MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5236–5243 Vol. 17, No. 9
0270-7306/97/$04.00+0


# Transient Expression of a Winged-Helix Protein, MNF-β, during Myogenesis

QUAN YANG, RHONDA BASSEL-DUBY, AND R. SANDERS WILLIAMS*

*Departments of Internal Medicine and Molecular Biology/Oncology, University of Texas Southwestern Medical Center, Dallas, Texas 75235*

Received 21 April 1997/Returned for modification 30 May 1997/Accepted 9 June 1997

**A novel winged-helix transcription factor, MNF-β, is expressed coincidentally with cell cycle withdrawal and differentiation of skeletal myogenic cells. MNF-β is closely related to the myocyte nuclear factor (MNF) protein previously described (now termed MNF-α), but expression of the two isoforms is differentially regulated, and they exhibit distinctive functional properties with respect to DNA binding in vitro and transcriptional regulatory activity in transient-transfection assays. A DNA sequence motif binding MNF-β with high affinity was selected from a library of random oligonucleotides and was found to be similar to but distinct from the cognate binding site for HNF-3β, a more distantly related winged-helix protein. The temporal pattern of MNF-β expression and the presence of MNF binding motifs within conserved promoter elements of several genes that modulate cell cycle progression support a working hypothesis that MNF proteins may modulate proliferation of myogenic precursor cells during development and muscle regeneration.**

Proteins of the forkhead/winged-helix family of transcription factors are expressed in many eukaryotic organisms and in many cell lineages of higher vertebrates, and several proteins of this class are known to function in the control of cellular differentiation and proliferation, pattern formation, and signal transduction (18, 19).

Among mammalian winged-helix proteins, the hepatocyte nuclear factor-3 (HNF-3) subgroup, which includes three closely related isoforms (α, β, and γ), has been most extensively characterized (20). HNF-3β is essential during early embryonic development for formation of the node and notochord (1, 29), and these proteins have additional functions in activation and maintenance of cell-type-specific gene expression in hepatocytes and other tissues of endodermal origin (8). The three isoforms of HNF-3 are encoded by separate genes, but the proteins are highly conserved within their winged-helix DNA binding domains, and they have correspondingly similar DNA binding properties in vitro (22). The different isoforms, however, exhibit distinctive temporal-spatial patterns of expression during embryogenesis (27), suggesting that they control discrete developmental events.

In a previous paper, we described the cloning of myocyte nuclear factor (MNF), which was identified as a winged-helix protein on the basis of the signature 110-amino-acid forkhead-like domain within the middle protein of the linear sequence (4). Subsequent studies demonstrated that MNF is the first member of this protein family to be expressed selectively in cardiac and skeletal muscle precursor cells during embryonic development, and expression of MNF is most abundant in satellite cells (14), a committed stem cell pool arrested at an early stage of myogenesis prior to the activation of basic helix-loop-helix proteins of the MyoD family (11, 21). The winged-helix domain of MNF is more closely related to the yeast forkhead proteins FKH1 and FKH2 than to the mammalian HNF-3 proteins. MNF also shares a second structural motif, the forkhead-associated (FHA) domain, with FKH1 and FKH2 from *Saccharomyces cerevisiae*, and it is the only mammalian member of this family known to do so (17). Outside the winged-helix and FHA regions, MNF bears no obvious resemblance to other members of this protein family or to other proteins in the database.

In our initial characterization of MNF (4), we noted that antibodies raised against the cloned recombinant protein recognized a second protein of smaller molecular size in immunoblots of nuclear proteins from differentiated skeletal myotubes in culture. We proposed that this smaller cross-reacting antigen, which was absent from proliferating myoblasts, was likely to represent a second isoform of MNF, and screening of cDNA libraries prepared from skeletal muscle yielded a novel cDNA encoding MNF-β. This paper presents an initial characterization of this second MNF isoform with respect to its expression, DNA binding properties, and potential functions as a transcriptional regulator. In conjunction with other studies reported elsewhere, these data support a working hypothesis for the role of MNF proteins in muscle development and regeneration.

## MATERIALS AND METHODS

**Cloning of MNF-β.** A 471-bp cDNA fragment (bp 796 to 1267) containing the winged-helix domain of MNF-α (4) was used to screen $10^6$ recombinants of a mouse skeletal muscle 5′-STRETCH PLUS λgt11 cDNA library (Stratagene). After three rounds of plaque purification, cDNA was isolated from lambda lysates of two clones, digested with *Eco*RI, subcloned into pGEM11Z (Promega), and sequenced by using Sequenase (United States Biochemical).

**RT-PCR and Southern blotting.** Total RNA was extracted from adult tissues, embryos, and cultured cells by using the TriPure Isolation kit (Boehringer Mannhem). Ten micrograms of total RNA was used in each reverse transcription (RT) reaction (Retro-Script; Ambion). Complementary DNA (5 μl) was then used as a template for the PCR in a 50-μl reaction volume including 200 ng of each primer, 2 mM $MgCl_2$, *Taq* buffer, and 1 μl of *Taq* polymerase (Gibco/BRL). The primers used for amplification are listed in Table 1. Fifteen microliters of the products of each PCR was loaded on a 2% agarose gel and analyzed by Southern blotting (26) with individual cDNA probes for detection of amplified sequences encoding MNF-α, MNF-β, myoglobin, or glyceradehyde phosphate dehydrogenase (GAPDH). Probes were generated by PCR with the appropriate primer pairs from plasmid templates and then radiolabeled by random priming (26). All of the RT-PCR experiments were performed within a range where the intensity of the hybridization signal varied as a linear function of the abundance of the input DNA (not shown). The amplified products produced by RT-PCR with primers specific for MNF-α or MNF-β were 307 and 216 nucleotides, respectively.

* Corresponding author. Mailing address: UT Southwestern Medical Center, 6000 Harry Hines Boulevard, NB11.200, Dallas, TX 75235-8573. Phone: (214) 648-1400. Fax: (214) 648-1450. E-mail: williams@ryburn.swmed.edu.

TABLE 1. Primers used in PCR

| Amplified product | 5′ primer | 3′ primer |
|---|---|---|
| MNF-α cDNA | TACTTCATCAAAGTCCCTCGGTC | GTACTCTGGAACAGAGGCTAACTT |
| MNF-β cDNA | TACTTCATCAAAGTCCCTCGGTC | GTGCGCGCGCGCATGTGGGGC |
| GAPDH cDNA | GTGGCAAAGTGGAGATTGTTGCC | GATGATGACCCGTTTGGCTCC |
| Myoglobin cDNA | ACCATGGGGCTCAGTGATGGGGAG | CAGGTACTTGACCGGGATCTTGTGC |

**Plasmids and fusion protein constructs.** To generate a fusion protein consisting of glutathione *S*-transferase (GST) fused to the winged-helix domain of MNF (GST-WH protein), a cDNA fragment encoding the DNA binding domain of MNF (amino acids 267 to 411) was generated by PCR with pBST-MNF (4) as a template, forward primer 5′-CTCACACCATGGCTGCGAAGGCCGCCTC C-3′ (underlined is a *Nco*I site), and reverse primer 5′-CTCGGATCCATCAGC CCTGGGTGGGT-3′ (underlined is a *Bam*HI site) and was subcloned into pGEX.CS (23). The GST-WH fusion protein was affinity purified by using glutathione-Sepharose beads (Pharmacia), eluted with 15 mM glutathione, and then dialyzed against Dulbecco's phosphate-buffered saline–10% glycerol–1 mM dithiothreitol in the presence of a protease inhibitor cocktail (10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 10 μg of aprotinin per ml, and 10 mM phenylmethylsulfonyl fluoride).

Site-directed mutagenesis was performed by using the Sculptor *In Vitro* Mutagenesis System (Amersham) with oligonucleotide 5′-GCTGAGCTGCAGGG CGAGCTCGGCAATGAAGCTCGAC-3′. Amino acids Ser-Arg-Arg-His at positions 130 to 134 of MNF-β were changed to residues Ala-Asp-Asp-Ala.

Expression vectors were generated by inserting MNF-α or MNF-β cDNA into the *Eco*RI site of pCI-neo (Promega) and either used to overexpress MNF protein in transfection studies or used in a TNT coupled reticulocyte lysate system (Promega) to synthesize in vitro MNF proteins. VP16 fusion proteins were constructed by using PCR to introduce an in-frame ATG at the amino end of the winged-helix domain of MNF (amino acids 279 to 389), and the VP16 activation domain (amino acids 413 to 490) was subsequently fused to the carboxyl end of the winged-helix domain by excising the VP16 domain from pSJJ1193(CRF3) (25) with restriction enzymes *Bgl*II and *Bam*HI and subcloning this fragment into the pGEM7Z-ATG+WH plasmid. The chimeric WH+VP16 fragment was excised and ligated to an expression vector driven by the cytomegalovirus (CMV) promoter. To construct MNF-β+VP16, an 860-bp fragment containing the amino region of MNF-β was excised from pBST-MNF (4) with *Sty*I and *Xba*I and subcloned into the pGEM7Z-WH+VP16 plasmid. The chimeric MNF-β+VP16 fragment was excised and ligated to an expression vector driven by the CMV promoter. A WH+eng fusion was generated by fusing the winged-helix domain (amino acids 279 to 389) of MNF in frame with the repression domain (amino acids 2 to 292) of the *Drosophila* Engrailed protein (2, 16).

**DNA binding site selection.** Affinity-purified GST-WH fusion protein or $His_6$-tagged recombinant MNF (rMNF) (4) was used to select high-affinity binding sites by a combination of previously described protocols (12, 22). For the GST-WH protein the degenerate oligonucleotide used was 5′-GAGCCGCGG ATACGTN$_{15}$CGAGCGTCTAGACGC-3′ (underlined are *Sac*II and *Xba*I sites), the amplification forward primer was 5′-GAGCCGCGGATACGT-3′, and the reverse primer was 5′-GCGTCTAGACGCTCG-3′. Double-stranded DNA templates were made by a single cycle of extension PCR with the corresponding reverse primer. The binding reaction was carried out with 0.4 μg of double-stranded DNA and 3 μg of affinity-purified GST-WH fusion protein in 100 μl of gel shift buffer [20 mM HEPES (pH 7.4), 40 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 0.2 mg of bovine serum albumin per ml, and 2 μg of poly(dI-dC) (Pharmacia) per ml] at room temperature for 20 min. The DNA-protein complex was collected by incubation with 20 μl of glutathione beads (prewashed in wash buffer plus 0.1% Nonidet P-40 [NP-40] for 30 min at 4°C) followed by a low-speed spin. The collected beads were washed three times with 500 μl of wash buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.2 mg of bovine serum albumin per ml) plus 0.1% NP-40. The selected DNA was released from the complex by four successive rounds of elution in which 50 μl of STE (1 M NaCl, 50 mM Tris [pH 8.0], 10 mM EDTA) was added, the mixture was heated at 100°C for 2 min, and the supernatant was collected and pooled. Finally, the DNA was precipitated with ethanol and resuspended in 20 μl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA), followed by standard PCR with the annealing temperature at 37°C for 30 cycles. About one-fourth of the PCR product was used for each subsequent selection. A total of five rounds of site selection binding were performed. The final selected DNA products were cloned into the pBluescript II KS plasmid (Stratagene) at the corresponding restriction enzyme sites (*Sac*II and *Xba*I) and sequenced. A total of 41 different DNA sequences isolated in this manner were compared to generate consensus binding requirements for MNF proteins.

The degenerate oligonucleotide for $His_6$-tagged rMNF was 5′-GCGTCGAC AAGCTTTCTAGAN$_{35}$GAATTCCGGATCCCTCGAGCG-3′ (underlined are *Hin*dIII and *Bam*HI sites), and the forward and reverse amplification primers were 5′-GCGTCGACAAGCTTTCTAGA-3′ and 5′-CGCTCGAGGGATCC GAATTC-3′, respectively. The binding reaction was carried out with 5 μg of double-stranded DNA, 3 μg of rMNF, and 1 μg of poly(dI-dC) in 20 μl of binding buffer (20 mM HEPES [pH 7.6], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.2 mg of bovine serum albumin per ml) at room temperature for 20 min. The DNA-protein complex was collected with 10 μl of nickel-agarose (Qiagen) and washed four times with 500 μl of isotonic saline containing 0.1% NP-40 and 0.1% bovine serum albumin. The recovered DNA served as a template for PCR with forward and reverse primers with the annealing temperature at 55°C for 30 cycles. A total of six rounds of site selection binding were performed. The recovered DNA was subcloned into the pBluescript II KS plasmid at the corresponding restriction enzyme sites (*Hin*dIII and *Bam*HI) and sequenced.

To monitor the site selection binding products, electrophoretic mobility shift assays (EMSA) were performed with a radiolabeled DNA probe generated by five cycles of PCR with 1 μl of each round of site selection binding product as the template in the presence of [$^{32}$P]dATP, dCTP, dGTP, and dTTP.

**EMSA.** Binding reactions were performed in 20 μl of gel shift buffer (20 mM HEPES [pH 7.4], 40 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol) containing 1 ng of radiolabeled double-stranded DNA ($10^4$ cpm), 1 μg of poly(dI-dC) (Pharmacia) (when GST fusion protein was used), or 1 μg of sonicated salmon sperm DNA (Promega) (when in vitro-translated proteins were used) with either 100 ng of GST-WH protein or in vitro-translated MNF proteins made with the TNT coupled reticulocyte lysate system (Promega), and the reaction mixtures were incubated at room temperature for 20 min. Competition studies were performed by including 50 or 100 ng of unlabeled double-stranded DNA prior to the addition of radiolabeled DNA probe. The DNA-protein complexes were resolved on 4% native polyacrylamide gels equilibrated in 45 mM Tris-borate–1 mM EDTA.

The specific MNF binding site sequences used for EMSA were 5′-GTACTG TAAATAAATAGTGCCGCGGTACCAGATC-3′ and 5′-CCCGGTCAAA ACAATAAACATCAACACTTGGTGTACA-3′. The double-stranded oligonucleotide binding sites were radiolabeled as previously described (15).

**Cell culture, transfections, and luciferase assay.** Monolayers of C2C12 or Sol8 myogenic cells were grown as recommended by the American Type Culture Collection. Cells were differentiated by withdrawing fetal bovine serum from the medium and supplementing the medium with 2% horse serum, 10 μg of insulin per ml, and 10 μg of transferrin per ml (Gibco/BRL Research). Transfections were performed on $10^5$ cells (as directed by Gibco/BRL Research) with 5 μl of Lipofectamine and 2 μg of plasmid DNA. Cells were harvested at 24 h post-transfection and lysed in reporter lysis buffer (Promega), and luciferase was measured with the luciferase assay system (Promega). Light emissions were integrated for the initial 10 s of emission at 25°C in a Berthoid LB9500C luminometer. The efficiency of transfection was determined by cotransfection of pCMV-lacZ as previously described (3).

**Nucleotide sequence accession number.** The sequence reported in this paper has been deposited in the GenBank database under accession number U95016.

## RESULTS

**Cloning of MNF-β, a distinctive MNF isoform.** A cDNA encoding MNF-β was cloned from a mouse skeletal muscle library. Within the amino-terminal and winged-helix domains (amino acid residues 1 to 409), MNF-β is identical to MNF-α, but the sequence diverges in the carboxyl terminus (Fig. 1A). The absolute identity of amino acid residues 1 to 409 in MNF-α and MNF-β suggests that the two forms arise by alternative splicing. This conclusion also is supported by preliminary characterization and partial sequencing of the MNF genomic locus on mouse chromosome 5 (syntenic to human chromosome 7) that demonstrates the presence of presumptive exons encoding sequences specific to each isoform (not shown). MNF-α (90 kDa) is expressed in committed myoblasts and differentiated myotubes in culture, while MNF-β (55 kDa) becomes detectable only in the transition from proliferative growth of undifferentiated myogenic cells to postmitotic myotubes (Fig. 1B to D).

Both RT-PCR and Western blot assays demonstrate a transient burst of MNF-β expression within the first day after Sol8

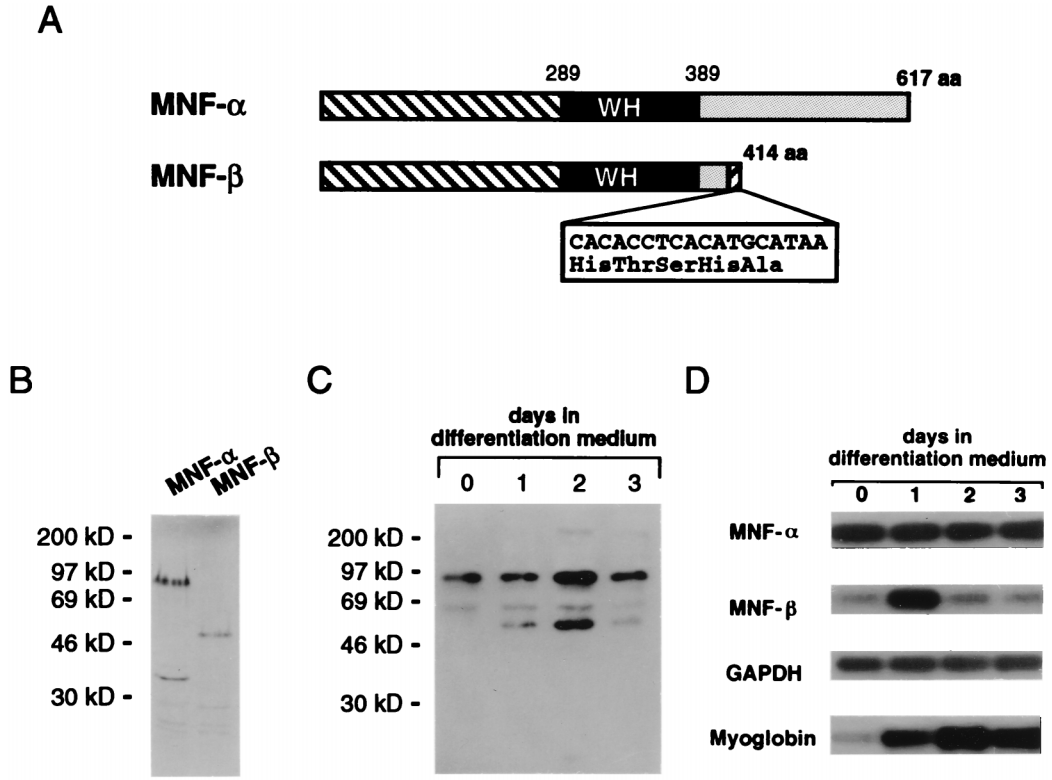


FIG. 1. Two isoforms of MNF exhibit distinctive expression patterns during myogenic differentiation. (A) Diagram of MNF isoforms MNF-α and MNF-β. WH, winged-helix/forkhead DNA binding domain. The enlarged box shows the C-terminal amino acid (aa) residues unique to MNF-β. (B) [$^{35}$S]methionine-labeled, in vitro-translated MNF-α and MNF-β analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (C) Western blot analysis of MNF proteins during sequential stages of myogenic differentiation (Sol8 cells). Protein extracts were prepared from proliferating myoblasts in growth medium (day 0) or at sequential time points after a shift to differentiation medium (1, 2, or 3 days). (D) RT-PCR–Southern blot analysis of MNF mRNA during different stages of myogenic differentiation. Probes for detection of GAPDH or myoglobin sequences were used to confirm equal amounts of input total mRNA and to monitor myogenic differentiation, respectively.

myogenic cells are placed under conditions favoring differentiation (Fig. 1C and D). An increased abundance of MNF-β mRNA is detected on day 1, and this is followed by peak expression of MNF-β protein on the second day of differentiation (Fig. 1D). This transient period of MNF-β expression corresponds temporally to several events that characterize the myogenic program: irreversible cell cycle withdrawal, fusion of mononucleated precursors into multinucleated myotubes, and activation of genes encoding terminal differentiation markers (e.g., myoglobin [Fig. 1D]). Expression of MNF-β mRNA and protein wanes, however, at later stages of the differentiation process (Fig. 1C and D).

Within mouse embryos, both isoforms of MNF are expressed, as assessed by RT-PCR (Fig. 2). The greatest expression of MNF-β, relative to total embryonic RNA, is observed at E9.5, a stage of active cardiomyocyte differentiation (Fig. 2A). Among tissues of adult animals, mRNA encoding MNF-β is most abundant in skeletal muscle, heart, and brain (Fig. 2B). These data indicate clearly that expression of mRNAs encoding the two MNF isoforms is differentially regulated during development and among adult tissues.

**Selection of DNA binding sites for MNF.** A consensus binding site for MNF was identified by using a modification of the site selection method developed by Funk and Wright (12). Two independent selection experiments were performed, using two different libraries of random deoxyoligonucleotides (35- versus 15-bp random regions), two different selection methods (glutathione versus nickel binding), and two different forms of MNF, containing identical DNA binding domains.

The consensus binding sequences derived from the two experiments were virtually identical (Fig. 3). The uniform choice of nucleotide G at position 1 in screen 1 is attributable to a G corresponding to position 1 in the nonrandom region of the deoxyoligonucleotide probe used in this selection.

**DNA binding specificity of MNF proteins in vitro.** The bindings of in vitro-translated MNF-α (amino acids 1 to 617), MNF-β (amino acids 1 to 414), and rMNF (amino acids 244 to 617 of MNF-α) proteins to the consensus binding motif were compared by EMSA (Fig. 4). All three MNF proteins contain identical winged-helix domains, and equivalent concentrations were generated in the in vitro translation reactions (Fig. 4A). We found that full-length MNF-α fails to bind the consensus motif under conditions where MNF-β or rMNF binds avidly (Fig. 4B). Since removal of either the N or C terminus of MNF-α enhances DNA binding, we conclude that the full-length protein assumes a configuration in which the winged-helix DNA binding domain is masked and that residues from both the N and C termini participate in this masking. We cannot at this time distinguish whether this is an artifact due to malfolding of recombinant protein or whether unmasking of the DNA binding domain by phosphorylation or protein-protein interactions is a bona fide mechanism by which the function of the endogenous protein is regulated.

Because the MNF consensus binding motif is similar but not identical to well-characterized motifs that bind HNF-3β, we used competitive binding assays to determine whether the binding properties of MNF-β and rMNF are similar to or distinct from those of this distantly related winged-helix tran-

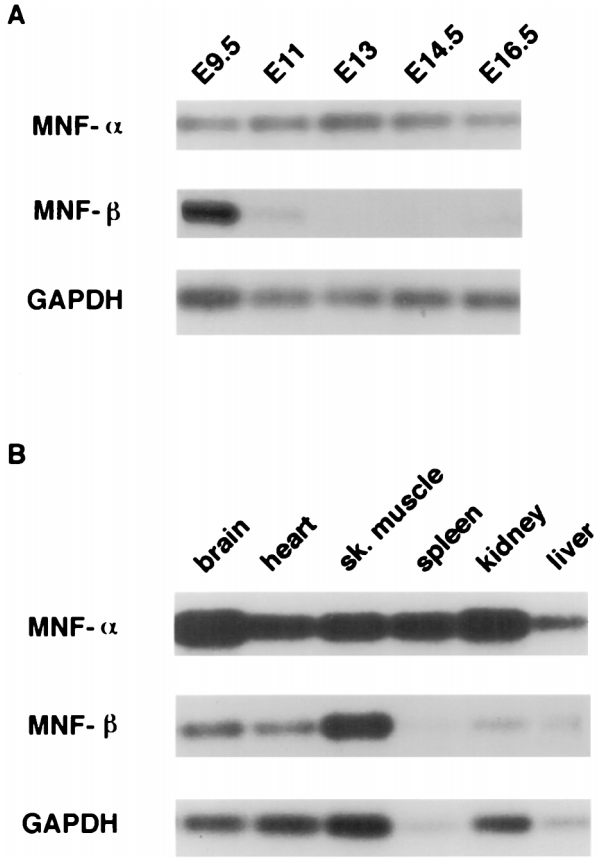


FIG. 2. Expression of MNF-α and MNF-β mRNAs in embryos and adult tissues. Total RNA (10 μg) was isolated from whole murine embryos or adult mouse tissues and analyzed by RT-PCR and Southern blotting. (A) MNF-α and MNF-β mRNA expression in the entire embryo at embryonic days E9.5 to E16.5 postcoitum. (B) MNF-α and MNF-β mRNA expression in adult mouse tissues.

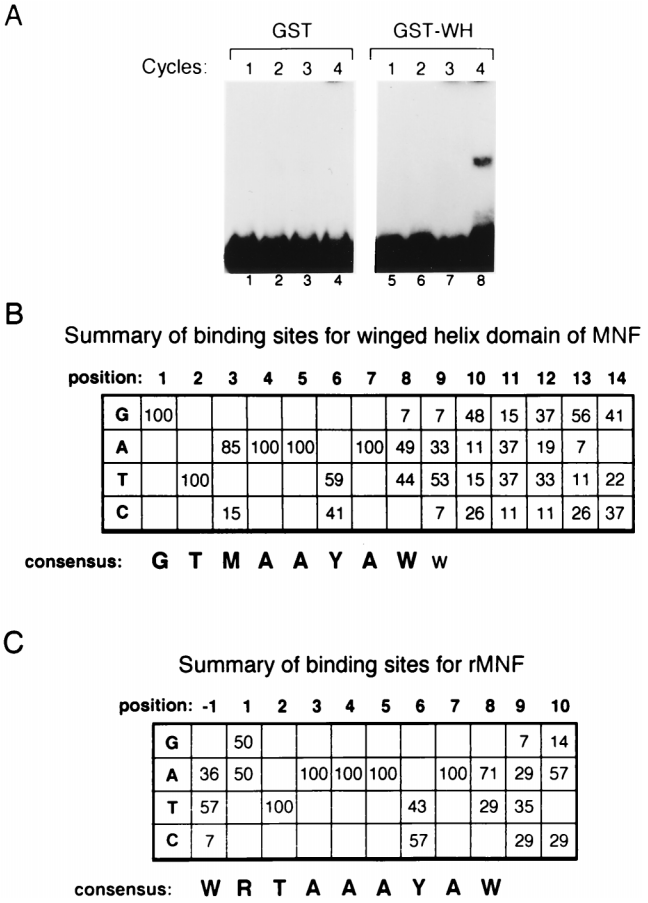


FIG. 3. Selection of binding sites from random-sequence oligonucleotides with MNF-derived proteins. (A) EMSA with radioactive deoxynucleotide probes obtained from each site selection cycle by including [$^{32}$P]dCTP during the cycles of PCR and 100 ng of bacterially expressed GST-WH. (B and C) Summary of selected binding sites for the winged-helix domain of MNF (B) and rMNF (C). Numbers represent the frequency percentage for each nucleotide at each position. This summary is based on analysis of 41 individual oligonucleotides identified in the selection procedure (27 for GST-WH and 14 for rMNF).

scription factor. The results (Fig. 4B) show clearly that MNF proteins can distinguish their cognate binding site from either of two well-characterized HNF-3β binding sites from the transthyretin (TTR) promoter, TTR-S (AGTCAATA) and TTR-W (CACAAATA) (9).

We initially cloned MNF (specifically, the form we now recognize as MNF-α) by screening expression libraries with a probe based on multimers of an upstream activation element from the human myoglobin gene promoter (CCAC sequence), and we observed that recombinant MNF would bind this sequence in vitro (4). We now recognize that the interaction between MNFs and the CCAC motif is of much lower affinity than the binding of MNF proteins to the WRTAAAYA sequence. A 200-fold molar excess of CCAC oligonucleotide fails to compete for binding in vitro of MNF-β or rMNF (a truncated form of MNF-α) to the newly defined MNF binding sequence (data not shown). These recent studies suggest strongly that the WRTAAAYA binding motif, rather than the CCAC element, represents the physiologically relevant cognate site at which MNF proteins bind DNA to modulate gene transcription.

**Transcriptional regulation by MNF proteins in vivo.** Forced overexpression of native MNF-β in several murine or human cell lines following transient transfection results in growth arrest variably followed by apoptotic cell death (11a). We observed, however, that C2C12 myogenic cells grown at high density were relatively resistant to this effect and that reproducible results from cotransfection assays could be obtained in this cell background with normalization of expression of luciferase reporter constructs to expression of a cotransfected *lacZ* expression plasmid. The specific promoter-luciferase constructs and the native and variant forms of MNF employed in the cotransfection assays are illustrated schematically in Fig. 5A. Reporter plasmids were constructed by engineering a minimal promoter (TATA element from the human *hsp70* gene) upstream of the firefly luciferase gene. To this minimal promoter, we attached either the consensus binding site for MNFs identified in the selection procedure (MNF site) and/or a Gal4p binding site (upstream activation sequence [UAS]) from the GAL1 promoter (four copies in a head-to-tail array). Expression of exogenous MNF proteins was driven by a CMV promoter, and these included (Fig. 5A) native MNF-α, native MNF-β, truncated MNF-α (rMNF), a fusion protein linking the DNA binding domain common to MNF-α and MNF-β to the potent transcriptional activation domain of the viral transactivator VP16 (WH+VP16 and MNF-β+VP16, respectively), and a fusion protein linking the DNA binding domain of MNF to the transcriptional suppression domain of the *Drosophila* Engrailed protein (WH+eng).

Immunoblots (not shown) confirmed that all of the exoge-

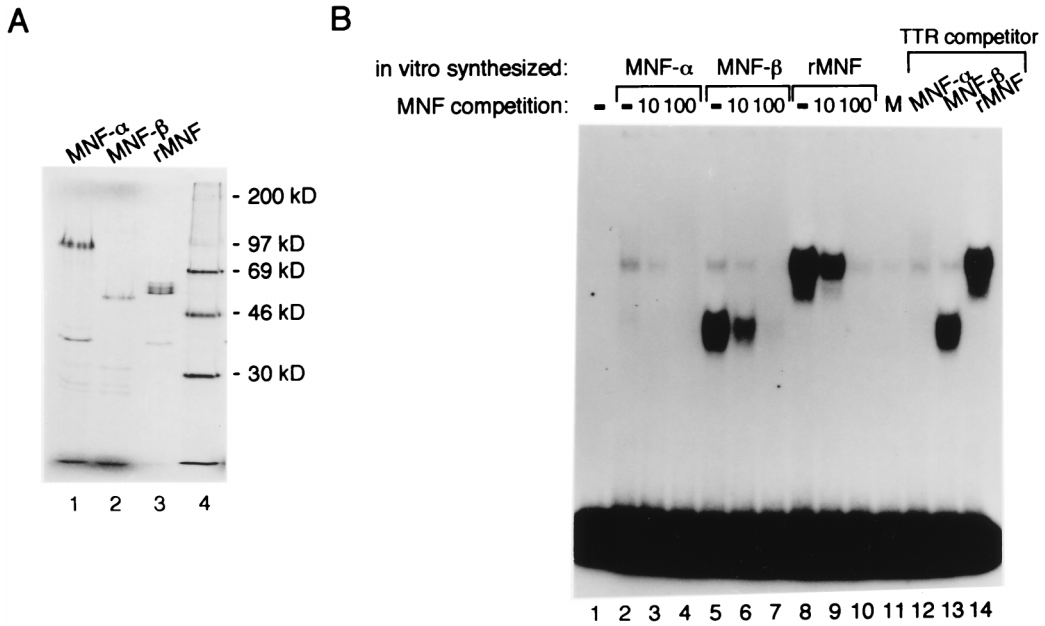


FIG. 4. Binding specificity of MNF protein for the MNF binding site. (A) [$^{35}$S]methionine-labeled, in vitro-synthesized MNF-α (lane 1), MNF-β (lane 2), and rMNF (lane 3) proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 4, protein markers. Lanes containing MNF-α and MNF-β are identical to those shown in Fig. 1B. (B) EMSA of $^{32}$P-labeled MNF binding site (5′-CCCGGTCAAAACATAAACATCAACACTTGTGTACA-3′) with 2 μl of in vitro-synthesized MNF-α (lanes 2 to 4 and 12), MNF-β (lanes 5 to 7 and 13), or rMNF (lanes 8 to 10 and 14) proteins or mock-in vitro-synthesized reticulocyte lysate (lane 11). Competition studies were performed with either 10 ng (lanes 3, 6, and 9) or 100 ng (lanes 4, 7, and 10) of unlabeled MNF binding site DNA or with 100 ng of TTR-S DNA (lanes 12 to 14).

nous MNF derivatives were expressed in vivo, and EMSA performed with nuclear extracts from transfected cells (data not shown) demonstrated that VP16 fusion proteins containing the winged-helix region from MNF bind to the MNF consensus binding sequence in vitro as avidly as native MNF-β. We observed potent transactivation of a luciferase reporter gene activity (>200-fold) by WH+VP16 only when the MNF binding site was included in the reporter construct (Fig. 5B), demonstrating that the winged-helix domain of MNF proteins is capable of binding the consensus binding motif in vivo as well as in vitro.

Neither MNF-α, MNF-β, nor rMNF exhibited direct transactivator function in our cotransfection assay system (Fig. 5B). However, if we stimulated transcription of the reporter gene via the Gal4p binding site by forced expression of a Gal4-VP16 fusion protein, we then observed reproducible transcriptional repression mediated by MNF-β (Fig. 5C). This transcriptional repression by MNF-β requires the presence of the MNF binding motif in *cis* within the reporter plasmid. Transcriptional repression also was produced by the WH+eng protein, which should direct the Engrailed repressor domain to the MNF binding site within the reporter construct. Neither MNF-α nor rMNF functions as a repressor under these assay conditions. We conclude from these experiments that native MNF-β can bind its cognate site in vivo and is capable, at least under some conditions, of functioning as a transcriptional repressor. Transcriptional repression by MNF-β requires amino acid residues within its amino terminus, distinct from the winged-helix domain, since rMNF fails to repress transcription. MNF-α also lacks this function, presumably due to its inability to bind DNA under the conditions of our assay. Remarkably, the transcriptional repressor function of MNF-β is sufficient to abolish, in *cis*, the transcriptional activating effects of the VP16 transactivation domain. It seems likely, therefore, that the repressor function of MNF-β mediated through its amino-terminal segment is so potent as to overcome transcriptional activation that otherwise would be conferred by the VP16 moiety.

**MNF includes an FHA domain.** The amino termini of both MNF-α and MNF-β include an amino acid sequence motif previously identified in yeast winged-helix proteins and termed the FHA domain (Fig. 6A). FHA domains have been identified in a number of other proteins that are not members of the winged-helix family, suggesting a more generalized, but currently uncharacterized, function, perhaps in mediating protein-protein interactions. Some FHA domain-containing proteins (e.g., MEK1, DUN1, and Ki67) have functions that are related to DNA replication or proliferative growth regulation, but this observation provides no definitive insights into the biological role of this structural motif.

We utilized the transient-cotransfection assay to determine whether an intact FHA domain is required for the activity of MNF-β as a transcriptional repressor. Amino acid substitutions that alter evolutionarily conserved residues of FHA domain were engineered into MNF-β, and this FHA mutant was less potent than native MNF-β as a transcriptional repressor in the cotransfection assay (Fig. 6B).

**The MNF binding motif is evolutionarily conserved in promoters of several genes encoding proteins that regulate proliferative cell growth.** The identification of a high-affinity binding motif for MNF proteins potentially provides an opportunity to identify target genes controlled by MNF during myogenesis. A search of the nucleotide database revealed approximately 10,000 entries with a match to the WRTAAAYA consensus binding site for MNF, a number obviously too large to represent authentic regulatory sites. When the search was constrained to rodent and primate sequences and limited by keyword descriptors (e.g., promoter, transcription, muscle, and cardiac) the field of candidate entries was reduced to about 100, each of which was examined in more detail. From among this set of genes, only the three shown in Table 2 met the

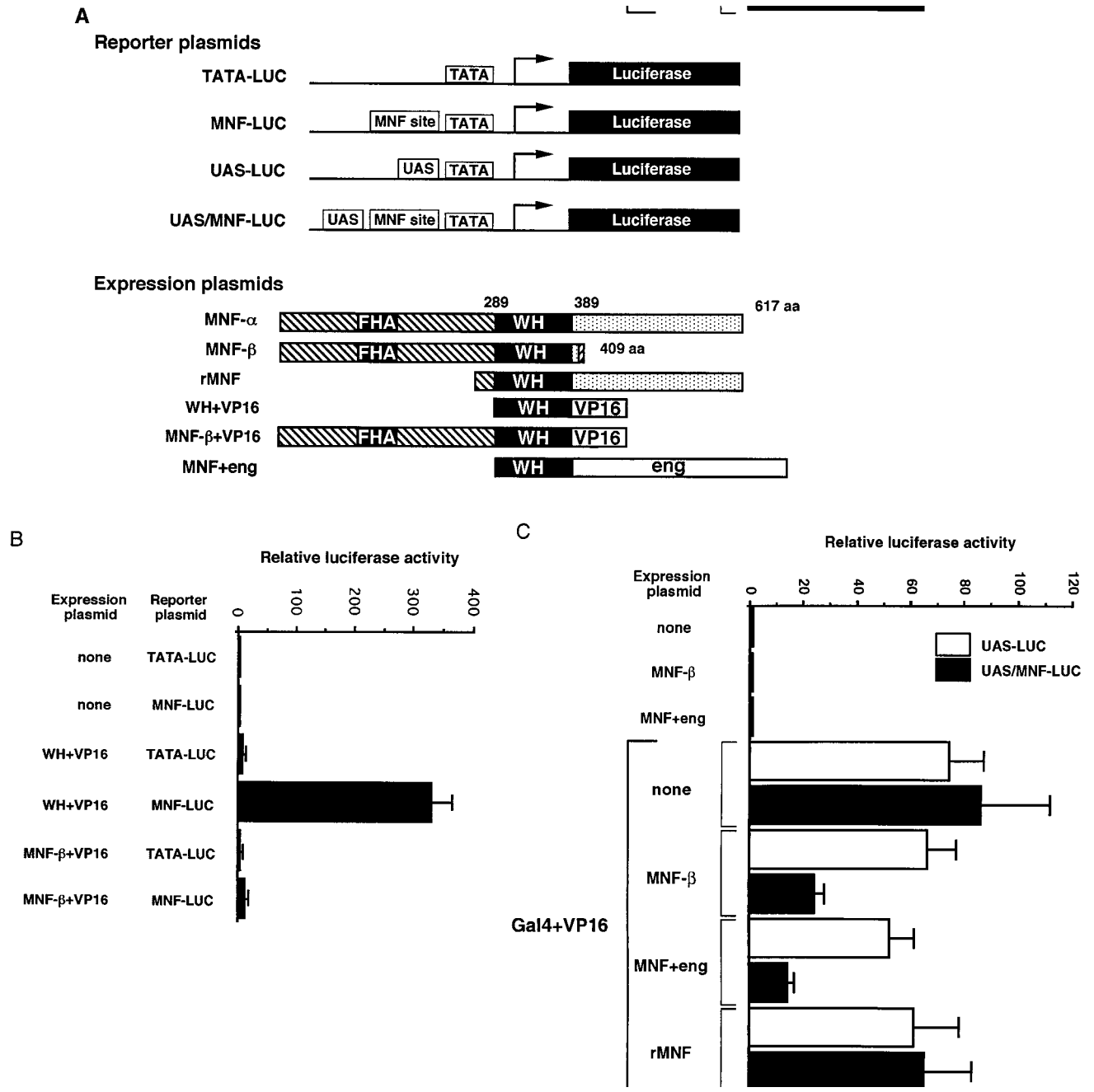


FIG. 5. Transcriptional regulatory functions of MNFs. (A) Schematic representation of promoter-reporter constructions and of native and variant forms of MNF used in the cotransfection assays. All reporter plasmids included a TATA box from the human *hsp70* promoter inserted in the pGL3-Basic luciferase reporter plasmid (Promega). MNF-LUC contains four copies of the MNF binding motif upstream of the TATA box. UAS-LUC contains four copies of the Gal4p binding site (UAS element) from the yeast GAL1 promoter adjacent to TATA box. UAS/MNF-LUC contains the multimerized UAS and MNF binding elements and a TATA box adjacent to them. rMNF lacks the N-terminal portion of MNF-α, and an initiation codon, ATG, was inserted at the 5′ end. WH+VP16 contains the winged-helix DNA binding domain of MNF fused in frame with VP16 transcriptional activation domain. MNF-β+VP16 contains the N-terminal portion (including the FHA domain) and the winged-helix domain of MNF-β fused with the VP16 activation region. MNF+eng contains the winged-helix domain of MNF fused with the transcriptional repression domain (amino acids 2 to 292) of the *Drosophila* Engrailed protein. aa, amino acids. (B) Transient-cotransfection assay for transcriptional activation. Luciferase activity was measured in whole-cell lysates prepared 24 h after transient transfection of C2C12 myogenic cells, corrected for expression of a cotransfected CMV-lacZ expression plasmid, and calculated relative to expression of the minimal promoter (TATA-LUC) in the absence of exogenous MNF proteins. Histograms depict mean values and standard deviations from three or more independent determinations. (C) Transcriptional repression of a promoter activated by exogenous GAL4+VP16. Data were calculated and are presented as for panel B.

further criteria of (i) evolutionary conservation of the MNF binding site in multiple mammalian species and/or (ii) presence of the MNF binding site within a limited (<5-kb) genomic segment shown previously to have transcriptional regulatory function. Electrophoretic mobility shift assays confirmed that recombinant MNF-β binds to an oligonucleotide (26-mer) based on the genomic sequence including and flanking the conserved WRTAAAYA motif from the mouse p21$^{WAF1/CIP1}$

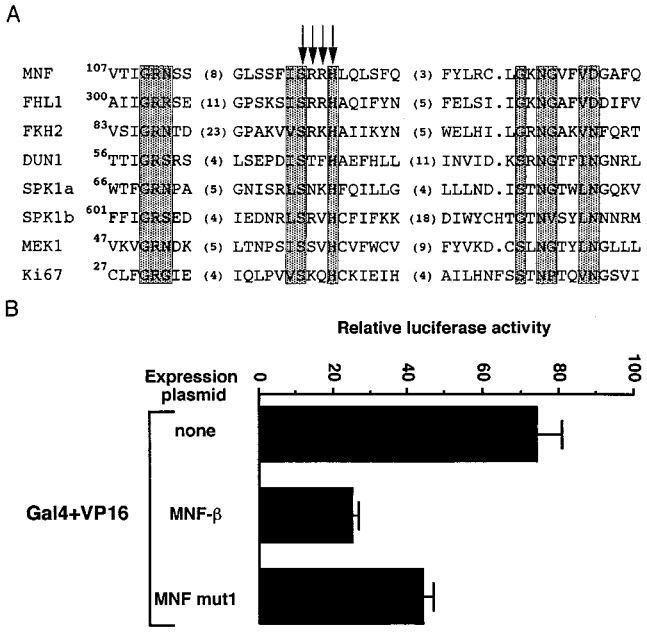


FIG. 6. The N terminus of MNF contains an evolutionarily conserved FHA domain. (A) Sequence alignment of some FHA-containing proteins. The shaded residues are conserved among those proteins. The arrows mark the mutated amino acids. Amino acids Ser-Arg-Arg-His at positions 130 to 134 of MNF-β were changed to residues Ala-Asp-Asp-Ala. The numbers in parentheses represent the lengths of omitted nonconserved regions. FHL1 an FKH2 are yeast forkhead/winged-helix proteins (accession numbers U32445 and L38850, respectively). DUN1, SPK1, and MEK1 are yeast kinases (accession numbers L25548, M55623, and Z71478, respectively). SPK1 contains two FHA domains. Ki67 is a human nuclear protein expressed selectively in proliferating cells (accession number X65550). (B) Mutation in a subregion of the FHA domain diminishes, but does not abolish, transcriptional repression by MNF-β. Cotransfection assays were performed and are presented as described for Fig. 5.

promoter (data not shown). The corresponding elements identified within the c-*myc* and *cdc2* promoters (Table 2) have not yet been examined directly in this manner, but they almost certainly represent high-affinity MNF binding sites based on the results of the site selection procedure.

## DISCUSSION

The major conclusion of this study is that striated myocytes express two functionally distinctive isoforms of MNF. The relative proportions of the two isoforms differ at sequential stages of the myogenic program. MNF-β, the newly identified isoform, is up-regulated contemporaneously with myogenic differentiation and cell cycle arrest, binds avidly to its cognate DNA recognition sequence, and can function as a transcriptional repressor. MNF-α, unlike the β isoform, is expressed in proliferating, undifferentiated myoblasts. Despite the presence of a functional DNA binding domain within the protein, full-length MNF-α fails to bind DNA with high affinity in vitro or to exhibit transcriptional regulatory functions in cotransfection assays in vivo.

The distinctive functional properties of these two MNF isoforms, and the developmentally regulated variation in their relative levels of expression, suggest that isoform switching is important for regulatory events controlled by these proteins during myogenesis. An appealing speculation, based on the transition in isoform expression found to be coincident with terminal differentiation, is that MNF-α and MNF-β may exert opposing functions and thereby comprise a genetic switch controlling reciprocal expression of a set of target genes in proliferating myoblasts versus differentiated myotubes. Our current studies demonstrate transcriptional repression by MNF-β, and while MNF-α was not observed to have a direct transactivating function, we hypothesize that MNF-α may function as a coactivator or corepressor in conjunction with as-yet-uncharacterized binding partners. The *Xenopus* winged-helix protein FAST-1 functions in precisely this capacity by reversibly binding XMADs under the control of transforming growth factor β signaling (6). In this heterodimer, FAST-1 contributes the DNA binding moiety, while XMAD provides the transactivation domain. Furthermore, it is possible that both isoforms of MNF can function as either transcriptional activators or repressors, depending on the availability of cofactors and the specific promoter context, a property attributed previously to HNF-3β (13, 24).

Other recent studies from our group lend additional support to the viewpoint that MNFs may serve unique and interesting functions during myogenesis. A detailed immunohistochemical analysis assessed spatial and temporal patterns of MNF expression during embryonic development and during muscle regeneration in adult mice (14). These studies demonstrated that MNF proteins are expressed in a pattern restricted to developing cardiac and skeletal muscles and the neural tube of the mouse embryo. In postnatal muscles, abundant expression of MNF is observed primarily in satellite cells, the quiescent myogenic stem cell pool. Because our current antibodies cannot discriminate between MNF-α and MNF-β, we can draw no conclusions as to the pattern of expression of the individual isoforms beyond the limited information provided by the RT-PCR assays and Western blots presented here. The selective expression of immunologically detectable levels of MNF in satellite cells has some intriguing parallels in relation to recent reports of two other mammalian winged-helix proteins ex-

TABLE 2. Putative MNF target genes

| Gene | Species | MNF binding site | Comment and reference | Accession number |
|---|---|---|---|---|
| *cdc2* | Human | $^{-589}$TGTAAATA | Myc-responsive region (5) | S72878 |
| p21$^{WAF1/CIP1}$ | Human | $^{-1599}$AGTAAACA | Evolutionarily conserved region (10) | U03106 |
| | Mouse | $^{-2252}$AGTAAACA | | U24173 |
| | Rat | $^{-2578}$AGTAAACA | | U24174 |
| c-*myc* | Human | $^{-633}$AATAAACA | Evolutionarily conserved region (7) | X00364 |
| | Mouse | $^{-627}$GATAAACA | | M12345 |
| **MNF consensus** | | WRTAAAYA | | |

pressed selectively in regenerating hepatocytes (30) or in embryonic stem cells (28). The latter protein, Genesis, was shown to have a transcriptional repressor function similar to that of MNF-β.

In this report we have emphasized the potential importance of MNFs within the myogenic program, but MNF proteins also may serve functions in other cell lineages. Although MNF-α mRNA is broadly distributed in many tissues, immunohistochemical studies (14) suggest that the protein is expressed only at low levels, if at all, in many cells where MNF mRNA is present. MNF protein is clearly detectable in neural tissue, within both the embryonic neural tube and the adult brain. It will be interesting to determine whether MNFs exert parallel or disparate functions in different cell types.

What downstream genes are regulated by MNFs? The results of our search of the nucleotide database do not, of course, establish any genes as authentic targets for regulation by MNFs. An extensive set of additional studies, beyond the scope of this report, will be required to determine whether the p21, c-*myc*, or *cdc2* gene, or other candidates, is indeed subject to regulatory control by MNFs. Moreover, many authentic target genes would not have been detected by our search of the current database or would have been eliminated by the restrictions we imposed in reducing the search to a manageable number of candidates. This approach was pursued and reported only to generate provisional hypotheses regarding downstream components of regulatory cascades involving MNFs.

In summary, new data presented in this paper support the hypothesis that MNF proteins have unique and important functions within the myogenic program, perhaps in the control of cell cycle progression. MNF-β, a novel winged-helix protein with transcriptional repressor activity, is expressed in the transition period when proliferating myoblasts withdraw from the cell cycle, and this protein binds DNA within conserved promoter elements of several genes that modulate proliferative cell growth. Experiments are under way to assess the functional significance in vivo of MNF binding for transcription of putative target genes, to identify binding partners for MNFs with coactivator or corepressor functions, and to generate null mutations at the MNF locus in transgenic mice.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant RO1 AR40849 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases and by American Heart Association Grant-in-Aid 95G-112.

We are grateful to Eduardo Fernandez, Daniel Garry, and Stephen Johnston for contributions to the design and interpretation of experiments.

## REFERENCES

1. **Ang, S. L., and J. Rossant.** 1994. HNF-3β is essential for node and notochord formation in mouse development. Cell **78:**561–574.
2. **Badiani, P., P. Corbella, D. Kioussis, J. Marvel, and K. Weston.** 1994. Dominant interfering alleles define a role for c-Myb in T-cell development. Genes Dev. **8:**770–782.
3. **Bassel-Duby, R., M. D. Hernandez, M. A. Gonzalez, J. K. Krueger, and R. S. Williams.** 1992. A 40-kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myoglobin promoter. Mol. Cell. Biol. **12:**5024–5032.
4. **Bassel-Duby, R., M. D. Hernandez, Q. Yang, J. M. Rochelle, M. F. Seldin, and R. S. Williams.** 1994. Myocyte nuclear factor, a novel winged-helix transcription factor under both developmental and neural regulation in striated myocytes. Mol. Cell. Biol. **14:**4596–4605.
5. **Born, T. L., J. A. Frost, A. Schonthal, G. C. Prendergast, and J. R. Feramisco.** 1994. c-Myc cooperates with activated Ras to induce the cdc2 promoter. Mol. Cell. Biol. **14:**5710–5718.
6. **Chen, X., M. J. Rubock, and M. Whitman.** 1996. A transcriptional partner for MAD proteins in TGF-beta signalling. Nature **383:**691–696.
7. **Corcoran, L. M., S. Cory, and J. M. Adams.** 1985. Transposition of the immunoglobulin heavy chain enhancer to the myc oncogene in a murine plasmacytoma. Cell **40:**71–79.
8. **Costa, R. H.** 1994. Hepatocyte nuclear factor 3/forkhead protein family: mammalian transcription factors that possess divergent cellular expression patterns and binding specificities, p. 183–206. *In* F. Tronche and M. Yaniv (ed.), Liver gene transcription. R. G. Landes Co., Austin, Tex.
9. **Costa, R. H., D. R. Grayson, and J. E. J. Darnell.** 1989. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α1-antitrypsin genes. Mol. Cell. Biol. **9:**1415–1425.
10. **el-Deiry, W. S., T. Tokino, T. Waldman, J. D. Oliner, V. E. Velculescu, M. Burrell, D. E. Hill, E. Healy, J. L. Rees, S. R. Hamilton, et al.** 1995. Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. Cancer Res. **55:**2910–2919.
11. **Feldman, J. L., and F. E. Stockdale.** 1992. Temporal appearance of satellite cells during myogenesis. Dev. Biol. **153:**217–226.

11a. **Fernandez, E., Q. Yang, R. Bassel-Duby, and R. S. Williams.** Unpublished results.

12. **Funk, W. D., and W. E. Wright.** 1992. Cyclic amplification and selection of targets for multicomponent complexes: myogenin interacts with factors recognizing binding sites for basic helix-loop-helix, nuclear factor 1, myocyte-specific enhancer-binding factor 2, and COMP1 factor. Proc. Natl. Acad. Sci. USA **89:**9484–9488.
13. **Furstenau, U., M. Schwaninger, R. Blume, I. Kennerknecht, and W. Knepel.** 1997. Characterization of a novel protein kinase C response element in glucagon gene. Mol. Cell. Biol. **17:**1805–1816.
14. **Garry, D. J., Q. Yang, R. Bassel-Duby, and R. S. Williams.** Persistent expression of MNF identifies myogenic stem cells in postnatal muscles. Dev. Biol., in press.
15. **Grayson, J., R. S. Williams, Y. T. Yu, and R. Bassel-Duby.** 1995. Synergistic interactions between heterologous upstream activation elements and specific TATA sequences in a muscle-specific promoter. Mol. Cell. Biol. **15:**1870–1878.
16. **Han, K., and J. L. Manley.** 1993. Functional domains of the Drosophila Engrailed protein. EMBO J. **12:**2723–2733.
17. **Hofmann, K., and P. Bucher.** 1995. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem. Sci. **20:**347–349. (Letter.)
18. **Kaufmann, E., and W. Knochel.** 1996. Five years on the wings of fork head. Mech. Dev. **57:**3–20.
19. **Lai, E., K. L. Clark, S. K. Burley, and J. E. Darnell, Jr.** 1993. Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biological function. Proc. Natl. Acad. Sci. USA **90:**10421–10423.
20. **Lai, E., V. R. Prezioso, W. Tao, W. S. Chen, and J. E. Darnell, Jr.** 1991. Hepatocyte nuclear factor 3α belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head*. Genes Dev. **5:**416–427.
21. **Megeney, L. A., B. Kablar, K. Garrett, J. E. Anderson, and M. A. Rudnicki.** 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. **10:**1173–1183.
22. **Overdier, D. G., A. Porcella, and R. H. Costa.** 1994. The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. Mol. Cell. Biol. **14:**2755–2766.
23. **Parks, T. D., K. K. Leuther, E. D. Howard, S. A. Johnston, and W. G. Dougherty.** 1994. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. Anal. Biochem. **216:**413–417.
24. **Philippe, J., C. Morel, and V. R. Prezioso.** 1994. Glucagon gene expression is negatively regulated by hepatocyte nuclear factor 3 beta. Mol. Cell. Biol. **14:**3514–3523.
25. **Regier, J. L., F. Shen, and S. J. Triezenberg.** 1993. Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. Proc. Natl. Acad. Sci. USA **90:**883–887.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Sasaki, H., and B. L. M. Hogan.** 1993. Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development **118:**47–59.
28. **Sutton, J., R. Costa, M. Klug, L. Field, D. Xu, D. A. Largaespada, C. F. Fletcher, N. A. Jenkins, N. G. Copeland, M. Klemsz, and R. Hromas.** 1996. Genesis, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. J. Biol. Chem. **271:**23126–23133.
29. **Weinstein, D. C., A. Ruiz i Altaba, W. S. Chen, P. Hoodless, V. R. Prezioso, T. M. Jessell, and J. Darnell, Jr.** 1994. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell **78:**575–588.
30. **Ye, H., T. F. Kelly, U. Samadani, L. Lim, S. Rubio, D. G. Overdier, K. A. Roebuck, and R. H. Costa.** 1997. Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues. Mol. Cell. Biol. **17:**1626–1641.