The winged-helix/forkhead protein myocyte nuclear factor β (MNF-β) forms a co-repressor complex with mammalian Sin3B

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Winged-helix/forkhead proteins regulate developmental events in both invertebrate and vertebrate organisms, but biochemical functions that establish a mechanism of action have been defined for only a few members of this extensive gene family. Here we demonstrate that MNF (myocyte nuclear factor)-β, a winged-helix protein expressed selectively and transiently in myogenic precursor cells of the heart and skeletal muscles, collaborates with proteins of the mammalian Sin3 (mSin3) family to repress transcription. Mutated forms of MNF-β that fail to bind mSin3 are defective in transcriptional repression and in negative growth regulation, an overexpression phenotype revealed in oncogenic transformation assays. These data extend the known repertoire of transcription factors with which mSin3 proteins can function as co-repressors to include members of the winged-helix gene family. Transcriptional repression by MNF-β–mSin3 complexes may contribute to the co-ordination of cellular proliferation and terminal differentiation of myogenic precursor cells.

Key words: myogenic cells, protein interactions, repression, transcription.

INTRODUCTION

Members of the winged-helix/forkhead family regulate tissue-specific gene expression and exert critical functions in development and carcinogenesis [1]. More than 80 proteins in this family have been identified in organisms as diverse as *Saccharomyces cerevisiae* and *Homo sapiens*. The signature motif that defines this family, termed the winged-helix domain, a 110-amino-acid region based on the structure derived from X-ray crystallography [2], functions as a sequence-specific DNA-binding domain, suggesting that winged-helix/forkhead proteins act primarily to regulate transcription. In adult mice, hepatocyte nuclear factor (HNF)-3 proteins function in concert with other transcription factors to transactivate liver-specific genes [3] but the biochemical mechanisms by which even these well-characterized members exert their important functions in the early embryo (e.g. notochord formation) remain largely undefined.

Biochemical functions, relevant cofactors and target genes subject to regulation have been characterized for only a few members of the winged-helix/forkhead family. Studies of the human winged-helix protein AFX [4] and its orthologue *Caenorhabditis elegans* DAF-16 [5] have delineated a pathway by which phosphatidylinositide 3-kinase signals to the nucleus. The serine/threonine kinase Akt, which phosphorylates and inactivates components of the apoptotic machinery, also regulates the activity of the forkhead protein FKHR1 (forkhead in rhabdomyosarcoma) [6]. Biochemical studies with *Xenopus laevis* forkhead activin signal transducer (FAST)-1 [7], human FAST-1 [8] and mouse FAST-2 [9,10] have shown winged-helix/forkhead proteins to mediate transcriptional responses to transforming growth factor-β by interacting with SMAD proteins. Reporter assays *in vivo* support the involvement of the winged-helix protein, HNF-3β, in direct activation of *sonic hedgehog* gene transcription within the central nervous system and axial mesoderm [11].

Myocyte nuclear factor (MNF)-β is the only member of the winged-helix/forkhead family of proteins currently known to be expressed selectively in cardiac and skeletal myogenic cells, within which it exhibits temporal and spatial patterns of expression that are unique in comparison with other transcription factors that govern myogenic developmental events [12,13]. Specifically, MNF-β is expressed in a transient burst as populations of proliferating myoblasts in cell culture withdraw from the cell cycle and express markers of terminal differentiation [12]. Within muscles of adult animals, sustained expression of MNF-β is observed only in quiescent, undifferentiated myogenic stem cells (satellite cells) [14]. This pattern of expression led to the hypothesis that MNF-β may participate in cell-fate decisions by which myogenic stem cells are established during development and renewed in the adult following muscle injury.

To elucidate more fully the biochemical and cellular functions of MNF-β, we conducted a screen for potential cofactors, and tested the hypothesis that MNF-β can affect the proliferation of mammalian cells using a well-established assay of oncogenic transformation. The results of these experiments demonstrate a physical and functional interaction between MNF-β and mammalian Sin3B (mSin3B) proteins as transcriptional co-repressors of promoters bearing the nucleotide-sequence recognition site for MNF-β. We observed also that forced overexpression of MNF-β suppresses oncogenic transformation driven by mammalian and viral oncogenes. Mutated forms of MNF-β lacking the mSin3-binding region are defective in both transcriptional re-

Abbreviations used: MNF, myocyte nuclear factor; rMNF, recombinant MNF; HNF, hepatocyte nuclear factor; mSin3, mammalian Sin3; GST, glutathione S-transferase; PAH, paired amphipathic helix domain; HA, haemagglutinin; EMSA, electrophoretic mobility-shift assay; RT, reverse transcription; GAL4-BD, DNA-binding domain of Gal4p; bHLH, basic helix-loop-helix.

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The sequence of mSin3B(293) reported has been deposited in the GenBank Nucleotide Sequence Database under accession number AF038848.

pression and in growth suppression of transformed cells. These data support the hypothesis that target genes transcriptionally repressed by MNF-β–mSin3 complexes are involved in control of proliferative cell growth, and that MNF-β may function to coordinate proliferation and differentiation of myogenic precursor cells.

MATERIALS AND METHODS

Yeast two-hybrid screen

Two-hybrid assays were performed as described previously [15] in yeast strain Y190 transformed with pAS1 plasmids expressing the Gal4p DNA-binding domain (GAL4-BD) fused to various fragments of MNF proteins (both MNF-α and MNF-β isoforms). Binding assays used bait plasmid consisting of GAL4-BD fused to amino acids 1–32, 1–206, 207–617 or 507–617 of MNF proteins. The screen was performed using an adult cardiac cDNA library fused to the GAL4 transactivation domain in a pACT plasmid. LacZ reporter activity was measured as described previously [15].

Plasmids, fusion-protein constructs and site-directed mutagenesis

Glutathione S-transferase (GST) was fused to amino acids 128–293 of mSin3B(293) (amino acids 1–293 of mSin3B) by ligation to the *Nco*I and *Bam*HI sites of pGEX.CS [16]. The full-length mSin3B(293) was obtained by PCR using mouse cardiac-cDNA library as the template, a forward primer complementary to a sequence within the pACT plasmid [15] and a reverse primer complementary to mSin3B sequence (5′-CACAGTCACCATG-GCTATGGGAGTT-3′). Mammalian expression plasmids were driven by the cytomegalovirus promoter derived from the pCI-neo plasmid (Promega) and included various fragments of MNF proteins, either alone or fused to the GAL4-BD [17]. Specific MNF segments employed in this study include: wild-type, full-length MNF-β (amino acids 1–409); a truncated form of MNF-β (amino acids 102–409) and a truncated form of MNF-α (amino acids 262–617), both of which lacked the N-terminal region necessary for binding to mSin3B; and a truncated form of MNF-β (amino acids 1–206) that contained the mSin3B-interaction domain but which lacked the winged-helix (DNA-binding) domain.

Construction of the activator plasmids GAL4-VP16 and WH-VP16 and the reporter plasmids UAS/MNF-TATA-luciferase and UAS-TATA-luciferase was described in a previous publication [12]. The luciferase reporter plasmids were constructed using pGL3 vector (Promega). Mutants of MNF-β, MNF-β(D330A/K331P) and MNF-β(L310P/G314P), bearing amino acid substitutions in conserved amino acids within the winged-helix (DNA-binding) domain were generated by a sequential-PCR step procedure [18] using the template pCI-MNF-β with the following primer sets (all 5′ → 3′): for MNF-β(D330A/K331P) were used primer 1, GCACCATCAGTGTCCCCAACTCC; primer 2, ACTGCCGCCCCGGGCTGGCAGAACTCTATC; primer 3, CCAGCCCGGGGCGGCAGTCCTGTAGTAAGG; and primer 4, TAACCCTCACTAAAGGGAAGCGGC. For MNF-β(L310P/G314P) were used primer 1, GCACCATCAG-TGTCCCCAACTCC; primer 2, ACACCAAGTCCCATCTA-CGCCCACATCACC; primer 3, GTAGATGGGACTTGGT-GTTAGCTGCCTGTCCTG; and primer 4, TAACCCTCAC-TAAAGGGAAGCGGC.

The final PCR products were separated on a 1% agarose gel, extracted from the gel by Qiaquick isolation kit (Qiagen, Valencia, CA, U.S.A.), subcloned using the TA cloning vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced to verify the mutation sites.

In vitro protein-interaction assays

GST–Sin3B protein (1 μg) expressed and purified from *Escherichia coli* was immobilized on glutathione-Sepharose 4B (Pharmacia) and preincubated with binding buffer [20 mM Hepes (pH 7.9)/40 mM KCl/1% Triton X-100/1 mM dithiothreitol/protease-inhibitor cocktail] with 2 mg/ml *E. coli* protein for 1 h at 4 °C as described previously [17].The immobilized protein was incubated with 2 μl of ^{35}S-radiolabelled *in vitro*-translated MNF proteins [12] for 1 h at 4 °C. The beads were then washed three times with binding buffer, mixed with 2 × SDS-loading dye and loaded on to an SDS/polyacrylamide (8%) gel. The radiolabelled proteins were visualized by exposing the dried gel to autoradiographic film.

Transcriptional reporter assays and immunoprecipitation procedures

Monolayers of mouse C_2C_{12} cells were grown as recommended by the A.T.C.C. Myoblasts were differentiated by withdrawing fetal bovine serum from the medium and supplementing the medium with 2% horse serum/10 μg/ml insulin/10 μg/ml transferrin (Gibco-BRL Research). Transfections were performed on 10^5 cells using 5 μl of Lipofectamine (Gibco-BRL Research) and a total of 2 μg of purified plasmid DNA. Cells were harvested 24 h after transfection, lysed and assayed for luciferase activity as described previously [12]. 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 co-transfection of pCMV-lacZ, and the expression of luciferase reporter plasmids, in the presence or absence of the various transcriptional activator and repressor proteins described in each of the Figure legends, was normalized to β-galactosidase activities determined in the same extracts, as described previously [19]. In each co-transfection experiment, expression of each truncated or mutated form of MNF was compared with that of the wild-type form by immunoblot analysis, using a polyclonal anti-MNF antibody described previously [12,13].

For immunoprecipitation experiments, C_2C_{12} cells were co-transfected with MNF expression vector (pCI-MNF-α, pCI-MNF-β or pCI-rMNF) and pCI-HA-mSin3B(293), a construct encoding full-length mSin3B(293) tagged with an influenza haemagglutinin (HA) epitope. The cells were lysed in Dulbecco's PBS/0.5% Nonidet P-40 containing protease-inhibitor cocktail (Boehringer Mannheim), frozen and thawed, and cleared by centrifugation at 12000 ***g*** for 10 min. The supernatant was incubated with anti-MNF [13] in binding buffer [50 mM Tris/HCl (pH 7.4)/5 mM EDTA/150 mM NaCl/0.25% gelatin/0.05% Nonidet P-40] for 1 h at 4 °C prior to addition of Protein A–Sepharose beads (Pharmacia). Proteins associated with the beads were precipitated by centrifugation in a microcentrifuge for 1 min, washed three times with binding buffer, boiled in gel-loading buffer and separated by SDS/PAGE (8% gel). Immunoblot analysis was performed as described previously [13] using rabbit anti-MNF or monoclonal anti-HA (Boehringer Mannheim) as primary antibody. Enhanced chemiluminescence (ECL®) reagents were purchased from Amersham and used as directed.

Electrophoretic mobility-shift assays (EMSAs)

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% (v/v) glycerol] containing 1 ng of radiolabelled double-stranded DNA (10^4 c.p.m.), 1 μg of poly(dI-dC) (Pharmacia) and 100 ng of MNF proteins prepared with the TNT-coupled reticulocyte lysate system (Promega). Reaction mixtures were incubated at room temperature for 20 min and DNA–protein complexes were resolved on 4% native polyacrylamide gels equilibrated in 45 mM Tris/borate/1 mM EDTA. The specific MNF-binding-site sequence used for EMSA was 5′-GTACTGTAAATAAATAGTGCCGCGGTACCAGATC-3′ and its complementary strand. The double-stranded oligonucleotide was radiolabelled as described previously [19].

Transformation assays

Transformation assays were performed as described previously [20]. Transfection of NIH 3T3 cells was performed with calcium phosphate precipitation using 200 ng of H-Ras, 600 ng of v-Myc and 600 ng of MNF expression plasmid. Then, 1 day after transfection the cultures were split 1:3 and maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. After 10–14 days the colonies were stained with Giemsa (Gibco-BRL) and focus formation was assessed visually.

Expression of mSin3B mRNA and protein

Total RNA was extracted from cultured cells or from tissues of adult mice using the TriPure Isolation kit (Boehringer Mannheim). Total RNA (10 μg) 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). Primers used for amplification of mSin3B(293) mRNA were 5′-CGTCCATTCCGTGGCATGTCTG-3′ and 5′-TCACTCCTCAGCTGTGCAGTAACC-3′, and for mSin3B(954) mRNA 5′-GTCTGAAGGTGGTAGAGCTGTACC-3′ and 5′-TACTGCTCCACATAGCGAGCCAG-3′. Primers used to amplify myogenin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were described previously [12]. Each RT-PCR reaction (15 μl) was loaded on a 2% agarose gel and analysed by Southern blotting for detection of amplified sequences encoding each of these proteins, as described previously [12]. Parallel PCR reactions and Southern-blot analyses using known quantities of cDNA were used to determine the number of PCR cycles necessary for experimental results to fall within a linear range of the detection assay.

Immunohistochemical methods using specific polyclonal antibodies raised in rabbits against recombinant mSin3B protein were used to assess the expression of mSin3B in cultured C_2C_{12} myoblasts and myotubes, and in tissues of murine embryos at several stages of development. Monolayers of C_2C_{12} cells were either grown as myoblasts or differentiated by withdrawing fetal bovine serum from the medium and supplementing the medium with 2% horse serum/10 μg/ml insulin/10 μg/ml transferrin. Myoblasts and myotubes were fixed with 2% paraformaldehyde for 5 min, washed three times with Dulbecco's PBS and processed for immunohistochemistry (described below). Frozen sections (7 μm thick) were hydrated in PBS, incubated with 10% normal goat serum for 30 min at room temperature to reduce background staining, and incubated overnight at 4 °C with the primary antiserum in a humid chamber. The following day the sections were washed with PBS (three washes, 10 min each) and incubated with the secondary antibody for 1 h at room temperature, after which they were washed further with PBS and mounted under coverslips with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). Substitution controls with either normal rabbit serum or PBS were performed, and in all instances the controls were negative. Rabbit mSin3B (A-20) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was diluted (1:100) in PBS containing 0.3% Triton X-100 and rabbit MNF antiserum was used as described previously [14]. The secondary antiserum used in these studies was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:50; Jackson Immunoresearch, West Grove, PA, U.S.A.).

RESULTS

mSin3 interacts with an N-terminal domain of MNF proteins

The two-hybrid screen identified three unique cDNA clones from a cardiac-cDNA library that was engineered to express proteins fused to the transcriptional activation domain of GAL4 (GAL4-AD). Two of the products of this screen interacted selectively with the C-terminal domain of MNF-α that is not shared with MNF-β, and these MNF-α-interacting proteins will be described elsewhere. The third cDNA encoded a novel isoform of mSin3B,

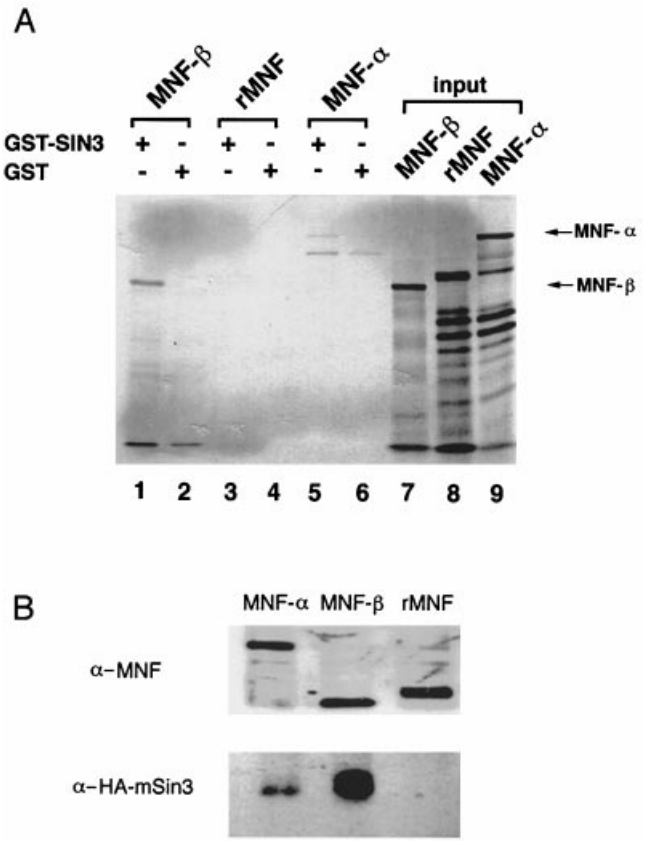

Figure 1 mSin3 binds the N-terminus of MNF proteins *in vitro* and in mammalian cells

(**A**) Full-length MNF proteins, or a truncated form that excludes the N-terminus (rMNF; amino acids 262–617), were transcribed and labelled with [^{35}S]methionine in reticulocyte lysates and incubated with bacterially expressed GST–mSin3B fusion protein or GST alone immobilized on glutathione–Sepharose beads. Bound proteins were compared with the total input protein after SDS/PAGE and visualized by autoradiography. (**B**) C_2C_{12} myogenic cells were transfected with expression plasmids encoding MNF-α, MNF-β or rMNF in combination with full-length mSin3B(293), epitope tagged with HA antigen. Proteins were immunoprecipitated from nuclear extracts using anti-MNF polyclonal antiserum, and the three recombinant MNF proteins were visualized in immunoblots (upper panel) probed with anti-MNF serum or probed with anti-HA monoclonal antibody (lower panel), which revealed co-immunoprecipitation of mSin3B(293) with MNF-α and MNF-β but not rMNF.

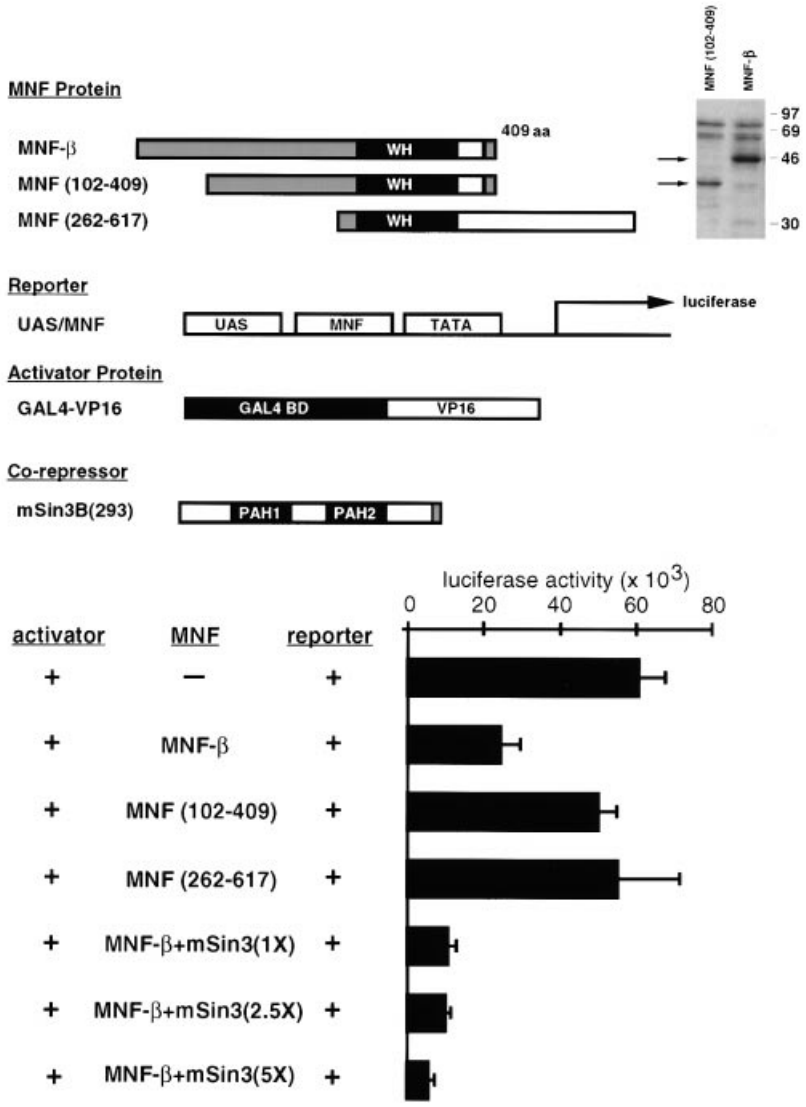

Figure 2 The mSin3-binding region of MNF-β is a transcriptional-repression module

A reporter gene controlled by a promoter containing binding sites for both MNF and GAL4 (UAS), and activated by a GAL4-BD-VP16 fusion protein, was repressed by MNF-β but not by variant MNF proteins that lacked the N-terminal mSin3-binding region. Co-transfection of mSin3B(293) in various doses (1–5 ×) augmented transcriptional repression by MNF-β. Results are presented as means ± S.E.M. of three or more individual transfection assays. The insert shows an immunoblot of recombinant MNF proteins expressed after transfection of C_2C_{12} myogenic cells. WH, winged helix.

which was found to interact specifically with an N-terminal region (amino acids 1–206) present in both MNF-β and MNF-α. In the yeast two-hybrid assay, the extreme N-terminus of MNF (amino acids 1–32) was also capable of binding mSin3B, although the interaction was weaker than with amino acids 1–206 (results not shown).

The original mSin3B cDNA (clone F7.2) isolated in the two-hybrid screen was a partial cDNA nearly identical to amino acids 136–274 of the *mSin3B* gene listed in GenBank (accession number L38622). Clone F7.2 contained sequences that matched the region encoding PAH2 (paired amphipathic helix domain 2) of mSin3B, but lacked the remaining three PAHs. This partial cDNA clone contained a 3′-untranslated region and poly-adenylation signal, suggesting that it was complete at its 3′ terminus, but the absence of the expected PAH1 region suggested that this clone was incomplete at its 5′ end. A PCR-based procedure was used to clone a full-length cDNA we termed mSin3B(293). The nucleotide sequence of mSin3B(293) was identical to the mSin3B sequence listed in GenBank (accession number L38622) from nucleotide base pairs 13 to 835, except for nucleotide base changes at 710 and 723, resulting in amino acid substitutions of glycine and proline at residues 230 and 233, respectively. The final 19 codons (57 nucleotides) before the stop codon and the 3′-untranslated region of mSin3B(293) have no similarity with the longer form, which we designated mSin3B(954). Full-length mSin3B(293) contains PAHs 1 and 2, but lacks the additional PAHs 3 and 4 that are present in mSin3B(954). We confirmed that mSin3B(293) is an authentic gene product expressed in cardiac cells, and not an artifactual product of the cloning procedure, using a RT-PCR assay with primers that selectively amplified cDNA encoding the different mSin3B(293) and mSin3B(954) isoforms.

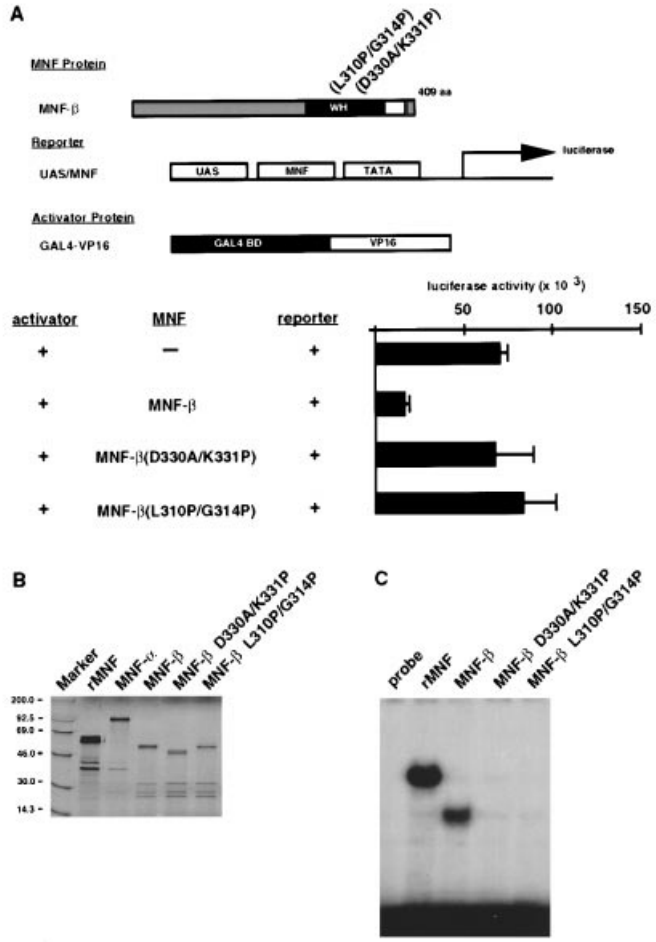

Figure 3 Amino acid substitutions in the winged-helix domain of MNF-β impair its function as a transcriptional co-repressor

(**A**) Site-directed mutagenesis was used to introduce amino acid substitutions at specific residues of MNF-β, D330A/K331P and L310P/G314P, which are conserved among other winged-helix/forkhead proteins [12] and that lie within α-helical structures predicted on the basis of X-ray crystallography of HNF-3 bound to DNA [2]. A reporter gene controlled by a promoter containing binding sites for both MNF and GAL4 (UAS), and activated by a GAL4-BD–VP16 fusion protein, was repressed by wild-type MNF-β but not by variant MNF proteins that were defective in DNA binding. Results are presented as means $\pm$ S.E.M. of three or more individual transfection assays. (**B**) Proteins were expressed in a coupled *in vitro*-transcription/translation reaction in the presence of ^{35}[S]methionine and resolved by SDS/PAGE. (**C**) DNA-binding assay using EMSA of wild-type and mutated forms of MNF-β.

An interaction between the N-terminus of MNF and mSin3B proteins was also observed *in vitro* using recombinant proteins. A fusion protein linking mSin3B (amino acids 128–293) to GST was expressed in *E. coli*, and purified GST–mSin3B immobilized on glutathione–Sepharose beads was incubated with MNF proteins, or segments thereof, expressed in reticulocyte lysates (Figure 1A). Full-length MNF-β or MNF-α bound specifically to GST–mSin3B, while a variant form of MNF-α (amino acids 262–617, identified as recombinant MNF, or rMNF) lacking the native N-terminal region failed to exhibit this interaction. As further confirmation that mSin3B interacts with the N-terminus of MNF, truncated *in vitro*-translation products arising from initiation at internal methionine codons, so as to delete the authentic N-terminus (rapidly migrating bands in lanes of Figure 1A labelled 'input'), also failed to bind in this assay.

A co-immunoprecipitation procedure was used to determine whether MNF and mSin3B proteins also form physical associations in mammalian cells. C_2C_{12} cells were co-transfected with expression plasmids leading to overexpression of full-length MNF-α, MNF-β or rMNF (amino acids 262–617) in conjunction with full-length mSin3B(293), into which an influenza HA-antigen segment was inserted at the N-terminus as an epitope tag. Nuclear extracts were immunoprecipitated with a polyclonal antibody that recognized all three of these forms of MNF, and examined by immunoblot analysis using anti-HA monoclonal antibody to identify HA-tagged mSin3B(293). The results (Figure 1B) demonstrate co-immunoprecipitation of mSin3B(293) with either MNF-α or MNF-β, whereas the rMNF variant lacking the N-terminal region failed to associate with mSin3B(293) in this assay.

The mSin3-binding domain of MNF functions as a transcriptional-repression module

We reported previously that MNF-β functions as a transcriptional repressor in mammalian cells when bound to its cognate DNA-recognition sequence [12]. Here, we used a similar co-transfection assay to demonstrate that transcriptional repression by MNF-β requires the mSin3-interaction domain (Figure 2, upper panel). Using a reporter gene containing binding sites for both GAL4 and MNF-β, and activated in *trans* by a GAL4–VP16 fusion protein, native MNF-β repressed transcription, as we have observed previously [12]. Co-transfection of mSin3B(293) in various doses (1–5 $\times$) augmented transcriptional repression by MNF-β. In contrast, neither a truncated MNF-β variant (amino acids 102–409) nor a truncated MNF-α variant (amino acids 262–617), each of which lacked the mSin3-binding region but was competent for DNA binding [12], functioned as a repressor in this assay. Immunoblot assays (Figure 2, insert) confirmed that both native and truncated forms of recombinant MNF are stable and accumulate to detectable levels following transfection. Transcriptional repression by MNF-β is dependent upon DNA binding, as evidenced by the requirement for both an MNF-binding motif within the reporter plasmid [12] and a functional DNA-binding domain (in addition to the mSin3B-interaction region) within MNF-β. Mutated forms of MNF-β, MNF-β(D330A/K331P) and MNF-β(L310P/G314P), which included the mSin3B-binding domain (amino acids 1–206), but which were defective in DNA binding due to amino acid substitutions within the winged-helix domain, lacked potency as transcriptional repressors (Figure 3).

The N-terminal segment of MNF-β that binds mSin3B can confer transcriptional silencing activity to a heterologous DNA-binding protein. A fusion protein linking the GAL4-BD to the mSin3-interaction domain of MNF-β (amino acids 1–206) functioned as a potent repressor of a reporter plasmid containing both GAL4- and MNF-β-binding sites that was activated in *trans* by a fusion protein linking the DNA-binding domain of MNF-β to the VP16 transactivation region (Figure 4). Transcriptional repression by the GAL4-BD–MNF-β fusion protein required DNA binding, since repression was not observed if the reporter plasmid lacked GAL4-binding sites.

MNF-β suppresses oncogenic transformation

Focus-forming assays were utilized to assess the effect of MNF expression on the frequency by which NIH 3T3 cells undergo oncogenic transformation following stable integration of transgenes expressing either Myc or E1A in combination with Ras (Table 1). Concomitant expression of MNF-β markedly reduced

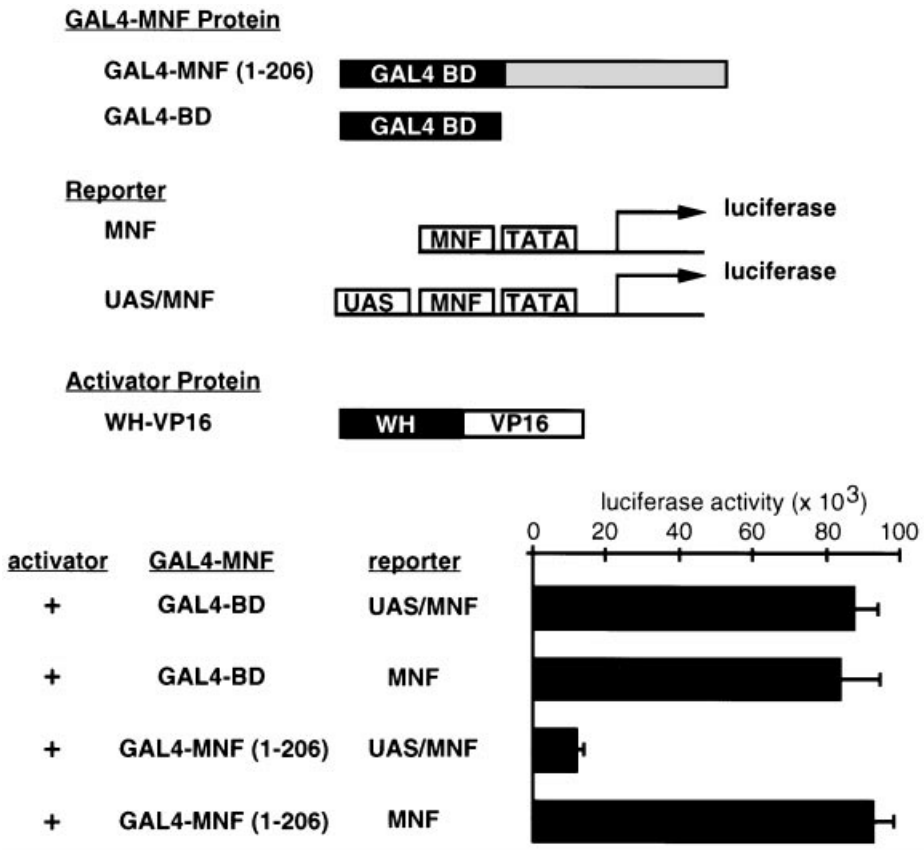

Figure 4 The terminal segment of MNF-β confers transcriptional repression to a heterologous DNA-binding protein

A reporter gene controlled by a promoter containing binding sites for both MNF and GAL4 (UAS), and activated via the MNF-binding site by a WH–VP16 fusion protein, was repressed by the N-terminus of MNF-β fused to the GAL4-BD only if the GAL4-binding site (UAS) was present in the reporter construct. Results are presented as means ± S.E.M. of three or more individual transfection assays.

Table 1 Effect of MNF on NIH 3T3-fibroblast transformation

		Total foci per transfection					
v-Myc + H-Ras	Experiment ...	1	2	3	4	5	6
pCI		566	563	1065	763	534	475
pCI-MNF-β		186	185	340	334	251	–
pCI-MNF (102–409)		–	–	–	560	516	504

		Total foci per transfection	
E1a + H-Ras	Experiment ...	7	8
pCi		330	317
pCI-MNF-β		94	113

the number of foci observed. A representative experiment is shown in Figure 5, but this result was repeated in six independent experiments using Myc + Ras and in two experiments using E1A + Ras. The transforming effects of Myc + Ras in NIH 3T3 cells were maintained, however, in the presence of a truncated form of MNF-β (amino acids 102–409) that lacked the mSin3-binding region. These results indicate that the mSin3-interaction surface of MNF-β is important both for its biochemical function as a transcriptional repressor and its cellular function as a negative growth regulator.

mSin3B is expressed in cardiac and skeletal myocytes

Based on a sensitive RT-PCR/Southern-blot assay, we have shown that transcripts encoding mSin3B(293) and mSin3B(954), are present in C_2C_{12} myoblasts and myotubes, and in tissues of adult mice including heart and skeletal muscle (Figure 6). Although there appeared to be some variation in the relative abundance of the two transcripts among different tissues, in no case was one isoform detected in the absence of the other. Immunohistochemical analyses using a polyclonal antibody that detects both forms of mSin3B demonstrated the presence of mSin3B protein(s) in cardiac muscle as early as E9.5 (day 9.5 of embryonic development), when MNF was also expressed in the developing heart (Figures 6E and 6F). Likewise, mSin3B protein was detected in developing skeletal muscles at E13.5 when MNF was also present (Figures 6G and 6H).

Interestingly, immunoreactive mSin3B in proliferating C_2C_{12} myoblasts is localized primarily to the cytoplasmic compartment (Figure 6C). Upon differentiation of these cells into multi-nucleated myotubes, however, mSin3B appears to translocate to the nucleus (Figure 6D). This finding suggests that the function of mSin3B as a transcriptional repressor at relevant target genes

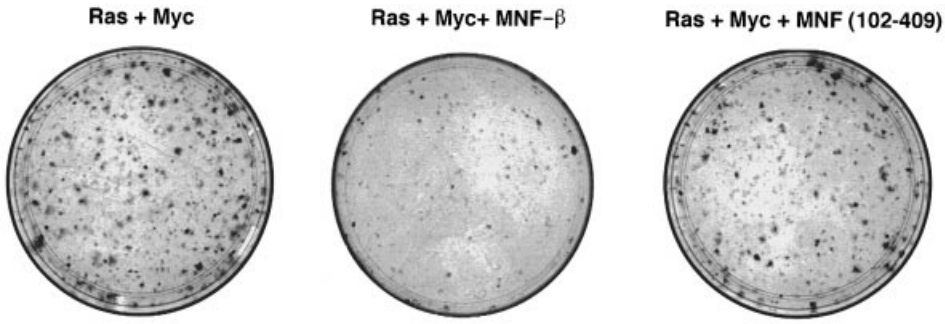

Figure 5 Negative growth regulation by MNF-β

Oncogenic transformation of 3T3 cells by Myc + Ras was suppressed by MNF-β but not by a truncated form of MNF-β defective for binding mSin3 and for transcriptional repression. Representative plates stained with Giemsa Blue are shown 10 days following transfection.

(in conjunction with MNF-β and/or other co-repressor proteins) is developmentally regulated, even in the absence of changes in the total cellular pool of mSin3B.

DISCUSSION

mSin3 proteins as co-repressors

mSin3 proteins were first described in 1995 [21,22], and have subsequently been the subject of intense interest [23–28]. mSin3 proteins form complexes with the Mad family of DNA-binding transcriptional repressors or nuclear-hormone-receptor families to repress transcription of downstream target genes and to regulate critical cellular activities, such as proliferation, differentiation or other responses to extracellular signals [29,30].

Under conditions of mitogen stimulation, Myc–Max heterodimers transactivate downstream genes such as cdc25 phosphatase that promote cell-cycle progression [31]. In response to signals that suppress cell growth, Mad proteins are up-regulated, and Max–Myc heterodimers are replaced by Mad–Max complexes that bind the same E-box-recognition sequences, to repress rather than activate transcription [32]. Transcriptional repression by the Mad–Max complex is mediated by an interaction between Mad and mSin3 [21,22], which in turn recruits histone deacetylases to the promoter regions of target genes [23,25,33]. The resulting deacetylation of core histones is believed to alter the local conformation of chromatin structure in a manner that prevents access of the basal transcriptional machinery.

MNF-β and mSin3 form a complex to repress transcription

The major finding of our current work is that the winged-helix/forkhead protein MNF-β forms a physical complex with members of the mSin3B family of proteins. This interaction appears to be important for the biochemical function of MNF-β as a transcriptional repressor, since deletion of the region of MNF-β required for binding mSin3 proteins leads to loss of repressor activity. The mSin3-binding domain of MNF-β functions as a transcriptional repression module that can be transferred to a heterologous DNA-binding domain so as to direct mSin3 to different cognate recognition sites. This effect is similar to repression mediated by the mSin3-interaction region from Mad fused to the N-terminus of a truncated form of the basic helix-loop-helix (bHLH) protein T-cell transcription factor EB (TFEB) [34]. We conclude that MNF-β and mSin3 collaborate as co-repressors to regulate transcription of downstream genes that are specified by the winged-helix DNA-binding domain of MNF-β. In this regard, MNF-β serves a role analogous to that ascribed to the Mad–Max heterodimer and recruits the co-repressor mSin3 to promoter regions of target genes. These data extend the known repertoire of mSin3-interacting proteins to include at least one member of the forkhead, winged-helix family of transcription factors.

While few downstream targets of winged-helix transcription factors have been identified, transcriptional repression appears to be a frequent consequence of the regulatory actions of members of this extended gene family. This is seen from the effects of *Drosophila* forkhead on *trachealess* [35], mammalian HNF-3β on *aldolase B* [36] and *C. elegans* pha-4 on *lin-26* [37]. It will be interesting to determine whether these other winged-helix proteins also utilize Sin3-related proteins as co-repressors.

Multiple isoforms of mSin3B

The specific mSin3 protein that we isolated from a cardiac-cDNA library on the basis of its interaction with MNF-β and termed mSin3B(293) was not described previously in the GenBank database, but represents an alternatively spliced product of the same gene previously designated mSin3B. Binding to MNF-β is mediated by the PAH2 region of mSin3B(293), so we expect MNF-β to also form complexes with other mSin3 proteins that contain this domain, in particular mSin3B(954). A short form of mSin3B, similar in size to mSin3B(293), was shown to interact with nuclear receptor co-repressor 1 (N-CoR) and to function as a co-repressor in transcriptional silencing by nuclear-hormone receptors [23]. Although the sequence of this truncated mSin3B isoform was not reported, it is likely to be identical to the mSin3B(293) protein that we describe here.

MNF-β can antagonize oncogenic transformation provoked by Myc or E1A in combination with Ras

Several prior observations suggested that MNF-β may be involved in negative regulation of cell proliferation. MNF-β is expressed transiently as myogenic cells undergo cell-cycle withdrawal in differentiating myogenic cells in culture [12], and is expressed stably in quiescent satellite cells in muscles of intact animals [14]. MNF-β is a transcriptional repressor, and binding sites for MNF-β are conserved in promoter regions of several genes that encode proteins required for cell-cycle progression [12]. In the present study, we assessed whether MNF-β could antagonize the ability of transforming oncogenes to promote mitogen-independent cell growth in a focus-forming assay. These experiments consistently demonstrated a growth-suppressive

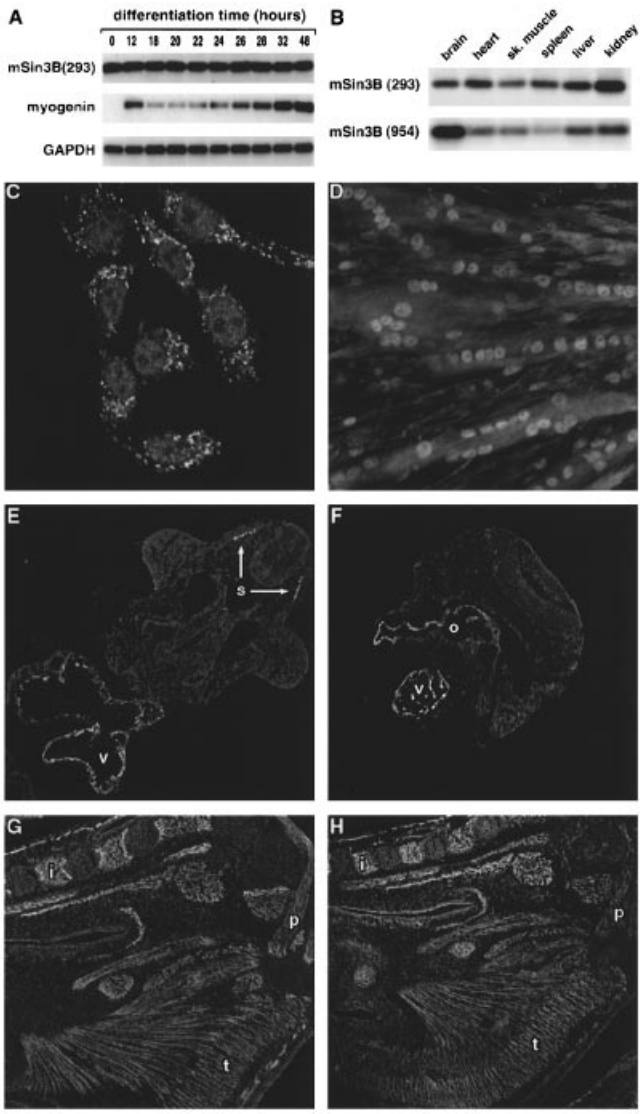

Figure 6 mSin3B proteins are expressed in cardiac and skeletal muscles

(**A**) RT-PCR assay demonstrating expression of mSin3B(293) mRNA in C_2C_{12} myogenic cells in the transition from proliferating myoblasts to differentiated myotubes. The induction of myogenin mRNA during differentiation is a biochemical marker of differentiation and the presence of GAPDH mRNA verifies the integrity of RNA samples and the uniformity of the RT-PCR reactions. (**B**) RT-PCR assay demonstrating expression of both mSin3B(293) and mSin3B(954) in tissues of adult mice, including heart and skeletal muscle. Subcellular localization of mSin3B proteins in cultured C_2C_{12} myoblasts (**C**) and myotubes (**D**). (**E–H**) Expression of mSin3B proteins (**E** and **G**) and MNF proteins (**F** and **H**) in tissues of murine embryos at E9.5 (**E** and **F**) and E13.5 (**G** and **H**). Embryonic structures: s, somite; v, ventricle; o, outflow tract; i, intercostal muscles; p, pharyngeal muscles, t, tongue.

effect of MNF-β that requires the mSin3-binding domain. From these results, we conclude that the interaction with mSin3B and transcriptional-silencing activity of MNF-β at specific downstream target genes is important for its ability to suppress cell growth.

A proposed model for the role for MNF-β–mSin3 complexes in muscle development

During development of skeletal muscles, terminal differentiation of myogenic precursor cells is associated with cell-cycle withdrawal, but the molecular mechanisms that co-ordinate these separate features of myogenesis are incompletely defined. Myogenic-determination genes of the MyoD group of bHLH transcription factors are essential for the expression of downstream genes that define the myogenic lineage, and can concomitantly suppress cell growth [38]. Activation of terminal-differentiation markers by myogenic bHLH factors is direct, since binding sites for these proteins, or for other transcription factors with which they directly associate (MEF2), are present within the promoters of most, if not all, genes that are expressed selectively in differentiated myocytes [39]. The mechanisms by which proliferative cell growth is arrested by myogenic bHLH proteins, in contrast, are less well defined. Synergistic interactions between MyoD family members and retinoblastoma tumour-suppressor protein (pRb) have been proposed [40], but remain incompletely characterized. Up-regulation of cyclin-dependent-kinase inhibitors accompanies myogenic differentiation, and can be provoked by forced expression of MyoD [41–44], but the molecular signals that induce this response appear to be indirect and have not been identified.

We hypothesize that transient expression of MNF-β during myogenic differentiation, and formation of mSin3B–MNF-β complexes, represses transcription of genes required for cell-cycle progression. In this manner, MNF-β serves as an effector molecule within the myogenic programme to co-ordinate cell-cycle withdrawal with terminal differentiation. Proteins of the mSin3B family are present within developing cardiac and skeletal muscles at stages where MNF proteins are also expressed (Figures 6E–6H), and therefore are available to function in the manner proposed by this hypothesis. Moreover, mSin3B appears to translocate from the cytoplasm to the nucleus during the transition of proliferating skeletal myoblasts into post-mitotic, differentiated myotubes (Figures 6C and 6D), an event that corresponds temporally with the expression of MNF-β in these cells [12].

A second function of MNF-β–mSin3 repressor complexes may be to promote or maintain the quiescent state of satellite cells, the undifferentiated myogenic stem cells that are capable of supporting regeneration of adult muscles [14]. The relative importance of MNF-dependent gene regulation by comparison with MNF-independent pathways that modulate cell proliferation during myogenic differentiation and satellite-cell formation cannot be ascertained at this time, but should be defined by analysis of mice bearing null mutations in the *MNF* gene, which are currently being developed. By analogy with the function of distantly related winged-helix transcription factors that serve as distal effectors in signalling pathways initiated by extracellular growth factors in *C. elegans* [5], *Xenopus* [7], mouse [9,10] and human [8], we speculate that MNF-β also may function downstream in signalling pathways triggered by extracellular factors that promote myogenic differentiation in vertebrates.

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