

A rainbow trout SRY-type gene expressed in pituitary glands

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Received 8 September 1995

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 AACAAAT 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 (Sex determining Region 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 (SRY-type HMG box) 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.

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 *EcoRI* adaptor, cDNA was ligated with *EcoRI*-digested λ ZAPII 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 -³²P]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 *NdeI* site and 5'-AGAATCG-GATCCGTTGAGCGGTAGATACTG-3' with a sequence of *BamHI* site. The PCR products digested with *NdeI* and *BamHI* was inserted between the *NdeI* and *BamHI* sites of pET15b (Novagen). The recombinant protein was expressed in *E. coli* BL21(DE3) plyS 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'-GGGAGAGAACAAATGGGTGCCCTAC-3'; HuSRY, 5'-GGGGTTAACGTAACAAAGAATCTGGTAGA-3'; HuAllmut, 5'-GGGGTTAACGTCCGCGGTAATCTGGTAGA-3'. The oligonucleotides were labeled with [α -³²P]dCTP by Klenow fragment described previously [14].

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GCTGCGTCCGAAGACTGTACCAACCTGCAGCAGCAGGACAGGACAGGACGAAAGAAAAACAACAAACAGAA	72
ACGGAAAACCACTGAGTTTTTTGTTGATGTTGTGAAGGAGTCTACCTACCATTGTTTCAGCGTGCGTAATAATGCGCAATTTGTTCTAGCTCAT	162
TATTTTCACTTGAAAAGTCGGAGACACAAAAAATAAATAAACACACTAAAGAATTTACACCGGATATAATCCTTTTAGGGCTGATCAA	252
ATGTTAAAAATGACCGCAGCATGACAGATCTTCATCAGCGACCCACCCTGTAGCCGGGGGACCACTGAGCTCCATGTGCCAGGATGAT	342
M L K N T A E H D K S I S D F C T S P A G T C S S M S Q D D	+30
TCCGACTCGGATCGCCCTCTGCGCCAACGGGATCAGATGGATCACTCCTCGCTGGTATAGGAAAAGAAATTGGACGGCGAGGATGATGAC	432
S D S D A P S S P T G S D G S L L A G I G K K L D G E D D D	+60
CGATTCCCAGCTGCATACGTGACCGGGTGTCCGAGGTTCTCAAGGGATATGACTGGTCCCTCTGCTCCCCATGCCAGTTTCGGGGAAATGGC	522
R F P A C I R D A V S Q V L K G Y D W S L V P M P V R G N G	+90
TCGCTGAAAAATAAACCCCATGTCTCAAAAGACCGATGAACGGCTTCATGGTGTGGGGCGGACCGCCAGAAAGCTTCGGGGCAATAC	612
S L K N K P H V K R P M N A F M V W A Q A A R R K L A D Q Y	+120
CCTCATCTGCACAATTCGGAACTGAGCAAGACTTTTGGGGAAGCTGTGGCGCTTACTCTCAGAGAAATGAGAAAGCGGCCATTGTGGAGGAA	702
P H L H N A E L S K T L G K L W R L L S E N E K R P F V E E	+150
CGCGAGAGACTTCGGGTGCGAGCACAAAAAAGACCATCCCAGCTACAGTACCAGCCTCGGCGTCCGAAAAGTGTGAAACCTGGGCGAGGT	792
A E R L R V Q H K K D H P D Y K Y Q P R R R K K S V K P G Q S	+180
GACTCCGACTGTGGTGCAGAGCTGGGAACTACATGTACAAAGCTGAACCAAGGATTGTGGCAGGCATAGCCGATGGACAGCACCCT	882
D S S B S G A E L G N H M Y K A E F P G L T L G A G I A D G H H H P	+210
GAACATGCAAGGCCAACCCACCGGTCACCCACACCCCCACCACCCCAAAACAGAGCTGCACACGGGGGCAAAATGGACATGAAGCAC	972
E H A G Q P H G P P T P P T T P K T D L H X G G K L D M K H	+240
GAGGAGCGGCGCTCTGCGACAGCGGCGGACAGAACATCGACTTCAGCAACGTGGACATCTCCGAGCTCAGCACCAGCGTCATCAGCAAC	1062
E G R R L R V S G R Q M I D F S N V D I S E L S T G D V I S	+270
ATGGAGGCGCTTCGACGTGTCATGAGTTTGACAGTATTACCGCTCAACGGGCTGCTCCACCGCTGGCGTGGAGTGGACACCGGC	1152
M E A F D V H E F D Q Y L P L N G H A S T A G V G V D H H G	+300
CACCATGGACAAAACCTGCTATCTGGTCTATCTCGTATACATCATACAGCCACGCCACAGCGAAGCGGTGCCGTTTGGAGCCGCAAGAGT	1242
H E G O P N P A S G A I S Y T S Y S H A T A M G A V W S R K S	+330
GCCACCTGTCTGCTCTTCTCCTCAACTCTAGCGAGCGCTCCACAGCAGCCGCGCCACATCAAGACGGAGCAGCTGAGCCGCCACGCCAC	1332
A T M S A S S T S S E A V P Q H R A N I K T E Q L S P S H	+360
TACAGCGGCGACAGTCACTCCGACAGCTCCCGCTCCCACTCAGATTACTCCCACTCTTCAACAGCCGACAGCTGCTGACGCTCATCCGCA	1422
X S G D S H S H S G S P S H S D Y S H S Y T A Q T C V T S S A	+390
GCTGCGGCTCTCTTCCAGTTCTCAGTGTGACTATACAGACCTCCAGAGTTCCAACTATTACAACCCCTACTCAGGCTACCTCTCCAGC	1512
A A A S F S G Q C D Y I T D L Q S S N Y I N P Y S G Y P S S	+420
CTGTACCAAGTACCCCTTCTTCACTCTCCAGAAAGGCGCTACCACGGGAGCCCCATCCTCAACACTTTGTCCATTCCCCCACCACAGT	1602
L Y Q I Y P Y F H S R R A Y H G S P I L N T L S I P P T H S	+450
CCCCCAGCTTCCAAGTGGGAGCAGCGGTGTACRACACCCCTTCTCAGAGCGGTAGGTGACTGTTTCATTACTCAGGAGAGCTGTTCATCA	1692
F P T S N W D Q P V I T T L S R P *	+467
ATATGTTTTGCAACGGACAAAGAGATGACCAATTTCTTATTCGACAAAGTCCAAAGTAGTCCCTCCCTTTTTTTGTTGTTGGTGGCCCAATG	1782
AACAGACCCCTGCTGTCATGGAAGATGAGAGAGTGGAGGACAGGAGTTGGAGGCTGCTAGGCTATCCCCGTGCAATTTGACATCATTTGA	1872
AGAAAAAATATCGAAGTGGCTGCTGATTTTATACAAACCTTTTTTTGTTTATCTACCTACCCATGGAGAGCAGAAAGACTGACTTTGACAA	1962
AGAAATCAAAATCAAAGGGCGAGCCAAAATTCCTCTGTGAGGAGTGGGACCCCCCCCCCCCCACACACACACACACACATCTAGTACAG	2052
CCACACCTTCTTCAAGTTCAGGGTTGTGTTTCATAGGGGACATCTGGAAGATGTATATGCAACGGAAATAGAGCTTTCTTATTTTC	2142
TTCTGTTTGGTGGCTTAATGACACAGACAGGCTGACATGT	2232
GGACTTGTGTAGGAGAGAGAGAGAAATACCGCTTAGGCGCCAGGCTCTTGT	2322
GGGATTAAGGATTTTTTTCAGAGGCTGAAATCCCATGGAACCCCTATTCCCTAAATAGTGCACACTTTTAAACAGGGGCCATAGGGGCTCT	2412
GGTCAAGTGTGCTACAGATGTAGGCTGAGCTGTATATCTAT	2502
GTGTAATGTGAATCTTAAATGTTCAATTTGTTGTTTATTTGATGTGAAGATCTTAAATATGTATTTATGTTTCATCATATTTATTTCC	2592
CCTGTTATTCCTGCTGATTTGTAAACACAGTAACAAAGCAGGGGTATATCCAAATGTTTGTACTTGGAGGTGGGTGGGTGGGTGGGTGGG	2682
TCTAAGTACAGTAGAAATTTCCATACCCATTACTACATTTTATATCATCATCTCCCTAGACGACCAATATGACACTGTGCTCCGATTTCCAA	2772
TGTGACGGAAAGAAAGTGGGACCAATAGATGATCTATTGAAATACATGATGCAACATGACCAATGACCAATGACCAATGACCAATGACCA	2862
TGATCCAAAGAGTGGCTGGAAAGCTATGCTTGACCAATTCGATTAAGTATGATGTGGGGTTTAAAGGAAAGTGGGGTAAAGATAACACAGAC	2952
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AGCATGAAAGAAAGTAAAGGACGAAAGCTGTTTGTGATGACTTAAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC	3132
TACAAAGAAAGACAGAGGCTTAAATCACTATGGCAGAAATCATGTGAGAACGCTTCATGTATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT	3222
TAAAGGTGCAATTCAGCTGTAAACCGATTTTCAAAATGCACAGAAAGGATTTGTGTGCTTCTTAAATGCTTTATCATCTCTTCCGAAATG	3312
TACCAAACTTCATGACAGCATACAGTAACTAGTAACTTCAACATCAGACATCAGAAATCAATATCTCATCAATCTTATCTGAGTGGCTTTGT	3402
AACAGGCTCAAGTGTGCTGCTCAAGTTTGTCTTTGTACAGAGCACCATACTCTACTTTATGTCCACAGAAATGAGTGTGTTTTCTTTATATTAT	3492
CGAGTGTATGTTTTTAAATGACTAAGCTGTGAAGTGTGATCTCATCTATATTAATCAAGGTCATCCACAGCCACAGCTAAACCCATTGCT	3582
TGGCGGCTTTCTGTGTAAGAAATAAATAAGAACTTTTCTCATG-polyA	

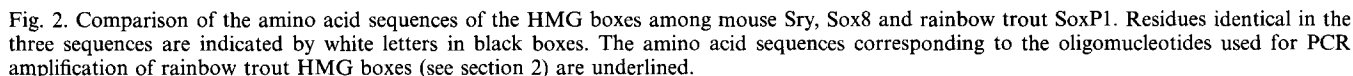
Fig. 1. Nucleotide sequence and deduced amino acid sequence of rainbow trout SoxP1 cDNA clone, pRTSX4. The HMG box is indicated by white letters in black boxes. An asterisk indicates the stop codon. The putative polyadenylation signal AATAAA (nucleotides 3603–3608) is underlined.

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 *EcoRV* 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



immature trout, while GTH-II β gene showed much higher expression in mature trout. The level of GTH-I β 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 ACAAT 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.

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

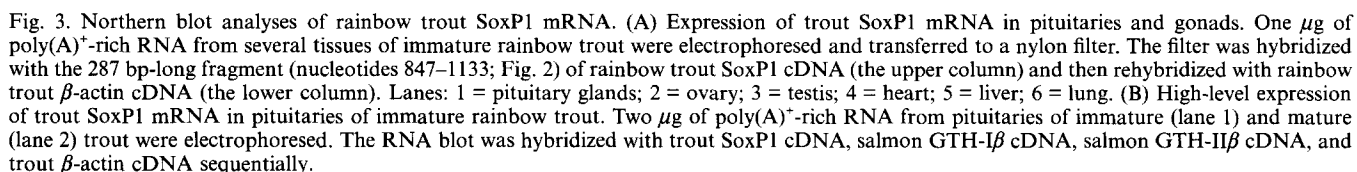




Fig. 4. Sequence-specific DNA binding property of rainbow trout SoxP1 HMG box protein. Gel shift mobility analysis was carried out with the soluble fraction of *E. coli* lysate in which recombinant trout SoxP1 HMG box protein was overproduced. The sequences of the oligonucleotide probes (Mut-11, HuSRY, 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 be 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-I β and GTH-II β) 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.

Acknowledgments: We wish to express our gratitude to Dr. S. Yamashita of Central Research Laboratory, Nippon Suisan Kaisha Ltd. and Dr. M. Ono of Kitasato Univ. for helpful discussions. This work was supported in part by a grant pioneering research project in biotechnology from the Ministry of Agriculture, Forestry and Fisheries, Japan, and a grant from the Kitasato Research Foundation and research grants of the Kanagawa Academy of Science and Technology to M.I.

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