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The basic helix-loop-helix transcription factor Mist1 functions as a transcriptional repressor of myoD.

Claudie Lemerrier^{1,2}, Robert Q To^{1,3}, Rosa A Carrasco, and Stephen F Konieczny*

¹ Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

² Present address: Laboratoire de Greffe de Moelle, UMR 5540, Université Bordeaux 2, 146 rue Leo Saignat, 33076 Bordeaux Cedex, France

³ Present address: The Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121, USA

* Corresponding author: Stephen F. Konieczny, sfk@bilbo.bio.purdue.edu

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Abstract

A good model system to examine aspects of positive and negative transcriptional regulation is the muscle-specific regulatory factor, MyoD, which is a basic helix-loop-helix (bHLH) transcription factor. Although MyoD has the ability to induce skeletal muscle terminal differentiation in a variety of non-muscle cell types, MyoD activity itself is highly regulated through protein-protein interactions involving several different co-factors. Here we describe the characterization of a novel bHLH protein, Mist1, and how it influences MyoD function. We show that Mist1 accumulates in myogenic stem cells (myoblasts) and then decreases as myoblasts differentiate into myotubes. Mist1 functions as a negative regulator of MyoD activity, preventing muscle differentiation and the concomitant expression of muscle-specific genes. Mist1-induced inhibition occurs through a combination of mechanisms, including the formation of inactive MyoD-Mist1 heterodimers and occupancy of specific E-box target sites by Mist1 homodimers. Mist1 lacks a classic transcription activation domain and instead possesses an N-terminal repressor region capable of inhibiting heterologous activators. Thus, Mist1 may represent a new class of repressor molecules that play a role in controlling the transcriptional activity of MyoD, ensuring that expanding myoblast populations remain undifferentiated during early embryonic muscle formation.

Introduction

During embryonic development, specific biochemical and morphological changes occur in response to extracellular signals that target individual cells within a particular organ or tissue. In many cases, the responses represent major changes in gene expression patterns. To ensure that gene activity is modulated correctly under these conditions, the transcription machinery must be regulated precisely by a number of positive- and negative-acting transcription factors. Among the different transcription factor families that have been identified, the basic helix-loop-helix (bHLH) family has been shown to be a key regulator of many different developmental pathways. In the case of skeletal muscle, four bHLH muscle regulatory factors (MRFs) (known as MyoD, myogenin, MRF4 and Myf-5) are involved in the differentiation and maintenance of the skeletal muscle phenotype (reviewed in Buckingham, 1994; Olson and Klein, 1994; Ludolph and

Konieczny, 1995). Forced expression of any one MRF in non-muscle cells results in the conversion of the cells to a myogenic stem cell population that can be induced to express genes involved in terminal differentiation. The MRFs, like most bHLH proteins, form homodimers, but preferentially heterodimerize with the widely expressed E2A gene products, E12 and E47 (Blackwell and Weintraub, 1990; Lassar *et al.*, 1991), or with the related bHLH protein HEB (Zhang *et al.*, 1991; Hu *et al.*, 1992). It is this high affinity, stable heterodimer complex that binds to a conserved DNA motif (E-box; -CANNTG-) which is present in the promoter or enhancer regions of many developmentally regulated genes, including genes involved in myogenic differentiation events.

The MRFs are classified as myogenic 'activator' proteins because each contains at least one activation domain that is able to induce gene transcription. However, not all bHLH proteins exhibit activator properties. Many function instead as negative regulators of gene transcription. For example, Id (Benezra *et al.*, 1990), which possesses a HLH motif but lacks a basic domain, is capable of forming heterodimers with bHLH proteins and thereby preventing DNA binding. Id inhibits MyoD activity *in vivo* by forming either transcriptionally inactive complexes of MyoD-Id or by forming heterodimers with E-proteins and effectively blocking the formation of active MyoD-E-protein complexes (Benezra *et al.*, 1990; Jen *et al.*, 1992). A second class of negative regulators are the HES proteins, which are bHLH factors that exhibit a repressor activity that manifests itself through binding to an E-box-related (N-box) DNA target sequence (Sasai *et al.*, 1992). Similarly, the mouse bHLH protein Twist has been shown to alter the activity of the MyoD protein family by blocking DNA binding, by titrating E-proteins and by inhibiting trans-activation of the MyoD co-factor, MEF2 (Spicer *et al.*, 1996; Hamamori *et al.*, 1997). Thus, both bHLH and HLH proteins can modulate effectively the transcriptional activity of the MyoD family and regulate directly how MyoD controls myogenic events during development.

The above studies have revealed the importance of bHLH proteins in regulating both gene transcription and cell fate determination. They have also demonstrated that active and negative strategies are involved in controlling the establishment and maintenance of a particular phenotype. In order to understand better how bHLH proteins interact and regulate each other's activities, we recently identified an additional member of the complex bHLH protein network, Mist1 (Mak *et al.*, 1996). The *Mist1* gene exhibits a complicated expression pattern, with transcripts being detected in several different tissues including skeletal muscle derivatives (Lemerrier *et al.*, 1997). Specifically, *Mist1* transcripts are expressed in skeletal muscle-forming regions during embryonic days E12.5 and E16.5, but disappear rapidly as muscle cells differentiate. This partially overlapping expression pattern with the MyoD protein family prompted us to examine whether Mist1 interacts with the muscle regulatory factors and whether it modulates MRF activity. In this report, we show that Mist1 protein accumulates in undifferentiated myoblasts, but levels rapidly decrease as cells begin to differentiate. In addition, although Mist1 associates with MyoD, the heterodimer complex of Mist1-MyoD is unable to bind to muscle-specific E-box elements. Mist1 also inhibits MyoD from inducing muscle fiber formation in transfected C3H10T1/2 fibroblasts and represses MyoD from activating gene transcription in myogenic cells. This repression mechanism is complex, however, involving DNA binding competition as well as titration of E-proteins and the presence of a strong repressor domain in the N-terminus of the Mist1 protein. Our results suggest that Mist1 inhibits the activity of the muscle regulatory factors in skeletal muscle and also raise the possibility that Mist1 may serve as a regulator of other bHLH proteins in additional developmental systems.

Results

Mist1 protein is co-expressed with MyoD in proliferating myoblasts

We previously reported the cloning and characterization of a cDNA encoding a novel bHLH protein, Mist1, which exhibits a unique expression pattern during embryonic development and in adult tissues (Lemerrier *et al.*, 1997). Although Mist1 is capable of binding to an E-box DNA target sequence as a homodimer, Mist1 protein complexes do not activate gene transcription when tested in a variety of experimental systems (Lemerrier *et al.*, 1997). Since the *Mist1* gene is expressed in skeletal muscle-forming regions during embryonic development, we set out to investigate the possibility that Mist1 may regulate members of the MyoD MRF family.

To address this possibility, a derivative of the C2 myogenic cell line (C2C7) was tested for Mist1 expression. C2 cells express MyoD in undifferentiated myoblasts as well as in differentiated myotubes (Vaidya *et al.*, 1989). Examination of Mist1 protein accumulation in these cells reveals that Mist1 is present at relatively high levels in undifferentiated myoblasts, but as differentiation proceeds over a 3 day period Mist1 protein accumulation becomes reduced, with only very low levels, if any, detected in the myotube cultures (Figure 1). Conversely, as differentiation progresses, high levels of skeletal myosin accumulate. Thus, Mist1 is co-expressed with MyoD in myoblasts but not in differentiated myotubes, suggesting the possibility that Mist1 and MyoD may interact within a common regulatory network in proliferating myogenic stem cell populations.

Mist1 forms homodimers and heterodimers with MyoD and E-proteins

In order to examine potential interactions between MyoD and Mist1, electrophoretic mobility shift assays were performed using *in vitro* translated proteins and a ³²P-labeled E-box oligonucleotide. As shown in Figure 2, Mist1 homodimers readily interact with the E-box sequence whereas MyoD homodimers do not bind to the DNA. When MyoD and Mist1 are co-incubated with the E-box probe, no new DNA-bound complexes are observed. Interestingly, addition of MyoD to these reactions produces a noticeable reduction in Mist1 homodimer binding, suggesting that MyoD interacts with Mist1, but that the MyoD–Mist1 heterodimer complex does not bind to the target DNA. Mist1 also forms a heterodimer with the E-protein E47, but in this instance the Mist1–E47 complex binds to the E-box site (Figure 2).

The reduction in Mist1–Mist1 DNA complexes observed in the presence of MyoD prompted us to investigate directly whether Mist1 interacts with this bHLH muscle regulatory factor. For these studies, *in vitro* protein–protein interaction assays were performed using a histidine-tagged Mist1 protein (His-Mist1) and a variety of ³⁵S-labeled *in vitro* translated proteins. Nickel beads, with or without His-Mist1, were incubated with the ³⁵S-labeled proteins, and bound proteins were subsequently resolved on an SDS–PAGE gel. When His-Mist1 is incubated with [³⁵S]Mist1, a strong, specific 24 kDa band is detected by autoradiography (Figure 3), confirming that Mist1 homodimers readily form under these conditions. Similarly, Mist1 forms heterodimers with the [³⁵S]MyoD and [³⁵S]E47 proteins (as well as with [³⁵S]MRF4, data not shown). However, no interactions are observed when His-Mist1 and [³⁵S]c-Fos or [³⁵S]MLP are tested. These *in vitro* studies demonstrate that Mist1 interacts with the bHLH proteins Mist1, MyoD, MRF4 and E47, and that formation of homodimers or heterodimers occurs independently of DNA target sites. In addition, Mist1 protein interactions are limited to bHLH factors since no interactions are observed with c-Fos (leucine zipper protein), MLP (LIM-domain protein) or with the MADS domain protein MEF-2 (data not shown).

Mist1 functions as a potent inhibitor of MyoD activity

Given that the *Mist1* and *MyoD* genes exhibit partial overlapping expression patterns (Lemerrier *et al.*, 1997) and the proteins directly interact with each other, we next examined whether *Mist1* influences *MyoD* activity when co-expressed in cells. C3H10T1/2 fibroblasts were transfected with *MyoD*, *Mist1* or a combination of expression plasmids, and the number of *MyoD*-generated myocytes was measured. As expected, *MyoD* efficiently induces the formation of muscle fibers that express skeletal muscle myosin (Figure 4A). However, when *Mist1* is co-transfected with *MyoD*, fiber formation and myosin expression are severely reduced (Figure 4A). Analysis of cell extracts prepared from these cultures confirmed that high levels of myosin protein accumulate in the *MyoD*-transfected cells but very little skeletal myosin is detected in cells co-transfected with the *MyoD* plus *Mist1* cDNA constructs (Figure 4B). Importantly, the reduction in fiber formation and myosin accumulation is not due to a *Mist1*-dependent decrease in *MyoD* protein levels. Cells transfected with the *MyoD* expression plasmid, with or without the *Mist1* expression plasmid, exhibit similar levels of *MyoD* (Figure 4B). Thus, *Mist1* inhibits *MyoD* activity without altering the synthesis or stability of the *MyoD* protein.

In an effort to identify the mechanism(s) by which *Mist1* represses *MyoD* activity, we also examined the effect *Mist1* has on *MyoD*-dependent transcription. For these studies, *MyoD* and/or *Mist1* expression plasmids were co-transfected into C3H10T1/2 cells with troponin I luciferase (TnI-Luc) or (E-box)₄-CAT (containing four copies of an E-box site) reporter genes. As expected, cells expressing *MyoD* generate high levels of TnI luciferase activity (Figure 5A). However, when *MyoD* and *Mist1* expression plasmids are tested at a 1:1 ratio, TnI-Luc expression is repressed by 85%. A similar *Mist1*-dependent repression (75% reduction) is obtained when the (E-box)₄-CAT reporter gene is tested (Figure 5A). At higher *Mist1*:*MyoD* ratios (5:1) (Figure 5B), TnI-Luc expression is completely absent, confirming that *Mist1* functions as a potent inhibitor of *MyoD* activity. Once again, this inhibition is specific since *Mist1* does not influence the transcriptional activity of non-bHLH factors nor does *Mist1* affect transcription from control SV40-Luc, CMV-LacZ and MEF2-CAT reporter genes (data not shown).

The repressor function of *Mist1* is somewhat analogous to the repressor activity associated with Id, a HLH protein that inhibits the activity of the MRFs by forming DNA-binding inactive complexes of Id-*MyoD* or Id-E-proteins (Jen *et al.*, 1992). In order to compare the ability of Id and *Mist1* to repress *MyoD* activity, we tested both proteins in *MyoD* transfection assays. When equal concentrations of Id and *MyoD* expression plasmids are tested in C3H10T1/2 cells, TnI-Luc activity is reduced ~50%, and at a 5:1 molar ratio, only 10% of *MyoD*-dependent activity remains (Figure 5B). In contrast, at these concentrations, *Mist1* represses TnI-Luc activity by 85 and 100%, respectively. Although it is difficult to compare directly the repressor activity associated with these two proteins, it is clear that *Mist1* inhibits *MyoD* activity at least as well as Id represses *MyoD* activity. Additionally, since *Mist1* is a bHLH factor with DNA-binding activity, and Id represents a HLH factor lacking DNA-binding activity, the mechanism of *Mist1*-induced inhibition may be substantially different from the mechanism by which Id represses the MRFs (see below).

Mist1 repression partially occurs through a DNA-binding mechanism

There are several mechanisms by which *Mist1* may inhibit *MyoD* activity, including: (i) titration of E-proteins from *MyoD*, resulting in a decreased number of transcriptionally active *MyoD*-E-protein complexes, (ii) occupancy of essential E-box sites, thereby preventing *MyoD*-E-proteins from binding to DNA or (iii) dimerization with *MyoD*, forming a DNA-binding inactive complex. To understand further the contribution of protein dimerization versus DNA binding involved in *Mist1* repression, C3H10T1/2 fibroblasts were transfected with expression plasmids encoding *MyoD*, E47 or a tethered *MyoD*~E47

protein (Neuhold and Wold, 1993) in the presence or absence of Mist1. As shown previously, Mist1 efficiently represses MyoD-dependent TnI-Luc expression, whereas the related bHLH factor, Mash1 (Johnson *et al.*, 1990), has little effect on MyoD activity (Figure 6A). Co-transfection of MyoD and E47 into the C3H10T1/2 cells produces the same high levels of TnI-Luc activity as observed with MyoD alone. Interestingly, inclusion of Mist1 in the MyoD + E47 group again generates an 85% reduction in reporter gene expression, suggesting that the additional E47 protein is not sufficient to reverse the Mist1-dependent inhibition of TnI-Luc gene expression. Thus, it seems likely that Mist1 repression is not dependent on a simple E-protein titration model. As a confirmation of this hypothesis, we also co-transfected Mist1 with a tethered MyoD~E47 construct (Neuhold and Wold, 1993). MyoD~E47 possesses the same activating properties that are associated with the individual MyoD and E47 proteins, but the tethered MyoD~E47 protein is highly resistant to Id repression since stable intramolecular MyoD-E47 heterodimer complexes rapidly form (Neuhold and Wold, 1993). Surprisingly, when Mist1 is co-transfected with MyoD~E47, a large decrease (90%) in reporter gene activity is observed, which also parallels the decrease in MyoD~E47-induced muscle fiber formation (Figure 6B). Conversely, overexpression of Id has little effect on MyoD~E47-induced myogenesis (Figure 6B), reinforcing the idea that Mist1 repression does not involve a strict titration of available bHLH factors but primarily involves a DNA-binding mechanism.

To test this latter hypothesis, electrophoretic mobility shift assays were performed using a ³²P-labeled E-box probe and *in vitro* translated proteins. Co-incubation of MyoD with E47 generates the predicted MyoD-E47 heterodimer complex that binds efficiently to the E-box site (Figure 7A). Incubation of Mist1 with E47 results in two distinct DNA-binding complexes, a Mist1-Mist1 homodimer and a Mist1-E47 heterodimer. When increasing amounts of Mist1 are added to the MyoD + E47 reactions, a progressive reduction in the formation of DNA-bound MyoD-E47 complexes occurs that coincides with an increase in the formation of DNA-bound Mist1-E47 heterodimers and Mist1-Mist1 homodimers. These results suggest that Mist1 is able to disrupt a transcriptionally active complex, such as MyoD-E47, to form two additional DNA-binding complexes (Mist1-Mist1, Mist1-E47) that, none the less, remain transcriptionally inactive on muscle genes. Interestingly, Mist1 has no effect on the DNA-binding activity associated with the MyoD~E47 tethered protein (Figure 7B), even though MyoD~E47 transcriptional activity is severely repressed by Mist1 (Figure 6A).

The experiments described above strongly support the idea that Mist1 represses skeletal muscle differentiation primarily through occupancy of E-box sites and not through titration of specific bHLH factors in the cell. In order to test this hypothesis, we generated a mutant Mist1 cDNA (Figure 8A) that contains a substitution of the basic domain amino acids RER with GGG (Mist1^{mut basic}) (see Materials and methods for details). We reasoned that an altered protein which retains dimerization but not DNA-binding activity would no longer function as a repressor if Mist1 repression occurs primarily through DNA interactions. As predicted, both the wild-type Mist1 and the Mist1^{mut basic} proteins efficiently form homodimers when tested in an *in vitro* binding assay (Figure 8B). However, Mist1^{mut basic} homodimers fail to bind to E-box sites whereas the wild-type Mist1 protein readily forms a Mist1-Mist1-DNA complex (Figure 8C).

In order to test the new Mist1^{mut basic} protein *in vivo*, we co-transfected Mist1 expression plasmids along with a MyoD expression plasmid and the TnI-Luc reporter gene into C3H10T1/2 fibroblasts. As shown in Figure 8D, wild-type Mist1 inhibits MyoD-induced activation of the TnI-Luc reporter whereas Mist1^{mut basic} has no effect, despite the fact that the Mist1^{mut basic} protein stably accumulates in the cell and translocates to the nucleus (data not shown). As a further test for *in vivo* activity, we examined the ability of Mist1 to block the activity of the endogenous MyoD protein in the myogenic cell line C2C7. As predicted, C2C7 cells expressing the wild-type Mist1 protein fail to activate expression of the TnI-Luc reporter gene (Figure 8E). In contrast, the Mist1^{mut basic} protein has no effect on C2C7 terminal differentiation, even though Mist1^{mut basic} is capable of dimerizing with

MyoD and E47 (unpublished data). Taken together, these data suggest that Mist1 primarily inhibits MyoD in proliferating myoblasts through a DNA-binding mechanism and that the contribution of dimerization with endogenous bHLH proteins is not a significant factor given that the Mist1^{mut basic} protein, which retains dimerization function, no longer represses MyoD in these myogenic cells.

Mist1 possesses an N-terminus repressor domain

The ability of Mist1 to repress MyoD activity could involve a passive mechanism of occupancy of E-box sites or involve an active mechanism by which a repressor domain inhibits E-box-dependent transcription. In order to distinguish between these possibilities, several Gal4 DNA-binding domain (Gal4 DB)-Mist1 fusion proteins were generated and their ability to repress expression of CAT reporter genes analyzed. For these studies, we took advantage of a CAT reporter system (Hollenberg *et al.*, 1995) in which five Gal4-binding sites are positioned downstream of eight LexA operators (L8G5-CAT) (Figure 9). This construct then was co-transfected into C3H10T1/2 cells with or without a LexA-VP16 activator and various Gal4-Mist1 fusion constructs. As predicted, when a plasmid containing only the Gal4 DB is tested, no CAT activity is detected, confirming that the Gal4 DB has no transcriptional activity in this system (Figure 9). However, when the LexA-VP16 expression plasmid is co-transfected with Gal4 DB, high levels of CAT activity are observed. When Gal4 DB-Mist1(1-197) (full-length Mist1) is tested alone, again no CAT activity is detected, confirming our previous results that Mist1 lacks a functional activation domain (Lemercier *et al.*, 1997). Interestingly, when LexA-VP16 is included in this group, the Gal4 DB-Mist1(1-197) represses LexA-VP16-induced CAT activity by ~90%. Similar repression is also obtained when Gal4 DB-Mist1(1-126) and Gal4 DB-Mist1(1-71) are tested, whereas no repression is observed with Mist1(131-197). The repression associated with Gal4 DB-Mist1(1-197), -Mist1(1-126) and -Mist1(1-71) is dependent on DNA binding since no repression occurs with a reporter gene lacking Gal4-binding sites (L8-CAT). To examine if the repression domain of Mist1 can be overridden, we also generated a Mist1 construct containing the VP16 transcriptional activation domain. As shown in Figure 9, the control Gal4 DB-VP16 efficiently activates expression of the L8G5-CAT reporter gene. Interestingly, when the Gal4 DB-Mist1(1-197)-VP16 expression construct is tested, high levels of CAT activity are also obtained. Similarly, Gal4 DB-Mist1(1-197)-VP16 no longer represses the activity of LexA-VP16 (Figure 9). Taken together, these results suggest that Mist1 inhibits gene transcription through a DNA-binding, active repression mechanism, with a potential repressor domain located at the N-terminus of the protein.

Discussion

Skeletal myogenesis is controlled by a number of transcription factors, including the four members of the MyoD family. Forced expression of the myogenic bHLH proteins in non-muscle cells often overrides pre-existing developmental fates by instructing cells to enter a myogenic lineage pathway. The dominance of the MRFs underscores the need to modulate their activity precisely in muscle-forming regions since overactivity may result in inappropriate muscle formation (Weintraub *et al.*, 1989; Miner *et al.*, 1992). Similarly, inhibition of MRF activity is critical to non-muscle tissues to ensure that the normal developmental potential of these cells is not adversely affected. In this study, we report that Mist1, a novel bHLH protein that is transiently expressed in embryonic skeletal muscle-forming regions, interacts with MyoD and inhibits the transcriptional activity associated with this MRF. The mechanism by which Mist1 represses MyoD activity is complex, and involves a combination of occupancy of E-box sites as well as use of an active repressor domain. This combination defines a new repressor protein that probably operates to modulate activity of bHLH factors in myogenesis as well as bHLH factors found in a variety of other developmental systems.

The need for negative regulators of MyoD activity is an obvious requirement when one considers how myogenic stem cells remain as an undifferentiated cell population in tissue culture model systems, in cells found within the developing limb buds and in regenerating satellite cells. In each case, proliferating myoblasts express high levels of MyoD and yet remain undifferentiated until appropriate environmental conditions are attained, conditions that are usually associated with the depletion of specific growth factors (e.g. fibroblast growth factors) from the local environment. Indeed, the role of growth factor-induced post-translational modifications associated with MyoD obtained from myoblasts versus differentiated myotubes has been examined extensively (reviewed in Ludolph and Konieczny, 1995). Many of these studies have failed to identify a specific modification(s) that is responsible for converting MyoD from an inactive to an active transcription factor complex. A second avenue of investigation has been to identify factors that interact directly with MyoD to block its activity. Candidate gene products are *Id* and *Twist*, both of which prevent MyoD activity in a variety of experimental situations. In this current study, we provide supporting data that *Mist1* similarly may serve as a negative regulator of MyoD. The relevance of *Mist1* acting as a MyoD negative regulator is even more compelling given that the *Mist1* protein accumulates to high levels in myoblasts, where MyoD activity needs to be kept in check, and then *Mist1* protein levels decrease in differentiated myotubes, where MyoD activity is required to be maximal.

Mist1 contains a central bHLH motif that is utilized for DNA binding to E-box regulatory elements and for protein dimerization with other bHLH factors. Unlike most bHLH factors, *Mist1* lacks a transcription activation domain and instead exhibits an inhibitory potential. The mechanism of inhibition is complicated by the fact that *Mist1* forms both homodimer and heterodimer bHLH complexes. For instance, *Mist1*-MyoD heterodimers form, but since these complexes do not bind to DNA, a net decrease in the active MyoD protein pool ensues. In this instance, *Mist1* resembles the HLH protein *Id*, which interacts with MyoD and E47, forming inactive heterodimers that no longer bind to DNA (Benezra *et al.*, 1990; Jen *et al.*, 1992). However, unlike *Id*, *Mist1* efficiently challenges a tethered MyoD~E47 complex (Neuhold and Wold, 1993) when assayed in an E-box-dependent reporter gene system or when examining MyoD~E47-induced myofiber formation. These results suggest that *Mist1* and *Id* utilize different molecular strategies for repressing MyoD activity. This is especially relevant for *Mist1*-E47 heterodimers, which continue to interact with E-box sequences without leading to transcriptional activation. Thus, although *Mist1* forms inactive heterodimer complexes with other bHLH factors, we favor a model by which *Mist1*-directed repression occurs primarily through muscle-specific E-box occupancy by *Mist1*-*Mist1* homodimers (see Figure 10). In this scenario, *Mist1* levels in proliferating myoblasts are sufficient to compete with MyoD for muscle-specific E-box sites. When cells commit to terminal differentiation, *Mist1* transcription ceases, leading to functional MyoD activity and activation of muscle-specific genes.

Mist1 shares several similarities with the HES-1 proteins, the mammalian homologs for *Drosophila hairy* and *enhancer of split*. Like *Mist1*, HES-1 is able to repress MyoD-induced fiber formation and inhibit expression of muscle-specific reporter genes, probably by preventing MyoD from binding to E-box sequences (Sasai *et al.*, 1992). In addition, HES-1 uses an N-box recognition motif (-CACNAG-) to trigger an active transcriptional repression mechanism. Although there are some similarities between *Mist1* and HES-1, *Mist1* cannot be classified as a HES family member since it is unable to bind to N-box sequences (unpublished data) and *Mist1* lacks the conserved C-terminal amino acid motif WRPW which is present in *hairy*, *enhancer of split* and HES proteins and which is involved in protein dimerization and transcriptional repression (Dawson *et al.*, 1995; Fisher *et al.*, 1996). Other HLH proteins which inhibit muscle development are *Id* (Jen *et al.*, 1992) and *Twist* (Rohwedel *et al.*, 1995; Spicer *et al.*, 1996; Hamamori *et al.*, 1997). The mechanism of myogenic inhibition associated with these factors often involves the disruption or titration of molecules that participate in myogenesis, namely members of the MyoD and E-protein families. However, these repressor proteins also interact with specific co-regulators of the MyoD family to inhibit muscle-specific transcription similarly.

For instance, Twist has been shown to form a heterodimer complex with E-proteins, which then represses the ability of MEF2 transcription factors to promote myogenesis (Spicer *et al.*, 1996). Since MEF2 cooperates with MyoD to generate high levels of muscle transcription, restricting MEF2 activity may ultimately modulate MyoD activity. We have not examined formally whether Mist1 blocks MyoD–MEF2 interactions. However, given the muscle and non-muscle expression patterns associated with the *Mist1* and *MEF2* genes, it is tempting to speculate that Mist1 may alter MEF2 activity in other developmental contexts by associating with additional bHLH proteins that may be part of different regulatory pathways. Further studies will be designed to examine these possibilities.

At this time, the mechanism by which Mist1 homodimers, when bound to specific E-box sites, inhibit gene expression remains unknown. Clearly, Mist1 utilizes an N-terminal repressor domain that is sufficient to influence negatively transcription of heterologous systems. Thus, one possibility is that the Mist1 repression domain directly interacts with a specific TATA box-binding protein (TBP)-associated factor (TAF) that is associated with TBP to inhibit polymerase II transcription. Similarly, Mist1 may function by recruiting deacetylases to potential transcription complexes, thereby promoting core histone deacetylation and transcriptional repression. Analogous transcription mechanisms have been proposed for several well characterized transcription factor complexes, including N-CoR, a known co-repressor for the thyroid hormone receptor (Hörlein *et al.*, 1995; Zamir *et al.*, 1996; Heinzl *et al.*, 1997) and mSin3, a co-repressor for the Mad–Max transcription complex (Ayer *et al.*, 1995; Alland *et al.*, 1997). Additional function/structure studies will be required to characterize the essential properties associated with the Mist1 repressor domain. Regardless of the precise mechanism, however, the specificity of the system relies on two variables; the embryonic expression pattern of the *Mist1* gene and specific E-box-dependent target genes.

Materials and methods

DNA constructions

All of the cDNAs tested in mammalian cells were subcloned into the pcDNA3 expression vector (Invitrogen). MyoD, E47 and MyoD~E47 cDNA inserts were isolated from pECE-MyoD, pECE-E47 and pECE-MyoD~E47, respectively (Neuhold and Wold, 1993) by digestion with *Hind*III and *Eco*RI and subcloned into pcDNA3 at the *Hind*III and *Eco*RI sites. The Mash1 cDNA was excised from pBS-Mash1 (Johnson *et al.*, 1990) using a *Kpn*I digest and ligated into the *Kpn*I site in pcDNA3. The mouse Id1 cDNA was digested from pE:Id (Benezra *et al.*, 1990) with *Sma*I and the isolated insert ligated into the *Eco*RV site in pcDNA3. In order to produce Gal4 DB fusion proteins, the Gal4 DB region, plus part of the polylinker from the Gal4 (1–147) plasmid (Sadowski *et al.*, 1988), was digested with *Hind*III and *Xba*I and ligated into pcDNA3. Point mutations were introduced into pcDNA3-Mist1 using the 'Quick Change Site Directed Mutagenesis' kit from Stratagene Cloning Systems. The Mist1 basic mutant (Mist1^{mut basic}) contains alterations in amino acids 80–82 from RER to GGG. Mutations were confirmed by direct DNA sequencing.

All Mist1 deletions were generated by PCR as described previously (Lemerrier *et al.*, 1997) using pcDNA3-Mist1 (HA epitope-tagged at the N-terminus) as a template and the following 5' primers containing an *Eco*RI site and 3' primers containing a *Xba*I site: Mist1(1–197), 5' primer 5'-GGAATTCATGTATCCTTATGACGT-3' (*Eco*RI site underlined), and 3' primer 5'-CGTCTAGAGCTCCCCTCTCTGAAG-3' (*Xba*I site underlined); Mist1(1–126), 5' primer as above, and 3' primer 5'-CGTCTAGAGGCTGTCAGCGACTTG-3'; Mist1(1–71), 5' primer as above, 3' and primer 5'-CGTCTAGAACGCTGTTCTCCCTG-3'; Mist1(131–197), 5' primer 5'-GGAATTCATGTCCAGCAGCCGCCT-3', and 3' primer 5'-CGTCTAGATTACCAGTCTGGG GCT-3'. All PCR products were digested with *Eco*RI and *Xba*I and then ligated in-frame into the pcDNA3-Gal4 DB plasmid at the *Eco*RI and *Xba*I sites.

pcDNA3-Gal4-VP16 was obtained by isolating the Gal4-VP16 insert from a Gal4-VP16 plasmid (Sadowski *et al.*, 1988) using *Hind*III and *Xba*I and subsequently subcloning the insert into the pcDNA3 vector. Gal4 DB-Mist1-VP16 was generated by ligation of Gal4 DB-Mist1 (1–197) into pcDNA3-VP16 (Lemerrier *et al.*, 1997) using the available *Hind*III and *Xba*I sites. Bacterial His-tagged Mist1 protein was obtained by digesting the Mist1 cDNA from pcDNA3-Mist1 with *Kpn*I and *Xho*I and ligating the 700 bp fragment in-frame into pQE31 (Qiagen, Inc.) at the available *Kpn*I and *Sa*I sites.

Cell culture and DNA transfections

The C2C7 mouse myoblast line was derived from the original C2C12 myogenic cell line (Blau *et al.*, 1983) and was obtained from Anne Fernandez (Montpellier, France). C2C7 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM):Ham F12 nutrient mix (1:1) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 µg/ml). Terminal differentiation into myotubes was induced by replacing the culture medium with high glucose DMEM containing 2% horse serum for 1–3 days.

For cell transfections, C3H10T1/2 fibroblasts were plated at a density of 1×10^5 cells/35 mm well in basal Eagle's medium (Gibco) containing 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 µg/ml). C2C7 myoblasts were similarly plated in DMEM:Ham F12 nutrient mix growth medium as described above. Luciferase or CAT assays involved transfecting cells with 0.2 µg of each test plasmid (unless otherwise stated) along with 1 µg of a troponin I luciferase or 2 µg of (E-box)₄-CAT reporter plasmid as described previously (Johnson *et al.*, 1996). When pcDNA3-MyoD was present in the DNA precipitate, cells were induced to differentiate in low glucose DMEM containing 2% horse serum. After 48–72 h, luciferase activities were measured using a luciferase kit (Luciferase Assay System, Promega) as described previously (Lemerrier *et al.*, 1997). Luciferase activities were normalized to the protein content of each sample. For LexA-CAT assays, 2×10^5 C3H10T1/2 fibroblasts/60 mm dish were transfected with 2 µg of each Gal4 DB fusion protein plasmid, 2 µg of L8G5-CAT reporter plasmid (eight LexA operators with five Gal4-binding sites, upstream of the CAT gene) or 2 µg of the control reporter L8-CAT (eight LexA operators upstream of the CAT gene), in the presence or absence of 0.4 µg of LexA-VP16 activator plasmid (Hollenberg *et al.*, 1995). After 72 h, cell extracts were prepared (Naidu *et al.*, 1995) and protein content determined. The amount of cell extract used for each CAT assay was normalized to the protein level of each sample.

Immunohistochemistry

C3H10T1/2 cells (5×10^5 cells/100 mm dish) were transfected as described above with 10 µg of the pcDNA3-Mist1 expression vector with or without 10 µg of pcDNA3-MyoD. DNA concentrations were normalized using the empty pcDNA3 vector as a control. After 72 h in differentiation medium, cells were rinsed three times in phosphate-buffered saline (PBS) and then fixed in a 20:2:1 solution of 70% ethanol/formalin/acetic acid for 1 min at 4°C as described by Kong *et al.* (1995). Following a 30 min permeabilization/blocking step (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP-40, 5% horse serum), the plates were incubated with an MF-20 hybridoma supernatant (specific for striated muscle myosin heavy chains) for 1 h under gentle shaking and then rinsed three times in PBS. The primary complexes were detected using a biotinylated anti-mouse antibody and a horseradish peroxidase (HRP)-streptavidin conjugate (Vector Laboratories). Specific immunocomplexes were visualized by DAB staining and examined under an Olympus inverted phase contrast microscope.

Western blots

C3H10T1/2 fibroblasts were transfected as described above and then cell extracts were prepared by lysing the cells in 25 μ l of 4 \times SDS-PAGE loading dye (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 4% β -mercaptoethanol). Extracts were sonicated for 20 s and kept at -80°C until analyzed further. Extracts were prepared similarly from myoblast and myotube cultures of C2C7 cells. SDS-PAGE and Western blots were performed according to standard protocols. Blots were saturated for 1 h with TBS (10 mM Tris pH 8.0, 150 mM NaCl) containing 10% non-fat dry milk (w/v) prior to incubation with the primary antibody. For myosin detection, the blots were incubated in the MF-20 supernatant diluted 1:20 in TBS-10% non-fat dry milk for 1 h and then rinsed for 20 min in TBS-0.1% Tween-20 (TBS-T). The immunocomplexes were detected using an anti-mouse IgG-HRP conjugate (Santa Cruz, Inc.) and a chemiluminescence detection kit (Amersham). For MyoD and Mist1 detection, the primary antibodies were as follows: rabbit polyclonal anti-MyoD C20 (Santa Cruz, Inc.), mouse monoclonal anti-HA epitope (Boehringer Mannheim) and rabbit polyclonal anti-Mist1 serum raised against a GST-Mist1 fusion protein (Lemerrier *et al.*, 1997). The immunocomplexes were detected as above using an anti-mouse or anti-rabbit IgG-HRP conjugate and chemiluminescence.

Electrophoretic mobility shift assays

The DNA probes used in the gel mobility shift assay experiments were oligonucleotides derived from the troponin I IRE E-box consisting of the wild-type sequence 5'-GATCCGTCTGAGGAGACAGCTGCAGCTCC-3' (E-box site underlined). *In vitro* translated proteins were prepared as previously described (Lin *et al.*, 1991; Lemerrier *et al.*, 1997) using the pcDNA3 plasmids. Typical binding reactions contained 5 μ l of 5 \times binding buffer [25 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 5 mM dithiothreitol (DTT) and 10% glycerol], 0.5 μ g of poly(dI-dC), 3 μ l of *in vitro* translated protein, 300 fmol of the labeled DNA probe (typically \sim 10 000 c.p.m.) in a total volume of 25 μ l. The entire binding reaction mixture was allowed to assemble at room temperature for 30 min. For the Mist1 titration experiments, increasing amounts of Mist1 ranging from 1 \times to 4 \times in volume were added to the binding reactions during the incubation. Following incubation, all reactions were analyzed by loading the entire mixture onto a 5% non-denaturing gel and electrophoresis at 100 V for 3 h at room temperature. After electrophoresis, the gel was dried at 80°C for 1 h and the bound complexes visualized by autoradiography.

In vitro protein-binding assays

Approximately 1 μ g of bacterial produced His-tagged Mist1 fusion protein was conjugated to nickel (Ni²⁺) agarose beads and mixed with 5 μ l of [³⁵S]Mist1, -Mist1^{mut basic}, -MyoD, -E47, -MRF4, -c-Fos (Rauscher *et al.*, 1988) or -MLP (Arber *et al.*, 1994; Kong *et al.*, 1997) and allowed to incubate in 250 μ l of binding buffer (10 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.25% NP-40, 20 mM imidazole) at 4°C for 2 h with gentle rocking. Following this incubation, the complexes were subjected to three 1 ml washes with binding buffer followed by two additional washes with binding buffer minus NP-40. As a negative control, the ³⁵S-labeled proteins were incubated with nickel beads alone (no His-tagged Mist1) in the binding reaction. The isolated pellets were heated to 95°C for 5 min in SDS sample buffer and subjected to 12% SDS-PAGE gel electrophoresis. Protein interactions were visualized by autoradiography.

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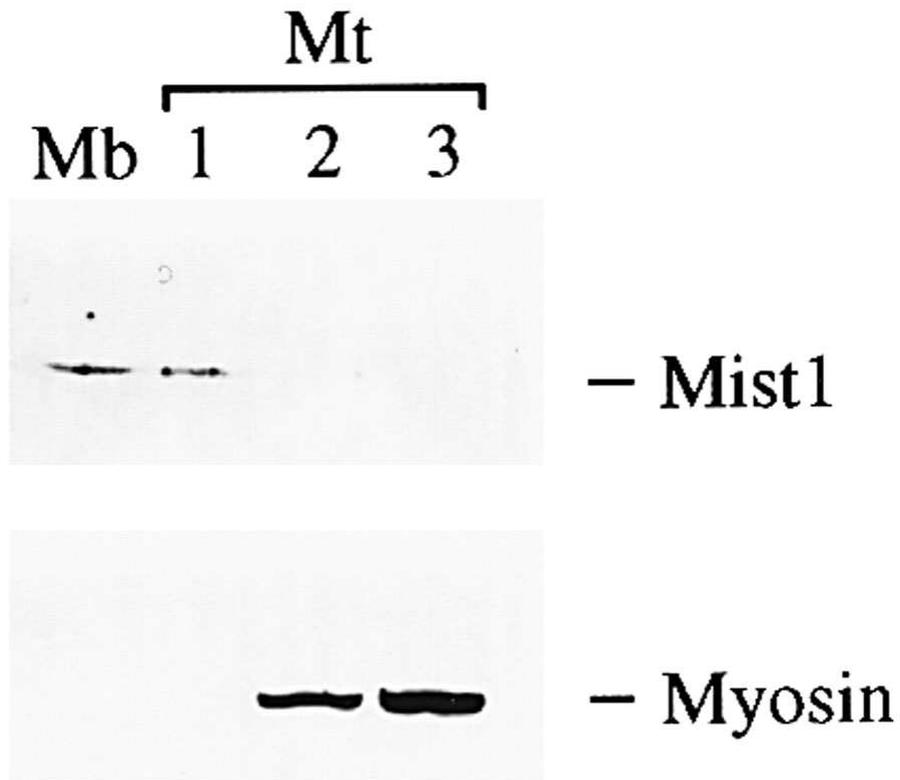


Figure 1: Mist1 protein accumulation decreases as myoblasts differentiate into myotubes. C2C7 myoblasts (Mb) were maintained in an undifferentiated state or induced to differentiate into myotubes (Mt) over a 3 day period (lanes 1-3) as described in Materials and methods. Protein extracts were harvested each day and then subjected to Western analysis using antibodies directed against Mist1 and skeletal myosin. Mist1 protein levels decrease as myoblasts differentiate into myotubes whereas skeletal myosin accumulates only in the myotube cultures.

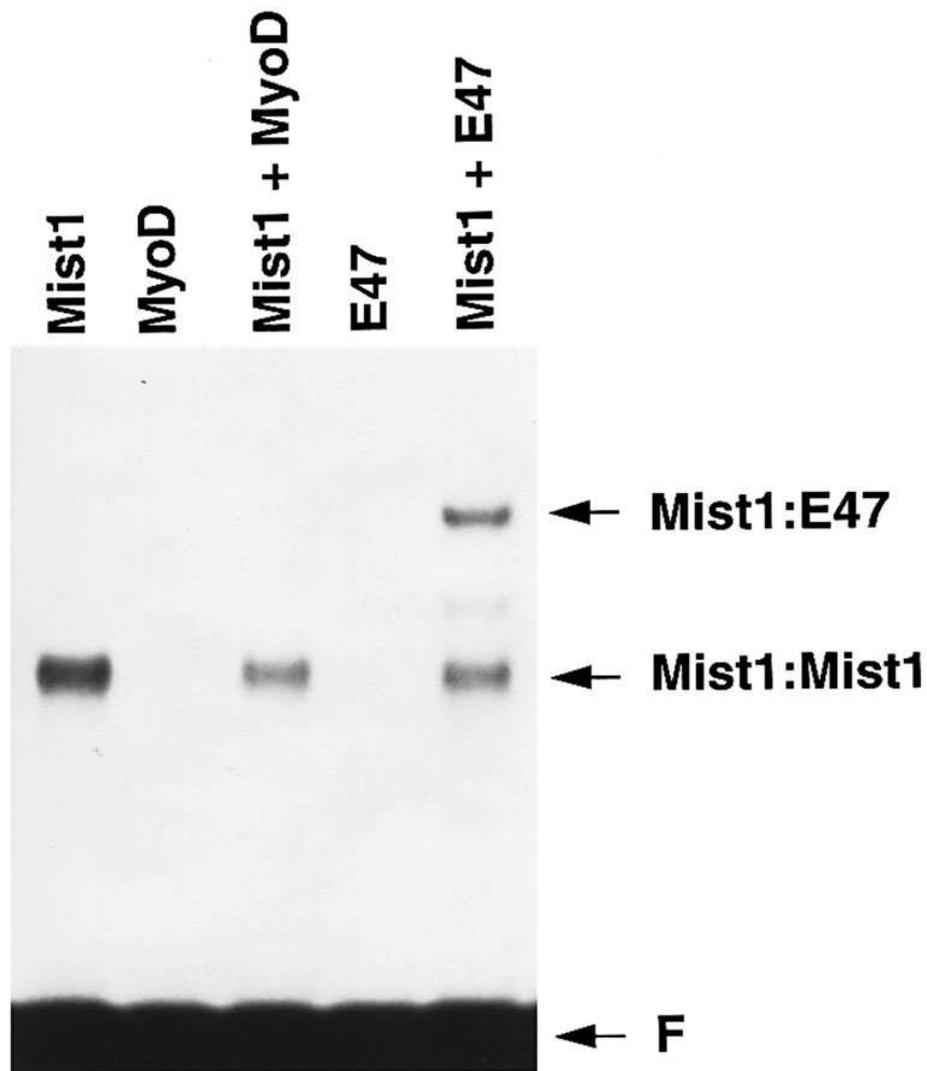


Figure 2: Mist1 homodimers interact with E-box DNA targets. *In vitro* translated Mist1, MyoD and E47 proteins were incubated with a ³²P-labeled TnI E-box oligonucleotide and subjected to electrophoretic mobility shift assays. Under these conditions, Mist1 forms homodimers which bind efficiently to the E-box site. In contrast, MyoD or E47 alone do not produce DNA-binding complexes. Incubation of Mist1 with MyoD leads to a decrease in Mist1 homodimer binding, suggesting that a Mist1-MyoD complex forms that does not bind to DNA. Similarly, Mist1-E47 heterodimers form but, in this instance, the heterodimer complex interacts with the E-box site. F, free probe.

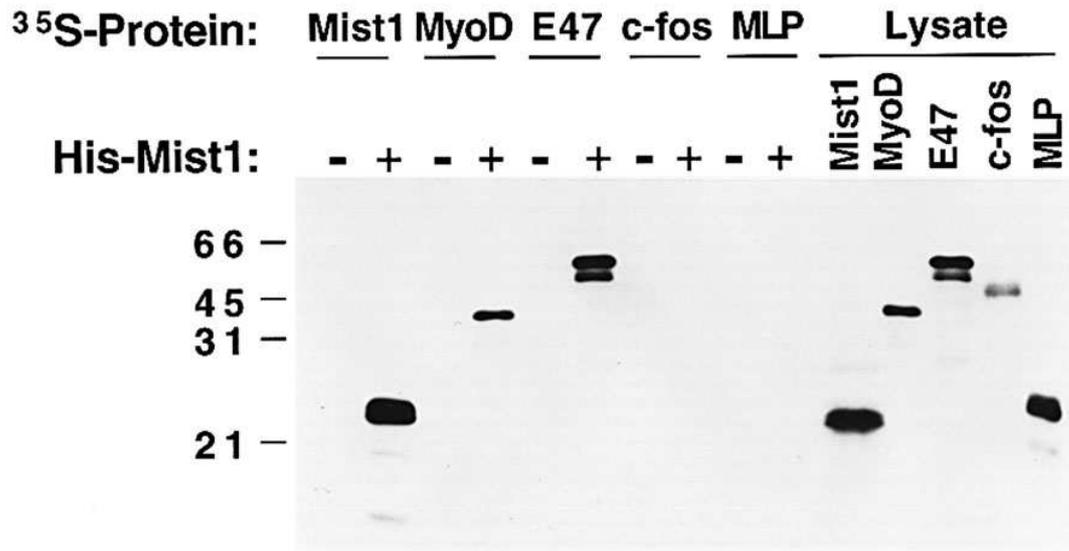


Figure 3: Mist1 forms both homodimers and heterodimers with MyoD and E47. A His-tagged Mist1 fusion protein (+) was conjugated to nickel agarose beads and mixed with [³⁵S]Mist1, -MyoD, -E47, -c-Fos or -MLP. The bound complexes then were resolved on an SDS-polyacrylamide gel and visualized by autoradiography. As a negative control, the ³⁵S-labeled proteins were incubated with nickel beads alone (-) in the binding reaction. Mist1 interacts efficiently with the bHLH proteins Mist1, MyoD and E47 but not with the control leucine zipper protein c-Fos or with the LIM domain protein MLP. The input [³⁵S]proteins are shown in the last five lanes for reference.

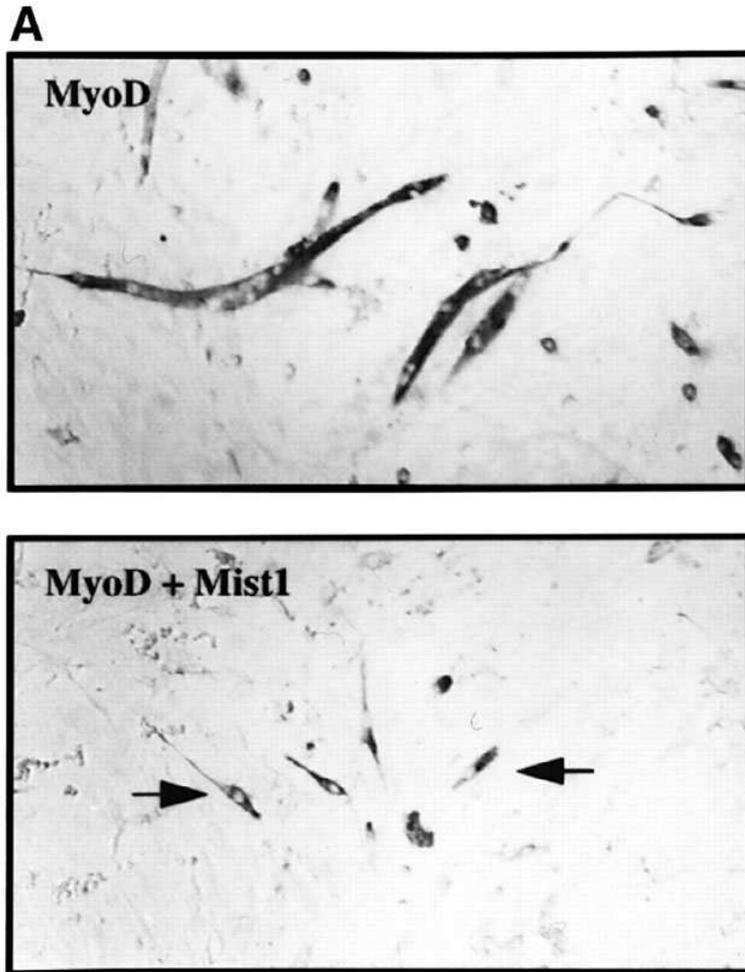


Figure 4: Mist1 inhibits MyoD-induced myogenesis. **(A)** C3H10T1/2 cells were transfected with expression plasmids containing MyoD or MyoD plus Mist1 and then induced to differentiate. Immunohistochemistry using a skeletal myosin-specific antibody reveals large differentiated myocytes in MyoD cultures whereas only rare, small myocytes (arrows) are detected in cultures co-expressing MyoD and Mist1. **(B)** Western analysis of protein extracts from cell cultures comparable with those in (A) reveal that skeletal myosin (MF-20) is detected in the MyoD-expressing cells but not in cells co-expressing MyoD and Mist1. Note that the level of MyoD protein (detected using an anti-HA antibody) is similar in both the MyoD and MyoD plus Mist1 groups.

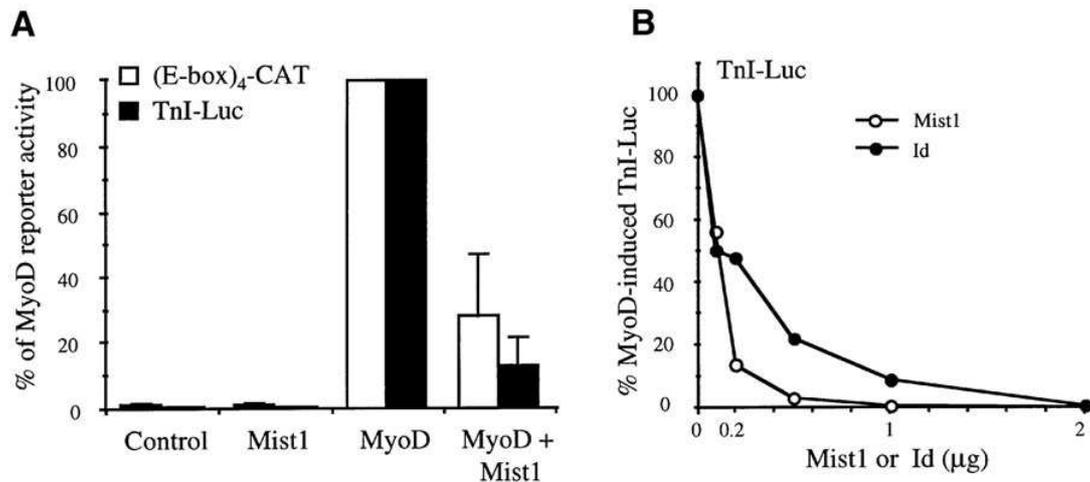


Figure 5: Mist1 inhibits MyoD from transcriptionally activating E-box-dependent reporter genes. **(A)** C3H10T1/2 cells were co-transfected with expression plasmids encoding MyoD or Mist1 and either the (E-box)₄-CAT or TnI-Luc reporter genes as described in Materials and methods. No reporter gene expression is detected in the control transfections (without MyoD or Mist1) or in the Mist1 alone group, whereas high levels of reporter gene expression are detected with MyoD. However, when MyoD and Mist1 (1:1) are co-transfected into the cells, reporter gene expression is reduced ~75–85%. Error bars indicate the standard deviation. **(B)** Mist1 and the HLH factor Id were co-transfected with MyoD (0.2 μg) into C3H10T1/2 cells as in (A) using increasing concentrations of expression plasmid DNA (0–2 μg). Although both Mist1 and Id inhibit MyoD-induced reporter gene expression, Mist1 is a more potent repressor in this assay system.

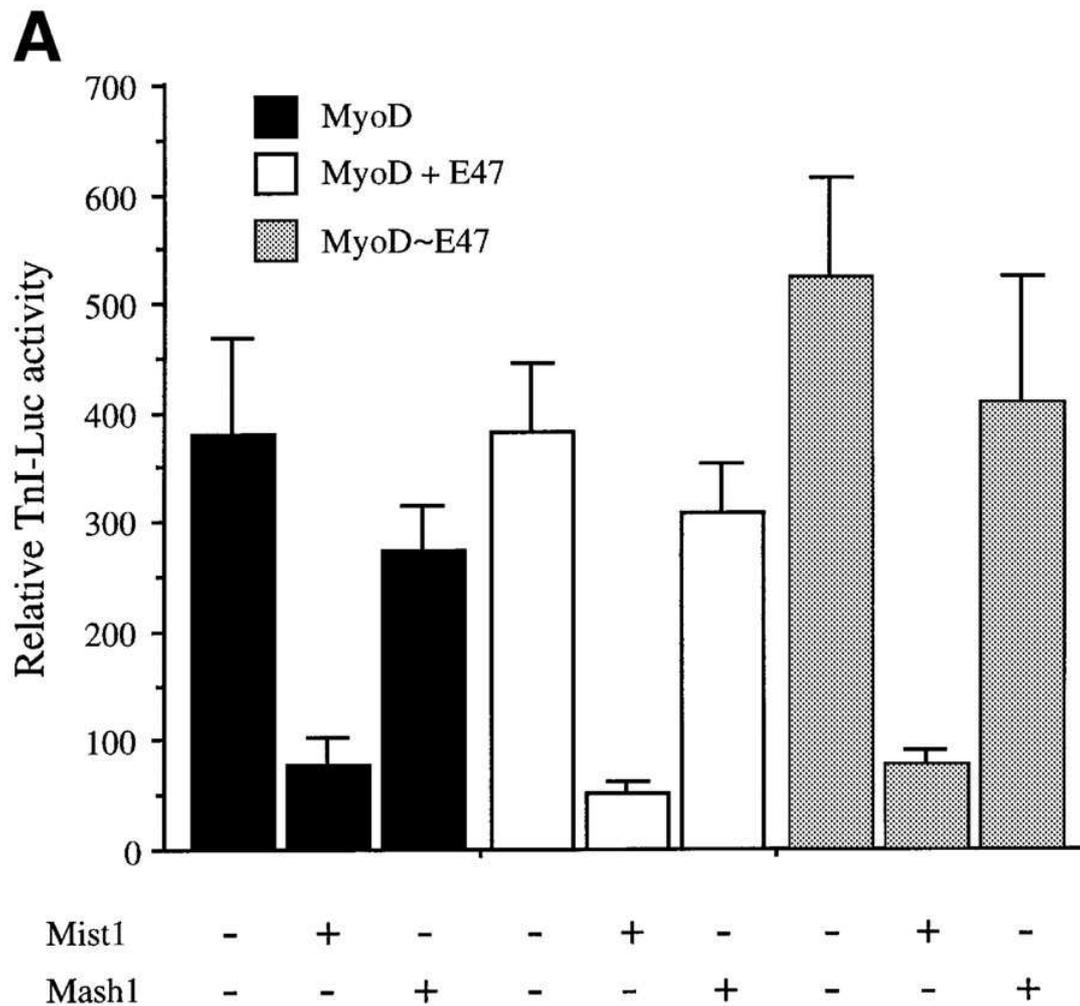


Figure 6: Mist1 inhibits MyoD activity even in the presence of an excess of E-proteins. **(A)** C3H10T1/2 cells were co-transfected with the indicated MyoD, E47 and MyoD~E47 expression plasmids, TnI-Luc and either Mist1 or Mash1. Analysis of TnI-Luc reporter gene activity reveals that Mist1 efficiently represses MyoD and the pre-formed tethered MyoD~E47 complex from activating the reporter gene. Similarly, Mist1 repression of MyoD is not altered by an excess of E47, suggesting that Mist1 inhibits MyoD activity through a mechanism that is independent of dimerization with E-proteins. The bHLH factor Mash1 serves as a negative control in these studies. Error bars indicate the standard error of the mean. **(B)** Western analysis of skeletal myosin and MyoD~E47 protein accumulation in C3H10T1/2 cells transfected with the MyoD~E47 expression plasmid, with or without the Mist1 or Id expression plasmids. As predicted, Mist1 represses MyoD~E47 function whereas Id has little effect on the myogenic-inducing activity associated with this pre-formed heterodimer complex. In all cases, the level of the MyoD~E47 protein, as detected with an anti-MyoD antibody, remains roughly equivalent.

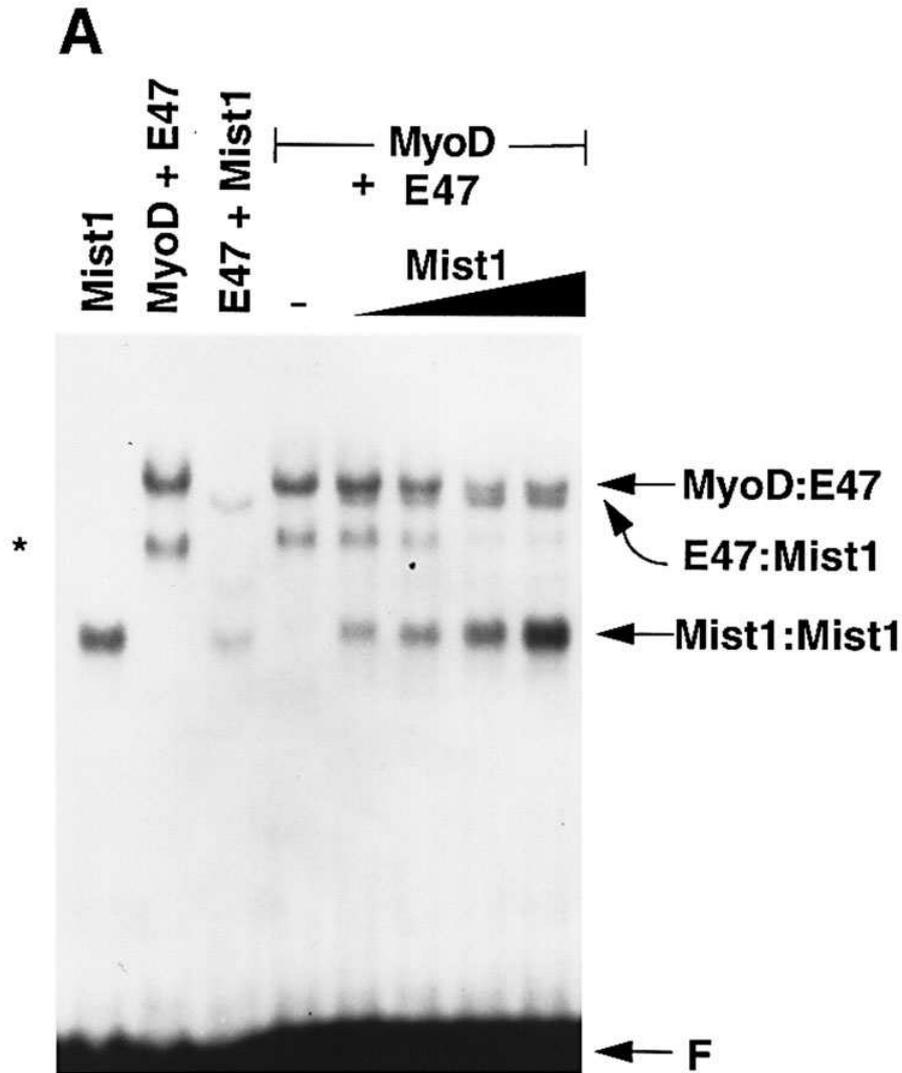


Figure 7: Mist1 inhibits MyoD-E47 complexes from interacting with E-box targets. **(A)** *In vitro* translated Mist1, MyoD and E47 proteins were incubated with a ^{32}P -labeled TnI E-box oligonucleotide and subjected to electrophoretic mobility shift assays as described in Figure 2. MyoD and E47 form heterodimers that interact with the E-box DNA site. Incubation with increasing concentrations of Mist1 inhibits the formation of a MyoD-E47-DNA complex. At the same time, a new E47-Mist1 complex is detected as well as an increase in Mist1-Mist1 homodimer binding. Each of the dimer-DNA complexes is indicated. DNA-protein complexes containing a truncated version of E47 (due to an internal translation initiation site) are indicated by (*). **(B)** Similar electrophoretic mobility shift assays as in (A) except that the pre-formed MyoD-E47 complex was competed with increasing concentrations of Mist1. In this instance, Mist1 fails to block MyoD-E47 DNA binding, even though Mist1 homodimers readily interact with the E-box site. F, free probe.

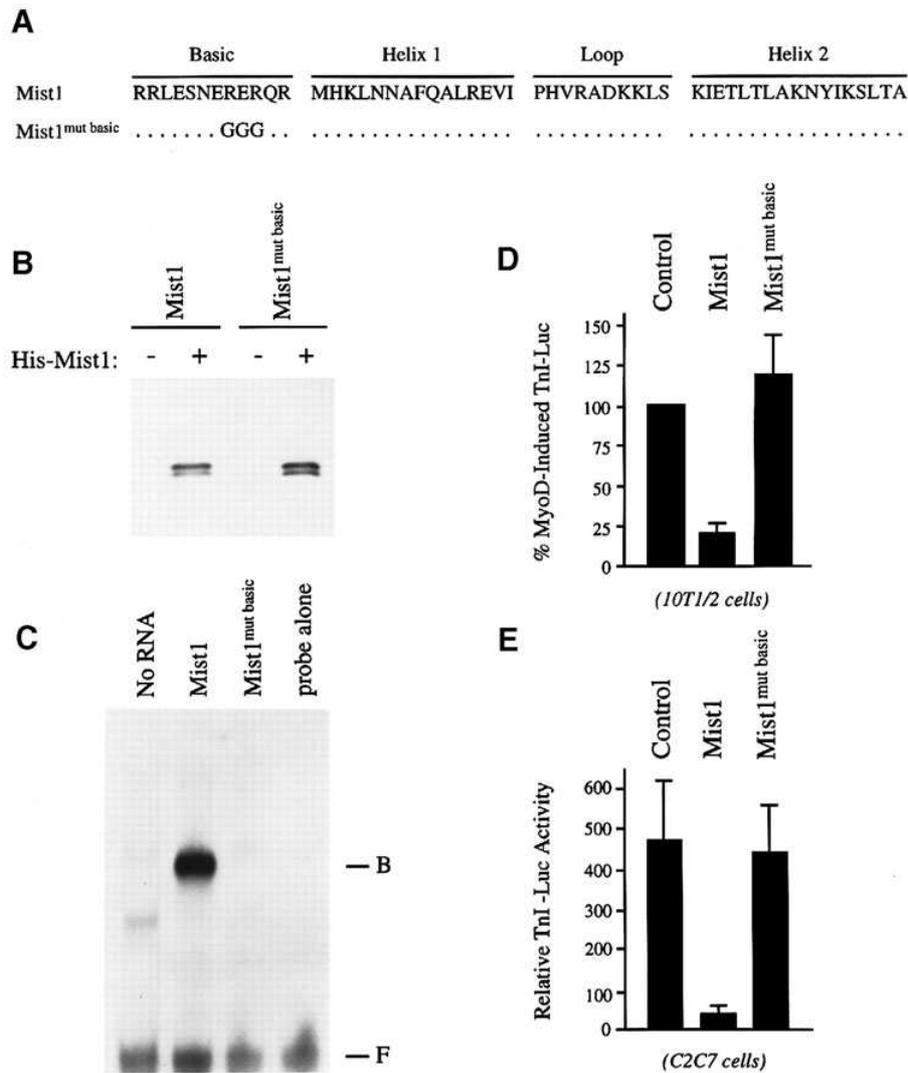


Figure 8: A basic domain mutant of Mist1 does not inhibit myogenesis. **(A)** The basic helix-loop-helix amino acid sequence of the wild-type (Mist1) and basic domain Mist1 (Mist1^{mut basic}) proteins. The altered amino acids (RER→GGG) are indicated. **(B)** Protein-protein interaction assay as described in Figure 3. Both Mist1 and Mist1^{mut basic} efficiently form homodimers in solution. **(C)** Electrophoretic mobility shift assay as described in Figure 2. In this instance, Mist1 homodimers interact with the targeted E-box oligonucleotide whereas Mist1^{mut basic} homodimers are unable to bind to the DNA (B, bound complexes, F, free oligonucleotide probe). **(D)** C3H10T1/2 cells were co-transfected with expression plasmids encoding MyoD, Mist1 or Mist1^{mut basic} and the TnI-Luc reporter gene as described in Figure 5. Mist1 efficiently inhibits MyoD from activating the TnI-Luc gene whereas Mist1^{mut basic} has no effect. **(E)** Similar *in vivo* assays were performed in the myogenic cell line C2C7. In this instance, C2C7 myoblasts were transfected with 1.5 μ g of TnI-Luc alone (control) or co-transfected with 0.5 μ g of pcDNA3-Mist1 (Mist1) or 0.5 μ g of pcDNA3-Mist1^{mut basic}. The cells were induced to differentiate for 72 h and then assayed for luciferase activity. Whereas Mist1 efficiently represses the TnI-Luc reporter, the Mist1^{mut basic} protein has no effect on C2C7 terminal differentiation. Error bars in (D) and (E) indicate the standard error of the mean.

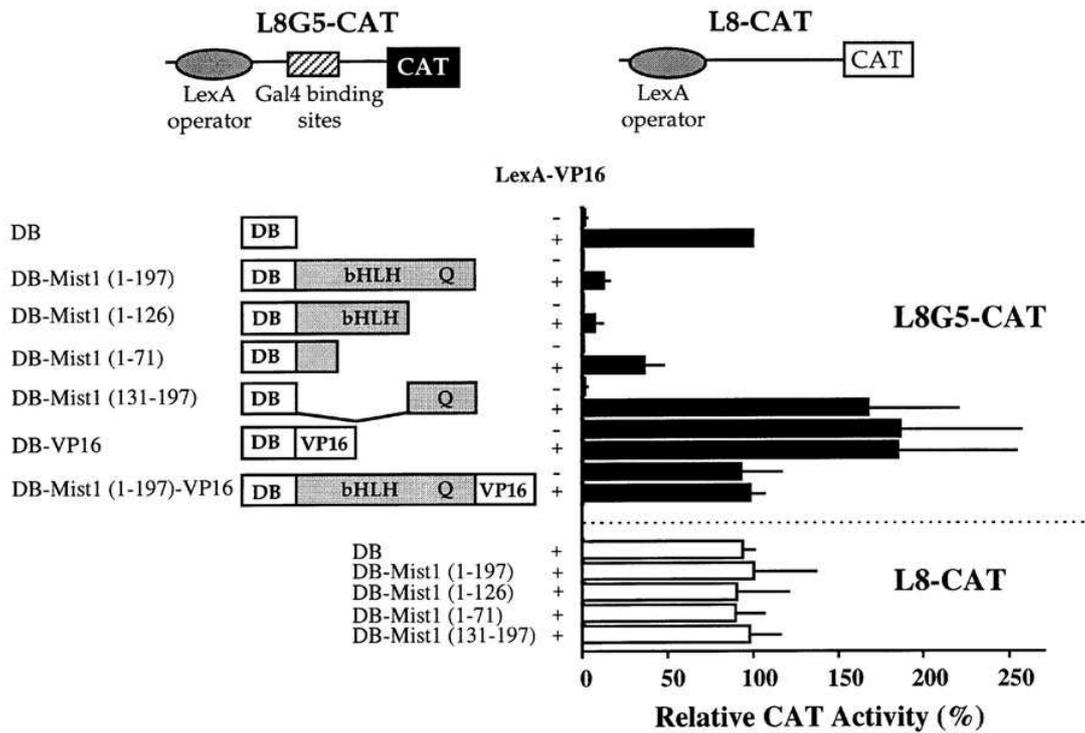


Figure 9: Mist1 acts as a potent repressor of LexA-dependent transcription. The L8G5-CAT reporter gene and Gal4-Mist1 expression plasmids were co-transfected into C3H10T1/2 cells in the presence (+) or absence (-) of a LexA-VP16 transcriptional activator. In the absence of activator, the Gal4-Mist1 proteins exhibit no transcriptional activity. When LexA-VP16 is added, Mist1 efficiently represses CAT expression. Analysis of different regions of the Mist1 protein reveals that the repressor function maps to amino acids 1-71 (see text for details). Mist1 has no effect on the control L8-CAT reporter gene. Error bars indicate the standard deviation.

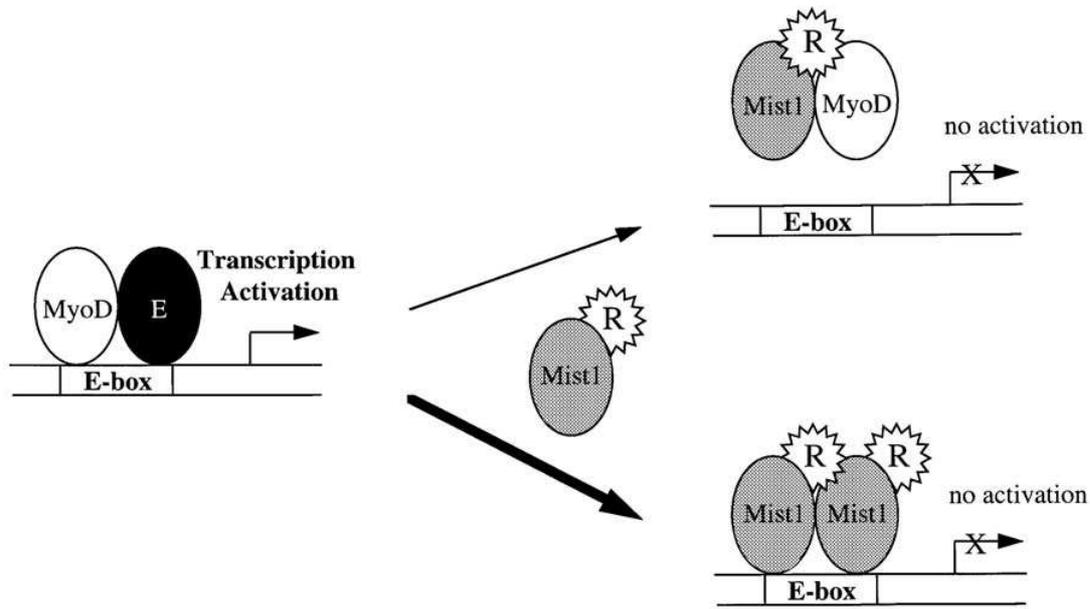


Figure 10: Model of Mist1-induced transcriptional repression of muscle-specific E-box-dependent genes. Mist1 can inhibit muscle gene expression either by forming inactive bHLH heterodimers with MyoD or by forming Mist1 homodimers which efficiently bind to E-box target sites. R represents the N-terminal repressor domain. See text for details.