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Casein Kinase II Increases the Transcriptional Activities of MRF4 and MyoD Independently of Their Direct Phosphorylation

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The myogenic regulatory factors (MRFs) are a subclass of a much larger group of basic helix-loop-helix transcription factors which includes members of the E protein family such as E47, E2-2, and HEB. Although the MRFs are unique in their ability to confer a myogenic phenotype on nonmuscle cells, they require E protein partners to form a MRF-E protein heterodimer, which represents the functional myogenesis-inducing complex. The mechanisms controlling homodimer and heterodimer formation in vivo remain largely unknown, although it is likely that posttranslational modification of one or both basic helix-loop-helix partners is critical to this regulatory event. In this respect, MyoD and MRF4, both members of the MRF family, exist in vivo as phosphoproteins and contain multiple consensus phosphorylation sites, including sites for casein kinase II (CKII) phosphorylation. In this study, we demonstrate that overexpression of CKII increases the transcriptional activities of MRF4 and MyoD in vivo. Interestingly, mutation of the individual CKII sites within MRF4 and MyoD does not alter the ability of CKII to enhance MRF transcriptional activity, suggesting that the effect of CKII expression on the MRFs is indirect. Given that the MRFs require dimerization with E protein partners to activate muscle-specific transcription, the effects of CKII expression on E protein function also were examined. Our studies show that E47 serves as an in vitro substrate for CKII and that CKII-phosphorylated E47 proteins no longer bind to DNA. These observations were confirmed by in vivo experiments showing that overexpression of CKII produces a dramatic reduction in E47 homodimer-directed transcription. We conclude from these studies that CKII may act as a positive regulator of myogenesis by preventing E protein homodimers from binding to muscle gene regulatory elements.

The myogenic regulatory factors (MRFs) MyoD, myogenin, Myf-5, and MRF4 form a subclass of a much larger group of transcription factors which are characterized by a common basic helix-loop-helix (bHLH) motif (reviewed in references 16, 23, and 30). The crystal structure of the MyoD-DNA complex (24) has revealed that the MyoD amphipathic α-helices, separated by a variable loop region, are responsible for protein dimerization and that the MyoD basic domain provides the contact points with DNA. For the bHLH MRF proteins, the consensus DNA sequence -CANNTG-, referred to as an E box, serves as the DNA target site although additional flanking nucleotides also have a role in DNA binding specificity (2, 42, 43). Binding of MRF-containing protein complexes to E-box elements located within the regulatory regions of muscle-specific genes often is sufficient to activate muscle gene expression.

Although the MRFs are capable of forming homodimers in vitro (25), they function in vivo as heterodimers with a second bHLH protein from the E protein family such as E47, E2-2, or HEB (34). In most instances, the E protein dimer partner is expressed ubiquitously, while MRF expression remains restricted to the skeletal muscle lineage. The E protein partner preferred by each MRF currently is unknown, but partner choice likely represents an important molecular mechanism for controlling MRF activity during myogenesis. Indeed, positive as well as negative regulatory partners are known to be involved in modulating MRF activity (13, 31).

An additional mechanism through which the activities of a variety of transcription factors are regulated is by differential phosphorylation. Each of the MRFs exists as a phosphoprotein in vivo, and analysis of the amino acid sequence of the various MRFs reveals several potential sites of phosphorylation, including consensus sites for cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), and casein kinase II (CKII) (10, 17, 25, 38, 41). All four of the MRFs are subject to phosphorylation by PKA in vitro, and the overexpression of PKA in myogenic cells inhibits differentiation (10, 19, 41). However, site-directed mutagenesis of the PKA site within the DNA binding domain of myogenin, MRF4, MyoD, and Myf-5 does not abolish the myogenic potential of the MRFs, nor does it alter their response to PKA expression, suggesting that PKA overexpression inhibits myogenesis by targeting molecules other than the MRFs. Each MRF also is subject to in vitro phosphorylation by PKC, and overexpression of PKC in vivo similarly inhibits myogenesis (10, 20). For myogenin, in vivo phosphorylation of the PKC site is triggered by the treatment of cells with basic fibroblast growth factor (FGF-2), leading to a block in the ability of myogenin to interact with its target DNA (20). In contrast, while PKC phosphorylation of both MRF4 and MyoD inhibits the ability of the proteins to bind target DNA in vitro, mutagenesis of the PKC site does not prevent MRF4 or MyoD activity from being inhibited following treatment with FGF-2 in vivo (reference 10 and unpublished results), demonstrating that the MRFs are not regulated similarly by PKC.

Currently, no data regarding the importance of CKII phos-

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phorylation to the function of MyoD and MRF4 are available. CKII is a ubiquitous enzyme found in the cytoplasm and in the nucleus of most mammalian cells (36), and its activity increases as myoblasts differentiate into myotubes (40a). The enzyme phosphorylates a variety of transcriptional regulators, including c-Myc, c-Jun, c-ErbA, and the serum response factor (reviewed in reference 14). In the case of c-Jun, phosphorylation by CKII inhibits DNA binding (21), which appears to be the consequence of electrostatic repulsion generated by the close proximity of the phosphorylated residues to the DNA binding domain of c-Jun (14). Similarly, in vitro CKII phosphorylation of Max, the cellular dimerization partner of c-Myc, promotes an increase in the on-off rates for DNA binding by Max homodimers, suggesting that CKII phosphorylation of Max in vivo has the potential to influence the transcription of genes regulated by the Myc-Max protein complex (1, 3).

The presence of consensus CKII phosphorylation sites in MRF4 and MyoD prompted us to investigate whether CKII also regulates the activities of the MRFs. Our studies show that overexpression of the catalytic subunit of CKII during myogenesis increases the transcriptional activity of both MRF4 and MyoD. In vitro phosphorylation experiments demonstrate that MRF4 and MyoD serve as substrates for CKII phosphorylation but that MRF4 is phosphorylated exclusively in the amino terminus, while the major sites of CKII phosphorylation in MyoD are located in both the amino and the carboxy termini. Site-directed mutagenesis of the CKII sites in MRF4 and MyoD does not result in a loss of myogenic potential, nor does it abolish the response to CKII expression, suggesting that direct phosphorylation by CKII is not the molecular mechanism underlying the increase in MRF activity observed during myogenesis. Instead, our studies show that the effect of CKII on MRF activity is likely to be mediated through direct alteration of the E proteins, the obligate dimerization partners of the MRFs. Overexpression of CKII inhibits the ability of E47 to activate transcription of reporter gene constructs controlled by an immunoglobulin enhancer, and in vitro DNA binding assays demonstrate that the phosphorylated form of E47 does not bind to DNA. These results suggest that a CKII signaling pathway enhances the activity of functional MRF-E protein complexes by a mechanism that does not require CKII-directed phosphorylation of the MRF4 and MyoD proteins.

MATERIALS AND METHODS

Cell culture and transient DNA transfections. C3H10T1/2 fibroblasts were maintained in basal Eagle medium supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded into six-well tissue culture plates for transient DNA transfections employing DNA calcium phosphate precipitates as described previously (15). Briefly, precipitates containing 0.25 μg of test plasmid and 1 μg of luciferase reporter plasmid were added to wells of 80 to 90% confluent C3H10T1/2 cells. For the overexpression of CKII, 2.5 μg of the cytomegalovirus expression plasmid or 2.5 μg of the cytomegalovirus-CKII plasmid (11) encoding the catalytic α-subunit of CKII was included in the precipitates. After 5 h, cells were washed with serum-free medium and provided a differentiation medium composed of Dulbecco modified Eagle medium plus 2% horse serum. At 72 h posttransfection, the cells were lysed by addition of 25 mM Tris (pH 7.8), 2 mM dithiothreitol, 1% Triton X-100, and 10% glycerol. Luciferase activity subsequently was measured with a Promega Luciferase Kit. All luciferase activities were normalized to the protein content of each sample and are reported as relative light units per microgram of protein. For each experimental group, a minimum of three independent transfections were performed.

Experimental gene constructs. The 245-bp proximal promoter region of the human cardiac α-actin gene (26) was ligated into the luciferase reporter pGL2-basic (Promega) to create the α-actin luciferase reporter gene. Similarly, a 2.3-kb promoter fragment of the quail troponin I (TnI) gene containing the entire first intron (22) was inserted into the pGL2-basic plasmid to create the TnI luciferase reporter (TnI-luc). The reporter plasmid (E-box)$_4$ luciferase (also known as 4R-TK luciferase) was constructed by inserting four copies of the muscle creatine kinase right E-box and a thymidine kinase gene promoter (39) into the pGL2-basic plasmid. A multimerized immunoglobulin E-box luciferase reporter also was constructed by inserting four copies of the μE5-μE2 E-box tandem from the immunoglobulin H (IgH) enhancer (32) into the pGL2-promoter plasmid.

Point mutations in the muscle regulatory factors MRF4 and MyoD were generated by oligonucleotide-directed mutagenesis (Amersham). Single-stranded DNA was produced from the appropriate pBluescript-MRF plasmid constructs. All mutations were verified by DNA sequencing prior to subcloning into the pEMscribeα2 eukaryotic expression vector (7, 18) or into a prokaryotic pGEX protein expression vector (Pharmacia). pGEX-MyoD and pGEX-MRF4 constructs encoding specific peptide fragments were generated by standard PCR techniques. Briefly, 5′ primers to the mouse MyoD cDNA or to the rat MRF4 cDNA were designed to include an *Eco*RI restriction site while 3′ primers were synthesized to include an *Xba*I restriction site. The resulting PCR-generated DNA fragments were cloned in frame into the appropriate *Eco*RI- and *Xba*I-digested pGEX vector. The ΔNΔC MyoD and ΔNΔC MyoD-VP16 constructs plus the pECE-MyoD~E47 construct were generous gifts of A. Lassar and B. Wold, respectively, and have been described previously (5, 29).

Fusion protein purification. *Escherichia coli* cells transformed with pGEX plasmids were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to produce glutathione *S*-transferase (GST) fusion proteins. After 3 h, the cells were lysed and supernatants were collected. Fusion proteins were affinity purified from the lysates by adsorption to glutathione agarose beads. Proteins were eluted from the beads with 50 mM Tris (pH 8.0) and 20 mM reduced glutathione. Similarly, *E. coli* cells transformed with a His-tagged pQE-MyoD expression vector were induced with 2 mM IPTG for 3 h. Bacterial cell pellets were collected and lysed with 6 M urea–10 mM Tris (pH 7.5)–20 mM imidazole. Supernatants containing MyoD-His proteins were incubated for 60 min at 4°C with Ni-agarose. The beads were washed, and the protein was eluted by addition of 6 M urea–10 mM Tris (pH 7.5)–250 mM imidazole. Protein concentrations were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purification of the ΔE47-His protein has been described previously (33), and the protein was a generous gift of T. Kadesch.

In vitro CKII assays. Purified GST-fusion or His-tagged MRF and E proteins were incubated in 50 mM Tris (pH 7.5)–200 mM NaCl–10 mM $MgCl_2$–20 μM ATP–20 μCi of [γ-^{32}P]ATP–25 ng of CKII (New England Biolabs) for 30 min at 30°C. Reactions were terminated by addition of SDS-PAGE sample buffer. Proteins were electrophoretically separated through SDS–10% or 12% polyacrylamide gels, dried, and visualized by autoradiography.

Phosphoamino acid analysis. GST-MRF4 and GST-MyoD fusion proteins were radiolabeled by CKII, electrophoretically separated through 10% polyacrylamide gels, and dried, and the subsequent labeled protein bands were excised. Proteins were eluted from the gel, precipitated, and hydrolyzed in the presence of 5.7 N HCl at 110°C for 60 min. Hydrolysates were dried, resuspended in distilled water, and applied to cellulose thin-layer chromatography plates (Whatman K2). Phosphoamino acids were separated by chromatography in pH 1.9 buffer (formic acid-glacial acetic acid-water, 50:156:1,794 [vol/vol/vol]) in the first dimension and pH 3.5 buffer (glacial acetic acid-pyridine-water, 100:10:1,890 [vol/vol/vol]) in the second dimension. Phosphoamino acid standards were visualized by ninhydrin staining while radiolabeled phosphoamino acids were detected by autoradiography.

E47 homodimer cross-linking. Purified ΔE47 was phosphorylated with CKII as described above in the presence or absence of ATP. Eighty nanograms of each reaction mixture was diluted in gel shift binding buffer (see below) and incubated at 37°C for 15 min and then additionally incubated at room temperature for 15 min. To irreversibly cross-link the protein complexes, glutaraldehyde was added at a final concentration of 0.0125% in 0.05 M KCl–0.025% Nonidet P-40. The reactions were allowed to proceed at room temperature for 3 min and stopped by the addition of SDS-PAGE sample buffer. The ΔE47 complexes were separated by SDS-PAGE and transferred to nitrocellulose with a Pharmacia semidry blotter. The protein blots were blocked in 5% nonfat dry milk in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) for 20 min and incubated with a rabbit polyclonal E2A antibody (Santa Cruz) diluted 1:100 in blocking solution for 1 h. The blot was developed for chemiluminescence by using goat anti-rabbit IgG-peroxidase and an enhanced chemiluminescence kit (Amersham).

Electrophoretic mobility shift assays. TnI and the immunoglobulin gene μE5 E-box probes were prepared by radiolabeling the positive strand with [γ-^{32}P]ATP and T4 polynucleotide kinase as described previously (22). Free label was removed by Sephadex G-50 spin columns. The labeled oligonucleotide was mixed with an excess of the respective complementary negative strand, heated to 95°C for 5 min, and slowly cooled to room temperature to allow the strands to anneal.

ΔE47-His was phosphorylated by CKII in the presence or absence of ATP as described above. An aliquot of the reaction mixture was analyzed by SDS-PAGE to ensure that the protein was sufficiently phosphorylated, as judged by a shift in electrophoretic mobility (see text for example). The remainder of the kinase reaction mixture was diluted in 8 M urea–10 mM Tris (pH 7.5) to a final concentration of 5 ng/μl. A second aliquot (100 ng) was incubated with 12 U of lambda phosphatase (New England Biolabs) for 1 h at 30°C prior to dilution in 8 M urea–10 mM Tris (pH 7.5). For the analysis of homodimer formation, 5 ng of ΔE47-His was diluted in binding buffer (25 mM Tris [pH 8.0], 50 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol) and incubated at 37°C for 15 min. Protein complexes were allowed to bind to a ^{32}P-μE5 E-box DNA probe in the presence of 2 μg of poly(dI-dC) for 15 min at room temperature prior to

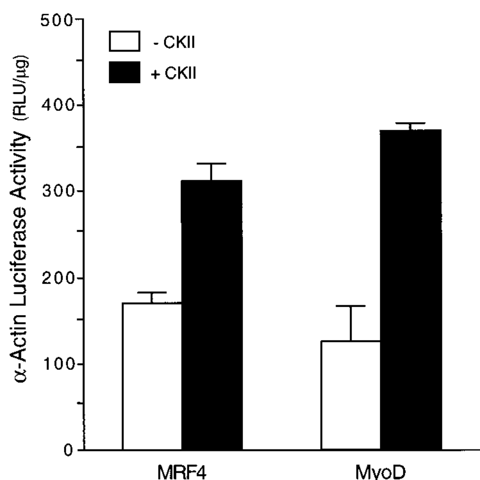


FIG. 1. CKII increases the transcriptional activity of MRF4 and MyoD. C3H10T1/2 fibroblasts were transiently transfected with the α-actin luciferase reporter gene and expression plasmids for MRF4 and MyoD in the absence (−) or presence (+) of the catalytic α-subunit of CKII. Cells were maintained for 72 h in differentiation medium prior to assaying for luciferase activity. Activity is presented as relative light units (RLU) per microgram of protein. CKII increases the transcriptional activity of both MRF4 and MyoD approximately two- to threefold over control (pCMV)-transfected cells. Error bars represent the standard error of the mean.

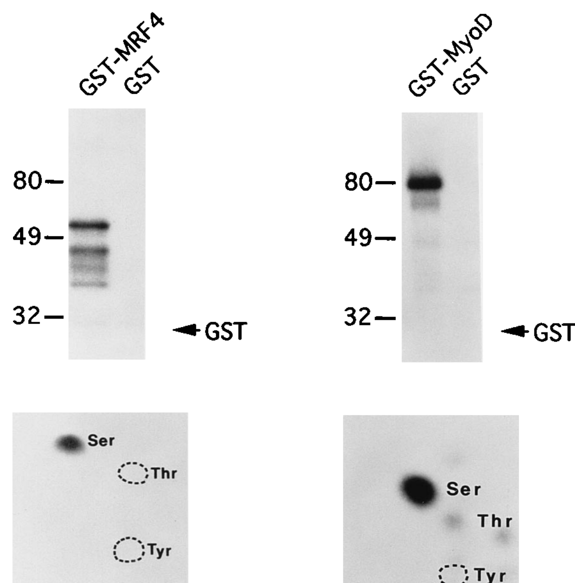


FIG. 2. MRF4 and MyoD are phosphorylated by CKII in vitro. Purified GST, GST-MRF4, and GST-MyoD were incubated in kinase buffer containing [γ-^{32}P]ATP and CKII. Proteins were separated by SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography (upper panels). Hydrolysis of the labeled GST-MRF4 and GST-MyoD proteins and separation of the resulting amino acids by thin-layer chromatography reveal that phosphorylation occurs on serine residues for MRF4 and on serine and threonine residues for MyoD (lower panels). The positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated. Numbers to the left of each panel are molecular masses in kilodaltons.

electrophoretic separation in 5% nondenaturing acrylamide gels. Similar methods were used to analyze heterodimer formation. Briefly, 10 ng of MyoD-His and ΔE47-His were allowed to renature and form heterodimers in binding buffer for 15 min at 37°C. The proteins were further incubated with a ^{32}P-TnI E-box DNA probe for 15 min at room temperature. Protein-DNA complexes were electrophoretically separated in nondenaturing acrylamide gels. Gels were dried, and the labeled complexes were visualized by autoradiography.

RESULTS

Overexpression of CKII increases MRF-mediated transcription of muscle-specific promoters. Previous studies have shown that MRF4 and MyoD exist as phosphoproteins in vivo and that these MRFs contain potential CKII phosphorylation sites (10, 38). In an effort to examine the role of CKII phosphorylation in MRF protein activity, C3H10T1/2 cells were transiently transfected with an α-actin luciferase reporter gene, pEM-MRF4 or pEM-MyoD, and a plasmid expressing the catalytic α-subunit of CKII. After 72 h in differentiation-inducing medium, cell extracts were prepared and analyzed for luciferase activity. As expected, in the absence of CKII overexpression, each MRF is able to drive high levels of α-actin luciferase expression (Fig. 1) and to induce a differentiated muscle phenotype (data not shown). However, inclusion of the CKII expression vector in the precipitates produces an approximately two- to threefold increase in MRF4- and MyoD-induced α-actin luciferase activity (Fig. 1). A similar increase also is obtained when an α-acetylcholine luciferase reporter gene is used in these assays (data not shown). The observed increase in MRF4 and MyoD activity suggests that modification of the MRFs by CKII plays a direct role in positively regulating their activity during myogenesis.

CKII is a serine/threonine protein kinase that phosphorylates a variety of transcription factors, some of which are structurally similar to the myogenic regulatory factors (reviewed in reference 14). To determine if the MRFs serve as substrates for CKII, control GST, GST-MRF4, and GST-MyoD fusion proteins were incubated with [γ-^{32}P]ATP and purified CKII as described in Materials and Methods. The proteins were resolved by SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography. As shown in Fig. 2, control GST protein is not phosphorylated by CKII, whereas GST-MRF4 and GST-MyoD exhibit intense labeling by this kinase. Subsequent phosphoamino acid analyses of the labeled proteins reveal that MRF4 and MyoD are phosphorylated predominantly on serine residues, although MyoD also contains a weak, but detectable, level of phosphothreonine (Fig. 2). As expected, no phosphotyrosine residues are detected in these assays.

Identification of the CKII phosphorylation sites within MRF4 and MyoD. Analysis of the MRF4 and MyoD primary amino acid sequences reveals five potential CKII phosphorylation sites (S/T-X-X-D/E) in MRF4 and five sites in MyoD (Fig. 3a). Both myogenic factors contain CKII consensus sites within their carboxy termini and within their transcription activation domains (TADs), but neither possesses sites within the conserved bHLH domain. Prior to examination of the functional relevance of these potential sites, truncated MRF proteins were used to map the location of each CKII phosphorylation event. Initial studies demonstrated that the CKII sites within the carboxy terminus of MRF4 are not modified by CKII in vitro since a truncated MRF4 protein (amino acids 148 to 242) is not phosphorylated in these assays (Fig. 3b). On the other hand, an MRF4 protein containing only amino acids 50 to 85 is phosphorylated readily by CKII (Fig. 3b), suggesting that the consensus CKII sites at serines 56 and 57 serve as CKII substrates. Further in vitro phosphorylation assays confirmed that MRF4 is modified by CKII exclusively on serines 56 and 57 since mutation of these serines to alanine residues (MRF4 A56/57) generates a protein which is refractile to CKII-directed [γ-^{32}P]ATP labeling (Fig. 3b). We also examined the effects of placing negatively charged amino acid residues (aspartic acid) on MRF4 at these positions by generating

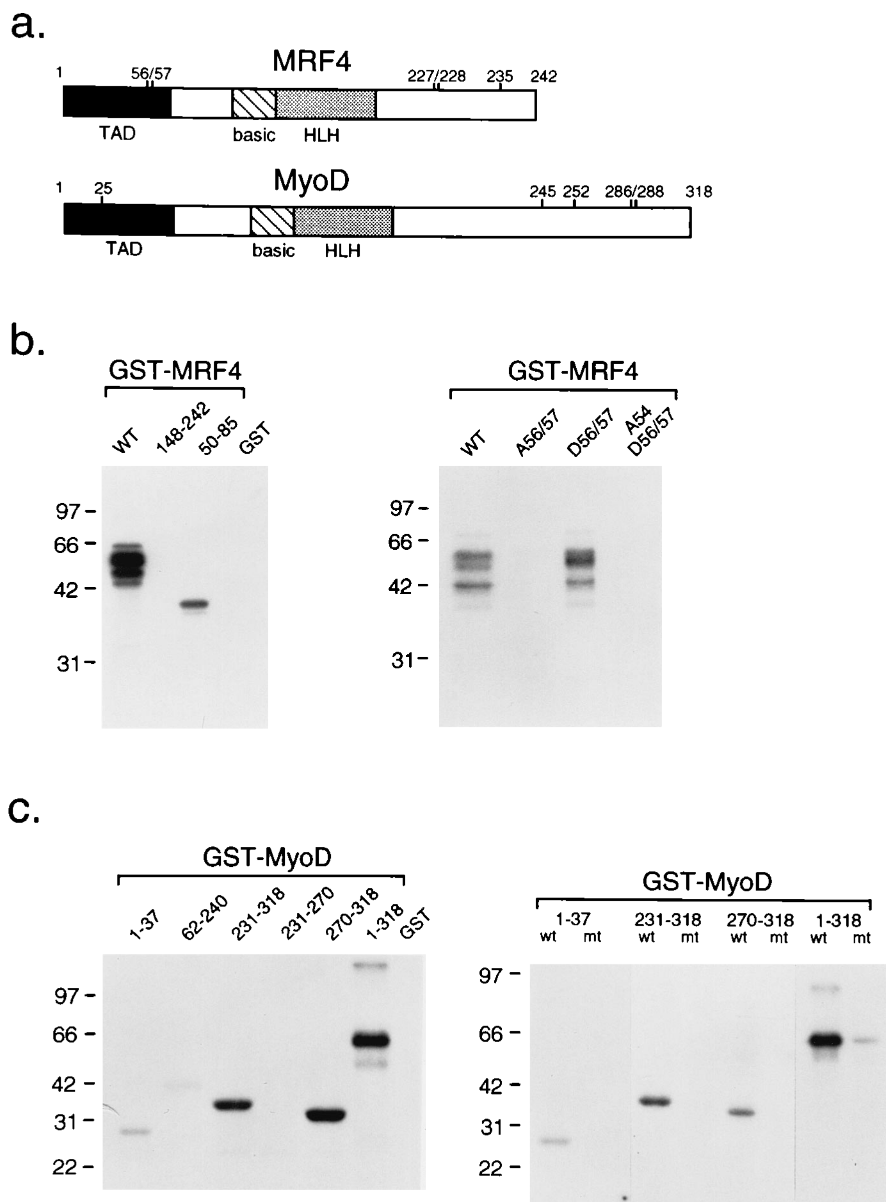


FIG. 3. Identification of the CKII phosphorylation sites in GST-MRF4 and GST-MyoD. (a) Schematic representations of the MRF4 and MyoD proteins showing the locations of the CKII consensus phosphorylation sites, TADs, and the bHLH regions. (b) Control GST, wild-type GST-MRF4 (WT), and various truncated and mutant GST-MRF4 proteins were phosphorylated in vitro by CKII and analyzed by SDS-PAGE and autoradiography. As indicated, GST-MRF4 is phosphorylated by CKII on serines 56 and 57. GST-MRF4 D56/57 is phosphorylated by CKII because of the generation of a novel CKII site at serine 54 (see text for details). (c) GST-MyoD peptides were phosphorylated by CKII in vitro and analyzed by SDS-PAGE and autoradiography as described for panel b. Consensus CKII sites within GST-MyoD peptides 1 to 37, 231 to 318, and 270 to 318 were mutated to alanine residues prior to phosphorylation by CKII. Autoradiography of the resolved wild-type (wt) and mutant (mt) peptides shows abolished radiolabeling of the mutant peptides and a dramatically reduced labeling of the full-length (1 to 318) GST-MyoD mutant. Numbers to the left of panels b and c are molecular masses in kilodaltons.

MRF4 D56/57. Surprisingly, incubation of MRF4 D56/57 with CKII results in the production of a labeled protein, suggesting that an additional CKII site exists in this mutant. Indeed, subsequent examination of the MRF4 D56/57 amino acid sequence revealed that the mutant protein now contains S-X-D-D, which serves as a new CKII site. An additional mutation of serine 54 to alanine in D56/57 (A54/D56/57) produces a protein that is resistant to CKII phosphorylation (Fig. 3b).

A similar in vitro analysis of MyoD was undertaken to identify the CKII sites within this MRF. Truncated MyoD proteins were used to establish that a relatively weak site for CKII phosphorylation exists within amino acids 1 to 37 of MyoD and that a major site(s) is contained within amino acids 270 to 318 of the MyoD carboxy terminus (Fig. 3c). Peptides 62 to 240 and 231 to 270 fail to incorporate significant levels of ^{32}P, demonstrating that the serines between amino acids 62 and 270 are not efficiently utilized as CKII substrates. To further localize the MyoD CKII phosphorylation sites, threonine 25 and serines 286 and 288 were changed to alanine residues and the mutant proteins were incubated with [γ-^{32}P]ATP and CKII. Mutation of these sites completely abolishes CKII-directed phosphorylation of the MyoD peptides (Fig. 3c), and incorpo-

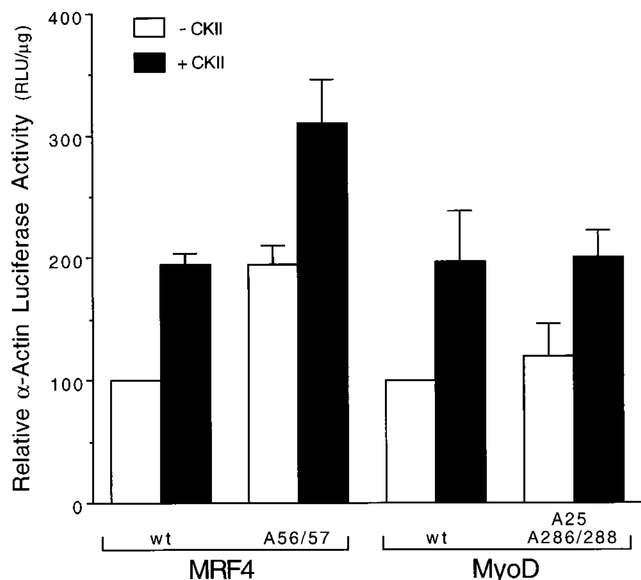


FIG. 4. Mutation of the CKII sites in MRF4 and MyoD does not affect their transcriptional activity. C3H10T1/2 cells were transiently transfected with the α-actin luciferase reporter gene and expression plasmids for CKII, MRF4, MRF4 A56/57, MyoD, or MyoD A25/286/288. After 72 h in differentiation medium, the cells were harvested and analyzed for luciferase activity. Activities are reported relative to the activities of the wild-type (wt) MRF4 or MyoD proteins in the absence of coexpressed CKII, which are set at 100. Mutation of the CKII sites in the myogenic factors does not affect their ability to be further activated in the presence of CKII. Error bars represent the standard error of the mean. RLU, relative light units.

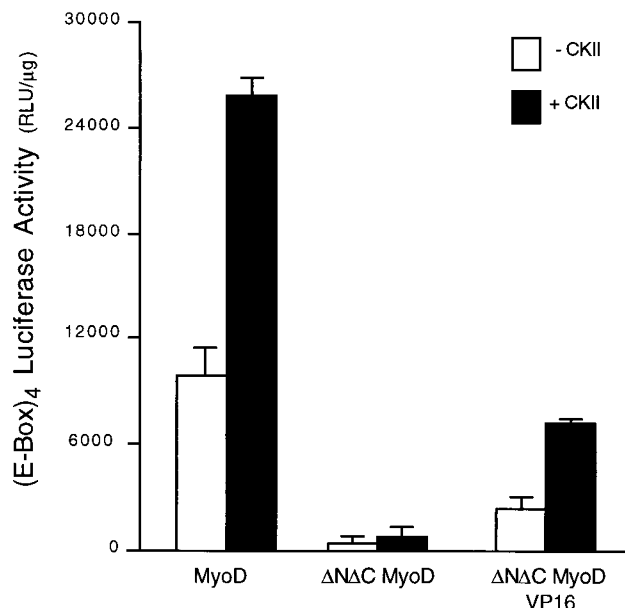


FIG. 5. CKII-directed enhancement of MyoD activity is targeted to the bHLH domain. C3H10T1/2 cells were transiently transfected with a multimerized E-box luciferase reporter gene and MyoD, ΔNΔC MyoD (containing only the MyoD bHLH domain), and ΔNΔC MyoD-VP16 in the presence or absence of the CKII expression plasmid as described for Fig. 1. Cells were harvested 72 h posttransfection, and luciferase activity was measured. CKII expression enhances ΔNΔC MyoD-VP16 activity in a similar fashion as with the wild-type MyoD protein. Error bars represent the standard error of the mean. RLU, relative light units.

ration of all three mutations into a full-length GST-MyoD protein (A-25, A-286, and A-288) significantly reduces phosphorylation by CKII, confirming that these residues in MyoD serve as the major targets for this kinase. Thus, both MRF4 and MyoD possess CKII sites within their amino-terminal TADs, while only MyoD contains CKII phosphorylation sites within the carboxy-terminal region of the protein.

Mutation of the MRF CKII sites does not alter MRF transcriptional activity. Studies from our laboratory have shown that MRF4 is phosphorylated in vivo on serines 56 and 57 (unpublished data) and that the overexpression of CKII in vivo stimulates the myogenic potential of both MRF4 and MyoD (Fig. 1). If the enhanced transcriptional activity of the MRFs results from the direct phosphorylation of the proteins by CKII, mutation of the CKII sites within these proteins should influence their transcriptional activity, most likely in a negative way. Furthermore, the coexpression of CKII with mutant MRFs should not result in an increase in MRF-mediated, muscle-specific gene transcription. To test these predictions, C3H10T1/2 cells were cotransfected with the α-actin luciferase reporter gene, expression plasmids encoding either MRF4 or MyoD mutant proteins, and a plasmid encoding the catalytic α-subunit of CKII. After 72 h in differentiation medium, cell extracts were prepared and analyzed for reporter gene activity. Unexpectedly, in the absence of CKII, MRF4 A56/57 is more effective than the wild-type MRF4 protein in initiating transcription from the α-actin reporter (Fig. 4), suggesting that in vivo phosphorylation of these sites decreases MRF4 activity or that these mutations generate a conformational change which allows for enhanced MRF4 transcriptional activity. Importantly, however, coexpression of the mutant MRF4 protein and CKII in C3H10T1/2 cells produces a level of transcriptional activity that is equivalent to the increase observed with the wild-type protein and CKII, demonstrating that the effect of CKII expression on MRF4 activity is indirect. Similar results also were obtained by using extracts prepared from cells overexpressing the MyoD proteins. The MyoD A25/286/288 protein initiates transcription from the α-actin luciferase reporter to a level comparable with that of wild-type MyoD, and the cotransfection of cells with CKII and MyoD A25/286/288 enhances reporter gene activity (Fig. 4). These results demonstrate that the observed increase in muscle-specific gene transcription produced by overexpression of CKII is not due to a direct modification of the myogenic regulatory factors, but rather to secondary, CKII-directed events.

While the positive influence of CKII expression on myogenic activity cannot be attributed to direct phosphorylation of the MRF CKII sites, MyoD and MRF4 are involved in mediating the observed increase in muscle-specific gene transcription. To establish which region of the MRF protein is affected by CKII overexpression, C3H10T1/2 cells were transfected with an (E-box)$_4$ luciferase reporter gene, plasmids expressing a series of truncated MyoD proteins, and the CKII expression vector. As expected, the full-length MyoD protein activates the luciferase reporter gene and this activity is enhanced by coexpression of CKII (Fig. 5). In contrast, expression of the bHLH region of MyoD (ΔNΔC MyoD) (5) produces a minimal level of transcription from the reporter which is not increased by the overexpression of CKII. Addition of the VP16 TAD to the bHLH region of MyoD (ΔNΔC MyoD-VP16) restores the transcriptional activity of the MyoD bHLH region, and like the wild-type protein, transcription by ΔNΔC MyoD-VP16 is enhanced in the presence of CKII. These studies demonstrate that CKII overexpression targets the bHLH region of MyoD and is independent of the structural regions of MyoD (i.e., the TAD and carboxy termini) that contain CKII phosphorylation sites.

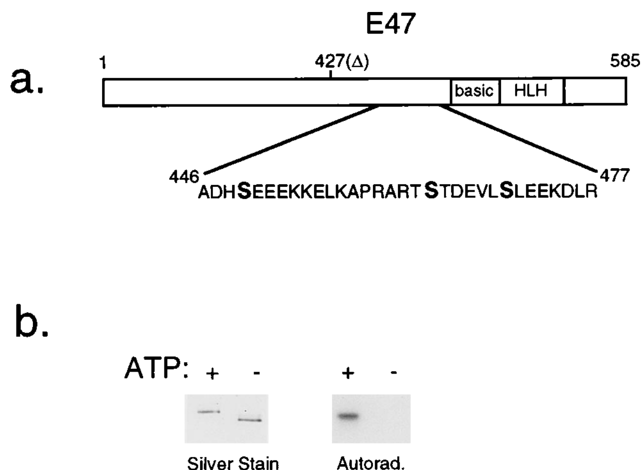


FIG. 6. E47 is phosphorylated by CKII. (a) Schematic representation of the structure of E47 illustrating the bHLH region and the adjacent acidic region. The amino acid sequence of the acidic region (amino acids 446 to 477) is given with the predicted CKII phosphorylation sites in boldface. (b) The ΔE47-His fusion protein (amino acids 427 to 585) was incubated with CKII in kinase buffer in the presence (+) or absence (−) of [γ-^{32}P]ATP. The proteins were separated by SDS-PAGE and visualized by silver staining (left panel) and autoradiography (right panel). Phosphorylation of E47 by CKII results in a more slowly migrating, labeled band.

Phosphorylation of E47 by CKII affects both transcriptional and DNA binding activity. Since the increase in the myogenic activity of both MRF4 and MyoD in response to CKII apparently targets the bHLH domain of the proteins by an indirect mechanism, we examined the possibility that CKII modulates the activity of the E proteins, which are known to be the obligate dimerization and DNA binding partners for the MRFs (17). The amino acid sequence of E47 contains multiple CKII consensus phosphorylation sites, the majority of which are located within an acidic region immediately upstream of the DNA binding domain (Fig. 6a). To establish whether these consensus sites serve as substrates for CKII in vitro, a truncated version of E47 (ΔE47-His) containing amino acids 427 to 585 was incubated with [γ-^{32}P]ATP and purified CKII. As shown in Fig. 6b, phosphorylation of ΔE47 results in the generation of a more slowly migrating protein that is labeled by ^{32}P, implying that at least one of the three CKII sites contained within this region (serines 449, 464, and 470) is phosphorylated by CKII. Recent studies by Sloan et al. (35) have confirmed that E47 exists as a phosphoprotein in 10T1/2 P2 cells, a myogenic cell line derived from the parental C3H10T1/2 cell line used in this current study. Furthermore, the major sites of E47 phosphorylation in vivo have been assigned to serines 449 and 464 (35), both of which are contained with the ΔE47 protein (Fig. 6).

To examine the effects of CKII phosphorylation on E47 activity in vivo, C3H10T1/2 fibroblasts were cotransfected with a multimerized immunoglobulin E-box reporter gene ([μE2+μE5] luciferase) and expression plasmids for E47 and CKII. In contrast to the results obtained with the MRFs (Fig. 1), overexpression of CKII dramatically reduces E47-directed transcription (Fig. 7a), suggesting that the phosphorylation of E47 by CKII alters its transcriptional activity, either by initiating changes in dimer formation or by altering DNA binding potential. To investigate the mechanism involved in inhibiting E47 activity, ΔE47 was incubated with CKII in the presence or absence of ATP. An aliquot of each reaction mixture was incubated with a ^{32}P-labeled μE5 E-box probe and analyzed by standard gel mobility shift assays. Consistent with our predictions, unphosphorylated ΔE47 (E^{OH}) forms homodimers and avidly binds to its cognate DNA site (Fig. 7b) while the ΔE47 protein phosphorylated by CKII (E^P) does not bind efficiently to the μE5 E-box DNA. The DNA binding activity of E^P is restored, however, when E^P is incubated with a phosphatase (lambda phosphatase), confirming that the failure of the ΔE47 protein to bind to DNA is directly related to its phosphorylation status. The remaining DNA binding activity observed with E^P in these experiments can be attributed to residual E^{OH}-E^{OH} and E^{OH}-E^P complexes in the reaction mixture since the band is only partially supershifted following treatment with an antibody to phosphoserine (data not shown).

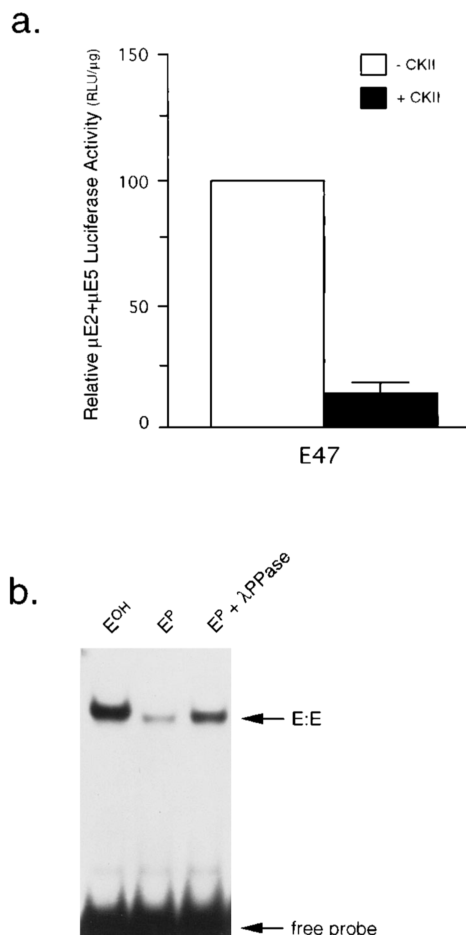


FIG. 7. CKII phosphorylation represses E47 transcriptional and DNA binding activity. (a) C3H10T1/2 cells were transiently transfected with the μE5 E-box luciferase reporter gene and the expression plasmids encoding E47 and CKII. After 48 h in differentiation medium, the cells were analyzed for luciferase activity, which is reported relative to the activity measured for E47 in the absence of CKII (100). CKII expression reduces the ability of E47 to activate transcription by ~80%. The error bar represents the standard error of the mean. RLU, relative light units. (b) Purified ΔE47-His was phosphorylated by CKII in the presence (E^P) or absence (E^{OH}) of ATP. E^P was further incubated with lambda phosphatase (λPPase) to remove phosphate groups. The proteins were allowed to bind to ^{32}P-μE5 probes, and the resulting protein-DNA complexes were separated by electrophoresis through a nondenaturing acrylamide gel and visualized by autoradiography. Phosphorylated E47 (E^P) exhibits a reduced DNA binding activity which can be restored upon phosphatase treatment.

While it is evident that phosphorylation inhibits the DNA binding activity of E47, it is unclear whether this is due to a disruption of the E47 homodimer or due to the formation of E^P homodimers that have lost their affinity for DNA. To re-

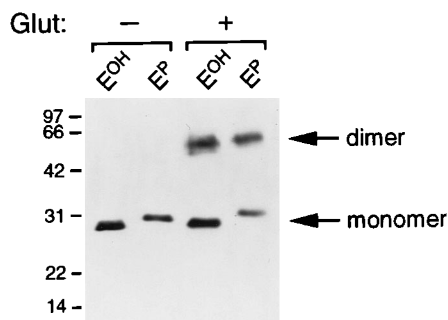
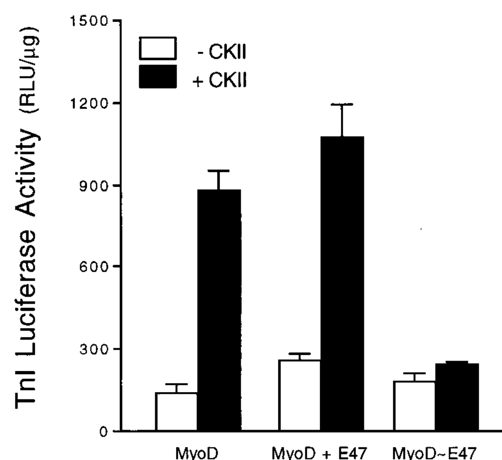


FIG. 8. Phosphorylated E47 is capable of forming homodimer complexes. Purified ΔE47-His was phosphorylated by CKII in the presence or absence of ATP. Phosphorylated (E^P) and unphosphorylated (E^{OH}) forms of ΔE47-His were allowed to form homodimers prior to addition of glutaraldehyde (Glut). Cross-linking was allowed to proceed at room temperature and stopped by addition of SDS gel loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with an E protein-specific antibody and developed by chemiluminescence. Both E^P and E^{OH} efficiently form homodimers. Numbers to the left are molecular masses in kilodaltons.

solve these possibilities, ΔE47-His was phosphorylated in the presence or absence of ATP and allowed to form dimers under the identical conditions used for the gel shift assays. Following homodimer formation, the ΔE47-His proteins were irreversibly cross-linked with glutaraldehyde and analyzed by SDS-PAGE and Western blots (immunoblots) with an E protein-specific antibody. As shown in Fig. 8, cross-linking E^{OH} and E^P in vitro produces new E47-specific protein complexes of 54 and 55 kDa, respectively, which correspond to the predicted sizes of the E47 dimer complexes. Taken together with the data shown in Fig. 6 and 7, these results demonstrate that both E^{OH} and E^P can produce homodimers in solution but that E^P-E^P homodimers fail to bind to μE5 E-box sites and to activate gene transcription.

Although we have shown that the CKII-mediated phosphorylation of E47 does not inhibit its ability to form homodimers, modification by CKII may indirectly enhance myogenesis by increasing the affinity of the E47 protein for the MRFs. To test this hypothesis, we utilized a construct (MyoD~E47) which expresses the MyoD protein tethered to the E47 protein (29). Since cells transfected with this construct will contain "preformed" MyoD-E47 heterodimers, we reasoned that if CKII enhances myogenesis by increasing the levels of MRF-E heterodimers in cells, CKII should have no effect on the transcriptional activity of a MyoD~E47 fusion protein. As expected, E47 alone does not activate the TnI luciferase reporter gene (data not shown), whereas MyoD produces the standard increase in TnI luciferase activity (Fig. 9a). Cotransfection of cells with the CKII expression vector generates a large increase in MyoD activity and yet has no effect on the inability of the E47 protein to activate muscle-specific reporter genes (data not shown). When the MyoD and E47 expression plasmids are coexpressed in C3H10T1/2 cells, TnI luciferase activity is increased above the level attained with MyoD alone and this activity is further stimulated by the overexpression of CKII. As previously reported (29), the tethered MyoD~E47 protein also activates muscle reporter genes to levels comparable with that of MyoD alone when the cells are maintained in differentiation medium. However, the MyoD~E47 protein does not respond to CKII overexpression in a way analogous to that of MyoD plus E47 (Fig. 9a), supporting the idea that CKII activity in cells may be favorable to the formation of MRF-E heterodimers. A similar pattern of transcription activation is obtained when $(E\text{-box})_4$ luciferase, a multimerized muscle creatine kinase E-box reporter gene, is tested in these assays (Fig. 9b), ruling out the possibility that the effects of CKII are targeted to additional transcription factors that bind to the promoter and/or enhancer elements of complex muscle-specific reporter genes, such as TnI.

The observation that the myogenic activity of a MyoD~E47 fusion protein is not enhanced by CKII is strong evidence to suggest that CKII enhances myogenesis by promoting the formation of MRF-E protein complexes. As an additional test of this hypothesis, we performed gel mobility shift assays with a ^{32}P-labeled TnI E-box probe, purified MyoD protein, and either the E^{OH} or E^P proteins. Similar to our earlier findings, E^P homodimers exhibit a greatly reduced affinity for the TnI E-box compared with the E^{OH} homodimers (Fig. 10). The addition of MyoD to the dimerization-binding reaction mixtures



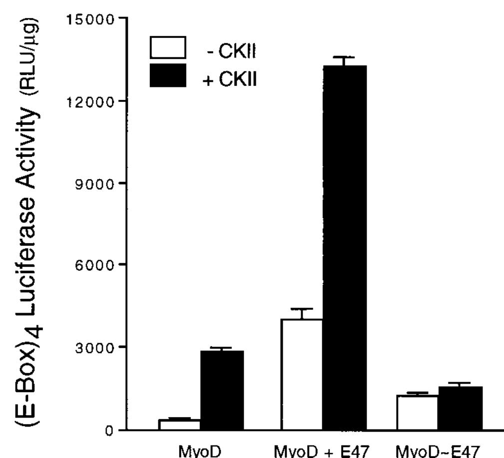


FIG. 9. CKII does not alter the transcriptional activity of a MyoD~E47 fusion protein. C3H10T1/2 cells were transiently transfected with a TnI luciferase reporter gene (a) or a multimerized $(E\text{-box})_4$ reporter gene (b) and expression vectors for CKII, MyoD, E47, MyoD plus E47, or a MyoD~E47 tethered protein construct. After 72 h in differentiation medium, the cells were harvested and luciferase activity was measured. CKII increases TnI and $(E\text{-box})_4$ luciferase activity in cells transfected with MyoD or in cells transfected with MyoD plus E47. In contrast, CKII does not alter the transcriptional activity of the MyoD~E47 fusion protein. Error bars represent the standard error of the mean. RLU, relative light units.

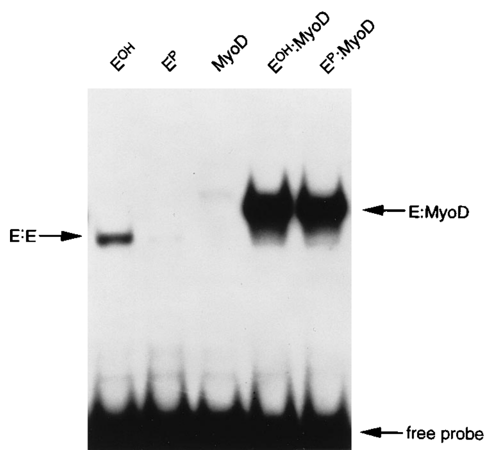


FIG. 10. CKII phosphorylation of E47 inhibits homodimer but not heterodimer formation and DNA binding. E^{OH} and E^{P} (10 ng) were incubated alone or with MyoD-His (10 ng) and a ^{32}P-TnI E-box DNA probe and then electrophoretically separated. No difference in the amount of heterodimer formation or DNA binding (MyoD-E^{OH} versus MyoD-E^{P}) was detected although a significant reduction in the amount of E^{P} homodimer formation and DNA binding (E^{OH} versus E^{P}) is observed.

led to the formation of the preferred MyoD-E protein heterodimer binding complex. However, regardless of whether E^{OH} or E^{P} was used as the binding partner for MyoD, heterodimer DNA binding activity was comparable. Thus, although the phosphorylation status of the E protein has a profound effect on homodimer DNA binding activity, there is no discernible effect on the formation or DNA binding activity of heterodimers in vitro. Additional experiments will be required to establish whether phosphorylated E47 forms a complex with MyoD at accelerated rates and/or whether this complex exhibits altered DNA binding kinetics.

DISCUSSION

Central to our understanding of the process by which proliferating myoblasts are converted to fully differentiated myotubes is defining the molecular mechanisms that modulate the expression and functional activity of the myogenic regulatory factors. The precise mechanism by which MRF activity is controlled during myogenesis remains unknown, although it is clear that posttranslational events play a critical role. Inhibition of MRF activity, for example, has been attributed to several distinct mechanisms including protein modification (10, 19, 20) and the interaction with dominant-negative HLH proteins (13, 31). Given that the MRFs exist in vivo as phosphoproteins (10, 17, 25, 38, 41), it is likely that reversible phosphorylation of the MRFs is involved in regulating aspects of DNA binding, dimerization preference, and transcriptional activity. Therefore, establishing the importance of individual phosphorylation events is crucial to understanding how MRF activity is controlled during myogenesis.

In this study, we have examined the effects of CKII phosphorylation on MRF4- and MyoD-directed myogenesis. In contrast to previous studies describing the negative regulation of myogenic differentiation by specific protein kinases (10, 19, 20, 41), our experiments have revealed that overexpression of the catalytic subunit of CKII increases the muscle-specific transcriptional activity of both MRF4 and MyoD. Since both MRF4 and MyoD contain consensus CKII sites which are targets of phosphorylation, we tested whether the direct modification of the MRFs by CKII was responsible for increasing the transcriptional activity of these proteins. Using mutant MRF proteins that were not substrates for CKII, we demonstrated that full-length MRF proteins, or chimeric MRF proteins containing GAL4 DNA binding domains (15a), activate reporter gene expression and, in the case of the full-length proteins, remain responsive to the positive influence of CKII activity. While these data suggest that phosphorylation of the MRFs by CKII does not directly enhance transcriptional activity, it is possible that phosphorylation of MyoD and MRF4 by CKII in vivo plays a more subtle role in modulating MRF activity by altering the conformation of the MRFs and thus regulating the dimerization and DNA binding activity of these proteins. Indeed, the CKII site in MRF4 may serve such a purpose since MRF4 A56/57 exhibits an enhanced transcriptional activity compared with the wild-type MRF4 protein. Additional studies will be required to rigorously establish whether CKII phosphorylation alters the structure of MRF monomers or alters MRF-containing dimeric and tetrameric (8) complexes in muscle cells.

The MRF bHLH domain represents the minimum structure required for myogenic activity, with the basic region necessary for DNA binding and the HLH motif essential for dimerization with other HLH proteins (6, 27, 28). Although the MyoD bHLH region is not phosphorylated by CKII, the transcriptional activity of protein complexes containing this minimum domain (ΔNΔC MyoD-VP16) is enhanced by CKII overexpression. CKII does not increase the transcriptional activity of all bHLH factors, however, since we have demonstrated that CKII inhibits the transcriptional activity of E47 homodimers by greatly reducing the affinity of these complexes for DNA. Sloan et al. (35) recently confirmed these results in B cells in which only unphosphorylated E47 homodimers are transcriptionally active. Interestingly, the phosphorylation status of E47 does not adversely affect complex formation with the MRFs nor does it alter the DNA binding activity of MRF-E47 heterodimers, implying that CKII activity in cells favors the occupation of E-box target DNA sites by MRF-E protein heterodimers (see below). A similar regulatory mechanism has been described for the Myc-Max protein complex, in which CKII phosphorylation of the Max protein has no effect on the formation of Max-Max homodimers but rather encourages the formation of Myc-Max DNA binding heterodimers (1).

Our results clearly indicate that the increase in MRF activity in response to CKII is indirect and probably is associated with effects exerted by CKII on the obligate MRF protein partners, the E proteins. E12 and E47 are expressed in myogenic cells and readily heterodimerize with the MRFs, and E47, at least, exists as a phosphoprotein in a variety of cell types (4, 17, 27, 28, 33). Although E12 and E47 are very similar in structure and function, E47, unlike E12, efficiently forms homodimers that are capable of binding to DNA (37). The capacity to bind to DNA is controlled by the amino acid composition of an acidic region found immediately upstream of the DNA binding motif in both proteins. In E12, this acidic region contains several proline residues which likely modify the overall α-helical structure of this domain. A chimeric E protein in which the E47 acidic region is substituted for the analogous residues in E12 efficiently binds DNA as a homodimer (37). More precise substitutions of serine 464, threonine 465, serine 470, and leucine 471 from E47 into the E12 acidic region also generate a protein with the ability to bind to DNA (37). Interestingly, these substitutions create consensus CKII sites within E12 that also exist in E47 (Fig. 6), suggesting that CKII may have a role in modulating the DNA binding activity of E proteins containing CKII sites. Indeed, we have found that wild-type E12 does not respond to CKII overexpression in a similar fashion as E47

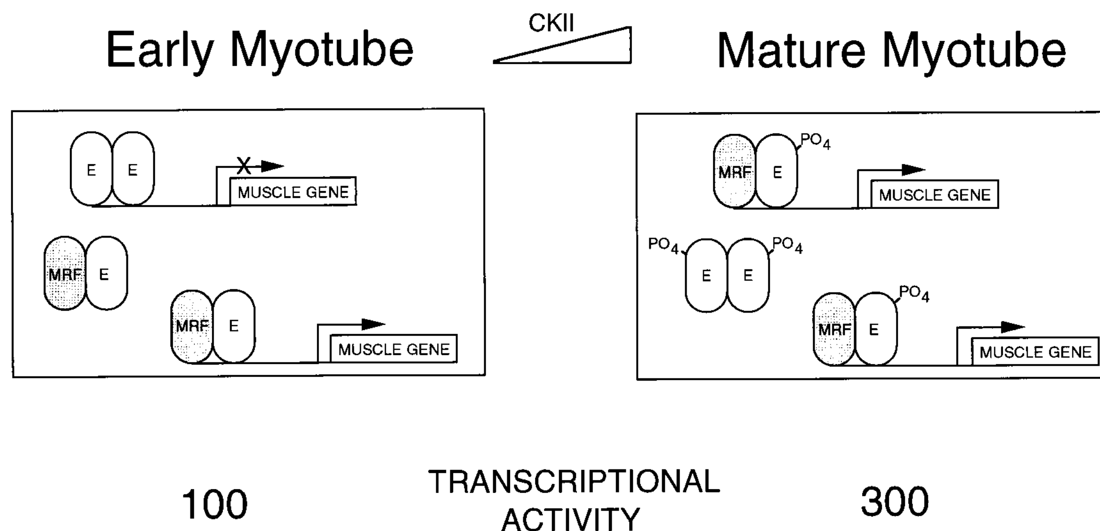


FIG. 11. Model of CKII regulation of myogenic transcription. In the absence of CKII, E47 forms homodimers that are capable of occupying muscle-specific E-box sites and thus preventing MRF-E heterodimers from binding. In the presence of CKII, phosphorylated E47 homodimers are prevented from binding to muscle-specific E-box sites, permitting MRF-E heterodimers to occupy these sites to maximally activate muscle gene expression.

(unpublished data). How additional E proteins, such as HEB and E2-2, respond to CKII activity remains unknown. Both HEB and E2-2 contain potential CKII sites within the region immediately upstream of their bHLH motifs (12), suggesting that HEB, E2-2, and E47 may be regulated through similar reversible phosphorylation events. In support of this notion, we have shown that an E47 protein phosphorylated by CKII in vitro no longer binds to DNA as a homodimer and yet retains the ability to form functional heterodimers with the MRFs. In addition, in vivo experiments using a tethered MyoD~E47 protein have suggested that modification of the E protein may increase its affinity for the MRFs. These observations lead us to speculate that CKII activity, by phosphorylating the acidic region of E47 and possibly other members of this superfamily, increases the pool of E proteins available to the MRFs and effectively increases the number of MRF-E protein complexes in cells. A direct test of this hypothesis will require a combination of electrophoretic mobility shift assays and binding competitions using dominant-negative mutant proteins (34) to establish whether phosphorylation of E proteins enhances MRF-E complex formation.

An additional mechanism for increasing the pool size of E proteins may involve phosphorylation of Id (13), the dominant-negative HLH regulatory protein. It is possible that phosphorylation of Id prevents its heterodimerization with E proteins, thus allowing an increase in the number of E proteins available for heterodimerization with the MRFs. This scenario seems unlikely, however, since Id1 is not phosphorylated by CKII in vitro (unpublished results).

Finally, CKII phosphorylation may increase MRF activity in cells by interfering with a potential repressor of E47 function. Studies by Genetta et al. (9) describe a model system for the IgH enhancer in which a repressor protein binds to the μE5 site located within the IgH enhancer and inhibits cell-type-specific transcription. In B cells, this repressor is displaced from the μE5 E-box within the IgH enhancer by E2A gene products. However, in myoblasts, the IgH enhancer is inactive, presumably since the repressor protein remains in place (40). If a similar repressor system operates to control the tissue-specific expression of muscle genes, it is formally possible that CKII activity could function to alleviate this constraint. In this model, a regulatory protein associated with the E-box sites of muscle-specific genes would maintain these genes in an inactive, or suboptimally active, transcriptional state (Fig. 11). CKII activity, which increases during the transition from myoblast to myotube (40a), would result in displacement of this repressor, leading to a concomitant increase in MRF-directed muscle-specific gene transcription during differentiation. Since we have shown that phosphorylated E proteins are unable to bind either to the μE5 E-box or to the TnI E-box, it is possible that the repressor protein described in this model is E47. Alternatively, additional E proteins, such as E2-2 and HEB, also may be involved. As an example, HEB, which is similar to E47, is found in skeletal muscle cells, forms homodimers as well as heterodimers with the MRF proteins, and can bind to both muscle and nonmuscle E-box DNA sites (12). Thus, it is possible that the proposed repressor system may actually be due to occupation of E-box sites by a class of E proteins and not due to a single protein per se. To rigorously test this model, future experiments will be designed to establish whether CKII functions as a positive regulator of myogenesis by preventing E protein homodimers from binding to muscle gene regulatory elements in vivo.

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