Muscle Differentiation Is Antagonized by SOX15, a New Member of the SOX Protein Family*

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SOX proteins belong to a multigene family characterized by a unique DNA binding domain, known as the high mobility group box, that is related to that of the testis determining gene SRY. cDNA sequences for more than 30 SOX genes have been identified, and some are known to have diverse roles in vertebrate differentiation and development. Here, we report the isolation and characterization of mouse Sox15 that was uncovered during a screen for high mobility group box containing transcription factors that are expressed at different levels during skeletal muscle differentiation. Sox15 cDNAs were found at a much higher frequency in myoblasts prior to their differentiation into myotubes. Electrophoretic mobility shift assays indicated that recombiant SOX15 protein was capable of binding to a consensus DNA binding site for SOX proteins. When overexpressed in C2C12 myoblasts, wild type SOX15, but not a C-terminal truncated form or the related protein Sox11, specifically inhibited activation of muscle-specific genes and expression of the basic helix-loop-helix myogenic factors myogenin and MyoD, resulting in a failure of the cells to differentiate into myotubes. These results suggest a specific and repressive role for SOX15, requiring the C-terminal domain, during myogenesis.

The sex determination gene, SRY, encodes a protein with a 79-amino acid DNA binding motif known as the high mobility group (HMG) box (1). Genes that encode proteins with more than 50% amino acid similarity with the SRY HMG box have been identified and classified as SOX genes (SRY box). SOX genes were first described in mammals but have now been isolated from many phyla, including insects, fish, amphibians, and birds (2). SOX genes belong to a multigene family classified into seven groups according to their amino acid sequence and genomic organization (for a review, see Ref. 3). SOX proteins can act as transcription factors and architectural components of chromatin. They bind in vitro to the same DNA consensus sequence as SRY ((A/T)(A/T)CAA(A/T)G) (4, 5). The majority of SOX cDNAs have been isolated using degenerate PCR primers specific for the DNA binding HMG domain and, as a result, exist only as incomplete forms. Consequently, relatively few full-length SOX cDNA sequences are available, but those that have been further characterized play critical roles in the regulation of fundamental developmental processes, and mutations in some SOX genes are associated with human diseases. SOX1, SOX2, and SOX3 are involved in the development of the central nervous system in chicken and regulate δ-crystallin gene expression in the eye (6, 7). SOX4 has been shown to regulate thymocyte and lymphocyte differentiation (8, 9). SOX9 was identified by positional cloning and is associated with the skeletal malformation syndrome campomelic dysplasia, in which two-thirds of XY individuals show sex reversal (10–12). SOX9 is expressed very early in the genital ridge and is involved in sex determination and testis development. More recently, SOX9 has been identified as the first transcription factor essential for chondrocyte differentiation and cartilage formation in mouse (13) and chick embryos (14). SOX10 is essential for the peripheral nervous system development and has been identified as the causative gene for Hirschsprung-Waardenburg syndrome (15–17).

Although SOX genes are known to play fundamental roles in diverse developmental processes, to date, none of them has been clearly involved in the control of skeletal muscle differentiation. Identification of many key transcription factors involved in myogenesis, such as the myogenic basic helix-loop-helix (bHLH) factors, has been possible because the formation of muscle fibers can be recreated ex vivo. Muscle differentiation is characterized by withdrawal of myoblasts from the cell cycle, induction of muscle-specific gene expression, and cell fusion into multinucleated myotubes. All of these events are coordinated by members of the MyoD family of myogenic bHLH proteins (MyoD, Myf5, myogenin, and MRF4) (for reviews, see Refs. 18 and 19).

To determine whether Sox genes were involved in skeletal muscle differentiation, we isolated Sox genes that are expressed at different levels in proliferating murine C2C12 myoblasts or fully differentiated myotubes. We used highly degenerate PCR primers that have previously been shown to amplify a broad range of SOX HMG box sequences (20). We isolated Sox4, Sox8, and Sox9 cDNAs at similar frequencies in both cell types, whereas the Sox15 cDNAs were found at a much higher frequency in proliferative myoblasts (9% of isolated cDNAs) than in differentiated cells (1%). Here, we describe the characterization of full-length murine Sox15, a novel member of the SOX family. Sequence analysis indicates that Sox15 is most likely the mouse homologue of human SOX20 and belongs to group G of the SOX gene family. We present data showing that Sox15 may function as a negative regulator of muscle differentiation. Indeed, myoblasts overexpressing Sox15, under conditions that normally lead to differentiation, (i) were not able to express the early differentiation marker myogenin, (ii) failed to activate muscle-specific gene expression as assessed by a MCK luciferase assay, and (iii) did not fuse into multinu-
cleated myotubes. Looking at earlier points in the muscle differentiation pathway, we found that (iv) SOX15 inhibited the expression of MyoD in proliferating myoblasts. The specificity of SOX15 inhibition was demonstrated by the incapacity of either a C-terminal deleted mutant of SOX15 or another member of the SOX protein family, SOX11, to inhibit differentiation.

MATERIALS AND METHODS

Cell Culture—C2C12 myoblasts (21) were grown in a proliferation medium composed of 50% Dulbecco’s modified Eagle’s medium (Sigma) and 50% nutrient mixture F12 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Euromedex). To induce differentiation, the medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum, and 48–72 h later, the cells were harvested for assays. COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Preparation of RNA and Reverse Transcription-PCR Analysis—Total RNA was extracted using the guanidine isothiocyanate/acid phenol method described by Chomczynski and Sacchi (22). Poly(A)+ RNA was prepared using the Dynabeads mRNA purification kit (Dynal). For the identification of Sox genes in C2C12 muscle cells, 500 ng of poly(A)+ RNA were reverse-transcribed using SuperScript™ II kit according to the instruction of the manufacturer (Life Technologies, Inc.). 1 μl of this reaction was used in the highly degenerate primers P5–1 and P3–1 as described in Ref. 20. Amplification products were subcloned into pUC18, using the SureClone ligation kit (Amersham Pharmacia Biotech) and sequenced. To isolate Sox15 full-length cDNA, 1 μl of cDNA from C2C12 myoblasts was amplified with primers specific for mouse Sox15, 5′-TAAATTGCGATCTGGTCCCGAAAGAACAGCAG-3′ and 5′-GTGGTATGGTGATTGCATTCGTTG-3′, yielding a 770-base pair product. PCR products were subcloned into the BamHI and EcoRI sites of the eucaryotic vector pRK5myc downstream and in-frame with the Myc epitope MEQKLISEEDL and then sequenced.

Poly(A)+ RNA from organs of adult mice and whole embryos was isolated directly on poly d(U) paper (Amer sham Pharmacia Biotech) as described in Ref. 23.

Semiquantitative analysis of the abundance of Sox15 mRNA was done by RT-PCR. Poly(A)+ RNA was isolated from myoblasts, and 10-fold dilutions were reverse-transcribed. Subsequent amplifications of the resulting cDNA were carried out with primers specific for mouse Sox15 and Myf5, a low abundance mRNA in myoblasts. The number of amplification cycles was adjusted such that the abundance of products reflected the dilutions used for reverse transcription. Parallel PCR amplification of known amounts of Sox15 and Myf5 mRNAs served as an internal control. All PCR products were analyzed on ethidium bromide stained 2% agarose gels, and their specific fluorescence quantified using the AlphaImager™ imaging system (Alpha Innotech Corp., San Leandro, CA). The sequences of specific primers for the detection of Myf5 were 5′-TGTCGCTGGCCGAAAGAACAGCA-3′ and 5′-TAGAGGTGACATCGGGGAGAG-3′.

DNA Constructs and Transfection Assays—Deletion mutants SOX15 (1–125) and SOX15 (1–181) were generated in the context of pRK5myc expression vectors. SOX15 (1–125) and SOX15 (1–181) were digested at the internal XhoI site (open reading frame position 372), filled in with DNA polymerase 1 Klenow fragment, and religated with T4 DNA ligase. This strategy introduced a stop codon, 8 amino acids behind the HMG domain of SOX15. The same approach was used to construct SOX15 (1–181) by digesting at the PstI site (open reading frame position 537) and religating the truncated fragment using a vector PstI site at the 3′ end.

C2C12 cells were transfected using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 24 h in proliferation medium, transfected cells were placed in differentiation medium for the indicated times and harvested for luciferase or immunofluorescence assays. Subconfluent COS7 cells were transfected with 1 μg of the expression plasmids and 7 μl of the liposome DC-Chol (24) in 200 μl of serum-free medium. After a 5-h incubation, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% serum, and the cells were harvested after 48 h.

DNA Binding Assays—Preparation of nuclear extracts from transfected COS7 cells and electrophoretic mobility shift assay were done as described previously (25, 26). The DNA probes used in this assay were complementary double-stranded DNA oligonucleotides, including the SOX binding site for SRY (5′-GGAGCTGAAACAATGGTGCTCTCA-3′) and T-cell factor (5′-GGAGA-CTGACCAAAAAGCCGCTC-3′), and two mutated versions of this site (Mut 1–2, 5′-GGAGACTGGAGCAAGGCCGCTC-3′; Mut 3–4, 5′-GGAGACTGGAGCAAGGCCGCTC-3′; mutated nucleotides are underlined). For competition or supershift experiments, unlabeled competitor oligonucleotide or antibody solution was incubated for 15 min at room temperature before the probe was added. The DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel in Tris-Borate-EDTA buffer at 4 °C and visualized by autoradiography after fixation with 10% methanol-10% acetic acid.

Western Blotting—Cells were lysed directly in Laemmli sample buffer (27). Proteins were separated by SDS-polyacrylamide gel electrophoresis in 15% acrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 μm) using a semidyblotting system (Bio-Rad). The membrane was blocked overnight in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and 5% skim milk powder (Fluka) (PBST-M). After an incubation of 1 h with 9E10 anti-Myc monoclonal antibody (TEBU, Le Perray-en-Yvelines France) diluted 1/2000 in PBST-M, the membrane was washed three times with PBST and incubated for 1 h with an anti-mouse horseradish peroxidase conjugate (Amersham Pharmacia Biotech) diluted 1/5000 in PBST-M. The membrane was washed three times with PBST and developed using the ECL detection kit (Amer sham Pharmacia Biotech) according to the manufacturer’s instructions.

Immunofluorescence—Cells were fixed in 3.7% formaldehyde in PBS for 5 min and incubated for 10–20 min for 1 min. Fixed cells were incubated for 30 min at 37 °C with anti-tag antibodies (either mouse anti-Myc 9E10 or mouse anti-HA) and rabbit anti-myo-genin (TEBU, France) diluted 1/100 in PBS-BSA. After three washes in PBS, cells were further incubated with biotinylated anti-rabbit antibody (A mer sham Pharmacia Biotech; dilution, 1/100) for 30 min at 37 °C. Staining was finally revealed after an incubation of 30 min with Texas Red-conjugated streptavidin (A mer sham Pharmacia Biotech; dilution, 1/100) and fluorescein conjugated anti-mouse antibody (Cappel; dilution, 1/40). DNA was stained with Hoechst 33258. Cells were washed and mounted in FluorSave reagent (Calbiochem). Images were collected and processed on a Zeiss Axioshot.

Luciferase Assays—Plasmids used for luciferase assays were pRL-TK (Promega, Charbonnieres France), and MCK-FLuc (muscle creatine kinase enhancer and promoter upstream of firefly luciferase gene; gift from Dr. S. Leibovitch, Institut Gustave Roussy, Villejuif, France).

Cells were transfected with 1 μg of total plasmid DNA composed of pPR5 or pRK5myc-tagged Sox15/MCK-FLuc/pRL-TK plasmids (ratio, 2/1/1). Following incubation for 24 h in proliferation medium and 36 h in differentiation medium, transfected cells were harvested for luciferase assays. Activities of firefly and Renilla luciferases were measured sequentially using the Dual-Luciferase™ reporter assay kit reagent as described by the manufacturer (Promega).

RESULTS

Cloning and Characterization of Sox15—In order to identify Sox genes involved in muscle differentiation, we used a RT-PCR screen to search for genes that are differentially expressed between murine C2C12 myoblasts and myotubes. The RT-PCR strategy used highly degenerate PCR primers designed from a multiple alignment of HMG domain sequences representative of the SRY/Sox protein family (20). These primers hybridize to a broad spectrum of Sox sequences and together amplify a 180-base pair sequence from the 240-base pair HMG box. C2C12 myoblasts or myotubes served as the source of template mRNA for RT-PCR. Two hundred independent cloned PCR products for each cell type were analyzed by automatic sequencing and/or cross-hybridization experiments. In myoblasts and myotubes, Sox4, Sox8, and Sox9 were the most frequently detected Sox genes, each representing approximately 30% of all clones. Analysis of the clones differentially expressed between the two cell stages, 18 clones (9% of all clones) corresponding to the Sox15 HMG box were detected in proliferating myoblasts whereas only 2 (1% of all clones) were isolated from differentiated C2C12 cells.

A BLAST sequence similarity search with the Sox15 HMG box sequence produced a match with a mouse genomic sequence derived from chromosome 11 and corresponding to the
Sox15 gene (GenBank™ accession number AB014474). We amplified the full-length Sox15 cDNA from C2C12 myoblasts RNA using 5' and 3' specific primers derived from the genomic sequence. The open reading frame of Sox15 encodes a 231-amino acid protein that is particularly rich in proline (11.2%), serine (13%), and glycine (8.6%) (Fig. 1A). The N-terminal domain of the protein contains putative casein kinase and protein kinase C phosphorylation sites (positions 7–10 and 78–80, respectively). Our sequence analysis shows a nucleotide difference (G3A) from the data base-derived sequence at nucleotide position 211 of the open reading frame, thus changing the coding sequence to the highly conserved lysine found in all SOX proteins described so far. Comparison of protein sequences revealed the best match with the recently cloned human SOX20 gene (74% amino acid identity) (Fig. 1B) (28, 29).

DNA Binding Activity of SOX15—SOX proteins have the ability to recognize and bind to specific DNA sequences via their HMG domain. Among the HMG box protein family, this ability for sequence-specific DNA recognition is unique to SOX proteins and the distantly related T-cell factor/LEF family (30). The consensus DNA binding site for SOX proteins has been defined as the heptameric sequence 5'-(A/T)(A/T)CAA(A/T)G-3'. To test whether SOX15 was able to bind the same target nucleotide sequence, nuclear extracts were prepared from COS 7 cells transfected with Myc-tagged sox15 cDNA. We performed gel mobility shift assays using 32P-labeled oligonucleotide probes corresponding to two SOX binding site consensus sequences (SRY and T-cell factor binding sites, respectively) (see under "Materials and Methods"). Similar results were obtained with the two probes. As shown in Fig. 2A, nuclear extracts from COS7 cells transfected with wild type Sox15 gave rise to a major protein-DNA complex (lane 1) in the presence of the labeled SOX binding site oligonucleotide. No band was observed in COS7 cells transfected with an empty eucaryotic expression vector (data not shown). We confirmed the identity of the retarded band detected in cells expressing SOX15 by supershift experiments. Preincubation of nuclear extracts in the presence of increasing quantities of 9E10 monoclonal antibody produced a supershift band in both cases (Fig. 2A, lanes 2 and 3). The specificity of the interaction was determined with unlabeled competitor oligonucleotides. Greatly reduced DNA binding was observed in the presence of an excess of wild type oligonucleotide (Fig. 2A, lanes 4 and 5), and the binding was partially inhibited with mutant oligonucleotides, as expected (lanes 6–9).

SOX proteins can function as either transcriptional activators or transcriptional repressors. These properties are medi-
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Fig. 2. A, binding of SOX15 to a consensus SOX DNA binding site. Nuclear extracts were prepared from transfected COS7 cells. In an electrophoretic mobility shift assay reaction, $^{32}$P-labeled SOX binding site probe and 2 μg of nuclear extract were incubated together (lane 1). Supershift experiments were performed after preincubation of either 200 ng (lane 2) or 1 μg (lane 3) of monoclonal 9E10 antibody. The specificity of retarded bands was controlled by the use of either an excess of cold SOX binding site probe (lanes 4 and 5, 200 ng and 1 μg, respectively) or of mutated forms of this oligonucleotide MUT 1–2 (lanes 6 and 7, 200 ng and 1 μg, respectively) and MUT 3–4 (lanes 8 and 9, 200 ng and 1 μg, respectively). The arrow indicates the SOX15-DNA complex, whereas the asterisk indicates the supershift (SOX15-DNA-antibody) complex. B, immunodetection of SOX15 full-length and C-terminal deleted forms. Total cellular extracts of COS7 cells transfected with either the expression vector pRK5myc alone or with the Myc-tagged wild type and deleted SOX15 forms were analyzed by Western blotting with the 9E10 anti-Myc monoclonal antibody. C, band shift experiment demonstrating the binding capability of wild type and the truncated form of SOX15. The $^{32}$P-labeled SOX binding site probe was incubated with nuclear extracts from COS7 cells transfected with wild type or deleted SOX15 (1–125) expression vectors, in the absence or presence of 1 μg of 9E10 monoclonal antibody.

Figure 2: Binding of SOX15 to a consensus SOX DNA binding site. Nuclear extracts were prepared from transfected COS7 cells. In an electrophoretic mobility shift assay reaction, $^{32}$P-labeled SOX binding site probe and 2 μg of nuclear extract were incubated together (lane 1). Supershift experiments were performed after preincubation of either 200 ng (lane 2) or 1 μg (lane 3) of monoclonal 9E10 antibody. The specificity of retarded bands was controlled by the use of either an excess of cold SOX binding site probe (lanes 4 and 5, 200 ng and 1 μg, respectively) or of mutated forms of this oligonucleotide MUT 1–2 (lanes 6 and 7, 200 ng and 1 μg, respectively) and MUT 3–4 (lanes 8 and 9, 200 ng and 1 μg, respectively). The arrow indicates the SOX15-DNA complex, whereas the asterisk indicates the supershift (SOX15-DNA-antibody) complex. B, immunodetection of SOX15 full-length and C-terminal deleted forms. Total cellular extracts of COS7 cells transfected with either the expression vector pRK5myc alone or with the Myc-tagged wild type and deleted SOX15 forms were analyzed by Western blotting with the 9E10 anti-Myc monoclonal antibody. C, band shift experiment demonstrating the binding capability of wild type and the truncated form of SOX15. The $^{32}$P-labeled SOX binding site probe was incubated with nuclear extracts from COS7 cells transfected with wild type or deleted SOX15 (1–125) expression vectors, in the absence or presence of 1 μg of 9E10 monoclonal antibody.

ated by a C-terminal domain of the protein, presumably by interacting with accessory proteins that help to stabilize the DNA binding of SOX proteins (3, 31). We therefore wished to study the effect of C-terminal deletions on SOX15 DNA binding activity. SOX15 (1–125) is truncated immediately after the DNA binding domain, whereas SOX15 (1–181) retains only one half of the C-terminal domain. Western blot analysis of Myc-tagged wild type and C-terminal deleted SOX15 proteins in total cellular extracts of transfected COS7 cells showed that the wild type and mutant SOX15 proteins migrate at their predicted molecular weights (Fig. 2B). Using nuclear extracts prepared from COS7 cells transfected with either wild type or the deleted forms of Sox15, encoding amino acids 1–125 and 1–181 (not shown), all of the SOX15 proteins bound the same DNA consensus sequence, yielding DNA-protein complexes with different mobilities, which were partially supershifted in the presence of 9E10 anti-Myc antibody (Fig. 2C). These results demonstrate that the C-terminal truncated proteins are still capable of binding DNA.

SOX15 Acts as a Negative Regulator of Myoblast Differentiation—Because Sox15 appeared to be enriched in proliferating myoblasts, we decided to study the effect of SOX15 overexpression on muscle differentiation. Vectors expressing Myc-tagged SOX15 or the deleted forms SOX15 (1–125) and SOX15 (1–181) were transfected into C2C12 myoblasts. After 24 h in proliferation medium, cells were switched into a medium promoting myotube formation for 48–72 h. Commitment of transfected cells into differentiation was assessed by immunofluorescence using the Myc-tagged SOX15-overexpressed proteins and observing the expression of myogenin as an early marker of differentiation. As shown in Fig. 3A, SOX15 localizes in the nucleus, in agreement with its expected role as a transcription factor. Furthermore, wild type SOX15 expressing cells do not undergo differentiation. They remain negative for myogenin and do not show any of the features associated with myotube formation i.e. multinucleation and membrane fusion. By marked contrast, cells transfected with the truncated form SOX15 (1–125) do express myogenin and can differentiate apparently normally into myotubes, demonstrating that the C-terminal domain is absolutely required to inhibit differentiation.

Surprisingly, we were not able to overexpress SOX15 (1–181) in myoblasts because it appeared to be toxic for myoblasts when they are placed in differentiation medium. We do not currently understand the basis for the observed cell death.

To assess the specificity of SOX15 inhibition of muscle differentiation, we overexpressed in C2C12 cells another member of the SOX family, human SOX11, thought to be involved in the maturation of the central nervous system (32). As shown in Fig. 3B, cells overexpressing HA-tagged SOX11 can fully differentiate into multinucleated myotubes that also express myogenin, supporting the view that inhibition of myogenesis is specific for SOX15.

To determine whether SOX15 was acting upstream or downstream of the master control gene MyoD, an immunofluorescence experiment was performed on C2C12 proliferating cells. As shown in Fig. 3B, the overexpression of wild type SOX15 significantly impairs the expression of MyoD, whereas SOX15 (1–125) has no effect. These immunofluorescence studies were quantified by evaluating the proportion of transfected cells expressing the muscle differentiation markers MyoD and myogenin. Fig. 4A shows that overexpression of SOX15 antagonizes muscle differentiation, acting at a very early stage, before the expression of MyoD in the myogenesis pathway.

To confirm the inhibitory role of SOX15 in myogenesis, we used transient co-transfection assays in C2C12 myoblasts with a muscle-specific promoter driving the expression of luciferase. The construct MCK-luc contains the upstream region from the muscle creatine kinase gene and has been shown to be directly transactivated by myogenic bHLH proteins during muscle differentiation (33). As such, activation of MCK-luc provides a qualitative measurement of the extent of differentiation. Differentiation-dependent activation of the reporter gene was significantly inhibited by full-length SOX15 protein (Fig. 4B); in contrast, the truncated form of SOX15 (1–125) has a smaller inhibitory effect on the reporter MCK-luc activity. The fact that in this assay the differentiation-dependent expression of the
MCK-luc reporter was not completely repressed by SOX15 may reflect co-transfection efficiency.

**Expression Pattern of the Sox15 Gene—**Northern blot analysis failed to detect Sox15 transcripts in several adult and fetal tissues. We therefore used RT-PCR to determine the tissue distribution of Sox15 mRNA. To avoid amplification of genomic DNA, PCR primers spanned an intron (Fig. 1A). Glyceraldehyde-3-phosphate dehydrogenase was used as a control to measure cDNA quality and abundance. By RT-PCR, Sox15 transcripts could be detected in all adult mouse tissues examined (brain, kidney, liver, heart, skeletal muscle, diaphragm, intestine, and testis), as well as in early mouse embryos (8.5 and 9.5 dpc) (Fig. 5). However, Sox15 transcripts appear to be most abundant in skeletal muscle and brain, with more expressed in 9.5 dpc embryos than in 8.5 dpc embryos.

In order to set the expression of Sox15 in the context of another transcription factor involved in myogenesis, we performed semiquantitative RT-PCR amplifications on myoblasts poly(A)+ RNA with Sox15- and Myf5-specific primers. In the linear range of the amplification reaction, we found that Sox15 transcripts were at least 100-fold less abundant than Myf5 transcripts (data not shown).

**DISCUSSION**

In this paper, we report the characterization of a novel SOX gene, mouse Sox15, isolated in a screen for HMG box containing transcription factors expressed at different levels during myogenesis. Together with the sequence similarity within the human SOX20 HMG box, the high degree of conservation in their N- and C-terminal domains suggests a close evolutionary relationship between mouse Sox15 and the recently characterized human SOX20 gene (28, 29). The two proteins share 74% homology in their coding sequence. Although most of the SOX proteins are encoded from a single exon, mouse Sox15 and human SOX20 are the only members of the SOX family having an intron located at the 3’ end and not interrupting the HMG domain. Based on this characteristic and on sequence homology within the HMG box, hSOX20 and Sox15 define a new group (G) of the SOX gene family.

Sox15 transcripts appear to be ubiquitously expressed in adult mouse tissue, although transcripts are more abundant in brain and skeletal muscle tissues. Levels of expression are low, as we were unable to detect Sox15 mRNA by Northern blot analysis. However, in the RT-PCR differential screen, cDNA

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**Fig. 3.** SOX15 but not a truncated form or SOX11 antagonizes myogenin and MyoD expression. C2C12 cells were transfected with plasmid DNA and maintained in 15% serum medium for 24 h before analysis (B, nondifferentiated cells) or after a further incubation of 48 h in differentiation medium (A). Cells expressing Myc-tagged SOX15 and HA-tagged SOX11 proteins were visualized by staining with anti-Myc 9E10 or anti-HA antibodies, respectively (green). Cells in A and B were stained with anti-myogenin and anti-MyoD antibodies, respectively (red).

**Fig. 4.** Inhibition of myogenesis by SOX15. A, more than 200 cells expressing either no exogenous protein (NT, nontransfected cells) or transfected wild type (wt) SOX15, SOX15 (1–125), or SOX11 were counted, and the percentages of proliferating myoblasts expressing MyoD (not determined for SOX11 expressing cells) and differentiated cells expressing myogenin were determined. B, C2C12 cells were transiently transfected with the MCK-luc reporter vector and expression vectors encoding either full-length SOX15 or the truncated form (1–125). Luciferase activity was measured as described under “Materials and Methods.”
encoding SOX15 were found at a higher frequency in myoblasts than in differentiated cells, suggesting a down-regulation of SOX15 during differentiation. This observation prompted us to investigate a potential role for SOX15 in myogenesis. C2C12 cells transfected with expression vectors coding for wild type SOX15 failed to differentiate and were not able to express the muscle promoting factor MyoD. This repressive role for SOX15 was specific because neither overexpression of a C-terminal deleted form of SOX15 nor the related SOX family member SOX11 caused a block to differentiation. That cells expressing a C-terminal deleted form of SOX15 were still able to differentiate into myotubes strongly suggests that C-terminal sequences are required for SOX15 inhibition of muscle differentiation. Previous analysis have shown that C-terminal domains of several SOX proteins are capable of activating transcription (9, 32, 34). More recently, the C-terminal sequences of chicken SOX14 and SOX21 were shown to act as repressor domains, providing evidence for a repressive activity of SOX proteins (31). In several attempts to characterize a transcriptional activity for SOX15 using a luciferase reporter gene under the control of seven consensus SOX binding sites (35), we were unable to detect any changes in reporter gene expression. However, we could only measure activation in this assay, and if SOX15 is a repressor, we would not be able to measure its activity. Direct target genes regulated by SOX15 remain to be identified but would be expected to be those expressed at very early stages of muscle differentiation.

SOX genes show diverse and dynamic patterns of expression throughout embryogenesis, and members of the SOX gene family are involved in many different aspects of development. In the RT-PCR differential screen that identified SOX15, other members of the SOX family were isolated from myoblasts and myotubes; some, such as SOX9, are known to be strong activators of transcription. We do not know what role, if any, these proteins play in myogenesis. Our RT-PCR expression analysis has shown that Sox15 gene transcripts could be detected in a wide variety of adult mouse tissues, as well as in early mouse embryos. This result suggests that Sox15 function is not restricted to muscle differentiation. Perhaps the function of SOX15 is to repress muscle development in all other tissues. Alternatively, an accessory factor may be required to promote SOX15 activity in myoblast progenitor cells. In support of this view, mis-expression of the b1-crystallin gene in lens cells depends on the interaction of SOX1/2/3 with the partner factor DEFS, which probably has a tissue-restricted expression (6).

Few proteins are known to inhibit the function of the bHLH myogenic factor MyoD in myoblasts (for a review, see Ref. 36). Tight regulation of MyoD activity ensures that myoblasts do not precociously enter into differentiation. We have shown that SOX15 acts as a powerful inhibitor of MyoD expression, explaining its repressive role on muscle differentiation. Because SOX15 may act as a transcription factor, we cannot rule out the possibility that other critical regulators of muscle differentiation, such as the MEF-2 transcription factors, or co-factors of MyoD, such as the retinoblastoma tumor suppressor protein (pRb), may also be regulated by SOX15. As such, SOX15 could interfere with muscle differentiation by regulating, in addition to MyoD, a set of genes involved in this process.

Whether SOX15 regulates directly or indirectly MyoD remains to be determined. To date, relatively little is known about the transcription factors that control MyoD expression. At least three transcription factors (Pax-3 (37, 38), Myf-5 (37), and the serum response factor SRF (38)) have been postulated as upstream regulators of MyoD. However, it is not known whether these factors or SOX15 act directly by binding to the MyoD regulatory regions. This may be due to the complexity of the MyoD gene, which is regulated by at least three different enhancers located up to 20 kilobases upstream from the transcription start site. One of these regions, the distal regulatory region, is unusual because it requires stable chromosomal integration for muscle-specific activity (39). Interestingly, the distal regulatory region contains several putative SOX binding sites, one of which is identical to the T-cell factor binding site used in our study. Whether SOX15 binds to such sites remain to be elucidated.

In light of recent results (40), we can propose another model to explain SOX15-induced down-regulation of myogenesis. Three members of the SOX family (Xsox17α, Xsox17β, and Xsox3) have been reported to repress Wnt signaling in Xenopus embryos by interacting with β-catenin. The C-terminal region of Xsox17β, without the DNA binding HMG box, is sufficient to inhibit β-catenin activity. Wnt signals modulate cell fate and proliferation in many different adult and embryonic cells and induce myogenic bHLH gene expression in somites (41). We have shown that the presence of the SOX15 C-terminal domain was necessary for myogenesis inhibition. We can speculate that SOX15 could down-regulate muscle differentiation by interacting with β-catenin via its C-terminal domain, thereby repressing Wnt signaling in muscle cells. SOX proteins may therefore play a general role in regulating tissue responses to Wnt signals.

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Mouse SOX15 Inhibits Myogenesis

![Diagram](image-url)
Mouse SOX15 Inhibits Myogenesis

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