Two *Sox9* messenger RNA isoforms: isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*

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Abstract ***Sox*** **is a family of** ***SRY*****-related testis-determining genes. We have isolated two different mRNA isoforms of the frog** ***Sox9*** **gene from adult frog testis cDNAs. One form (*Sox9* α) encodes a 482 amino acid protein containing the HMG box, whereas the other form (*Sox9* β), which completely lacks the HMG box, is a truncated 265 amino acid protein of *Sox9* α. Sox9 α is 82% similar to mouse, 86% to chicken, and 77% to trout Sox9 at the amino acid level. *Sox9* expression was up-regulated in embryos after stage 16, and was seen in both developing testes and ovaries. The size of *Sox9* transcripts was determined to be 7.8 knt by Northern blot analysis. In addition, *Sox9* α expression was found prominently in the testis and brain among various tissues of adult frogs examined, and was considerably higher than *Sox9* β. The fact that *Sox9* is expressed in both sexes suggests that this gene is involved in gonadal development of male and female frogs. This is dissimilar to the pattern in birds and mammals, in which *Sox9* expression is male-specific.**

Key words: Frog; Sox9; Isoform; cDNA cloning; Expression in the gonad

1. Introduction

The Y chromosome-linked gene *SRY* is a dominant inducer of testis development in mammals [1,2]. The *SRY* gene has a DNA-binding domain of 79 amino acid motif, known as the high mobility group (HMG) box which is present in some of the classes of non-histone proteins [3]. After the discovery of the *SRY* gene, a number of the *SRY*-related HMG box (*Sox*) genes have been isolated from a wide variety of organisms (see reviews [4,5]). Sox proteins have now been classified into seven subgroups, A to G [5]. Members of the same subgroup share an identity of greater than 80% within the HMG domain, and also regions of significant homology outside the HMG box. For instance, members of group D contain a leucine zipper motif in addition to an HMG domain. *Sox 1–3* in group B and *Sox11* in group C are expressed during lens development and in the nerve system in chicken, respectively [6,7]. *Sox5* and *6* in group D are expressed in chondrogenesis and cooperatively activate the type II collagen gene in mouse embryos [8]. Taken together, Sox proteins have diverse functions in various tissues of vertebrates.

Sox9 in group E, which contains a transactivation domain at the extreme C-terminus, is a transcription factor. The *Sox9* gene expression occurs at sites of chondrogenesis in mouse embryos [8]. In human beings, *Sox9* mutations have been shown to cause bone dysmorphology syndrome campomelic dysplasia and partial or complete sex reversal [9,10]. Recently, the *Sox9* expression was found to follow differentiation of Sertoli cells in the mouse testis, in experimental sex reversal when fetal ovaries were grafted to adult kidneys, and in the chick where there is no evidence for a *SRY* gene [11,12]. Thus, it is likely that the *Sox9* gene plays an important role in sex determination in at least birds and mammals, and that it may be critical for differentiation of Sertoli cells in all vertebrates. However, the *Sox9* gene has been isolated in only fish, reptiles, birds and mammals. To understand the precise role(s) of *Sox9* for sex determination in vertebrates, molecular cloning of the *Sox9* gene in amphibians is required. In this study, we report the isolation of two isoforms of *Sox9* cDNAs and their expressions during gonadal development in the frog *Rana rugosa*.

2. Materials and methods

2.1. Animals

The frog *R. rugosa* was used for all experiments. Frogs were primed by injection of the extract of pituitaries of *Rana catesbeiana* into the body cavity 20 h before obtaining unfertilized eggs, as described elsewhere [13]. Tadpoles stages were differentiated according to Shumway [14], and Taylor and Kollros [15].

2.2. Reverse transcription and PCR

Total RNA was isolated from unfertilized eggs, embryos and gonads of tadpoles [16]. Adult male frogs were also used to isolate total RNA of various tissues except ovaries. To isolate cDNA encoding Sox9, all total RNAs were treated with 6 U of RNase-free DNase I (Promega) for 1 h at 37°C to avoid genomic DNA contamination. cDNA was synthesized by incubating 2 μg of RNA in 20 μl of the first strand buffer (Gibco-BRL), which was supplemented with 200 U of Superscript II (Gibco-BRL), 0.5 mM dNTPs (Takara), 10 mM DTT (Gibco-BRL) and 0.4 μM oligo-dT25 primers at 42°C for 50 min. Samples were then incubated at 70°C for 10 min to inactivate the reverse transcriptase. One tenth of the first strand mixture was added to a 50 μl PCR buffer containing 200 μM of dNTPs, 0.2 μM of forward and backward PCR primers corresponding to nucleotides 436–457 (5′-TGCTGAAGGGTTACGACTGGAC-3′) and 1629–1648 (5′-TGGAGAGCTGTGTGTAGAC-3′) of trout *Sox9* cDNA [17], respectively. The PCR products obtained were cloned into the pCR2.1 cloning vector (Invitrogen). The nucleotide sequence of the PCR product had 74% identity with that of trout *Sox9* cDNA. Thus, this PCR fragment was used as a probe to isolate cDNA encoding the frog Sox9.

2.3. cDNA cloning and sequence analysis

The *Sox9* cDNA fragment (1.1 kb) was labeled with [α-^{32}P]dCTP (Amersham) using a random primer DNA labeling kit (Amersham)

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and used to screen an adult frog testis cDNA library in λ gt10 vector by plaque hybridization. Hybridization was carried out at 65°C in 0.5% SDS, 6×SSC, 5×Denhardt's solution and 100 mg/ml of denatured salmon sperm DNA according to Yamamoto et al. [18]. Filters were washed in 0.2×SSC and 0.1% SDS at 65°C, and autoradiographed. Positive cDNAs were subcloned into a pUC19 vector. The sequence analysis was performed using ABI 373A automated DNA sequencer by the manufacturer's guide (Perkin Elmer).

2.4. Northern blot analysis

Fifteen μg of total RNA isolated from various tissues of adult frogs were electrophoresed on 1.2% denaturing formaldehyde agarose-MOPS gel and transferred onto nylon membranes (Hybond N, Amersham). In this study, both the adrenals and kidneys were excised, since the adrenals cannot be completely separated from the kidneys surgically. RNA was then hybridized with DIG-labeled 1.0 kbp *Pst*I/*Eco*RI fragments of *Sox9* α cDNA as probes which recognizes both forms according to the manufacturer's guide (Amersham).

2.5. Analysis of the Sox9 expression by RT-PCR

RT-PCR was employed for the *Sox9* expression analysis. All total RNA isolated from unfertilized eggs, embryos, gonads with mesonephros of tadpoles, and various tissues of frogs were used as the initial templates for RT-PCR. 196 bp and 110 bp fragments of the *Sox9* α and β cDNAs were amplified by PCR using the forward and backward primers, 5′-CTGGAAGGACTGTGCATG-3′ corresponding to base pairs 3–20 and 5′-GCGAATGAGATGGGGAGT-3′ corresponding to base pairs 181–198 of the *Sox9* α cDNA, and 5′-CATGTAGAGTTTGCAGTGTG-3′ corresponding to base pairs 77–96 and 5′-CCCTTTGTTTACCTACTGCT-3′ corresponding to base pairs 167–186 of the *Sox9* β cDNA, respectively. A 374 bp fragment of the frog *R. rugosa EF1* α was also amplified as a control by RT-PCR using the forward and backward primers, 5′-TCCACCAC-CACCGGCCACCT-3′ and 5′-CTCCCACACCAGCAGCAACAAT-3′, respectively. The PCR reaction consisted of 3 min at 94°C, followed by 26, 28 or 32 cycles of 74°C (30 s), 65°C (30 s), and 72°C (1 min), ending with 7 min of extension at 72°C.

2.6. Photography

Images were scanned and adjusted in Adobe Photoshop for brightness and contrast.

3. Results

3.1. Cloning of two isoforms of frog Sox9 cDNAs

To isolate the *Sox9* gene, we made use of PCR to amplify the *Sox9* cDNA, using the forward and backward primers corresponding to regions conserved in the known Sox9 amino acid sequence, or in that of rainbow trout [17]. By RT-PCR, the frog *Sox9* cDNA fragment (1.1 kb) was obtained and used to screen positive cDNAs in a λ gt10 library constructed from an adult frog testis mRNA. This procedure allowed us to obtain 1.7 and 1.8 kb cDNAs. They were subcloned into the pUC19 vector and sequenced. Because of space, nucleotide sequences of two *Sox9* cDNAs are not presented here. One isoform, *Sox9* α (GenBank Accession No. AB035887) was found to have no putative poly(A) signal upstream of the poly(A)-attached 3′-end of the cDNA in its 3′-UTR. An ORF encodes a protein of 482 amino acids (a calculated molecular mass of 53 946) with the HMG box from amino acid 104 to 182. There was no proline- and glutamine-rich segment found in human [9] and mouse [19] Sox9s, which is compatible with the finding on trout Sox9 [17]. The frog Sox9 α is highly homologous with human, mouse, chicken, turtle and trout Sox9s (82%, 82%, 86%, 84% and 77% overall amino acid sequence identity, respectively). When the phylogenetic tree was constructed at the level of nucleotide sequences from the pairwise matrix of genetic distance of frog and other known *Sox9*s by the UPGMA method [20] using the program

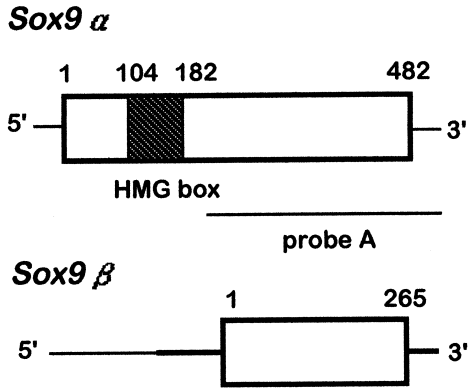

Fig. 1. Schematic representation of *Sox9* α and β cDNAs. The box indicates the ORF (hatched box, HMG box), and the bar indicates the non-coding regions in each isoform. A bold line in *Sox9* β indicates the 1.2 kbp segment that is identical to that of *Sox9* α. A line below *Sox9* α indicates a position of probe A (1.0 kbp) which was used for Northern blot analysis.

induced with PHYLIP [21], it was found that frog *Sox9* α was diverged from trout *Sox9* as expected, and that turtle, chicken and mammalian *Sox9*s were separated from frog *Sox9* α (data not shown).

On the other hand, the other isoform (Sox9 β; GenBank Accession No. AB035888) with no HMG box is a truncated 265 amino acid protein (a predicted molecular mass of 29 392) of Sox9 α. The segment of *Sox9* β cDNA (nucleotide positions 513–1706) was identical to *Sox9* α cDNA. Schematic representation of Sox9 α and β is shown in Fig. 1.

When the comparison of amino acid sequences of different Sox9s with frog Sox9 α was made, all sequences were found to be extremely similar (Fig. 2). In particular, only one out of 79 amino acids in the HMG box of frog Sox9 differed from that of mammals (human, boar and mouse), chicken and turtle (98.7% identity). The transactivation domain residues in the C-terminal 83 amino acids in frog Sox9 also had 87% identity with that in human [22] and 84% in mouse [23] Sox9s, suggesting that the frog Sox9 also functions as a transcription factor.

3.2. Northern analysis

When tissue-specific expression of *Sox9* was examined by Northern analysis using the probe which recognizes both α and β forms, only one band was found. The transcript was 7.8 knt in length (Fig. 3). The result was the same when probes which recognize each isoform were used (data not shown). The testis was the most prominent among various tissues from adult male frogs except ovaries. The low level of transcripts was also seen in the brain. However, the ovary produced no *Sox9* mRNA.

3.3. Stage- and tissue-specific expression of the Sox9 gene

The expression of *Sox9* was examined by RT-PCR in order to confirm whether the ovary of adult frogs does not express both types of *Sox9* α and β mRNAs. As shown in Fig. 4, *Sox9* α and β mRNAs were observed in the brain and testis, which was compatible with the result from Northern blot analysis. In addition, the ovary showed no expression of the *Sox9* mRNA.

When *Sox9* α and β expressions during development of embryos were investigated, no appreciable amount of the

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Frog     MNLLDPFLKM TEEQEKCLSG APSPSMSEDS AGSPCPSGSG SDTENTRPQE N---TFTKGD QDLKKETEDE KFPVCIREAV   77
Human    MNLLDPFMKM TDEQEKGLSG APSPTMSEDS AGSPCPSGSG SDTENTRPQE N---TFPKGE PDLKKESEED KFPVCIREAV   77
Mouse    MNLLDPFMKM TDEQEKGLSG APSPTMSEDS AGSPCPSGSG SDTENTRPQE N---TFPKGE PDLKKESEED KFPVCIREAV   77
Boar     MNLLDPFMKM TDEQEKGLSG APSPTMSEGS RGSPCPSGSG SDTENTRPQE N---TFPKGE PDLKKESEED KFPVCIREAV   77
Chicken  MNLLDPFMKM TEEQDKCISD APSPTMSDDS AGSPCPSGSG SDTENTRPQE N---TFPKGD PDLKKESDED KFPVCIREAV   77
Turtle   MNLLDPFMKM TEEQDKCISG APSPTMSDDS AGSPCPSGSG SDTENTRPQE N---TFPKGD PDLKKESDQD KFPVCIREAV   77
Trout    MNLLDPFLKM TDEQEKCFSD APSPSMSEDS VGSPCPSGSG SDTENTRPSD NHLLLGPDGV LGEFKKADQD KFPVCIRDAV   80
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Frog     SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ETEKRPFVEE  157
Human    SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ESEKRPFVEE  157
Mouse    SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ESEKRPFVEE  157
Boar     SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ESEKRPFVEE  157
Chicken  SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ESEKRPFVEE  157
Turtle   SQVLKGYDWT LVPMPVRLNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN ESEKRPFVEE  157
Trout    SQVLKGYDWT LVPMPVRVNG SSKNKPHVKR PMNAFMVWAQ AARRKLADQY PHLHNAELSK TLGKLWRLLN EGEKRPFVEE  160
         ********** ******* ** ********** ********** ********** ********** ********** * ********

Frog     AERLRIQHKK DHPDYKYQPR RRKSVKNGQS EQEDGSDQTH ISPNAIFKAL -QADSPHSAS SMSEVHSPGE HSGQSQGPPT  236
Human    AERLRVQHKK DHPDYKYQPR RRKSVKNGQA EAEEATEQTH ISPNAIFKAL -QADSPHSSS GMSEVHSPGE HSGQSQGPPT  236
Mouse    AERLRVQHKK DHPDYKYQPR RRKSVKNGQA EAEEATEQTH ISPNAIFKAL -QADSPHSSS GMSEVHSPGE HSGQSQGPPT  236
Boar     AERLRVQHKK DHPDYKYQPR RRKSVKNGQA EAEEATEQTH ISPNAIFKAL -QADSPHSSS GMSEVHSPGE HSGQSQGPPT  236
Chicken  AERLRVQHKK DHPDYKYQPR RRKSVKNGQS EQEEGSEQTH ISPNAIFKAL -QADSPQSSS SISEVHSPGE HSGQSQGPPT  236
Turtle   AERLRVQHKK DHPDYKYQPR RRKSVKNGQS E-QEGSEQTH ISPNAIFKAL -QADSPQSSS SMSEVHSPGE HSGQSQGPPT  235
Trout    AERLRVQHKK DHPDYKYQPR RRKSVKNGQS E-PEDGEQTH ISSGDIFKAL QQADSPASS- -MGEVHSPSE HSGQSQGPPT  237
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Frog     PPTTPKTD-V QPGKPDLKRE GRPLQES-GR QPPHIDFRDV DIGELSSEVI SNIETFDVNE FDQYLPPNGH PGVAST----  310
Human    PPTTPKTD-V QPGKADLKRE GRPLPEG-GR QPP-IDFRDV DIGELSSDVI SNIETFDVNE FDQYLPPNGH PGVPATH---  310
Mouse    PPTTPKTD-V QAGKVDLKRE GRPLAEG-GR QPP-IDFRDV DIGELSSDVI SNIETFDVNE FDQYLPPNGH PGVPATH---  310
Boar     PPTTPKTD-V QPGKADLKRE GRPLPEG-GR QPP-IDFRDV DIGELSSDVI SNIETFDVNE FDQYLPPNGH PGVPATH---  310
Chicken  PPTTPKTDAQ QPGKQDLKRE GRPLAEG-GR QPPHIDFRDV DIGELSSDVI SNIETFDVNE FDQYLPPNGH PGVPATH---  312
Turtle   PPTTPKTD-V QPGKQDLKRE GRPLQEG-GR QPPHIDFRDV DIGEFSREVI SNIETFDVNE LDQYVPPNGH GGVPATHGQP  313
Trout    PPTTPKTD-L AVGKADLKRE GRPLQEGTGR QLN-IDFRDV DIGELSSDVI SNIEAFDVHE FDQYLPPHGH PGMPGIN---  312
         ********     ** ***** **** *  ** *   ****** **** *  ** **** *** *  *** ** **  *

Frog     --QVT-YTGS Y-GISSAAGG PAGA-GHAWM PKQ------P QQ-------- ---------Q PQQP-----Q QQQHGLPTLS  357
Human    G-QVT-YTGS Y-GISSTAAT PASA-GHVWM SKQQAPPPPP QQPPQAPPAP QAPPQPQAAP PQQPAAPPQQ PQAHTLTTLS  386
Mouse    G-QVT-YTGS Y-GISSTAPT PATA-GHVWM SKQQAPPPPP QQPPQAPQAP QAPPQ-QQAP PQQPQA-PQQ QQAHTLTTLS  384
Boar     G-QVT-YTGS Y-GISSTAAT PAGA-GHVWM SKQQAPPPPP HPPQQPPPVP QAPAQPQAAL PQQPQAPPQQ PQAHTLTTLS  386
Chicken  G-QVTTYSGT Y-GISSSASS PAGA-GHAWM AKQ------- ---------- ----QPQ--P PQPPAQPPAQ ---HTLPALS  363
Turtle   G-QVT-YSGR Y-GISSTSAT QGGA-GPVWM SKQ---PPQP QQ--QPPPAP --------GP A--PAA---- --AHTMTTVS  368
Trout    GAQTS-YTGS YRGISSNSIG QVGAGGHGWM SKQQ------ ---------- ---------- -QQP-----I SILSGGGGTG  359
           *   * *  * ****        * *  **  **

Frog     NEQSQAQQ-- RTHIKTEQLS PSHYSDQQQQ HSP------- QQLNYTSFNL QHYG-STYPT ITRSQYDYTE HQ-GSNSYYS  426
Human    SEPG-QSQ-- RTHIKTEQLS PSHYS-EQQQ HSP------- QQIAYSPFNL PHYS-PSYPP ITRSQYDYTD HQ-NSSSYYS  453
Mouse    SEPG-QSQ-- RTHIKTEQLS PSHYR-EQQQ HSP------- QQISYSPFNL PHYR-PSYPP ITRSEYDYTD HQ-NSGSYYS  451
Boar     SEPG-QSQ-- RTHIKTEQLS PSHYS-EQQQ HSP------- QQIAYSPFNL PHYS-PSYPP ITRSQYDYTD HQ-NSGSYYS  453
Chicken  GEQGPAQQ-- RPHIKTEQLS PSHYS-EQQQ HSPQQQQQQQ QQLGYGSFNL QHYG-SSYPP ITRSQYDYTE HQ-NSGSYYS  438
Turtle   SEQGQSQQ-- RTHIKTEQLS PSHYS-EQQQ HSP------- QQVNYRSFNP QQYS-SSYPT ITGWQYDYTD HQ-SSNSYYS  436
Trout    GEQG-QSQGR TTQIKTEQLS PSHYS-EQQG SPP------- QHVTYGSFNL QHYSASSYPS ITRTQYDYSD HQGGANSYYS  430
          *     *    * ******* ****   **    *        *   *  **    *    **  **   ***   **    ****

Frog     HAAGQSSSLY STFSYMNPSQ RPMYTPIADT TGVPSIP-QT HSPQHWE-QP VYTQLTRP                          482
Human    HAAGQGTGLY STFTYMNPAQ RPMYTPIADT SGVPSIP-QT HSPQHWE-QP VYTQLTRP                          509
Mouse    HAAGQGSGLY STFTYMNPAQ RPMYTPIGDT SGVPSIP-QT HSPQDWE-QP VYTQVTRP                          507
Boar     HARSQGSVLY STFTYMNPAH GPMYTPIADT SGVPSIP-QT HSPQHWE-QP VYTQLTRP                          509
Chicken  HAAGQSGGLY STFTYMNPTQ RPMYTPIADT SGVPSIP-QT HSPQHWE-QP VYTQLTRP                          494
Turtle   HAASQSTSLY STFTYMNPAQ RPMYTPIADS TGVRSIP-QT HSPQHWE-QP VYTQLTRP                          492
Trout    HAGAQGSGLY SFSSYMSPSQ RPMYTPIADP TGVPSVPTQT HSPQHWEQQP VYTQLSRP                          488
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Fig. 2. Comparison of the amino acid sequences of frog, human, mouse, boar, chicken, turtle and trout Sox9s. Identical amino acids are indicated by asterisks. Gaps are introduced in the sequence to optimize the alignment by dashes. The HMG box is boxed. The proline- and glutamine-rich (P/Q-rich) domain in mammals is lined above the sequence. The sources of the *Sox9* sequences are human [9], mouse [19], boar (GenBank Accession No. AF029696), chicken (U12533), turtle [33] and trout (AB006448).

Sox9 α and β mRNAs was found in embryos before stage 12 (Fig. 5). However, *Sox9* mRNA was detected in embryos after stage 16, and in gonads of tadpoles at stage II to X where the onset of morphological differentiation is known to occur [24]. There was no significant difference in the *Sox9* α mRNA level in the testis and ovary of tadpoles at stages XVI and XXV. But, its level was higher in the testis than in the ovary of 2 month post metamorphosis and adult frogs. In addition, neither *Sox9* α nor β mRNA was found in the ovary of adult frogs.

4. Discussion

In this study, we isolated the cDNAs encoding two Sox9

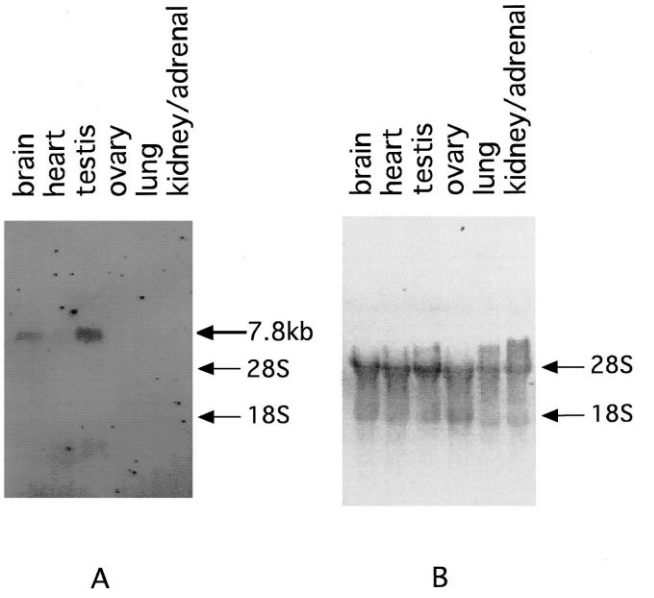

Fig. 3. Northern blot analysis of frog *Sox9* α and β. Fifteen μg of total RNA isolated from various tissues of adult frogs were subjected to Northern analysis for frog *Sox9* gene expression. All tissues except ovaries were obtained from male frogs. A 1.0 kb *Sox9* α cDNA fragment (see Fig. 1, nucleotides 817–1824) was used as a probe. A: Northern blot analysis of *Sox9* α and β mRNAs in frog tissues. The RNA size markers were 28 and 18S ribosomal RNAs. B: Total RNA stained with ethidium bromide.

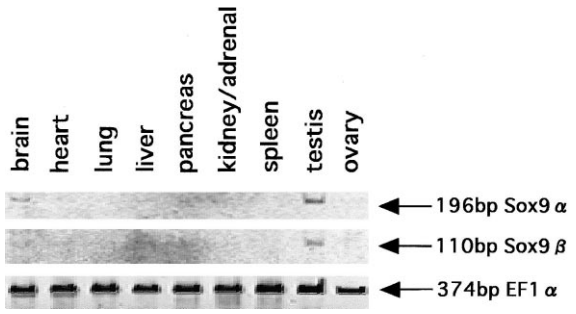

Fig. 4. RT-PCR analysis of *Sox9* α and β from various tissues of adult frogs. RT-PCR was carried out as described in Section 2. One half of the PCR reaction mixture (20 μl) was electrophoresed on a 6% polyacrylamide gel. DNA was visualized by ethidium bromide staining. Amplification was carried out for 28 cycles.

isoforms (α and β) in vertebrates for the first time. Both *Sox9* α and β were expressed during gonadal development of frogs. The expression of the former was greater at all stages examined than that of the latter. It is not unusual that Sox9 protein has two isoforms. Kanai et al. [25] isolated two different mRNA isoforms of the mouse *Sox17* gene. One form is expressed in spermatogonia and the other is highly expressed in pachytene spermatocytes and round spermatids, which led them to make a conclusion that one form may function as a transcriptional activator in the premeiotic germ cells, and that a splicing switch into the other form may lead to the loss of its function in the postmeiotic germ cells [25]. This study showed that *Sox9* β had 100% identity at the nucleotide level to *Sox9* α, except for the first 512 bp segment. The nucleotide position of 513 in *Sox9* β cDNA, which is the first nucleotide of the segment of *Sox9* β cDNA identical to *Sox9* α, matches the splicing site of the second exon of rainbow trout *Sox9* gene [17]. These results suggest that *Sox9* β is probably produced by splicing out the first exon of *Sox9* α containing the initiation methionine and the HMG box.

What is the function of *Sox9* during gonadal development in frogs? Non-mammalian vertebrates lack a *SRY* gene [26,27]. *Sox9* appeared to be the earliest testis-specific gene in chicken. Recently, the *Sox9* gene was also cloned and sequenced in fish [17]. The sequence analysis showed that the trout *Sox9* gene appeared to be male-specific, and that the gene structure is highly conserved through evolution [17]. These results suggest that *Sox9* is an important gene for the testis development in birds and fish. However, it may not act as a substitute for *SRY*, since the *Sox9* gene is expressed after the first signs of sexual dimorphism are observed and is not located on either of the sex chromosomes [12,28]. Nevertheless, *Sox9* must be involved in the regulation of testis development in chicks because of absence of *SRY*. According to de Silva et al. [11], *Sox9* transcripts are present in the genital ridge of both male and female chick embryos at stage 35, a

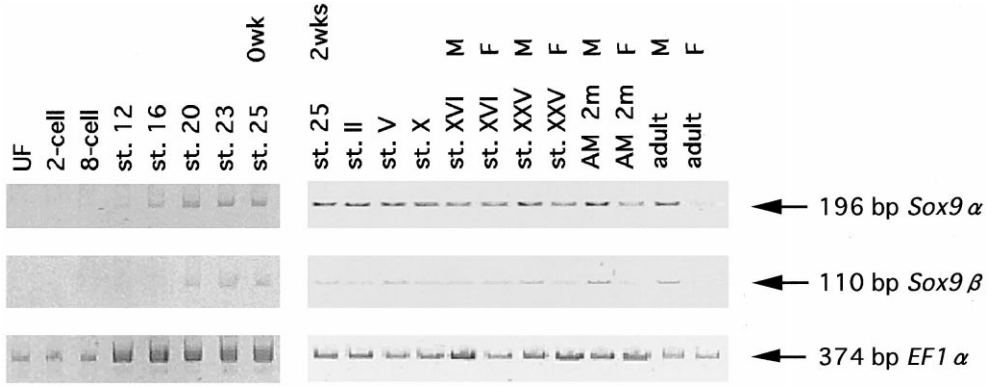

Fig. 5. RT-PCR analysis of *Sox9* α and β expressions in embryos and gonads at different developmental stages. For this analysis, whole eggs and developing embryos up to stage 25 at 2 weeks (wks), gonads with mesonephros of tadpoles at stage II to X, and testes (M) and ovaries (F) with mesonephros of tadpoles at stage XVI and XXV, and testes (M) and ovaries (F) of frogs 2 months (AM 2m) and 2 years (adult) after metamorphosis were used to extract total RNA. Two μg of DNase treated RNA were reverse transcribed in 20 μl at 42°C for 50 min, using 200 U of Superscript II (Gibco-BRL), 0.5 mM dNTPs (Takara), 10 mM DTT (Gibco-BRL) and 0.4 μM oligo-dT25 primer. The primers used to amplify frog *Sox9* α and β are described in Section 2. RT-PCR was carried out by 32 cycles to amplify *Sox9* α and β cDNAs, or 26 cycles to amplify *EF1* α cDNA.

stage at which the genital ridges look morphologically identical, and the levels become much lower in developing testes at later stages. The *Sox9* gene is expressed during the process of gonadal development in mouse and chicks, and the *Sox9* mRNA and protein are seen in Sertoli cells, but not in any other type of cells in the mouse testis [11]. These results strongly suggest that *Sox9* plays an important role in Sertoli cell differentiation and in the maintenance of Sertoli cell functions in the testis of mouse and chicks. *Sox9* α and β may also play an important role in differentiation of Sertoli cell in the testis of frogs. In mice, the *Sox17* isoform is expressed at a low level in the testis throughout postnatal development, while the *t-Sox17* form is expressed abundantly in the testis, predominantly in postmeiotic germ cells [25]. Based on these results, Kanai et al. [25] concluded that a switch from *Sox17* to the *t-Sox17* isoform may alter the function of Sox17 at the meiotic and postmeiotic phases during spermatogenesis in mice. However, *Sox9* α and β were expressed in a parallel fashion during gonadal development of frogs. Therefore, there may not be a switch from one to the other type of Sox9 in frogs. We do not know their roles in gonadal development of frogs presently. *Sox9* was also expressed in the brain of male frogs. However, its function in the brain remains unclear.

It should be noted that the expression of *Sox9* α and β was detected in the developing testis and ovary of the frog *R. rugosa*, suggesting that they are not a determinant for the testis development at least in this species. Then, is the gene isolated in this study truly a *Sox9* gene? It is unlikely that our gene is not *Sox9*, because of the high similarity with other Sox9s at the amino acid level. This is also supported by the facts that there is only one amino acid difference in the HMG box between frog Sox9 α and mammalian Sox9, and that the transactivation domain residues at the C-terminus are highly conserved. Yet, these findings cannot account for the difference in the function between amphibian and mammalian Sox9s. In addition to the HMG box and transactivation domain, the frog Sox9 may have another active site(s) working commonly for gonadal differentiation. We are unable to exclude the possibility, however, that the active site interacts with another factor(s) to determine the sex during gonadal development. Through evolution, SRY may have diverged from Sox proteins to inhibit the activity of an ovary inducer during gonadal development in mammals. Graves [29] proposed a hypothesis that the sex determination in mammals is controlled by the interaction of *SRY* and *Sox* genes. According to the hypothesis [29], the product of autosomal gene *Sox9* is required to determine testis. In females, *Sox9* is inhibited by a product of related X-born gene, *Sox3* (from which *SRY* evolved). Therefore, no testis forms in this case. In males, on the contrary, *Sox3* is inhibited by the product of its Y-born paralogue *SRY*, so that *Sox9* is able to function and determine testis. This might be true in mammals, but not in non-mammalians, since neither *SRY* nor the interaction between an SRY-like testis determinant and Sox proteins has been reported in animals other than mammals. Needless to mention, sex is also differentiated in non-mammalians. Then, what is a dominant factor(s) for the sex determination in amphibians? No candidate has been found so far. But, there must be an exclusive factor, which may interact with *Sox9* α and β, for the sex determination in amphibians. The interaction between a testis determinant and Sox9 α might be regulated by Sox9 β, since these two isoforms have the identical transactivation domain at the C-terminus.

Finally, *Sox9* is known to be expressed in a sex- and tissue-specific manner during embryogenesis in mammals [11,12,19]. Mutations in *Sox9* lead to XY sex reversal and skeletal abnormalities [9,10]. However, sex reversal is not seen in 25% of XY infants showing the skeletal abnormalities, and sex reversal without skeletal abnormalities has not been found in cases of *Sox9* mutations [30,31], which imply that *Sox9* by itself can not determine the testis development. Undoubtedly, other genes such as *SRY*, *SF-1*, *WT-1* and *DAX-1* must be involved in the sex determination in mammals [32], although interactions of the products of these genes remain unclear. Many genes are probably involved in the sex determination in amphibians as well. Isolation of such genes in amphibians is urgent.

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