Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară, TOM VII, 2006

THE CLONING AND TISSUE EXPRESSION OF SOX8 IN *RAMISGURNUS DABRYANUS*

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Keywords: *Paramisgurnus dabryanus, PdSox8*

Abstract: Sox8 gene is one of important transcription factors in developments of many tissues and organs. In this article, The *Sox* genes of *P. dabryanus* were amplified by degenerate primers within HMG-box. Three amplification bands with the length 220,550 and 1500bp respectively were observed in all individuals. A novel gene fragment was obtained from the 550bp band. It coded 53 amino acid residues, the other part of the fragment may be a intron. The identity of possible amino acid sequence to mouse *Sox*8, 9 and *SRY* is 96%, 94%, and 47% respectively. The identity to human *SOX*8, 9 and *SRY* is 64%, 94%, and 40% respectively. So, it was named *PdSox*8. Northern blotting result showed that it overexpresses in adult liver and brain.

INTRODUCTION

An important advance in the research of sex determination mechanism was made in 1990 when Sinclair and colleagues cloned the human testis-determining gene—sex-determining region of the Y chromosome (*SRY*), which has been regarded as the gene of testis determining factor (TDF)[1]. *SRY* genes have been found only in male mammals including marsupials. However, the HMG-box region appears to be the only conserved part of the protein. The difference outside this domain is striking even among very relative species. The gene that plays the same role has not been found in the other vertebrate. Because of the *SRY* gene discovery, a new gene family has been known, which is SYR-related HMG-box genes(*Sox)* [2]. Every *Sox* gene has a conservative HMG-box domain compared with the *SRY*. The *Sox* genes are very different from the *SRY*, although *SRY* is the founder member of this gene family. *Sox* genes are very conservative in the evolution. The different *Sox* genes isolated from different species have very high homologue. Another characteristics of *Sox* genes is that they are present in both male and female individuals. The *SRY* is believed to have evolved from *SOX-3*, based on several lines of evidence, including their sequence similarity, the location of *SOX-3* on the X chromosome and some aspects of their expression. This model should be supported directly from the phylogenetic tree of the *SOX* family, but no evidence has been provided to date. *Sox*3 is a transcription factor and plays roles in both sex during the embryogenesis. This fact implies that the up-stream genes of the sex differentiation cascade were acquired in the later stage and varied highly among the different species; the down-stream genes were possibly conservative, acquired in the early stage and played similar role. *Sox* gene family is conservative during evolution and is the only one which is related to sex development among the found gene families.

Fishes serve as a key link on the view of the systematic evolution of vertebrate. Comparing with the other vertebrates, Its sex determination is primitive, varied and polymorphic. To better understand the sex determination and differentiation mechanism, it is essential to study extensively the structure and role of *Sox* genes in fish. The importance of research achievement in mammals to direct the similar studies in fishes is becoming increasingly apparent. Processes in sex determination of fish have been made on the basis of hormone treatment to fry, hybridization, karyotype and chromosome banding results. Heteromorphic sex chromosomes have been found only in several species. *Paramisgurnus dabryanus* is common Cypriniformes fish in china. By studying the karyotype with the C-banding and silver staining methods, we have found that it is of ZZ/ZW type. There are no heteromorphic sex chromosomes in its relative species—*Misgurnus auguillicaudatus*[3]. In this study we examined the sequence and expression characteristics of *Sox*8 in *P. dabryanus*.

MATERIALS AND METHODS

Materials

P. dabryanus was obtained from Wuhan markets. All fish were sexed by examining gonads. The degenerate primer set was designed within HMG-box motif. Primer I is 5'GATGGATCCATGAA (C/T)GC(A/T/C)TT(C/T)AT(G/A/T)T(A/G/C)GG3'; primer II is 5'GCGCGAATTCGG (A/G/T/C) C/T(G/T)(A/G)TA(C/T)TT(A/G)TA(A/G)T(C/T)(G/A/T)3'.

Preparation of genomic DNA

DNA was extracted from fish blood samples. 0.1-0.5ml total blood, anti-coagulated by ACD, was centrifuged at 3000rpm for 10mins, then rinsed several times with 0.85% NaCl. The blood cells were diluted in suitable volume lye's buffer(0.5MEDTA,pH8.0;0.5%SDS). Proteinase K was added at a final concentration of 150ug/ml and the solution was incubated overnight at 50^0C.This was followed by three phenol-chloroform and chloroform extractions. After precipitation with ethanol, the pellet was rinsed in 70%ethanol, moderately dried and dissolved in TE.

Degenerate primer PCR

The degenerate PCR was performed in a volume of 20μl with about 200ng genomic DNA, 1μmol/L of each primer, 200μmol/L dNTP and 2u of Taq DNA polymerase. Thirty-five amplification cycles were performed with annealing temperature 52^0C for 40seconds and extension at 72^0C for 1minutes. The amplification products were electrophorosed on 1.5% agarose gels to check the amplification bands.

Cloning and nucleotide sequence

PCR-amplified products were digested with *EcoRI* and *BamHI*, then electrophoresed in 1.2% low melting-point agarose gels. PCR amplification bands were excised from gel, ligated into the pBluescript plasmid digested with same restriction enzymes. Cybersym Com. was entrusted to sequence the clones with dideoxy-mediated chain-termination method using T3/T7 promoter sequence as general primers. Searching Genbank determined the similarity and possible amino acids sequence of the DNA sequences. The cloned DNA fragments were named after the sequence that has the highest similarity with them.

Preparation of total RNA and Northern blotting

The total RNA was extracted from adult tissues (including heart, liver, kidney, brain, testis and ovary) using total RNA isolation system of Promega Corp. Place 50mg tissues sample into the denaturing solution and disrupt the tissue for 15-30seconds. Add 2mol NaAc(pH4.0) and mix thoroughly by inverting the tube 4-5 times. Add 900μl phenol: chloroform: Isoamyl alcohol, carefully mix by inversion 3-5 times and then shake vigorously for 12 seconds. Chill on ice for 20 minutes. Centrifuge at 11000rpm for 10 minutes at 4^0C. Transfer the top aqueous phase to a fresh tube, add an equal volume of the isopropanol and incubate the sample at -20^0C overnight to precipitate the RNA. Pellet the RNA by centrifugation at 11000rpm for 0minutes at 4^0C. Resuspend the RNA pellet in 900μl denaturing solution and then add equal volume of phenol: chloroform: Isoamyl alcohol to purify the RNA . Dissolve the RNA in 11μl H_2O.

Add 4.4μl formaldehyde buffer, 6.6μl formaldehyde and 22μl formamide, mix thoroughly. Add 4μl mixture to 2μl formaldehyde loading buffer then treated at 65^0C for 15minutes. Then load on a 1% MOPS formaldehyde-agarose gel to resolve RNA samples by electrophoresis. Wash gels for 4-5 times with DEPC-treated water. Transfer RNA to a neutral nylon membrane in 20×SSC for 20 hours. The membrane then was baked at 80^0C for 2 hours. Keep at 4^0C.

Probe labeling: the 400μg purified DNA fragments was denatured thoroughly at 100^0C for 10minutes, then chilled on ice. Add 2μl d(GTP,ATP,CTP) mixture with the final concentration 5mmol/L,klenow DNA polymerase 2μl,50uCiα-^{32}P-dCTP,adjuest the final volume to 20μl. Incubate at 37^0C for 3-12hours. The membrane was prehybridized for 3hr at 65^0C. Replace the prehybridization solution. Denature the labeling probe at 100^0C for 10 minutes, then add to hybridization box after chilled on ice. Hybridized 12-18hr at 65^0C. Following stringency washes of 5×SSC,1%SDS and 0.1SSC,0.1%SDS,for 15minutes at 65^0C each. The blot was exposed to X-film for 7 days at -20^0C with an intensifying screen.

RESULTS

Conservative part cloning and sequencing of *PdSox*8

Degenerate primer set was designed on the basis of published *Sox* gene sequences from

different species, which was specific to the HMG-box motif. With genomic DNA of *P.dabryanus* as template, three bands were observed with the lengths of 220,550 and 1500bp respectively. A very weak band was usually obvious on the negative film(Figure 1). The results were same between male and female individuals. No sex-specific bands were observed. The results showed that the *Sox* genes in *P.dabryanus* be very different from each other in the length of the conservative regions. To determine the structure feature of amplification products with different lengths, the 550bp band was cloned and sequenced. The amplification products of genomic DNA were electrophoresed with 1.2% low melting-point agarose gels excising 550bp band which was cloned into pBluescript plasmid. 5 recombinant plasmids were screened. They confirmed containing 550bp-insert fragment by digestion of restriction enzyme and PCR amplification. With dideoxy-mediated chain-termination method using T3/T7 promoter sequence as general primers, two gene fragments with different DNA sequences were gained. The DNA sequence and possible amino acids sequence of one among them were showed in figure 3. By similarity comparison, the former 100 sequences with high score were *Sox* genes from that of fruitfly to human. This showed that the cloned fragment was part of one *Sox* gene.

The first three genes with the highest identity of possible amino acid sequence to the novel gene fragment among the 100 sequences and the identity to *SRY*/*SRY* were showed in figure 4. The identity of possible amino acids sequence to mouse *Sox*8 gene was the highest one to 96%, the next was mouse *Sox*9.

The gene fragment was named as *PdSox*8 according to the usual name practice of *Sox* genes. The cloned part of *PdSox*8 is within HMG-box region length 500bp and coding 53 amino acids. The others is one intron possibly with length 340bp and in keeping with the "GT…AG" principle. The *Sox*8 data were only about that of human and mouse without intron. So, the *PdSox*8 was very different from M*Sox*8 and H*Sox*8 in gene structure. But by the identity of amino acid sequence comparison, the identity to M*Sox*8 is very high, and to H*Sox*8 is lower relatively.

The expression of *PdSox*8 in adult tissues

Total RNA was electrophoresed in a 1.2%(w/v) agarose gel containing 1% formaldelyde and transferred to Hybond-N membrane. The cloned fragment of *PdSox*8 was labeled with random primer elongation method. Northern blotting results showed that *PdSox*8 overexpressed in adult brain and liver, no obvious signals were observed in the other tissues(Figure 2). M*Sox*8 and H*Sox*8 expressed during embryogenesis. This work did not involve *PdSox*8 expression in embryo tissues.

1 2 3 4 5 6

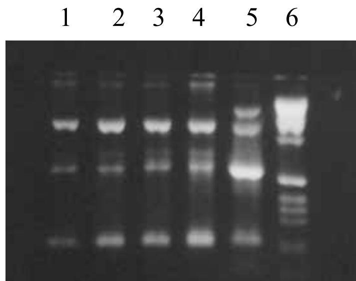

Fig.1 The PCR amplification results of *Sox* genes in *P.dabryanus*
lane 1-2, male individuals; lane 3-5, female individuals; lane 6, DNA molecular weight marker

1 2 3 4 5 6

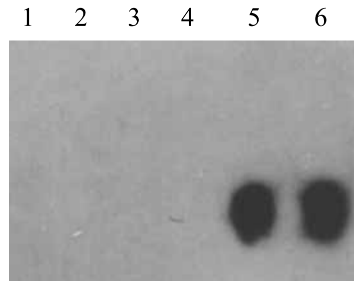

Fig.2 The express analysis of *PdSox*8
lane 1-6 represents adult Ovary, Kidney, Testis, Heart, liver and Brain

A Q A A R R K L A D Q Y P H L H N A E L S
GCGCAGGCTGCGCGCAGAAAACTGGCAGATCAGTACCCCCATCTTCATAACGCA
GAGCTCAGC
K T L G K L W R
AAGACCTTGGGCAAGTTGTGGAGgtaagtgggtacatttttgaaattctatttttgtaaaact
atttaatatgatgttatgaatgtatttgcatgtaacatgaacaatcccctgcaagaacttttg
gtcttactacatgaagtctaactgtgatatttgtagttggtaatataaatgataatcagtctg
caaatctgccatggttactgcacttttactacggttgtaaattgtaatgtgcagtaatctcat
caaacaaaacttgttttgctcttatttttcaagtcaactattttatttattgcacaacatttc
L L S E
aattggataataaaaactacattcaatcctgtaatttgtaaatgtccagGCTGCTCTCTGAG
N E K R P F I E E A E R L R V Q H K K D
AATGAGAAGAGACCGTTTATAGAAGAGGCAGAAAGACTGAGGGTCCAGCACAA
GAAAGAC

Fig.3 The DNA sequence and possible amino acid sequence of *PdSox*8 (Genbank acception number: AF116250)in *P. dabryanus*

gene	amino acids sequences	similarity
*PdSox*8	AQAARRKLADQYPHLHNAELSKTLGKLWRLLSENEKRPFIEEAERLRVQHKKD	
M*Sox*8	................................S.....V............	96%
M*Sox*9	...............................N.S.....V............	94%
H*SOX*9	...............................N.S.....V............	94%
M*Sox*10	....P.........................N.SD............M....	90%
H*SOX*8	.KDE.KR..Q.N.D....V...M...A.KE.NAA.....V..........LR.	64%
H*SOX*10	SR.Q...M.QEN.KM..S.I..R..A..K..T.S......D..K...AM.M.E	58%
M*SRY*	SRGE.H...Q.N.SMQ.T.I..Q..CR.KS.T.A.....FQ..Q..KTL.REK	47%
H*SRY*:	SRDQ...M.LEN.RMR.S.I..QD.YQ.KM.T.A..W..FQ..QK.QAM.REK	40%

Fig.4 The amino acid identity of *PdSox*8 to *Sox*8,*Sox*9,*Sox*10 and *SRY*/*Sry* of human and mouse within the corresponding conservative region

M, H represents mouse, human respectively

DISSCUSION

Many *Sox* genes had been separated in fish according to the published data so far[4]. The cloned *PdSox*8 fragment was within HMG-box motif. The noticeable characteristic of it was the

intron. Only *Sox*9 and *Sox*17 had one intron within the HMG-box among the found 33 *Sox* genes so far, but the sites of them were different from that of *PdSox*8.

Several amplification bands were found when the same primer set was used to amplify the genomic DNA of *Mononpterus albus* and *Mastacebelus aculeatus*[5]. The fact showed intron(s) present in some *Sox* genes of fish. Major genes are with introns in enkaryotic organism, minor are without. The length of introns varies considerably among the same genes of different species, but the number and site are same usually. It was suggested that the new recombination of intron and exons play important role in the large scope gene evolution. Intron(s) contain little genetic information. The exon shuffling within these sites is dangerous to functional domain of exons. So, it is possible that the entire organism contained introns. Because the prokaryotes genomes replicate fast, the introns would be a load of fast replication and disappear in the evolution. The difference of *PdSox*8 to *MSox*8 and *HSox*8 support this viewpoint seemingly. Moreover, someone’s viewpoints were justly opposite. They suggested that the genomes have no intron originally. Introns stemmed from the false recombination of exons and accumulated in the genomes during the evolution. So genes with intron(s) are minor in prokaryotes and low enkaryotic organism, but major in high enkaryotic organism. It is difficult to judge which one is true. What can be affirmed is introns are not parasite of genomes. It is doubtless that there is an intron within the HMG-box of *PdSox8*. Perhaps the intron is indispensable in the regulation of gene expression of *PdSox8* through the proper splicing ways.

Sox8 belongs to a family of transcription regulators characterized by a unique DNA-binding domain known as the high mobility group box[]6,7,8,9]. Many Sox proteins play fundamental roles in vertebrate development and differentiation processes. Expression of Sox8 is strong during embryonic development of muscle, spinal cord, bone formation, the peripheral nervous system and brain[10]. SOX8 is closely related to SOX9, a critical gene involved in mammalian sex determination and differentiation[11]. Expression studies indicate that the two genes have largely overlapping patterns of activity during mammalian embryonic development. In particular, both genes are expressed in the developing Sertoli cell lineage of the developing testes in mice. It was suggested that SOX8 may substitute for SOX9 in species where SOX9 is expressed too late to be involved in sex determination[8]. However, Sox8 is expressed at similar levels in gonads of both sexes during the sex determining period in lower vertebrate, suggesting that SOX8 is no responsible for sex determination or gonad differentiation in that species[12,13]. So, the significance of SOX8 for sexual and splicing way of *PdSox*8 in development will need to be uncovered in the future.

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