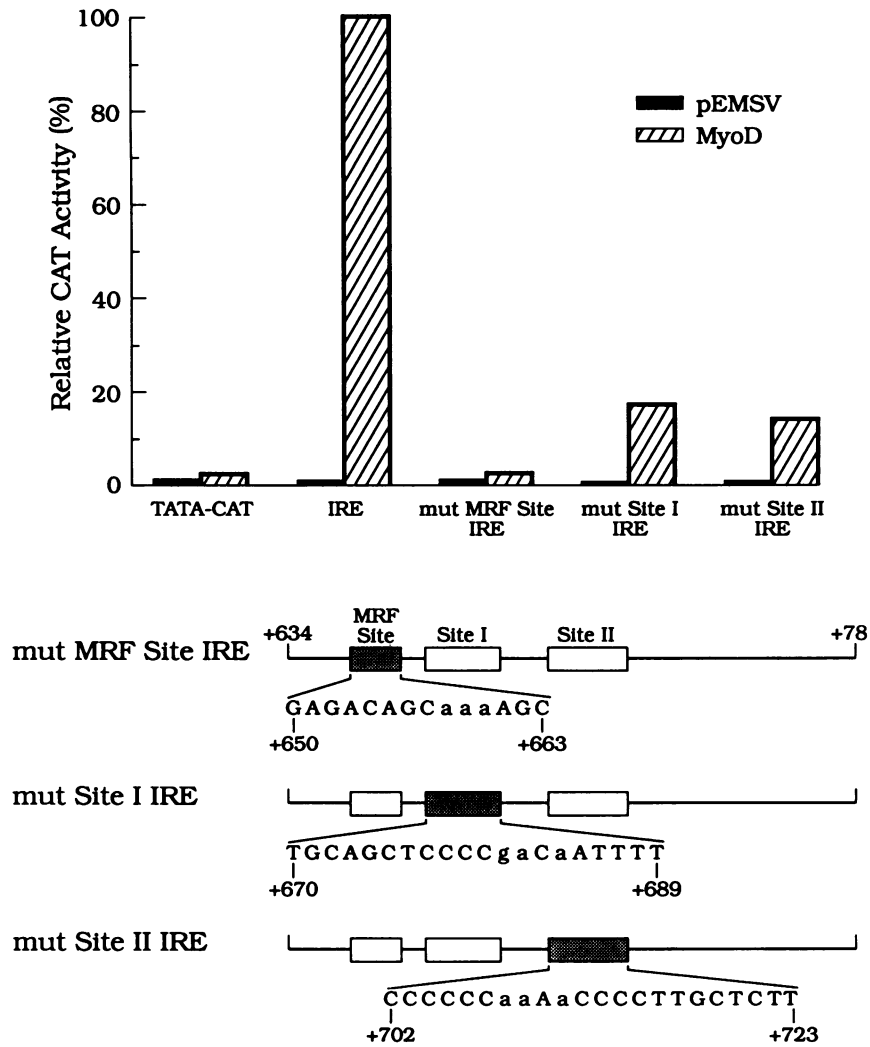

FIG. 8. *Trans*-activation of the TnI IRE and IRE mutants by MyoD. C3H10T1/2 cells were cotransfected with the indicated CAT genes and either the control pEMSVscribeα2 expression vector (pEMSV) or the MyoD expression vector (MyoD). TATA-CAT represents the control CAT gene lacking IRE inserts. CAT activity was calculated as the percent acetylated chloramphenicol per 30 μg of protein and is presented as a percentage of the CAT expression obtained with the MyoD-activated wild-type IRE gene, which was set at 100%. Each value represents the average from three independent transfections. The specific IRE mutations introduced into the MRF site, site I, and site II are indicated by lowercase letters. The basal levels of CAT expression in the control pEMSV transfections are as follows: TATA-CAT, 1.1%; IRE, 0.6%; mutant (mut) MRF site IRE, 0.9%; mutant site I IRE, 0.4%; mutant site II IRE, 0.6%.

MRF site located within the IRE, we were interested in determining whether site I and site II also contributed to the MyoD-dependent *trans*-activation response. Utilizing site-directed mutagenesis, we tested additional IRE mutants in which three nucleotides within the MRF site, site I, and site II were changed (Fig. 8). Gel mobility shift assays demonstrated that the introduced mutations within the MRF site, site I, and site II abolished the respective protein-DNA interactions that were detected with the wild-type IRE (data not shown). As expected from the DNA-binding results, MyoD was unable to *trans*-activate the cotransfected mutant MRF site IRE gene (Fig. 8). Remarkably, the mutant site I IRE and the mutant site II IRE genes were activated to only 17 and 14% of the level obtained with the wild-type IRE gene. In both cases, these gene constructs contained an intact MRF site (Fig. 8). Our results demonstrate that although the IRE MRF site binds the muscle regulatory factors and is required for TnI gene expression, it is not sufficient for *trans*-activation by MyoD or for expression in normal skeletal myofibers (Table 1). Instead, the proteins that bind to sites I and II, in conjunction with the muscle regulatory factors that bind to the MRF site, are required to regulate TnI gene expression.

DISCUSSION

Muscle-specific expression of the TnI gene is regulated primarily through the tissue-specific IRE enhancer located within the first intron (51). Similar to the enhancers associated with the MCK (8, 9), MLC (16), and α-acetylcholine receptor (40) genes, the TnI IRE also contains an muscle regulatory factor-binding consensus sequence. As shown in this study, MyoD, myogenin, Myf-5, and MRF4, in the presence of the b/HLH immunoglobulin enhancer-binding protein E12, recognize and bind to identical nucleotides within the TnI MRF site. Interestingly, under the same

binding conditions, MyoD, myogenin, Myf-5, and MRF4 homo-oligomers or MyoD-myogenin, MyoD-MRF4, and myogenin-MRF4 hetero-oligomers did not interact with the MRF site. These results agree with previous studies demonstrating that MyoD-E12 (25) and myogenin-E12 hetero-oligomers (8) bind to the MCK MRF site and suggest that the muscle regulatory factors constitute at least one component of the transcription complex regulating muscle-specific gene expression.

Although each of the muscle regulatory factors converts fibroblasts to stable myogenic lineages, functional differences exist among the four proteins. MyoD, myogenin, and Myf-5 efficiently *trans*-activate cotransfected TnI and MCK genes, whereas MRF4 produces only a weak *trans*-activation response (52). Since MRF4 is capable of interacting with the TnI MRF site, it is unclear why MRF4 does not efficiently *trans*-activate TnI gene expression. One possibility is that the complex formed between MRF4 and the IRE is less stable than the complexes formed by MyoD, myogenin, and Myf-5. Although the binding constants for each muscle regulatory factor have not been determined formally, there are data to suggest that the MRF4 complex exhibits a lower affinity for MRF sites than the MyoD or myogenin complexes (6, 27a). Alternatively, the unique activation specificities of the muscle regulatory factors may reside in the diverged amino and carboxy regions found within each protein. For example, it is possible that the diverged regions interact with different transcription factors and that the regulation of individual muscle-specific genes depends upon the interaction of an muscle regulatory factor with a preformed transcription complex that is associated with the enhancer region. Our observation that the TnI IRE relies on additional DNA-binding proteins to generate a normal transcription pattern supports this hypothesis.

As expected, MRF site-binding activities are present in nuclear extracts derived from the 23A2 myogenic cell line. Muscle nuclear extracts produced at least three specific protein-DNA complexes (M1, M2, and M3), whereas nonmuscle extracts lacked detectable muscle regulatory factor-binding activity. Since anti-MyoD antibodies specifically interrupted formation of the M2 complex and antimyogenin antibodies specifically recognized the M3 complex, it is likely that MyoD and myogenin bind to the MRF site in vivo. In gel mobility shift assays, M2 and the MyoD-E12 complex, as well as M3 and the myogenin-E12 complex, did not migrate to identical positions (Fig. 4B), suggesting that MyoD and myogenin also may interact with proteins other than E12. In this regard, MyoD, myogenin, Myf-5, and MRF4, when complexed with the b/HLH protein E47 (34), efficiently bind to the TnI MRF site as well (27a). An alternative possibility for the observed difference in mobilities is that MyoD, myogenin, and E12 undergo posttranslational modifications in vivo that alter their migration in the gel mobility shift assays. The identification of additional proteins and the role of specific protein modifications that may be essential in forming the M1, M2, and M3 complexes will require further study.

We have identified three functional regions within the TnI IRE—the MRF site, site I, and site II. The MRF site is recognized by hetero-oligomers between E12 and the muscle regulatory factors MyoD, myogenin, Myf-5, and MRF4, while sites I and II bind proteins that are present in both muscle and nonmuscle cell types. It is clear from our studies that the muscle regulatory factors and the MRF site, although essential for TnI gene expression, are not sufficient to generate the complete enhancer activity. Mutagenesis of site I dramatically inhibits the ability of MyoD to *trans*-activate expression of a reporter gene, even though the mutated IRE contains a functional MRF site and site II. Similar results were obtained when mutations were introduced into site II alone. In addition, IRE constructs that contain only sites I and II (such as mutant 5′Δ3) retain differentiation-specific expression (50a). On the basis of these data, we propose that muscle-specific transcription of the TnI gene requires RNA polymerase II (22, 23) and at least four protein complexes that bind to three distinct regions within the IRE.

Recently, Weintraub et al. (48), using synthetic oligonucleotides linked to TKCAT, suggested that two adjacent MCK MRF sites are required and sufficient to activate gene expression by MyoD. This is in contrast to the results obtained with the TnI gene. For example, the TnI IRE contains a single MRF site, while the MCK enhancer contains two MRF sites. However, both genes are *trans*-activated to similar levels by MyoD (52). Additionally, TnI IRE constructs containing only the single MRF site or two adjacent MRF sites exhibit very low levels of *trans*-activation (50a). When the MRF site is positioned upstream or downstream of sites I and II, however, full enhancer activity is restored, suggesting that MyoD cooperates with the ubiquitous proteins that bind to site I and site II to generate an active muscle-specific transcription complex. Future studies are required to determine whether the ubiquitous proteins that bind to the MCK enhancer (19) similarly interact with the muscle regulatory factors to control MCK gene expression.

TnI gene expression is restricted to myofibers despite the observation that MyoD and the protein factors that interact with site I and site II in the TnI IRE are present in both proliferating and differentiated 23A2 cells. Therefore, additional regulatory mechanisms must control the binding or functional activity of these protein complexes. Previous studies from our laboratory have shown that growth factors play important roles in modulating the activity of the muscle regulatory factors. C3H10T1/2 cells transfected with MyoD, myogenin, Myf-5, or MRF4 expression vectors and maintained in the presence of basic fibroblast growth factor do not convert to a myogenic phenotype (46) and do not express cotransfected contractile protein genes (52). This suggests that the proteins which interact with muscle regulatory elements are regulated posttranslationally through cellular events triggered by growth factor-mediated signal transduction pathways. In support of our findings, a recent study by Mueller and Wold (33) utilized in vivo footprinting to demonstrate that the MRF site within the MCK enhancer is unoccupied in myoblasts but becomes occupied in differentiated myofibers, despite the presence of MyoD in both cell types. In these experiments, the adjacent DNA sites which bind ubiquitous protein factors also remained unoccupied in undifferentiated myoblasts. Together with our studies, these results suggest that expression of the TnI gene and additional contractile protein genes rely on specific signal transduction pathways to initiate the modification(s) of both muscle regulatory factors and ubiquitous transcription factors prior to their binding to muscle-specific enhancers. Future studies will address how growth factors modulate the activity of the proteins that bind to the TnI enhancer. In addition, we are interested in establishing the individual roles of the TnI IRE binding proteins in initiating and/or maintaining the high TnI expression levels associated with differentiated skeletal myofibers.

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