Differential Roles for Sox15 and Sox2 in Transcriptional Control in Mouse Embryonic Stem Cells*\(^\ddagger\)\(^\S\)

Received for publication, February 7, 2005, and in revised form, April 5, 2005 Published, JBC Papers in Press, April 29, 2005, DOI 10.1074/jbc.M501423200

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Sox family transcription factors play essential roles in cell differentiation, development, and sex determination. Sox2 was previously thought to be the sole Sox protein expressed in mouse embryonic stem (ES) cells. Sox2 associates with Oct3/4 to maintain self-renewal of ES cells. In the current study, digital differential display identified transcripts for an additional Sox family member, Sox15, enriched in mouse ES cells. Reverse transcription-PCR confirmed that Sox15 expression is highest in undifferentiated ES cells and repressed upon differentiation. Sox15 is expressed at low levels in several tissues, including testis and muscle. In vitro studies showed that Sox15, like Sox2, associated with Oct3/4 on DNA sequences containing the octamer motif and Sox-binding site. Gel mobility shift assays and SELEX analyses showed that Sox15 binds similar DNA sequences as Sox2 but with weaker affinity. In contrast to the early embryonic lethality observed in Sox2-null mice, Sox15-null ES cells and mice were grossly normal. DNA microarray analyses revealed that Otx2, Ctgf, Ebf, and Hrc are dysregulated in Sox15-null ES cells, however. Chromatin immunoprecipitation showed that Sox15, but not Sox2, bound to a Sox consensus binding site within the Hrc gene. Taken together, these data demonstrate differential roles for Sox15 and Sox2 in transcriptional control in mouse ES cells.

Embryonic stem (ES)1 cells are derived from the inner cell mass of blastocysts and proliferate indefinitely while maintaining pluripotency, the ability to differentiate into all cell types within an organism (1). Mouse ES cells were first established in 1981 and led to the development of knockout mouse technology.

* This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Uehara Memorial Foundation, the Naito Foundation, the Sumitomo Research Foundation, the Mitsubishi Foundation, and a Toray Science and Technology Grant (to S. Y.). This work was also supported in part by a Grant-in-Aid for 21st Century Centers of Excellence Research from the Ministry of Education, Culture, Sports, Science, and Technology. The costs of publication of this article were supported in part by a Grant-in-Aid for 21st Century Centers of Excellence Research from the Ministry of Education, Culture, Sports, Science, and Technology.

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\(^\S\) The abbreviations used are: ES, embryonic stem; EC, embryonic carcinoma; HMG, high mobility group; EST, expressed sequence tag; CHIP, chromatin immunoprecipitation; SELEX, systematic evolution of ligands by exponential enrichment; RT, reverse transcription; HA, hemagglutinin A; WT, wild type.
with Oct3/4 are essential for the establishment and maintenance of pluripotent embryonic cells. To better understand the processes governing mouse ES cell self-renewal, we analyzed expressed sequence tag (EST) data bases with digital differential display and identified several genes that are highly enriched in early mouse embryos and ES cells (12, 25, 28). Interestingly, ESTs encoding Sox15 (29) were found specifically in ES cells, indicating that Sox15 is expressed in ES cells in addition to Sox2. Sox15 expression has also been reported in muscle (30) and testis (31). Sox15 is the single member of Sox group G, but its HMG domain is closely related to the group B Sox proteins, including Sox2 (32), suggesting that the two Sox proteins may play similar roles in ES cells.

In the current study, we compared the expression, protein-protein interactions, and DNA binding of Sox15 and Sox2 in mouse ES cells. Our data showed that Sox15 and Sox2 behave similarly in vitro. However, Sox15-deficient mice developed normally, and Sox2 and Sox15 appear to regulate different sets of genes in vivo. Our results highlight the unique DNA recognition mechanisms for each Sox family transcription factor.

**EXPERIMENTAL PROCEDURES**

**RT-PCR—**Total RNA was isolated with Trizol (Invitrogen). First-strand cDNA was synthesized from total RNA with ReverTra Ace (Toyobo, Japan), and PCR was carried out with Ex Taq DNA polymerase (Takara, Japan). Primers used for Sox15 were described by Sarraj et al. (31). Other primers used were Sox2-gw-as (5'-CACCATGTTACATGATGGGACG-3'), and Sox2-gw-as (5'-CCACCGCTCTGACGTTGCGA-3') for Sox2, Oct2, Otx2-as (5'-CTTACAGATGAAAACTTCAGCAAGTCC-3') and Oct2-as (5'-GGGACAGCTTGGTCTTTCCTTACC-3') for Oct2, Ctgf-s (5'-GGGATGCTCAGAGCTGCAATG-3') and Ctgf-as (5'-GGGATGCTCAGAGCTGCATGG-3') for Ctgf, Ebaf-s (5'-GCTTTGTGAGACTGCTGACCTCA-3') and Ebaf-as (5'-GCTTTGTGAGACTGCTGACCTCA-3') for Ebaf, and Hrc-s (5'-GAGCACTTCTTACTGATTGGTGGCCCAAGAG-3') and Hrc-as (5'-CCCTTCATCCTTCTGTGGCTATGGG-3') for Hrc.

**Generation of Anti-Sox15 and Anti-Sox2 Antibodies—**The C-terminal regions of Sox15 and Sox2, which are devoid of the HMG domains, were amplified by PCR. The primers used were anti-Sox15-as (5'-CACTTCGGAGCCACGGGTGTCTCCC-3') and anti-Sox15-as (5'-TAAAGGTTGTGTTACTGCGAACAGG-3') for Sox15 and anti-Sox2-as (5'-CACCCTCATGGAAGAAGATAACGTAC-3') and anti-Sox2-as (5'-CTACCTGAGGCTGACATTAGAC-3') for Sox2. PCR products were subcloned into pENTR/D-TOPO (Invitrogen) to construct pENTR-Sox15 and pENTR-Sox2. These entry vectors were recombined with the destination vector pCAG-IP-Flag-HA-gw and pCAG-IP-Myc-gw by LR reaction to construct pCAG-IP-Flag-HA-Sox15, pCAG-IP-Flag-HA-Sox2, and pCAG-IP-Myc-Sox15. The membranes were blocked with 5% skim milk in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) and incubated with primary antibodies in TBST with 0.5% skim milk overnight at 4 °C. Antibodies used were anti-Sox15 serum (1:1000 dilution), anti-Sox2 serum (1:1000), anti-Myc antibody (1:600), and anti-HA (1:600). The secondary antibody used was horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:3000, SC2030, Santa Cruz Biotechnology). Signal was detected with ECL Western blotting Detection Reagents (RPN2106, Amersham Biosciences).

**Sox2-Sox15 Chimeric Protein—**To construct Sox2/15 chimera proteins, three fragments (N terminal, HMG, and C terminal of Sox2 or Sox15) were amplified with primers containing annealing sites to each other. The primers used were Sox2-gw-as (5'-CACCATGTTACATGATGGGACG-3') and Sox2HM1-g-hmg-as (5'-ATGGGACGAGAAGACGCGGAGAAGTGGAAGCGGAGCCAT-3') and Sox2HM1-g-hmg-as (5'-ATGATGCTGAGAACTCGCGGAGCCG-3') for Sox2 N terminal, Sox2HM1-g-hmg-as (5'-CACCATGTTACATGATGGGACG-3') and Sox2HM1-g-hmg-as (5'-ATGGGACGAGAAGACGCGGAGAAGTGGAAGCGGAGCCAT-3') for Sox15 HMG, Sox2HM1-g-hmg-as (5'-CACCATGTTACATGATGGGACG-3') and Sox2HM1-g-hmg-as (5'-ATGGGACGAGAAGACGCGGAGAAGTGGAAGCGGAGCCAT-3') for Sox15 C terminal. To make Sox2-15, in which the HMG domain of Sox2 was replaced with that of Sox15, three PCR products (Sox2-N terminal, Sox2HM1-HMG, and Sox2-C terminal) were mixed and reamplified with the primers Sox2-gw-as and Sox2-gw-as. To make Sox15-2-15, in which the HMG domain of Sox15 was replaced with that of Sox2, three PCR products (Sox15-N terminal, Sox15HM1-HMG, and Sox15-C terminal) were mixed and reamplified with the primers Sox15-gw-as and Sox15-gw-as.

**Immunoprecipitation—**For immunoprecipitation, 10 μg of each antibody were added.

**Chromatin Immunoprecipitation (CHIP) Assays—**Formaldehyde was added directly to the culture medium to a final concentration of 1% (v/v), and the dishes were gently shaken on a shaker at room temperature for 10 min. Glycine was added to a final concentration of 125 mM, and the dishes were returned to the shaker. After 5 min, dishes were washed three times with ice-cold phosphate-buffered saline and harvested by scraping into 3 ml of cold phosphate-buffered saline. Cells were collected by centrifugation at 2000 rpm for 5 min at 4 °C, and the supernatants were discarded. Cell pellets were resuspended in 10 ml of PBS (PBS: 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 1.0 mM Na2EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.4 μM/ml pepstatin, 1 μM/ml leupeptin). Samples were allowed to swell on ice for 10 min before homogenization with three strokes of a glass Dounce homogenizer to release nuclei. Nuclei were collected by centrifugation at 7000 g for 10 min at 4 °C, and the supernatants were discarded. Nuclei were resuspended in 50 mM Tris, pH 7.6, 10 mM EDTA, 1% SDS, and the protease inhibitors (50 μl/mg of cell of dish). Samples were incubated on ice for 10 min, and immunoprecipitation dilution buffer A (0.01% SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 7.6, 167 mM NaCl) was added to a final volume of 750 μl. Samples were sonicated for 30 s 10 times with 1-min intervals using a Bioruptor (Cosmo bio). Chromatin samples (500 μl) were first precleared with normal mouse IgG (5 μl) in the presence of protein G-Sepharose bead slurry (60 μl of a 50/50 slurry of beads in TBS (16.7 mM Tris, pH 7.6, 167 mM NaCl) supplemented with 1 mg/ml bovine serum albumin and 200 μg/ml salmon sperm DNA). Samples were incubated for 2 h at 4 °C on a rotator, and beads were collected by centrifugation at 2000 rpm for 5 min at 4 °C. The supernatant (500 μl) was transferred to a new tube, and 5 μg of each antibody were added. Samples were incubated overnight at 4 °C on a rotator, 60 μl of blocked protein G slurry were added, and incubation was continued on the rotator for an additional 2 h at 4 °C. Beads were collected by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. Beads were first washed twice with 500 μl of ice-cold buffer B (0.05% (v/v) SDS, 1% (v/v) Triton X-100, 20 mM Tris, pH 7.6, 2 mM EDTA, 150 mM NaCl) and then
washed once sequentially with buffer D (0.05% (v/v) SDS, 1% (v/v) Triton X-100, 20 mM Tris, pH 7.6, 2 mM EDTA), and buffer C (0.1% (v/v) Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.6, 2 mM EDTA). Beads were transferred to a microtube, and bound material was eluted by incubating the beads with 75 µl of elution buffer (0.1 mM sodium bicarbonate, 1.0% (w/v) SDS) with vigorous shaking for 10 min. The elution was repeated four times. The four eluates were pooled, 30 µl of which was added, and samples were heated at 65 °C for 6 h to reverse the S1 nuclease linkage. DNA was collected by ethanol precipitation and resuspended in 20 µl of water. A portion of this (0.5 µl) was used for PCR amplification with ExTaq polymerase. Primers used were Hrc-R1-U (GTCTACACCAACCTTCTTTCTACCAAC) and Hrc-R1-L (GGTGTTCTGCGAGGTTGCACA) for the fragment S1, Hrc-R2-U (TAAAGAGGAGCAGCAGAGAAAGAA) and Hrc-R2-L (CAGTGTCTTCTAAGTTGGAGACCGT) for S2, Hrc-R5-U (AGACAGACAGAACAGAGACCAGC) and Hrc-R5-L (ATACATACGAGCGGTTGTTGTTTGC) for S5.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX)—pENTR-Sox15 or pDONR-Sox2 was recombined with pHi1119-gw to construct pH1119-Sox15 and pH1119-Sox2 for producing a fusion protein containing maltose-binding protein and Sox15 or Sox2. Purification of maltose-binding protein fusion proteins and SELEX were performed as previously described (12).

Construction of Sox15 Targeting Vectors—To disrupt the mouse Sox15 gene, we inserted a cassette carrying the internal ribosome entry site (IRES) followed by a segment of the β-galactosidase and neomycin resistance genes (βgeo) into the single exon of the gene, upstream of the HMG domain (34). A 1.5-kbp 5′ arm of the targeting vector was amplified by the Expand long template PCR system (Roche Applied Science) with primers sox15–5arm-s-NotI (5′-CGCGCGCGATGAGGGAAGTATTATG-3′) and sox15–5arm-as-SpeI (5′-ACTAGTCCCCCAGACGCTCCA-3′) and sox15–3arm-as-XhoI (5′-CTCGAGTTGTTGCTTAAACCTCTC-3′). The internal ribosome entry site-βgeo cassette was ligated between the two PCR fragments. A diphertheria toxin A cassette was placed downstream of the 3′ arm. The resulting targeting vector was linearized with SacII and introduced into RF8 ES cells by electroporation (35). Genomic DNAs from G418-resistant colonies were screened for homologous recombination by Southern blot analyses.

Genotyping of ES Cells and Mice—For 5′ recombination, genomic DNA was digested with EcoRI, separated on a 1% agarose gel, and transferred to nylon membrane. A 550-bp 5′ probe was amplified with sox15–5′-5′-5′-3′ and sox15–5′-5′-5′-3′ and sox15–3′-3′-3′-3′. Hybridization with this probe resulted in an 8.0-kbp band from the wild-type locus and a 4.3-kbp band from the targeted locus.

For 3′ recombination, genomic DNA was digested with MluI. An 870-bp 3′ probe was amplified with sox15–3′-3′-3′-3′ and sox15–5′-5′-5′-5′ and sox15–3′-3′-3′-3′ and sox15–3′-3′-3′-3′ and sox15–3′-3′-3′-3′. Hybridization with this probe resulted in an 8.0-kbp band from the wild-type locus and an 8.8-kbp band from the targeted locus.

After identifying ES cell clones that were correctly targeted, we determined genotypes of mice and ES cells with three-primer PCR. A 4.3-kbp band from the targeted locus.

Generation of Sox15-null and Rescued ES Cells—ES cells deficient in Sox2 were obtained by culturing cells heterozygous ES cells with a high concentration (2–6 mg/ml) of G418 (36). To obtain rescue cells, pCAG-IP-Sox15 was transfected into Sox15-deficient ES cells by electroporation. To identify clone carrying Sox15 expressing, we screened colonies resistant to 2 µg/ml puromycin by Northern blot and Western blot analyses.

DNA Microarray—Total RNA from Sox15 heterozygous ES cells and homozygous ES cells were labeled with Cy3 and Cy5 hybridized to Mouse Development Microarray (Agilent) according to the manufacturerv's protocol. The arrays were scanned with G2565BA Microarray Scanner System (Agilent). Hybridization was repeated with different clones. Data were analyzed with GeneSview (Silico Genetics).

RESULTS

To identify candidate ES cell-specific genes, we compared EST libraries derived from mouse ES cells (three libraries, 33,077 clones) and various somatic tissues (103 libraries, 1,040,493 clones) by digital differential display. Twenty Uni gene clusters were found exclusively in ES cell-derived libraries. One of them (Mm. 176369) encoded Sox15. This was unexpected, since Sox2 had been recognized as the sole Sox family protein expressed in mouse ES cells. Sox15 expression is found in testis and muscle, but its expression in ES cells had not been studied.

Sox15 expression was highest in two independent ES cell lines, RF8 and MG1.19, as assessed by RT-PCR (Fig. 1A). Its expression decreased following retinoic acid-induced differentiation. Weaker expression was also detected in stomach and skin. We confirmed the expression of Sox15 in undifferentiated ES cells at a protein level (Fig. 1B). These data show that Sox15, in addition to Sox2, is expressed in mouse ES cells.

Despite occupying different Sox family groups, Sox2 and Sox15 have a high degree of sequence homology. Thus, we first examined whether Myc-Oct3/4 associates with Sox15 as well. We next performed a gel mobility shift assay to examine
and an Oct3/4-Sox2 complex were observed, respectively. Bands corresponding to an Oct3/4 monomer, a Sox2 monomer when we incubated $^{32}$P-labeled Fgf4 enhancer fragments with CAAAG), to which Sox2 and Oct3/4 bind synergistically (23). This suggests that the weaker affinity of Sox15 for the Fgf4 and Fbx15 enhancer is, at least in part, attributable to small differences within the HMG domain compared with Sox2.

We then performed gel mobility shift assays with these COS7 cell extracts and either the Fgf4 enhancer (Fig. 4B) or the Fbx15 enhancer (Fig. 4C). With both enhancers, we found that Sox2-15-2 showed weaker affinity than Sox2, whereas Sox15-2-15 showed stronger affinity than Sox15. When Oct3/4-expressing extracts were included in the reaction, the differences between these Sox proteins became smaller. These data indicate that the weaker affinity of Sox15 for the Fgf4 and Fbx15 enhancer is, at least in part, attributable to small differences within the HMG domain compared with Sox2.

The decreased binding of Sox15 compared with Sox2 to the Fgf4 and Fbx15 enhancers suggests that the recognition sequences may differ between these proteins. To test this possibility, we performed SELEX analysis. We prepared affinity purification columns with recombinant maltose-binding protein-tagged Sox15 or maltose-binding protein-tagged Sox2. Oligonucleotides with random sequences were purified on these affinity columns, amplified by PCR, and reapplied to the affinity column. After repeating this procedure five times, the eluted DNA was cloned and sequenced. As expected, Sox2 bound to AACAAATG (Fig. 5), and Sox15 preferentially bound to nearly identical sequences. Thus, the decreased affinity of Sox15 for the examined enhancers is not attributable to an increased preference of an alternative binding site.

We have shown that Sox15 binds to DNA in a synergistic manner with Oct3/4, but we next wished to examine the functional outcomes of this interaction. We used a reporter gene in which luciferase cDNA was driven by the Fgf4 enhancer and promoter (23). This reporter gene was introduced into COS7 cells together with expression vectors encoding Oct3/4 and/or Sox proteins.

When both Sox15 and Oct3/4 were introduced, the Fgf4 enhancer was activated ~4-fold (Fig. 6A), but neither Sox15 nor Oct3/4 alone had an effect. However, luciferase activity induced by Sox15 and Oct3/4 was lower than that induced by Sox2 and Oct3/4, which showed ~7-fold enhancement. We obtained a similar result with the Fbx15 reporter construct (25), in which the luciferase cDNA is driven by the minimum thymidine kinase promoter plus five copies of the Fbx15 enhancer (Fig. 6B). These data indicate that Sox15 synergistically activates the Fgf4 and Fbx15 enhancers with Oct3/4 but to a lesser extent than Sox2.

To study the possible function(s) of Sox15 in ES cells, we inactivated Sox15 by homologous recombination. The Sox15 gene consists of two exons, and we constructed a targeting vector in which a cassette consisting of internal ribosome entry site and β-geo (a fusion of β-galactosidase and the neomycin-resistant gene) replaced the HMG domain (Fig. 7A). This vector was introduced into RF8 ES cells by electroporation. Screening of 250 G418-resistant clones identified three positive clones by
both PCR and Southern blot analyses (Fig. 7B).

One of the positive clones was injected into blastocysts of C57/BL6 mice, and germ line transmission was obtained. Sox15-null mice were born with the expected Mendelian ratio (\(+/+/+/--;-- = 30:57:25\)). They were grossly normal in appearance and fertile. This is in great contrast with the observed peri-implantation embryonic lethality of Sox2-null mice.

To better study the function of Sox15 in ES cells, we established homologous mutant ES cells by selecting heterozygous cells with a high concentration of G418. We obtained 48 colo-
nies with 2–3 mg/ml of G418. PCR and Southern blot analyses revealed that 4 of 48 clones were homozygous for the Sox15 deletion. PCR and Southern blot analyses demonstrated that Sox15 transcripts were absent in these clones (Fig. 7C). This was again in great contrast to Sox2 mutants, of which homozygous mutant ES cells could not be obtained.

Sox15-null ES cells were normal in morphology and proliferation when maintained in an undifferentiated state on STO feeder cells (Fig. 7D). They were also competent in differentiation after LIF removal, retinoic acid treatment, and teratoma formation (not shown). Northern blot analyses showed that the expression levels of Sox/Oct target genes, such as Fgf4, UTF1, and Fbx15, were indistinguishable between wild-type, Sox15-heterozygous, and Sox15-null ES cells (Fig. 7E).

We next performed DNA microarray analyses to study the effect of Sox15 deletion on gene expression. We used Agilent mouse development arrays that contain ~20,000 genes expressed in early embryos. Comparison between Sox15-heterozygous ES cells and Sox15-null cells showed that Fgf4 and Fbx15 are normally expressed in Sox15-null ES cells, consistent with the results of Northern blot analyses (not shown). In addition, ES cell-specific genes, such as Nanog and Oct3/4, are also normally expressed in Sox15-deficient cells.

However, the expression of Otx2 (37), Ctgf (38), and Ebf1 (39) were significantly decreased, whereas Hrc (40) was increased in Sox15-null ES cells. RT-PCR analyses confirmed these changes (Fig. 8A), and wild-type expression levels were restored by the introduction of Sox15 cDNA into Sox15-null ES cells.

To study whether the expression of these genes was directly regulated by Sox15, we performed chromatin immunoprecipitation (ChIP) analysis. We identified six putative Sox binding sequences (S1–S5; Fig. 8B) in the flanking regions of the mouse Hrc gene. We precipitated formalin-fixed nuclear extracts of
sequence, we performed gel mobility shift assays with a 32P-labeled probe containing the Sox15 recognition fragment and the indicated genotypes. The targeting vector contains the β-geo cassette in place of the HMG domain. The length of the diagnostic EcoRI (E) or MluI (M) restriction fragments and the locations of the 5’ or 3’ probes for Southern blot analysis are shown. B, Southern blot analysis. Specific hybridization with the 5’ probe produces an 8.0-kb band from the wild-type locus and a 4.3-kb band from the target locus. Hybridization with the 3’ probe produces an 18.0-kb band from the wild-type locus and a 3.8-kb band from the target locus. +/+, +/−, and −/−, genotypes of Sox15+/+, Sox15−/−, and Sox15−/− cells, respectively. C, Northern blot analysis. Total RNA was isolated from indicated ES cells and hybridized with either Sox15 probe or Sox2 probe. D, morphology of Sox15-null ES cells. ES cells of the indicated genotypes were cultured at a low density to produce single cell-derived colonies. E, expression of Fgf4, Fbx15, and UTF1 in Sox15-knockout ES cells. Total RNA from ES cells of the indicated genotypes was analyzed with Northern hybridization for the expression of known target genes of Oct3/4 and Sox2. IRES, internal ribosome entry site.

RF8 ES cells with anti-Sox15 antibody and performed PCR to amplify fragments S1–S5.

All five fragments were amplified from extracts before CHIP (Fig. 8C). However, only S1 and S5 were amplified from the anti-Sox15-purified extracts. When we performed CHIP with nuclear extracts from Sox15-null ES cells, S1, but not S5, was amplified. This suggests that Sox15 antibody may cross-react with other protein(s) with the binding and washing conditions we used. However, our data did show that S5 was specifically co-purified with Sox15.

When CHIP was performed with anti-Sox2 antibody, none of the five fragments were amplified (Fig. 8C). In contrast, the proximal enhancer of the mouse Nanog gene was co-purified with anti-Sox2 antibody, but not with anti-Sox15 antibody. Thus, Hrc is regulated specifically by Sox15 in vivo, whereas Nanog is regulated by Sox2, but not Sox15.

The S5 sequence does not contain the octamer motif. ChIP analysis showed that S5 was not co-purified with anti-Oct3/4 antibody (Fig. 8C). In contrast, the Nanog enhancer containing the octamer motif was co-puritated. These data indicated that Oct3/4 does not associate with Sox15 on the S5 sequence of the Hrc gene.

In order to better understand Sox15 binding to the Hrc S5 sequence, we performed gel mobility shift assays with a 32P-labeled probe containing this sequence. When this probe was incubated with ES cell nuclear extracts, a shifted band was observed (Fig. 8D). This band was identical to that observed with nuclear extracts of COS7 cells expressing Sox2. In contrast, a shifted band corresponding to Sox15 was only observed with extracts of COS7 cells expressing Sox15, but not with ES cell nuclear extracts. These data demonstrated that the S5 Sox15 binding site is preferentially bound by Sox2 in vitro, in contrast to the in vivo situation revealed by CHIP.

DISCUSSION

Sox2 was previously thought to be the sole Sox protein expressed in mouse ES and EC cells (23). However, we now report that Sox15 is also expressed in mouse ES cells. Our study demonstrated that Sox15 and Sox2 regulate different sets of genes in vitro, despite similar protein-protein interactions and DNA recognition in vitro; Sox2 regulates Fgf4 and Fbx15, whereas Sox15 regulates Hrc, Otx2, Ctgf, and Ebf.

It remains elusive how the specificity of each Sox protein is determined. Sox15 and Sox2 recognize and bind similar DNA sequences, and the binding of both is enhanced by the presence of Oct3/4 binding. This is not wholly unexpected, since the HMG domains, which bind to DNA and Oct3/4, are 78% identical between the two Sox proteins.

In contrast, the identity outside the HMG domains is less than 30%. Some Sox proteins bind to other proteins through non-HMG domains (18). For example, the non-HMG motif PLNLSSR is required for binding of Sox6 to the co-repressor CtBP2 (41). Sox15 and Sox2 may bind to different transcription regulators through non-HMG domains and therefore regulate unique sets of genes. Consistent with this notion, we found that Oct3/4 did not associate with Sox15 on the Hrc gene.

The finding that Sox15 shares the same DNA recognition sequence with Sox2 suggests that Sox15 may interfere binding of Sox2 to its target genes such as Fgf4 and Fbx15. However, we found that the expression levels of Fgf4 and Fbx15 were not increased in Sox15-deficient ES cells (Fig. 7E). Furthermore, overexpression of Sox15 in ES cells did not decrease the expression of these genes (not shown). These data showed that Sox15 does not affect DNA binding of Sox2.

Recently, Kuroda et al. (42) reported that Nanog contains an adjacent octamer motif and Sox-binding site in the 5’-flanking region. They showed that Sox2 bound to this site in EC cells and embryonic germ cells. However, an undefined factor preferentially bound to the same site in ES cells they examined. Further studies are required to determine whether Sox15 binds to the Nanog gene in those cells.

During the preparation of this manuscript, the generation of Sox15-null mice was reported (43). Consistent with our data,
these Sox15-deficient mice were normal in development, gross appearance, and fertility. However, cultured Sox15-deficient myoblasts displayed a marked delay in differentiation in vitro (43). Expression of the early myogenic regulated factors MyoD and Myf5 was altered in Sox15-deficient myoblasts. These results suggest another specific role for Sox15 that cannot be compensated by other Sox family members.

Sox15 is the sole member of Sox family group G, and it is only found in mammals (32). Other organisms, including Fugu, *Drosophila melanogaster*, and *Caenorhabditis elegans*, do not have Sox15 orthologs. These data indicate that the Sox15 gene evolved relatively recently. This might account for its relatively minor roles compared with other members of the Sox family transcription factor.

Acknowledgments—We thank Yukiko Ikekuchi, Chihiro Takigawa, Junko Iida, and Masako Shirasaka for technical and administrative assistance. We are grateful to Dr. Hitoshi Niwa and Jun-ichi Miyazaki for CAG promoter-containing plasmids and Dr. Robert Farese Jr. for RF8 ES cells.

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