mSharp-1/DEC2, a basic helix-loop-helix protein functions as a transcriptional repressor of E-box activity and Stra13 expression

Sameena Azmi, Hong Sun, Anne Ozog and Reshma Taneja*

Brookdale Department of Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine, New York, NY 10029-6574

*author for correspondence

e-mail: Reshma.Taneja@mssm.edu
Tel:  212 241 9413
Fax: 212 860 9279

Running title: Mechanisms of transcriptional repression mediated by mSharp-1.
Summary

Transcription factors belonging to the basic helix-loop-helix (bHLH) family play critical roles in 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, 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.
Introduction

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 (HLH) 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 development of brain, eye, as well as in differentiation and activation of T cells (24-26). The DNA-binding properties of this subfamily are quite divergent. While 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 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 several mechanisms including competition for DNA-binding and protein-protein interactions with MyoD and E47. Moreover, using gel mobility shift experiments and chromatin immunoprecipitation assays we also demonstrate both in vitro and in vivo, that mSharp-1 negatively regulates Stra13 expression. Transcriptional repression of Stra13 expression depends, in part, on binding of mSharp-1 to three E-box sites in the proximal promoter, as well as by direct interaction with the transactivator Sp1 resulting in inhibition of Sp1 activity and Stra13 expression. Our observations indicate that mSharp-1 functions as a repressor both by E-box 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 $5 \times 10^5$ plaques were plated, transferred to Hybond N+ membrane (Amersham Pharmacia Biotech) and prehybridized in a solution containing 50% formamide, 5X SSPE, 5X Denhardt’s solution, 0.5% SDS, and 0.05 mg/ml denatured salmon sperm DNA for 3-5 hr at 42°C. The rat Sharp-1 cDNA was used as a probe. Hybridization was carried out at 42°C for 16 hr in the same buffer containing $^{32}$P-labeled probe. Filters were washed 4 times at 65°C with 2X SSC + 0.1% SDS. Four phage clones that were positive after two rounds of screening were subcloned into pTZ18R in the EcoRI site and sequenced on both strands. All the clones obtained from library screening were partial and lacked the 5’ end of the cDNA including the translation start site. One of these clones, which contained the longest coding sequence was used to further clone full length mouse Sharp-1 cDNA. A PstI-EcoRI fragment from this clone was subcloned in pTZ18R. A BLAST sequence similarity search of Genbank EST database with rat Sharp-1 resulted in the identification of several mouse ESTs. One EST (Accession No. BE985179), which contained the 5’ UTR and coding sequences for N-terminal region was restricted with EcoRI and PstI to release a fragment of about 600bp. This fragment was ligated to the EcoRI-PstI partial cDNA clone obtained from the library screening to obtain the full length mouse Sharp-1 cDNA. The complete nucleotide sequence was determined on both strands.

Generation of anti-mSharp-1 antibody

Polyclonal anti-mSharp-1 antibody was raised against a C-terminal peptide (Sigma GenoSys). The serum was tested for antigenicity by ELISA with coated synthetic peptide according to the
manufacturer's protocol, and purified by caprylic acid and ammonium sulfate precipitation. The serum was tested for specificity by western-blot using in vitro translated mSharp-1 and Stra13, and positive reactivity was obtained with mSharp-1 only.

**GST Pull-down Assay**

GST pull-down assays were done as described (28). 20 µl of Glutathione Sepharose 4B beads containing 4 µg of GST-mSharp-1 protein was incubated with 5 µl of [35S] methionine-labeled E47 or MyoD translated in rabbit reticulocyte lysate using the TNT coupled in vitro transcription-translation system (Promega). The samples were incubated in a total volume of 200 µl of GST binding buffer (50mM Tris-HCl, pH8.0, 100mM NaCl, 0.3mM DTT, 10mM MgCl2, 10% glycerol, 0.1% NP-40) and complete proteinase inhibitor cocktail (Roche) for 3 hr at 4°C, followed by three washes with the same buffer. Bound proteins were eluted by boiling for 10 min in sample buffer, separated on 12% SDS-PAGE gels and visualized by autoradiography.

**RNA Extraction and RT-PCR**

Total RNA was extracted from various organs of 8-week old mice with Trizol (Invitrogen). Semi-quantitative RT-PCR was performed as previously described (16). A 280 bp mSharp-1 fragment was amplified using primers RT8 (5’-AGGCACTCTAACTGGTTCCG-3’) and RT9 (5’-AGAGTAAGAGATGCTCTGCT-3’). The amounts of RNA were normalized to the ubiquitous 36B4 transcripts (31) with primers RT6 (5’-CAGCTCTGGAGAAACTGCTG-3’) and RT7 (5’-GTGTACTCAGTCTCCACAGA-3’). RT-PCR products were detected by southern blot analysis with 32P-labeled mSharp-1 and 36B4 cDNA probes.

**Plasmid Constructs**

Plasmid p355 which contains the full length mSharp-1 cloned in pTZ18R in the EcoRI site was used for in vitro translation. A mSharp-1 expression vector p359 was constructed by subcloning
the insert from p355 in the EcoRI site of mammalian expression vector pCS2. To generate GST-Sharp-1 fusion construct, a BamHI restriction site was introduced by PCR in plasmid p421, which contains the Sharp-1 EST BE985179 subcloned in KS+. The insert was released with BamHI and introduced in the same site of pGEX-2TK (Pharmacia) to generate plasmid p422. Plasmid p440 containing the entire GST-Sharp-1 construct was generated by a triple ligation using the BamHI-SphI fragment from p422 and the SphI-EcoRI fragment from p359 cloned into the BamHI-EcoRI sites of pGEX-2TK. The Stra13 expression vector pCS2-Stra13 and Stra13 promoter construct pGL3PmN have been previously described (28).

**Site-directed mutagenesis**

Mutations in the E-box sequence of the Stra13 promoter construct pGL3PmN were introduced using the Quick-change Mutagenesis kit (Stratagene, La Jolla CA). Oligonucleotide primers used to generate mutations in E-box 1 (E-box M1), E-box 2 (E-box M2) and in E-box3 (E-box M3) 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 (DMEM) containing 10% fetal bovine serum. 5 X 10^4 cells were plated one 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 50ng of the β-galactosidase plasmid pCH110 (Amersham Pharmacia). All transfections were performed in duplicate at least three times.
Myogenic differentiation assays

10T1/2 cells were seeded at a density of 2 X 10^5 cells/well in 6-well plates one day prior to transfection. Cells were transfected as described above with 2 µg of MyoD in the presence or absence of mSharp-1. After five days in differentiation medium (DMEM containing 2% horse serum) cells were rinsed three times in PBS and then fixed in methanol for 20 min at 4^0C. Following a 30 min blocking step (5% goat serum in PBS), 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 4^0C. The primary complexes were detected using a biotinylated anti-mouse antibody and a horseradish peroxidase (HRP)-streptavidin conjugate (Vector Laboratories).

Nuclear Extracts

10T1/2 cells (2X10^6) cultured on 100-mm culture dishes were transfected with 5µg each of MyoD and E47 for 48 hr, washed twice with phosphate-buffered saline and resuspended in 400 µl of buffer A (10mM HEPES [pH 7.8], 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol, and 1mM PMSF). After incubation on ice for 5 min, NP-40 was added to a final concentration of 0.6%. Nuclei were pelleted and the cytoplasmic proteins carefully removed. Nuclei were then resuspended in buffer C (20mM HEPES [pH 7.9], 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol and 1 mM PMSF). After vortexing and stirring for 30 min at 4^0C, the samples were centrifuged and nuclear proteins in the supernatant were transferred to a fresh vial. Protein concentrations of nuclear extracts were determined by Bio-Rad protein assay using bovine serum albumin as a standard.

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 was 5'-GGCCGCAGCAGCACGTGGCACAGCAGCACGTGGGCACAGCAGCACGTGGGCAGC-3'. A typical binding reaction contained 20,000 cpm probe, 5 µl of *in vitro* translated protein or 5 µg of nuclear extract, 1 µg poly[dI-dC], 5 mM HEPES [pH 7.8], 50 mM KCl, 0.5 mM DTT, and 10% glycerol. The reaction was incubated at room temperature for 20 min and fractionated on 5% polyacrylamide gels in 0.5X TBE buffer. Gels were dried and exposed to X-ray film. For competition experiments, 200- or 400-fold excess of competitor DNA 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.

**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 (approximately 30x10^6) 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 PBS and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1). The lysate was sonicated to shear DNA to lengths between 200-1000bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM Tris-HCl pH 8.1, 150 mM NaCl). An equivalent of 10^7 cells was incubated with 10 µl anti-mSharp-1 antibody or with 10 µl 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 hr at 4°C and the agarose complex was pelleted by brief centrifugation at 4°C.
After extensive washing, the pellet was dissolved in 250µl of elution buffer and spun to remove the agarose. The supernatant was treated with 250µl 5M NaCl and heated at 65°C for 4 hr to reverse protein-DNA crosslinks. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover DNA. Eluted DNA was resuspended in 30µl TE. For PCR reactions, 2µl of DNA was amplified using primers RT31 (5'-TCTCATTCACTTGGCTCGCA-3') and RT46 (5'-ATGTTCTATGAGCTAGCCTGAGCTTCC-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 8µg of the triple E-box mutant construct [E-boxM(1+2+3)] using Lipofectamine Plus (Invitrogen). 48hr after transfection, cells were crosslinked and incubated with 10µl anti-mSharp-1 or 2µl anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) as described above. The protein A/antibody/histone/DNA complex was then used for both immunoprecipitation and PCR. 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 PCR reactions, DNA was eluted as described above and was amplified using a 5' primer from pGL3 [RT145 (5'-CTAGCAAAATAGGCTGTCCC-3')] and a 3' primer corresponding to the mutant E-Box2 site [RT40 (5'-TGCGAGCACAGGTAAGAGA-3')] that allowed for specific amplification of the exogenously transfected DNA. 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.

$^{35}$S Met-labeled mSharp-1 protein was generated using the TNT coupled transcription-translation system (Promega). For immunoprecipitation, lysates were incubated with 2µg of rabbit polyclonal Sp1 antibody (PEP2, Santa Cruz Biotechnology) in 1:1 mix of two buffers (20mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 420 mM NaCl, 0.2 mM EDTA) and (20mM HEPES, pH 7.9, 10mM KCl, 0.2 mM EDTA, 20% glycerol) for one hour on a rotating wheel at 4$^0$ C. The immunocomplexes were absorbed to proteinA/G-Agarose (Santa Cruz Biotechnology) overnight at 4$^0$C and washed three times in the buffer described above. Associated proteins were then eluted in SDS sample buffer containing DTT by boiling for 5 min and loaded on a 12% polyacrylamide gel. Gel was fixed and dried before detection of $^{35}$S-labeled mSharp-1 by autoradiography.
Results

cDNA Cloning and Expression analysis of mSharp-1

To understand the biological properties of Sharp-1 and 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 DEC2 (Accession No. AB044090) but contains a longer 3’ UTR (33). Domain analysis (Fig. 1B) indicated that mSharp-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, mSharp-1 also contains a proline residue in the basic region and lacks the WRPW motif. mSharp-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 mSharp-1 expression, we performed RT-PCR using total RNA from embryos between E9.5 to E16.5 as well as various tissues from adult mice (Fig. 1C). Consistent with our previous report, mSharp-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.

mSharp-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 motif (CANNTG) or N-box motif (CACNAG). To investigate the transcriptional
properties of mSharp-1, we tested whether mSharp-1 can bind to either the E-box or the N-box sequences in vitro. Electrophoretic gel mobility-shift (EMSA) assays were performed using $^{32}$P-labeled oligonucleotide probes containing three E-box sequences or containing the N-box sequence. Addition of in vitro translated mSharp-1 to the E-box oligonucleotide resulted in a DNA-protein complex (Fig. 2A, lane 1). In contrast, mSharp-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 further assess the specificity of mSharp-1 binding to the E-box, we generated polyclonal antibodies specific to mSharp-1. Addition of anti-mSharp-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 4R-tk-luc reporter that contains four tandem E-boxes from the 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 required recruitment of HDAC1. However, neither addition of trichostatin A (TSA), a HDAC inhibitor, nor co-
transfection of HDAC1 expression vector had any effect on mSharp-1 mediated repression of E-box activity (data not shown). Thus transcriptional repression of the synthetic E-box reporter 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 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 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 $^{35}$S 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. 3C, 3D).

**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 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 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. 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, 3 and 4), but not with oligonucleotides containing a mutation in the E-box sites (Fig. 5A, lanes 5, 6 and 7. To further assess binding of endogenous mSharp-1 on the Stra13 promoter in vivo, ChIP assays were performed. Since 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 (pi) 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 pre-immune 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 Stra13 promoter. In vitro translated mSharp-1 bound to 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 further increasing amounts of mSharp-1 resulted in its binding to Stra13 promoter (lanes 7, 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 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, 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 unlabelled fragment containing the native promoter (Fig. 6C, lanes 2, 3) but not by the triple mutant promoter fragment (Fig. 6C, lanes 4, 5). These results demonstrate that while 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). Since 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). Since 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 inhibited Sp1 induced activation of the triple E-box mutant promoter in a dose-dependent manner suggesting that mSharp-1 may impair Sp1 mediated transactivation (Fig. 7B).

To examine whether mSharp-1 could inhibit the transactivation domain of Sp1, we used a Gal-
Sp1 fusion construct that contains the Gal4-DNA binding domain fused to the transactivation domain of Sp1 (41). 10T1/2 cells were transfected with a Gal-luciferase reporter along with Gal-Sp1 in the absence or presence of mSharp-1. The Gal-Sp1 fusion protein transactivated the reporter, and co-transfection of mSharp-1 inhibited Sp1-induced transactivation (Fig. 7C). These results indicated that the transactivation domain of Sp1 is a target for mSharp-1 repression. Taken together, these results indicate that suppression of Stra13 expression by mSharp-1 is caused, at least in part, by inhibition of Sp1 activity.

Given that mSharp-1 inhibited Sp1 mediated activation of the Stra13 promoter, we investigated whether mSharp-1 and 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 mSharp-1 indicating that mSharp-1 directly interacts with Sp1. To further confirm 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.

While 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 [E-boxM(1+2+3)] and 48 hr after transfection, cells were crosslinked 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 flanking Sp1 sites. To avoid detection of mSharp-1 bound to E-box sites on the endogenous Stra13 promoter, we used primers that allowed for specific detection of the transfected triple E-box mutant construct by using a 5’ primer corresponding to a sequence in the vector pGL3, and the other to the mutant E-box 2 site. As shown in Fig. 7F, immunoprecipitation of chromatin with either anti-Sp1 or with mSharp-1 antibodies resulted in amplification of the Stra13 mutant promoter containing the Sp1 sites. Taken together, these data indicate that while 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) 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 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 previously demonstrated 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 Sin3A/HDAC co-repressor complex.
through the bHLH domain (42). Since mSharp-1 mediated repression of a synthetic E-box reporter is not sensitive to TSA nor is it augmented by co-transfection of HDAC1, our data suggest that this repression is independent of HDAC recruitment. Nevertheless, these studies do not exclude the possibility that in context of natural promoters that harbor E-box sites or by interaction with other transcription factors, mSharp-1 can recruit Sin3A/HDAC co-repressors to mediate transcriptional repression. We have not examined the mechanism by which mSharp-1 inhibit 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. Since 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. Since 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 both during embryonic development and
in adult tissues. Moreover, during mouse embryogenesis, mSharp-1 is temporally expressed earlier than Stra13 (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. Since 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, VHL, p107, and PML 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 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. While 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 repress Stra13 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. Since Stra13 promoter lacks a TATA box, it is likely that the basal activity of the promoter is dependent 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 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.

While 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 prx1 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 HLH domain of dHand being critically required for its function. These studies indicate that while 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 (54). 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. Since both Stra13 and mSharp-1 can dimerize with several bHLH factors as well as with other DNA-bound transcription factors (16; this study; and our unpublished observations) 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 are 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.
Acknowledgements

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*Development* **129**, 3077-3088


FOOTNOTES:

Abbreviations used here are: bHLH, basic helix-loop-helix; TSA, trichostatin A; HDAC, histone deacetylase; E(spl), Enhancer of Split; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assays; tk, thymidine kinase; ChIP, chromatin immunoprecipitation.
Figure Legends

**Fig.1. Sequence and expression of mSharp-1.** *A*, The nucleotide sequence and the deduced amino acid sequence of mSharp-1 are indicated. The bHLH domain is underlined and the orange domain is underlined in bold. The stop codon is indicated by an asterix. *B*, Amino acid sequence alignment of mSharp-1 and Stra13 shows that the two proteins share 96% identity in the bHLH domain and about 66% identity in the orange domain. *C*, RT-PCR analysis from various tissues of an adult mouse shows widespread expression of mSharp-1. The expected PCR product of mSharp-1 (280bp) is shown. The amounts of RNA in each reaction were normalized with transcripts of the 36B4 gene.

**Fig.2. mSharp-1 binds to the E-box sequence and represses E-box reporter activity.** *A*, An oligonucleotide containing three E-box sequences was used as a probe for EMSA with *in vitro* translated mSharp-1. A specific complex indicated by an arrow was detected in the absence of any added antibody (*lane*1) and was supershifted (SS) with specific anti-mSharp-1 polyclonal antibody (*lane*2). Binding of mSharp-1 was specifically competed out using a 200-fold excess of E-box containing oligonucleotide (*lane*4). *B*, mSharp-1 inhibits E-box activity in a dose dependent manner. 10T1/2 cells were transfected with the indicated amounts of mSharp-1 expression vector and 0.5µg of the reporter 4R-tk-luc. Empty expression vector was added to normalize the amount of DNA in each well. Cells were harvested for luciferase activity 48 hr after transfection. The extent of repression in the presence of mSharp-1 was measured relative to the activity of 4R-tk-luc in the presence of an equivalent amount of the empty vector (pCS2) alone.
Fig.3. mSharp-1 inhibits MyoD/E12 induced activation of the E-box reporter and myogenic differentiation. A, 10T1/2 cells were transiently transfected with 100 ng of 4R-tk-luc reporter and 50 ng of β-galactosidase plasmid pCH110 (Amersham Pharmacia) along with 250 ng of expression vectors for MyoD, E12 and mSharp-1. Increasing amounts of MyoD and E12 (125, 250 and 375 ng) were also co-transfected with mSharp-1. The data shown is representative of three independent experiments performed in triplicate. B, 35S-labeled E47 and MyoD were translated in vitro and incubated with GST alone or with GST-mSharp-1 fusion protein. 10% of the input was run on the gel as control. C, 10T1/2 cells were transfected with MyoD in the presence or absence of mSharp-1. Differentiated cells were identified by immunostaining using an antibody against skeletal muscle myosin heavy chain (MHC). D, Total number of skeletal muscle specific myosin positive cells after transfection with MyoD alone, and with MyoD+mSharp-1 counted in six 40X fields.

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 pGL3PmN and increasing amounts of mSharp-1 expression vector as indicated. Cells were harvested 48 hr after transfection for luciferase activities. The activity in the absence of mSharp-1 was assigned a value of 100%. The data shown is an average of three independent experiments.

Fig.5. mSharp-1 binds to the E-box sequences in the Stra13 promoter in vitro and in vivo and competes with MyoD for binding. A, Gel shift assays were carried out with the Stra13
promoter containing three E-boxes as a probe and in vitro translated mSharp-1. mSharp-1 can bind to the E-box sequences in the Stra13 promoter (lane 1). The binding was competed out with a 400-fold excess of each of the three E-box sequences (lanes 2-4) but not with oligonucleotides harboring a mutation in the E-box sites (lanes 5-7). The protein-DNA complex is indicated by an arrow. Free probe is indicated by FP. B, C2C12 cells were chemically cross-linked and subjected to chromatin immunoprecipitation as described under “Experimental procedures”. Immunoprecipitation was performed with anti-mSharp-1 antibody or preimmune serum (pi) as a control. DNA was recovered from the chromatin immunoprecipitates was purified by phenol-chloroform extraction, and PCR amplification was conducted using primers flanking the E-box sites 1 and 2 in the Stra13 promoter. Input represents amplification of the Stra13 promoter from DNA-protein complexes before immunoprecipitation. 1% of input was run on the gel as a positive control. Amplified products were run on agarose gels and visualized by ethidium bromide staining. C, Gel shift assays were performed with in vitro translated mSharp-1 or with nuclear extracts from 10T1/2 cells transfected with MyoD and E47. mSharp-1 binding to Stra13 promoter (lane 1) was inhibited by addition of anti-mSharp-1 antibody (lane 2). Binding of MyoD and E47 to Stra13 promoter (lane 3) was not affected in presence of anti-mSharp-1 antibody (lane 4) but was competed out with increasing amounts of mSharp-1 (lanes 6-8). The complex formed by mSharp-1 was specifically blocked with anti-mSharp-1 antibody (lane 9). FP indicate free probe.

Fig.6. Transcriptional repression of Stra13 expression by mSharp-1 is dependent on both binding to the E-box elements as well as non DNA-binding dependent mechanisms. A, The wild type Stra13 promoter construct pGL3PmN was used as a template to generate single and triple E-box mutants [E-boxM1, E-boxM2, E-boxM3 and E-boxM(1+2+3)] schematically shown.
on the left. 0.5µg of wild type or mutant reporters were transfected into 10T1/2 cells with 0.25µg of mSharp-1 expression vector. The activity of the wild type and mutant promoter constructs in the absence of mSharp-1 (filled bars) was taken as 100% and the relative extent of repression by mSharp-1 was determined (open bars). B, EMSA analysis using wild type and triple E-box mutant promoter fragments as probe with in vitro translated mSharp-1. The protein-DNA complex is indicated by an arrow. C, Binding of mSharp-1 to pGL3PmN (lane 1) was competed with 200 and 400 fold excess of unlabelled wild type probe containing the unmutated promoter (lanes 2, 3) but not with E-box mutant promoter fragment (lanes 4, 5). FP indicate free probe.

**Fig.7. mSharp-1 interacts with Sp1 to repress Stra13 expression.** A, Stimulation of Stra13 promoter activity by Sp1. 10T1/2 cells were transfected with 50ng of pGL3PmN or E-BoxM(1+2+3) in the presence or absence of 50ng of a Sp1 expression vector (pCMV-Sp1) as indicated. Luciferase activity was determined 48 hr later. The data shown is an average of three independent experiments. B, mSharp-1 inhibits Sp1 induced activation in a dose-dependent manner. 10T1/2 cells were transiently transfected with 50ng of a Sp1 expression vector, 50ng of the reporter E-BoxM(1+2+3) and the indicated amounts of mSharp-1. Cells were harvested for luciferase activity 48 hr after transfection. Sp1 induced transcriptional activity was assigned a value of 100%. The luciferase activities are the average of three independent experiments. C, mSharp-1 represses the transactivation domain of Sp1. 10T1/2 cells were transfected with 100ng of Gal4-reporter and equivalent amounts of mSharp-1 expression vector in the absence and presence of Gal-Sp1. Cells were harvested 48 hr after transfection for luciferase activities. The transactivation activity of Sp1 was assigned a value of 100%. D, Sp1 interacts with mSharp-1 in vitro. 35S-labeled mSharp-1 was translated in vitro and incubated either with Sp1 antibody or control IgG. 10% of the input was run on the gel as control. E, mSharp-1 is associated with Sp1
in vivo. 10T1/2 cells were transfected with E-BoxM(1+2+3) and 48 hr after transfection were chemically cross-linked and subjected to chromatin immunoprecipitation. Immunoprecipitation was performed with or without anti-Sp1 antibody, followed by immunoblotting with polyclonal mSharp-1 antibody. F, mSharp-1 interacts with Sp1 bound to the Stra13 mutant promoter E-BoxM(1+2+3). 10T1/2 cells were transfected with E-BoxM(1+2+3) for 48 hr. Chromatin immunoprecipitation was performed as described above with either anti-mSharp-1 or anti-Sp1 antibodies. For a negative control, a no-antibody immunoprecipitation was performed. DNA was eluted from chromatin immunoprecipitates and subjected to PCR using primers specific for the transfected construct. 1% of input was run on the gel as a positive control.

**Table I.** Double stranded synthetic oligonucleotides used as probes in electrophoretic mobility shift assays. The oligonucleotide sequences are 5' to 3'. The E-box sequences are underlined. The mutated base pairs are shaded.

**Table II.** Oligonucleotide primers used to mutagenize E-box binding sites in the Stra13 promoter. The three E-box sites in the Stra13 promoter were mutated by changing CANNTG to ACNNGT. The sequence of each set of primers used for mutagenesis is shown.
**Fig. 1**

### C

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![Gene Expression](http://www.jbc.org/content/journal/jbc/1/1/fig1)

mSharp-1

36B4
Fig. 2

(A) 

Antibody – + Competitor – +

SS

FP

1 2 3 4

(B) 

Relative Luciferase Activity

4R-tk-luc + + + +
mSharp-1(ng) 0 100 250 500
pCS2(ng) 500 400 250 0
Fig. 3

A

Relative Luciferase Activity

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B

C

MyoD

MyoD+mSharp-1

D

# MHC Positive Cells

MyoD

MyoD + mSharp-1
A

CGTGAGGCT CATGTGATGA AGCCGGGGAA GGCCGGCCAG GTGCTCCTCT CCCTCCCGGG 481

E-BOX 3

Pm1I

Sp1

CAGCCGGCCAG ACCTGCTTTG AGTCACAGGG TAGAACACGT AGCTTCTACC CACCCACTCG 421

CTCCCATTTA ACCAGCCCG CAGCCTCTCC TTACTCCTCG GCTGTCGCCCT TCCAGCAACT 361

Sp1/Sp1

Sp1

CCCCCCCCCC GGGGCCCCCG CTCGCCCTG AGCACCCCCC CTTCCCCCTC CGCCTGTCCG 301

Sp1/Sp1

E-BOX 2

TTTCATTCA CTTGCTTCGC ACGCCGCAAGA CAGGCGAGCG AGACACACCC 241

GCCAGTCTGT GTGCAGAGCG GGACCGAGAG GCCGCTGCGG CACCCGGCCA TGCACGCCCC 181

CAACTGAAAGC GACACCTCAA AGGCCGCTGCT CCTGGCATCT CACGCCATTG TAAGGAAGCT 121

E-BOX 1

CAGGCTAGCT CATAGGAACT TACCTTTGGA GACCCCTGGA TCTCCCCTTC CAGGCCAGCC 61

NheI

CTTTTCAGA CTCTACTAAA GTGCAGACAG GAGGCACAG TGCCCCTGTC TCGCCGACTC 1

B

C

Fig.4
Fig. 6
Fig. 7
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mSharp-1/DEC2, a basic helix-loop-helix protein functions as a transcriptional repressor of E-box activity and Stra13 expression
Sameena Azmi, Hong Sun, Anne Ozog and Reshma Taneja

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