Positive and Negative Regulation of Myogenic Differentiation of C2C12 Cells by Isoforms of the Multiple Homeodomain Zinc Finger Transcription Factor ATBF1*

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The ATBF1 gene encodes two protein isoforms, the 404-kDa ATBF1-A, possessing four homeodomains and 23 zinc fingers, and the 306-kDa ATBF1-B, lacking a 920-amino acid N-terminal region of ATBF1-A which contains 5 zinc fingers. In vitro, ATBF1-A was expressed in proliferating C2C12 myoblasts, but its expression levels decreased upon induction of myogenic differentiation in low serum medium. Forced expression of ATBF1-A in C2C12 cells resulted in repression of MyoD and myogenin expression and elevation of Id3 and cyclin D1 expression, leading to inhibition of myogenic differentiation in low serum. In contrast, transfection of C2C12 cells with the ATBF1-B isoform led to an acceleration of myogenic differentiation, as indicated by an earlier onset of myosin heavy chain expression and formation of a higher percentage of multinucleated myotubes. The fourth homeodomain of ATBF1-A bound to an AT-rich element adjacent to the E1 E-box of the muscle regulatory factor 4 promoter mediating transcriptional repression. The ATBF1-A-specific N-terminal region possesses general transcription repressor activity. These results suggest that ATBF1-A plays a role in the maintenance of the undifferentiated myoblast state, and its down-regulation is a prerequisite to initiate terminal differentiation of C2C12 cells.

Skeletal muscle differentiation proceeds through the initial commitment of mesoderm cells toward the myogenic lineage followed by irreversible withdrawal from the cell cycle and concomitant differentiation to multinucleated muscle cells. Several groups of muscle-specific and ubiquitous regulatory proteins play major roles in establishing these processes. The MyoD family of muscle regulatory factors (MRFs), 1 which belong to the basic helix-loop-helix class of transcription factors act in a hierarchic manner with MyoD and myf5 involved in the determination of the myogenic lineage and myogenin and MRF4 in the execution of the differentiation program (for review, see Refs. 1 and 2). These MyoD family MRFs form heterodimers with ubiquitously expressed basic helix-loop-helix proteins, known as E proteins, to bind and regulate E-box elements found in the regulatory regions of muscle-specific genes (1, 2). Forced expression of MyoD in certain non-muscle cells drives these cells to the myogenic lineage leading to activation of muscle-specific genes (3, 4). Myogenic activity of the MyoD family genes has been shown to be enhanced by the myocyte enhancer factor-2 proteins, which belong to the MADS-box family of transcription factors (5). These proteins are expressed at the onset of myogenic differentiation and interact physically with MyoD family MRFs and bind to AT-rich elements often positioned in close proximity to E-boxes in the control regions of muscle-specific genes (6, 7).

In addition to positive action of the MyoD-MRFs described above, myogenesis is controlled by proteins acting in a negative manner. The Id family of proteins, which contain the helix-loop-helix motif but lack the basic DNA binding domain, interacts with E proteins or the MyoD family proteins forming complexes incapable of binding to E-boxes (8–10). Id genes are expressed in proliferating myoblasts but not in differentiating myotubes, and forced expression of Id in myoblasts can delay myogenic differentiation (11). In addition, there are several transcription factors that inhibit myogenic differentiation through active transcriptional repressive mechanisms. MyoR and Mist1, which belong to the basic helix-loop-helix family of transcription factors, form heterodimers with E proteins and bind to E-box elements in the promoters of myogenic genes to repress their transcription actively (12–14). MyoR and Mist are expressed in undifferentiated myoblasts, but their expression is down-regulated during differentiation to allow the MyoD family MRFs to execute the myogenic program. A zinc finger homeodomain protein, ZEB, also binds to E-box elements and inhibits transcription of muscle-specific genes (15, 16). ZEB is expressed in undifferentiated myoblasts, and as muscle differentiation is induced, the MyoD family MRFs accumulate to high levels to displace ZEB from E-boxes alleviating the transcriptional repression (15). Thus, progression of muscle differentiation depends on a balance between the positive and...
negative regulatory factors.

ATBF1 belongs to a family of proteins containing both homeodomains and zinc finger motifs that are believed to play roles in the growth and differentiation of mesoderm and neuroectoderm tissues in both vertebrates and invertebrates (15–24). The ATBF1 gene encodes two isoforms, which are generated by alternative splicing and the use of independent promoters (19, 24). ATBF1-A is a 404-kDa protein containing 4 homeodomains, 23 zinc finger motifs, and a number of segments believed to be involved in transcriptional regulation (24). ATBF1-B is a 306-kDa protein that carries the same 4 homeodomains but 5 fewer zinc finger motifs because of the absence of 920 amino acid residues at the N terminus (19). ATBF1-B binds to an AT-rich element in the enhancer and promoter of the human α-fetoprotein (AFP) gene and down-regulates their activities (25). Analysis of ATBF1-A expression in developing mice showed that ATBF1-A mRNA levels are high in the 13.5-day fetal brain and decrease subsequently through postnatal development (26). In P19 murine embryonal carcinoma cells, ATBF1-A is not detected in undifferentiated cells but is expressed when neuronal differentiation is induced by treatment with retinoic acid (22, 24). Induction of muscle cells from P19 cells by treatment with dimethyl sulfoxide is also accompanied by ATBF1-A mRNA expression (22). Although this expression is not as prominent as that observed in neural differentiation, this raised the possibility that ATBF1-A is also involved in myogenesis. This is of interest considering that muscle and neuronal differentiation employ similar regulatory mechanisms, such as the use of a hierarchy of basic helix-loop-helix transcription factors, the myocyte enhancer factor-2 family of cofactors (2, 27), and likely Id proteins (28).

In this study, we examined the role of ATBF1 isoforms in myogenesis using C2C12 myoblasts, which can be induced to undergo terminal differentiation by low serum conditions. We found that forced expression of ATBF1-A and ATBF1-B in C2C12 cells resulted in inhibition and promotion of myogenic differentiation of these cells, respectively. In addition we show that ATBF1-A extinguishes MyoD and myogenin expression and inhibits MyoD-induced activation of the MRF4 promoter. Our results suggest that ATBF1-A is an inhibitor of myogenesis, and its down-regulation is essential for terminal differentiation of C2C12 cells.

MATERIALS AND METHODS

Plasmids—pPOP-MEF, ATBF1-A expression vector, and pPOP-MER antisense ATBF1 expression vector were constructed by cloning the full-length human ATBF1-A DNA into pPOP (29). ATBF1-B expression vectors, pPOP-E and pATBF1-B, were constructed by cloning the full-length human ATBF1-B cDNA into pPOP and pCDNA1 (Invitrogen), respectively. pPOP, pgkMyoD (mouse MyoD expression vector), and pPOP-MER were used to transfect mammalian cells. For MyoD and myogenin expression vectors, pPOP-E and pcATBF1, pPOP, or pcDNA1 along with 4 μg of pc1-Neo (Strategene) were transfected into C2C12 cells using the high efficiency calcium phosphate method (31). Cells were incubated in GM containing 400 μg/ml G418, and colonies were isolated and propagated. For myogenic conversion assays, 10T1/2 fibroblasts in GM were transiently transfected with 1 μg of pPGK-MyoD and 1 μg of pPOP-ME, pPOP-E, or pPOP-MER using Superfect transfection reagent (Qiagen) according to the manufacturer’s protocol, transferred to DM, and incubated for 4 days. For MRF4 promoter assays, 10T1/2 cells were transfected with 0.5 μg of pMRF4-luc, 0.5 μg of pPGK-MyoD, 0.5 μg of pCH110, and 0.5 μg of the respective ATBF1 expression vector using Superfect, as described above. Cells were transferred to DM, incubated for 2 days, and analyzed for luciferase and β-galactosidase activities using the luciferase assay system (Promega) and β-galactosidase assay system (Promega), respectively, according to the manufacturer’s protocols.

The yeast Gal4 DNA binding domain (Gal4BD) was fused in-frame to the N terminus of human ATBF1-A and -B proteins to generate the vectors ATBF1-A-Gal4BD and ATBF1-B-Gal4BD, respectively. In addition the Gal4DBD was attached to the ATBF1-A N terminus to create N-terminus Gal4DBD contains four Gal4 BD binding sites upstream of the thymidine kinase promoter. C2C12 cells were transfected in GM with 0.5 μg of 4xGal4-luc, 0.5 μg of Gal4BD, 0.5 μg of pCH110, and 0.5 μg of empty pPO expression vector. Cells were incubated in GM for 48 h, and luciferase activity was analyzed as described above.

Immunocytochemistry—Cells were grown on 6- or 24-well plates, washed three times with phosphate-buffered saline (PBS), and then fixed in a 70% ethanol, 10% formaldehyde, 5% acetic acid solution at −20 °C for 10 min. The cells were rehydrated with PBS and incubated for 2 h at 37 °C with a monoclonal antibody against myosin heavy chain (MHC) (MF-20, Developmental Studies Hybridoma Bank) (32), at a final concentration of 5 μg/ml in PBS supplemented with 5% goat serum. The cells were then washed twice with PBS and incubated with peroxidase-coupled anti-mouse IgG (1:500; Santa Cruz Biotechnology) for 1 h at 37 °C. After two washes with PBS, the cells were incubated with 0.3% diaminobenzidine and 0.15% H2O2 in PBS for 10 min at room temperature. The cells were then washed with PBS for 5 min and stained with 0.05% Cresyl Violet.

Production of Bacterially Expressed ATBF1-A Proteins—ATBF1-A cDNA fragments were subcloned in-frame with the histidine tag sequence into pET30 (a, b, or c) vectors (Novagen). The recombinant proteins were expressed in the Escherichia coli strain BL21 (DE3LyS3), and the expression of recombinant proteins was induced by the addition of 2 μM isopropyl-1-thio-β-D-galactopyranoside. Three hours after induction, the bacteria were collected by centrifugation, resuspended in NF2 column binding buffer containing 2 μM MgCl2, 30 μM α-2, 1 μM leupeptin, 20 μM benzamide, 20 μM pepstatin, and 1 μM pepstatin, and lysed by freezing and thawing in the presence of 1% Triton X-100. The host DNA was digested with 5 mg/ml DNase I on ice for 20 min. The bacterial lysate was clarified by centrifugation for 20 min at 10,000 × g. The His-tag recombinant proteins were purified by Ni-nitrolotriacetic acid-agarose beads according to the manufacturer’s protocol (Novagen). One ml of the eluted protein solution was
concentrated to 50 μl with a Centricron-30 microfiltration column (Amicon) in 50 mM Tris (pH 7.5). The recombinant protein was stored at -80 °C.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared from C2C12 and 10T1/2 fibroblasts essentially as described by Lassen et al. (33). Nuclear extracts (10 μg) or bacterially expressed proteins (5 ng) were incubated in a 20-μl binding reaction containing 20 mM HEPES (pH 7.6), 5% glycerol, 50 mM NaCl, 1.5 mM MgCl2, and 1 μg of poly[dI-dC]-poly[dI-dC] for 10 min at room temperature. The appropriate probes were added at a concentration of 5 pg/μl (30,000 cpm total), and the reaction mixture was incubated further for 20 min at room temperature. The reaction mixture was resolved on a 5% polyacrylamide gel in 0.5× TAE buffer. Gels were dried and exposed to x-ray film at -70 °C. The probe used was A/E1, 5'-ACGTTTATTAAATGCATCTGGGTG-3' (A), corresponding to the region of the mouse MRF4 promoter containing an E-box (underlined) and an AT rich sequence (double underlined) (39), with a 4-base pair overhang to facilitate labeling with [α-32P]dCTP using Klenow. For supershift assays, 1 μl of a D4, a rabbit polyclonal raised against the fourth homeodomain of ATBF1, 1 μl of C20, a MyoD polyclonal antibody (Santa Cruz Biotechnology), or 5 μl of F5D myogenin hybridoma supernatant (7) was added to the reaction mixtures prior to the addition of probe DNA.

**Mutagenesis of the MRF4 Promoter**—A HindIII-Xhol fragment of the MRF4 promoter was subcloned from pMRF4-CAT vector into pBluescript KS(+) . The AT-rich element, located at positions 11–19, was mutated in the pBSMRF4 construct by PCR mutagenesis. The MRF4/AT mutant was created by replacing nucleotides 11 (T→G), 16 (A→G), and 17 (A→C) using mMRF4/AT primer 5'-GGGTCGACT-TATGTCACCGACgAATTgcATGC-3' (G) and the T7 primer. The PCR conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles. The mutations were verified by sequencing. The wild-type and mutant MRF4 promoter constructs were digested with SalI and XhoI, [32P]dCTP labeled with Klenow polymerase, and used as probes in gel mobility shift assays.

**RESULTS**

**ATBF1-A Inhibits Myogenic Differentiation of C2C12 Cells**—Analysis of ATBF1-A mRNA by RNase protection assay showed that ATBF1-A mRNA was expressed in C2C12 myoblasts proliferating in GM (Fig. 1A). Upon transfer of the cells to DM to induce myogenic differentiation, ATBF1-A mRNA expression declined markedly within 24 h and remained low for 72 h (Fig. 1A). Consistent with these observations, ATBF1-A mRNA was barely detectable in differentiated skeletal muscle tissue isolated from a 7-day-old mouse fetus (Fig. 1B). These results, both in vitro and in vivo, demonstrate that ATBF1-A is expressed preferentially in undifferentiated muscle and suggest that down-regulation of its expression may be required for terminal differentiation of skeletal muscle. ATBF1-B mRNA could not be detected by Northern blotting or RNase protection assay either before or after differentiation of C2C12 cells. We therefore performed RT-PCR analysis of ATBF-B mRNA levels in undifferentiated and differentiated C2C12 cells. We found that ATBF1-B mRNA levels were essentially unchanged after differentiation compared with 90% decrease in ATBF1-A mRNA levels after differentiation (Fig. 1C). These results show that the relative levels of ATBF1-B increase during myogenic differentiation and suggest that the ATBF1 isoforms may play opposing roles in this differentiation pathway.

To examine further whether ATBF1-A regulates myogenic differentiation, we transfected C2C12 cells with a vector expressing the human ATBF1-A cDNA. Three stably transfected clones, A2, A6, and A19, which expressed high, medium, and low levels of ATBF1-A mRNA, respectively (Fig. 2A), were selected and then analyzed for their ability to undergo myogenic differentiation in DM. After 4 days of incubation in DM, control C2C12 cells that were not transfected or transfected with the empty expression vector showed extensive formation of multinucleated myotubes, positive for MHC expression (Fig. 2B, panels b and c). In contrast, ATBF1-A-transfected cells showed either no MHC-positive cells (Fig. 2B, panels d and e) or only a small number of mononucleated MHC-positive cells (Fig. 2B, panel f). In terms of the fusion index, representing the percentage of nuclei present in multinucleated myotubes per field of view (34), myogenic differentiation was reduced 99, 90, and 80% in A2, A6, and A19 cells, respectively, compared with control cells (Fig. 2C). These results indicate that forced expression of ATBF1-A inhibits myogenic differentiation of C2C12 cells.

The progression of myogenic differentiation is accompanied by the induction of positive-acting MRFs including MyoD and myogenic (2, 35), whereas expression of the inhibitory factor Id3 is down-regulated (9). In control C2C12 cells, after transfer from GM to DM, levels of MyoD and myogenin mRNA increased, whereas Id3 mRNA levels decreased as differentiation proceeded (Fig. 3, lanes 1–4). In A6 cells, MyoD and myogenin mRNA were not expressed in cells grown in GM, and no increase in MyoD and myogenin mRNA levels was observed after the transfer of the cells to DM to induce differentiation (Fig. 3, lanes 5 and 6). Expression of Id3 mRNA in A6 cells in GM was much higher than that in control cells (Fig. 3, lane 5), and although its expression level was reduced after transfer to DM, it remained comparable or higher than that in undifferentiated control cells in GM (Fig. 3, lane 6).

The initiation of C2C12 differentiation is also accompanied by changes in expression cycle regulatory factors, including the induction of p21Cip1/Waf1 and the reduction of cyclin D1 mRNA levels (36–38). We confirmed that levels of p21Cip1/Waf1 mRNA were elevated, and cyclinD1 mRNA levels were reduced in control cells following transfer from GM to DM (Fig. 3, lanes 1–4). No induction of p21Cip1/Waf1 mRNA was observed in A6 cells when transferred from GM to DM (Fig. 3, lane 6). In
differentiation of C2C12 cells, we stably transfected C2C12 cells with a vector expressing the human ATBF1-B cDNA. Three clonal cell lines, B3, B5, and B6 (Fig. 4A), were selected and analyzed for the ability to differentiate in DM. We found that these cells exhibited enhanced myogenic differentiation, showing 25–40% higher fusion indices than control cells after 4 days in DM (Fig. 4B). Time course analysis of MHC expression showed that B6 cells generated multinucleated MHC-positive cells within 24 h after the transfer to DM (Fig. 4C, middle right panel) and formed well advanced myotubes at 36 h (Fig. 4C, bottom right panel). In contrast, control cells transfected with the empty vector were devoid of MHC-positive cells at 24 h (Fig. 4C, middle left panel) and formed early myotubes at 36 h (Fig. 4C, bottom left panel). These results show that the ATBF1-B isoform promotes myogenic differentiation of C2C12 cells.

ATBF1-A Inhibits and ATBF1-B Promotes Transdifferentiation of 10T1/2 Fibroblasts Induced by MyoD—10T1/2 fibroblasts transdifferentiate to the myogenic lineage when transfected with MyoD (3). To examine whether ATBF1-A or -B affects MyoD-dependent myogenic conversion of 10T1/2 cells, we transfected these cells with a MyoD expression vector with or without the ATBF1-A or -B expression vector. The cells were then transfected to DM, incubated for 4 days, and stained for MHC expression (Fig. 5A). We found that transfection of ATBF1-A with MyoD at a ratio of 1:1 resulted in a 60% decrease in the number of MHC-positive cells compared with control cells transfected with MyoD alone (Fig. 5B). Transfection of ATBF1-A with MyoD at a ratio of 5:1 resulted in a 80% reduction of MHC-positive cells (Fig. 5B). In contrast, transfection of ATBF1-B with MyoD at a ratio of 1:1 resulted in a 40% increase in MHC-positive cells (Fig. 5B). RNase protection assays revealed that ATBF1-A mRNA is expressed in 10T1/2 cells (data not shown), and the transfection of antisense ATBF1-A and MyoD led to a similar increase in the number of MHC-positive cells. These results indicate that ATBF1-A inhibits MyoD-dependent myogenic conversion of 10T1/2 cells, whereas ATBF1-B promotes this process, which suggests that ATBF1-B may promote myogenic conversion by counteracting the negative effect of endogenous ATBF1-A.

ATBF1-A Inhibits Activation of the MRF4 Promoter by MyoD—As indicated in Fig. 3, the impaired expression of MyoD and myogenin may underlie the myogenic inhibitory action of ATBF1-A. To determine whether ATBF1-A directly inhibits transcription of MyoD family of MRF genes, we analyzed the effect of ATBF1-A on the activation of the MRF4 promoter. The MRF4 promoter contains two E-boxes (E1 and E2), a myocyte enhancer factor-2 site that overlaps with the TATA-box, and an AT-rich sequence adjacent to the E1-box (Fig. 5C) (39). The AT-rich sequence bears similarity to the ATBF1 binding site in the MRF4 promoter and myogenin may promote myogenic conversion by counteracting the negative effect of endogenous ATBF1-A. We therefore investigated the possibility that the AT-rich element is involved in transcriptional repression of the MRF4 promoter by ATBF1-A. First, we performed gel mobility shift assays to analyze the binding of ATBF1-A to the AT-rich element. Because of its large size, we expressed addition, cyclin D1 mRNA levels remained elevated in A6 cells compared with those in control cells in DM (Fig. 3, lane 6). These results show that forced expression of ATBF1-A in C2C12 cells affects the expression of the regulatory factors that control the cell cycle progression as well as myogenic differentiation.

The ATBF1-B Isoform Enhances Myogenic Differentiation of C2C12 Cells—The ATBF1-B isoform differs from ATBF1-A in that it lacks 920 amino acid residues at the N terminus (24). To analyze whether this isoform has any function in myogenic differentiation, we transfected C2C12 cells stably transfected with ATBF1-A. Three clones of ATBF1-A transfected cells, A2, A6, and A19, untransfected C2C12 cells (U), and cells transfected with the empty expression vector (V) were grown in GM (G) or incubated in DM for 4 days (D) and analyzed for ATBF1-A mRNA by RNase protection assay. Probe indicates undigested probe. Panel A, human ATBF1-A mRNA in C2C12 transfected cells grown in GM (U), vector-transfected (V), and untransfected (U). Panel B, relative fusion indices of ATBF1-A-transfected cells. Untransfected and vector-transfected C2C12 cells and A2, A6, and A19 cells were incubated in DM for 4 days, and their fusion indices were expressed as percentages of that of vector-transfected cells. The values are based on the number of nuclei in myotubes in five random fields. Error bars correspond to the S.E. of the mean. This graph is a representation of a single experiment consisting of three plates/sample and was repeated two additional times.

FIG. 2. Forced expression of ATBF1-A inhibits myogenic differentiation of C2C12 cells. Panel A, human ATBF1-A mRNA in C2C12 cells stably transfected with ATBF1-A. Three clones of ATBF1-A transfected cells, A2, A6, and A19, untransfected C2C12 cells (U), and cells transfected with the empty expression vector (V) were grown in GM (G) or incubated in DM for 4 days (D) and analyzed for ATBF1-A mRNA by RNase protection assay. Probe indicates undigested probe. Panel B, ATBF1-A transfected cells (A2, A6, A19) were grown GM or in DM for 4 days and immunostained for MHC expression. U and V, as in panel A. Panel C, relative fusion indices of ATBF1-A-transfected cells. Untransfected and vector-transfected C2C12 cells stably transfected with ATBF1-A; A2, A6, and A19 cells were incubated in DM for 4 days, and their fusion indices were expressed as percentages of that of vector-transfected cells. The values are based on the number of nuclei in myotubes in five random fields. Error bars correspond to the S.E. of the mean. This graph is a representation of a single experiment consisting of three plates/sample and was repeated two additional times.

FIG. 3. Analysis of mRNAs for myogenic and cell cycle regulatory factors in ATBF1-A transfected cells. Total RNA (10 μg) isolated from untransfected (U), vector-transfected (V), and ATBF1-A-transfected (A6) C2C12 cells grown in GM (G) or incubated in DM for 4 days (D) was analyzed by Northern blotting using 32P-labeled cDNA probes for MyoD, myogenin, Id3, p21CIP1/WAF1, cyclin D1, and GAPDH mRNAs.
ATBF1-A in six segments in *E. coli* (Fig. 6A) and incubated each polypeptide with a $^{32}$P-labeled A/E1 probe containing both the AT-rich sequence and the E1-box. As indicated in Fig. 6A, polypeptide V bound to the A/E1 probe. This 60-kDa protein contains homeodomain 4 (HD4), which has also been demonstrated to bind to the AT-rich sequence of the human AFP enhancer. Polypeptide V will be referred to as HD4 because homeodomain 4 is the only functional motif in this segment. The binding of HD4 to the A/E1 probe was reduced by the addition of an excess amount of cold probe (Fig. 6C, lane 3). The addition of anti-HD4 antibodies (Fig. 6C, lane 4), but not anti-IgG antibodies (Fig. 6C, lane 5), resulted in a supershift of the HD4-A/E1 band. Introduction of a mutation to the AT-rich element (Fig. 6D) abolished HD4-A/E1 complex formation (Fig. 6D). In transient transfection assays, the MRF4 promoter carrying the mutated AT-rich element was refractory to transcriptional repression by ATBF1-A (Fig. 6E). These results show that ATBF1-A HD4 binds to the AT-rich element of the MRF4 promoter and mediates transcriptional repression.

The E1 E-box in the A/E1 probe is a potential binding site for the MyoD family MRFs and is separated by the AT-rich element (Fig. 7). Inhibition of MyoD-induced MRF4 Promoter Activation Requires the N-terminal Region and the DNA Binding Domain—The experiments presented above suggest that ATBF1-A may repress activity of the basal transcription complex through the N-terminal region specific to ATBF1-A. The transcriptional regulatory activity of this region was studied by analyzing the effect of deleting short segments within the ATBF1-A-specific region (Fig. 7). Expression of an ATBF1-A construct with a deletion of the first 113 amino acids (plasmid Δ1–113) had no effect on the activation of the MRF4 promoter by MyoD. Similarly, a deletion of the last 345 amino acids of the ATBF1-A-specific region (plasmid Δ113–550) did not affect activation of the MRF4 promoter by MyoD. In contrast, deletion of 438 internal amino acids of the ATBF1-A-specific region (plasmid Δ550–894) inhibited reporter activity to the same extent as the wild-type ATBF1-A. Expression of the ATBF1-A N-terminal region alone is not sufficient to inhibit MyoD-dependent activation of MRF4. These results show that two ATBF1-A-specific regions, amino acids 1–113 and amino acids 550–894, together with the remainder of the ATBF1-A protein, are involved in inhibition of MRF4 promoter activity.

**ATBF1-A Is a Transcriptional Repressor**—The experiments described above show that ATBF1-A-specific N-terminal region is associated with transcriptional inhibitory activity. To characterize this repressive activity further, we transfected C2C12 cells with expression vectors, ATBF1-A-Gal4DB or ATBF1-B-
Effects of ATBF1-A and ATBF1-B expression on the myogenic conversion of 10T1/2 cells and MyoD-dependent transcriptional activation of the MRF4 promoter. Panel A, expression of MHC in 10T1/2 cells transfected with the MyoD expression vector and empty expression vector or with vectors expressing ATBF1-A or ATBF1-B. The transfected cells were incubated in DM for 48 h and then immunostained for MHC expression. Panel B, effects of ATBF1-A and ATBF1-B on MHC expression in 10T1/2 cells. 10T1/2 cells were transfected with the MyoD expression vector and the indicated amount of ATBF1-A or ATBF1-B. In each case, the total amount of DNA transfected was adjusted to 6 μg with the empty expression vector. Five random fields of view from each group were scored for the presence of MHC-positive cells. Values obtained in the control group were set at 100%. Panel C, cis-acting elements present in the mouse MRF4 promoter used in MRF4-luc. Panel D, effects of ATBF1-A and ATBF1-B on MyoD-dependent activation of the MRF4 promoter. 10T1/2 cells were transfected with MRF4-luc with either the empty expression vector or MyoD expression vector along with an equal amount of the vector expressing ATBF1-A or ATBF1-B. The total amount of DNA transfected was adjusted to be the same by the addition of the empty expression vector. After transfection, cells were allowed to differentiate in DM for 2 days, and then luciferase activity was analyzed. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the activity of cells transfected with MRF4-luc alone. Values expressed are the average of three independent experiments. Error bars correspond to the S.E. of the mean.

DISCUSSION

We show in this report that forced expression of ATBF1-A resulted in inhibition of terminal differentiation of C2C12 myoblasts in low serum. Genes that are affected in expression by ATBF1-A transfection include positive and negative myogenic regulatory factors, cell cycle regulatory factors, and muscle structural genes. In vivo, expression of ATBF1-A mRNA was low in differentiated muscle compared with undifferentiated myoblasts. These results, taken together, suggest that ATBF1-A plays a role in the maintenance of the undifferentiated myoblast state, and its down-regulation is essential for terminal muscle differentiation.

Differential Activity of ATBF1 Isoforms—We have demonstrated that ATBF1-A functions as a negative regulator of C2C12 myogenic differentiation, whereas the ATBF1-B isoform does not. ATBF1-B may act in an inhibitory manner toward the A isoform because it shares the DNA binding domain with ATBF1-A but lacks the repressor domain associated with N terminus of ATBF1-A. This possibility is supported by the observation that antisense ATBF1 exhibited similar effects on the MyoD-induced myogenic conversion of 10T1/2 cells (data not shown). A number of genes are known to generate more than one mRNAs through alternative splicing and/or alternative promoter usage, often yielding functionally different protein isoforms. In the case of transcription factors, isoforms with opposing regulatory activities can be produced because of modulation of DNA specificity or affinity, transactivation, or protein-protein interaction (40–43). The ATBF1 gene may belong to this group of transcription factors having isoforms with opposite functions.

We have not been able to detect ATBF1-B mRNA in C2C12 cells directly by Northern blotting and RNase protection assays. ATBF1-B expression has been detected in all cell types that express ATBF1-A analyzed so far but at much lower levels than the ATBF1-A isoform (19, 22, 24, 26, 44). Using RT-PCR we observed that the levels of expression of ATBF1-B relative to ATBF1-A increased after differentiation. This suggests that the ATBF1-B isoform may help decrease the anti-myogenic activity of the ATBF1-A isoform in a dominant negative manner. The decline of ATBF1-A expression may also increase in MyoD and myogenin expression. Although ATBF1-B-expressing C2C12 cells exhibited an accelerated onset of myogenic differentiation, expression of ATBF1-B in 10T1/2 cells did not enhance MyoD-dependent transcriptional activation of the MRF4 promoter. Because MRF4 is activated later in the myogenic program (1), it is possible ATBF1-B may act to...
FIG. 6. ATBF1 binds to the AT-rich element of the MRF4 promoter through homeodomain 4 and mediates transcriptional repression. Panel A, polypeptide V, the HD4-containing segment of ATBF1-A, binds to the MRF4 promoter. Bacterially expressed polypeptides I–VI (5 ng) corresponding to the various parts of the ATBF1-A molecule shown at the top were incubated with labeled A/E1 probe and analyzed by gel electrophoresis. Panel B, nucleotide comparison of the ATBF1 binding site of the AFP enhancer element (AFP AT site) with the wild-type and mutated AT-rich sequences of the MRF4 promoter. Panel C, characterization of HD4-A and E1 interaction. HD4-containing polypeptide V (5 ng) was incubated with labeled A/E1 probe (lane 2). To each binding reaction, an excess amount of unlabeled probe (lane 3), anti-HD4 antibodies (lane 4), or anti-IgG antibodies (lane 5) was added. Panel D, mutation of the MRF4 AT-rich element abolishes binding of HD4. HD4 (5 ng) was incubated with labeled wild-type A/E1 probe (lane 2) or A/E1 probe carrying mutated AT-rich sequence (lane 4) and analyzed by gel electrophoresis. Lanes 1 and 3 contain only labeled wild-type or mutated probes, respectively. Panel E, effect of mutation of the AT-rich element in the MRF4 promoter on transcriptional repression by ATBF1-A. 10T1/2 cells were transfected with MRF4-luc carrying the wild-type or mutated AT-rich element along with the MyoD expression vector, with or without the ATBF1-A expression vector. Values expressed are the average of three independent experiments. Error bars correspond to the S.E. of the mean. Panel F, MyoD is not present in a differentiation-specific complex. Nuclear extracts prepared from C2C12 cells treated with GM (G) or with DM (D) for 4 days were incubated with the A/E1 probe. A differentiation-specific (A) and a non-differentiation-specific (B) protein-DNA complex are noted with arrows. Panel G, myogenin is present in the differentiation-specific complex and is displaced by HD4. Nuclear extracts from C2C12 cells treated with DM for 4 days were incubated with labeled A/E1 probe.

antagonize ATBF1-A activity at events earlier than the onset of MRF4 expression. In addition, our attempts to transfet C2C12 cells stably with antisense ATBF1-A failed to generate viable colonies, consistent with the notion that the reduction of ATBF1-A expression leads to cell differentiation.

FIG. 7. The N-terminal region and DNA binding domain of ATBF1-A are required to inhibit MyoD-induced activation of the MRF4 promoter. 10T1/2 cells were transfected with MyoD expression vector and MRF4-luc reporter with or without vectors expressing the full-size ATBF1-A or ATBF1-B or various portions of these proteins. In all cases the β-galactosidase expression vector pCH110 was cotransfected as an internal control. After transfection, cells were induced to differentiate in DM for 48 h and then analyzed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the activity of cells transfected with MyoD and MRF4-luc alone. Values expressed are the average of three independent experiments. Error bars correspond to the S.E. of the mean. The ATBF1-A protein with homeodomain (boxes) and zinc fingers (ovals) is shown in the upper left. The bars below indicate the parts of the ATBF1-A molecule expressed.

myogenin gene products in A6 cells may explain the inability of these cells to differentiate into myotubes. At present, we do not know whether ATBF1-A regulates MyoD expression directly. The MyoD regulatory regions are complex, and little is known about how this gene is regulated. However, an AT-rich region in the distal core enhancer of the human MyoD gene has been identified (46, 47) and consequently may be subject to regulation by ATBF1-A. In this study, we also demonstrated that ATBF1-A inhibits MRF4 promoter, and this may account at least in part for the lack of MyoD and myogenin expression in A6 cells. MRF4 is the last member of the MyoD family MRFs expressed during myogenic differentiation. However, in mice homozygous for an inactive MRF4 allele, expression of MyoD
and myogenin is greatly reduced on embryonic day 10 when MRF4 is normally expressed (48), suggesting that MRF4 may have effects on the expression of the upstream factors.

We found that in addition to the suppression of the positive MRFs described above, ATBF1-A increased the expression of a negative regulatory factor, Id3, in GM. Furthermore, the down-regulation of Id3 which normally occurs upon serum deprivation was not observed in A6 cells. Forced expression of Id3 has been shown to inhibit myogenic differentiation (49). The suppression of the MyoD family MRF genes and the continued, elevated expression of Id3 support the idea that ATBF1-A prevents the onset of myogenic induction.

**Inhibition of MRF4 Promoter Activity by ATBF1-A**—The MRF4 promoter has been shown to be activated preferentially by myogenin acting on the E1 E-box element (39, 50). We found that HD4 of ATBF1-A binds to an AT-rich sequence adjacent to the E1 E-box. HD4 or the remaining portions of ATBF1-A, including the zinc fingers, were unable to interact with an E-box probe in mobility shift assays, and ATBF1-A had no effect on the activation by MyoD of a luciferase reporter construct consisting of five E-box elements (data not shown). Thus the transcriptional inhibition of MRF4 mediated by ATBF1-A was directed specifically to the AT-rich element and not to E-box sites. However, the binding of ATBF1-A to the AT-rich element is not sufficient to inhibit MRF4 promoter activity because HD4 is shared by ATBF1-B which is unable to inhibit the MRF4 promoter. In addition to the DNA binding domain (HD4), two N-terminal regions specific for ATBF1-A were required for inhibition of the MRF4 promoter. One region from amino acid 1–113 contains a short stretch of residues rich in proline, a characteristic of some transcriptional repressor domains (51–54). The other region from amino acid 550–894 contains four zinc finger motifs unique to ATBF1-A, which may provide a surface for protein–protein interactions (55, 56). This ATBF1-A-specific N-terminal region alone does not inhibit MRF4 promoter activity. However, the fusion of this region to the yeast Gal4 DNA binding domain yielded a protein having 60% inhibitory activity of full-length ATBF1-A. These results suggest that for the N-terminal region to possess transcriptional repressor activity it must be combined with a DNA binding domain. The reason for such a fusion protein being unable to achieve 100% inhibition may be the failure to form a three-dimensional structure similar to the intact ATBF1-A molecule. The thyroid hormone receptor repression domain is also composed of several regions that contribute to its transcriptional repressor activity, and all are required for its maximal activity (57). Similarly, the ZEB protein containing a homeodomain and seven zinc fingers requires the whole molecule carrying both a DNA binding domain and a repressor domain to inhibit myogenic differentiation (15, 16). Unlike ATBF1-A, however, ZEB exerts transcriptional repression through a subset of E-box elements (15, 58, 59) in conjunction with the corepressor protein CtBP (60–62). It is not surprising that an intact ATBF1-A molecule is required for its full activity, considering that the structural organization of ATBF1, including the arrangement of a large number of putative structural domains, is evolutionarily conserved among human, mouse, and Drosophila orthologs (17, 23, 63).

There are several possibilities for how ATBF1-A might inhibit activation of the MRF4 promoter by MyoD. It is possible that the ATBF1-A strictly blocks the basal transcription of this promoter by preventing the formation of the preinitiation complex, possibly through interactions with TATA-binding protein or other basal transcription factors. Alternatively, ATBF1-A may bind in close proximity to activator proteins such as myogenin to antagonize their action to prevent MRF4 induction without affecting the basal transcriptional machinery. Our data demonstrate that ATBF1-A HD4 binds to the AT-rich element and displaces myogenin from binding to the adjacent E-box element. However, this displacement may only contribute to a small proportion of the ATBF1-A inhibitory activity because ATBF1-B also contains HD4, yet does not significantly affect MyoD activation of the MRF promoter. Since HD4 does not interact with E-box sequences (Fig. 6D), myogenin can still bind to the upstream, E2 E-box of the MRF4 promoter and activate transcription in the presence of ATBF1-B. However, binding of ATBF1-A to the AT-rich element through HD4 may place the MRF4 promoter under transcriptional constraint because of the general transcriptional inhibitory activity of ATBF1-A. Therefore inhibition of the MRF4 promoter by ATBF1-A requires both a trans-repression domain as well as its DNA binding domain. The experiments employing ATBF1-A-Gal4DB fusion proteins indicate that ATBF1-A is capable of inhibiting basal transcription, although the possibility that ATBF1-A interferes directly with activation by MyoD or myogenin cannot be ruled out. Finally, ATBF1-A may recruit corepressor proteins possessing histone deacetylase activity to the to the MRF-4 promoter. It is not known whether ATBF1-A associates with these corepressor proteins; however, interactions between ATBF1-A and other proteins may be a key in establishing a functional complex. The repressive activity of ATBF1-A may be mediated through interactions between the ATBF1-A-specific N-terminal domain and regions common to both isoforms with other transcription factors. Recently, it has been reported that an interaction between the extreme C-terminal portion of both ATBF1-A and ATBF1-B and the proteocorepressor c-Myb represses activation of Myb-responsive promoters (64). We are currently examining whether ATBF1-A can interact with corepressor or coactivator complexes.

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