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Myocyte Nuclear Factor, a Novel Winged-Helix Transcription Factor under both Developmental and Neural Regulation in Striated Myocytes

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A sequence motif (CCAC box) within an upstream enhancer region of the human myoglobin gene is essential for transcriptional activity in both cardiac and skeletal muscle. A cDNA clone, myocyte nuclear factor (MNF), was isolated from a murine expression library on the basis of sequence-specific binding to the myoglobin CCAC box motif and was found to encode a novel member of the winged-helix or HNF-3/*fork head* family of transcription factors. Probes based on this sequence identify two mRNA species that are upregulated during myocyte differentiation, and antibodies raised against recombinant MNF identify proteins of approximately 90, 68, and 65 kDa whose expression is regulated following differentiation of myogenic cells in culture. In addition, the 90-kDa form of MNF is phosphorylated and is upregulated in intact muscles subjected to chronic motor nerve stimulation, a potent stimulus to myoglobin gene regulation. Amino acid residues 280 to 389 of MNF demonstrate 35 to 89% sequence identity to the winged-helix domain from other known members of this family, but MNF is otherwise divergent. A proline-rich amino-terminal region (residues 1 to 206) of MNF functions as a transcriptional activation domain. These studies provide the first evidence that members of the winged-helix family of transcription factors have a role in myogenic differentiation and in remodeling processes of adult muscles that occur in response to physiological stimuli.

Myoglobin is a cytosolic hemoprotein that is expressed selectively in cardiac and skeletal myocytes (57, 64). During embryonic development, myoglobin is expressed at low levels in fetal ventricular myocytes and in skeletal muscles but not in the fetal atria (46). Following birth, however, myoglobin expression is markedly and progressively upregulated in all chambers of the heart and in most skeletal muscles (46, 64), coincident with the increased respiratory demand placed upon the heart during postnatal life and with increased respiratory and locomotor activity. Even in adult animals, myoglobin expression is subject to physiological regulation in response to changing demands for mitochondrial respiration (57). These temporal and spatial patterns of myoglobin expression are distinct from those of sarcomeric proteins, suggesting that myoglobin transcription may be controlled, at least in part, by factors different from those involved in expression of other markers of terminal differentiation in striated muscles.

In previous studies we have defined control elements within the 5′-flanking region of the human myoglobin gene required for transcriptional activity in both skeletal and cardiac muscle (1, 2, 10). Results from gene transfer experiments with cultured skeletal myogenic cells, direct injection of plasmid constructs into the myocardial wall of living animals, and germ line transgenic mice are consistent and indicate that a 380-bp upstream region of the myoglobin gene (−373 to +7) is sufficient to recapitulate the spatial and temporal pattern of expression of the endogenous myoglobin gene.

An extensive mutational analysis of this region demonstrated that a canonical TATA box and two upstream sequence elements within this region are necessary for muscle-specific transcriptional activity (2, 60). One of these upstream elements is rich in adenine and thymidine bases (A/T element) and functions in vitro as a binding site for members of the MEF2 family of transcriptional activators, as well as for other nuclear factors that remain to be identified. A second site is characterized by a CCCACCC motif (CCAC box) and binds specifically to nuclear protein factors present in skeletal and cardiac myocytes. Cooperative interactions between factors binding at the CCAC box and A/T element are required for transcriptional activation of the myoglobin gene.

In the present study, we report the cloning of a novel protein, termed myocyte nuclear factor (MNF), that is expressed in cardiac and skeletal myocytes and exhibits sequence-specific DNA-binding activity for the myoglobin CCAC box. The predicted amino acid sequence of MNF reveals it to be a member of the winged-helix family of DNA-binding proteins, defined by a 110-amino-acid domain first identified in the *fork head* gene of *Drosophila melanogaster* and in mammalian hepatocyte nuclear factor 3α (HNF-3α), HNF-β, and HNF-γ, which are involved in liver-specific expression of the transthyretin and α_1-antitrypsin genes (34, 35, 61, 62). *fork head* functions in the *Drosophila* embryo as a region-specific homeotic gene that promotes terminal, in contrast to segmental, development. Mutations in *fork head* result in the replacement of the foregut and hindgut by ectopic head structures. *fork head* is a nuclear protein expressed in gut, yolk nuclei, salivary glands, and the nervous system of developing flies. Recently, seven additional *fork head*-related genes have been isolated and identified in *Drosophila* species (20).

Other members of this family have been identified in organisms as diverse as *Saccharomyces cerevisiae* and humans

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(11, 18, 29, 34–37, 41, 48, 55, 56, 62, 67). HCM1 functions in yeast cells in a calmodulin-dependent signal transduction cascade (67), while human ILF was identified on the basis of sequence-specific DNA binding to an interleukin-2 response element within the terminal repeat region of the human immunodeficiency virus (36). Rat BF-1 is expressed selectively in the developing brain (56), whereas avian *qin* functions as a dominant oncogene (37). A chromosomal translocation that generates a gene fusion between *PAX3* and a *fork head*-related gene encoded on chromosome 13 in human alveolar rhabdomyosarcoma cells was recently identified (13). Thus, in a manner analogous to the extensive basic helix-loop-helix family of transcription factors, the family of proteins related to *fork head* and HNF-3 includes many members with a multiplicity of functions during development and in mediating responses to extracellular stimuli in a variety of cell types (6, 53).

A crystal structure of the signature motif that characterizes this family was recently determined (5). Three α-helical regions are separated by short β-sheets, and two loops within the DNA-bound form resemble the wings of a butterfly, resulting in the descriptor "winged helix" to define this family (33). An interesting feature of known winged-helix proteins is the diversity of apparently dissimilar DNA sequence motifs recognized by various members of this family. Our current results identify a novel winged-helix protein that recognizes a sequence motif unlike those to which other members of this family bind. In addition, our results provide the first evidence that winged-helix proteins are involved in control of gene expression within myogenic lineages.

The cDNA we have termed MNF (to highlight the structural relationship to HNF-3) encodes a protein with a predicted molecular size of 66 kDa that binds specifically to an essential transcriptional control element from the human myoglobin gene and is expressed in cardiac and skeletal myocytes in which the endogenous myoglobin gene is transcriptionally active. In addition to the winged-helix domain required for DNA binding, MNF includes a transcriptional activation domain, as assessed by fusion of an amino-terminal region to the DNA-binding domain of *GAL4* and transfection into *S. cerevisiae*. Probes based on the MNF sequence detect two major mRNA species in skeletal muscle of approximately 4.3 and 2.4 kb, both of which are upregulated during myocyte differentiation. Antibodies raised against recombinant MNF identify three major polypeptides of approximately 90, 68, and 65 kDa. The abundance of these MNF proteins is upregulated during muscle differentiation in culture, as well as in muscles of intact animals in which myoglobin expression is induced by chronic motor nerve stimulation. MNF is encoded by a single-copy gene and maps to distal mouse chromosome 5.

MATERIALS AND METHODS

Cell culture. sol8 myogenic cells were derived by Christian Pinset from a primary culture of mouse soleus muscle (9) and were obtained from Vijak Mahdavi, Boston, Mass. The sol8 monolayers were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum. Myotube formation was induced by growing the cells to confluence and switching the medium to differentiation medium (DMEM supplemented with 2% horse serum, 10 μg of insulin [Gibco/BRL Research, Gaithersburg, Md.] per ml, and 10 μg of transferrin [Gibco/BRL] per ml).

Isolation and sequencing of MNF cDNA clones. A modified version of the λgt11 expression cloning procedure (58) was used to isolate clones that bind to the CCAC transcriptional control element of myoglobin. A radiolabeled probe consisting of three CCAC elements (5′-CAACCACCCCACCCCCT GTGG-3′) flanked by *Bam*HI and *Bgl*II restriction enzyme sites (2) was used to screen a λgt11 cDNA library derived from mouse 266-6 cells (44) and obtained from Galvin Swift and Raymond MacDonald. Two unique clones survived three rounds of plaque purification, and cDNA inserts from these clones were amplified by PCR (95°C for 1 min, 52°C for 0.5 min, and 72°C for 2 min done for 35 cycles) with forward and reverse λgt11 primers (New England Biolabs, Beverly, Mass.). PCR products were ligated into the pCRII vector by the TA cloning procedure (Invitrogen, San Diego, Calif.). One of these, designated pCRII-MNF, contained a unique 1,605-nucleotide sequence and was used in further screening of a λZAP (Stratagene, La Jolla, Calif.) cDNA library prepared from mouse 266-6 cells. Overlapping clones that extended the known sequence of MNF to 2,394 bp were obtained.

Activation domain assay. Fusion constructs encoding the Gal4 DNA-binding domain (28) linked to MNF cDNA encoding either amino acids 1 to 206 or 207 to 617 were constructed by ligating a 630-bp *Bam*HI-*Bgl*II DNA fragment or an 1.8-kb *Bgl*II-*Sal*I DNA fragment, respectively, into the pAS1 vector (12). Vector pAS1 or the fusion construct MNF cDNA encoding amino acids 1 to 206 or amino acids 207 to 617 was transformed by the lithium acetate method (25) into yeast strain Y190 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 + URA*::*GAL-lacZ LYS2*::*GAL-lacZ HIS3 cyh*^{r}) (22) that contains a chromosomally integrated reporter gene, *Escherichia coli lacZ*, under the control of the *GAL4* promoter. Whole-cell extracts were prepared by the glass bead method, and β-galactosidase activities were assayed (4, 26). Results were plotted relative to those obtained with vector alone in four independent experiments.

Construction and purification of histidine-tagged MNF fusion protein. A 1.6-kb DNA fragment of MNF cDNA was excised from pCRII-MNF by cleavage with restriction enzyme *Nsi*I and was subcloned into the *Pst*I site of the expression vector pQE-30 (Qiagen, Studio City, Calif.). This construct was used to transform *E. coli* M15(pREP4). Induction of transformants with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) generated recombinant MNF (rMNF) protein fused to six histidine residues. Protein purification was achieved by affinity chromatography involving the binding of the histidine-tagged rMNF to immobilized nickel and elution of the protein with a step gradient of imidazole (40 to 320 mM). rMNF was eluted from the nickel column with 160 mM imidazole and was concentrated and dialyzed with Dulbecco phosphate-buffered saline by using a Centricon unit (Amicon, Danvers, Mass.). One liter of induced cultures yielded 3 to 5 mg of purified protein.

Production of polyclonal antibody against rMNF protein. Polyclonal antibody was obtained by using 0.5 mg of purified rMNF mixed with TiterMax adjuvant (CytRx Corp., Norcross, Ga.) and injected subcutaneously into a New Zealand White rabbit. Blood samples were acquired by standard methods (21).

Electrophoretic gel mobility shift assay. Oligonucleotide probes were incubated with purified rMNF protein and analyzed by electrophoretic gel mobility shift assay as previously described (2) with 500 ng of rMNF. Oligonucleotides corresponding to both strands of the CCAC box region (−226 to −205 upstream region of myoglobin) were synthesized with an added GATC nucleotide overhang at the 5′ terminal of each oligonucleotide. The complementary strands were annealed and were then end labeled with the Klenow fragment of DNA polymerase I (Promega) and [α-^{32}P]dATP (3,000 Ci/mmol). Competition studies were performed with unlabeled, preannealed oligonucleotides corresponding to the CCAC box re-

gion or to functionally inactive CCAC box mutants, mut 3 and mut 4 (2). The sequences of these oligonucleotides were as follows: CCAC sense, 5′-GATCACGCACAACCACCCCAC CCCCTGTG-3′; CCAC antisense, 5′-GATCCACAGGGGG TGGGGTGGTTGTGCGT-3′; CCAC mut 3 sense, 5′-ACG CACAACCACCCCGGTACCTGTGGCCTGAGC-3′; CCAC mut 3 antisense, 5′-GTGCTCAGGCCACAGGTACCGGGG TGGTTGTG-3′; CCAC mut 4 sense, 5′-ACGCACAACCAC CCCACCGGTACCGGCCTGAGCTGTCC-3′; CCAC mut 4 antisense, 5′-GTGGACAGCTCAGGCCGGTACCGGTGG GGTGGTTGTG-3′.

Chromosome localization. C3H/HeJ-*gld* and *Mus spretus* (Spain) mice and (C3H/HeJ-*gld* × *M. spretus*)F_1 × C3H/HeJ-*gld* interspecific backcross mice were bred and maintained as previously described (54). *M. spretus* was chosen as the second parent in this cross because of the relative ease of detection of informative restriction fragment length variants in comparison with crosses obtained by using conventional inbred laboratory strains.

DNA isolated from mouse organs by standard techniques was digested with restriction endonucleases, and 10-μg samples were electrophoresed in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.), hybridized at 65°C, and washed under stringent conditions (50). Probes used included a 475-bp fragment encoding the winged-helix (*fork head*) region of MNF, mouse platelet-derived growth factor (*Pdgfa*), (49), the erythropoietin (*Epo*) clone MSEP1.2 (38), and β-glucuronidase (*Gus*) (14).

Gene linkage was determined by segregation analysis (17). Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group. This method resulted in determination of the most likely gene order (3).

Northern (RNA) blot analysis. Total RNA was obtained from sol8 myoblasts and differentiated myotubes and from mouse skeletal muscle and heart by using RNA STAT-60 solution as described by the supplier (Tel-Test B Inc., Friendswood, Tex.). A 20-μg portion of RNA was loaded onto 1% agarose–formaldehyde gels, and following electrophoresis at 96 V for 3 h, the RNA was blotted to nitrocellulose paper (50). Filters were prehybridized in 50% formamide–0.8 M NaCl–50 mM Na_2HPO_4 (pH 7.5)–2.5× Denhardt's solution–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS)–250 μg of heat-denatured sonicated herring sperm DNA per ml for 4 h prior to addition of radiolabeled riboprobe (10^6 cpm/ml). Hybridization was performed at 55°C for 18 h, and then the filters were washed twice at room temperature and twice at 60°C in wash buffer (20 mM Na_2HPO_4 [pH 7.5], 30 mM NaCl, 1 mM EDTA, 0.1% SDS) prior to autoradiography.

Plasmid pGEM5-FkhMNF, consisting of a 475-bp fragment containing the *fork head* domain inserted into pGEM5 (Promega), was linearized with *Sac*II, isolated on an agarose gel, and incubated with [α-^{32}P]UTP (Amersham Corp., Arlington Heights, Ill.) in the presence of SP6 RNA polymerase (Promega) to generate an antisense radiolabeled RNA probe.

Immunoblot analysis. Cellular extracts from sol8 cells were prepared at various times during differentiation by transferring the cells to ice, removing the medium, and washing the cells in Dulbecco phosphate-buffered saline prior to the addition of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 100 μg of aprotinin per ml). The cellular lysate was frozen, thawed, and cleared by centrifugation at 12,000 × *g* for 15 min. The protein concentration in the supernatant was measured by the Bradford protein assay (Bio-Rad, Richmond, Calif.). A 10-μg sample of each protein extract was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose paper by using the Bio-Rad protein blotter. Immunoblotting was performed at 100 mA for 16 h at 4°C. Primary antibodies used were either rabbit anti-rMNF or goat anti-myoglobin (Cappel Research Products, Durham, N.C.) at dilutions of 1:5,000 and 1:1,000, respectively. Control antibodies were preimmune serum from rabbits or goats. Secondary antibodies were either goat anti-rabbit immunoglobulin G (Bio-Rad) or rabbit anti-goat immunoglobulin G (Cappel), used at a 1:10,000 dilution. Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Corp. Exposure times were 1 and 10 min for the rMNF and myoglobin immunoblots, respectively.

Immunoprecipitation of MNF protein metabolically labeled with ^{32}P. After 1 day in differentiation medium, sol8 myotubes were rinsed with phosphate-deficient DMEM (D-DMEM) (Sigma Chemical Co., St. Louis, Mo.) and then incubated for 18 h at 37°C with D-DMEM containing 2% dialyzed fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 mCi of $^{32}P_i$ (Amersham Corp.). Cell lysates were solubilized in 0.6 ml of lysis buffer, frozen, thawed, and centrifuged at 12,000 × *g* for 15 min. The supernatant was cleared by incubation with a 1/100 dilution of preimmune rabbit serum and 100 μl of a 15% solution of protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) for 30 min at 4°C and centrifuged for 5 min at 12,000 × *g*. The supernatant was divided into two tubes each containing 0.5 ml of immunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Nonidet P-40), 100 μl of a 15% solution of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.), and either 1 μl of antiserum raised against MNF or 1 μl of preimmune rabbit serum. After incubation for 30 min at 4°C, the immunocomplexes were centrifuged for 5 min at 12,000 × *g* and washed three times with immunoprecipitation buffer, and the protein was eluted from the complex by adding SDS-PAGE sample buffer, boiling the sample for 5 min, and centrifuging the sample for 5 min at 12,000 × *g*. The samples were separated by SDS-PAGE, and after electrophoresis, the gel was dried and exposed to film.

Chronic electrical stimulation of muscle. Pulse generators were implanted in adult New Zealand White rabbits, and stimulating electrodes were applied to the common peroneal nerve, as previously described (65). Continuous electrical stimulation at 10 Hz was applied, and tibialis anterior muscles were harvested after periods of stimulation ranging from 45 min to 3 days. Tissue samples were quickly frozen in liquid nitrogen and stored at −70°C. Soluble protein extracts were prepared as described previously (39) and analyzed by the immunoblotting procedure described above.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank data base under accession number L26507.

RESULTS

Isolation and sequence analysis of a cDNA encoding MNF. We used a probe consisting of three repeats of the myoglobin CCAC element (nucleotides −225 through −205 upstream of the transcriptional start site of the human myoglobin gene) to isolate λgt11 clones expressing proteins that bind to this sequence motif. The CCAC box was identified previously as an element required for transcription of the myoglobin gene in both cardiac and skeletal muscle (1, 2, 10). Here we report the isolation and characterization of a unique cDNA that encodes a CCAC box-binding protein, termed MNF. The cDNA clone initially isolated consisted of 1,605 bp. Further screening

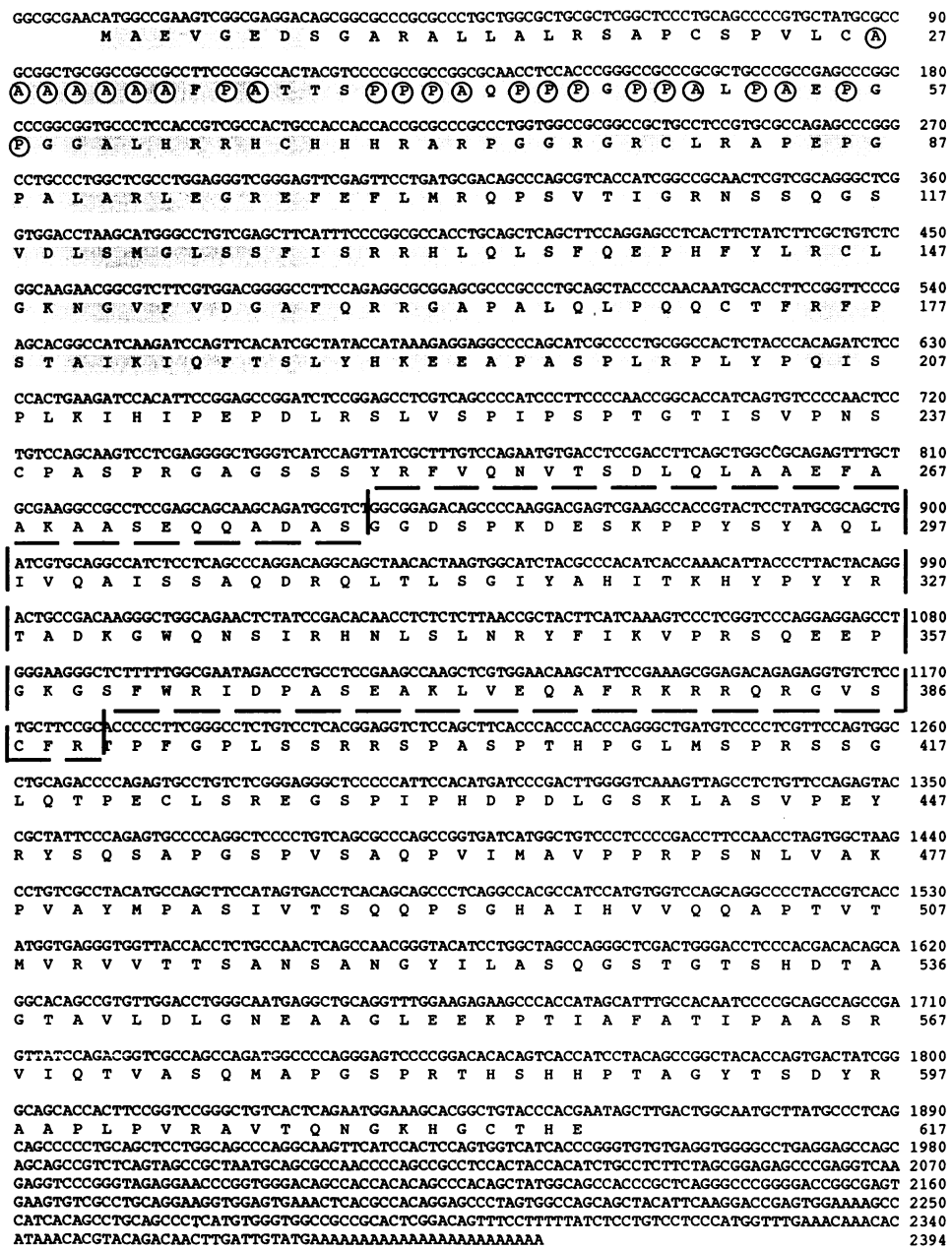

FIG. 1. Nucleotide and predicted amino acid sequence of MNF cDNA. The *fork head* domain is boxed. Shaded amino acids outline the activation domain of MNF. Alanines and proline clusters within the activation domain are highlighted with circles.

yielded additional cDNA clones containing overlapping sequences extending to 2,394 bp (Fig. 1). This cDNA includes an open reading frame initiated from a methionine codon that fulfills Kozak's criteria (32) and encodes a 617-amino-acid protein with a predicted molecular mass of 66 kDa.

Searches of protein data bases revealed that a 110-amino-acid region within MNF is closely related to the winged-helix domain (35, 62) common to members of the HNF-3/*fork head* family of transcription factors (boxed in Fig. 1). The winged-helix domain of MNF exhibits 35 to 89% sequence identity by comparison with known members of this multigene family (Fig. 2). MNF appears to be most closely related to ILF (36), a protein identified on the basis of binding affinity to an interleukin-2 response element within the long terminal repeat of human immunodeficiency virus. MNF is, however, a unique protein, and exhibits less than 50% identity to ILF or to any other known protein outside of the winged-helix region.

MNF includes a transcriptional activation domain within an amino-terminal region. Nucleotide sequences encoding the first 206 amino acids of MNF (lacking the winged-helix region) or residues 207 to 617 were fused to the DNA-binding domain-encoding sequences of the yeast transcriptional activator *GAL4* in the expression vector pAS1 (12). Transfection of the amino-terminal MNF-*GAL4* chimera resulted in 10-fold *trans*-activation of a reporter gene (β-galactosidase) controlled by the *GAL1* promoter (Fig. 3A). However, the *GAL4* chimera

A

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          1        10        20        30        40        50
MNF       GGDSPKDESKPPYSYAQLIVQAISSAQDRQLTLSGIYAHITKHYPYYRTADKGWQNSIRH
ILF       .......D.............TM·P·K····N···T····N···············
BF-1      ·DKKNGKYE·····NA··MM··RQSPEKR···N···EF·M·NF····ENKQ·····
FKHR      KSS·SRRNAWGNL···D··TK··E·SAEKR····Q··EWMV·SV··FKDKG*··K·····
HNF 3β    TYRRSYTHA·····IS··TM··QQSPNKM····E··QW·MDLF·F·RQNQQR·····
FKH       TTRRSYTHA·····IS··TM··QNNPT·M····E··QF·MDLF·F··QNQQR·····

2° struc:              HHHHHHHHHHH     BBBHHHHHHHHHH         HHHHHHHHHHH

          60        70        80        90        100       110
MNF       NLSLNRYFIKVPRSQEEPGKGSFWRIDPASEAKLVEQAFRKRRQRGVSCFR
ILF       ····························S··I·····P···P···
BF-1      ·····KC·V····HYDD····NY·ML··S·DDVFIGGTTG·L·R·STTSRA
FKHR      ····HSK··R·QNEGT--··S·W·MLN·------EGGKSG·SPR·RAASMD
HNF 3β    S··F·DC·L·····PDK·····TLH·D·GNIFENGCYLR·QK·FKCEKQ
FKH       S··F·DC·V·I··TPDK·····TLH·D·GNMFENGCY-L···KRFKDEK

2° struc: HHHH   BBBBBBB         BBB
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B

Percent identity to MNF forkhead domain	
ILF	89%
BF-1	47%
FKHR	35%
HNF-3β	42%
FKH	43%

FIG. 2. Comparison of the MNF *fork head* domain sequence with other *fork head* proteins. (A) Alignment of MNF *fork head* domain with *fork head* domains from ILF (36), BF-1 (56), HNF-3β (35), FKHR (13), and FKH (62). Dots represent identical alignment of amino acids with the MNF sequence. The asterisk indicates the omission of five amino acids (SNSSAG). A vertical line corresponds to a gap in the sequence. Gaps have been introduced to allow maximal sequence alignment. The secondary structural elements are extrapolated from the known crystal structure of the HNF-3/*fork head* domain (5) and denoted by H for α-helix and B for β-strand. (B) The degree of sequence identity between each of the *fork head* domains and the MNF *fork head* domain is given as a percentage.

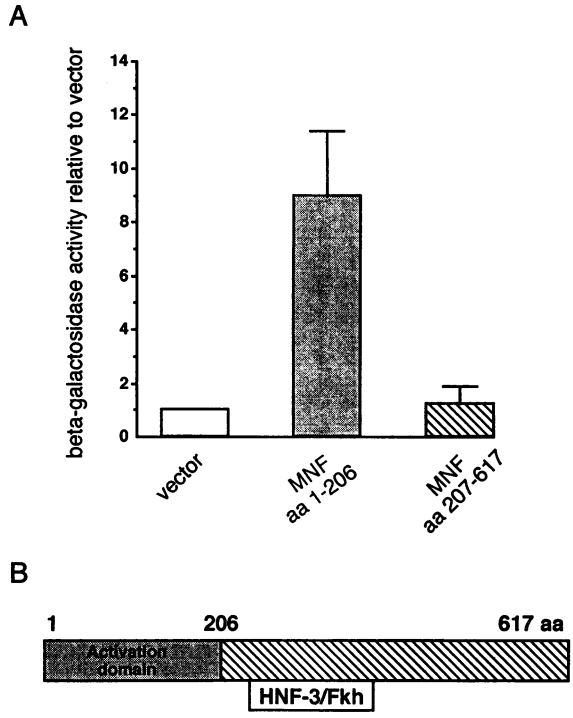

FIG. 3. An activation domain resides within the first 206 amino acids (aa) of MNF. (A) β-Galactosidase activity measured from a protein extract of yeast cells transformed with fusion constructs encoding the *GAL4* DNA-binding domain linked either to amino acids 1 to 206 (shaded area) or 207 to 617 (hatched area) of MNF. Results were expressed relative to vector pAS1 (12) and represent mean values (± standard error of the mean) from at least four independent observations. (B) Diagram of MNF protein. HNF-3/Fkh illustrates the position of the winged-helix domain. The shaded area is the amino terminus of the protein and contains the activation domain.

that includes amino acids 207 through 617 of MNF exhibited no *trans*-activation function (Fig. 3A). These results indicate that a transcriptional activation domain resides within the first 206 amino acids of MNF (Fig. 3B, shaded region), a region rich in alanine and proline residues (circled in Fig. 1).

Purified rMNF containing the winged-helix region binds specifically to the myoglobin CCAC box motif. A histidine tag was placed at the amino terminus of MNF by using the pQE expression vector system (Qiagen), to facilitate purification of recombinant protein, rMNF (Fig. 4D), after expression in *E. coli* M15 cells (Fig. 4A). Southwestern (DNA-protein) blot analysis showed that purified rMNF binds the myoglobin CCAC box sequence (Fig. 4B), and immunoblot analysis demonstrated that rabbit polyclonal antibodies raised against purified rMNF recognize the protein at a dilution of 1:40,000 (Fig. 4C).

Binding of rMNF to the myoglobin CCAC box was sequence specific, as assessed by electrophoretic mobility shift assays with purified protein (Fig. 5, lane 1). An excess of unlabeled oligonucleotides containing the native CCAC sequence disrupted binding of rMNF to the labeled CCAC box probe (Fig. 5, lane 2), whereas nonfunctional CCAC box mutants, mut 3 and mut 4 (2), failed to inhibit binding (Fig. 5, lanes 3 and 4).

Chromosomal mapping of MNF. Southern blot analysis of genomic DNA digested with several different restriction enzymes indicated that MNF is encoded by a single gene in the mouse genome. Chromosomal localization of the MNF gene was performed by analyzing a panel of DNA samples from an interspecific cross that has been characterized for over 600 genetic markers throughout the mouse genome. The genetic markers included in this map span between 50 and 80 centimorgans (cM) on each mouse autosome and the X chromosome. Initially, DNA from the parental mice was digested with various restriction enzymes and hybridized with MNF cDNA probe to determine restriction fragment length variants (RFLVs) for haplotype analyses. Informative RFLVs were detected with *Taq*I-restricted DNAs, and each of the 114 *Taq*I-restricted DNAs from the interspecific backcross mice displayed either the homozygous or heterozygous F_1 pattern when hybridized with the MNF probe.

Comparison of the haplotype distribution of MNF indicated that in all of the 114 meiotic events examined, the *Mnf* locus cosegregated with the *Pdgfa* locus (Fig. 6) previously mapped to the distal mouse chromosome 5 (31, 49). In the most likely gene order, the *Mnf* locus was located 1.8 ± 1.2 cM distal to *Epo* (38) and 3.5 ± 1.8 cM distal to *Gus* (14).

MNF is expressed in tissues and cells in which the myoglobin gene is transcriptionally active. Northern blot analysis was performed with RNA extracted from skeletal and cardiac muscle of adult mice (Fig. 7A) and from mouse sol8 myogenic cells at various times during differentiation induced by withdrawal of growth factors (Fig. 7B). Under conditions of high stringency and using a probe complementary to nucleotide sequences 805 to 1266 of the MNF cDNA, we detected mRNA transcripts of approximately 4.3 and 2.4 kb in skeletal muscle (Fig. 7A, lane Sk) and slightly larger molecular size in heart muscle (Fig. 7A, lane H). Following differentiation of sol8

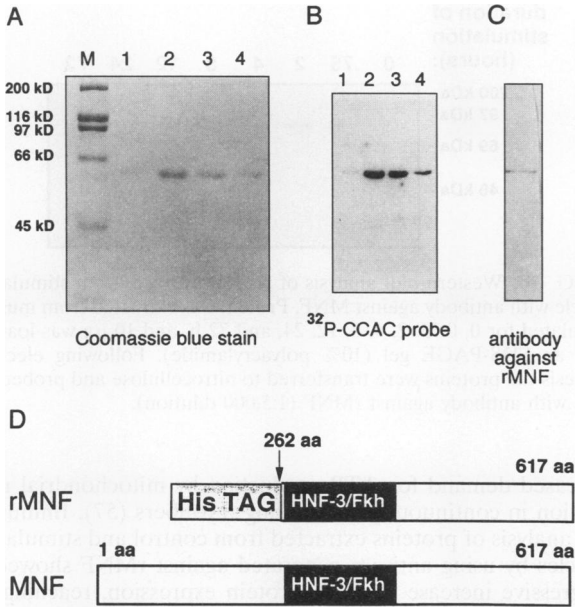

FIG. 4. Characterization of rMNF. (A) rMNF was bound to a Ni-agarose column and eluted with four 1-ml fractions containing 160 mM imidazole. A 10-μl sample from each fraction was analyzed by SDS-PAGE (10% polyacrylamide) (lanes 1 to 4). Protein was detected by Coomassie blue staining. (B) Protein-DNA (Southwestern) blot analysis of rMNF with [^{32}P]CCAC. Lanes 1 to 4 correspond to the protein samples in panel A. (C) Western immunoblot analysis of rMNF with antibodies (1:40,000 dilution) generated against rMNF. (D) Illustration of rMNF linked to the histidine tag and MNF.

myoblasts to multinucleated myotubes that express the endogenous myoglobin gene, both the 4.3- and 2.4-kb transcripts increase in quantity by 1.8- and 5.8-fold, respectively (Fig. 7B).

MNF expression is not limited, however, to striated myocytes. RNase protection assays (results not shown) indicate that at least one form of MNF mRNA can be detected in other mouse tissues (kidneys and brain).

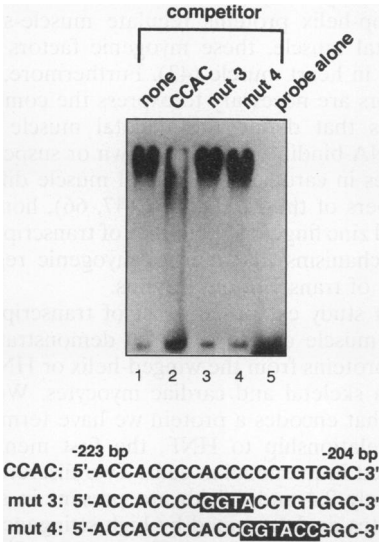

FIG. 5. rMNF binds specifically to the CCAC box element. ^{32}P-labeled CCAC box probe was used in a gel mobility shift assay to assess specific binding of rMNF (lane 1). Competitive-binding studies included 100 ng of unlabeled CCAC box sequence (lane 2) or 100 ng of functionally inactive CCAC box mutants (2), mut 3 (lane 3), or mut 4 (lane 4). The DNA sequences of CCAC, mut 3, and mut 4 are shown.

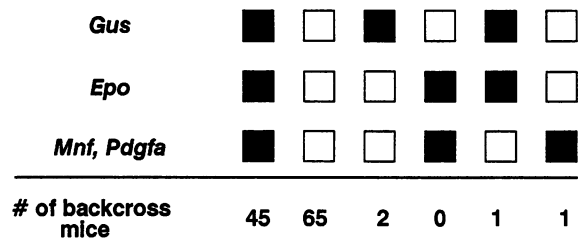

FIG. 6. Segregation of MNF among mouse distal chromosome 5 loci in (C3H/HeJ-*gld* × *M. spretus*)F_1 × C3H/HeJ-*gld* interspecific backcross mice. Solid boxes represent the homologous C3H pattern, and open boxes represent the F_1 pattern. The informative RFLVs for MNF are described in the text. For the other markers, the following RFLVs were used: *Gus* (14), *Bam*HI (C3H 2.0 and 1.3 kb; *M. sprefus* 4.3 kb), *Epo* (38), *Taq*I (C3H 1.8 and 1.0 kb, *M. sprefus* 2.0 and 1.2 kb).

MNF and other closely related proteins are present in myocytes, and become more abundant during muscle differentiation. The relationship between expression of myoglobin and proteins recognized by antibodies raised against rMNF was examined by using sol8 myogenic cells in the proliferative phase and at various stages of differentiation (Fig. 8). After 1 day in differentiation medium, these cells can be distinguished from myoblasts morphologically (rod shaped) and by expression of sarcomeric proteins (data not shown). Myoglobin expression is, however, limited or absent at this stage (Fig. 8A, lanes 1 and 2). After 2 days in differentiation medium, multinucleated myotubes are abundant and myoglobin protein is detectable (lane 3). By day 3 in differentiation medium, myotubes are increased in size and myoglobin protein is expressed to higher levels (lane 4). These results are corroborated by immunohistochemical studies that demonstrate uniform expression of myosin heavy-chain protein as early as day 1 in differentiation medium but uniform expression of myoglobin only after 3 days in differentiation medium (data not shown). Other investigators also have observed this delayed expression of myoglobin during differentiation of other myogenic cell lines in culture (19).

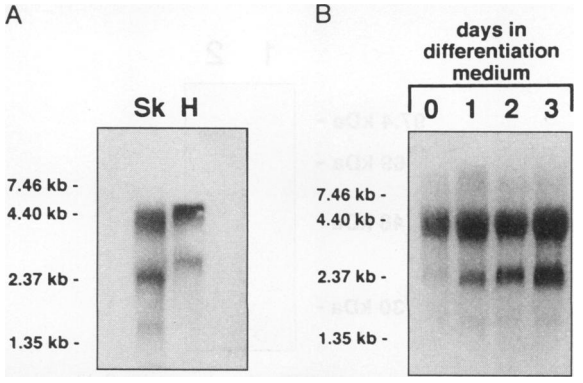

FIG. 7. Northern blot analysis of MNF. A radioactive riboprobe encoding the HNF-3/*fork head* domain of MNF was used to probe Northern blots with RNA extracted from mouse skeletal muscle (lane 1) or heart tissue (lane 2) (A) or RNA extracted from sol8 cells grown in differentiation medium for 0 (lane 1), 1 (lane 2), 2 (lane 3), or 3 (lane 4) days (B).

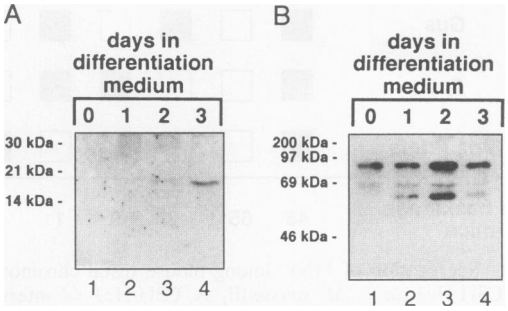

FIG. 8. Western blot analysis of MNF-related proteins in sol8 myoblasts and myotubes. Protein extract (10 μg) from sol8 cells in growth medium (lane 1) or differentiation medium for 1 day (lane 2), 2 days (lane 3), or 3 days (lane 4) was loaded onto an SDS-PAGE gel (10% polyacrylamide), transferred to nitrocellulose, and probed by ECL with antibody against myoglobin (1:1,000 dilution) (A) or MNF (1:5,000 dilution) (B).

Expression of several polypeptides recognized by anti-MNF antibodies is regulated in a manner parallel to, but temporally in advance of, the pattern of myoglobin expression observed in sol8 cells. A polypeptide of approximately 90 kDa represents the major band detected by anti-MNF sera in sol8 myoblasts (Fig. 8B, lane 1). As the cells undergo differentiation, this protein becomes more abundant and a different 65-kDa polypeptide becomes detectable in immunoblots (lanes 2 to 4). This pattern of expression of MNF and related proteins is consistent with that predicted for a physiological regulator of myoglobin gene expression.

MNF protein is a phosphoprotein. Differentiated sol8 cells were incubated for 18 h with $^{32}P_i$, and soluble nuclear proteins were extracted and immunoprecipitated with antibodies raised against rMNF. As illustrated in Fig. 9, the 90-kDa form of MNF incorporates ^{32}P in these myoglobin-expressing cells.

Chronic electrical stimulation of skeletal muscle augments expression of the 90-kDa form of MNF. Miniature pulse generators were implanted in adult New Zealand White rabbits, and the pacing electrodes were placed adjacent to the common peroneal nerve of one hind limb. Continuous electrical stimulation was applied, and rabbit muscle was harvested at 0.75, 2, 4, 8, 12, 24, and 72 h. This intervention induces expression of myoglobin mRNA as an adaptive response to the increased demand for ATP production by mitochondrial respiration in continuously contracting myofibers (57). Immunoblot analysis of proteins extracted from control and stimulated muscles by using antisera generated against rMNF showed a progressive increase in MNF protein expression, reaching at least a 17-fold increase by 24 h, in response to this physiological stimulus (Fig. 10). This induction is consistent with the hypothesis that MNF is a physiological regulator of myoglobin gene expression in these cells.

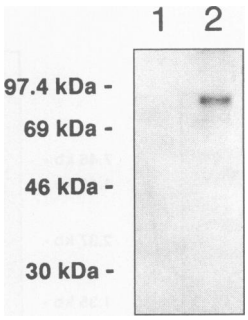

FIG. 9. Phosphorylation of MNF-related protein. Sol8 myotubes grown for 1 day in differentiation medium were incubated with $^{32}P_i$ for 18 h. Immunoprecipitation was done on protein extract with either preimmune serum (lane 1) or antibody generated against rMNF protein (lane 2). The bound material was boiled and loaded onto an SDS-PAGE gel (8% polyacrylamide). After electrophoresis the gel was dried and exposed to film for 18 h.

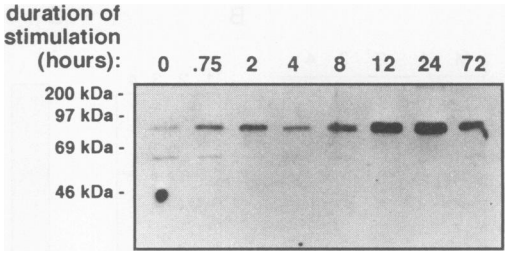

FIG. 10. Western blot analysis of protein extracts from stimulated muscle with antibody against MNF. Protein was extracted from muscle stimulated for 0, 0.75, 2, 4, 8, 12, 24, and 72 h, and 10 μg was loaded onto an SDS-PAGE gel (10% polyacrylamide). Following electrophoresis the proteins were transferred to nitrocellulose and probed by ECL with antibody against rMNF (1:5,000 dilution).

DISCUSSION

Several different classes of transcription factors are involved in commitment and differentiation of skeletal and cardiac myocytes. Within the skeletal muscle lineage, members of the basic helix-loop-helix family, including MyoD, myogenin, MRF4, and Myf5, can activate and maintain muscle-specific gene transcription (42, 63). Forced expression of any one of these genes can induce nonmuscle cells to express muscle-specific proteins (reviewed in reference 63), and disruption of the myogenin gene in transgenic mice leads to a nearly complete deficiency of myogenic differentiation (23). Although basic helix-loop-helix proteins regulate muscle-specific proteins in skeletal muscle, these myogenic factors have never been detected in heart muscle (43). Furthermore, other transcription factors are necessary to express the complete repertoire of genes that define the skeletal muscle phenotype. Additional DNA-binding proteins known or suspected to play important roles in cardiac and skeletal muscle differentiation include members of the MADS-box (47, 66), homeo-domain (8, 15, 30), and zinc finger (51) families of transcription factors. Thus, the mechanisms that control myogenic regulation involve an array of transcription proteins.

Our present study extends this list of transcription factors implicated in muscle development by demonstrating expression of novel proteins from the winged-helix or HNF/*fork head* gene family in skeletal and cardiac myocytes. We isolated a cDNA clone that encodes a protein we have termed MNF to highlight its relationship to HNF, the first member of this family identified in mammalian cells. Two discrete transcripts of approximately 2.4 and 4.3 kb are present in skeletal and cardiac myocytes as determined by high-stringency hybridization to a probe based on the MNF sequence. Since MNF is encoded by a single-copy gene in the mouse, we presume that the 4.3-kb form of MNF mRNA arises as a splice variant, but the precise identity of this larger transcript remains to be determined.

Antibodies raised against recombinant MNF protein pro-

duced in bacteria detect three discrete polypeptides of approximately 90, 68, and 65 kDa in immunoblots prepared from differentiated mouse sol8 myotubes, indicative of posttranslational modifications and/or alternative splicing of exons within protein-coding sequences of the gene. Our finding that the 90-kDa form is phosphorylated provides direct evidence for posttranslational modifications of MNF, and the aforementioned Northern blot analysis suggests that alternative splicing also occurs.

Several findings support the premise that MNF has a physiological function during muscle development. MNF exhibits sequence-specific DNA binding to an upstream activation sequence (CCAC element) that is required for transcriptional activity of the human myoglobin promoter in both skeletal and cardiac myocytes. In addition, MNF is regulated in parallel to expression of myoglobin in two different experimental systems in which the myoglobin gene is upregulated. The abundance of MNF mRNA and of immunoreactive MNF polypeptides is increased during muscle differentiation in culture. The induction of the 65-kDa polypeptide in sol8 myotubes is particularly striking, since this form of MNF is virtually undetectable in myoblasts. Also, MNF is induced during fiber-type transformation that occurs as a consequence of continuous motor nerve stimulation in skeletal muscles of adult animals.

Our current data also demonstrate that MNF includes a functional *trans*-activation domain, but further studies are required to define more completely its physiological functions in the intact cell. Other winged-helix proteins bind DNA as monomers, and recombinant MNF is capable of DNA binding in vitro in the absence of accessory proteins. Our current findings are insufficient, however, to define the active form(s) of MNF and the potential regulatory effects of phosphorylation. Likewise, we cannot exclude a requirement for other proteins in hetero-oligomeric complexes in transcriptional activation mediated by MNF. To the contrary, multimerization of the myoglobin CCAC box motif (to which MNF binds) upstream of a TATA element is insufficient to direct muscle-specific expression of a reporter gene following transfection into cultured cells, unless the A+T-rich upstream activation sequence from the myoglobin gene is included in the construction (16). Thus, it is likely that MNF may require protein-protein interactions with other heterologous transcription factors to effect efficient *trans*-activation in muscle cells.

Although MNF was isolated on the basis of sequence-specific binding to the myoglobin CCAC box motif, additional studies are necessary to determine in a definitive manner whether MNF is indeed a physiological *trans*-activator of the myoglobin gene and of other genes that require closely related CCAC elements for transcriptional activity. Promoters or enhancers from certain other muscle-specific genes including muscle creatine kinase (24), cardiac α-actin (51), β-myosin heavy chain (7) and slow cardiac troponin C (45) also contain CCAC motifs, mutations of which alter transcription.

Sp1, a ubiquitously expressed transcription factor, is capable of binding to the CCAC motif (52), which differs from a consensus Sp1-binding site (27) largely by the substitution of a guanine base in the position occupied by an adenine in the CCAC box. Our current data suggest, however, that MNF binds the myoglobin CCAC element with higher affinity than Sp1 does. Several proteins within the erythroid lineage that bind a CACCC motif from the β-globin gene have been described (40), but the expression of these factors in developing muscle and their relative affinity for binding the myoglobin CCAC box have not been assessed. Other data from our own laboratory also suggest that additional CCAC box-binding factors, distinct from MNF and Sp1, are present in developing myotubes. When multimerized CCAC sequences are used to probe protein blots prepared from nuclei isolated from sol8 myotubes, we observe prominent binding to a 40-kDa protein (2), as well as to a larger polypeptide consistent with the molecular mass of MNF (data not shown). These data are particularly interesting in view of the recent report by Wang et al. (59) of a 40-kDa protein termed htβ, isolated from a human T-cell lymphoma-derived cell line on the basis of binding to a CCAC box sequence of the human T-cell receptor Vβ8.1 promoter element from positions −72 to −92. Studies are under way in our laboratory to determine the relationship between CBF40 and htβ and the relative importance of these proteins and MNF for physiological *trans*-activation of myoglobin expression.

In summary, we have identified a novel member of the winged-helix or HNF-3/*fork head* family of transcription factors on the basis of sequence-specific DNA binding to an upstream activation sequence from the human myoglobin gene. This protein, MNF, includes an amino-terminal *trans*-activation domain and is expressed in adult skeletal and cardiac myocytes in which the myoglobin gene is transcriptionally active. MNF is encoded by a single locus on chromosome 5 in the mouse genome, but alternative splicing and/or posttranslational modifications give rise to two forms of MNF mRNA and three forms of MNF protein. The abundance of MNF polypeptides is developmentally regulated during differentiation of skeletal myotubes in culture and is responsive to neural regulation in adult skeletal muscle fibers. These data provide the first evidence for participation of winged-helix proteins as transcriptional regulators in mammalian striated myocytes and suggest a role for MNF in both muscle development and postnatal physiological adaptations to changing work demands.

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