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PCR AMPLIFICATION AND SEQUENCE ANALYSIS OF MOLE RAT (*SPALAX LEUCODON*) *Sox2* GENE

T. TERZIN and MILENA STEVANOVIĆ

Institute for Molecular Genetics and Genetic Engineering,
11000 Belgrade, Yugoslavia

Abstract - *Sox2* gene is involved in control of eye development and it has been shown that in mammals this gene is a transcriptional activator of γF-crystallin in lens. The mole rat (*Spalax leucodon*), which is an extreme example of natural visual degeneration, has strongly rudimented eyes with undeveloped, disorganised and vacuolated lens nuclei. Here we present a partial characterisation of the mole rat *Sox2* gene which is cloned using PCR - based approach (and primers specific for human *SOX2* gene). Our results have shown no significant changes in the sequence within the open reading frame of the mole rat *Sox2* gene and have revealed that mole rat *Sox2* gene is strongly conserved compared with orthologue gene in humans, sheep and mouse. Our results indicate that the mole rat *Sox2* gene remains under selective pressure since it probably has, apart from the control of eye development, the other important roles in the embryonic development.

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INTRODUCTION

Cloning a gene for determination of male sex in mammals (SRY), led to discovery of a whole family of related genes. Homology between them is restricted to HMG box, therefore these genes have been named *Sox* genes (SRY related HMG box; the same genes in the humans have been called *SOX* genes) (S i n c l a i r *et al.* 1990).

Several different *SOX* genes are expressed in human foetal brain supporting the hypothesis that these genes play important roles in nervous system development (S t a n o j č i ć *et al.* 1998). Therefore it is predicted that *Sox* genes are transcriptional factors that play a major role in development of central nervous system.

The main feature of the *Sox* genes is the presence of a highly conserved HMG-box type of DNA-binding domain. Using PCR, a large number of *Sox* HMG-boxes in different animals were identified. Apart from mammals, *Sox* genes were identified in birds, reptiles, amphibians, fishes, insects and nematodes. (G r i f f i t h s 1991; F u k a d a *et al.* 1995). In the case of *Sox2* gene, extremely high degree of sequence homology within the HMG-box is also extended to the rest of the protein among different higher vertebrate species (P a y e n *et al.* 1997). Comparisons of nucleic acid sequences revealed a strong homology within mammals, 94% between the ovine and murine open riding frames (ORF)s and 96% between sheep and man. The percentage of homology between different Sox2 polypeptides is very high: 99% between sheep and humans, and 98% between sheep and mice (P a y e n *et al.* 1997). This feature was utilised for PCR amplification of rodent *Sox2* gene with primers designed from the human *SOX2* gene sequence.

Sox2 has been shown to play a critical role in vertebrate eye development. It is expressed in the early developing lens and implicated in the regulation of crystallin gene expression. In mice SOX2 protein is detected in the nuclei of the cells of both the optic cup and the lens pit at 10.5 dpc. At 12.5 dpc, SOX2 protein could be detected in the cytoplasm of the lens fibers, although the protein is sometimes detected in the nuclei of the less differentiated lens fiber cells around the equatorial region and in the anterior epithelium (N i s h i g u c h i 1998). In chicken, it has been demonstrated that *Sox2* gene is highly expressed in the lens placode which parallels δ-crystallin gene activation (P i a t i g o r s k y 1981). Besides lens, *Sox2* gene expression was significant in the developing central nervous system and retina.

Crystallins constitute 90% of the soluble proteins in lens fiber cells (d e J o n g 1981) and they have been divided into several classes. Although there are many conserved and diverse crystallin genes, they are all expressed in the lens with spatial and temporal expression patterns characteristic for each class (M a c A v o y 1978;

W i s t o w 1988). Several transcription factors have been implicated in the crystallin gene regulation, including SOX1 and SOX2 proteins. SOX proteins binding is essential for lens-specific activity of the chicken δ-crystallin enhancer, as well as of the mouse γF-crystallin promoter (K a m a c h i 1995). *Sox2* is, therefore, a critical gene for mammalian lens development, at least partly through its direct action on crystallin gene expression.

Mole rat (*Spalax leucodon*) has strongly rudimented eyes located under the skin that do not respond to light stimuli. In the developing lens of this animal the elongation of the lens fibers leads to the formation of a rudimentary lens nucleus that becomes disorganised and vacuolated (S a n y a l *et al.* 1990). Therefore, the mole rat is providing a suitable model system for studying possible evolutionary changes in genes involved in the control of eye development. Since *Sox2* gene is implicated in the control of lens development, the aim of this work was to investigate the status of the *Sox2* gene in mole rat. The mole rat *Sox2* gene was cloned and sequenced and the sequence was analysed for the presence of mutation and/or rearrangement.

MATERIALS AND METHODS

PCR amplification

Primers used for amplification are as follows:
F3: CAGATGCAGCCCATGCACCGCTA;
F4:CTACATGAACGGCTCGCCCACCTA;
F7: CACAGCGCCCGCATGTACAACATG;
F9: CCGGCGGCAACCAGAAAAACAGCCCG;
R2: CAGATACATGCTGATCATGTCC;
R5: CTAGGTGGGCGAGCCGTTCATGTA;
R9:GCGGATCGAGCGTACCGGGTTTTCTCCATGC;
R10: CTTATCCTTCTTCATGAGCGTCTTGG.

Primers are designed based on the sequences of human *SOX2* gene (S t e v a n o v i ć *et al.* 1994) and artificial *Eco*RI and *Bam*HI sites are introduced in primers F9 and R10 for the purpose of cloning. PCR amplifications were performed on the genomic DNA prepared by standard methods (S a m b r o o k *et al.* 1989) from mole rat (*Spalax leucodon*), mice (*Mus musculus*), hamster (*Cricetus cricetus*), and rat (*Rattus norvegicus*) using the following combinations of primers: F3R5, F4R2, F4R9, F7R2 and F9R10. PCR amplifications were carried out in the final volume of 20 mL containing 20 ng of genomic DNA and 10 pmols of primers. The thermal profile was: hot start and denaturation 30 sec at 94°C, annealing 30 sec at 60°C (F7R2), 56°C (F4R9), 61°C (F3R5 and F4R2) and 63°C (F9R10), followed by an extension of 2 min at 72°C for 35 cycles. PCR amplifications were conducted on a Techne Genius thermal cycler. The PCR products were visualised on 2% agarose gels stained by ethidium bromide.

Southern hybridisation

PCR products were capillary transferred from agarose gel on Hybond N+ membrane. Hybridisation was carried out with radiolabeled *SOXA* probe (S t e v a n o v i ć *et al.* 1993) overnight at 65°C according to the manufacturers protocol (A m e r s h a m) Filter was washed at a final stringency of 0.1xSSPE, 0.1%SDS for 20 min at 65°C.

Cloning and sequencing

The mole rat F7R2 PCR product was digested by *Eco*RI and *Bam*HI restriction enzymes and cloned in pBluescript//KS+ vector. Clones were sequenced using Sequenase Version 2.0 (USB) on double stranded template.

RESULTS AND DISCUSSION

We have used the published sequence of the human *SOX2* gene (S t e v a n o v i ć *et al.* 1994) to design primers for PCR amplification of *Sox2* gene from different rodents. The positions of primers and the length of expected PCR products are presented in Fig.1. The amplifications were carried out on genomic DNAs obtained from mole rat (*Spalax leucodon*), mouse

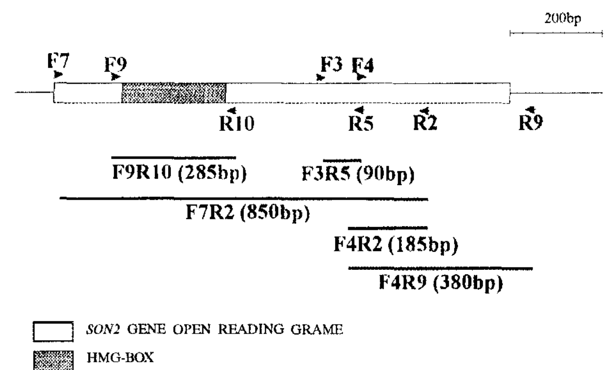

Fig.1. Schematic presentation of the predicted amino acid sequence of the mole rat *Sox2* gene. Positions of primers are presented by arrows. Primer combinations, as well as the lengths of PCR products, are indicated by bars

(*Mus musculus*), hamster (*Cricetus cricetus*) and rat (*Rattus norvegicus*). PCR products of expected size were obtained on all genomic DNA tested, except for the primer combination F4R9 which worked on human and rat DNA only (Fig. 2-a). The presence of PCR products with primers that are designated based on the

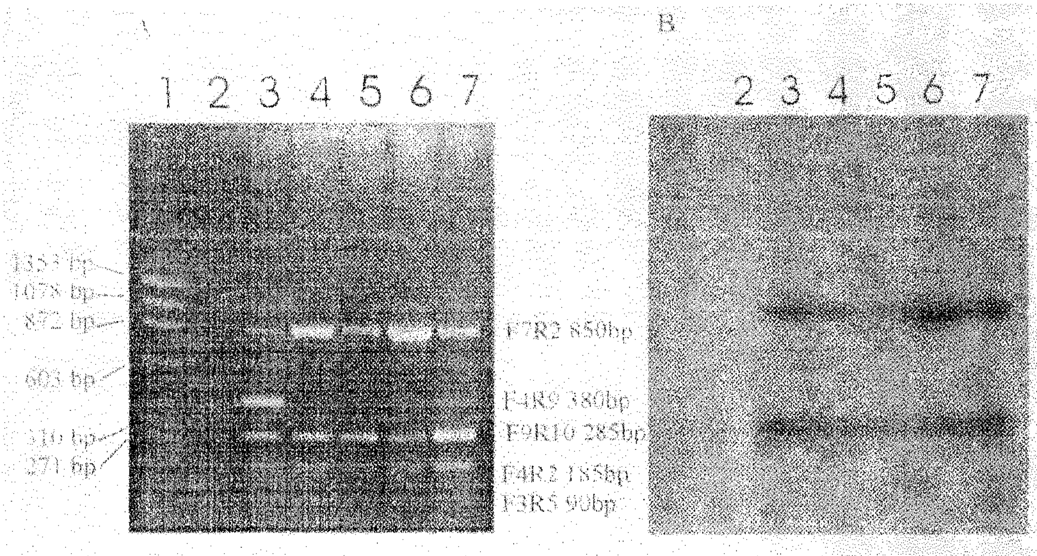

Fig.2. PCR analysis of the *Sox2* genes.

A: Ethidium stained gels of PCR products. B: Gel presented in (A) is blotted and hybridized with human HMG-box containing *SOX*A probe. Each lane shows mix of five single PCR reactions that are loaded together.Lane 1: DNA length marker; lane 2: negative control; lane 3: positive control done on human genomic DNA; lanes 4-7: PCR products obtained on mouse, hamster, mole rat and rat genomic DNAs.

sequence of the human *SOX2* gene indicates a high sequence conservation amongst *Sox2* gene in mammals. Primer R9 have recognition sequence in the 3 untranslated region of the human *SOX2* gene. The published 5 and 3 nontranslated sequences of the *Sox2* genes in different species show a lower degree of conservation (P a - y e n *et al.* 1997). The presence of F4R9 PCR product obtained on human, as well as, on rat genomic DNA indicates a higher sequence similarity in 3' untranslated region in these two species as compared to other examined rodent species.

To confirm that PCR products represent specific amplification of the *Sox2* gene we performed hybridisation with the probe that contains HMG box of the human *SOX2* gene (Fig. 2-b). Our results confirmed that F7R2 and F9R10 PCR products obtained from different rodent species contain the sequence homologous with the human *SOX2* box probe.

Our PCR analysis has shown no significant changes in the length of sequence within the open reading frame of the mole rat *Sox2* gene. In order to analyse the sequence, we have cloned and sequenced the longest F7R2 PCR product of the mole rat *Sox2* gene.

Table 1. Sequence similarity at nucleotide level (upper part) and amino acid level (lower part) between mole rat (sl) and murine (mm), ovine (oa) and human (hs) *SOX2* genes.

	PERCENT SIMILARITY AT NUCLEOTIDE LEVEL			
	sl *Sox2*	mm *Sox2*	oa *Sox2*	hs *SOX2*
sl *Sox2*		95.4	93.3	94.0
mm *Sox2*	97.1		93.0	94.2
oa *Sox2*	96.4	97.5		95.7
hs *SOX2*	96.0	97.5	98.9	
	PERCENT SIMILARITY AT AMINO ACID EVEL			

Comparison of nucleic acid sequences of the *Sox2* gene has revealed a strong homology within mammals ranging from 93.3% similarity between mole rat and ovine to 95.4% similarity between mole rat and murine gene (Table 1). The deduced amino acid sequence of

the mole rat *Sox2* open reading frame was compared with published *Sox2* amino acids sequences of different species (Fig. 3). The comparison has shown a high level of similarity ranging from 96%-97.1% (Table 1). The difference in the length of the amino acid sequence observed between indicated species is due to variable number of glycine residues in the N-terminal region.

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MYNMMETELKPPGPQQASGGGGTG----TAAASGGNQKNS     slSox2aa
........................G-NA....T.......     mmSox2aa
................T.....G.GGNS....A.......     oaSox2aa
................T.....G---NS....A.......     hsSOX2aa

PDRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGA     slSox2aa
........................................     mmSox2aa
........................................     oaSox2aa
........................................     hsSOX2aa

EWKLLSETEKRPFIDEAKRLRALHMKEHPDYKYRPRRKTK     slSox2aa
........................................     mmSox2aa
........................................     oaSox2aa
........................................     hsSOX2aa

TLMKKDKYTLPGGLLAPGGNSMASGVGVGAGLGAGVNQRM     slSox2aa
........................................     mmSox2aa
........................................     oaSox2aa
........................................     hsSOX2aa

DSYAHMNGWSNGSYGMMQEQLGYPQHPGLNAHGAAQMQPM     slSox2aa
..............S.........................     mmSox2aa
..............S...D.....................     oaSox2aa
..............S...D.....................     hsSOX2aa

HRYDVSALQYMSMTSSQTYMNGPPTYSVSYSQQGTPGMAL     slSox2aa
......................S....M............     mmSox2aa
......................S....M............     oaSox2aa
......................S....M............     hsSOX2aa

GSMGSVVKSEASSSPPVVTSSSHSRAPCQAGDLRDMISMYL    slSox2aa
.........................................    mmSox2aa
.........................................    oaSox2aa
.........................................    hsSOX2aa
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Fig.3. Alignment of mole rat Sox2 polypeptide sequence with murine, ovine and human homologue. The HMG domain is boxed. Abbreviations: sl (*Spalax leucodon*); mm (*Mus musculus*); oa (*Ovis aries*); hs (*Homo sapiens*).

The sequence comparison indicates that mole rat *Sox2* gene is strongly conserved as compared to orthologue gene in humans, sheep, mouse and chicken. A partial sequence analysis of mole rat *Sox2* has revealed the absence of any mutation within the open reading frame that would make Sox2 protein inactive. However, the presence of the mutation within the sequences that are not included in this analysis can not be ruled out, although a high sequence conservation indicates that mole rat *Sox2* gene remains under selective pressure.

CONCLUSIONS

Sox2 gene is implicated in the control of lens development. It has been shown that chicken Sox2 activates δ1-crystallin enhancer core and that Sox2 protein binding is essential for lens-specific promoter activity of the mouse γF-crystallin gene (Kamatchi *et al.* 1995). Since the mole rat (*Spalax leucodon*) has strongly rudimented eyes that posses rudimentary disorganised and vacuolated lens nuclei the aim of this work was to investigate the status of the *Sox2* gene in mole rat. Here we presented cloning and partial sequence analysis of the mole rat *Sox2* gene. Our results indicate the absence of mutation and rearrangement within the mole rat *Sox2* open reading frame. We have shown that mole rat *Sox2* gene is strongly conserved comparing to orthologue gene in humans, sheep and mouse. Our results suggest that mole rat *Sox2* gene remains under selective pressure since it probably has, apart from the control of eye development, the other important roles in the embryonic development.

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PCR УМНОЖАВАЊЕ И АНАЛИЗА СЕКВЕНЦЕ *SOX2* ГЕНА КОД СЛЕПОГ КУЧЕТА (*SPALAX LEUCODON*)

Т. ТЕРЗИН и МИЛЕНА СТЕВАНОВИЋ

Институт за молекуларну генетику и генетичко инжењерство, 11000 Београд, Југославија

Sox2 ген је укључен у контролу развића ока и код сисара је показано да је транскрипциони активатор γФ-кристалина у сочиву ока. Слепо куче (*Spalax leucodon*), је екстремни пример природне дегенерације ока. Ова животиња нема спољашње очи, а сочиво је неорганизовано и вакуолизовано. У овом раду смо приказали парцијалну карактеризацију *Sox2* гена слепог кучета. Ген је клониран коришћењем приступа заснованог на PCR технологији (употребом "прајмера" специфичних за *SOX2* ген ч овека). Наши резултати указују да у отвореном оквиру читања *Sox2* гена код слепог кучета нема значајних промена у односу на до сада окарактерисане *Sox2* гене код сисара. Запажа се снажна конзервисаност секвенце у поре-ђењу са ортологним генима човека, овце и миша. Наши резултати указују да је *Sox2* ген слепог кучета под селективним притиском, што указује да сем у развићу ока вероватно има и друге важне функције у ембриогенези.