

A Splice Variant of the ITF-2 Transcript Encodes a Transcription Factor That Inhibits MyoD Activity*

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Proteins of the basic helix-loop-helix (bHLH) family are transcription factors that bind DNA containing the E box motif (CANNTG) found in the promoters of many muscle-specific genes. ITF-2 is a bHLH protein with widespread expression that is thought to form active heterodimers with MyoD, a muscle-specific bHLH transcription factor. We have isolated cDNAs derived from two alternatively spliced forms of mouse ITF-2, termed MITF-2A and -2B. These proteins differ in their N termini. Neither MITF-2A nor -2B transactivated the *cardiac α -actin* promoter, which contains an E box, when transfected into nonmuscle cells. In fact, MITF-2B inhibited MyoD activation of the *cardiac α -actin* promoter. This inhibitory activity required the N-terminal 83 amino acids since MITF-2A showed no inhibitory activity, and a mutant MITF-2B with deletion of the N-terminal 83 amino acids failed to inhibit MyoD-mediated transcriptional activation. MyoD activity was also inhibited by Id, a HLH protein, and this inhibition was reversed by the addition of excess E12 or MITF-2A. However, the inhibition of MyoD activity by MITF-2B was not reversed with E12 or MITF-2A. While Id is thought to inhibit MyoD by binding and sequestering potential dimerization partners, MITF-2B appears to inhibit MyoD activity by forming an inactive heterodimer with MyoD. Thus, differentially spliced transcripts of mouse ITF-2 encode different proteins that appear to dimerize with MyoD and activate or repress transcription.

During skeletal muscle development, a family of transcription factors of the bHLH¹ class plays a pivotal role in inducing and maintaining the differentiated character of skeletal muscle (Olson, 1990; Weintraub *et al.*, 1991; Sassoon, 1993). These transcription factors, MyoD, myf-5, myogenin, myf-6/MRF-4/herculin (Davis *et al.*, 1987; Braun *et al.*, 1989; Edmondson and

Olson, 1989; Olson, 1990; Rhodes and Konieczny, 1989; Wright *et al.*, 1989; Braun *et al.*, 1990; Miner and Wold, 1990) activate the transcription of many muscle-specific genes by binding to DNA at E box sites (CANNTG) within their promoters (Lassar *et al.*, 1989).

The active forms of these transcription factors are thought to be heterodimers comprised of a myogenic bHLH protein and a ubiquitous E type bHLH protein (Lassar *et al.*, 1991). The latter proteins are derived from alternatively spliced transcripts from two genes, one called E12 (also called E2A, E2-5, or ITF1) (Murre *et al.*, 1989a; Henthorn *et al.*, 1990b; Nourse *et al.*, 1990) and the other called ITF-2 (also called E2-2, SEF2, or TFE) (Henthorn *et al.*, 1990a; Corneliussen *et al.*, 1991; Javaux *et al.*, 1991). The basic region of these transcription factors is responsible for binding to the E box sequence in DNA, while the HLH domain mediates dimerization. Homodimers of E12 are able to transactivate some E box-containing promoters, while homodimers of ITF-2 cannot (Henthorn *et al.*, 1990a). Although homodimers of MyoD are inactive, heterodimers of MyoD and E12 or MyoD and ITF-2 can activate promoters containing E box sequences (Lassar *et al.*, 1991). Thus, MyoD requires heterodimerization with ubiquitous E type proteins for activity. All of the E type bHLH proteins contain a conserved motif, designated the loop-helix (LH) motif, which is responsible for most of the transcriptional activation activity of these proteins (Quong *et al.*, 1993).

Lineage-specific bHLH proteins exist in tissues other than muscle. MASH1 and MASH2 are bHLH proteins that are restricted to neuronal lineages (Johnson *et al.*, 1990). They are mammalian homologues of the *Drosophila achaete-scute* genes. Tal-1, tal-2, and lyl-1 are bHLH proteins that are restricted to hematopoietic tissues (Chen *et al.*, 1990; Xia *et al.*, 1991; Mellentin *et al.*, 1989). These three proteins have been implicated in the development of human lymphoid malignancies. The neurogenic and the hematopoietic bHLH proteins heterodimerize with E12 protein (Murre *et al.*, 1989b; Hsu *et al.*, 1991; Voronova and Lee, 1994).

The protein Id has an HLH motif, but it lacks the basic domain. Id is believed to act as a negative regulator of other bHLH proteins through the formation of heterodimeric complexes that fail to bind DNA (Benezra *et al.*, 1990). Id mRNA levels decrease upon terminal differentiation of several cell lines, including myoblasts, consistent with the idea that transcription from E box containing promoters is regulated by both activating and inhibitory transcription factors. Because no correlation is evident between the order of expression of the myogenic bHLH proteins and the appearance of specific contractile proteins (Ontell *et al.*, 1993), it is possible that the presence of E type bHLH and inhibitory HLH proteins may participate in regulating myogenesis.

We have examined the effects of bHLH proteins on the activity of the muscle-specific *cardiac α -actin* promoter. The first

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U16321 and U16322.

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¹ The abbreviations used are: bHLH, basic helix-loop-helix; bp, base pair(s); PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; CAT, chloramphenicol acetyltransferase.

440 bp of the human *cardiac α -actin* promoter contains one E box, which is essential for *cardiac α -actin* expression in skeletal muscle (Skerjanc and McBurney, 1994). MyoD transactivates the *cardiac α -actin* promoter in transient transfection experiments (Pari *et al.*, 1991; Sartorelli *et al.*, 1990; Skerjanc and McBurney, 1994). We cloned cDNAs derived from mRNAs of two alternatively spliced forms of mouse *ITF-2*, termed MITF-2A and -2B. Neither MITF-2A nor -2B transactivated the *cardiac α -actin* promoter. However, MITF-2B inhibited the transactivation of the *cardiac α -actin* promoter by MyoD while MITF-2A did not.

MATERIALS AND METHODS

Polymerase Chain Reaction Amplification of *ITF-2*—The mouse *ITF-2* probe used to screen the library was prepared as described previously (Skerjanc and McBurney, 1994). Briefly, PCR was performed with the following degenerate oligonucleotides: 1) AAAGAATTCA(A/C)(T/C)A(A/C)C(G/A)C(T/T)(G/C)CG(G/C)GAGCG and 2) AAAGGATC-CA(C/T)(G/A)GC(C/G)T(G/T)(G/C)(T/C)G(C/G)AGGAT. Oligonucleotide 1 represents a consensus for the basic domain of E12 (Murre *et al.*, 1989a), HEB (Hu *et al.*, 1992), *ITF-2* (Henthorn *et al.*, 1990a), and MyoD (Davis *et al.*, 1987), and the boldface sequence is an *EcoRI* site. Oligonucleotide 2 represents a consensus for helix 2, and the boldface sequence is a *Bam*HI site. PCR was performed with 30 ng of cDNA, prepared as described below and 2 μ g of each oligonucleotide in 100 μ l with *Taq* polymerase (Life Technologies, Inc., BRL, Burlington, Canada) under standard conditions with annealing at 55 $^{\circ}$ C.

Fragments of several bHLH cDNAs were amplified, subcloned, and sequenced, as described previously (Skerjanc and McBurney, 1994). The amplified cDNA fragment encoding *ITF-2* encompasses the conserved bHLH domain and has the following sequence: AACACCGC-CCGGGAGCGCCTGAGGGTCCGAGATATCAACGAGGCTTTCAAGG-AGCTTGCCCGTATGGTGCAGCTCCACCTGAAGAGCGACAAGCCC-CAGACCAAGCTCCTGATCCTCCACAACGCCGT.

The MITF-2 probes were prepared by replacing cold dCTP with 50 μ Ci of [α - 32 P]dCTP during a PCR amplification with 10 ng of the amplified MITF-2 cDNA fragment.

cDNA Library Construction and Screening—Total RNA was isolated (Auffray and Rougeon, 1980) from day 6 Me₂SO-treated P19 cells, which contained abundant cardiac muscle. Poly(A)⁺ RNA was selected by standard protocols (Sambrook *et al.*, 1989). cDNA was primed with an oligo(dT) *NotI* primer-adaptor and was synthesized with the superscript λ system for cDNA synthesis and cloning (Life Technologies, Inc.). *SalI* adapters were ligated to the ends. The cDNA was subsequently digested with *NotI* and size fractionated by column chromatography, yielding 300 ng of size-selected cDNA. Some of this cDNA (30 ng) was used in a PCR reaction with the degenerate oligonucleotides to generate the *ITF-2* probe, as described above. Part of this cDNA (20 ng) was ligated into λ gt22A phage arms, which had been digested with *NotI* and *SalI* and packaged using the BRL λ packaging system (Life Technologies, Inc., BRL, Burlington, Canada). A total of 2×10^6 plaques were obtained.

Standard procedures (Sambrook *et al.*, 1989) were used to plate 500,000 plaques onto 10×150 -mm plates. Plaques were transferred to Hybond-N and denatured by autoclaving for 2 min at 100 $^{\circ}$ C. DNA was cross-linked by UV irradiation, and the filters were hybridized for 16 h at 42 $^{\circ}$ C with the radiolabeled *ITF-2* PCR product. Washing was performed for 30 min at room temperature in $2 \times$ SCC, 0.2% SDS and for 15 min at 65 $^{\circ}$ C in $0.2 \times$ SCC, 0.2% SDS. Hybridization was visualized by autoradiography. Ten positive plaques were identified from the 500,000 plaques screened, and six were isolated after three rounds of plaque purification.

***ITF-2* Sequence Analysis**—Standard procedures (Sambrook *et al.*, 1989) were used to purify the phage DNA and ligate the cDNA inserts into the *NotI/SalI* sites of pBluescript (KS orientation, Stratagene Cloning Systems, La Jolla, CA). The ends of all clones were sequenced with the T7 Sequencing kit from Pharmacia Biotech Inc. *RsaI* and *AluI* fragments of three of the cDNAs were subcloned, and the miniprep DNA was sequenced (Kraft *et al.*, 1988). Gaps in the sequence were completed by using synthetic oligonucleotides as internal primers. All three cDNAs were completely sequenced on both strands.

DNA sequence alignment was performed using DNASTAR software (DNASTAR Inc., Computer systems for molecular biology and genetics, Madison, WI). The two DNA sequences were aligned (Wilbur and Lipman, 1983) with *k*-tuple size of 3, range of 20, and gap penalty of 3. The similarity index is reported in Fig. 2.

Plasmid Constructs—All of the eukaryotic expression constructs contained the mouse *pgk-1* promoter (Adra *et al.*, 1987) driving the specified cDNA. PGK-MITF-2B contains a 2.3-kb *SspI/NotI* fragment of clone 1 encompassing the entire open reading frame and 478-2763 bp of the sequence. PGK-MITF-2A contains the entire open reading frame in the 1.8-kb *SalI/NotI* fragment of clone 3. PGK-MITF-2B Δ is identical to PGK-MITF-2B except that a 129-bp *EcoRV* fragment from 2143-2272 bp was exchanged from clone 3 into clone 1. This removed the RSRS encoding sequence found from 2161 to 2172 bp in clone 1. Myc-MITF-2B contains six tandem copies of the 11-amino acid human c-myc epitope (Evan *et al.*, 1985) fused in frame to the N terminus of MITF-2B, adding a total of 89 amino acids upstream of MITF-2B. Myc-MITF-2B Δ N contains the same c-myc tag fused to a *XhoII* site in codon 83 of MITF-2B. Thus MITF-2B Δ N lacks the first 83 amino acids found in MITF-2B. PGK-Id contains a 930-bp *BamHI/XhoI* fragment of Id cDNA (Benezra *et al.*, 1990). PGK-E12 contains a 1.4-kb *EcoRI* fragment of a partial E12 cDNA (Murre *et al.*, 1989a). The partial E12 cDNA was previously subcloned downstream of and in frame with an initiator methionine (Murre *et al.*, 1989a) creating an expression construct missing the first 208 amino acids of the full-length E12. PGK-MyoD and PGK-CAT contain the *pgk-1* promoter sequences driving MyoD and CAT cDNAs, respectively, and CA-LacZ contains 440 bp of the human *cardiac α -actin* promoter driving the *Escherichia coli lacZ* gene (Pari *et al.*, 1991).

Cell Culture and Transfections—P19 embryonal carcinoma cells were cultured as described previously (Rudnicki and McBurney, 1987). Cells were transfected by the calcium phosphate method (Chen and Okayama, 1987). For transient transfections, 10^6 cells in 5 ml of medium in a 60-mm dish were exposed to a DNA precipitate for 6–8 h containing 5 μ g of CA-LacZ, 1 μ g of PGK-CAT, with or without 1.5 μ g of PGK-MyoD and specified amounts of PGK-MITF-2B, PGK-MITF-2A, PGK-MITF-2B Δ , PGK-E12, or PGK-Id. Each transfection contained a total of 15 μ g of DNA, completed by including sufficient quantities of pGEM vector DNA (Pharmacia). Cells were harvested 24 h after transfection.

β -Galactosidase and CAT assays were performed as described previously (Norton and Coffin, 1985; Sleight, 1986). Each β -galactosidase activity was normalized for transfection efficiency with the CAT activity from that transfected culture.

The P19[MyoD] cell line stably expresses MyoD and has been described previously (Skerjanc *et al.*, 1994). Differentiation was induced by plating 5×10^5 P19 or P19[MyoD] cells into 60-mm bacterial dishes containing either 1 μ M retinoic acid or 0.8% Me₂SO. Cells were cultured as aggregates for 5 days and then plated in tissue culture dishes and harvested for RNA on day 6. Me₂SO treatment under these conditions induces cardiac muscle in P19 cells and skeletal muscle in P19[MyoD] cells. Retinoic acid treatment induces neuroectoderm in P19 cells and a mixture of neuroectoderm and skeletal muscle in P19[MyoD] cells (Skerjanc *et al.*, 1994).

RNAse Protection—In order to produce the desired antisense RNA for RNase protection, a 1206-bp *SalI/SstI* fragment of MITF-2B (clone 1) was subcloned into Bluescript vector (KS⁺ orientation, Stratagene Cloning Systems, La Jolla, CA) and called MITF-2B(1,1206). MITF-2B(1,1206) was linearized with *SspI* at 478 bp so that the resulting riboprobe was complementary to nucleotides 478-1206 of MITF-2B and carried some vector sequences. Antisense RNA was synthesized from MITF-2B(1,1206) linearized with *SspI* and from pTRI- β -actin-mouse plasmid DNA using T7 RNA polymerase, as described in the MAXIScript *in vitro* transcription kit protocol (Ambion Inc, Austin, TX). Incorporation of radioactivity was checked by trichloroacetic acid precipitation, and the RNA probes were purified from a 5% acrylamide-8 M urea gel as described in RPA II, ribonuclease protection assay kit (Ambion Inc). The MITF-2B and β -actin probes migrated on the acrylamide/urea gel as single bands of 765 and 330 bp, respectively, and incorporated [α - 32 P]CTP to a specific activity of about 5×10^8 cpm/ μ g RNA.

The RNase protection was performed as described in the RPA II kit (Ambion Inc.) by hybridizing 2.2 fmol of riboprobe with 5 μ g of total sample RNA or 10 μ g of torula yeast RNA. Total RNA was isolated (Auffray and Rougeon, 1980) from P19 and P19[MyoD] cells on day 0 and on day 6 after treatment with Me₂SO and retinoic acid as well as from mouse brain, liver, heart, and leg muscle. After RNase digestion of the hybridized probe and sample RNAs, protected fragments were separated on a 5% polyacrylamide, 8 M urea gel and visualized by autoradiography.

To estimate the size of the bands protected from RNase digestion, the 1-kb ladder was labeled with [γ - 32 P]ATP using the exchange reaction with bacteriophage T4 polynucleotide kinase, as described previously (Sambrook *et al.*, 1989). The labeled 1-kb ladder was subjected to

electrophoresis alongside the RNase protection samples. A graph of log molecular weight *versus* distance from the origin was used to determine the sizes of the RNase-resistant fragments. The calculated molecular weights agreed with the predicted molecular weights within an error of 10%.

RESULTS

Cloning Mouse *ITF-2*—We set out to determine the spectrum of bHLH proteins present in differentiating cultures of P19 cells by using degenerate oligonucleotide primers from the bHLH region to amplify bHLH cDNAs from a library of cDNA made from P19 cultures (Skerjanc and McBurney, 1994). No cDNAs encoding novel bHLH proteins were found; only cDNAs encoding E type bHLH proteins were identified. One class of amplified cDNA was derived from the mouse homologue of *ITF-2*. This *ITF-2* probe was used to screen a cDNA library in bacteriophage λ made from Me₂SO-treated P19 cells. Three cDNA clones were isolated, subcloned, and sequenced on both strands. They derive from two alternatively spliced forms of mouse *ITF-2* mRNA, and the relationship of the three clones to these alternatively spliced forms is summarized in Fig. 1A.

Clone 1 contains both upstream and downstream stop codons along with an open reading frame predicting a 670-amino acid protein. It has a truncated 3'-untranslated region, probably because the oligo(dT) cDNA primer initiated reverse transcription at an internal A-rich sequence. Clone 2 contains a partial coding region and an extended 3'-untranslated region. The nucleotide and amino acid sequence for clones 1 and 2 were designated MITF-2B and are shown in Fig. 1B.

Clone 3 also contains both upstream and downstream stop codons and an open reading frame identical to that of clone 1 except in two regions. The first 159 bp of clone 3 is different from that of clone 1 and predicts a protein with a different N terminus that we designated MITF-2A (Fig. 1C). In addition, clone 1 contained 12 nucleotides inserted 50 bp upstream of the bHLH domain that were absent from clones 2 and 3. These 12 nucleotides encode the four amino acids, RSRS, indicated in Fig. 1, A and B. Clones 1 and 3 contain an LH motif, which is the presumptive transcriptional activation domain (Quong *et al.*, 1993).

Comparison of *MITF-2* with Other E Type bHLH cDNAs—cDNAs derived from the human and dog homologues of *MITF-2* have been cloned previously. A comparison of nucleic acid sequence between these *ITF-2* clones and *MITF-2* is outlined in Fig. 2. The nucleotide similarity index for various regions of the cDNAs has been calculated by the method of Wilbur and Lipman (1983). *MITF-2B* appears to be the same splice variant as *SEF2-1B* (Corneliusson *et al.*, 1991), while *MITF-2A* appears to be the same splice variant as *SEF2-1A* (Corneliusson *et al.*, 1991). However, *MITF-2A* contains 142 bp of novel 5' sequence, including the first 17 amino acids, an initiator methionine, and an upstream stop codon. *HUMITF-2* (Henthorn *et al.*, 1990a) is a partial cDNA and appears to be identical to *MITF-2B* and *SEF2-1B* but missing the first 49 amino acids. *SEF2-1D* and *TFE* (Javaux *et al.*, 1991) appear to be different splice variants.

The RSRS sequence is found in three of the seven cDNAs from the *ITF-2* gene and appears to derive from a mini-exon that may or may not be included.

The 3'-untranslated region of *MITF-2A/B* is about 1 kb longer than the sequences cloned previously. A remarkable sequence identity (93–95%) was found in the 3'-untranslated regions of *ITF-2* from the different species. The 5' sequences are the least conserved with only 39–45% homology for *SEF2-1A*, B, and D with *MITF-2B*.

The extent of nucleic acid homology between the related but distinct gene products, HEB (Hu *et al.*, 1992) and E12 (Nourse *et al.*, 1990), with *MITF-2B* is also shown in Fig. 2. These

cDNAs show less homology in the coding region (61–80%), with the greatest similarity in the bHLH domain. The 5'- and 3'-untranslated regions have very low homology (39–48%) with *MITF-2B*. Neither cDNA contains the RSRS domain.

Activity of *MITF-2*—*MITF-2A* and -2B were examined for their abilities to transactivate the cardiac α -actin promoter when transfected into cells with or without MyoD. MyoD alone transactivated the cardiac α -actin promoter 10-fold over background, while neither *MITF-2A* nor *MITF-2B* activated the cardiac α -actin promoter significantly (Fig. 3A). Co-transfecting *MITF-2A* with MyoD had no effect on MyoD-induced cardiac α -actin promoter activity; however, co-transfection of *MITF-2B* with MyoD resulted in less promoter activity than that induced by MyoD alone. A similar inhibition of MyoD activity was found by co-transfection of Id with MyoD (Fig. 3A). Thus, despite their similarity, it seems that *MITF-2A* and -2B do not have the same activity as transcription factors.

MITF-2A and -2B encode proteins identical but for two regions. The N termini are different, and *MITF-2B* contains the 4 amino acids, RSRS, just upstream of the bHLH domain. In order to determine which of these two regions is responsible for the *MITF-2B* inhibition of MyoD activity, the 12 bp encoding the RSRS sequence were removed from *MITF-2B*, in a construct called *MITF-2B Δ* . *MITF-2B Δ* , like *MITF-2B*, inhibited MyoD activity (Fig. 3A). Since *MITF-2B Δ* is identical to *MITF-2A* in all regions except the first 182 amino acids, the inhibitory activity of *MITF-2B* must require the amino-terminal domain.

To further define the inhibitory domain, the first 83 amino acids of *MITF-2B Δ* were deleted and replaced with six copies of the 11-amino acid c-myc epitope (Evan *et al.*, 1985) creating Myc-*MITF-2B Δ N*. As a control, this c-myc tag was fused to the N terminus of intact *MITF-2B* creating Myc-*MITF-2B*. The MyoD activation of the cardiac α -actin promoter was reduced to 17% by *MITF-2B* but to only 78% by Myc-*MITF-2B Δ N* (Fig. 3B). This suggests that the inhibitory domain resides in the first 83 amino acids of *MITF-2B*. The Myc-*MITF-2B* reduced promoter expression by an intermediate amount (47%), suggesting that the c-myc tag may interfere with the region near the N terminus that mediates transcriptional inhibition.

Id is thought to inhibit MyoD activity by forming inactive heterodimers with E type bHLH proteins, sequestering them away from MyoD (Benezra *et al.*, 1990). In agreement with this model, an excess of E12 reversed the Id inhibition (Fig. 4). An excess of *MITF-2A* also reversed Id inhibition (Fig. 4), suggesting that E12 and *MITF-2A* can heterodimerize with Id and/or MyoD. However, neither E12 nor *MITF-2A* was able to reverse the inhibition created by *MITF-2B* (Fig. 4).

Distribution of *MITF-2* Splice Products—Since *MITF-2B* appears to inhibit E box-mediated transactivation (Fig. 3) while *MITF-2A* is an activator (Fig. 4), we set out to determine if the expression of these forms was regulated in a tissue-specific manner. RNase protection was performed by hybridizing various RNA samples with an antisense riboprobe comprising nucleotides 478–1206 of *MITF-2B* (Fig. 1A). The *MITF-2A* transcript is predicted to protect a 134-nucleotide fragment, while the *MITF-2B* transcript is predicted to give a 728-nucleotide protected fragment. When compared with [γ -³²P]ATP-labeled 1-kb ladder standards, the calculated molecular sizes of the protected bands obtained agreed with the predicted sizes.

We examined RNA from several cell types and tissues. We found that *MITF-2B* transcripts, indicated as the labeled band 1, were present in all cell types examined, including P19 stem cells (lane 1 and 2), P19-derived cardiac muscle (lane 3), P19[MyoD]-derived skeletal muscle (lane 4), P19- (lane 5), and P19[MyoD]- (lane 6) derived neuroectoderm, mouse brain (lane

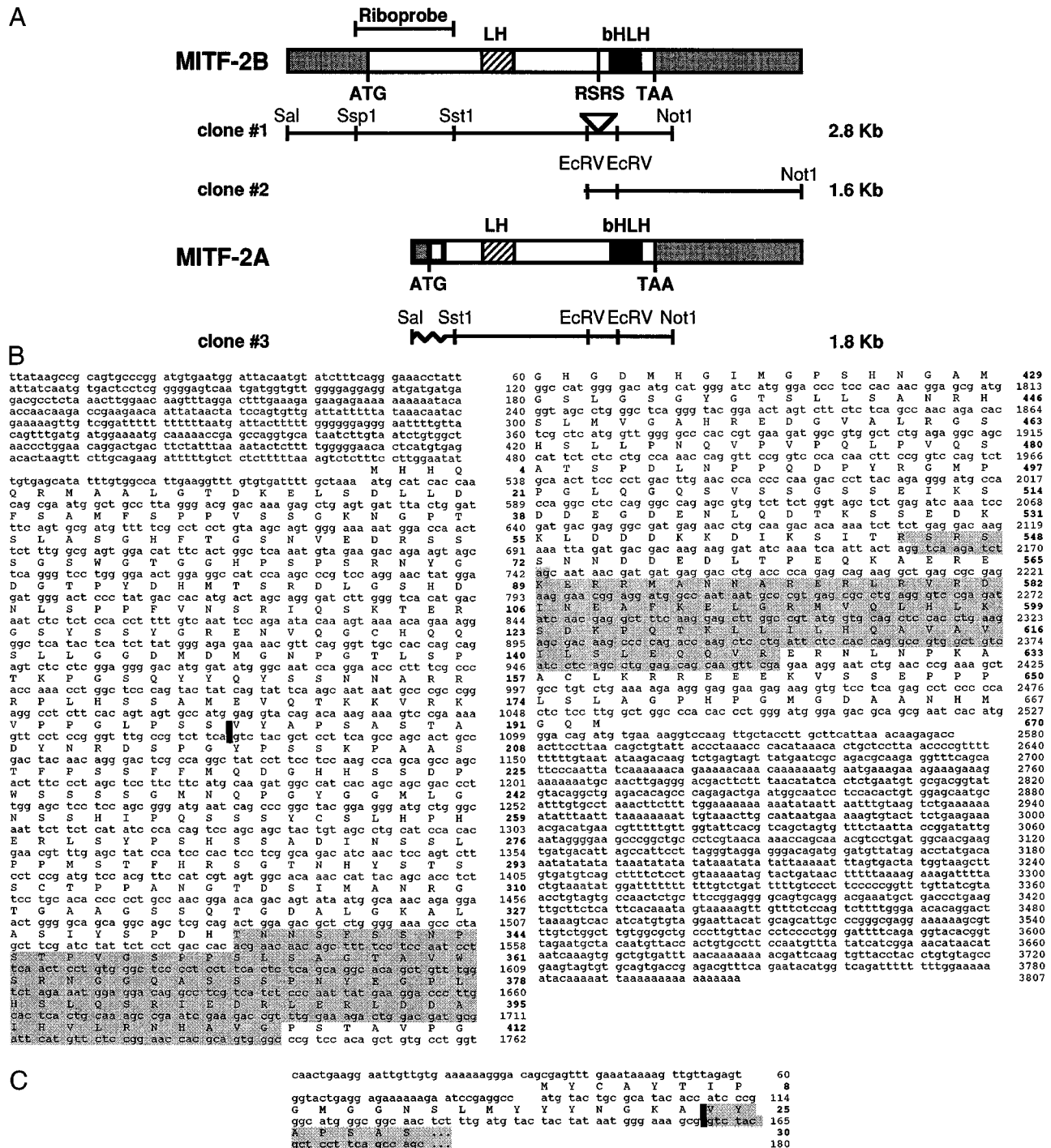


FIG. 1. Panel A, schematic outline of the relationship between the three cloned cDNAs and MITF-2A and MITF-2B. Untranslated regions are indicated as gray boxes, the LH domain as a striped box, and the bHLH domain as a black box. The RSRS domain is shown as a triangle in clone 1. The unique 5' sequence of MITF-2A is shown as a wavy line in clone 3 and as a boldface box in MITF-2A. Restriction sites used in subcloning are indicated. Panel B, the nucleotide and amino acid sequences of MITF-2B. The LH, RSRS, and bHLH domains are indicated by shaded areas between nucleotides 1532 and 1741, 2160 and 2172, and 2222 and 2401. The position at which MITF-2A becomes identical to MITF-2B is shaded at nucleotide 1072. Amino acid residues are presented in capital letters using the single-letter code, and nucleotides are in small letters. Panel C, the unique 5' nucleotide and amino acid sequence of MITF-2A. The sequence of MITF-2A that is identical to MITF-2B is shaded, starting at nucleotide 60.

7), mouse liver (lane 8), and mouse leg muscle and heart (data not shown). Protection of a riboprobe prepared from β -actin cDNA indicated that all of the samples contained equal amounts of mRNA (Fig. 5B).

The level of MITF-2A mRNA was variable in the samples analyzed. MITF-2A transcripts were present in all samples containing neurons (Fig. 5A lanes 5–7, band 5). Barely detectable levels were found in liver (Fig. 5A, lane 8, band 5) and the

transcript was undetectable in P19 stem cells, P19-derived cardiac muscle and P19-derived skeletal muscle (Fig. 5A, lanes 1p4, band 5). Thus, MITF-2A seems to be expressed at high levels only in cells of the neuroectoderm lineage.

Band 3 appears to derive from MITF-2 transcript spliced to yield the D isoform represented by SEF2-1D (Fig. 2). This band was detected in all cell types and was elevated about 7-fold in brain tissue (lane 7).

FIG. 2. Summary of the homologies between various E type bHLH cDNAs. The cDNAs are named in the left-hand column, while their sources are named in the right-hand column. Untranslated regions are indicated as gray boxes, the LH domain as a striped box, and the bHLH domain as a black box. Initiator methionine sequences and stop codons are shown as ATG and TGA, respectively. The nucleotide identity between the clone and MITF-2B is indicated in percent by the series of numbers in the boxes. The top seven cDNAs derive from the *ITF-2* gene, while HEB and E12 are from different genes.

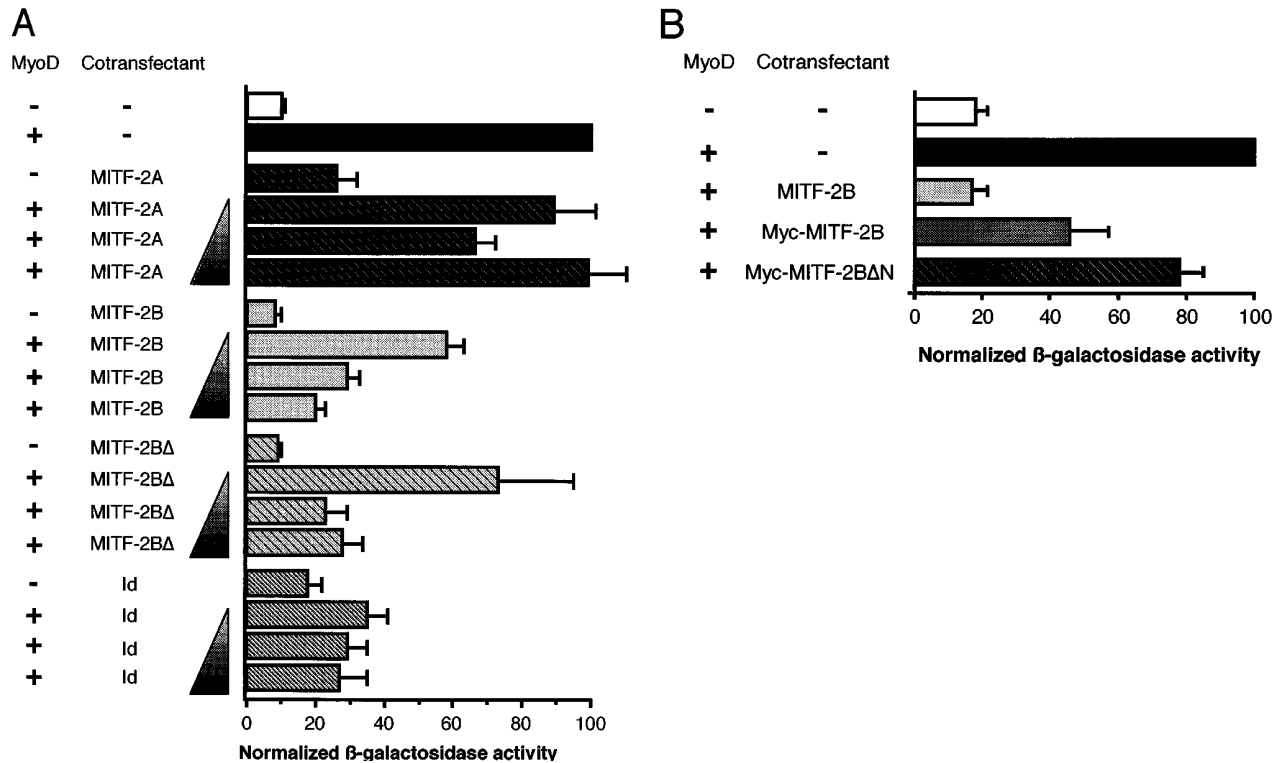
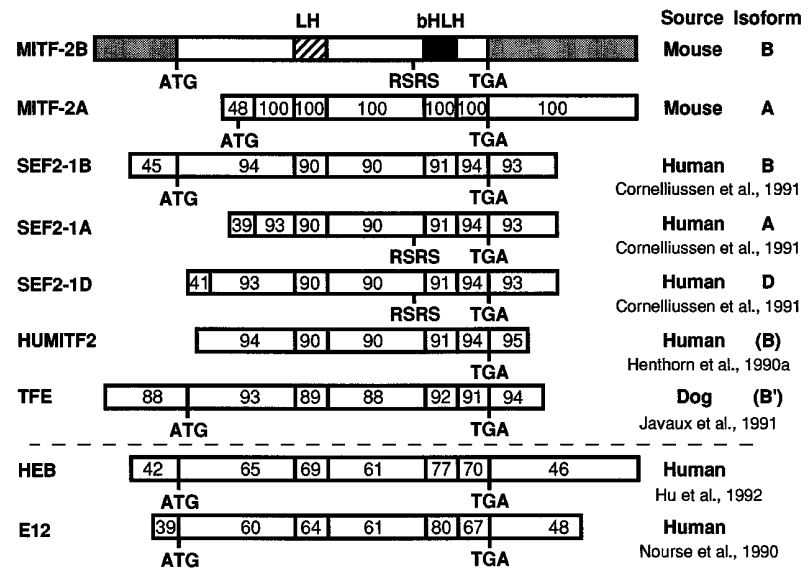


FIG. 3. MITF-2B inhibits MyoD activity. Panel A, MITF-2B, MITF-2B Δ , and Id inhibit MyoD activity, while MITF-2A does not. P19 cells were transfected with 5 μ g of the reporter construct CA-LacZ and 1 μ g of the standardization reporter PGK-CAT, along with expression constructs encoding bHLH proteins. MyoD indicates the presence or absence of 1.5 μ g of PGK-MyoD. The expression constructs for MITF-2A, MITF-2B, MITF-2B Δ , and Id were present at concentrations of 1.5, 3, and 6 μ g/transfection. Panel B, the inhibition of MyoD activity requires the N-terminal 83 amino acids of MITF-2B. P19 cells were co-transfected as in panel A with CA-LacZ, PGK-CAT, and PGK-MyoD along with 6 μ g of plasmid containing the *pgk-1* promoter driving the indicated construct. β -Galactosidase activities were assayed and normalized for transfection efficiency against CAT activity and to the activity of the positive control (MyoD alone). Error bars represent standard error calculated from between three and 10 different experiments.

Two additional bands, 2 and 4, were detected in all cells, representing MITF-2 transcripts with 5'-ends derived from as yet unidentified exons.

DISCUSSION

We cloned 3 cDNAs containing sequences encoding the bHLH region of the mouse *ITF-2* protein. These cDNAs were derived from two alternatively spliced transcripts called MITF-2A and MITF-2B. Neither MITF-2A nor MITF-2B encoded a protein that by itself activated expression of the *car-*

diac α -actin promoter. In fact, the MITF-2B protein inhibited MyoD-induced expression from the *cardiac α -actin* promoter, while the MITF-2A protein did not. The inhibitory activity of MITF-2B requires the first 83 amino acids at its amino terminus. The MITF-2A protein is a transcription activator because it activated *cardiac α -actin* promoter expression when co-expressed in cells along with MyoD and Id. Thus, the MITF-2A and MITF-2B proteins arise from the same gene by differential splicing or different promoter usage and encode transcription factors that activate and repress expression, respectively.

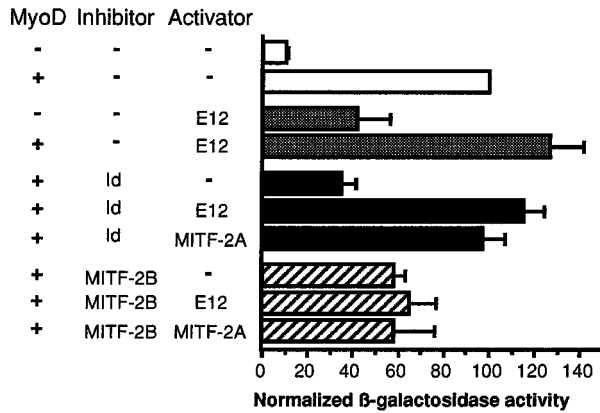


FIG. 4. **E12 and MITF-2A reverse the inhibition of MyoD activity by Id but not by MITF-2B.** P19 cells were transfected as described in the legend to Fig. 3 with and without 1.5 μ g of PGK-MyoD, 1.5 μ g of PGK-Id or PGK-MITF-2B, and 6 μ g of PGK-E12 or PGK-MITF-2A, as indicated. Error bars represent standard error calculated from between four and 10 different experiments.

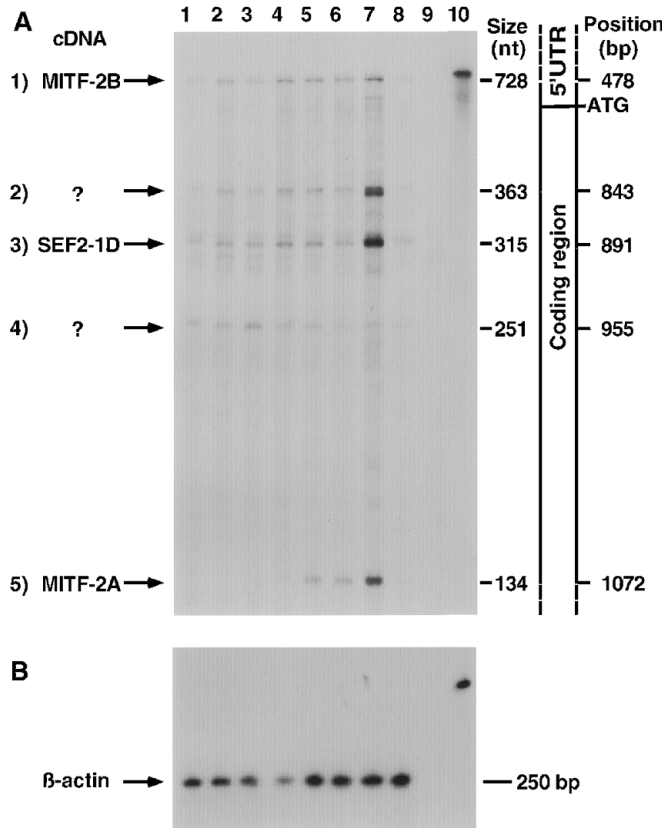


FIG. 5. **RNase protection demonstrates that MITF-2B is widely expressed.** Radiolabeled antisense riboprobes for MITF-2B (panel A) or β -actin (panel B) were hybridized to 5 μ g of RNA from P19 stem cells (lane 1), P19[MyoD] stem cells (lane 2), P19-derived cardiac muscle (lane 3), P19[MyoD]-derived skeletal muscle (lane 4), P19-derived neuroectoderm (lane 5), P19[MyoD]-derived neuroectoderm and skeletal muscle (lane 6), mouse brain (lane 7), mouse liver (lane 8), and torulla yeast (lane 9). All samples were digested with RNase, except lane 10, which is a control for riboprobe integrity. Samples were separated by electrophoresis on an acrylamide/urea gel and visualized by autoradiography. The sizes of the bands, determined by comparison with a radiolabeled 1-kb ladder, are indicated on the right, and the corresponding cDNAs are indicated on the left.

Both MITF-2B and Id inhibited MyoD activity, but they appear to do so by different mechanisms. While the inhibitory activity of Id was effectively lost in the presence of excess E12 or MITF-2A, these latter two proteins were unable to reverse

the inhibition of MITF-2B. We interpret our results as suggesting that Id inhibits primarily by sequestering the "active" E type proteins from MyoD, while MITF-2B seems to inhibit by forming stable heterodimers directly with MyoD.

Although we found that MITF-2B inhibited MyoD activation of the cardiac α -actin promoter, human ITF-2 protein cooperated with MyoD to activate promoters containing an E box in COS cells (Lassar *et al.*, 1991). The HITF-2 used in these experiments was encoded by a cDNA that was not full-length and contained a synthetic initiation codon resulting in a protein, which lacked 49 amino acids from the N terminus when compared with MITF-2B. Since the inhibitory activity of MITF-2B resides in the N-terminal 83 amino acids, the critical inhibitory domain may be absent from the HITF-2 used in these experiments. Alternatively, P19 stem cells may contain a different complement of bHLH proteins to those in COS cells, resulting in inhibition by ITF-2 in one system and activation in the other. Evidence that the two cell types contain different bHLH proteins derives from the finding that MyoD transfected alone is not active in COS cells but is active in P19 cells (Lassar *et al.*, 1991; Pari *et al.*, 1991; Skerjanc and McBurney, 1994).

Other transcription factors are alternatively spliced to create one form that activates and another form that inhibits transcription. However, the splicing usually involves the removal of an activation domain, resulting in a shorter form, which is an inhibitor, and a longer form, which is an activator. For example, FosB undergoes alternative splicing to remove a C-terminal transcriptional activation domain, creating Δ FosB, which is a transcriptional repressor (for review, see Foulkes and Sassone-Corsi (1992)). TFE3 is a bHLH leucine zipper transcription factor that binds to the intronic enhancer of the immunoglobulin heavy chain gene. Alternative splicing at the N terminus removes the first 105 nucleotides and creates an inhibitor of transcription (Roman *et al.*, 1991). Finally, the retinoic acid receptors γ 1 and γ 2 undergo alternative splicing in which different N-terminal amino acids produce transcriptional repressors or activators (Husmann *et al.*, 1991).

Apart from differences at their 5'-ends, cDNAs from mouse and human ITF-2 may or may not contain a 12-bp sequence in the coding region 50 bp upstream of the bHLH domain. These 12 bp encode the 4 amino acids RSRS, but the role, if any, of this tetrapeptide in MITF-2 activity remains unclear.

The transcripts encoding MITF-2A and MITF-2B appear to be present in most cell types and tissues investigated, making it difficult to deduce the roles of these transcription factors in regulating expression of E box containing genes. Given that many transcription factors are regulated post-transcriptionally, such as by phosphorylation or protein turnover, it seems possible that the inhibitory and activation effects of MITF-2 isoforms might be subject to additional means of modulation.

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