A rainbow trout SRY-type gene expressed in pituitary glands

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Abstract A Sox (SRY-type HMG box) gene, designated SoxP1, was isolated from a cDNA library made from pituitaries of immature rainbow trout. Sequence analysis indicated that the cDNA had an open reading frame encoding 467 amino acid residues containing a DNA binding motif, known as the high mobility group (HMG) box. Northern blot analysis showed trout SoxP1 mRNA was detected in pituitaries and gonadal tissues, but not in liver, spleen, and heart. In pituitaries, trout SoxP1 mRNA was more abundant in immature fish than in mature fish. Gel shift retardation analysis indicated that the recombinant HMG box protein of SoxP1 produced in *E. coli* had a DNA binding property for an AACAAT or AACAAAG sequence. These findings suggest that the trout SoxP1 protein may play certain roles in growth or maturation in pituitaries as a transcription factor.

Key words: SRY; Sox; HMG box; Pituitary; Rainbow trout

1. Introduction

The SRY (*S*ex determining *R*egion of the *Y* chromosome) gene is responsible for testis formation during embryonic development in mammals [1–5]. This gene encodes a protein with a DNA binding motif known as the high mobility group (HMG) box, found in some transcription factors [6]. After the discovery of SRY, several genes encoding a protein with an SRY-type HMG box have been identified in various vertebrates [7], and termed Sox (*S*RY-type HMG b*ox*) genes [7,8]. Although human SOX9 that is related to autosomal sex reversal and campomelic dysplasia has been shown to be expressed in various tissues [9,10], most of the Sox genes have been shown to be expressed in limited tissues. Chicken Sox2 and Sox3 genes are expressed in the developing nervous system [11]. Mouse Sox4 is a transcriptional activator in T lymphocytes [12], and mouse Sox5 is related to spermatogenesis [13]. Recently, we have cloned SOX cDNAs encoding a protein with a leucine zipper motif (SOX-LZ) from mouse and rainbow trout [14], which are both expressed in later stages of spermatogenesis.

Pituitary glands regulate a wide variety of biological processes such as development, growth, sexual maturation, and metabolism by secreting various hormones in animals. To investigate whether a member of the Sox family may play a role in growth or sexual maturation as a regulatory gene in pituitary glands, we have cloned a Sox gene (SoxP1) from a pituitary cDNA library of immature rainbow trout. SoxP1 mRNA is more abundant in pituitaries of immature trout than in those of mature trout.

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2. Materials and methods

2.1. Animals

Immature rainbow trout, *Oncorhynchus mykiss* (mean body weight: 100 g) were obtained in June, and mature rainbow trout (mean body weight: 300 g) were obtained in January from Iwate Prefectural Experimental Station.

2.2. Purification of RNA and construction of cDNA library

Total RNA was extracted with guanidium isothiocyanate and purified by CsCl ultracentrifugation [15]. Poly(A)$^+$ RNA selection from total RNA was performed as described previously [16]. cDNA was synthesized using a commercial kit according to the manufacturer's instructions (Amersham). After ligation with an *Eco*RI adaptor, cDNA was ligated with *Eco*RI-digested λZAPII DNA (Stratagene) and packaged using Gigapack extracts (Stratagene).

2.3. Isolation of cDNA fragments encoding SRY-type HMG boxes

To obtain Sox cDNA fragments, the polymerase chain reaction (PCR) was performed using the rainbow trout pituitary cDNA as template. Primers used for PCR were degenerate oligonucleotides corresponding to the conserved amino acid sequences of the SRY type HMG boxes as follows: *a forward primer,* 5′-ATGAA(CT)GC(ACGT)-TT(CT)ATGGT(ACGT)TG-3′; *a reverse primer,* 5′-GG(TC)TG-(AG)TA(TC)TT(AG)TA(AG)TT(ACGT)GG-3′. The PCR amplification was carried out by *Thermus thermophilus* DNA polymerase (Toyobo) for 30 cycles (95°C for 1 min, 55°C for 30 s, and 70°C for 2 min).

2.4. Screening of the cDNA library

cDNA fragments encoding SRY-type HMG boxes were labeled with [α-^{32}P]dCTP by the random priming methods and used to screen the cDNA library. Hybridization was performed at 42°C for 18 h (50% (v/v) formamide/5 × standard saline citrate (SSC)/0.1% SDS/1 × Denhardt's solution/denatured calf thymus DNA (0.1 mg/ml)). Filters were washed in 0.1 × SSC/0.1% SDS once at room temperature for 5 min, and then twice at 65°C for 60 min.

2.5. RNA blot analysis

Poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon filter. The hybridization condition was the same as that for the cDNA library screening.

2.6. Expression of recombinant protein in Escherichia coli

Using the trout SoxP1 cDNA as template, the DNA fragment (nucleotides 502–1144) encompassing the HMG box region was obtained by PCR. The PCR primers were 5′-GGCAGCCATATGCCAGTTCGG-GGAAATGGC-3′ with a sequence of *Nde*I site and 5′- AGAATCG-GATCCGTTGAGCGGTAGATACTG-3′ with a sequence of *Bam*HI site. The PCR products digested with *Nde*I and *Bam*HI was inserted between the *Nde*I and *Bam*HI sites of pET15b (Novagen). The recombinant protein was expressed in *E. coli* BL21(DE3) plysS by addition of isopropylthiogalactopyranoside at a final concentration of 1 mM.

2.7. Gel retardation analysis

Using the soluble fraction of the *E. coli* lysate which contained the recombinant protein, gel retardation analysis was performed as described previously [14]. The sequences of duplex oligonucleotide probes are as follows: Mut-11, 5′-GGGAGAGAACAATGGGTGCCCTAC-3′; HuSRY, 5′-GGGGTTAACGTAACAAAGAATCTGGTAGA-3′; HuAllmut, 5′-GGGGTTAACGTCCGCGGTAATCTGGTAGA-3′. The oligonucleotides were labeled with [α-^{32}P]dCTP by Klenow fragment described previously [14].

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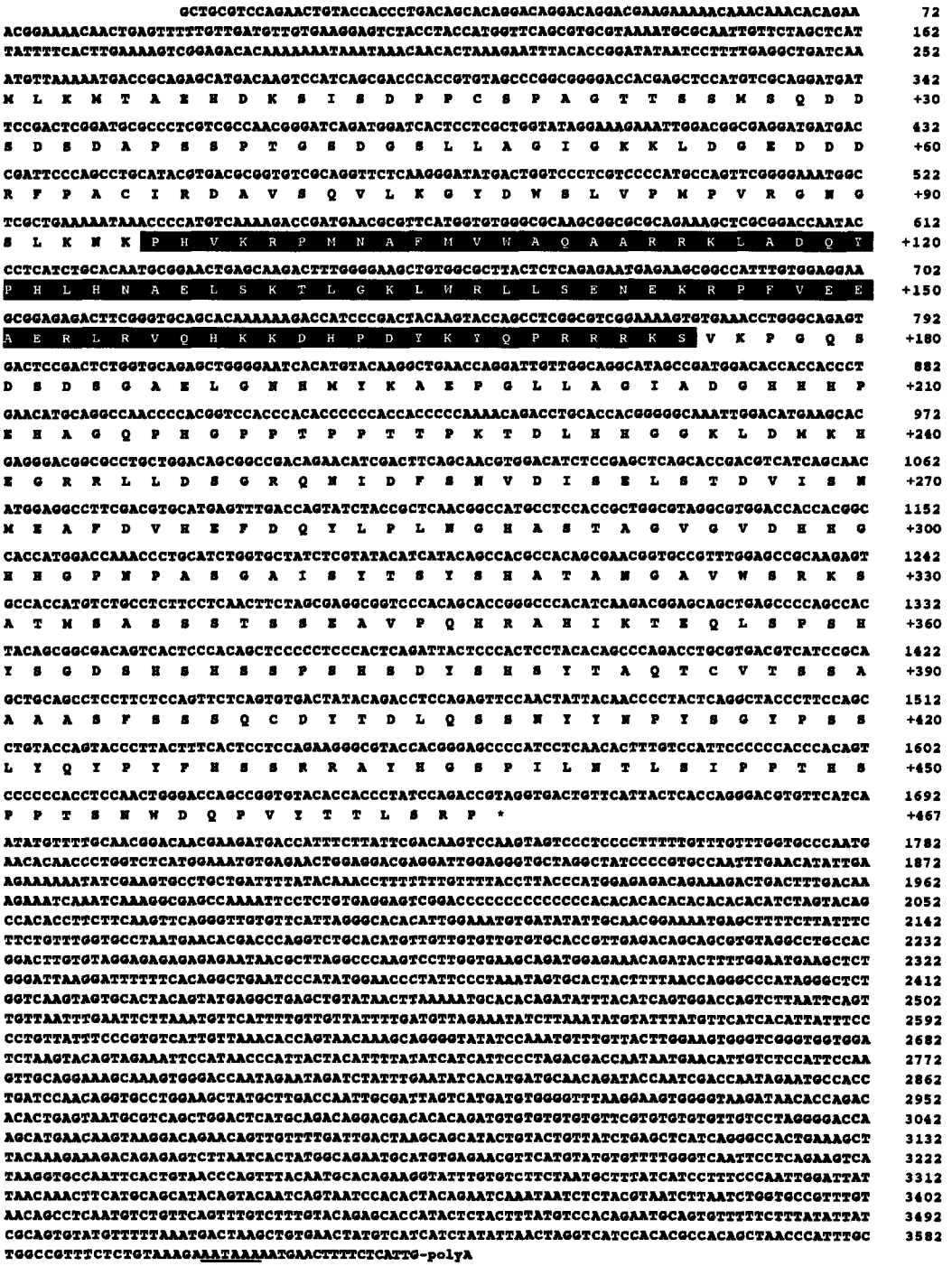

Fig. 1. Nucleotide sequence and deduced amino acid sequence of rainbow trout SoxP1 cDNA clone, pRTSX4. The HMG box is indicated by white letters in black boxes. An asterisk indicates the stop codon. The putative polyadenylation signal AATAAA (nucleotides 3603–3608) is underlined.

3. Results

3.1. Isolation of rainbow trout SoxP1 from pituitary cDNA library

PCR was carried out using pituitary cDNA from immature rainbow trout as a template and degenerate oligonucleotide primers based on the conserved amino acid sequences of the HMG boxes of Sox genes (see section 2). An approximately 200 bp-long fragment was isolated and cloned into the *Eco*RV site of pBluescript II (Stratagene). Using this fragment as a probe, the pituitary cDNA library of immature rainbow trout was screened, and two positively hybridizing phages were isolated. Positive clones were rescued as pBluescript plasmid by in vivo excision, and the nucleotide sequence of the clone containing the longer cDNA insert, pRTSX4, was determined for both strands by the dideoxy methods. The nucleotide and deduced

Fig. 2. Comparison of the amino acid sequences of the HMG boxes among mouse Sry, Sox8 and rainbow trout SoxP1. Residues identical in the three sequences are indicated by white letters in black boxes. The amino acid sequences corresponding to the oligomucleotides used for PCR amplification of rainbow trout HMG boxes (see section 2) are underlined.

amino acid sequence of trout SoxP1 are shown in Fig. 1. The cDNA was 3626 bp in length, not including the poly(A) tail, and contained an open reading frame encoding 467 amino acid residues. The amino acid sequence of the HMG box of trout SoxP1 was identical to that of mouse Sox8 except for one amino acid residue (Fig. 2).

3.2. Northern blot analyses of rainbow trout SoxP1

To determine the expression pattern of the rainbow trout SoxP1 gene, Northern blot of poly(A)$^+$-RNA from various tissues of immature rainbow trout was hybridized with the 287 bp-long fragments of pRTSX4 (nucleotides 847–1133), which does not contain the sequence encoding the HMG box (Fig. 3A). An intense band of about 4 kb was detected in pituitaries. SoxP1 mRNA was also detected in ovary and testis, but not in any other organ examined. Further, its expression in pituitaries was examined using immature and mature trout by Northern blot analysis (Fig. 3B). The filter was then sequentially re-hybridized with cDNA of salmon ganadotropic hormones, GTHIβ and GTHIIβ [17], and rainbow trout β-actin cDNA. The β-actin cDNA was isolated from a rainbow trout pituitary cDNA library with mouse β-actin cDNA [18] as a probe. The rainbow trout SoxP1 gene was expressed predominantly in immature trout, while GTH-IIβ gene showed much higher expression in mature trout. The level of GTH-Iβ mRNA was similar in immature and mature trout.

3.3. Sequence specific DNA binding of rainbow trout SoxP1 HMG box protein

To test sequence-specific DNA binding of trout SoxP1, the HMG box region protein was produced in *E. coli* using the expression vector, pET15b. Gel retardation assay was carried out using three kinds of synthetic duplex DNA : HuSRY contains an AACAAAG sequence, a high affinity site for human SRY, while HuAllmut contains a CCGCGGT sequence in exchange for the AACAAAG sequence within HuSRY [19]; Mut11 contains an AACAAT sequence, a high affinity site for mouse Sry and Sox5 [20]. Fig. 4 indicates the sequence-specific DNA binding property of trout SoxP1 by showing that the SoxP1 protein bound with Mut11 efficiently and also with HuSRY, but not with HuAllmut.

4. Discussion

We have isolated an Sox cDNA (trout SoxP1) from a pituitary cDNA library of immature rainbow trout. The amino acid

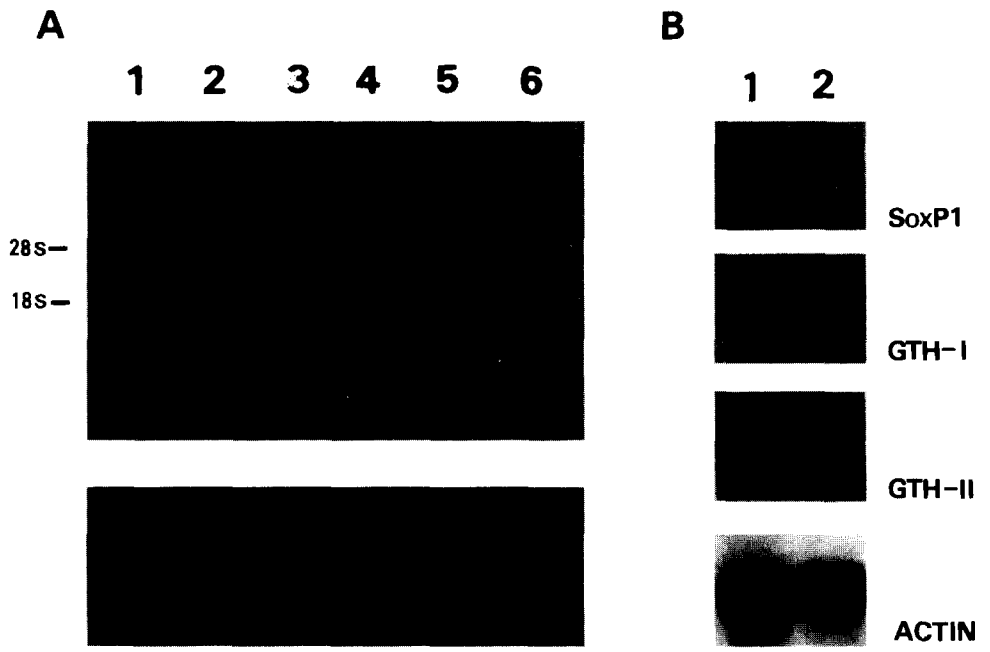

Fig. 3. Northern blot analyses of rainbow trout SoxP1 mRNA. (A) Expression of trout SoxP1 mRNA in pituitaries and gonads. One μg of poly(A)$^+$-rich RNA from several tissues of immature rainbow trout were electrophoresed and transferred to a nylon filter. The filter was hybridized with the 287 bp-long fragment (nucleotides 847–1133; Fig. 2) of rainbow trout SoxP1 cDNA (the upper column) and then rehybridized with rainbow trout β-actin cDNA (the lower column). Lanes: 1 = pituitary glands; 2 = ovary; 3 = testis; 4 = heart; 5 = liver; 6 = lung. (B) High-level expression of trout SoxP1 mRNA in pituitaries of immature rainbow trout. Two μg of poly(A)$^+$-rich RNA from pituitaries of immature (lane 1) and mature (lane 2) trout were electrophoresed. The RNA blot was hybridized with trout SoxP1 cDNA, salmon GTH-Iβ cDNA, salmon GTH-IIβ cDNA, and trout β-actin cDNA sequentially.

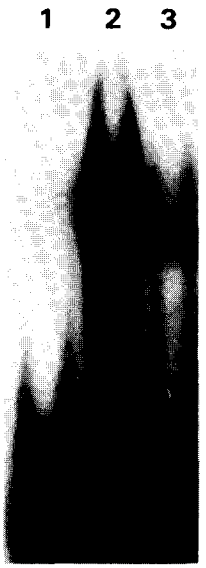

Fig. 4. Sequence-specific DNA binding property of rainbow trout SoxP1 HMG box protein. Gel shift mobility analysis was carried out with the soluble fraction of *E. coli* lysate in which recombinant trout SoxP1 HMG box protein was overproduced. The sequences of the oligonucleotides probes (Mut-11, HuSRY, and HuAllmut) were described in section 2. Lanes: 1 = Mut-11; 2 = HuSRY; 3 = HuAllmut.

sequence of the trout SoxP1 HMG box showed high homology (98.2%) with that of mouse Sox8 which was expressed in 12.5 day embryo [8]. As mouse Sox8 has been cloned only in the HMG box region, it is still unknown whether mouse Sox8 protein has overall homology with rainbow trout SoxP1 protein. To judge if rainbow trout SoxP1 is the homologue of mouse Sox8, it is necessary to know the primary structure and expression pattern of mouse Sox8. Anyway, it is interesting to investigate whether the trout SoxP1 may be conserved in structure and function during vertebrate evolution.

The trout SoxP1 gene showed differential expression in pituitaries during maturation, and the mRNA level was much higher in immature fish than in mature fish. Besides pituitaries, trout SoxP1 gene was shown to be expressed in ovary and testis, but not in liver, kidney, and lung. Thus, the trout SoxP1 gene expression seems to limited to endocrine tissues. In gonadal tissues, however, trout SoxP1 gene expression was not regulated differentially during maturation (data not shown). When salmon RNA prepared from anterior and posterior pituitary was probed with the trout SoxP1 cDNA, the hybridizing band was more detected in anterior pituitary (data not shown), where hormones involved in growth and maturation such as gonadotropin, growth hormone, and prolactin are produced. We examined whether the expression of the trout SoxP1 in pituitaries of immature trout may have relation to gonadotropic hormone expression. However, similar expression patterns of the trout SoxP1 and gonadotropic hormone genes (GTH-Iβ and GTH-IIβ) in pituitaries of immature and mature fish was not observed (Fig. 3B). Also, sexual dimorphism of the trout SoxP1 transcripts in pituitary gland was not observed by Northern blot analysis (data not shown). Considering the sequence-specific DNA binding of the HMG box region protein of the trout SoxP1 protein, trout SoxP1 protein is considered to function as a transcription factor. Identification of target genes of the trout SoxP1 protein in pituitaries and gonads is necessary to understand the biological roles of the SoxP1 protein.

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