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Molecular cloning and expression of buffalo *SRY* gene: A mammalian sex determination protein

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A number of proteins are expressed specifically in X- and Y-chromosome bearing spermatozoa. The Sry protein is known to be expressed only in Y-chromosome bearing spermatozoa and also in other tissues like brain, kidney and adrenal gland of adult males. Although expression of buffalo Sry protein has not been reported earlier, the recombinant Sry can be used to understand its role in the tissues. Further, protein based test is needed for assessment of sorted semen. The present study was undertaken to clone and express the buffalo *SRY* gene. The recombinant protein was expressed as inclusion bodies. The expressed fusion protein showed a mol wt of about 27.19 kDa. However, Western blotting with sperm cell lysate showed a band at about 31 kDa. The HMG-box of Sry protein had shown 6 amino acid substitutions as compared to the *Bos taurus* and these would probably make buffalo Sry functionally more active compared to its closely related species. The recombinant Sry could be used to raise monoclonal antibodies for labelling the Y-chromosome bearing spermatozoa, which can be used for assessing the enrichment of Y-chromosome bearing spermatozoa in sex sorted semen.

Keywords: Buffalo, sex determination protein, *SRY* gene, sperm specific marker, Y-spermatozoa

Introduction

A number of techniques have been developed for separation of X- and Y-chromosome bearing spermatozoa. Currently, flowcytometry is the most followed method. This technology requires the development of a method to verify the purity of the sexed sperms. Usually, sexed semen purity is verified by flowcytometry reanalysis[1]. However, a method for validation of data that does not rely on the same instrumentation is required for the practical use or the improvement of such technology. In 2011, Pillai[2] had reported a duplex Taqman PCR for quantification of X- and Y-chromosome bearing spermatozoa in buffalo semen, targeting *PLP* and *SRY* genes. But development of a protein based test is also needed for the assessment of sorted semen.

Several marker proteins have been reported from both X- and Y-chromosome bearing spermatozoa, such as, Ubelx, MHR6A[3] and AKAP82 (a major sperm fibrous-sheath protein) in the mouse[4], and SPAN-X (a nuclear associated protein) in the human[5]. Further, marker proteins present either on X-chromosome bearing spermatozoa, such as, PLP[6], ESX-1[7] and SREBP2, or present on Y-chromosome bearing spermatozoa, such as, SRY[8], Zfy-1, Zfy-2[9], Ubely[3] and Y353iB[10], have also been reported.

SRY (sex-determining region on the Y chromosome) gene has been discovered in mouse about 20 years back, which is known to initiate mammalian sex differentiation through *SOX9*, a master regulator of sertoli cell differentiation into testis[11]. The Sry protein binds to the testis-specific enhancer of Sox9 core element and activates the expression of downstream processes of testis development[12]. Utsumi and Iritani[13] have reported a PCR methodology for embryo sexing using a male-specific (SRY) primers. Rao *et al* have developed a method for buffalo sex identification by amplifying a Y-chromosome-specific sequences[14]. Further, a Loop-mediated isothermal amplification (LAMP) for sexing buffalo embryos has also been reported[15].

So far, the expression of the buffalo *SRY* gene has not been reported. Therefore, objective of the present study was to amplify, sequence, express and characterize buffalo *SRY* gene.

Material and Methods

Total RNA Isolation and cDNA Synthesis

Buffalo (*Bubalus bubalis*) testes were collected from the local slaughterhouse. Total RNA was isolated from

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100 mg of testis samples using RNAgents following the method described by the manufacturer (Promega, USA). The absorbance of RNA was measured at 260 nm/280 nm in Nanodrop spectrophotometer (Nanodrop®, USA) to check the purity. The cDNA was synthesized using Revert Aid™ H-Minus First Strand cDNA synthesis kit (Fermentas, USA) using oligo(dT) primer.

Primers

Primers were designed from the published sequence of *Bos taurus* (Acc. No. DQ 119747) using Gene Tool BioLite with *Bam*HI *and Kpn*I restriction sites at the 5′ end of the forward and the reverse primers, respectively. Thus the designed forward primer was 5′-AGGATCCGCGGTGGTACAG CAACAAAATATTC-3′ and the reverse primer was 5′-AGGTACCGAGTGCCTTTGAGAGCGAGA GTAA-3′.

PCR Amplification, Cloning and Sequencing of *SRY* Gene

The cDNA synthesized from buffalo testis was used for amplification of the *SRY* gene. The PCR was standardized with 1× buffer containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Non-idet P40 and 2.5 mM $MgCl_2$, 200 µmoles of each dNTP, 25 pmoles of each primer and 2.0 U of Taq DNA polymerase, using 50 ng of cDNA in a final volume of 25 µL. The thermal cycling conditions of PCR were: initial denaturation 95°C for 5 min, followed by cycle denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min for 35 cycles and final extension at 72°C for 10 min. Amplified PCR product was confirmed for their size on 2.0% agarose gel. The *SRY* gene and pPROEXHTb vector (Invitrogen, Life Technologies, USA) were overnight digested with *Bam*HI and *Kpn*I. The double digested PCR product and vector were set for ligation. The ligated product was transformed into the freshly prepared DH5α competent cells using the standard protocol of Sambrook and Russell[16]. Two recombinant clones were sent for custom sequencing of both strands using gene specific forward and reverse primers (GCC Biotech Pvt. Ltd., Kolkata). The sequence obtained was analyzed by using DNAStar, and the translated data of the sequence was analyzed using ExPaSy tools (http://www.cbs.dtu.dk/services/NetPhos/; web.expasy.org/protparam/).

Expression and confirmation of Sry protein

One of the recombinant clone was chosen for the expression of Sry protein. The mid-log phase culture (OD_{600} reaches 0.5 to 1.0) was induced with 2 mM IPTG. 1 mL of the culture was collected from the tube before induction and kept as an un-induced control. The bacterial culture was collected after 1, 2 and 3 h post induction. All the cultures collected were pelleted by centrifugation at 13000 rpm and stored at −20°C until further analysis. A 12% SDS-PAGE was run with the whole cell lysate of recombinant and non-recombinant clones along with pre-stained marker[17]. Later, the gel was transferred to the nitrocellulose membrane and membrane was developed using Ni-activated HisProbe-HRP conjugate (Thermo Scientific, USA)[18].

Purification of Sry Protein

The 500 mL of over-night grown LB broth culture was induced with 2 mM IPTG and kept for incubation at 37°C for another 2 h. The culture was centrifuged at 3500 rpm for 25 min and the pellet was stored at −20°C for overnight. The pellet was then thawed and resuspended in 4 volumes of the lysis buffer [20 mM sodium phosphate (pH 7.4), 0.3 M NaCl and 1 mg/mL Lysozyme], mixed and kept at room temperature for 1 h on a magnetic stirrer. The lysed bacterial pellet was centrifuged at 12000 rpm for 30 min at 4°C. Both the pellet and supernatant were processed for protein expression. We tried first to purify protein from soluble fraction. The supernatant was passed through the HisPur™ Cobalt Resin (Pierce, USA) after mixing for 1 h. The column was washed with excess of wash buffer [50 mM sodium phosphate (pH 7.4), 0.3 M NaCl]. The bound protein was eluted with 10, 20, 40, 60, 80 and 150 mM imidazole containing wash buffer.

Separately, the pellet was washed 3-4 times with double distilled water. The pellet was mixed in inclusion body solubilisation buffer [50 mM sodium phosphate (pH 7.4), 0.3 M NaCl, 8 M urea] for 1-2 min. The lysate was centrifuged at 12000 rpm at 4°C for 30 min. The supernatant fraction of the pellet was run through the HisPur™ Cobalt Resin (Pierce, USA), the column was washed with excess of wash buffer [50 mM sodium phosphate (pH 7.4), 0.3 M NaCl, 8 M urea]. The bound protein was eluted with 10, 20, 40, 60, 80 and 150 mM imidazole containing wash buffer. Fractions of 1 mL were collected. The fractions showing 0.3 OD_{280} are loaded on SDS-PAGE. The purity of the protein was checked by electrophoresis on 8-12% gradient SDS-PAGE as described earlier and purification of protein was confirmed by Western blotting.

Production of Hyperimmune Sera in Rabbit

The hyperimmune serum was raised by immunizing rabbit with purified recombinant protein. About 100 µg protein in Freund's incomplete adjuvant was inoculated subcutaneously in rabbits over the back and rump. Inoculation was repeated at 14th and 21st d with Freund's incomplete adjuvant. Blood was collected on 28th d and serum was separated. The specificity of the hyper immune serum was confirmed by Western blot. The purified recombinant protein and sperm cell lysate were run on SDS-PAGE and the gel was then transferred to the nitrocellulose membrane. The membrane was then cut into two parts. The first part was developed with Ni-activated HRPase conjugate (His Probe™-HRP, Thermo Scientific, USA) and other part of the blot was developed using Sry hyper immune sera as primary antibody and peroxidase conjugated goat anti-rabbit IgG as a secondary antibody.

Results and Discussion

PCR Amplification, Sequencing and Computational Analysis of *SRY* Gene

The size of the PCR amplified product was approx 680 bp (Fig. 1). The sequence was submitted to the NCBI GenBank database (acc. no. JQ619659). The sequence obtained was aligned and analyzed using Gene Tool software with the published sequences of the SRY gene of *B. taurus* (cattle; acc. no. NM_001014385.1), *B. grunniens* (yak; acc. no. AB077320.1), *Equus caballus* (horse; acc. no. NM_001081810.1), *Ovis aeris* (sheep; acc. no. AY604733.1) and *Canis lupus familaris* (dog; acc. no. NM_001002978.1). The results show that nucleotide sequence of the buffalo *SRY* gene has a homology of 92.7, 99.7, 75.2, 78.4 and 72% with *B. taurus*, *B. grunniens*, *E. caballus*, *O. aeris* and *C. lupus familaries*, respectively. All Sry proteins identified from different species contained an evolutionarily conserved HMG (high-mobility group) DNA-binding domain. And for studying structure, function and evolution of the *SRY* gene, the HMG-box has gained much importance. The published HMG-box sequence of *B. taurus* (acc. no. NM_001014385.1) was blasted with the buffalo *SRY* sequence located at 79 to 315 nucleotides that showed 97% homology. Further, the ExPaSy (web.expasy.org/protparam/) tool was used to predict the composition of the recombinant protein. The recombinant protein was found to have 215 amino acid residues with a predicted mol wt of 28.268 kDa and iso-electric point 9.63. The peptide sequence contained the highest percentage of the serine residue (14%) and was rich in positively charged amino acids (16.74%). Further, the sequence was also analyzed in ExPaSy (http://www.cbs.dtu.dk/services/NetPhos/) for the post-translational modification, which showed 14 serine, 1 threonine and 3 tyrosine phosphorylation sites (Fig. 2). In comparison to cattle (*B. Taurus*), the HMG-box of Sry protein in buffalo (*B. bubalis*) had also shown amino acid substitutions of Val to Ile at 3rd, Val to Leu at 12th, Val to Leu at 21st, Asp to Glu at 32nd, Ala to Ser at 61st, Arg to Lys at 77th positions (Fig. 3).

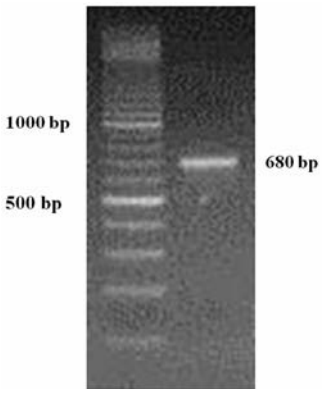

Fig. 1—1% Agarose gel electrophoresis showing ~680 bp amplified product of *SRY* gene. [Lane M: 100 bp DNA ladder; & Lane 1:~680bp amplified product]

The peptide sequence of HMG-box of buffalo *SRY* gene showed complete homology with yak and sheep sequences. On the other hand, HMG-box of Sry protein in *B. bubalis* when compared with *B. Taurus*, it showed amino acid substitutions like leucine for valine, glutamic acid for aspartic acid and lysine for argenine, which have similar chemical properties and structures. Therefore, substitutions seldom alter the function of a protein. However, Persson *et al*[19] have shown that substitution of valine for leucine-305 in FVIIa mutants, mimicking the effect of TF (tissue factor), increases the intrinsic enzymatic activity. Further, substitution of serine for alanine, which has hydroxyl oxygen and forms a hydrogen bond with the protein backbone, are generally a part of protein active sites. Here, a serine accompanied by a histidine and an aspartate (Fig. 4) act as a nucleophile to hydrolyze substrates[20]. This substitution probably indicates more active role of the protein segment. The Sry is a transcription factor, thus the expression of Sry in any cell or tissue has the potential to modify the genes expressed in that cell. If a mutation causes a transcription factor to be expressed in a tissue, the

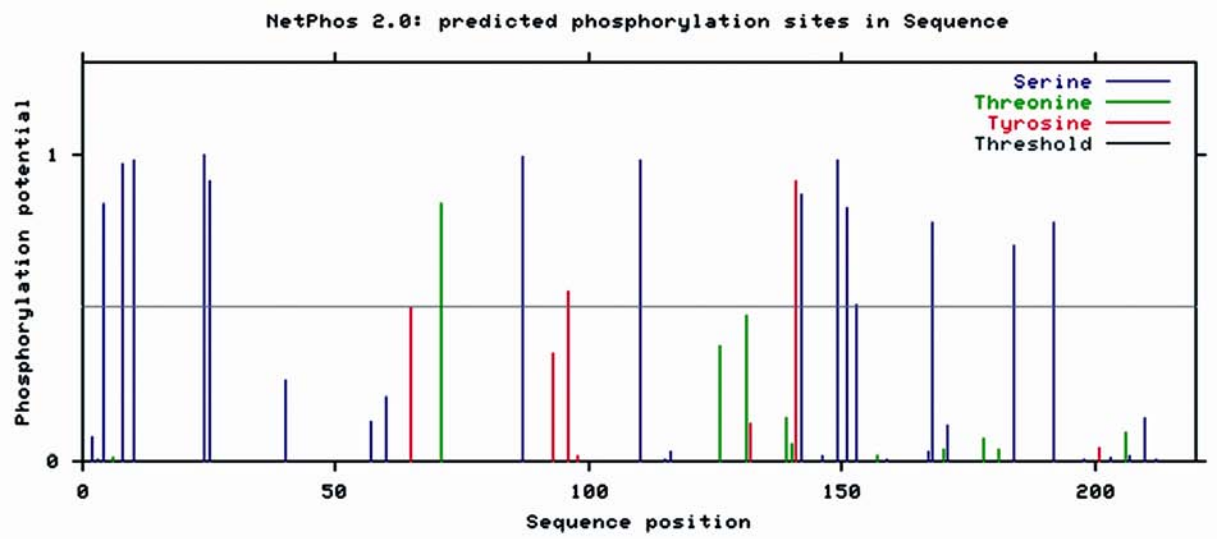

Fig. 2—Post-translational modification showing phosphorylation sites in *SRY* sequence.

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Query 1    DHIKRPMNAFILWSRERRRKLALENPKMKNSEISKQLGYEWKRLTDAEKRPFFEEAQRLL 60
           DH+KRPMNAFI+WSRERRRK+ALE PKMKNS+ISKQLGYEWKRLTDAEKRPFFEEAQRLL
Sbjct 52   DHVKRPMNAFIVWSRERRRKVALEYPKMKNSDISKQLGYEWKRLTDAEKRPFFEEAQRLL 111

Query 61   SIHRDKYPGYKYRPRRKA  78
           +IHRDKYPGYKYRPRR+A
Sbjct 112  AIHRDKYPGYKYRPRRRA  129
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Fig. 3—Alignment report of SRY-HMG box of *B. taurus* (Query 1-78) with *B. Bubalis* (Subject 52-129).

transcriptome of that cell or tissue may change and this change might have either subtle or large effects on the phenotype of that cell or tissue. Thus the substitution of serine for alanine in HMG-box would probably make buffalo Sry functionally more active than cattle Sry protein

Expression and Purification of Recombinant Protein

Colonies with recombinant plasmid vector were checked for protein expression (Fig. 4a) and the expression of protein was confirmed by Western blotting (Fig. 4b). The recombinant fusion protein Sry showed a mol wt of 27.19 kDa. The expressed recombinant protein formed inclusion bodies as it was coming in cell pellet after lysis of the bacteria. The pellet after cell lysis got dissolved in inclusion body solubilization buffer that was then loaded onto the Hispur Cobalt Resin (Thermo Scientific, USA). The recombinant protein got eluted in wash buffer containing 10 mM imidazole (Fig. 4c). Further confirmation of the purified protein was done by Western blotting (Fig. 4d).

The hyper immune serum was raised against the recombinant Sry protein and an SDS-PAGE was run with recombinant Sry protein and sperm cell lysate. The gel blotted on nitrocellulose membrane showed a band of 27.19 kDa with recombinant protein and a 31 kDa band with sperm cell lysate (Fig. 5). The native Sry in sperm cell lysate reacted with hyper immune sera that showed a band at 31 kDa. The lesser mol wt of the recombinant protein might be because of the loss of few N-terminal amino acid residues while designing expression primers. Another possibility of getting higher mol wt of the native protein might be the post-translational modifications of the naturally occurring Sry protein in the spermatozoa. However, the cited present literature did not show any evidence of the post-translation modifications of Sry protein. Further, analysis of sequence for post-translational modification using bio-informatics tool ExPASy (http://www.cbs.dtu.dk/services/NetPhos) had shown 14 serine, 1 threonine and 3 tyrosine phosphorylation sites but none of the glycosylation sites. The proteins without signal peptide

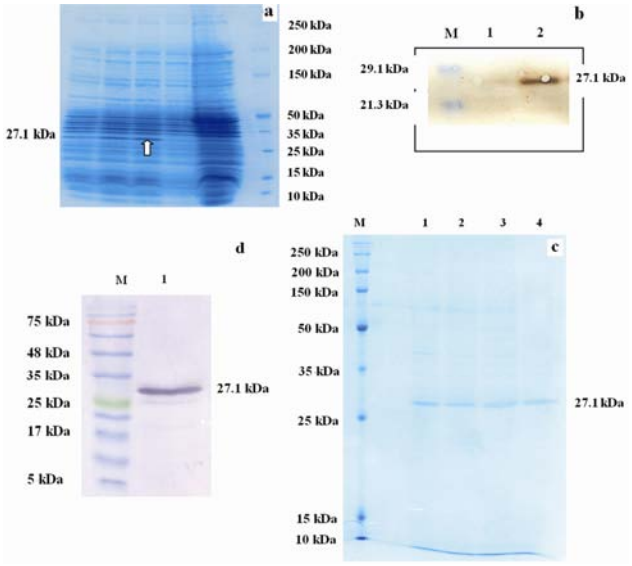

Fig. 4 (a-d)—a. 12% SDS-PAGE showing expression of recombinant Sry protein in DH5α cells at different time intervals induced with 2 M IPTG [Lanes 1-3: Culture pellet after 3, 2 and 1 h post induction, respectively; Lane 4: Non-recombinant clone induced with 2 mM IPTG for 1 h; Lane 5: Un-induced culture pellet; & Lane M: Mol wt marker (NOVAGEN)]; b. Western blot analysis of expressed Sry protein using HisProbe-HRP conjugate [Lane M: Pre-stained mol wt marker (NOVAGEN); Lane 1: Non-recombinant cell pellet; & Lane 2: Recombinant cell pellet]; c. 12% SDS-PAGE showing purified recombinant Sry fusion protein [Lane M: Pre-stained mol wt marker (NOVAGEN); & Lanes 1-4: Purified Sry protein]; d. Western blotting of purified recombinant Sry fusion protein [Lane M: Pre-stained mol wt marker (Biogene); & Lane 1: Purified Sry protein].

are unlikely to be exposed to the glycosylation machinery. Moreover, *SRY* is a marker gene present on Y-chromosome and it may be used for differentiation of Y-chromosome from X-chromosome bearing spermatozoa. By amplification of the SRY HMG-box, Ng *et al*[21] and Lu *et al*[22] have developed a rapid PCR for sex determination of embryo in ovine and bovine, respectively.

Further studies are required to raise monoclonal antibodies against the recombinant Sry protein that can be used for labelling the Y-chromosome bearing spermatozoa, which can be used for assessing the enrichment of Y-chromosome bearing spermatozoa in sex sorted semen. Previous studies clearly show that testes determination may not be the only function of Sry protein as it is expressed in brain, kidney and adrenal gland of adult males[23]. Therefore, the recombinant protein can be used for studies like protein-protein and protein-DNA interaction to understand other functions of Sry in different tissues and organs.

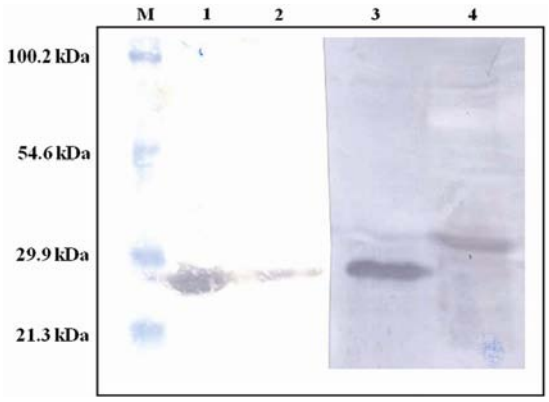

Fig. 5—Western blotting of purified recombinant Sry fusion protein and native sperm Sry protein. [Lane M: Mol wt marker (GBioscience); Lane 1: Pellet containing Sry protein of size ~27.1 kDa; Lanes 2 & 3: Purified recombinant Sry protein of size ~27.1 kDa; Lane 4: Sry protein from buffalo sperm cell (~31 kDa)]

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