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Similar Gene Structure of Two *Sox9a* Genes and Their Expression Patterns During Gonadal Differentiation in a Teleost Fish, Rice Field Eel (*Monopterus albus*)

RONGJIA ZHOU,[1]* LI LIU,[1] YIQING GUO,[1] HONGSHI YU,[1] HANHUA CHENG,[1] XIAO HUANG,[1] TERRENCE R. TIERSCH,[2] AND PHILIPPE BERTA[3]

[1]*Department of Genetics and Center for Developmental Biology, College of Life Sciences, Wuhan University, Wuhan, People's Republic of China*
[2]*Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana*
[3]*Human Molecular Genetics Group, Institut de Génétique Humaine, Montpellier Cedex, France*

ABSTRACT The *Sox9* gene encodes a transcription factor that is critical for testis determination and chondrogenesis in vertebrates. Mutations in human *SOX9* cause campomelic dysplasia, a dominant skeletal dysmorphology syndrome often associated with male to female sex reversal. Here we show that the *Sox9a* gene was duplicated during evolution of the rice field eel, *Monopterus albus*, a freshwater fish which undergoes natural sex reversal from female to male during its life, and has a haploid genome size (0.6–0.8 pg) that is among the smallest of the vertebrates. The duplicated copies of the gene (named *Sox9a1* and *Sox9a2*) fit within the *Sox9* clade of vertebrates, especially in the *Sox9a* subfamily, not in the *Sox9b* subfamily. They have similar structures as revealed by both genomic and cDNA analysis. Furthermore, both *Sox9a1* and *Sox9a2* are expressed in testis, ovary, and ovotestis; and specifically in the outer layer (mainly gonocytes) of gonadal epithelium with bipotential capacity to form testis or ovary, suggesting that they have similar roles in gonadal differentiation during sex reversal in this species. The closely related gene structure and expression patterns of the two *sox9a* genes in the rice field eel also suggest that they arose in recent gene duplication events during evolution of this fish lineage. *Mol. Reprod. Dev. 66: 211–217, 2003.* ● 2003 Wiley-Liss, Inc.

Key Words: sex reversal; *Sox9a*; gene duplication; gene dosage effect; rice field eel

INTRODUCTION

Campomelic dysplasia (CD) is a severe human dwarfism syndrome characterized by malformations of cartilage-derived structures (Maroteaux et al., 1971; Houston et al., 1983). Most campomelic patients (around 75%) show male-to-female XY sex reversal. Positional cloning and subsequent mutational analysis have shown that *SOX9* haploinsufficiency forms a molecular basis for this disorder (Foster et al., 1994; Wagner et al., 1994). This gene encodes an autosomal transcription factor that contains a DNA binding domain of the high mobility group (HMG) family that recognizes both chromatin structure and a specific binding sequence. Through this HMG box it appears related to the mammalian testis-determining factor *SRY*. It also contains a transcription activation domain located at the carboxyl terminus of the protein. Evidences have been provided that during chondrogenesis the chondrocyte-specific *Col2al*, the gene encoding type II collagen, and *aggrecan*, a gene encoding a sulfate proteoglycan are direct targets for SOX9, and SOX9 is also implicated in the transcription regulation of the gene for anti-Müllerian hormone during the sex determination process (Bell et al., 1997; de Santa Barbara et al., 1998; de Crombrugghe et al., 2000). The *KIAA0800*, a novel *Sox9*-activated gene, is evolutionarily conserved and potentially involved in sexual differentiation (Zhao et al., 2002a). Our recent results show that human SOX9 interacts with TRAP230, which adds a new transcription factor to the short list of

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*Correspondence to: Prof. Rongjia Zhou, Department of Genetics and Center for Developmental Biology, College of Life Sciences, Wuhan University, Wuhan 430072, P.R. China.
E-mail: rizhou@public.wh.hb.cn

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activators described so far targeting the TRAP complex to communicate to the general transcription machinery (Zhou et al., 2002b). As expected during embryonic life, *SOX9* is expressed predominantly at sites of chondrogenesis and in genital ridges where it appears to be upregulated in males precisely at a time when sex determination is engaged (Kent et al., 1996). Moreover, *SOX9* appears to be expressed in heart, kidney, nervous system, and otic vesicles of mouse embryos, and also in scrotum and mammary gland primordia and proliferating Wolffian duct epithelium of marsupial (Pask et al., 2002), suggesting that the Sox9 has additional roles and Sox9 pathways remain to be established in each case.

Efforts have been made to explore the functions of *SOX9* through comparative study of different species. The ortholog of *SOX9* from lizard (Choudhary et al., 2000), frog (Takase et al., 2000), turtle (Spotila et al., 1998), alligator (Western et al., 1999), zebrafish (Chiang et al., 2001), rainbow trout (Takamatsu et al., 1997), medaka (Yokoi et al., 2002), chicken (Kent et al., 1996; Morais da Silva et al., 1996), pig (accession number AF029696), marsupial (Pask et al., 2002), mouse (Wright et al., 1995; Kanai and Koopman, 1999), human (Foster et al., 1994), and six other primate species (Patel et al., 2001) have been identified, demonstrating conserved roles for this gene. Expression pattern analysis as reported in each case shows that common expression sites are gonads of embryo and adult, along with other distinct sites including brain, muscle, and regions of chondrogenesis, implying that *SOX9* is critical in sex determination and differentiation. This was confirmed by the recent experiment demonstrating that *Sox9* expression is sufficient to induce Sertoli cell differentiation and testis development after transgenesis in XX mice (Vidal et al., 2001).

The chondrogenic expression of *Sox9* is conserved among vertebrates. Because teleost fish has a mineralized skeleton, studies in evolution of teleostean osteogenesis mechanisms will help to understand skeletal development in vertebrates. The rice field eel, *Monopterus albus*, is an economically important freshwater fish of southeast Asia, which undergoes natural sex reversal from female to male during its life. This species with a small genome [all chromosomes are telocentric ($2N = 24$) and the haploid genome size is among the smallest of the vertebrates ($0.6 \sim 0.8$ pg)] provides a good model to study evolution and developmental processes, especially sex determination/differentiation and chondrogenesis processes. A little is known about molecular mechanisms of sex determination in this species. Two *Sox9* cDNAs, *Sox9a* and *Sox9b*, were recently cloned in zebrafish (Chiang et al., 2001), while only one *Sox9* gene was found in land vertebrates. Mechanisms of the *Sox9* gene evolution during vertebrate lineage still need to be explored. To contribute to this analysis, the two *Sox9a* genes from the rice field eel and the corresponding cDNA products were cloned along with the characterization of their expression patterns during the sex differentiation process.

MATERIALS AND METHODS

Fish Samples

The rice field eels *Monopterus albus* (a teleost fish belongs to the Synbranchoidae of the Synbranchiformes) were obtained from the rice field in Wuhan area in China. Phenotypic sex was verified by histological sectioning of gonads and microscope analysis.

Cloning of the *Sox9a* Genes of Rice Field Eel

Genomic libraries were constructed using DNA extracted from blood, and were partially restricted with *Sau*3AI. The partially digested DNA fragments of 9 to 23 kb were recovered by sucrose gradient centrifugation, ligated to a Lambda DASH II/*Bam*HI vector, and packaged with Glgapack II extracts (Stratagene, La Jolla, CA) following the protocol supplied by the Stratagene. A 0.6-kb fragment of *Sox9* flanking the HMG box cloned by degenerate PCR amplification of genomic DNA of the rice field eel (Zhou et al., 2002a) was labeled with (^{32}P) dCTP and used as a probe to screen the genomic library. A total of 1×10^6 phage-forming units were screened. After three rounds of filter hybridization, positive Lambda clones were digested by *Eco*RV or *Eco*RI, and subcloned into pBluescript (*Eco*RV or *Eco*RI digested) and sequenced on both strands using an ABI 377 autosequencer.

Isolation of the *Sox9a* cDNAs and RT-PCR Analysis

Total RNA was isolated from the testis of rice field eel. Reverse transcription was performed using the Superscript system (Gibco-BRL, Invitrogen, Carlsbad, CA) with 0.5 μg of oligo $(dT)_{12-18}$ and 5 μg of total RNA in a 20 μl reaction. The cDNA products (1μl) were amplified in PCR with primers for *Sox9a1*, *Sox9a2*, or *β-actin*. The reaction conditions were 35 cycles at 94°C for 30 s, 55°C or 60°C for 40 s, and 72°C 1.5 min. The primers for *Sox9a1* were: 5′TGGCTGTCACTGCAGTCTTT3′ and 5′CCAGCTGGCAGAAAGAAGTT3′, those for *Sox9a2* were 5′ACGCGTAACTTGGCACCAAAC3′ and 5′ATGTGTGGCTGTACATGTCA3′, and those for *β-actin* were 5′AAGAAAGATGGCTGGAAGAGG3′ and 5′GGGTGTGATGGTTGGCATGG3′. The PCR products were blunted by the Klenow and subcloned into pBluescript (*Eco*RV digested) and sequenced on both strands using an ABI 377 autosequencer.

Phylogenetic Analysis

All sequences were analyzed using the Blast (http://www.ncbi.nlm.nih.gov) and CLUSTALW (http://www.igh.cnrs.fr) programs to determine similarity. Neighbor-joining trees were constructed using Phylogenetic Analysis Using Parsimony (PAUP*). Bootstrap analyses (1,000 runs) were performed and the confidence values were calculated. Accession numbers of the gene sequences used for comparisons were: zebrafish *Sox9a*, AF277096; zebrafish *Sox9b*, AF277097; rainbow trout *Sox9*, AB006448; alligator *Sox9*, AF106572; frog *Sox9a*, AB035887; chicken *Sox9*, AB012236; pig *Sox9*,

AF029696; mouse *Sox9*, S52469; and human *SOX9*, Z46629.

Molecular Modeling

Homology modeling was used to generate structures of Sox9 proteins by use of a three-dimensional position-specific scoring matrix method (3D-PSSM) (Kelley et al., 2000) (http://www.bmm.icnet.uk/servers/3dpssm). Molecules were rendered using the 3D-Mol Viewer of the Vector NTI program 6.0.

In Situ Hybridization

Antisense and sense RNA probes were prepared respectively from subclones (linearized by *Sac*I or *Sac*II, repectively) of the *Sox9a1* and *Sox9a2* cDNAs, 3′-coding region after HMG box, and also containing some 3′-untranslated region, which is specific to each gene, labeled with digoxigenin-UTP, using SP6 or T7 RNA polymerase. Gonadal tissues were cryosectioned (Leica CM1850), and the sections were immediately hybridized with the probe. Hybridization signals were detected by use of the NBT/BCIP system according to the manufacturer's instructions (Roche, Germany).

RESULTS AND DISCUSSION

Two *Sox9a* Genes Arose in a Recent Gene Duplication Event

To search for homologs of *Sox9a* in the rice field eel, we used a 600-bp genomic fragment containing the HMG box of rice field eel *Sox9* isolated by degenerate PCR (Zhou et al., 2002a) as a probe to screen a genomic library. Two positive clones were obtained, which were used for further characterization. A positive *Eco*RV-generated fragment of ~6 kb from the Lambda clone A and a positive *Eco*RI-generated fragment of 2.5 kb from the Lambda clone B were subcloned into the pBluescript vector for sequencing. These clones showed the highest sequence similarity to mouse, rainbow trout *Sox9*, and zebrafish *Sox9a*, but lower similarity to *Sox9b* of zebrafish, and each had two introns and three exons. We therefore named them as *Sox9a1* and *Sox9a2* (Fig. 1A). The deduced amino acid sequences of the coding regions of the two *Sox9a* genes of rice field eel were similar to each other, which are apart from that of the *Sox9b* of zebrafish (Fig. 1B). Phylogenetic analysis of *Sox9* genes of diverse species further showed that the two *Sox9a* genes of the rice field eel were similar to each other. The relationships among the *Sox9* genes of the rice field eel, frog, alligator, chicken, and mammals were determined by phylogenetic analysis (Fig. 1C). Five groups were observed (A–E) which was consistent with established taxonomic relationships. The two *Sox9a* genes of the rice field eel clustered with the *Sox9* genes from diverse species, and fit especially well into the *Sox9* clade of group A, the fish group. Furthermore, because the two *Sox9a* genes of the rice field eel were distinct from the related group of *Sox* genes, such as *Sox8* and *Sox10*, the two *Sox9a* genes were not orthologs of *Sox8* and *Sox10*. Two copies of *Sox9* genes have also found in zebrafish and rainbow trout, but only one *Sox9* gene has been found in the genome of mammals and birds, which suggests that the *Sox9* gene was duplicated during the evolution of some fish lineages. Zebrafish *Sox9b* was proposed to be a duplicate of zebrafish *Sox9a*, when duplication events involving large chromosome sections occurred, consistent with the occurrence of a third genome duplication event in ray-finned fishes (Amores et al., 1998; Postlethwait et al., 1998; Chiang et al., 2001). After being duplicated, the *Sox9b* would get its diversified roles in neural crest (Li et al., 2002). As *Sox9a* is apart from *Sox9b*, two closely related *Sox9a* genes would arise in a recent duplication event in the rice field eel lineage. Interestingly, only one *Sox9* gene was found in a teleost fish, medaka (Yokoi et al., 2002). It is possible that the duplicated *Sox9* has been lost, or become another *Sox* gene, or a pseudogene by mutation in the medaka lineage.

Cloning and Characterization of the *Sox9a* cDNAs

Based on the genomic sequence information of the two *Sox9a* genes, we used RT-PCR to isolate the two *Sox9a* cDNAs from testis RNA. After subcloning, sequencing and comparison with genomic sequences, two *Sox9a* cDNAs were identified. Their intron-splicing sites were further confirmed (Fig. 1A). The deduced protein sequences were 482 aa for *Sox9a1* and 488 aa for *Sox9a2* (Fig. 1B). The Sox9 protein sequence alignment of the rice field eel, chicken, and mammals revealed high level of homology, especially in the eight evolutionarily conserved regions (A–H) (Fig. 1B). Adjacent to Region A (at the N-terminus), was the most conserved, domain B, which included the HMG box. This region is responsible for DNA binding, and as SOX9 carries signals for nuclear import at each end of its HMG domain (Südbeck and Scherer, 1997), the regions at each end of Domain B would presumably be involved in nuclear import functions. Human SOX9 has a major transactivation domain (TA) in the C-terminus (Südbeck et al., 1996). Due to a low conservation of this region among the diverse species studied, it appears that the full transactivation function of Sox9 requires other domains. Indeed, four other conserved regions were observed (Regions C–F) in the internal region. Moreover, the highest transactivation function was observed in the region from Region C to the 3′-end, where region from Region C, D to E could be the major activation domain, whereas the C-terminus (Regions F, G, and H) corresponding to the TA domain of human SOX9 only plays a minor role in transactivation function in zebrafish Sox9 (Chiang et al., 2001).

The HMG domains of Sox9 of diverse species were also strongly conserved. The HMG domain binds and bends DNA in a sequence-specific manner, allowing Sox9 to function as a transcription factor. Most known missense point mutations in human SOX9 occur in the HMG domain, with altered DNA binding compared to the wild type (Meyer et al., 1997; Preiss et al., 2001). When Sox9 HMG sequences of diverse species were

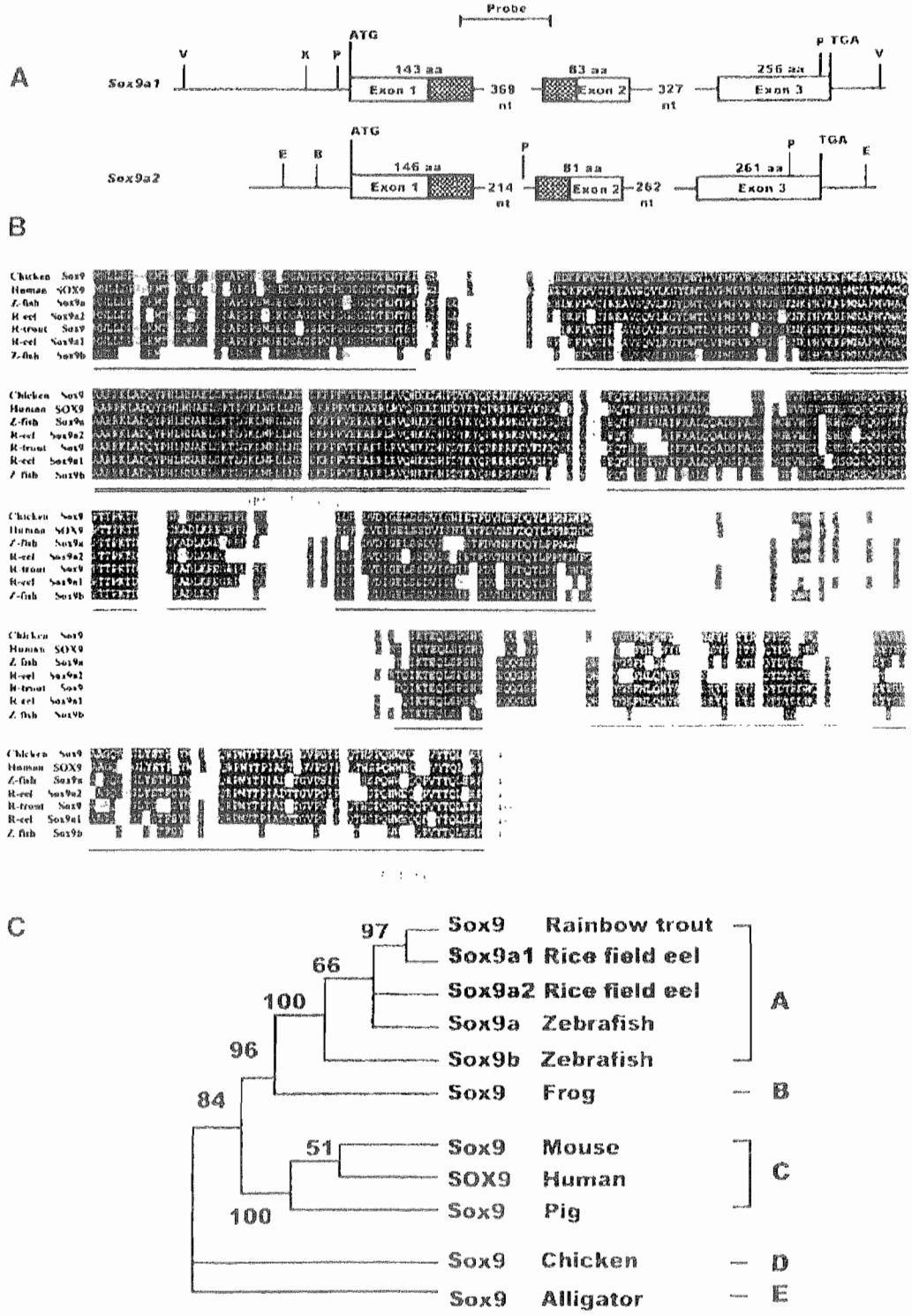

Fig. 1. **A**: Schematic representation of the gene structure of *Sox9a1* and *Sox9a2* of the rice field eel. The boxes indicate exons (shaded box, HMG domain). The numbers on the boxes indicate the numbers of amino acids (aa) encoded within the exon, and the numbers between the lines indicate the nucleotide (nt) numbers of the intron. The start code (ATG), stop code (TGA), and recognition sites for various restriction endonucleases (E, *Eco*RI; B, *Bam*HI; V, *Eco*RV; X, *Xba*I; P, *Pst*I) are shown. The solid line represents the probe used for library screening. **B**: The amino acid sequences of Sox9a1 and Sox9a2 of rice field eel (R-eel) and amino acid alignments of the Sox9 proteins of rice field eel, rainbow trout (R-trout), zebrafish (Z-fish), chicken, and human. The eight conserved regions are underlined (A–H). The HMG domain is double underlined. The transactivation domain in human SOX9 is indicated as "TA domain" (dotted line). GenBank database accession numbers: rice field eel *Sox9a1*, AF378150; *Sax9a2*, AF378151. **C**: Phylogenetic tree of the Sox9 protein of various vertebrate species constructed by the Neighbor-joining method. The numbers on each branch represent the bootstrap values in a thousand runs. The Sox9a1 and Sox9a2 of the rice field eel are shown in bold. Five groupings are indicated as A–E.

aligned, we observed that all other amino acids were identical, except for an amino acid variation in the HMG domain: S (serine) in human and chicken Sox9; G (glycine) in rice field eel Sox9a2, rainbow trout Sox9 and zebrafish Sox9b; V (valine) in rice field eel Sox9a1 and zebrafish Sox9a (Fig. 1B). In three-dimensional models of the HMG domains of rice field eel Sox9a1, Sox9a2, and human SOX9 we found that the structure of the domain was not changed although there was amino acid variation (Fig. 2). The HMG domain comprised three alpha helices which came together in an L-shape in which the short arm was formed by helices 1 and 2 and the long arm was formed by helix 3 and the N-terminal strand (Preiss et al., 2001).

Expression Patterns of the Duplicated *Sox9a* Genes in Gonads of the Rice Field Eel

Expression patterns of the *Sox9a1* and *Sox9a2* of the rice field eel were further analyzed. We examined expression of *Sox9a1* and *Sox9a2* by RT-PCR analysis using total RNA prepared from various tissues. Both *Sox9a1* and *Sox9a2* were expressed in testis, ovary, and ovotestis of intersex rice field eel (Fig. 3A). Transcripts from *Sox9a1* were also expressed in brain, whereas *Sox9a2* was detected in brain and heart. The expression of both *Sox9a* genes in brain of the rice field eel further supports that the distribution of SOX9 protein in rat and mouse brain correlated with the sites of reported brain abnormalities in campomelic dysplasia (CD) patients (Pompolo and Harley, 2001). In the rice field eel, natural sex reversal from female to male occurs during its life, while its gonad transforms from ovary to ovotestis to testis during sex transformation. In order to gain insight into the role of the two *Sox9a* genes in sex differentiation in this species, we analyzed their expression patterns in the three forms of gonads by in situ hybridization. Both *Sox9a1* and *Sox9a2* were expressed in the outer layer (mainly gonocytes) of gonadal epithelium, but not in the inner layer of gonadal epithelium (mainly consisting of growing and differentiated germ cells) in male, intersex, and female; and signals appeared to be identical in the three sexual forms (Fig. 3B). Ovarian structures in the gonad of the rice field eel were

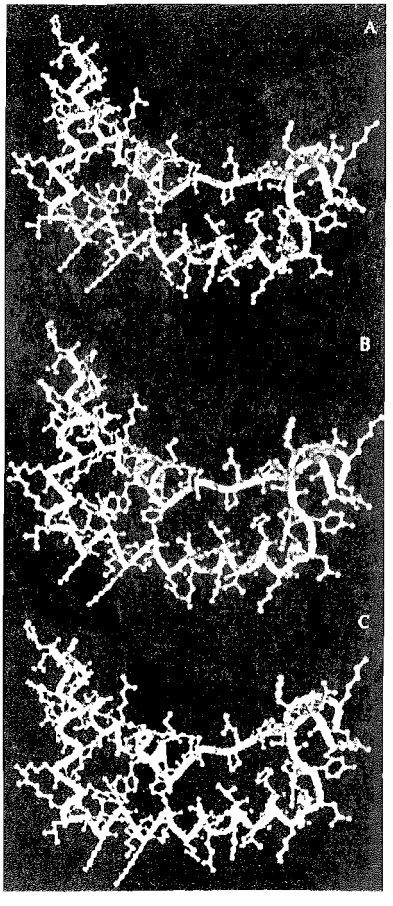

Fig. 2. The three-dimensional molecular structure of the HMG domains of rice field eel Sox9a1 (**A**), Sox9a2 (**B**), and human SOX9 (**C**). Sites of variation among the Sox9 proteins of fish, chicken, and human are indicated in green.

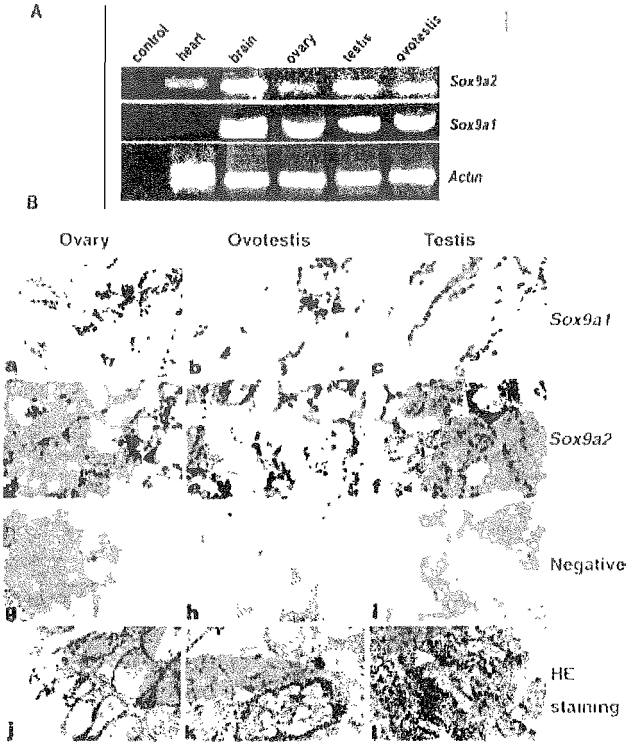

Fig. 3. **A**. Expression of rice field eel *Sox9a1* and *Sox9a2* in different adult tissues detected by RT-PCR. Ovotestis indicates the gonad of intersex rice field eels. *β-actin* gene primers were used to determine whether equal amounts of RNA were used for each reaction. A negative control was utilized by preparing reactions without inclusion of cDNAs. **B**: Expression analysis of *Sox9a1* and *Sox9a2* by in situ hybridization in gonads of female, intersex, and male rice field eel. (a), (b) and (c), and (d), (e) and (f) antisense probes for *Sox9a1* and *Sox9a2*, respectively, show expression of the two genes in gonadal epithelium of female, intersex, and male. Sense probing as control (g), (h), and (i) and H&E staining (j), (k), and (l) in the gonad samples of the three sexes are shown below. The gonadal epithelium is indicated by arrowheads.

replaced by testicular tissue during gonadal differentiation. The formation of testicular lobules in the gonad occurred later in the life cycle by rapid multiplication of spermatogonia derived from gonocytes (Chan and Phillips, 1967). The fact that both *Sox9a* genes were specifically expressed in the bipotential gonad in the three sexual forms, suggests that the double dose of the *Sox9a* genes may play a role in gonadal differentiation from female to ovotestis to testis during sex reversal. One copy of *Sox9* gene may be sufficient for determination of gonadal differentiation in birds and mammals, but we infer that two copies of *Sox9* may be essential for transformation of gonadal tissue from female to male within rice field eels. This kind of dose effect has been observed for *Dax1* which is an anti-testis gene involved in sex determination. When the *Sry* gene is present, one copy of *Dax1* is not sufficient to produce an anti-testis effect, but extra copies of *Dax1* can reverse male to female in the presence of *Sry* (Swain et al., 1998). This presents opportunities for study of functions of *Sox9*. It is difficult to study the null function of mouse *Sox9* by gene targeting, because heterozygosity of *Sox9* is lethal (Bi et al., 1999). The similar expression of the two *Sox9a* genes of the rice field eel may facilitate gene function ablation for further evaluation of the roles of *Sox9* genes in development and evolution.

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REFERENCES

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH. 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711–1714.

Bell DM, Leung KKH, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS. 1997. *SOX9* directly regulates the type-II collagen gene. Nat Genet 16:174–178.

Bi W, Deng JM, Zhang Z, Behringer RR, Crombrugghe De B. 1999. *Sox9* is required for cartilage formation. Nat Genet 22:85–89.

Chan STH, Phillips JG. 1967. The structure of the gonad during natural sex reversal in *Monopterus albus* (Pisces:Teleostei). J Zool Lond 151:129–141.

Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung B. 2001. Two *sox9* genes on duplicated zebrafish chromosomes, expression of similar transcription activators in distinct sites. Dev Biol 231: 149–163.

Choudhary B, Ganesh S, Raman R. 2000. Evolutionary conservation of the gene *Cvsox9* in the lizard, Calotes versicolor, and its expression during gonadal differentiation. Dev Genes Evol 210:250–257.

De Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. 2000. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 19:389–394.

De Santa Barbara P, Bonneaud N, BoizetDesclozeaux BM, Moniot B, Südbeck P, Scherer G, Poulat F, Berta P. 1998. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol 18:6653–6665.

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature 372:525–530.

Houston CS, Opitz JM, Spranger JW, Macpherson RI, Reed MH, Gilbert EF, Herrmann J, Schinzel A. 1983. The campomelic syndrome, review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al. in 1971. Am J Med Genet 15:3–28.

Kanai YA, Koopman P. 1999. Structural and functional characterization of the mouse *Sox9* promoter, implications for campomelic dysplasia. Hum Mol Genet 8:691–696.

Kelley LA, Maccallum RM, Sternberg MJE. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol 299:499–520.

Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. 1996. A male-specific role for SOX9 in vertebrate sex determination. Development 122:2813–2822.

Li M, Zhao C, Wang Y, Zhao Z, Meng A. 2002. Zebrafish *sox9b* is an early neural crest marker. Dev Genes Evol 212:203–206.

Maroteaux P, Spranger J, Opitz JM, Kucera J, Lowry RB, Schimke RN, Kagan SM. 1971. The campomelic syndrome. Presse Med 79: 1157–1162.

Meyer J, Sudbeck P, Held M, Wagner T, Schmitz ML, bricarelli FD, Eggermont E, Friedrich U, Haas OA, Kobelt A, Leroy JG, Van Maldergem L, Michel F, Mitulla B, Pfeiffer RA, Schinzel A, Schmidt H, Scherer G. 1997. Mutational analysis of the *SOX9* gene in campomelic dysplasia and autosomal sex reversal, lack of genotype/phenotype correlations. Hum Mol Genet 6:91–98.

Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R. 1996. *Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 14:62–68.

Pask AJ, Harry JL, Graves JA, O'Neill RJ, Layfield SL, Shaw G, Renfree MB. 2002. *SOX9* has both conserved and novel roles in marsupial sexual differentiation. Genesis 33:131–139.

Patel M, Dorman KS, Zhang YH, Huang BL, Arnold AP, Sinsheimer JS, Vilain E, McCabe ER. 2001. Primate *DAX1, SRY*, and *SOX9*, evolutionary stratification of sex-determination pathway. Am J Hum Genet 68:275–280.

Pompolo S, Harley VR. 2001. Localisation of the SRY-related HMG box protein, SOX9, in rodent brain. Brain Res 906:143–148.

Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly JS, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL, Talbot WS. 1998. Vertebrate genome evolution and the zebrafish gene map. Nat Genet 18: 345–349.

Preiss S, Argentaro A, Clayton A, John A, Jans DA, Ogata T, Nagai T, Barroso I, Schafer AJ, Harley VR. 2001. Compound effects of point mutations causing campomelic dysplasia/autosomal sex reversal upon *SOX9* structure, nuclear transport, DNA binding, and transcriptional activation. J Biol Chem 276:27864–27872.

Spotila LD, Spotila JR, Hall SE. 1998. Sequence and expression analysis of *WT1* and *Sox9* in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:417–427.

Südbeck P, Scherer G. 1997. Two independent nuclear localization signals are present in the DNA-binding high-mobility group domains of SRY and SOX9. J Biol Chem 272:27848–27852.

Südbeck P, Schmitz ML, Baeuerle PA, Scherer G. 1996. Sex reversal by loss of the C-terminal transactivation domain of human *SOX9*. Nat Genet 13:230–232.

Swain A, Narvaez V, Burgoyne P, Camerino G, Lovell-Badge R. 1998. *Dax1* antigonizes *Sry* action in mammalian sex determination. Nature 391:761–767.

Takamatsu N, Kanda H, Ito M, Yamashita A, Yamashita S, Shiba T 1997. Rainbow trout *SOX9*, cDNA cloning, gene structure and expression. Gene 202:167–170.

Takase M, Noguchi S, Nakamura M. 2000. Two *Sox9* messenger RNA isoforms, isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. FEBS Lett 466:249–254.

Vidal VP, Chaboissier MC, De Rooij DG. 2001. Sox9 induces testis development in XX transgenic mice. Nat Genet 28:216–217.

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene *SOX9*. Cell 79:1111–1120.

Western PS, Harry JL, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination, upregulation of *Sox9* expression after commitment to male development. Dev Dyn 214:171–177.

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P. 1995. The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. Nat Genet 9:15–20.

Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H, Araki K, Morohashi K, Ozato K. 2002. sox9 in a Teleost Fish, Medaka (*Oryzias latipes*): Evidence for diversified function of Sox9 in gonad differentiation. Mol Reprod Dev 63:5–16.

Zhou R, Cheng H, Zhang Q, Guo Y, Cooper RK, Tiersch TR. 2002a. *SRY*-related genes in the genome of the rice field eel (*Monopterus albus*). Genet Sel Evol 34:129–137.

Zhou R, Bonneaud N, Yuan CX, de Santa Barbara P, Boizet B, Schomber T, Scherer G, Roeder RG, Poulat F, Berta P. 2002b. SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic Acids Res 30:3245–3252.