Asian-Aust. J. Anim. Sci.
Vol. 24, No. 10 : 1358 - 1364
October 2011

www.ajas.info
http://dx.doi.org/10.5713/ajas.2011.11062

Detection of the SRY Transcript and Protein in Bovine Ejaculated Spermatozoa*

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ABSTRACT : The sex-determining region on the Y (SRY) gene is important in mammalian sex determination and differentiation. We report a study of the abundance of SRY gene products in bovine ejaculate. RT-PCR experiments using RNA extracted from bovine spermatozoa with SRY-specific primers yielded a 456 bp product, but the amount of SRY mRNA in sperm was lower than that in the testes ($p<0.01$). A protein of approximately 27 KDa was detected by western blotting. The SRY transcript was detected in the midpiece of approximately half the spermatozoa by *in situ* hybridization, and the SRY protein was detected in the heads of half the spermatozoa by immunofluorescence, indicating that SRY mRNA and protein may only be present in Y-bearing spermatozoa. These results suggest that the SRY transcript and protein are present in bovine ejaculated Y-sperm. The roles of the SRY gene in spermatogenesis, sperm motility, and the sperm-oocyte interaction merit further investigation. (**Key Words :** SRY Gene, Bovine Spermatozoa, *In situ* Hybridization, Bovine)

INTRODUCTION

Mammalian ejaculated spermatozoa are highly differentiated terminal cells with extremely compact nuclei and haploid genomes. They are regarded as dormant cells, because they are incapable of transcribing DNA or translating encoded mRNA. However, recent advances in molecular biology, suggests that despite this, they contain specific and complex mixtures of RNAs (Gilbert et al., 2007). Although the presence of mRNA in spermatozoa is well established, little is known about its function (Dadoune et al., 2005). Given the inability of the spermatozoon to synthesize RNA, it is assumed that its RNA content is residual material synthesized during spermiogenesis. Because of the delay between transcription and translation during spermatogenesis, it has been proposed that the mRNA population present in spermatozoa should be representative of the past events of spermatogenesis (Ostermeier et al., 2002; Dadoune, 2003; Steger, 2003; Lambard et al., 2004).

Mammalian sex determination and differentiation are a complex processes that depend on the expression of specific genes in a spatially and temporally well-defined manner. With the advance of molecular cloning techniques, some of the Y-chromosome genes associated with spermatogenesis have been identified. The SRY gene is on the short arm of the Y chromosome, which is expressed in the genital ridges at the onset of gonadal differentiation in both mouse and man (Hacker et al., 1995; Hanley et al., 2009). The gene is mutated in a subset of 46XY females and, when transgenic techniques were used to introduce the mouse SRY gene into XX karyotype mouse embryos, phenotypic male mice were generated (Koopman et al., 1991). It is now generally accepted that SRY is the testis-determining factor (TDF) or

* This work was supported by the National High Technology Research and Development Program ("863" Program) of China (No.2008AA101003), the National Science & Technology Pillar Program of China (No.2008BADB2B09), and the Project for Science and Technology Development of Jilin Province, P.R. of China (No. 20070210). Science and Technology Development Project of Changchun City (08GH08).
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Received March 10, 2011; Accepted May 10, 2011

switch for the male phenotype in mammals (Goodfellow and Lovell-Badge, 1993). Although SRY has been unequivocally identified as the TDF in mammals, its roles beyond sex determination are unclear. Recently, it has been recognized that mRNAs of genes that are expressed in the testis are also present in the mature spermatozoa (Ostermeier et al., 2002), these transcripts seem to be stored and it has been hypothesized that they fulfill some function after fertilization (Ostermeier et al., 2004). SRY and DAZL mRNA have been detected in mature human spermatozoa. In light of these results, it was deemed worthy to see whether this phenomenon occurs in other mammals. To answer this question, we sought to determine whether SRY transcripts are present in mature bovine spermatozoa.

MATERIAL AND METHODS

Spermatozoa preparation

Semen in 0.25 ml straws was thawed for 60 s at 37.5°C and motile spermatozoa were collected by swim-up (Parrish et al., 1986). In brief, four 0.25 ml aliquots of thawed semen were layered under l ml aliquots of the medium Sp-TALP medium (Parrish et al., 1986) in 12×55-mm plastic tubes. After 1 h at 37°C under an atmosphere containing 5% CO_2 incubation, the top 0.85 ml of medium from each tube was removed and pooled in a 15 ml conical centrifuge tube. The swim-up separated spermatozoa were then diluted with Sp-TALP to a final volume of 5 ml and centrifuged at 120 g for 10 min. The supernatant was discarded and the sperm pellet was diluted to 5 ml with Sp-TALP, then centrifuged again at 120 g for 10 min. The supernatant was once again discarded, yielding a sperm pellet with a concentration of $>20\times10^6$/ml.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted using an RNAprep Micro Kit (TIANGEN, China), and then frozen at -80°C until used. Total RNA sample pellets were dissolved in water and then adsorbed on an RNA extraction column that was subjected to treatment with RNAse-free, DNAse I treatment (TIANGEN, China) to eliminate contaminating genomic DNA from the samples. Reverse transcription of all RNA samples was carried out with a commercial kit (Promega) and the cDNA was frozen until used. Amplification of SRY transcripts was achieved by PCR using the SRY gene-specific primers listed in Table 1. The PCR was carried out in a final volume of 25 μl containing 100 ng cDNA, 2.5 μl of 10×Buffer, 0.2 μM of each primer, and 0.20 units of rTaq polymerase (TaKaRa Biotechnology). The basic PCR amplification profile consisted of an initial 4 min denaturation period at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C, with a finally 6 min extension period at 72°C. 10 μl of the product solution was run on a 1% agarose gel, stained with ethidium bromide and observed under a UV transilluminator. RNA samples incubated without reverse transcriptase served as negative controls. The PCR products were gel eluted and sequenced with an ABI 377 DNA sequencer using the M-13F and M-13R primers.

Detection of Leukocyte contamination

Leukocyte contamination was evaluated by PCR using a set of primers targeting the CD45 antigen sequences (Gilbert et al., 2007). Total RNA was also extracted from a blood sample using Trizol (Invitrogen) in accordance with standard procedures. 18s RNA was used as a positive control in all experiments. The primers used are listed in Table 1.

Quantitative real-time PCR

Real-time PCR was carried out using an ABI PRISM 7000 instrument according to the manufacturer's instructions. The amplification reactions were performed in

Table 1. List of primers used for PCR

Primer name	Sequence 5'-3'
Normal PCR	
SRY-F	AAGGGGAGAACATGTTAGGGAGAG
SRY-R	GCATCGGGTTGCATAGTAT
CD45-F	GACATCGCAGTGTTTGTTGC
CD45-R	GGAGGTTCACATTCCTCTCG
18S-F	GCTCGCTCCTCTCCTACTTG
18S-R	GATCGGCCCGAGGTTATCTA
Real time-PCR	
SRY-F	GAAAATAAGCACAAGAAAGTCCAGG
SRY-R	CAAAAGGAGCATCACAGCAGC
SRY-probe	TCCGCCGAAATCCGTGTAGCCA
GAPDH-F	GGCGCCAAGAGGGTCAT
GAPDH-R	GGTGGTGCAGGAGGCATT
GAPDH-probe	TACTTCTCGTGGTTCACGCCCATCACA

a final volume of 25 μl containing 100 ng of cDNA obtained from ejaculated sperm, 0.15 μl of Ex Taq HS (TakaRa), 2.0 μl of 10×Ex Taq Buffer (TakaRa), 2.0 μl of dNTP Mixture (containing each dNTP at a concentration of 2.5 mM) (TakaRa), 0.5 μl of primers (10 μM), 0.5 μl of TAMRA-FAM (10 μM), toped up with distilled water. The basic PCR amplification profile consisted of a 6 min denaturation period at 95°C followed by 48 cycles of 30 s at 94°C, 30 s at 59°C. The primers and probes using in the Real-time PCR experiments are listed in Table 1. To normalize the amount of expressed SRY mRNAs, the internal housekeeping gene GAPDH was also amplified, each cDNA product was tested in triplicate.

Sperm fluorescent ISH

The hybridization probe was synthesized and labeled using DIG-dUTP as described by the supplier (TBD Science, China). Before hybridization, spermatozoa were spread on 3-aminopropyltriethoxysilane-coated glass slides, air-dried and stored at -20°C until used. The slides were fixed using a PBS solution containing 2% acetic acid and 2.5% formaldehyde for 20 min at room temperature. The slides were dehydrated in ethanol and incubated in xylenes for 15 min at room temperature. The cellular proteins were digested with 0.01% pepsin (PH 4.0; Sigma) for 20 min at 37°C. The cells were then refixed in 2% formaldehyde and dehydrated. These cells were used for fluorescent *in situ* hybridization as described previously (Modi et al., 2003a; Modi et al., 2003b). Briefly, the probe was diluted at a concentration of 2 μg/μl in hybridization cocktail (50% formamide, 4×SSC, 10% dextran sulphate, 0.25% yeast tRNA and 0.25% Herring sperm DNA), and applied to the slides. The cover slip was sealed with rubber cement and kept at 37°C for 10 min for hybridization. Hybridization was carried out at 37°C overnight in a humidified chamber. The slides were stringently washed in 0.4×SSC at 70°C for 3 min followed by a wash in 2×SSC for 3 min at room temperature. Blocking was carried out in 0.2% normal sheep serum (Sigma) for 1 h followed by incubation with a diluted (1:100) solution of fluorescein isothiocyanate (FITC)-conjugated anti-digoxienin anti body (Roche) for 2 h at 37°C. The slides were washed extensively to remove unbound antibody and then subjected to signal amplification using the FITC Signal Amplification Kit (Roche). The signals were visualized using a fluorescence microscope with appropriate filters (Olympus, Japan).

Western blotting

Total protein was extracted from the spermatozoa using a kit (APPLYGEN, China), according to the manufacturer's instructions. Approximately 50 μg proteins were boiled for 5 min, separated by SDS-PAGE on 10% polyacrylamide Tris-glycine under denaturing conditions, and transferred to PVDF membranes (Millipore) using a transfer system at 90 V for 2 h. The membranes were washed with Tris-buffered saline containing 0.5% of Tween 20 (TBS-T), and then blocked with 5% nonfat dry milk in TBS-T for 1 h at room temperature. The membranes were incubated overnight with SRY monoclonal antibody (Santa Cruz, USA) diluted 1:300 in PBS at 4°C. The membranes were washed three times for 15 min each with TBST, and then incubated with HRP-conjugated goat anti-mouse IgG secondary antibody diluted 1:5,000 in TBS for 2 h at room temperature. The membranes were then washed several times, visualized using the SuperSignal substrate (Pierce), and then exposed using X-ray films.

Immunolocalization

Spermatozoa collected by the swim-up technique were smeared on microscope Slides, air dried at room temperature, fixed with absolute methanol for 10 min at -20°C, and permeabilized with phosphate-buffered saline-Triton X-100 (0.3%) (Sigma) for 5min at room temperature. The slides were blocked with normal goat serum (10%) in PBS at room temperature for 30 min. They were then incubated with mouse antihuman SRY monoclonal antibody diluted 1:100 in PBS at 4°C for 24 h. The slides were washed three times with PBS-T (0.5% Tween 20 in PBS) for 5 min each, and then exposed to goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (1:100) (BOSTER, China). Sperm cells incubated with BSA were used as negative controls. The slides were examined under a fluorescence microscope (Olympus). All experiments were carried out in duplicate and at least 200 cells per slide were counted.

Statistical analyses

The data for real-time PCR were presented as the means ±SEM. The differences in mean values were calculated using one-way ANOVA (as implemented in SPSS 13.0. software), and Dunnet's test was applied for multiple comparison. A value of $p<0.05$ was taken to indicate a statistically significant difference between means.

RESULTS

Expression of SRY transcripts in the ejaculated spermatozoa

SRY mRNA was detected in RNA from mature spermatozoa by RT-PCR. A specific band of the predicted size was observed in the sperm and testis samples (positive control). No bands were visible when reverse transcriptase was omitted from the reaction mixture (Figure 1), and cDNA of CD45 was not detected in the sperm RNA

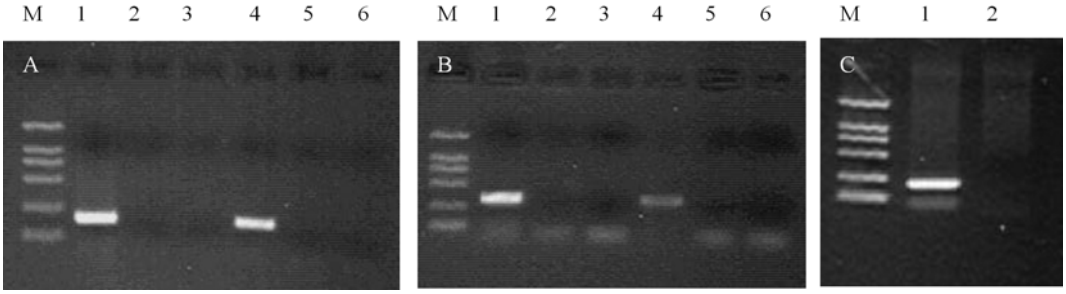

Figure 1. A. The 18S product was detected in the testes (lane 1) and spermatozoa (lane 2); no bands were detected in the negative controls (without reverse transcriptase) in lanes 2 and 5 or in lanes 3 and 6 (in which water was used as the template). B. The SRY product was detected in the testes (lane 1) and spermatozoa (lane 4); no bands were detected in the negative controls (without reverse transcriptase) in lanes 2 and 5 or in lanes 3 and 6 (in which water was used as the template). C. The test for CD45 contamination: a band was detected in the blood RNA (lane 1) but not in the spermatozoal RNA (lane 2).

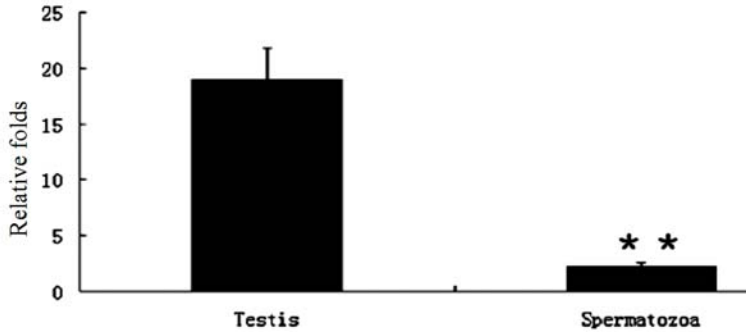

Figure 2. Expression of the SRY transcript in testis and spermatozoa. The amount of SRY mRNA in sperm was lower than that in testis ($p<0.01$). The asterisks indicate significant differences ($p<0.01$).

preparation, indicating that the SRY transcript detected in the sperm RNA by RT-PCR did not originate from somatic cell contamination.

The sequence of these RT-PCR products was found to be completely homologous to that of the corresponding region in the SRY gene (NM_001014385), suggesting that SRY mRNA is expressed in mature bovine spermatozoa. A real-time PCR assay was used to quantify the amount of SRY mRNA in cattle sperm and testes; it was found to be less abundant in the sperm ($p<0.01$) (Figure 2).

Cellular localization of SRY transcripts

In order to confirm the presence of the SRY transcript in bovine mature spermatozoa and to determine the cellular localization of these transcripts, sperm smears were subjected to fluorescent *in situ* hybridization with a dig-labeled cDNA probe. After signal amplification, positive signal s were detected in the midpiece region of approximately 50% of the spermatozoa (Figure 3). No signal was detected when the probe was omitted from the experiments (Figure 3). These results suggest that SRY transcripts are stored in the midpiece region of Y-bearing

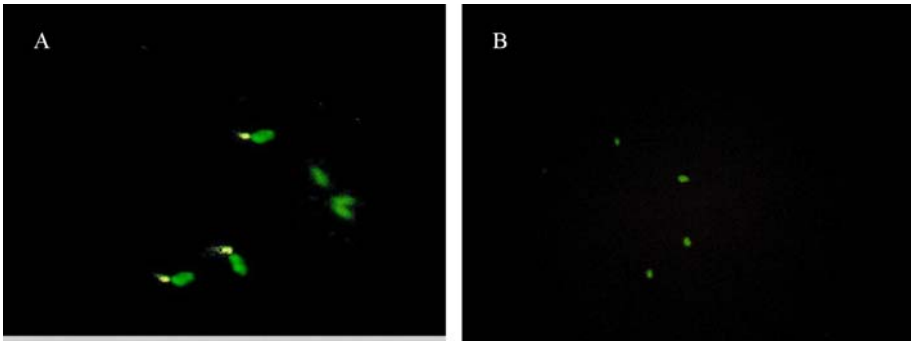

Figure 3. *In situ* localization of SRY in ejaculated spermatozoa. SRY mRNA was detected in the midpiece region of approximately half the spermatozoa by fluorescent *in situ* hybridization (A yellow signals). No signals were detected in the negative controls (B).

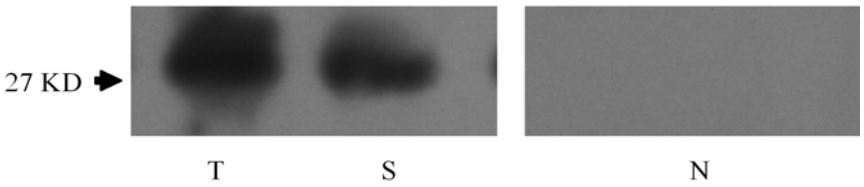

Figure 4. Western blotting of SRY proteins. A specific band was detected in the bovine ejaculated spermatozoa (S). T indicates positive control bands, corresponding to protein from the testes. A negative control was performed in which the antibody was replaced with normal goat serum (N).

spermatozoa.

Western blotting of the SRY protein

The presence of the SRY protein in sperm was investigated by western blotting with mouse antihuman SRY monoclonal antibody. A single band was detected in sperm protein extracts (Figure 4), no bands were visible in negative controls in which the antibody was replaced by normal goat serum (Figure 4).

Immunolocalization of SRY protein in bovine sperm

Immunofluorescence experiments using sperm smears and mouse antihuman SRY monoclonal antibody were performed to investigate the abundance and localization of the SRY protein in bovine mature spermatozoa. The SRY protein was identified in the heads of 50% of the mature bovine spermatozoa examined (Figure 5). No staining was visible in X-sperm or negative controls in which the primary antibody was omitted from the reaction mixture (Figure 5).

DISCUSSION

The SRY gene plays an important role in sex determination in mammals (Koopman et al., 1991; Goodfellow and Lovell-Badge, 1993). It has previously been shown that SRY transcripts can be detected in human midtrimester fetal, infantile, and adult testes and in ejaculated spermatozoa, indicating that SRY has other roles beyond sex determination (Modi et al., 2005). The results reported herein constitute the evidence demonstrating that SRY mRNA and protein are present in bovine ejaculated spermatozoa.

RT-PCR experiments using sperm RNA with specific primers for the SRY gene produced a single band of the expected size. The sequence of the RT-PCR product was completely homologous to that of the corresponding region of the full-length SRY cDNA sequence. The product did not originate from any contaminants in the semen samples, because the RNA was extracted from a motile sperm population collected using a swim-up protocol, and the sample was subjected to RT-PCR using specific primers for CD45 to rule out the possibility of any cellular contamination. Comparing to testis, the abundance of SRY mRNA in spermatozoa seems to be low. In order to corroborate the RT-PCR results and determine the cellular localization of SRY transcript, the bovine ejaculated spermatozoa were subjected to ISH; it was found that the SRY mRNA was expressed in the midpiece of nearly 50% of the ejaculated spermatozoa. These results are consistent with those of a study on the SRY gene in humans (Modi et al., 2005), in which it was suggested that the 50% of cells positive for SRY mRNA might be the Y-bearing spermatozoa. Thus, both our results and those of others suggest that the mRNA that is present in mammalian spermatozoa is located in the midpiece (Modi et al., 2005; Shah et al., 2005). However, it has also been reported that suggest that mRNA may be present throughout the entirety of the heads of the spermatozoa (Dadoune et al., 2005). It may be that the mRNA of different genes exhibit different

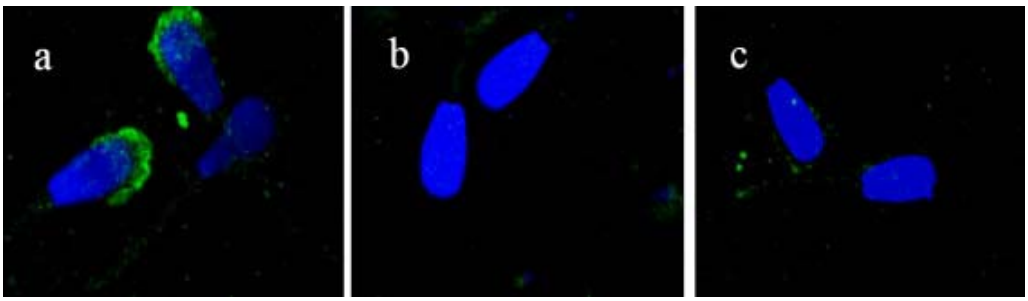

Figure 5. Immunolocalization of SRY proteins in bovine ejaculated spermatozoa. SRY was expressed in the acrosome region of bovine ejaculated spermatozoa (a). No signal was detected in the X-sperm (b) or the negative control (c), indicating that SRY was only expressed in the acrosome regions of Y-sperm.

localization patterns within the spermatozoa, in keeping with the different functions of the corresponding genes.

Although it is now widely accepted that under normal circumstances, spermatozoa are transcriptionally silent, reverse transcriptase activity has been observed in mature spermatozoa (Giordano et al., 2000), as has active translation of stored mRNAs (Gur and Breitbart, 2006). Some researchers have detected both mRNAs and the corresponding proteins in mature spermatozoa (Goodwin et al., 2000a; Goodwin et al., 2000b; Lin et al., 2002; Shah et al., 2005; Zuccarello et al., 2007). The SRY transcript has been detected by ISH and RT-PCR in the spermatogonia, spermatocytes and round spermatids, and the SRY protein in the germ cells, indicating that SRY may play a role in spermatogenesis in addition to functions in testicular development and maturation during fetal life and infancy (Modi et al., 2005). In the study, described herein, the SRY protein was detected in the spermatozoa by western blotting; immunolocalization experiments showed that it was only present in the acrosome region of Y-containing sperm. It still remains to be determined whether this SRY protein originates from translation of the SRY mRNA in ejaculated spermatozoa or whether it is residual material from spermatogenesis. Human ejaculated spermatozoa have been observed to express and secrete insulin and leptin, demonstrating that they can regulate their metabolism by means of autocrine signaling (Aquila et al., 2004; Aquila et al., 2005). Insulin and leptin can increase the activity of the phosphoinositide-3 kinases (PI3K) which are important intracellular mediators of cell survival and anti-apoptotic signals (Parsons et al., 2004). We have recently shown that bovine ejaculated spermatozoa can secrete BDNF, which plays an important role in sperm physiology (Li et al., 2010). These results suggest that complex events (transcription and translation) can occur in the ejaculated spermatozoa to sustain the survival and motility of sperm. It is clear that much remains to be learned about the origin and roles of SRY gene products in bovine ejaculated spermatozoa. Many studies have shown that certain special RNAs in the spermatozoa influence embryonic development (Ostermeier et al., 2002), and that sperm motility is related to the mRNA content of the spermatozoa (Ostermeier et al., 2002; Lambard et al., 2004); these findings suggest that spermatozoal RNA could be used as a molecular marker for studying infertility.

In conclusion, we have demonstrated the presence of SRY transcripts and protein in bovine ejaculated Y-spermatozoa. Our results suggest that SRY may have important roles in spermatogenesis and the survival of Y-sperm above and beyond its roles in sex differentiation. The importance of SRY in male reproductive physiology thus clearly merits further investigation.

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