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Structure and Function of the Upstream Promotor of the Human *Mafbx* Gene: The Proximal Upstream Promotor Modulates Tissue-Specificity

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Abstract Muscle loss has been linked to increased expression of an ubiquitin ligase termed muscle atrophy F-box (MAFbx), a nuclear protein involved in degradation of MyoD. To gain insights into mechanisms by which the human *MAFbx* gene is controlled, the structure of its upstream promotor were studied, and its expression in cultured cells was characterized. Expression of MAFbx was found only in cells of muscle lineage. A reporter gene controlled by 948 bases of human MAFbx upstream promotor displayed similar cell-type selectivity. MAFbx levels were greatly enhanced upon myogenic differentiation of C2C12 myoblasts, and differentiation markedly increased activity of a reporter gene constructed with 400 bp of upstream promotor from the *MAFbx* gene. The core promotor spanned approximately 160 bases beginning at −241 bp upstream of the first codon, included potential binding sites for MyoD and myogenin, and was highly conserved among mouse, rat, and human*MAFbx* genes. The major transcription start site for the human *MAFbx* gene was 340 bases upstream of the ATG and was localized the highly conserved region of 140 bp. The findings indicate an important role for the immediate upstream promotor of the human *MAFbx* gene in mediating its developmental expression and tissue specificity. J. Cell. Biochem. 96: 209–219, 2005. © 2005 Wiley-Liss, Inc.

Key words: muscle atrophy; ubiquitin ligase; promotor structure; developmental regulation

Muscle loss is an important medical consequence of spinal cord injury, burns, chronic illness, injury, and aging [Spungen et al., 2000; Yeh et al., 2002; Janssen et al., 2004]. The resulting weakness reduces mobility and independence, and increases risks of falls and fractures. Muscle loss results primarily from accelerated degradation of muscle proteins by caspases and the ubiquitin-proteasome system [Furuno et al., 1990; Tiao et al., 1994]. In this system, proteins are marked by the covalent attachment of the 76 amino acid protein ubiquitin by the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ-amino group of a lysine in the substrate protein [Pickart, 2001]. Additional ubiquitin molecules are attached to the first one, forming a polyubiquitin chain that is recognized by the 26S proteasome. This giant protease complex then degrades the substrate.

Conjugation of ubiquitin to appropriate substrate proteins is catalyzed by E3s (ubiquitin ligases) [Pickart, 2001]. The most common form of ligase is a multimeric complex comprised of an E2 (ubiquitin conjugase), one or more proteins providing substrate recognition, and structural proteins. Studies employing DNA microarray analysis or differential display have shown that a gene called muscle atrophy F-box (*MAFbx*) is greatly upregulated in muscle loss

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states [Bodine et al., 2001; Gomes et al., 2001; Lecker et al., 2004]. MAFbx is a component of an SCF family ubiquitin ligase that is expressed selectively in skeletal muscle and heart, where it is localized to the nucleus and has been linked to degradation of MyoD [Bodine et al., 2001; Tintignac et al., 2004]. Moreover, disruption of the *MAFbx* gene in mice greatly reduces rates of muscle loss [Bodine et al., 2001]. The *MAFbx* gene is upregulated in all muscle loss states studied to date, including paralysis, starvation, diabetes, renal failure, sepsis, and glucocorticoid excess [Bodine et al., 2001; Gomes et al., 2001; Wray et al., 2003; Lecker et al., 2004]. Consequently, understanding of the regulation of MAFbx expression has been of great interest.

Little is known about how expression of this gene is controlled. Muscle specific expression of many other genes is accomplished through muscle differentiation factors such as myogenin and MyoD acting at regulatory elements in promotor regions of such genes [Rawls and Olson, 1997]. These are transcription factors expressed early in the program of muscle differentiation that continue to be expressed in fully differentiated muscle. Such elements have been found in upstream regulatory regions, in non-coding sequences within the first exon, and in introns [Catala et al., 1995; Smith et al., 1998; Storbeck et al., 1998; Wheeler et al., 1999; Cheng et al., 2002; Gilley et al., 2003]. Core promotors or their immediate upstream regions also confer tissue selectivity [Smith et al., 1998]. Some insight into how MAFbx expression is upregulated in muscle loss states comes from findings that the forkhead family transcription factor Foxo3A is activated in muscle loss states such as starvation and glucocorticoid toxicity, and that in the mouse *MAFbx* gene, this transcription factor upregulates MAFbx expression by interactions with forkhead transcription factor elements within the upstream promotor and untranslated region of the first exon [Lee et al., 2004; Stitt et al., 2004]. The studies described below analyze the expression of MAFbx in cells of different developmental lineage, and characterize the effects of myogenic differentiation on expression of this gene. Additional studies examine the role of changes in activity of the *MAFbx* upstream promotor in tissue-selective expression of this gene and in the increase in its expression found during myogenesis. Studies of MAFbx upstream promotor structure suggest that a highly conserved core promotor encodes much of the developmental and tissue-specific expression of this gene.

MATERIALS AND METHODS

Plasmids

pMAF3.1: a 3.1 kb upstream region of the human MAFbx was cloned using sequence information from GeneBank to design the following PCR primers: upper strand—5′-CCGACAACATAGCAAGACCCCATCTCTC-3′; lower strand—5′-GAGAGGATCTCAAGCGTTGCAGGCTCCG-3′. The product was amplified by PCR using Pfx polymerase (Invitrogen, Carlsbad, CA) with human kidney genomic DNA as a template then cloned into TOPO-TA pCR2.1 (Invitrogen). The insert was excised with SacI and XhoI and subcloned into the same sites in pGL2-Basic (Promega). pMAF2.4*:* a 2.4 kb fragment from the *MAFbx* gene was cloned by PCR using 5′-TAACACATCTGTGAGGTCAACGGGAGTG-3′ as a primer for the upper strand, with the above primer for the lower strand. Cloning and subcloning of the PCR product were as above. pMAF948: a 948 bp fragment of the upstream region was amplified by PCR using 5′-CTTAGAGGGTTCGGGTAGGATA-3′ (upper strand) and 5′-GACTAGACGGATGGGGAGAC-3′ (lower strand). The fragment was cloned into TOPO-pTRC-His, excised with BamH1 and HindIII, then ligated into the BglII and HindIII sites of pGL2-Basic. pMAF948-Luc was the starting point for several 5′ deletions and 3′ deletions that were prepared by excision of fragments by restriction enzyme digestion using enzymes as indicated in Figure 3, followed by filling in of overhangs and re-ligation.

To prepare a full length cDNA clone of the MAFbx mRNA coding region, a cDNA library was prepared by reverse transcription (Omniscript, Invitrogen) using total human muscle RNA as a template (BD Biosciences; Palo Alto, CA) by PCR amplification using Pfx polymerase with 5′-CACCATGCCATTCCTCGGGCAGGACT-3′ and 5′-GAACTTGAACAAGTTGATAAAGTC-3′ as primers for upper and lower strands, respectively. This product was ligated into pcDNA3.1D/V5-His-TOPO (Invitrogen) to make pcDNA3.1-hMAFbx.

The pCMV5 construct expressing rat glucocorticoid receptor (GR) was a gift from Dr. Diane Robins (U-Michigan).

Primer Extension Analysis

Ten picomole of primer (5′-GAC TAG ACG GAT GGG GAG AC-3′) was annealed to 2 μg of total RNA from human skeletal muscle (BD Biosciences). A radiolabeled reverse transcript was then prepared using the superscript first—strand synthesis system (Invitrogen) at 42°C for 50 min after adding 1 μl of [λ-^{32}P]-dATP (3,000 Ci/mmol). Sequence ladders were generated using an M13mp18 DNA template and M13 sequencing primer (Sequenase DNA sequencing kit, v2.0, USB Cleveland, OH). The reactions were then analyzed by electrophoresis on 6% polyacrylamide gels, and the dried gels were visualized using a phosphorimager.

Cell Culture and Luciferase Assays

C2C12, L6, CHO, HeLa, PC12, HepG2, CV-1, and COS7 cells (American Tissue Type Collection) were maintained in DMEM supplemented with 10% fetal bovine serum. For determination of MAFbx promoter activity in the above cell lines, cells at 80%–95% confluence were co-transfected with reporter genes as indicated in the figures together with pCMV-Renilla which served as a transfection control. Cells were transfected with a total of 0.2 μg DNA per well using Lipofectamine Plus. Three hours later, cells were covered with an additional 0.8 ml of growth media. Twenty-four hours thereafter, activity of firefly and renilla luciferase was quantified in cell lysates using commercially prepared luciferase substrates (Promega). Luminescence was quantified with a LB 960 Microplate Lumonomiter (Berthold Technologies, Bad Wildbad-Germany).

Northern Blotting

Cells were seeded into 100 mm plates and incubated until confluent. Northern blot analysis was performed using 20 μg total RNA (RNAeasy Mini kit, Qiagen, Valencia, CA) after resolution by electrophoresis on agarose gels and transferred onto GeneScreen membranes (PE Biosciences, Boston, MA). The DNA probe was generated by random priming with [λ-^{32}P]dCTP (MP Biomedicals, Inc., Irvine, CA) using full length human MAFbx cDNA as a template. Blots were hybridized at 68°C for 1 h in QuikHyb solution (Stratagene, La Jolla, CA) then washed. Northern blots were visualized by phosphorimaging. The scanned images were edited using Adobe Photoshop 7.0. Intensities of bands on Northern blots were quantified with ImageJ version 1.31V.

RESULTS

In an early report of the cloning of MAFbx, it was found that the gene was expressed selectively in skeletal muscle and heart [Bodine et al., 2001]. To determine whether tissue selectivity extended to cultured cell lines, total RNA was isolated from cell lines having muscle and non-muscle lineage and was used for the determination of MAFbx mRNA levels by Northern blot (Fig. 1A). Expression was highest in muscle-derived cell lines (L6 and C2C12 cells) while being low or undetectable in cells of epithelial origin (HeLa, CV1, COS7), or in cells derived from liver (HepG2) or ovary (CHO).

Muscle specific expression arises through the concerted action of regulatory mechanisms that include expression of tissue specific transcription factors that interact with regulatory elements in the promotor regions of muscle genes [Rawls and Olson, 1997]. To begin to localize the upstream promotor of the human *MAFbx* gene and to determine whether this region conferred tissue-specific expression, a 948 bp

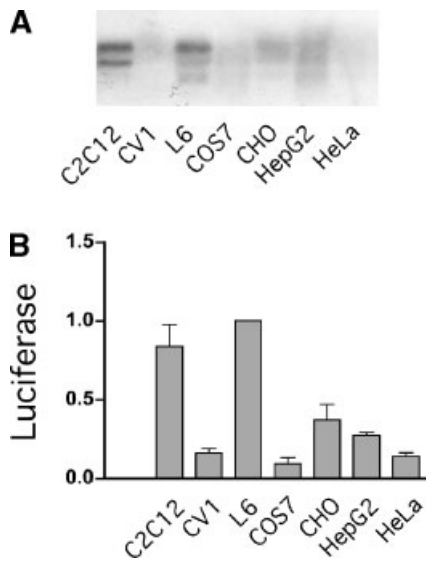

Fig. 1. Expression of muscle atrophy F-box (MAFbx) depends on cell lineage (muscle, C2C12 and L6; epithelial, CV1, COS7, and HeLa; other, HepG2 and CHO). **A**: Total RNA was used to assess MAFbx expression by Northern blotting. **B**: Cells were seeded into wells of 24-well plates (5×10^4). After incubation overnight, cells were transfected with pMAF948 and pCMV-Renilla. After 3 h, 0.8 ml of growth medium containing 10% CDS was added. Twenty-four hours later, luciferase activity was measured. Values were normalized relative to those for L6 cells. Data are mean values ± SEM for at least three experiments.

segment of the 5′ flanking sequence of the human *MAFbx* gene that began 14 bases upstream from the ATG was cloned by PCR using kidney genomic DNA as a template. Sequencing of the PCR product revealed two differences from the corresponding GeneBank sequence: deletion of G and C/T change at −681 and −339 bases upstream of the ATG, respectively (GeneBank accession # AY929855). These differences were present in three separate PCR clones from kidney DNA and were absent in those clones that were from the sequences for genomic DNA from lung, spleen, and skeletal muscle. These sequence variations appear to represent polymorphisms in the human MAFbx promotor region.

The PCR product obtained from amplification of kidney genomic DNA was ligated into pGL2-Basic, a promotorless reporter plasmid expressing firefly luciferase. Activity of the resulting reporter gene (pMAF948) was determined after transfection into cells of muscle and non-muscle lineage (Fig. 1B). Activity was greatest in the two muscle-derived cell lines (C2C12 and L6). As compared to activity in muscle cell lines, that in cells of epithelial lineage was approximately fivefold lower, and that in other cell types threefold lower. Thus, there was a good overall agreement between reporter gene activity and MAFbx levels observed in these same cell types (Fig. 1A,B) indicating that promotor sequences within the first 948 bp upstream of the ATG contribute to cell-type selective expression of MAFbx.

Tissue-specific expression of genes is an important part of the program of differentiation by which embryonic cells mature into their mature, fully functional counterparts. For example, the muscle-differentiation factors myogenin and MyoD become expressed early in the program of muscle differentiation, and in turn induce expression of other muscle-specific genes [Rawls and Olson, 1997]. To determine whether expression of MAFbx was induced during differentiation, we examined expression of the gene during differentiation of C2C12 myoblasts into myotubes that was induced by incubation in media containing 2% horse serum. Differentiation begins within hours and is nearly complete within 24–36 h [Bains et al., 1984]. When levels of MAFbx mRNA in differentiating cells were assessed by Northern blotting, increased MAFbx expression was readily apparent within 24 h and had reached a maximum by 72 h (Fig. 2A). Intensities of the bands present on the Northern blots were quantified by densitometry scanning and normalized relative to intensities of the bands for GAPDH. Levels of MAFbx had increased fourfold within 24 h and reached levels ninefold higher than undifferentiated cells by 72 h of differentiation. By contrast, levels of MAFbx mRNA did not change appreciably over this period in undifferentiated myoblasts (Fig. 2A).

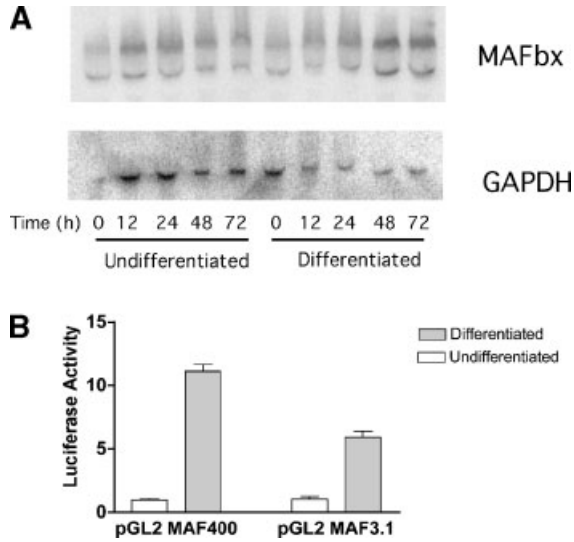

Fig. 2. Induction of MAFbx expression during differentiation of C2C12 cells. **A**: Cells were seeded into 100 mm plates, grown until 80% confluent then maintained in growth medium (DMEM supplemented with 10% FBS) or differentiation medium (DMEM supplemented with 2% horse serum) for the indicated periods at which time MAFbx mRNA levels were quantified by Northern blotting. **B**: Cells at 80%–95% confluence were transfected overnight with pMAF948 and CMV-Renilla then maintained for 24 h in media (undifferentiated cells, DMEM + 10% FBS, differentiated cells, DMEM + 2% HS), at which time luciferase activity was quantified. Differentiation was induced by incubation for 48 h in DMEM supplemented with 2% horse serum. Data are means ± SEM for six replicates from a representative experiment.

To determine whether MAFbx upstream promotor activity increased upon differentiation, C2C12 cells were transfected with reporter genes either without differentiation, or after differentiation for 48 h. We first tested expression of a reporter under the control of 3.1 kb of upstream promotor prepared by PCR amplification followed by ligation of the product into pGL2-Basic (pMAF3.1). This reporter was active in both undifferentiated and differentiated cells (Fig. 2B). Differentiation led to an approximately sixfold increase in activity. In additional experiments, cells were transfected with a reporter gene constructed by 5′truncation of pMAF948. This construct con-

tained 400 bp of upstream sequence, beginning 14 bases upstream of the first codon (pMAF400). It was also expressed in undifferentiated cells, and displayed over a 10-fold increase in activity upon differentiation. The findings indicate that MAFbx promotor activity is increased by the differentiation program and suggest that signals that are responsible for this increase act in large part through sequences located within 400 bases upstream of the ATG.

This 400 bp region begins just upstream of the first codon, suggesting that the 400 bp fragment is approaching the minimal promotor for this gene. The boundaries the minimal promotor were further defined by the construction of additional truncations of pMAF948 and the determination of their activity after transfection into C2C12 cells. Constructs used for this analysis are shown in Figure 3A. Removal of 100 bp from pMAF400 (pMAF300) dramatically reduced activity, suggesting that key elements for basal expression are contained within the 100 bp that were removed. Several 3′ truncations of pMAFbx948 were also constructed. Deletion

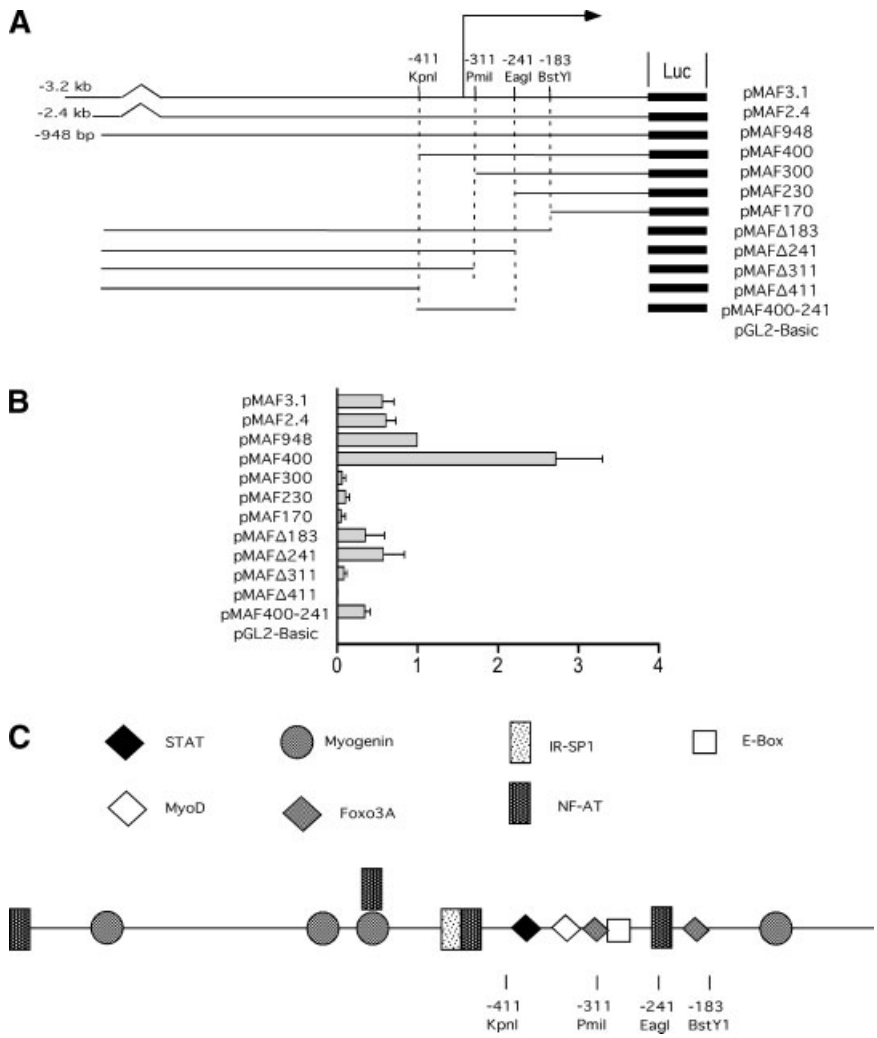

Fig. 3. Effects of truncations on activity of the MAFbx promotor. **A**: Map of reporter constructs used together with the location of restriction sites employed to generate the 5′ and 3′ truncations of pMAF948. **B**: C2C12 cells at 80% confluence were transfected for 3 h with the indicated reporter constructs and pCMV-Renilla then maintained in medium supplemented with 10% FBS. Twenty-four hours later, luciferase expression was quantified. Data were normalized relative to the values for the pMAF948 construct, and are mean values ± SEM for three separate determinations, each performed in triplicate. **C**: Selected, putative transcription factor bindings sites within the MAFbx 948 bp upstream region. The transcriptional start site (see Fig. 4) is shown as an arrow. Locations of Foxo3A sites are predicted from those in mouse MAFbx [Sandri et al., 2004], while that of the E-Box was from manual inspection of the sequence. Additional searches for potential transcription factor binding sites were conducted with Match (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi).

from pMAF948 of 170 or 230 bp (pMAFΔ183 and pMAFΔ241, respectively) reduced expression modestly (less than 50%, Fig. 3B). However, a marked decrease of activity was found when an additional 70 bp were removed from the 3′ end (pMAFΔ311), and activity was lost completely when 400 bp were removed from the 3′ end (pMAFΔ400). The above findings suggested that a region consisting of 184 bp between −227 and −411 relative to the ATG was essential for MAFbx expression. To confirm this interpretation, an additional construct was prepared containing only this region (pMAFbx400-241). When this reporter was introduced into C2C12 cells, luciferase expression was similar to that for pMAFbxΔ241 (Fig. 3B).

Additional experiments tested how promotor sequences further upstream influenced activity. The experiments compared activity of constructs having approximately 1 kb up sequence upstream of the ATG (pMAF948) with that of reporters having 2.4 and 3.1 kb of upstream promotor (pMAF2.4 and pMAF3.1). Importantly, the inclusion of additional upstream sequences (pMAF2.4 and pMAF3.1; Fig. 3B) did not appear to enhance activity, indicating the absence of additional enhancing elements within 3 kb upstream of the first codon.

To better understand how activity of the core promotor and its flanking sequences were regulated, the first 962 bases upstream of the first codon were searched for possible transcription factor binding sites (summarized in Fig. 3C). Several sites were predicted for the basal transcription factors NF-1 and SP-1. Some SP-1 sites were organized into a motif previously linked to insulin responsiveness (SP-IR in Fig. 3C) [Araki et al., 1991]. Sites were also predicted for binding of NF-AT, linked to muscle hypertrophy [Crabtree, 1999], and STAT3, which is activated by the muscle-trophic hormone IGF-1 [Zong et al., 2000]. Several sites were predicted for the muscle differentiation factor myogenin, together with one site binding another such factor, MyoD. Of considerable interest, potential binding sites for myogenin, MyoD, and NF-AT were found within the core promotor region (Fig. 3B). Two binding sites for forkhead factors such as Foxo3A were identified; both were located within the core promotor. Their location was similar to that of sites that were previously found to be important in regulation of the mouse MAFbx [Sandri et al., 2004], as discussed in more detail below. One E-box was identified in this region as well representing an additional potential binding site for muscle differentiation factors such as myogenin and MyoD [Krempler and Brenig, 1999].

The localization of the core promotor raised questions regarding the location of the transcriptional start site and the relationship of this site to the core promotor region. To address these questions, a primer extension analysis was performed using human muscle mRNA as a template (Fig. 4), and employing a primer beginning 14 bases upstream of the ATG. This analysis revealed a single major product of 327 bases based upon comparison of its mobility to that of sequencing standards, from which the major transcriptional start site can be inferred to be 340 bases upstream of the ATG. This determination adds 147 bases to the length of the 5′ UTR obtained from the original cDNA clone [Bodine et al., 2001]. Inspection of the sequences immediately upstream of the transcription start site revealed no TATA or CAAT box.

The presence of two forkhead binding sites in the human *MAFbx* gene in a similar location to that previously described for the mouse *MAFbx* gene suggested that these promotors may be more broadly evolutionarily conserved, at least in certain regions. Inspection of the nucleotide composition of mouse and rat MAFbx upstream promotor regions revealed a high GC content (66% and 62.8%, respectively). However, the human MAFbx upstream promotor region had an even higher GC content (73.8%), largely, because of a particularly GC-rich region in the 5′UTR. Alignment of the sequences for the first 1 kb of the upstream promotor for mouse, rat, and human *MAFbx* genes revealed that, overall, the sequences of the three species were 62.6% identical. The three genes contained a highly conserved region spanning approximately 140 bp and containing the transcriptional start site for the human MAFbx promotor. Sequences within this region were approximately 78% identical, while those beyond it were 52% identical. Sequences flanking the transcriptional start site of human MAFbx were absolutely conserved.

Of interest, the conserved region involved largely sequences downstream of the transcriptional start site, and contained two elements for forkhead transcription factors shown to be important for upregulation of the mouse *MAFbx*

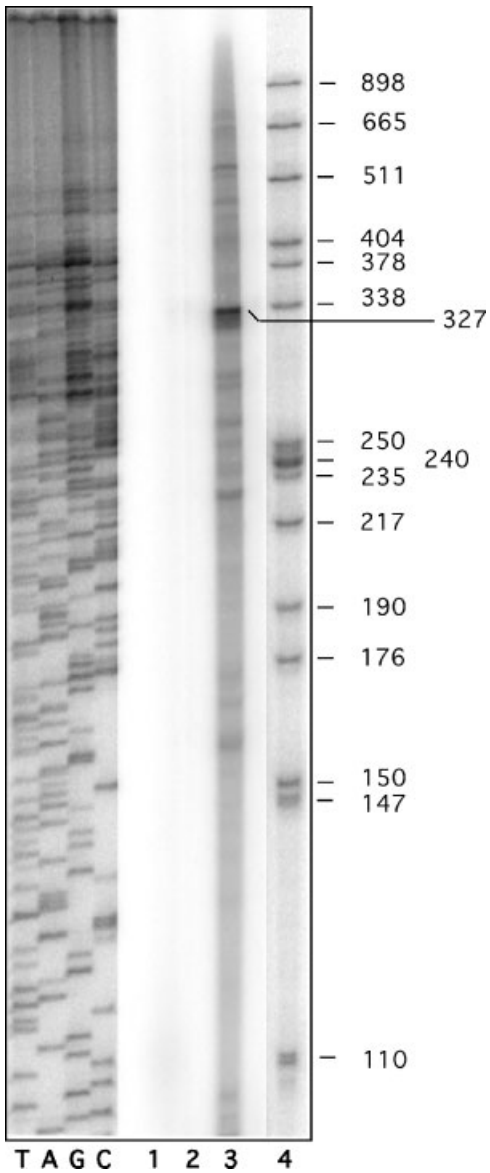

Fig. 4. Primer extension analysis of the 5′ UTR of human MAFbx mRNA. Radiolabeled products generated by reverse transcription of human muscle total RNA ATG were resolved by PAGE (**lane 3**). The primer used began 14 bases upstream of the ATG. Identical reactions without RNA (**lane 1**) or containing yeast mRNA (**lane 2**) were used as controls. T, A, G, and C indicate lanes for the corresponding sequencing standards (see Materials and Methods). **Lane 4**: λP[32]-dATP-labeled size standards generated by digestion of pGL2-basic with Hpa II.

gene by starvation or glucocorticoids [Sandri et al., 2004]. These forkhead transcription factor consensus sites (double underlined, Fig. 5) were present in essentially identical locations in the three genes, which fell within the core promotor of the human MAFbx upstream promotor. Both sites were situated downstream of the transcriptional start site for the human *MAFbx* gene. The sequences of these sites match exactly those for two corresponding sites in mouse and rat MAFbx upstream promotor regions. Sequences flanking these sites were also highly conserved.

Several forkhead sites have been found in upstream regions of the mouse *MAFbx* gene [Sandri et al., 2004]. To determine whether this was the case for the human MAFbx upstream promotor, a computer-assisted search of the corresponding region of the human MAFbx upstream promotor performed. This analysis revealed only one additional forkhead binding site located at −1,604 bp relative to the ATG).

The high degree of sequence conservation in the region of forkhead sites near the core promotor suggested conservation of function of these sites in regulation of MAFbx expression. The paucity of upstream forkhead sites in the human MAFbx promotor, however, provided evidence that regulation of human MAFbx expression may differ in some ways from that for mice. The conserved forkhead sites have been linked to glucocorticoid-induced activation of the mouse MAFbx promotor [Sandri et al., 2004]. To gain insight into whether this was also true for the human MAFbx promotor, C2C12 cells were co-transfected with a vector expressing the GR and MAFbx promotor constructs, then incubated with dexamethasone (Fig. 6). Incubation with dexamethasone increased activity of the MAFbx 3.1 kb promotor segment by approximately twofold. Similar activation was observed for the 2.4 and 400 bp truncations, while activation of the 948 bp truncation was reduced.

DISCUSSION

The findings above indicate that the muscle-specific expression of MAFbx described in studies of mouse tissues extends to myocyte-specific expression in cultured cells, where it is a part of the program of differentiation by which progenitors are committed to skeletal muscle cells. Mechanisms by which promotors mediate muscle-specific gene expression are varied with several different mechanisms employed. Upstream elements containing an E-box and several CArG boxes enhance expression of mouse myosin light-Chain 1A in a tissue-specific manner, while E-Boxes within the first exon enhance both levels of expression and muscle selectivity of the myotonic dystrophy protein kinase gene [Catala et al., 1995; Storbeck et al.,

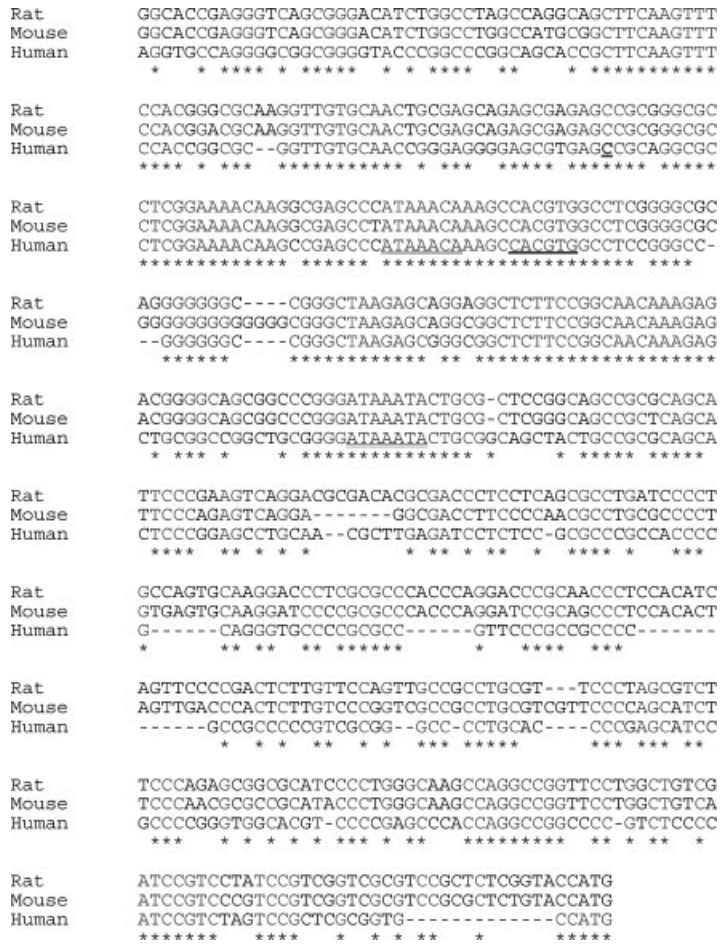

Fig. 5. Aligned sequences of the first 1 kb upstream of the ATG for mouse, rat, and human *MAFbx* genes. Forkhead binding sites are marked with a double underline. A conserved E-Box is shown as a bold underline. The transcriptional start site is bolded with a single underline. Sequence alignments were performed using the ClustalW function of Biology Workbench 3.2 (http://seqtool.sdsc.edu/CGI/BW.cgi).

1998]. In some cases, the core promotor itself also confers tissue-specificity, as is the case for the myotonic dystrophy protein kinase genes [Smith et al., 1998; Storbeck et al., 1998]. Intronic transcriptional elements also have been implicated in tissue-specific expression of muscle genes [Angus et al., 2001].

Tissue-specific expression of the human *MAFbx* gene appears to be dependent upon activity of the upstream promotor of this gene. This conclusion is based on the cell-type expression of reporter gene under the control of a 948 bp portion of upstream promotor beginning just 5′ to the first codon. Several lines of evidence indicate that elements of the immediate upstream promotor, and possibly within the first exon, confer such muscle-specific expression. Activity of a 400 bp region beginning just upstream of the ATG was strongly enhanced after differentiation of C2C12 myoblasts into myotubes. Such enhancement is taken by some authors as a test for muscle-specific expression [Catala et al., 1995]. The 5′ end of this promotor region corresponds to the 5′ end of the core promotor determined by truncation analysis. The insert is extended 228 bases beyond the 3′ boarder of the core promotor, and removal of this additional sequence substantially reduces activity, possibly through removal of a myogenin binding site. In contrast to some muscle genes, tissue specific expression was not enhanced by elements up to 3.1 kb upstream of the core promotor. Specifically, myogenesis increased activity of reporters having 3.1 kb of upstream promotor to a similar degree as the 400 bp sequence, and normalized activities of the promotors were also similar. While the above data does not identify transcription factors that mediate tissue-specific

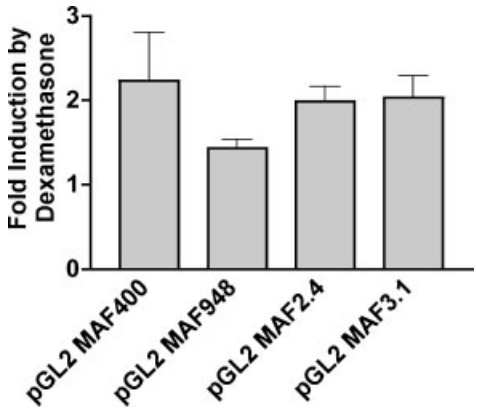

Fig. 6. Induction by dexamethasone of activity of the MAFbx promotor, and analysis of how truncations alter activation. C2C12 cells were transfected overnight with MAFbx reporters as indicated, pCMV-renilla, and a vector expressing rat GR. Cells were incubated overnight in DMEM containing 10% charcoal-dextran-stripped FBS supplemented with vehicle or dexamethasone (100 μM in ethanol). Ethanol concentrations were less than 0.1%. Data are expressed as fold-induction by dexamethasone as compared to activity for vehicle, and are the mean ± SEM for six replicates from a representative experiment.

expression of MAFbx, insights into their possible identity are available from the analysis of sequences for potential transcription factor binding sites. This analysis indicated potential binding sites for several myogenic factors including myogenin and MyoD as well as for factors known to regulate muscle hypertrophy (NF-AT) or the action of extrinsic factors promoting such hypertrophy such as IGF-1 acting via STAT3.

Truncation analysis indicated that the core promotor was localized between −241 and −411 bases upstream of the ATG. A striking feature of the core promotor of the human *MAFbx* gene was its high homology to sequences of the corresponding rat and mouse genes. The presence of such a highly conserved sequence within the upstream promotor suggests a critical function for these sequences in regulation of expression of the *MAFbx* gene. Evidence supporting this conclusion comes from findings that for both human MAFbx (Fig. 6) and mouse MAFbx [Sandri et al., 2004], activation of the promotor by dexamethasone requires only a 400 bp region consisting of the core promotor and downstream sequences containing two forkhead sites.

Another interpretation of the high sequence homology of the core promotor and initiation site of the human MAFbx promotor is that a common initiation site is used in all three species. If this were the case, the 5′ UTR of mouse and rat MAFbx mRNA would be approximately 389 bases as compared to 340 bases for human MAFbx mRNA determined by primer extension analysis (Fig. 4). These analyses suggest that the major transcriptional start site is further upstream than previously appreciated from clones of MAFbx mRNA for human and mouse MAFbx, which predict transcriptional start sites 192 and 326 bases upstream of the ATG, respectively [Bodine et al., 2001; Gomes et al., 2001].

The presence of two absolutely conserved forkhead bindings sites and an E-box within this conserved region argue that expression of human MAFbx is regulated, at least in part, by forkhead factors such as Foxo3A acting at these elements. Also remarkable is the presence of a conserved E-Box located in this sequence between the two forkhead binding sites. This finding provides additional indirect support for interactions of basic helix–loop–helix factors such as myogenin and MyoD with sequences within the first exon of MAFbx. When considering this possibility, it is of interest that promotor activity is almost completely lost after deletion of sequences between −240 and −311 bases upstream of the ATG because such deletions sequentially remove the first forkhead binding sites and E-Box followed by the second forkhead site (Fig. 3). This interpretation is consistent with findings from studies of the regulation of the mouse *MAFbx* gene, which indicated through mutational analysis that both of the forkhead sites located in the non-coding region of the first exon contributed to upregulation of this gene in muscle loss states [Sandri et al., 2004]. E-boxes capable of recruiting myogenic factors have been identified within, however additional studies are needed to test whether muscle differentiation factors bind and mediate transactivation at such elements.

The 5′ UTR of the *MAFbx* genes from three mammalian species revealed a relatively high-GC content with the frequent occurrence of CpG islands. This was particularly true for the human *MAFbx* gene. This finding initially suggested methylation as a means of suppression of the human *MAFbx* gene in non-muscle tissues. However, no evidence of methylation within this region was observed upon sequencing of PCR products generated after bisulfate treatment of genomic DNA from kidney, lung, or spleen (S. Guo, W. Zhao, unpublished),

tissues in which MAFbx expression is low [Bodine et al., 2001].

Despite the many similarities of the core promotor and immediate downstream regulatory sequences of the MAFbx promotor from rat, mouse and human, and regulation of activity of such sequences by glucocorticoids, caution should be used before assuming that overall regulation of this gene is identical in these difference species. Sequences upstream of the core promotor showed low homology as compared to those within the core promotor with direct implications for understanding how the *MAFbx* gene may be regulated in muscle loss states. Whereas the core promotor includes two forkhead binding sites in all species, only one additional forkhead site was found within 3.5 kb upstream of the ATG. By contrast, prior analysis of the mouse *MAFbx* gene has found many potential forkhead regulatory elements in these upstream sequences, and has provided evidence that these upstream elements enhance effects of Foxo3A on MAFbx expression [Sandri et al., 2004].

These differences in promotor structure suggest several interpretations. One is that there may be differences in regulation of human and mouse MAFbx expression, at least with respect to the relative magnitude of changes in expression resulting from the action of specific transcription factors such as Foxo3A. In addition, these differences suggest that regulatory mechanisms beyond activation of Foxo3A are important modulators of the expression of human MAFbx. For example, upstream regions of the MAFbx promotor contain binding sites for many other transcription factors that may play important roles in modulating MAFbx expression. For example, levels of MyoD rise markedly after denervation and loss of PKC-dependent phosphorylation of myogenin is an important component driving upregulation of this gene in denervation [Hyatt et al., 2003; Blagden et al., 2004]. Modulation of the phosphorylation state of myogenin or other muscle differentiation factors could be important contributors to regulation the of the expression of *MAFbx* and other genes critical to the genesis of muscle loss. Modulation of muscle loss by IGF-1 may involve transcription factors other than Foxo3A. Human MAFbx upstream promotor regions contain multiple binding sites for STAT3, which is a target of IGF-1 action through activation of junk-activated kinases [Zong et al., 2000]. Achieving a better understanding of how these pathways modulate MAFbx expression in development, health, and disease is an important goal for future studies.

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