mSharp-1/DEC2, a Basic Helix-Loop-Helix Protein Functions as a Transcriptional Repressor of E Box Activity and Stra13 Expression*

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Transcription factors belonging to the basic helix-loop-helix (bHLH) family play critical roles in the regulation of cellular differentiation of distinct cell types. In this study, we have characterized the DNA-binding and transcriptional properties of the bHLH factor mSharp-1/DEC2. mSharp-1 belongs to the Hairy/Enhancer of Split subfamily of bHLH factors and exhibits the highest structural and sequence identity with Stra13. We show that mSharp-1 specifically binds to the E box motif (CANNTG) as a homodimer and acts as a potent transcriptional repressor of MyoD- and E12-induced E box activity and differentiation. The inhibitory activity of mSharp-1 occurs through several mechanisms, including occupancy of E box sites by mSharp-1 homodimers and by direct physical interaction with MyoD and E proteins. Furthermore, by using gel mobility shift assays and chromatin immunoprecipitation experiments, we have identified Stra13 as a target for mSharp-1-mediated repression. We demonstrate that transcriptional repression of Stra13 depends, in part, on binding of mSharp-1 to three conserved E box motifs in the Stra13 proximal promoter. Moreover, mSharp-1 directly interacts with the transcriptional activator Sp1 and impairs Sp1 induction of Stra13 promoter. Our results suggest that mSharp-1 functions as a transcriptional repressor by DNA binding dependent and independent mechanisms.

Members of the basic helix-loop-helix (bHLH)† superfamily of transcription factors are expressed in a wide range of tissues during development and are involved in the regulation of cell fate determination, myogenesis, neurogenesis, and hematopoiesis (1, 2). The common structures shared among the members of this superfamily are the basic domain, which is required for DNA binding, and the helix-loop-helix domain, which is involved in dimerization (3, 4).

Based on the dimerization properties, tissue distribution, and the transcriptional activities, bHLH proteins can be categorized into three classes (5). Class A bHLH factors contain the mammalian “E” proteins, which include the two E2A gene products E12 and E47 as well as E2-2 and HEB. E proteins are ubiquitously expressed and can form homodimers or heterodimers with bHLH factors of the same class as well as with other classes. Class B bHLH proteins tend to be expressed in a tissue or cell type-specific manner and function as heterodimers with the class A bHLH factors. bHLH factors involved in tissue-specific differentiation generally belong to the class B subfamily and include the myogenic factors MyoD and myogenin, the neurogenic factors Mash1, NeuroD, and neurogenins, as well as the bHLH proteins SCL/TAL which are important for hematopoiesis (6–14). Both class A and class B bHLH factors bind to a common DNA sequence called the E box (CANNTG) commonly found in the promoter or enhancer regions of numerous developmentally regulated genes (15) and function as transcriptional activators. Class C bHLH factors (2) contain the Drosophila Hairy and Enhancer of Split [E(Spl)] proteins, the mammalian Hes proteins, as well as the more recently identified mammalian proteins including Stra13/DEC1/Sharp-2 (16–18), Sharp-1 (18), and the HRT/Hey/CHF/Hesr/Gridlock family (19–23). All members contain a characteristic motif called the orange domain and are distinctive in their function as transcriptional repressors. Some members of this subfamily are expressed ubiquitously, whereas others are tissue-restricted. Functional analysis by gene disruption studies has revealed novel roles for some of these genes in the development of brain and eye as well as in the differentiation and activation of T cells (10, 24, 26). The DNA-binding properties of this subfamily are quite divergent. Whereas Hairy/[E(Spl)]/Hes1 bind the N box sequence (27), which is a variant of the canonical E box sequence motif, some Hes proteins as well as a few members of the HRT/Hey/CHF/Gridlock subgroup bind the E box.

The C class bHLH factor Sharp-1, which was first cloned from adult rat brain (18), is more closely related to Stra13 than to the remaining family members. Both Stra13 and Sharp-1 lack the WRPW motif for recruitment of the co-repressor Groucho and function as transcriptional repressors through both histone deacetylase (HDAC)-dependent and -independent mechanisms (28, 29). The high degree of sequence conservation within the bHLH domain and the related mechanisms of transcriptional repression suggest that Stra13 and mSharp-1 possess unique functions and repress similar downstream regulatory targets. Sharp-1 is expressed during mouse embryonic development in tissues overlapping with Stra13 suggesting a cross-regulatory interaction between these two genes (30). We have previously characterized the transcriptional and functional properties of Stra13 (16, 26, 28), but the DNA-binding and transcriptional properties of Sharp-1 within the bHLH regulatory network have not been characterized and its biological targets not defined.

To better understand the molecular basis of Sharp-1 function, we have undertaken the analysis of Sharp-1 activity. We
report here the cloning of the mouse Sharp-1 (mSharp-1) cDNA and characterization of its transcriptional activity. We demonstrate that mSharp-1 represses transcriptional activation of E box containing synthetic and natural promoters. mSharp-1 binds to the E box motif as a homodimer and can repress E12/MyoD-mediated transcriptional activation and myogenic differentiation. mSharp-1-mediated repression of E box activity occurs via both non-DNA-binding dependent and non-DNA-binding dependent mechanisms. Taken together, our results provide a mechanistic basis for mSharp-1 function in the BHLH regulatory network and identify it as a regulator of StrA13 expression.

EXPERIMENTAL PROCEDURES

Isolation of mSharp-1 cDNA—mSharp-1 was isolated from a mouse embryo E11.5-Stretch Plus cDNA library (Clontech) using the rat Sharp-1 cDNA as a probe. Approximately 5×107 plaques were plated, transferred to Hybond N+ membrane (Amersham Biosciences), and prehybridized in a solution containing 50% formamide, 5×SSPE, 5×Denhardt’s solution, 0.5% SDS, and 0.05 mg/ml denatured salmon sperm DNA for 3–5 h at 42°C. The rat Sharp-1 cDNA was used as a probe. Hybridization was carried out at 42°C for 16 h in the same buffer containing 32P-labeled probe. Filters were washed 4 times at 65°C with 2×SSC + 0.1% SDS. Four phage clones that were positive after two rounds of screening were subcloned into pTiZ18R and sequenced on both strands. All the clones obtained from library screening to obtain the full-length mouse Sharp-1 cDNA were partial and lacked the 5′-untranslated region containing the entire GST-Sharp-1 construct was generated by a triple digestion using the BamHI-SphI fragment from p222 and the SphI-EcoRI fragment from p359 cloned into the BamHI-EcoRI site of pGEX-2T2K. The StrA13 expression vector pCS2-StrA13 and the StrA13 promoter construct pGp3PmN have been previously described (28).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA using in vitro translated mSharp-1 was performed as described (32). The sequence of the top strand of the probe (E box is underlined) was 5′-GGCCGCAGCCTTACAGTGGGGAGTGGTATGGA-3′. A typical binding reaction contained 20,000 cpm probe, 5 μl of in vitro translated protein or 5 μl of nuclear extract, 1 μg of poly(dI-dC), 5 mM HEPES, pH 7.8, 50 mM KCl, 0.5 mM dithiothreitol, and 10% glycerol. The reaction was incubated at room temperature for 20 min and fractionated on 5% polyacrylamide gels in 0.5×TBE buffer. Gels were dried and exposed to x-ray film. For competition experiments, 200- or 400-fold excess of competitor DNA (Table I) was added to the reaction on ice for 20 min prior to addition of the labeled probe. Antibody supershift assays included incubation of the binding components with 2 μl of anti-mSharp-1 antibody for 10 min at room temperature prior to addition of the labeled probe.

Site-directed Mutagenesis—Mutations in the E box sequence of the StrA13 promoter construct pGp3PmN were introduced using the Quickchange Mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers used to generate mutations in E box 1 (E boxM1), E box 2 (E boxM2), and E box 3 (E boxM3) are listed in Table II. The sequence of all mutant constructs was confirmed by sequencing.

Cell Culture and Transfections—10T1/2 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. 5×104 cells were plated 1 day before transfection in 24-well plates and transiently transfected with plasmids as indicated using LipofectAMINE Plus (Invitrogen). Empty expression vectors were added to normalize the amount of DNA in each well. Cells were harvested with passive lysis buffer, and luciferase assays were performed according to the manufacturer’s instructions (Promega, Madison, WI). Transfection efficiencies were normalized by co-transfecting 50 ng of the β-galactosidase plasmid pCH110 (Amersham Biosciences). All transfections were performed in duplicate at least three times. 

Myogenic Differentiation Assays—10T1/2 cells were seeded at a density of 2×104 cells/well in 6-well plates 1 day prior to transfection. Cells were transfected as described above with 2 μg of MyoD in the presence or absence of mSharp-1. After 5 days in differentiation medium (Dulbecco’s modified Eagle’s medium containing 2% horse serum), cells were rinsed three times in phosphate-buffered saline and then fixed in methanol for 20 min at 4°C. Following a 30-min blocking step (5% goat serum in phosphate-buffered saline), the plates were incubated with 1:400 dilution of MY-32, a monoclonal anti-skeletal myosin antibody (Sigma) specific for the myosin heavy chain, overnight at 4°C. The
primary complexes were detected using a biotinylated anti-mouse antibody and a horseradish peroxidase-strepavidin conjugate (Vector Laboratories).

Nuclear Extracts—10T1/2 cells (2 × 10^8) cultured on 100-mm culture dishes were transfected with 5 μg each of MyoD and E47 for 48 h, washed twice with phosphate-buffered saline, and resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 5 min, Nonidet P-40 was added to a final concentration of 0.6%. Nuclei were pelleted, and the cytoplasmic proteins were carefully removed. Nuclei were then resuspended in buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After vortexing and stirring for 30 min at 4 °C, the solution was centrifuged, and the supernatant was transferred to a fresh vial. Protein concentrations of nuclear extracts were determined by Bio-Rad protein assay using bovine serum albumin as a standard.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were done essentially as described (Upstate Biotechnology Protocols). For analysis of endogenous mSharp-1 binding to the E box sites in the Stra13 promoter, proliferating C2C12 (~30 × 10^8) cells were grown on 10-cm dishes, and the proteins bound to DNA were cross-linked by addition of formaldehyde (final 1%) to the culture medium. Cells were washed with cold phosphate-buffered saline and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). The lysate was sonicated to break DNA to lengths between 200 and 1000 bp. Sonicated supernatant was diluted 10-fold with ChIP dilution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). The lysate was treated with 1 μl of anti-mSharp-1 antibody or with 1 μl of preimmune serum overnight at 4 °C. DNA-mSharp-1 antibody complexes were collected by addition of salmon sperm DNA/protein A-agarose slurry. Samples were incubated 1 h at 4 °C, and the agarose complex was pelleted by brief centrifugation at 4 °C. After extensive washing, the pellet was dissolved in 250 μl of elution buffer and spun to remove the agarose. The supernatant was treated with 250 μl of 5 × NaCl and heated at 65 °C for 4 h to reverse protein-DNA cross-links. After treatment with EDTA and proteinase K, the eluted DNA was sonicated with phenol/chloroform and precipitated with ethanol to recover DNA. Eluted DNA was resuspended in 30 μl of TE. For PCR, 2 μl of DNA was amplified using primers RT31 (5' -ATGTTCCTAT-3') and RT46 (5' -ATGGTTCTAT-GAGCTAGCTGAGCTCTCC-3') that span the E box sites 1 and 2 in the Stra13 promoter. Thirty cycles of PCR were performed, and the amplified products were analyzed on a 2% agarose gel.

To investigate Sp1 and mSharp-1 interaction on the mutant Stra13 promoter, 10T1/2 cells were transfected with 8 μg of the triple E box mutant construct [E boxM1( 1 + 2 + 3)] using LipofectAMINE Plus (Invitrogen). 48 h after transfection, cells were cross-linked and incubated with 10 μl of anti-mSharp-1 or 2 μl of anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) as described above. The protein-DNA immunocomplexes were collected using a 4% agarose gel. For immunoprecipitation with Sp1, proteins bound to protein-A-agarose were eluted by boiling in SDS sample buffer and resolved on a 9% SDS-PAGE gel. Immunoblotting was performed with mSharp-1 antibody. Proteins were detected using the ECL kit (Amersham Biosciences). For PCRs, DNA was eluted as described above and precipitated with ethanol to recover DNA. Eluted DNA was resuspended in 30 μl of TE. For PCR, 2 μl of DNA was amplified using primers RT31 (5' -TCTCTATTCCTGCGTCTCGA-3') and RT46 (5' -ATGTTCTAT-GAGCTAGCTGAGCTCTCC-3') that span the E box sites 1 and 2 in the Stra13 promoter. Thirty cycles of PCR were performed, and the amplified products were analyzed on a 2% agarose gel.

Immunoprecipitation of in Vitro Translated mSharp-1 Protein with Sp1 Antibody—150 μl of mSharp-1 protein was generated using the TNT coupled transcription-translation system (Promega). For immunoprecipitation, lysates were incubated with 2 μg of rabbit polyclonal Sp1 antibody (PEP2, Santa Cruz Biotechnology) in 1:1 mix of two buffers (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA) and (20 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 20% glycerol) for 1 h on a rotating wheel at 4 °C. The immunocomplexes were absorbed to protein A/G-agarose (Santa Cruz Biotechnology) overnight at 4 °C and washed three times in the buffer described above. Associated proteins were then eluted in SDS sample buffer containing dithiothreitol by boiling for 5 min and loaded on a 12% polyacrylamide gel. Gel was fixed and dried before detection of 35S-labeled mSharp-1 by autoradiography.

RESULTS
cDNA Cloning and Expression Analysis of mSharp-1—To understand the biological properties of Sharp-1 and to determine the molecular basis of its function, we screened an E11 mouse embryonic cDNA library using the rat Sharp-1 cDNA as a probe in an effort to clone the mouse homologue of Sharp-1. Sequence analysis indicated that the cDNA contains a single open reading frame encoding a 410-amino acid polypeptide (Fig. 1A). The amino acid sequence is identical to the recently reported mouse DECG (GenBank™ accession number AB044909) but contains a longer 3'-untranslated region (33). Domain analysis (Fig. 1B) indicated that Sharp-1 contains a bHLH motif located in the N terminus and an orange domain which is a characteristic feature seen in all repressive bHLH factors such as Hairy/E(Spl)/Hes/Stra13. Similar to Stra13, Sharp-1 also contains a proline residue in the basic region and lacks the WRPW motif. Sharp-1 and Stra13 share the highest homology within bHLH domain (96%) and an overall homology of 50% over the entire length of the two proteins.

To analyze the temporal pattern of Sharp-1 expression, we performed RT-PCR using total RNA from embryos between E9.5 and E16.5 as well as various tissues from adult mice (Fig. 1C). Consistent with our previous report, Sharp-1 expression was detected at all embryonic stages tested (30). A high level of expression was also seen in a number of tissues including thymus, skeletal muscle, brain, stomach, uterus, and pancreas. Heart, spleen, lung, kidney, and eye exhibited intermediate levels of expression, whereas the lowest levels were seen in liver and in small intestine.

Sharp-1 Binds to the E Box and Mediates Transcriptional Repression of E Box Activity—Most bHLH proteins are thought to function as transcription factors by binding to either E box motifs (CANNTG) or N box motifs (CACNAG). To investigate the transcriptional properties of Sharp-1, we tested whether Sharp-1 can bind to either the E box or the N box sequences in vitro. EMSAs were performed using 32P-labeled oligonucleotide probes containing three E box sequences or containing the N box sequence. Addition of in vitro translated Sharp-1 to the E box oligonucleotide resulted in a DNA-protein complex (Fig. 2A, lane 1). In contrast, Sharp-1 did not exhibit any detectable binding to an oligonucleotide harboring the N box, whereas Hes1, which is known to bind the N box sequence, clearly bound to and was supershifted using an anti-Hes antibody (data not shown). To assess further the specificity of Sharp-1 binding to the E box, we generated polyclonal antibodies specific to Sharp-1. Addition of anti-Sharp-1 antibody to the binding reaction resulted in a supershift of the
complex (Fig. 2A, lane 2). In addition, mSharp-1 binding was competed with a 200-fold excess of an unlabeled oligonucleotide containing E boxes (Fig. 2A, lane 4). Taken together, these results demonstrate that mSharp-1 specifically binds to E box site likely as a homodimer.

We then tested whether binding of mSharp-1 to the E box resulted in transcriptional activation or repression of a synthetic E box reporter. Transient transfection experiments were performed in 10T1/2 cells using the 4R-tk-luc reporter that contains four tandem E boxes from the muscle creatine kinase (MCK) enhancer upstream of the thymidine kinase basal promoter (34), which are binding sites for MyoD, E12/E47, and several other bHLH proteins (35–38). Co-transfection of increasing amounts of mSharp-1 resulted in a dose-dependent inhibition of 4R-tk-luc reporter activity indicating that mSharp-1 functions as a transcriptional repressor of E box activity (Fig. 2B). mSharp-1 like Stra13 lacks the WRPW motif for recruitment of the co-repressor Groucho. We therefore investigated whether repression of E box activity by mSharp-1 appears to be independent of HDAC1 recruitment.

**mSharp-1 Inhibits MyoD and E12-induced Transcriptional Activity**—In an effort to identify the mechanism(s) by which mSharp-1 acts as a transcriptional repressor of E box reporter activity, we examined the effect of mSharp-1 on bHLH factors such as MyoD and E12 that are known to activate the E box reporter (Fig. 3A). Transient transfection assays were performed in 10T1/2 fibroblasts with the E box-dependent reporter 4R-tk-luc in the presence of activators of E box activity. Transfection of MyoD or E12 resulted in elevated luciferase activity, and as expected, co-transfection of E12 along with MyoD significantly enhanced MyoD transactivation activity (Fig. 3A). Transfection of mSharp-1 along with E12 resulted in a repression of E12-mediated transactivation, and addition of increasing amounts of E12 almost completely relieved this repression. As seen with E12, co-transfection of mSharp-1 inhibited MyoD-induced transactivation, and addition of increasing amounts of MyoD partially relieved mSharp-1 mediated repression. Furthermore, MyoD + E12-induced activation of the reporter was also reduced in presence of mSharp-1. These findings suggested that mSharp-1 may inhibit MyoD- and E12-driven E box reporter activity by competition for DNA binding or by dimerization with MyoD and E proteins.
mSharp-1 Heterodimerizes with MyoD- and with E47—In view of these transcriptional effects on E box reporter activity, we examined the dimerization properties of mSharp-1. In order to examine potential interactions of mSharp-1 with MyoD and E proteins, we performed GST pull-down assays. Equivalent amounts of GST-mSharp-1 fusion protein were used for interaction with 35S-labeled in vitro translated MyoD and E47. GST protein alone was used as a negative control. As shown in Fig. 3B, GST-mSharp-1 strongly interacted in vitro with E47 as well as with MyoD, whereas the control GST protein exhibited no interaction with either protein. These results indicate the specificity of mSharp-1 dimerization with MyoD and E47.

mSharp-1 Inhibits MyoD-induced Myogenic Differentiation—Given that mSharp-1 and MyoD exhibit a direct physical interaction, we examined if this interaction resulted in an inhibition of MyoD-induced myogenic differentiation. Transient transfections were performed in 10T1/2 cells with MyoD alone or along with mSharp-1. MyoD alone was sufficient to convert fibroblast cells into myotubes as determined by immunostaining for skeletal muscle-specific myosin (Fig. 3C), whereas mSharp-1 alone did not have any myogenic activity (data not shown). However, co-transfection of mSharp-1 along with MyoD strongly suppressed the number of differentiated myotubes indicating that co-expression of mSharp-1 represses MyoD-induced myogenic differentiation (Fig. 3, C and D).

mSharp-1 Binds to the E Boxes in the Stra13 Promoter in Vitro and in Vivo and Represses Stra13 Expression—Sequence analysis of the Stra13 promoter construct pGL3PmN (28) revealed the presence of three potential E box sites that conform to the core hexanucleotide consensus sequence of an E box motif CANNTG (Fig. 4A). These E box sequences are also present in the human Stra13 promoter (39), and alignment of the mouse and human sequences indicated that these sites are conserved, suggesting that they may be important in regulation of Stra13 promoter activity. We tested whether the E box sequences mediate transcriptional activation by positive bHLH factors. The promoter construct pGL3PmN was co-transfected with vectors expressing E47 and MyoD in 10T1/2 cells. As shown in Fig. 4B, E47 and MyoD up-regulated Stra13 promoter activity, suggesting that E47 + MyoD can activate the Stra13 promoter through the E box sites. Consistent with the effects seen on the E box reporter, co-transfection of mSharp-1 along with E47 + MyoD almost completely abolished transcriptional activation mediated by these positive bHLH factors on the Stra13 proximal promoter. Furthermore, co-transfection of increasing amounts of mSharp-1 resulted in a dose-dependent inhibition of basal Stra13 promoter activity (Fig. 4C). To investigate whether mSharp-1 could bind to the E box sites in the Stra13 promoter, we then performed EMSAs and ChIP assays. The Stra13 promoter pGL3PmN (Fig. 4A) was radiolabeled and used as a probe for EMSA. By using in vitro translated mSharp-1 for binding, a complex was detected on the promoter fragment (Fig. 5A, lane 1). To evaluate the specificity of this interaction, we designed oligonucleotides harboring each of the three E box sequences from the Stra13 promoter, as well as those containing a mutation in the E box sites (Table I). Competition experiments were carried using 400-fold excess of wild type and mutant oligonucleotides in EMSA analysis. The complex formed was specific as it was competed by an excess of each of the three wild type E box containing oligonucleotides (Fig. 5A, lanes 2–4) but not with oligonucleotides containing a mutation in the E box sites (Fig. 5A, lanes 5–7). To further assess binding of endogenous mSharp-1 on the Stra13 promoter in vivo, ChIP assays were performed. Because mSharp-1 is abundant in skeletal muscle (Fig. 1C), myoblast C2C12 cells were used for immunoprecipitation with anti-mSharp-1 antibody or with preimmune serum as a control. The immunoprecipitated DNA was subjected to PCR using primers that amplify a fragment flanking E box sites 1 and 2. As shown in Fig. 5B, immunoprecipitation of chromatin with anti-mSharp-1 antibody but not with preimmune serum resulted in amplification of the Stra13 promoter. Taken together, these results indicate that mSharp-1 binds to the Stra13 promoter in vitro and in vivo.
We then examined whether mSharp-1 competed with MyoD for binding to the Stra13 promoter. In vitro translated mSharp-1 bound to the Stra13 promoter, and its binding was blocked by anti-Sharp-1 antibody (Fig. 5C, lanes 1 and 2). Nuclear extracts from 10T1/2 cells transfected with MyoD + E47 also showed the presence of a single complex on the Stra13 promoter (Fig. 5C, lanes 3 and 5). Binding of MyoD + E47 was inhibited by addition of anti-MyoD (data not shown) but not anti-Sharp-1 antibody (lane 4). Addition of in vitro translated mSharp-1 to MyoD + E47 resulted in inhibition of MyoD binding (lane 6). Addition of increasing amounts of mSharp-1 resulted in its binding to the Stra13 promoter (lanes 7 and 8), and this complex was inhibited with anti-mSharp-1 antibody (lane 9). Taken together, these results indicate that mSharp-1 competes with MyoD + E47 for binding to the E box sites on the Stra13 promoter.

To determine whether mSharp-1-mediated repression of the Stra13 promoter occurs solely through DNA binding to the E box sites, constructs were generated containing a mutation in each of the three E box sites, as well as one with mutations in all three E boxes (Fig. 6A). Mutation of each of the E box sites reduced the basal activity suggesting that the Stra13 promoter activity is critically dependent on E box binding factors. Co-transfection of mSharp-1 resulted in 98% repression of the wild type promoter activity and the constructs E boxM1, E boxM2, and E boxM3 were repressed by 96, 95, and 87%, respectively (Fig. 6A). Remarkably, the construct E boxM(1/2/3) containing all three mutant sites was more resistant to repression by mSharp-1 (77% inhibition) but, nevertheless, exhibited only a partial decrease of mSharp-1-mediated repression. We therefore tested whether mSharp-1 could bind to the triple mutant construct at a non-E box site. Wild type and triple mutant Stra13 promoter fragments were radiolabeled and used as probes for EMSA with in vitro translated mSharp-1. A complex was formed on the wild type promoter but not on the triple mutant promoter fragment (Fig. 6B). Moreover, mSharp-1 binding was competed with an excess of unlabeled fragment containing the native promoter (Fig. 6C, lanes 2 and 3) but not by the triple mutant promoter fragment (Fig. 6C, lanes 4 and 5). These results demonstrate that although mSharp-1 binds...
only to the E box sites in the Stra13 promoter, transcriptional repression of Stra13 expression by mSharp-1 is mediated in part by binding to the E box and also occurs through non-DNA binding mechanisms.

**mSharp-1 Directly Interacts with Sp1 and Impairs Its Transactivation Domain Leading to Repression of Stra13 Promoter Activity**—The Stra13 promoter lacks a TATA box but is GC-rich and contains several Sp1-binding sites (Fig. 4A). Sp1 is important for transcription of many genes, and several transcription factors exert their effects on promoter activity through interaction with Sp1 (40). Because mSharp-1 strongly represses basal Stra13 expression in the absence of E box binding and the promoter contains several Sp1 sites, we investigated whether mSharp-1 could inhibit Sp1 activity and thereby repress Stra13 expression in a DNA-binding independent manner.

We first examined whether Sp1 activated Stra13 expression. The unmutated Stra13 promoter containing intact E box sites as well as the triple E box mutant promoter construct was transfected in 10T1/2 cells along with an expression vector for Sp1. As predicted from the presence of Sp1 sites, expression of Sp1 resulted in transcriptional activation of both promoter constructs (Fig. 7A). Because we were interested in determining the mechanism by which mSharp-1 represses Stra13 expression in the absence of E box binding, we focused our further studies on the triple E box mutant promoter construct. Interestingly, co-transfection of increasing amounts of mSharp-1 expression vector as indicated. Cells were harvested 48 h after transfection for luciferase activities. The activity in the absence of mSharp-1 was assigned a value of 100%. The data shown are an average of three independent experiments.

**FIG. 4. Stra13 proximal promoter contains three E boxes and is repressed by mSharp-1.** A, the location of the three conserved E box sequences in the Stra13 proximal promoter (PmlI-NheI) is indicated by open boxes, and the Sp1 sites are underlined. B, 10T1/2 cells were transfected with 0.5 μg of Stra13 promoter construct pGL3PmN with or without E47 and MyoD expression vectors (0.25 μg each), in the absence or presence of 0.5 μg of mSharp-1 expression vector. C, 10T1/2 cells were transfected with 0.5 μg of pGL3PmN and increasing amounts of mSharp-1 expression vector as indicated. Cells were harvested 48 h after transfection for luciferase activities. The activity in the absence of mSharp-1 was assigned a value of 100%. The data shown are an average of three independent experiments.
Sp1 interacted in vitro and in vivo. To examine a direct physical interaction between the two proteins, mSharp-1 was translated in vitro and labeled using [35S]methionine. The rabbit reticulocyte lysate was then immunoprecipitated using anti-Sp1 antibody. The immunoprecipitates were subjected to SDS-PAGE and visualized for 35S-labeled mSharp-1 by autoradiography. The results, as shown in Fig. 7D, indicated that immunoprecipitation with Sp1 clearly pulled down 35S-labeled mSharp-1 indicating that mSharp-1 directly interacts with Sp1. To confirm further that endogenous mSharp-1 interacts with Sp1,
10T1/2 cell extracts were immunoprecipitated using polyclonal anti-Sp1 antibody. The immune complexes were analyzed by SDS-PAGE and detected using polyclonal mSharp-1 antibody. Consistent with the results above, a strong association was seen between endogenous Sp1 and mSharp-1 (Fig. 7E), indicating that these two proteins strongly and directly interact in mammalian cells.

Although these results demonstrate that mSharp-1 can directly interact with Sp1 and inhibit its activity, they do not prove that endogenous Sp1, bound to the mutant Stra13 promoter, interacts with mSharp-1. To address this question, we performed chromatin immunoprecipitation experiments using the triple mutant Stra13 promoter construct and antibodies against Sp1 and mSharp-1. 10T/12 cells were transfected with triple E box mutant promoter construct and antibodies against Sp1 and mSharp-1. After transfection, cells were cross-linked, and chromatin was immunoprecipitated using polyclonal anti-Sp1 or anti-Sharp-1 antibodies. The immunoprecipitated DNA was subjected to PCR with primers that amplify a fragment containing the Sp1 sites. Amplification of the Stra13 mutant promoter containing the Sp1 sites was detected. Taken together, these data indicate that although mutation of the E box sites prevents mSharp-1 binding on the Stra13 promoter through E box sequences, mSharp-1 can still be recruited to Stra13 promoter via interaction with endogenous DNA-bound Sp1 and therefore inhibit Sp1-mediated transactivation of Stra13 expression.

**DISCUSSION**

In the present study, we describe the molecular cloning and functional characterization of the class C bHLH factor mSharp-1/DEC2. We show that mSharp-1 specifically binds to the E box motif and represses MyoD and E12-mediated transcriptional activation through several mechanisms including occupancy of E box sites and physical association with MyoD and E proteins. Furthermore, we demonstrate that Stra13 is a target for mSharp-1-mediated repression. mSharp-1 can negatively regulate Stra13 promoter activity by three distinct mechanisms: (i) by directly binding to conserved E box motifs, (ii) by sequestration of other positive bHLH factors such as MyoD and E proteins, and (iii) by interaction with the DNA-binding transcription factor Sp1 and inhibition of its transcriptional activity. Our data suggest that mSharp-1 can function as a transcriptional repressor via both DNA-binding dependent and independent mechanisms.

At the amino acid level, mSharp-1 exhibits the highest se-
sequence identity with Stra13 and shares 96% identity within the bHLH domain and an overall identity of 50%. Similar to Stra13, mSharp-1 also contains a proline residue in the basic region. In both mSharp-1 and Stra13, the position of this proline residue is displaced relative to its position in the basic domain of Hes/Hairy/E(Spl), which is thought to confer N box binding specificity. Consistent with this, mSharp-1 binds to the E box sequence but not to N box which is a target for the Hairy/E(Spl)/Hes proteins.

Most repressors mediate transcriptional repression by recruitment of co-repressors. The Hairy/E(Spl)/Hes family utilizes the WRPW motif for recruitment of the co-repressor Groucho to mediate transcriptional repression. We have demonstrated previously that Stra13 transcriptionally represses its own expression by recruitment of HDAC/Sin3A co-repressors (28), and subsequently Herp1 and -2 were also reported to recruit the Sin3A-HDAC co-repressor complex through the bHLH domain (42). Because mSharp-1-mediated repression of a synthetic E box reporter is not sensitive to trichostatin A nor is it augmented by co-transfection of
studies indicate that mSharp-1 can repress Stra13-mediated repression of Sp1-mediated transcriptional activity (49). Additionally, mSharp-1 can recruit Sin3A/HDAC co-repressors to mediate transcriptional repression. We have not examined the mechanism by which mSharp-1 inhibits gene expression when bound to the E box site. However, regardless of the specific DNA binding dependent repression mechanisms that may require co-repressor recruitment, our studies indicate that mSharp-1 may function as a repressor through several additional mechanisms.

mSharp-1 blocks transcriptional activation mediated by MyoD and E12. This repression of E box activation is reversed to a large degree by increasing amounts of E12 and partially by MyoD. Because mSharp-1 binds to the E box as a homodimer and also physically interacts with MyoD and E47, our data suggest that mSharp-1 acts as a negative regulator of myogenic bHLH factors in muscle differentiation by at least three mechanisms including occupancy of E box sites and thereby competition for DNA binding with MyoD and E12 heterodimers, by sequestration of E proteins, and by direct physical interaction with MyoD. These repressive effects of mSharp-1 on E box activation are similar to several negative bHLH factors including Mist1 (43), MyoR (44), ABF-1 (45), Dermo-1 (46), and N-Twist (47), which also utilize several overlapping mechanisms to regulate E box activity. Because mSharp-1 is expressed in a number of tissues, it is likely that it forms heterodimers not only with MyoD and E proteins but also with other positive factors expressed in a tissue-specific manner to regulate their functional activity as well.

mSharp-1 and Stra13 exhibit a high degree of sequence conservation, and interestingly, their expression patterns overlap in a number of tissues during both embryonic development and in adult tissues. Moreover, during mouse embryogenesis, mSharp-1 is temporally expressed earlier thanStra13 (30), suggesting that it may regulate Stra13 expression. This contention is supported by our findings that Stra13 promoter activity is indeed negatively regulated by mSharp-1. Our studies provide in vitro evidence that mSharp-1 can specifically bind to the E box sites in the Stra13 promoter and are supported in vivo by detection of endogenous mSharp-1 on the Stra13 promoter by ChIP experiments. Nevertheless, repression of Stra13 expression by mSharp-1 is not entirely dependent on E box binding, as mutation of all the E box sites in the Stra13 proximal promoter only partially abrogates mSharp-1-mediated repression. Because mSharp-1 does not directly bind to the triple E box mutant construct (Fig. 6B) but can still repress Stra13 expression, our results suggest that mSharp-1 regulates Stra13 expression by both DNA binding dependent and independent mechanisms.

Transcriptional regulation can occur as a result of direct protein-protein interactions between diverse transcription factors. For instance, myogenic bHLH proteins have been reported to physically interact with the ubiquitous transcriptional activator Sp1 through juxtaposed GC and E box sites and synergistically activate the expression of promoters expressed in skeletal muscle (48). Conversely, Von Hippel Lindau, p107, and promyelocytic leukemia protein repress transcription by impairment of Sp1-mediated transcriptional activity (49–51). Our studies indicate that mSharp-1 can repress Stra13 expression via interaction with Sp1, which constitutes a novel mechanism of mSharp-1-mediated repression. This is based on our observations that in the absence of E box binding, mSharp-1 represses Stra13 proximal promoter activity through a region containing several Sp1 sites and is a potent suppressor of Sp1-induced activation of Stra13 expression. mSharp-1 inhibits a Gal-Sp1 fusion construct, which contains only the transactivation domain of Sp1. Moreover, expression of increasing amounts of Gal-Sp1 can override mSharp-1-mediated inhibition of the Stra13 mutant promoter (data not shown). Together, these results suggest that mSharp-1 may target only the transactivation domain of Sp1 without affecting its DNA binding ability. The inhibition of Sp1 activity by mSharp-1 appears to be a result of a direct physical interaction, which is supported not only by co-immunoprecipitation of both endogenous proteins but also in vivo by ChIP experiments. Whereas mSharp-1 fails to bind directly to the E box mutant Stra13 promoter on its own indicating that its DNA binding specificity is likely E box sites, it is also associated with Sp1 on Sp1-binding sites on Stra13 promoter. Thus, mSharp-1 may utilize three distinct mechanisms to repressStra13 expression. It may repress Stra13 expression by binding to E box sites, sequester other positive E box binding factors, as well as interact with Sp1 to inhibit Sp1 activity. Because Stra13 promoter lacks a TATA box, it is likely that the basal activity of the promoter is dependent on a GC-rich region that contains several Sp1 sites. This is also consistent with our observations that both basal as well as Sp1-induced activation of Stra13 expression is repressed by mSharp-1. Sp1 is implicated in the expression of genes required for terminal differentiation as well as those required for growth (40). Given the strong association with Sp1, it is likely that in addition to Stra13 expression, mSharp-1 may regulate other promoters that are Sp1-dependent and required for differentiation or cell cycle regulation.

Although a vast number of biochemical studies have demonstrated clear DNA binding specificities, there is increasing evidence that several bHLH factors function in vivo by non-DNA binding dependent mechanisms. For instance, in a recent study, the bHLH factor dHand was reported to induce ectopic digits when expressed under the proxl promoter (52). Interestingly, the same phenotypic effects were seen with dHand mutants lacking the DNA binding domain as well as the transactivation domain, with only the helix-loop-helix domain of dHand being critically required for its function. These studies indicate that whereas dHand can bind in vitro to an E box site, some of its biological effects in vivo are independent of DNA binding. Similarly, ectopic expression of Hes6 in Xenopus embryos was found to promote neurogenesis, and mutant forms of Hes6 lacking the DNA binding domain or the WRPW domain had the same effect as wild type Hes6 (53). Moreover, defects in primitive hematopoiesis (13, 14) in SCL-/- embryoid bodies were rescued by DNA-binding defective SCL protein (25). These studies and ours support the view that the biological effects of bHLH factors are to a large degree regulated through dimerization and protein-protein interactions rather than solely by transcriptional regulatory mechanisms exerted by DNA binding. Because both Stra13 and mSharp-1 can dimerize with several bHLH factors as well as with other DNA-bound transcription factors (Ref. 16 and this study) it is likely that in vivo they regulate the functional activity of a number of bHLH factors through distinct mechanisms. The delineation of such regulatory mechanisms is required to understand the interactions not only between mSharp-1 and Stra13 but to also investigate their functions within the bHLH network during cellular differentiation and apoptosis.

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