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# A Gene That Is Related to *SRY* and Is Expressed in the Testes Encodes a Leucine Zipper-Containing Protein

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***SRY*-related cDNA encoding a protein with a high-mobility-group (HMG) box and a leucine zipper motif, which was designated *SOX-LZ*, was isolated from a rainbow trout testis cDNA library. Comparison of this cDNA with the mouse homologous cDNA isolated from a testis cDNA library exhibits an overall amino acid sequence identity of 77%, which is in striking contrast to the abrupt loss of amino acid sequence homology outside the HMG box found among mammalian *SRY* genes. In both rainbow trout and mice, Northern (RNA) blot analyses have revealed the presence of a testis-specific 3-kb-long *SOX-LZ* mRNA, and this transcript appeared coincidentally with the protamine mRNA, suggesting its expression in the germ line. A recombinant HMG box region protein encoded by *SOX-LZ* could bind strongly with an oligonucleotide containing an AACAAT sequence, which is also recognized by mouse Sry and Sox-5. Upon cotransfection into CHO cells, *SOX-LZ* transactivated transcription through its binding motif when the region including the leucine zipper motif was deleted [SOX-LZ (D105-356)]; however, the intact SOX-LZ failed to transactivate. The intact SOX-LZ could form homodimers through the leucine zipper, which resulted in inhibition of DNA binding by the HMG box, while SOX-LZ (D105-356), which was incapable of dimerization, showed specific binding with the AACAAT sequence. Thus, the repressed transactivation of the intact SOX-LZ in CHO cells was primarily attributable to the low level of DNA binding of SOX-LZ homodimers.**

In fish, sex determination and differentiation are attributed to both genetic and environmental factors (4, 7, 45). With regard to salmonids, male heterogamety has been demonstrated cytologically for several species (30, 39, 40), and a Y chromosome-specific DNA fragment has been isolated (11), although their sex chromosomes show little morphological differentiation, suggesting an early stage of heteromorphic evolution. Genetic evidence has also indicated that an XY system operates to control sex determination (6, 21, 24). However, in contrast to mammals, sex determination does not appear to be so strictly bound to the sex chromosomes, and functional sex inversion can be induced in both directions by treatment with exogenous sex steroids (12, 20). Thus, fish provide a particularly intriguing biological system in which to study sex determination and differentiation.

*SRY*, a candidate gene responsible for testis determination, has been isolated from the sex determining regions of human and mouse Y chromosomes (16, 36). Analyses of XY sex-reversed females (2, 18, 19, 23) and testis formation of mice transgenic for *Sry* (27) have equated SRY and the testis-determining factor. *SRY* encodes a protein with a DNA-binding motif known as the high-mobility-group (HMG) box (16, 36). Surprisingly, comparison of mammalian *SRY* sequences reveals that sequence conservation is largely confined to the HMG box, suggesting its rapid evolution (13, 16, 36, 41, 43). In addition to *SRY*, genes encoding an *SRY* type HMG box (*SOX* genes) have been identified by PCR with primers based on the conserved amino acid sequences of the HMG boxes of mammalian SRY (5, 9, 10, 15, 29, 42, 44). To investigate sex determination and differentiation in fish, we have isolated cDNA clones for a rainbow trout *SOX* gene (*SOX-LZ*) expressed in testes. From a phylogenetic interest, we have also isolated mouse homologous cDNA clones. *SOX-LZ* genes of both species show not only an overall structural conservation but also similar mRNA expression patterns.

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## MATERIALS AND METHODS

**Amplification of rainbow trout *SRY*-related HMG box sequence.** Total RNA was extracted from rainbow trout testes by the guanidium isothiocyanate method (33), and poly(A)$^+$ RNA was purified by using Oligotex-dT30 (Takara Shuzo). cDNA was synthesized by oligo(dT) priming and was used as the PCR template. PCR primers were designed on the basis of the conserved amino acid sequences of mammalian *SRY* gene (16, 36): P-1, 5′-CC(ACGT)ATGAA(CT)GC(ACGT)TT(CT)AT(ACGT)GT-3′; P-2, 5′-TA(CT)TT(AG)TA(AG)TT(ACGT)GG(AG)AA(CT)TT-3′. An approximately 200-bp-long PCR product was subcloned into pBluescript (Stratagene) and sequenced.

**Isolation of rainbow trout *SOX-LZ* cDNA clones.** To isolate cDNA clones corresponding to the testis-specific 3-kb-long *SOX-LZ* transcript, rainbow trout testis poly(A)$^+$ RNA was fractionated by sucrose gradient centrifugation (38), and its fraction containing only the testis-specific 3-kb-long *SOX-LZ* transcript as the *SOX-LZ* mRNA species was used to synthesize cDNA by oligo(dT) priming. cDNA was ligated into λZAPII DNA (Stratagene) via an *Eco*RI adaptor and packaged with Gigapack Gold II (Stratagene). With the PCR-amplified *SRY*-related cDNA fragment as a probe, the testis cDNA library was screened and positively hybridizing phages were isolated. Positive clones were rescued as pBluescript plasmid by in vivo excision. Single-stranded DNA was prepared (3), and the nucleotide sequence was determined by the dideoxy method (34) with T7 DNA polymerase.

**Screening of a mouse testis cDNA library.** Testis poly(A)$^+$ RNA was purified from 4-week-old C57BL/6 mice, and a cDNA library was constructed with unfractionated testis poly(A)$^+$ RNA by oligo(dT) priming. The DNA fragment corresponding to the leucine zipper region of the rainbow trout *SOX-LZ* cDNA (nucleotides 759 to 1082, which corresponds to amino acid residues 149 to 256) was amplified by PCR and used to screen the mouse testis cDNA library. Probe DNA was $^{32}$P labeled by the random-priming method, and hybridization was performed at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium

citrate)–5× Denhardt's solution–50 mM sodium phosphate buffer (pH 7.0)–0.1 mg of denatured calf thymus DNA per ml. The final wash was at 45°C with 2× SSC–0.1% sodium dodecyl sulfate (SDS). Positive clones were isolated and characterized as described above.

**Southern blot analysis.** High-molecular-weight chromosomal DNA from liver was prepared, and a total of 10 μg of male and female DNA was digested with *Eco*RI. After electrophoresis on a 0.7% agarose gel, DNA was blotted to a nylon filter and fixed by Stratalinker (Stratagene). The filter was hybridized with the PCR-generated leucine zipper region fragment of rainbow trout or mouse *SOX-LZ* cDNA (corresponding to amino acid residues 149 to 256), and washed at 65°C with 0.1× SSC–0.1% SDS.

**Northern (RNA) blot analysis.** Total RNA was prepared by the guanidium isothiocyanate method, and poly(A)$^+$ RNA was isolated by Oligotex-dT30. Rainbow trout testis cytoplasmic RNA was prepared as described elsewhere (33). RNA was fractionated on a 1.0% agarose–formaldehyde gel, transferred to a nylon filter, and fixed by Stratalinker. The filters were hybridized in 50% formamide–50 mM sodium phosphate buffer (pH 7.0)–1× Denhardt's solution–0.1% SDS–0.1 mg of denatured calf thymus DNA per ml at 42°C for 16 h and washed at 65°C with 0.1× SSC–0.1% SDS, with the exception of the rainbow trout RNA probed with the mouse β-actin cDNA, for which hybridization was done at room temperature, and the final wash was at 60°C with 2× SSC–0.1% SDS. The rainbow trout protamine cDNA probe (from nucleotides −4 to +174 [32]) was generated by PCR with the rainbow trout testis cDNA as the template. The mouse protamine 1 cDNA probe (from nucleotides −4 to +292 [26]) and the mouse β-actin cDNA probe (from nucleotides +48 to +778 [1]) were generated by PCR with the mouse testis cDNA as the template.

**Production of recombinant proteins in *Escherichia coli*.** pRTSOX-LZ1 was digested with *Sma*I at nucleotide 1956 or *Bal*I at nucleotide 1873 in the rainbow trout *SOX-LZ* cDNA, and then ligated with *Nde*I linker and digested with *Nde*I and *Bam*HI downstream of the cDNA insert. The DNA fragments encompassing the HMG box were inserted between the *Nde*I and *Bam*HI sites of pET15b (Novagen), and the resulting plasmids were named pET15b/HMG-B for the *Bal*I digest and pET15b/HMG-S for the *Sma*I digest. The recombinant proteins were produced in *E. coli* BL21 (DE3) pLysS and were purified from the soluble fraction by using His-Bind resin (Novagen) as previously described (46). Finally, the purified protein was dialyzed against 1× gel retardation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol) and stored at −80°C.

**Gel retardation assay.** Annealed oligonucleotides were labeled with the Klenow fragment of DNA polymerase I with [α-$^{32}$P]dCTP. Approximately 10 ng of purified recombinant protein or 6 μl of programmed reticulocyte lysate was preincubated with 1 μg of poly(dI-dC) in 1× gel retardation buffer for 5 min at room temperature and then 1 ng of $^{32}$P-labeled, annealed oligonucleotides was added, and the mixture was incubated for a further 30 min at room temperature. The samples were electrophoresed through a nondenaturing 4% polyacrylamide gel in 0.25× Tris-borate-EDTA at room temperature. The oligonucleotides used were as follows: HuSRY, 5′-gggGTTAACGTAACAAAGAATCTGGTAGA-3′ annealed to 5′-TCTACCAGATTCTTTGTTACGTTAAC-3′; HuAllmut, 5′-gggGTTAACGTCCGCGGTAATCTGGTAGA-3′ annealed to 5′-TCTACCA GATTACCGCGGACGTTAAC-3′ (17); HMG Mut-11, 5′-GTAGGGCACC CATTGTTCTCT-3′ annealed to 5′-gggAGAGAACAATGGGTGCCCTAC-3′ (14). Lowercase characters indicate residues added to allow fill-in labeling by the Klenow fragment.

**CAT assay.** A total of 6 × 10$^5$ CHO-K1 cells were transfected with 2.5 μg of a chloramphenicol acetyltransferase (CAT) reporter plasmid, 5 μg of an effector plasmid, and 2.5 μg of pSVβ-gal (Promega) by the standard calcium phosphate method (33). Cells were harvested 48 h later and freeze-thawed three times in 200 μl of 0.25 M Tris-HCl (pH 7.5). After removal of cellular debris by 5 min of microcentrifuge centrifugation, an aliquot of the cell lysate was used to assess β-galactosidase activity for normalization of transfection efficiency, and then the rest was treated at 60°C for 10 min and used for CAT assays (33). Since the SOX-LZ derivatives carrying the transactivation domain of herpes simplex virus VP16 exhibited much lower β-galactosidase activities, normalization was carried out separately with the SOX-LZ derivatives with and without the VP16 sequence. CAT assays were done four times, and they gave reproducible results.

**Effector plasmids.** pCMV/SOX-LZ was constructed by inserting the whole cDNA fragment of pRTSOX-LZ1 into the *Hin*dIII site of pRc/CMV (Invitrogen). The DNA fragment containing the transcriptional activation domain of herpes simplex virus VP16 (nucleotides 1691 to 2034, which includes the coding region for the C-terminal 77 amino acid residues and 108 nucleotides of the 3′ noncoding region [8]), was amplified from pGAL4VP16 (31) (a kind gift of M. Ptashne) by PCR (primers used, 5′-AACCCCCCCCGACCGATGTCAGC-3′ and 5′-TGATCGATCtctagaCGACAACCGCAGGTTTT-3′, in which the *Xba*I site is indicated by lowercase characters) and then digested with *Xba*I and phosphorylated. On the other hand, pCMV/SOX-LZ was digested with *Hpa*I at nucleotide 2489 in the rainbow trout *SOX-LZ* cDNA and *Xba*I in the downstream of the cDNA insert, and the larger fragment was ligated with the PCR-generated VP16 fragment to construct pCMV/SOX-VP16. Deletion constructs are designated by the amino acid numbers of both deletion borders. pCMV/SOX-LZ (D105-356) and pCMV/SOX-VP16 (D105-356) were made by religation of the end-filled *Apa*I digests of pCMV/SOX-LZ and pCMV/SOX-VP16, respectively. pCMV/SOX-LZ (D184-207), which has a specific deletion of the leucine zipper motif (amino acid residues 184 to 205), and pCMV/SOX-LZ (D104-209) were constructed by assembling PCR fragments, and pCMV/SOX-LZ (D184-207) has an additional Arg-Gly sequence instead.

**Reporter plasmids.** pB-CAT and pC-CAT, corresponding to pSV00CAT and pSV2CAT, respectively, were constructed by cloning the 1.6-kb *Hin*dIII-*Bam*HI fragment and the 0.8-kb *Bam*HI-*Eco*RI fragment, in which *Eco*RI site was converted to a *Sal*I site by the usage of a *Sal*I linker, of pSV00CAT into the *Bam*HI-*Sal*I backbone of pGV-B or pGV-C (Toyo Ink), respectively. p4×SRY/B-CAT was constructed as follows. An artificial promoter consisting of four copies of an AACAAT sequence in addition to the TATA box of the adenovirus major late promoter (nucleotides from −51 to +10) was synthesized by PCR with pML($C_2$AT) (35) as the template with oligonucleotides 5′-AGACTGGAT TCagatctGGCCCAACAATATTGTCCAACAATAGATTCCAACAAT ATTGTCCAACAATAGGGTGTTCCTGAAGGGGGGCTATAAAA-3′ and 5′-CTCTaagcttGGAAGAGAGTGAGGACGAACGCGC-3′, in which the *Bgl*II and *Hin*dIII sites are indicated by lowercase characters. The PCR product was digested with *Bgl*II and *Hin*dIII, and then the digests were inserted between the *Bgl*II and *Hin*dIII sites of pB-CAT to construct p4×SRY/B-CAT.

**Immunoprecipitation assays.** To produce SOX-LZ with the influenza virus hemagglutinin (HA) epitope (YPYDVPDYA) recognized by anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) at the carboxy terminus (*SOX-HA*), pCMV/SOX-HA was constructed from pCMV/SOX-LZ by replacing the sequence between the translation termination codon and the *Xba*I site, which is downstream of the cDNA insert, with the annealed oligonucleotides 5′-TTATA TCCATATGATGTTCCAGATTATGCTTAAT-3′ and 5′-CTAGATTAAGCA TAATCTGGAACATCATATGGATATAA-3′. $^{35}$S-labeled proteins were produced in a reticulocyte lysate-coupled transcription-translation system (Promega) from pCMV/SOX-HA plus pCMV/SOX-LZ or its derivative plasmids. A 10-μl volume of programmed lysates was diluted with 400 μl of buffer A (20 mM HEPES [pH 7.9], 60 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mg of bovine serum albumin per ml), and 2 μg of anti-HA antibody was added. The mixture was incubated at 4°C for 60 min. Then, 40 μl of protein A-agarose (Pierce) was added. After incubation at 4°C for 60 min, the immune complexes were precipitated by centrifugation and washed three times with buffer A. Associated proteins were eluted by boiling in Laemmli buffer and were separated on an SDS–6% polyacrylamide gel.

## RESULTS

**Isolation of *SRY*-related sequences expressed in rainbow trout testes.** To isolate rainbow trout *SRY*-related cDNA to investigate sex determination and differentiation in fish, we conducted a PCR using rainbow trout testis cDNA as the template and primers based on the conserved amino acid sequences of the HMG boxes of mammalian *SRY* genes (16, 36). An approximately 200-bp-long fragment was produced, which was subcloned and sequenced, revealing an HMG box with 55% amino acid sequence homology to that of human *SRY*. This fragment could detect a 3-kb-long RNA and a smaller amount of a 10-kb-long RNA in testes and only a 10-kb-long RNA in ovaries (see below). To isolate cDNA clones corresponding to the testis-specific 3-kb-long RNA, testis poly(A)$^+$ RNA was fractionated by sucrose gradient centrifugation, and the fraction containing only the 3-kb RNA species was used to construct a cDNA library. Using the PCR-generated fragment as a probe, we isolated cDNA clones that contain an approximately 3-kb-long cDNA insert. This cDNA clone (pRTSOX-LZ1) contains a 2,304-bp-long open reading frame (nucleotides 315 to 2618) that encodes a polypeptide of 767 amino acid residues, with the following structural features suggestive of transcriptional regulators: (i) a leucine heptad repeat (leucine zipper) (residues 184 to 205); (ii) a 39-amino-acid glutamine-rich stretch (50%) (residues 223 to 261); and (iii) an HMG box (residues 555 to 633) (Fig. 1), and we termed this protein SOX-LZ.

**Comparison of rainbow trout *SOX-LZ* with its mouse homolog.** Mammalian *SRY* genes show almost no significant homology outside the HMG box (13, 16, 36, 41, 43). To examine whether *SOX-LZ* also underwent rapid divergence during evolution, we carried out screening of a mouse testis cDNA library using the DNA fragment corresponding to the leucine zipper region of the rainbow trout *SOX-LZ* cDNA (nucleotides 759 to 1082) under a low-stringency condition. The mouse cDNA

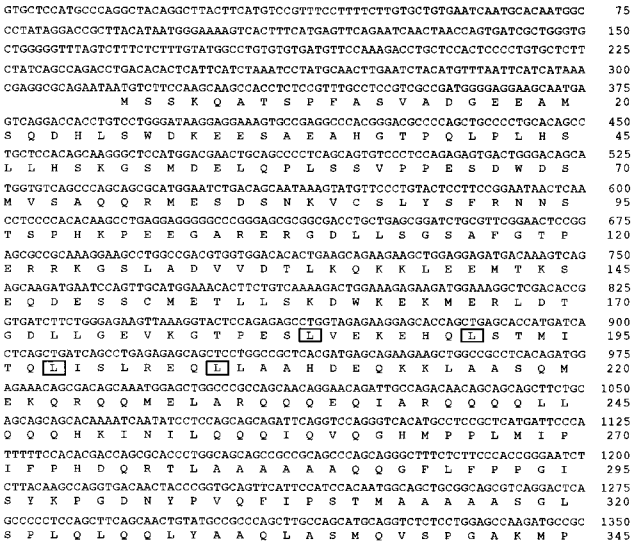

FIG. 1. The nucleotide and deduced amino acid sequence of rainbow trout *SOX-LZ* cDNA (pRTSOX-LZ1). The HMG box is shaded. The individual leucines of the leucine zipper are boxed. The putative polyadenylation signal (AATAAA) is underlined.

clones that were isolated (pMSOX-LZ1 and pMSOX-LZ2) encode a polypeptide of 786 amino acid residues and also contain a leucine zipper, a glutamine-rich region, and an HMG box in an arrangement similar to the arrangement of those of rainbow trout SOX-LZ. The predicted amino acid sequences of the rainbow trout and mouse SOX-LZs are well conserved and show a significant overall homology (77% identity and 85% similarity) (Fig. 2). SOX-LZ also shows some similarities to other members of the SOX family. In the HMG box, mouse SOX-LZ differs from mouse Sox-6 (9) by only one nucleotide, which results in a conservative amino acid change: Lys at 591 in mouse SOX-LZ and Arg in mouse Sox-6. The Lys residue is conserved between mouse and rainbow trout SOX-LZs, and the Arg residue is also conserved between mouse Sox-6 and human SOX-6 (9). In addition, the C-terminal 400 amino acid residues of mouse SOX-LZ show an amino acid sequence identity of about 55% with the 392-amino-acid-long mouse Sox-5 (10), and the homology extends even outside the HMG box (data not shown).

**The *SOX-LZ* gene is not Y linked.** The original *SRY* genes identified are known to be Y linked (16, 36), whereas the subsequently isolated *SRY*-related genes are autosomal (16). To determine whether the *SOX-LZ* gene is Y linked, genomic Southern blot analyses were performed using the leucine zipper region fragments as a probe (Fig. 3). Southern blots of male and female DNA revealed that the *SOX-LZ* gene is not Y linked in either rainbow trout or mice.

**Developmental regulation of testis-specific *SOX-LZ* mRNA expression.** To examine the expression pattern of *SOX-LZ* in rainbow trout, poly(A)$^+$ RNA from several tissues was analyzed for the presence of the *SOX-LZ* mRNA with the leucine zipper region fragment as a probe (Fig. 4A). Northern blot analysis revealed a testis-specific 3-kb-long *SOX-LZ* transcript. In addition, a smaller amount of an approximately 10-kb-long *SOX-LZ* transcript was observed in all tissues examined with the longer exposure (data not shown). The 10-kb transcript would be generated by alternate promoter usage or alternate RNA processing. To determine when the testis-specific 3-kb *SOX-LZ* mRNA appeared during testis development, testis cytoplasmic poly(A)$^+$ RNA was prepared from fish at different maturation stages, as judged by the amounts of the protamine mRNA (Fig. 4B). In trout, synthesis of the protamine mRNA is known to occur during the primary spermatocyte stage (22). The testis-specific 3-kb *SOX-LZ* mRNA increased in quantity as the protamine mRNA.

To compare the mouse *SOX-LZ* mRNA expression profile with that of rainbow trout, total RNA was prepared from various tissues of 4-week-old mice and analyzed by using the leucine zipper region fragment of the mouse *SOX-LZ* cDNA as a probe (Fig. 5A). Testis-specific expression of a 3-kb-long *SOX-LZ* mRNA was also observed for mice. The developmental expression pattern of the mouse *SOX-LZ* was further examined by using animals of different ages (Fig. 5B). In 3-week-old mice, a faint band of a 10-kb transcript was detectable, and the testis-specific 3-kb transcript could not be detected. From 4 weeks on, the pattern changed dramatically, and an intense 3-kb-long *SOX-LZ* transcript appeared as the protamine mRNA. In mice, the protamine mRNA is synthesized during the spermatid stage (25).

**Sequence-specific DNA binding of the SOX-LZ HMG box.** To test whether SOX-LZ binds DNA in a sequence-specific manner similar to that of SRY, the C-terminal portions of SOX-LZ including the HMG box region were produced in *E. coli* with the expression vector pET15b (Novagen) (37). On the other hand, three synthetic duplex DNA fragments were prepared as follows. HuSRY contains an AACAAAG sequence, the binding site for human SRY, and this sequence was replaced with CCGCGGT in HuAllmut (17); Mut-11 shows a high affinity for mouse Sry and contains an AACAAT sequence (14), which can also be recognized by mouse Sox-5 (10). The binding of the recombinant SOX-LZ HMG box peptides with these double-stranded oligonucleotides was tested by gel retardation assays (Fig. 6). As expected, these proteins formed complexes with HuSRY and Mut-11, whereas they did not bind HuAllmut. When incubated with a recombinant SOX-LZ HMG box protein produced as a fusion with glutathione *S*-transferase, most of the chromosomal DNA fragments recovered as DNA-protein complexes contained at least one AACAAT sequence (data not shown).

**Transactivation activity of SOX-LZ through the AACAAT binding motif.** To test the ability of SOX-LZ to transactivate transcription through the AACAAT binding motif, a full-length rainbow trout *SOX-LZ* cDNA was inserted into the


FIG. 2. Comparison of the amino acid sequences of rainbow trout and mouse SOX-LZs. Identical amino acids are shown in white against black, and conservative amino acids are shaded. Gaps introduced in the sequences to optimize the alignment are represented by dashes. The individual leucines of the leucine zipper are indicated by triangles. Conservative amino acids are classified as follows: D and E; H, K and R; S and T; I, L, M, and V; and F and Y.

eukaryotic expression vector pRc/CMV. The resulting plasmid, pCMV/SOX-LZ, was cotransfected into CHO cells with p4×SRY/B-CAT, a reporter plasmid carrying a synthetic promoter containing four copies of the AACAAT sequence upstream of the TATA box of the adenovirus major late promoter. As shown in Fig. 7, almost no effect was observed with pCMV/SOX-LZ. Since leucine zippers are often involved in homodimer or heterodimer formation, to eliminate the effects of any possible interaction through the leucine zipper, we constructed pCMV/SOX-LZ (D105-356), which lacks the sequence from nucleotides 626 to 1381 (amino acid residues 105 to 356) in the *SOX-LZ* cDNA. This deletion resulted in a significantly high level of CAT activity. A similar correlation was observed with pCMV/SOX-VP16 and pCMV/SOX-VP16 (D105-356): the former should direct production of a SOX-LZ–VP16 fusion protein which carries the transactivation domain of herpes simplex virus VP16 instead of the C-terminal 40 amino acids of SOX-LZ, and the latter is its deletion version as pCMV/SOX-LZ (D105-356). Compared with pCMV/SOX-VP16, pCMV/SOX-VP16 (D105-356) showed a much higher level of CAT activity (Fig. 7).

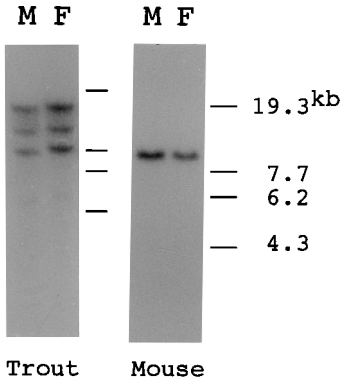


FIG. 3. Southern blot analyses of rainbow trout and mouse DNA. A total of 10 μg of male (M) and female (F) chromosomal DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel, and blotted to a nylon filter. The filter was hybridized with the PCR-generated leucine zipper region fragment of rainbow trout or mouse *SOX-LZ* cDNA (corresponding to amino acid residues 149 to 256) and washed at 65°C with 0.1× SSC–0.1% SDS.

**Dimer formation by SOX-LZ and its effect on DNA-binding activity.** Transfection experiments suggest the potency of SOX-LZ as a transcriptional regulator and the presence of a negative regulatory domain in the deleted region of SOX-LZ (D105-356). Since this deleted region contains a leucine zipper motif, we examined the ability of SOX-LZ to form homodimers. To this end, we constructed pCMV/SOX-HA, which should direct production of carboxy-terminally HA epitope-tagged SOX-LZ (SOX-HA). We also constructed

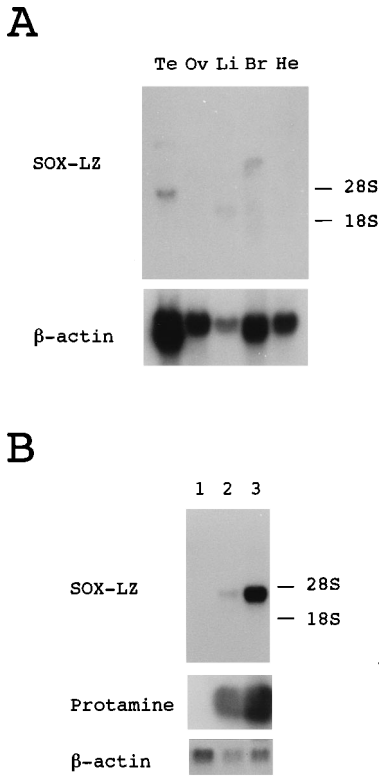


FIG. 4. *SOX-LZ* mRNA expression in rainbow trout. (A) Tissue-specific expression of *SOX-LZ* mRNA. RNA blots were prepared with 8 μg of testis (Te), ovary (Ov), liver (Li), brain (Br), and heart (He) poly(A)$^+$ RNA and probed with the leucine zipper region fragment of rainbow trout *SOX-LZ* cDNA (nucleotides 759 to 1082). Then, the filter was reprobed with the mouse β-actin cDNA. The positions of 18S and 28S rRNAs are indicated on the right. (B) Developmental regulation of *SOX-LZ* mRNA expression in testes. RNA blots were prepared with 5 μg of testis cytoplasmic poly(A)$^+$ RNA of 15 (lane 1)-, 18 (lane 2)-, and 21 (lane 3)-month-old fish and probed with the rainbow trout *SOX-LZ* cDNA (nucleotides 759 to 1082). The filter was reprobed with rainbow trout protamine cDNA and mouse β-actin cDNA.

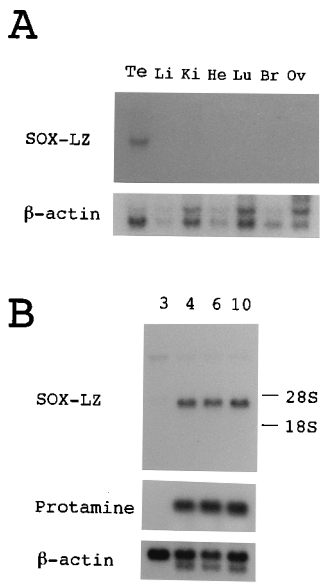


FIG. 5. *SOX-LZ* mRNA expression in mice. (A) Tissue-specific expression of *SOX-LZ* mRNA. RNA blots were prepared with 7.5 μg of testis (Te), liver (Li), kidney (Ki), heart (He), lung (Lu), brain (Br), and ovary (Ov) total RNA of 4-week-old mice and were probed with the leucine zipper region fragment of the mouse *SOX-LZ* cDNA (corresponding to amino acid residues 149 to 256). The filter was reprobed with the mouse β-actin cDNA. (B) Developmentally regulated expression of *SOX-LZ* mRNA in testes. RNA blots were prepared with 5 μg of testis poly(A)$^+$ RNA from 3-, 4-, 6-, and 10-week-old mice. The filter was probed with the leucine zipper region fragment of mouse *SOX-LZ* cDNA and was then reprobed with the mouse protamine and β-actin cDNA.

pCMV/SOX-LZ (D184-207), which carries the specific deletion of the leucine zipper motif (amino acid residues 184 to 205). SOX-LZ, SOX-LZ (D105-356), and SOX-LZ (D184-207) were produced together with SOX-HA by using an in vitro transcription-translation system, and their complex formation with SOX-HA was examined by immunoprecipitation with anti-HA antibody (Fig. 8). As expected, only SOX-LZ was coprecipitated with SOX-HA, indicating that SOX-LZ dimerizes through the leucine zipper.

Indeed, the recombinant HMG box region proteins of SOX-LZ bound the AACAAT sequence; however, it was still possible that the homodimerization of SOX-LZ affected the activity of DNA binding by the HMG box. We then tested the DNA-binding activities of SOX-LZ, SOX-LZ (D105-356), and SOX-LZ (D184-207) using in vitro-translated proteins (Fig. 9). In vitro transcription-translation reaction mixtures were set up in two aliquots; in one aliquot, [$^{35}$S]methionine was included to monitor the synthesis of the full-size proteins (Fig. 9A), and the other unlabeled aliquot was used for gel retardation assays (Fig. 9B). Incubation of the programmed lysates of pRc/CMV with the $^{32}$P-labeled Mut-11 double-stranded oligonucleotides resulted in retardation of the probe (Fig. 9B). This binding appeared to be due to an endogenous DNA-binding factor, which also recognizes the Mut-11 sequence. When the translation lysates were programmed with pCMV/SOX-LZ, no additional binding to the probe was detected. On the other hand, retardation of the Mut-11 probe was observed with the two SOX-LZ mutants with the leucine zipper deleted, SOX-LZ (D105-356) and SOX-LZ (D184-207), and the complexes were inhibited with unlabeled Mut-11 oligonucleotides, demonstrating its sequence-specific DNA binding. Thus, it is concluded that the dimerization of SOX-LZ through the leucine zipper inhibits DNA binding by the HMG box, leading to failure of the intact SOX-LZ to transactivate in CHO cells. However, in contrast to SOX-LZ (D105-356), SOX-LZ (D184-207) could hardly transactivate transcription through the binding of DNA by the HMG box, and SOX-LZ (D104-209) showed a medium

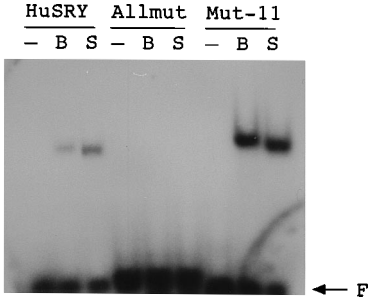


FIG. 6. Sequence-specific DNA binding of rainbow trout SOX-LZ HMG box domain peptides. Gel retardation analysis was performed using $^{32}$P-labeled oligonucleotides with purified SOX-LZ HMG box domain peptides (HMG-B [B] and HMG-S [S]) or no protein (−) as described in Materials and Methods. The position of the free probe (F) is indicated. The sequences of the oligonucleotide probes are as follows: HuSRY, 5′-gggGTTAACGTAACAAAGAATCTGGTAGA-3′; HuAllmut, 5′-gggGTTAACGTCCGCGGTAATCTGGTAGA-3′; and HMG Mut-11, 5′-gggAGAGAACAATGGGTGCCCTAC-3′ (lowercase letters indicate residues added to allow fill-in labeling by the Klenow fragment).

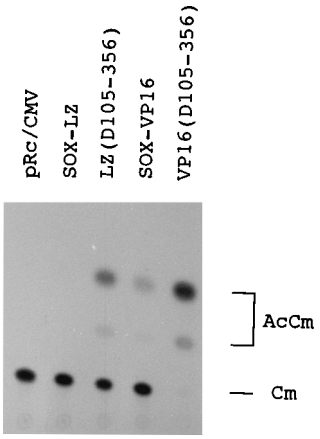


FIG. 7. Transactivation activities of rainbow trout SOX-LZ and its derivatives through its binding motif in a transient CAT assay. p4×SRY/B-CAT, a reporter plasmid carrying four copies of the AACAAT binding sequence, was transfected into CHO-K1 cells together with pCMV/SOX-LZ or its derivative effector plasmids, pCMV/SOX-LZ (D105-356), pCMV/SOX-VP16, or pCMV/SOX-VP16 (D105-356), or with pRc/CMV. A typical CAT assay by thin-layer chromatography separating the acetylated forms of chloramphenicol (AcCm) and chloramphenicol (Cm) is shown.

level of transactivation (Fig. 10). Taking into account the fact that the specific deletion of the leucine zipper motif restores the DNA-binding activity of the HMG box, these results suggest that in addition to the leucine zipper, negative regulatory domains that function in CHO cells are contained in the deleted region of SOX-LZ (D105-356).

## DISCUSSION

In this paper, we describe the cDNA cloning of a novel *SRY*-related mRNA (*SOX-LZ* mRNA) encoding a protein

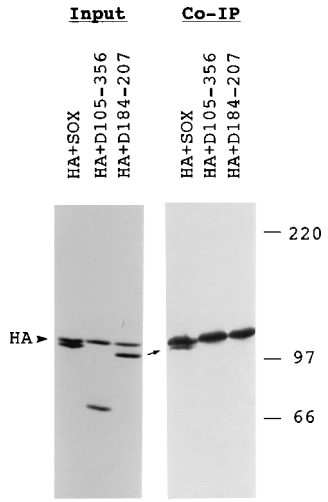


FIG. 8. Dimer formation of SOX-LZ. (Left gel) [$^{35}$S]methionine-labeled proteins were synthesized in a reticulocyte lysate-coupled transcription-translation system by cotranslation from pCMV/SOX-HA plus pCMV/SOX-LZ (HA+SOX), pCMV/SOX-LZ (D105-356) (HA+D105-356), or pCMV/SOX-LZ (D184-207) (HA+D184-207) and separated on an SDS–6% polyacrylamide gel. (Right gel) SDS-PAGE analysis of proteins recovered from programmed lysates by immunoprecipitation (Co-IP) with anti-HA antibody. The position of SOX-HA (HA) is indicated on the left. The arrow indicates SOX-LZ recovered by immunoprecipitation. Molecular mass markers (in kilodaltons) are indicated on the right.

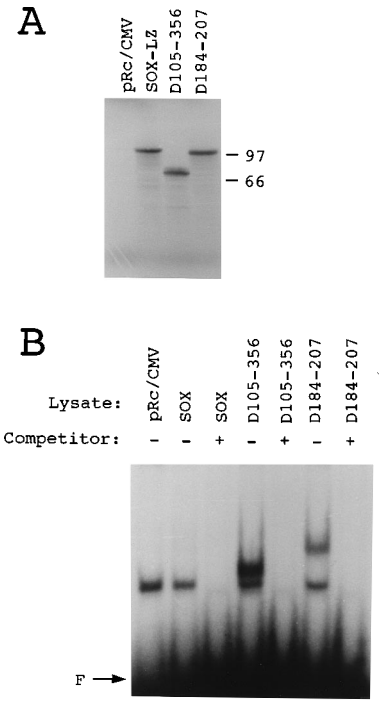


FIG. 9. DNA-binding activity of SOX-LZ. In vitro transcription-translation was carried out with pRc/CMV, pCMV/SOX-LZ, pCMV/SOX-LZ (D105-356), or pCMV/SOX-LZ (D184-207) in two aliquots: one aliquot was labeled with [$^{35}$S]methionine and subjected to an SDS–10% polyacrylamide gel to confirm the synthesis of the full-size proteins (A), and the other unlabeled aliquot was used for gel retardation analysis with a $^{32}$P-labeled Mut-11 oligonucleotide probe (B). As a control, competition experiments with a 1,000-fold excess of unlabeled Mut-11 probe are shown. The position of the free probe (F) is indicated.

with an SRY type HMG box and a leucine zipper motif. Comparison of rainbow trout and mouse *SOX-LZ* genes reveals an overall structural conservation. Moreover, *SOX-LZ* mRNA expression in testes is regulated in a developmental manner in rainbow trout that is similar to that of regulation in mice. These results indicate the conserved role of SOX-LZ during evolution and its importance in spermatogenesis.

The proteins encoded by mammalian *SRY* genes show very little homology outside the HMG box domain, and the high rate of nonsynonymous nucleotide exchanges in *SRY* genes leads to a conclusion that the portions outside the HMG box have no functional significance or have undergone species-specific divergent selection (41, 43). On the contrary, SOX proteins SOX-2, SOX-3, and SOX-4 are well conserved between humans and mice (42, 43). Moreover, SOX-LZ proteins exhibit an overall amino acid sequence identity of 77%, even between rainbow trout and mice, suggesting that portions other than the HMG box are also functionally important. Functional involvement of non-HMG box regions is also supported by the observation that the deletion of the leucine zipper region affected the transactivation activity of SOX-LZ.

Since the deletion construct of SOX-LZ could activate transcription from a minimal promoter linked to concatemerized SOX-LZ binding sites, SOX-LZ is considered a typical transcriptional activator. The repressed transactivation activity of the intact SOX-LZ in CHO cells is likely to result from the low level of DNA-binding activity imposed by homodimerization. Nerlov and Ziff have proposed the following function for a leucine zipper in cell-type-specific gene expression: the leucine zipper of the CCAAT/enhancer-binding protein α, which is

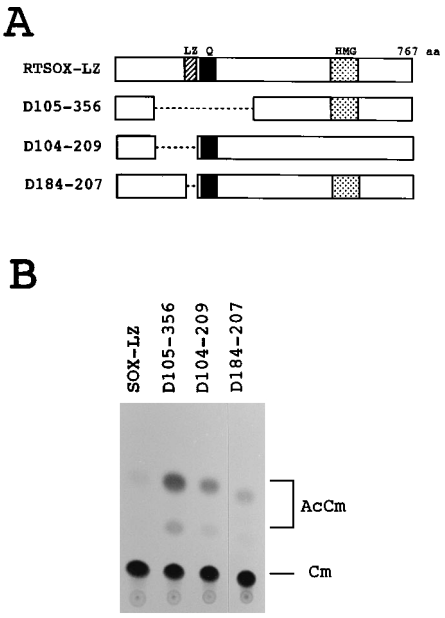


FIG. 10. Transactivation activities of deletion mutants of rainbow trout SOX-LZ. (A) Schematic representation of SOX-LZ mutants from which the leucine zipper region has been deleted. LZ, leucine zipper; Q, glutamine-rich region; HMG, HMG box. aa, amino acids. (B) Transient CAT activity assays showing the effect of deleting the leucine zipper motif. CHO-K1 cells were transfected with p4×SRY/B-CAT and pCMV/SOX-LZ or its deletion derivatives, pCMV/SOX-LZ (D105-356), pCMV/SOX-LZ (D104-209), or pCMV/SOX-LZ (D184-207), and CAT assays were carried out as described in the legend to Fig. 7. AcCm, acetylated chloramphenicol; Cm, chloramphenicol.

abundant in liver and adipose tissues, exerts a strong negative effect on albumin promoter activation in the nonhepatic HeLa cell line but not in HepG2 hepatoma cells (28). In this context and considering that the 3-kb-long *SOX-LZ* mRNA was detected only in testes, the leucine zipper of SOX-LZ is likely to have a somewhat cell-type-specific role that contributes to the repressed transactivation of the intact SOX-LZ in CHO cells. In addition, the fact that the transactivation activities of SOX-LZ (D184-207) and SOX-LZ (D104-209) are lower than that of SOX-LZ (D105-356) implies that the deleted region of SOX-LZ (D105-356) is involved in further negative interactions. In the glutamine-rich region, isoleucine (including one leucine) repeats every seven residues (amino acid residues 237 to 258), which could be an interaction site. Isolation of the interacting partner protein(s) of SOX-LZ may give a clue to understanding of the role of SOX-LZ in spermatogenesis.

In both rainbow trout and mice, the testis-specific 3-kb-long *SOX-LZ* mRNA was shown to appear coincidentally with the protamine mRNA, and in mice, it was detected from 4 weeks on, suggesting its expression in spermatids. Mouse *Sox*-5 expression is restricted to postmeiotic germ cells, mainly round spermatids (10), and mouse *Sry* transcript is also detected in adult testes (16). On the other hand, whereas SOX-LZ has potency as a transcriptional activator, SRY is not likely to be capable of transactivation. Like LEF-1, SRY induces DNA bending by sequence-specific DNA binding and is assumed to participate in transcriptional regulation by serving as an architectural element to accommodate formation of higher-order nucleoprotein complexes (14). This may also be the case with Sox-5 (10). Thus, although these proteins have similar DNA-binding affinities and their expression may show overlapping cell type specificities, it is most likely that they regulate distinct genes. To understand the role of SOX-LZ in spermatogenesis, its target gene(s) must be identified.

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