

# Ontogeny and cellular localization of SRY transcripts in the human testes and its detection in spermatozoa

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## Abstract

The sex-determining region on the Y (SRY) gene is unequivocally designated as the testis-determining factor in mammals; however, its roles beyond sex determination, if any, have been hitherto unknown. To determine whether SRY has any roles beyond sex determination, herein the expression of SRY mRNA was investigated in the midtrimester human fetal, infantile and adult testes as well as in ejaculated spermatozoa. High levels of SRY transcripts were *in situ* localized to the Sertoli cells of the developing testis at 9 weeks of gestation, and the expression persisted at comparable levels throughout the midtrimester (until 22 weeks) and also in the testis of an infant at 3 months of age. The germ cells and other somatic cells in the testes of fetuses and the infant were negative for SRY expression. The mRNA for SRY was detected in the spermatogenic cells, particularly the spermatogonia and the round spermatids; the expression was negligible in the meiotic stages. A single transcript of ~1.2 kb was detected in the adult testes and isolated spermatogonial cells. In the adult testis, *in situ* hybridization (ISH) studies revealed a switch in the cellular localization of SRY transcripts. SRY transcripts were also demonstrable by RT-PCR of RNA from ejaculated human spermatozoa. ISH revealed the presence of SRY transcripts in the midpiece of 50% of ejaculated sperm. These results suggest that SRY may have extensive roles in male reproductive physiology, such as maturation of fetal testis, spermatogenesis, sperm maturation and early embryonic development.

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## Introduction

Mammalian sex determination and differentiation is a complex process that depends on coordinated expression of specific genes in a strictly temporal and spatial manner. Extensive molecular and cytogenetic studies in man and other mammals have identified the SRY (sex-determining region on Y) gene on the short arm of the Y chromosome, which fulfills the genetic and conceptual requirement of a testis-determining factor (TDF). SRY is appropriately expressed in the genital ridges at the onset of gonadal differentiation in both mice and man (Hacker *et al.* 1995, Hanley *et al.* 2000); the gene is mutated in a subset of 46XY females and is detected in the genome of most XX males (reviewed in Haqq & Donahoe 1998, Harley *et al.* 2003). That XX mice transgenic for the SRY gene develop as males and exhibit male mating behavior (Koopman *et al.* 1991) is the ultimate validation of equating SRY to the TDF.

Although SRY has been unequivocally designated as the TDF in mammals, its roles beyond sex determination, if any, are unclear. As an architectural transcription factor, SRY has been demonstrated to bind and bend DNA to

initiate transcription (Haqq *et al.* 1994, Haqq & Donahoe 1998, Harley *et al.* 2003); it has also been reported to play a role in mRNA splicing (Ohe *et al.* 2002), indicating that it may have multiple functions.

Evidence is now accumulating of the additional roles of SRY in the human testis. Unlike in the mouse, the temporal expression of SRY in man is not restricted to a strict window period during testicular organogenesis. Studies in a limited number of human fetal and adult testicular samples have demonstrated the presence of SRY transcripts (Clepet *et al.* 1993, Hanley *et al.* 2000, Olesen *et al.* 2001). However, the cellular localization of the mRNA, particularly in the adult testis, is unknown. Although the expression of SRY protein has been reported in the Sertoli cells and the germ cells of fetal and adult testis (Salas-Cortes *et al.* 1999, 2001), the number of tissues examined has been limited, and the detailed description of the ontogeny and cellular localization is not available.

Thus, the present study aimed to examine the ontogeny and cellular localization of SRY mRNA in the male human gonads. Recently, it has been recognized that the

mRNA of genes that are expressed in the testis is also present in the mature spermatozoa (Ostermeier *et al.* 2002); these transcripts are apparently stored and are hypothesized to have functions after fertilization (Ostermeier *et al.* 2004). In view of this concept, the study was extended to determining the presence of SRY transcripts in mature spermatozoa.

## Materials and Methods

The ethics committee of King Edward Memorial Hospital (KEMH), Mumbai, India, where the tissues/semen samples were collected, approved the study. These tissues were collected after informed consent and were a part of previous studies (Modi 2001, Modi *et al.* 2003a, 2003b, Shah *et al.* 2005).

### Tissues

The collection of the fetal samples has been detailed previously (Modi *et al.* 2003b). Normal human fetal gonads were collected from legal abortions done at the Obstetric and Gynecology Department of KEMH. In all cases, anthropometric measurements were done according to the published instructions; the age of the fetus was estimated on the basis of the date of last menstrual period and/or the foot length (Robinson 1995).

Neonatal ( $n = 1$ ) and adult testes ( $n = 6$ ) were obtained from deceased subjects (aged 3 months–55 years) undergoing autopsy at KEMH. The gonads were collected within 3–6 h of death; the reproductive history of any of the deceased was not available. Semen samples were pooled from healthy male volunteers that had normal sperm count and morphology.

### Tissue processing

Testes from all fetuses were immediately fixed in 10% buffered formaldehyde for 24 h and processed for routine paraffin embedding and sectioning as described previously (Modi *et al.* 2003b); at all stages, care was taken to avoid RNase contamination. Sections 5  $\mu\text{m}$  thick were mounted on 3-aminopropyltriethoxysilane-coated, nuclease-free glass slides and dried at 37 °C for 24 h prior to use.

Adult testis was freed of tunica, and pieces were fixed in formaldehyde and processed as above. The remaining testicular tissue was immediately frozen in aliquots at –70 °C until use. Spermatogenic cells were isolated from the adult testes according to published protocol (Salhanick & Terner 1979). Briefly, the seminiferous tubules were separated and rinsed several times in autoclaved distilled water. The tubules were then lightly homogenized in TEMGED buffer (10 mM Tris, pH 7.4; 1.5 mM EDTA; 10% glycerol; 25 mM sodium molybdate; and 1 mM DTT), and the homogenate was passed through glass wool. The filtrate obtained was centrifuged at 3000 r.p.m., and the pellet was washed twice in 0.01 M PBS. The pellet contained

spermatogenic cells and was frozen in aliquots at –70 °C until use.

The semen samples were processed as described previously (Sachdeva *et al.* 2000). Briefly, 60 million spermatozoa were overlaid with 1 ml Dulbecco's Modified Eagle's Medium (Sigma) and incubated at 37 °C for 60 min, and the supernatant was centrifuged to obtain motile spermatozoa. The samples were checked under a microscope to rule out any contamination from leukocytes or other cellular material, and then frozen at –70 °C until use.

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from spermatozoa ( $n = 12$ ), spermatogenic cells ( $n = 4$ ) and whole testis ( $n = 4$ ) with the TRIZOL reagent (Gibco BRL, NY, USA), according to the manufacturer's instructions, and processed as detailed in the earlier study (Sachdeva *et al.* 2000). Briefly, 5  $\mu\text{g}$  RNA sample were treated with Rnase-free DNase 1 solution (Roche) for 1 h at 37 °C followed by heat inactivation of the enzyme. RNA from spermatozoa collected from four men (after swim up) was pooled for reaction. Three independent pools were analyzed for the present study. Reverse transcription of all RNA samples was carried out with a commercial kit (Gibco BRL) in a 20  $\mu\text{l}$  reaction volume containing 2.5 mM random hexamers and 200 U superscript reverse transcriptase for 1 h at 42 °C. Reverse transcriptase was denatured at 99 °C for 5 min, and the cDNA was frozen until use.

Amplification of SRY transcripts was achieved by PCR with the cDNA as template and commercially synthesized SRY gene-specific primers, as described in Table 1. These primers span 472 bp of the SRY gene, encompassing the HMG box of the SRY protein. For PCR, 2  $\mu\text{l}$  cDNA (8–10  $\mu\text{l}$  for sperm) were amplified in a final volume of 25  $\mu\text{l}$  PCR mix containing 10 mM Tris/HCl buffer, pH 8.3; 50 mM KCl; 1.5 mM  $\text{MgCl}_2$ ; 30 pmol of each primer; and 2 U of Taq polymerase (Gibco BRL). Amplification was carried out in a PCR machine (MJ Research, MA, USA) for 30 cycles, using the following cycling conditions: 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 5 min. A volume of 15  $\mu\text{l}$  of the products was run on 2% 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 automated fluorescence-based sequencer at the core sequencing facility at the institute.

The purity of spermatogenic cells was analyzed by determining the mRNA expression of follicle-stimulating hormone receptor (FSHR) and steroid acute regulatory (StAR) protein as Sertoli and Leydig cell markers respectively. The primers (Table 1) used for amplification of FSHR and StAR were commercially synthesized (Banglore Genie, India, or Gibco BRL). PCR was carried out with

**Table 1** Sequences of the primers/probes used for detection of SRY/FSHR/Star/18S genes.

Genes	Sequence	Annealing temperature (°C)	Predicted size (bp)
SRY (F)	5' GAATATCCCGCTCTCCGGA 3'	53	472
SRY (R)	5' GCTGGTCTCCATTCTTGAG 3'		
SRY (AS probe)	5' AGTGGCCTAGCTGGTCTCCATTCTTGAGTGTGTG 3'	42	–
SRY (S probe)	5' CACACACTCAAGAATGGAGCACCAGCTAGGCCACT 3'		
FSHR (F)	5' GCGAATTCAGATGGCTGAATAAG 3'	53	86
FSHR (R)	5' GCGGATCCGCTCATCTAGTTGGGGTTC 3'		
Star (F)	5' GTGGTAGACCAGCCCATGGA 3'	53	592
Star (R)	5' GCTCTGATGACACCACTCTGC 3'		
18S (F)	5' CGATGCTCTTAGCTGAGTGT 3'	55	315
18S (R)	5' GGAACACTACGCGTATCTGA 3'		

F: forward primer; R: reverse primer; bp: base pair; AS: anti-sense; S: sense.

testicular and spermatogenic cell cDNA as template, as described above. Leukocyte contamination of the sperm RNA preparation was ruled out by amplification of CD44, as described previously (Sachdeva *et al.* 2000). 18S RNA was used as positive control in all experiments.

### Northern hybridization

Twenty micrograms of testicular and spermatogenic RNA were fractionated in 1.2% formaldehyde gels and transferred to nylon membrane (Roche). The membranes were prehybridized in dig-easy hybridization granules (Roche) at 50 °C for 5 h. The probe for hybridization was prepared by PCR amplification of male genomic DNA with SRY gene-specific primers (described above). PCR was performed as described above except that 500 ng male genomic DNA were used for the template, and amplification was carried out for 35 cycles. The PCR products were gel eluted (Millipore, MA, USA) and labeled with <sup>32</sup>P (BRIT, Hyderabad, India) by random prime labeling (BRIT). After labeling, free nucleotides were removed by the nucleospin columns (Clontech). Hybridization was carried out overnight in a shaker incubator at 50 °C with <sup>32</sup>P-labeled PCR product. After hybridization, the membranes were washed thrice in 2 × SSC with 0.1% SDS at room temperature, and then thrice for 15 min in 0.5 × SSC with 0.1% SDS at 50 °C. The membranes were exposed to radiographic film at –80 °C for 96 h and developed.

### In situ hybridization (ISH)

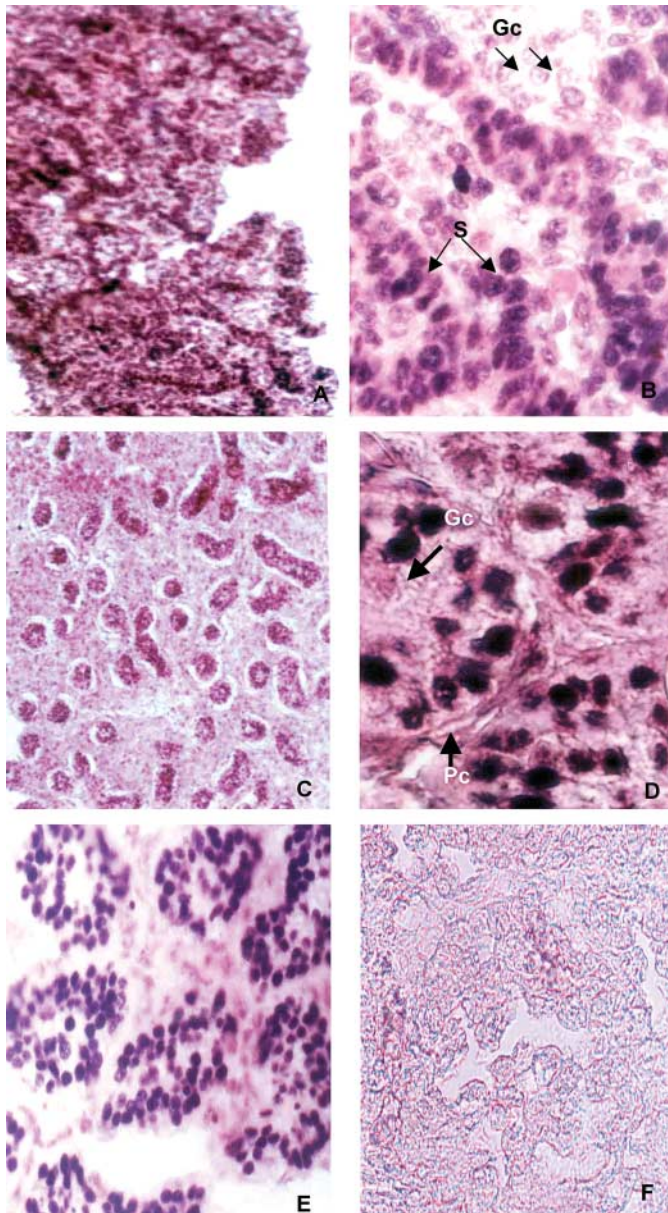
Expression of SRY transcripts was studied in paraffin sections of 18 fetal, one neonatal and six adult testes by non-radioactive, *in situ* hybridization (ISH), as detailed previously (Sheikh *et al.* 2001, Shah *et al.* 2005a). All the reagents used for ISH were purchased from Roche Molecular Biochemical and Sigma.

All the glass and plastic wares used were autoclaved and baked, and all solutions and water were treated with diethylpyrocarbonate (DEPC) to inactivate RNase prior to use. For ISH, the sections were deparaffinized in xylene, hydrated and refixed in 2% paraformaldehyde. After washing in PBS, the slides were incubated in 2 × SSC

(1 × SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7) for 15 min at room temperature. Prehybridization was carried out at room temperature for 1 h in a prehybridization cocktail containing 50% formamide, 4 × SSC, 5 × Denhardt's solution, 0.25% yeast tRNA, 0.5% sheared salmon sperm DNA and 10% dextran sulfate. The oligo probe used in this study was synthesized commercially (Gibco BRL) and tail-labeled with digoxigenin with a commercial kit (Roche Molecular Biochemicals). The probe quantity was determined according to the manufacturer's instructions (Roche Molecular Biochemicals). After prehybridization, the sections were hybridized overnight at 42 °C with the labeled probe diluted in the same cocktail at a concentration of 1 pmol/μl. Next day, the sections were stringently washed in varying concentrations of SSC (4 × SSC for 20 min, 2 × SSC for 10 min and 1 × SSC for 5 min at 45 °C) and blocked for 2 h at room temperature in blocking solution containing 2% normal sheep serum and 0.1% TritonX-100 in 0.1 M Tris–HCl buffer, pH 7.5. The sections were then incubated overnight at 4 °C in alkaline phosphatase conjugated anti-digoxigenin antibody diluted (1:500) in the above blocking solution. The slides were then extensively washed in 0.1 M Tris–HCl, pH 7.5, and equilibrated in 0.1 M Tris–HCl, pH 9.5, for 10 min. The color was developed at pH 9.5 for 35 min at room temperature in a solution of nitro blue tetrazolium and 5-bromo-4-chloro-2-indolyl phosphate containing 0.2% levamisole, and slides were mounted as detailed previously (Modi *et al.* 2003a). The sections were viewed light microscopically (BX-60 microscope; Olympus, Tokyo, Japan), and representative areas were photographed with a PM10SP camera (Olympus). Sections incubated with sense SRY probe and fetal ovarian samples served as negative controls.

### Sperm fluorescent ISH

The probe for hybridization was prepared by PCR amplification of male genomic DNA with SRY gene-specific primers (as used for Northern hybridization). Gel eluted (Millipore) PCR products were labeled with digoxigenin by the random prime labeling method according to the manufacturer's (Roche) instructions. After labeling, free



**Figure 1** Representative photographs of *in situ* localization of SRY transcripts in fetal and neonatal human testis. Intense mRNA signals were detected in the Sertoli (S) cells of the fetal testis from 9 weeks (A and B), 15 weeks (C) and 20 weeks (D) of development. Transcripts were also detectable in the testis of a 3-month-old boy (E). The germ cells (Gc) and the peritubular cells (Pc) did not stain for SRY mRNA. Signals were not detected when the sense probe was used as negative control (F). Magnification: A and C:  $\times 33$ ; E:  $\times 66$ ; B, D and F:  $\times 132$ .

nucleotides were removed by ethanol precipitation. cDNA probe specific to beta-actin was purchased from Clontech (USA) and labeled as above.

Spermatozoa from three men were spread on 3-aminopropyltriethoxysilane-coated glass slides, air-dried and stored at  $-20^{\circ}\text{C}$  until use. Before ISH, the slides were fixed in 2% acetic acid and 2.5% formaldehyde in PBS for 20 min. The slides were dehydrated in ethanol and incubated in xylene for 15 min at room temperature. After rehydration, the slides were incubated in 0.01% pepsin (pH 4.0; Sigma) for 20 min at  $37^{\circ}\text{C}$ . The cells were washed in RNase-free water and dehydrated. Fluorescent ISH was carried out as described previously (Modi *et al.* 1999, Modi *et al.* 2003a, 2003b). Briefly,  $2\ \mu\text{g}/\mu\text{l}$  of the probe were diluted in a hybridization cocktail (50% for-

amide,  $4\times$  SSC, 10% dextran sulfate, 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  $42^{\circ}\text{C}$  for 10 min for prehybridization. The probe and the target were codenatured at  $78^{\circ}\text{C}$  for 5 min. Hybridization was carried out at  $42^{\circ}\text{C}$  overnight in a humidified chamber. Next day, washing was carried out in  $1\times$  SSC containing 0.1% NP-40 at  $68^{\circ}\text{C}$  for 1 min followed by a wash in  $2\times$  SSC for 3 min at room temperature. Blocking was carried out in 2% normal sheep serum (Sigma) for 1 h followed by incubation in diluted (1:100) fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody (Roche) for 2 h at  $37^{\circ}\text{C}$ . The slides were washed extensively to remove unbound antibody, and this was followed by signal amplification with a FITC signal amplification kit

(Roche). The cells were mounted in antifade-containing medium with DAPI as counterstain (Vysis, Toulouse, France). The signals were viewed under a fluorescence microscope with appropriate filters (Nikon 90I, Kawasaki, Japan), and the images were captured and digitized by image-analysis software (Image Pro Plus 3.1, Image Pro, Media Cybernetics, Republic of Singapore).

## Results

### *Expression of SRY in the testis of human fetuses and one infant*

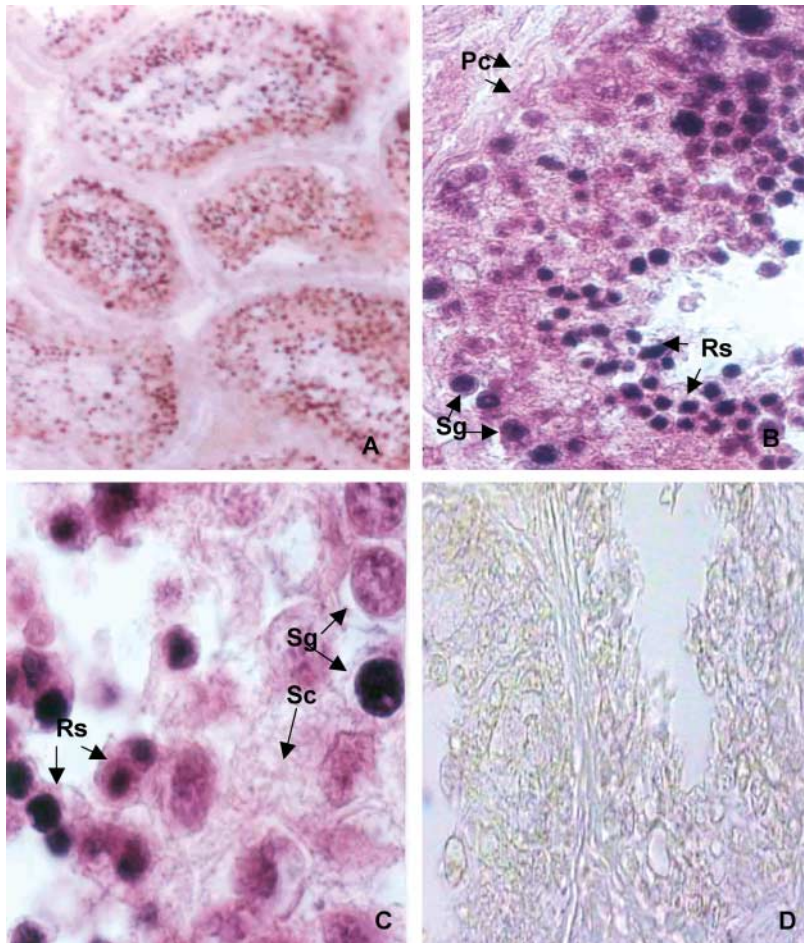
A total of 18 fetal testes (gestation age 9–22 weeks) and one testis from an infant of 3 months were analyzed for the present study. SRY mRNA was expressed in the Sertoli cells of the fetal testes and infant testis. SRY transcripts were *in situ* localized to the nucleus and cytoplasm of the Sertoli cells in all the developing testes studied from 9 weeks until 22 weeks (Fig. 1). At 9 weeks of development (Fig. 1A and B), the Sertoli cells were seen to be scattered along the gonadal parenchyma that was organizing to form the seminiferous tubules. Intense SRY mRNA staining was detected in the differentiating Sertoli cells (S)

that were seen to be surrounding the germ cells (Gc) and gave the appearance of incomplete tubules (Fig. 1B). At 12 weeks and later (up to 22 weeks) when the testis had well-formed seminiferous tubules (Fig. 1C and D), SRY transcripts were detected only in the Sertoli cells (Fig. 1D). Detectable SRY mRNA was not evident in the germ cells (Gc), peritubular cells (Pc), stromal cells and Leydig cells. Testis sections obtained from the infant at 3 months of age had SRY expression in the Sertoli cells similar to the fetal testes (Fig. 1E). No signals were detected in the negative control sections when a sense probe (Fig. 1F) or fetal ovarian sample (data not shown) was used for hybridization. Although quantitative analysis was not performed, the level of SRY mRNA did not seem to vary during the second trimester or even in the neonatal period.

### *Gene expression of SRY in the adult testis*

#### *ISH*

Testes from six adult men (age 23–55 years) were examined by nonradioactive ISH to determine the cellular localization of SRY mRNA. Strong positive signals for SRY transcripts were detected in all the testicular samples studied (Fig. 2A). However, in the adult testis, the cellular



**Figure 2** Expression of SRY mRNA in the adult testis. By nonradioactive *in situ* hybridization, SRY mRNA was localized in the germ cells within the seminiferous tubules (A). The staining was specifically detected in the spermatogonia (Sg) and round spermatids (Rs). The meiotic cells (Sc) stained weakly or were negative for SRY mRNA (B and C). Signals were not detected in the negative control sections, where a sense probe was used for hybridization (D). Magnification: A:  $\times 33$ ; B and D:  $\times 132$ ; C:  $\times 330$ .

localization of the mRNA differed from that observed in the fetal testis. In the adult testis, SRY transcripts were detected in the spermatogenic cells (Fig. 2B). The spermatogonial cells (Sg) at the base of the tubules stained positive for SRY mRNA (Fig. 2B and C). However, the expression of SRY was reduced or switched off in the meiotic stages, particularly at pachytene (Fig. 2C). After meiosis, the expression of SRY was switched on (Fig. 2B and C), and intense signals were detected in a subset of round spermatids (Rs). Apparently, in certain areas of the tubule, intense SRY mRNA localization was observed in the differentiating germ cells, and the same cells in the adjacent areas were weakly positive or negative for SRY transcripts (Fig. 2B). This pattern of staining in the adult testis indicates that SRY expression may be stage specific during spermatogenesis. However, the precise stages at which the intensity of staining increases could not be determined, as the sections were not counterstained. All negative control sections (sense probe hybridization) were without staining (Fig. 2D), confirming the specificity of the signal.

#### Northern blotting

Northern blotting was carried out to determine the size of the SRY transcripts in testis and spermatogenic cells. A single band of  $\sim 1.2$  kb was detected in total RNA from both testes and spermatogenic cells (Fig. 3E, lanes 2 and 1 respectively) when hybridized with a SRY gene-specific,  $^{32}$ P-labeled cDNA probe.

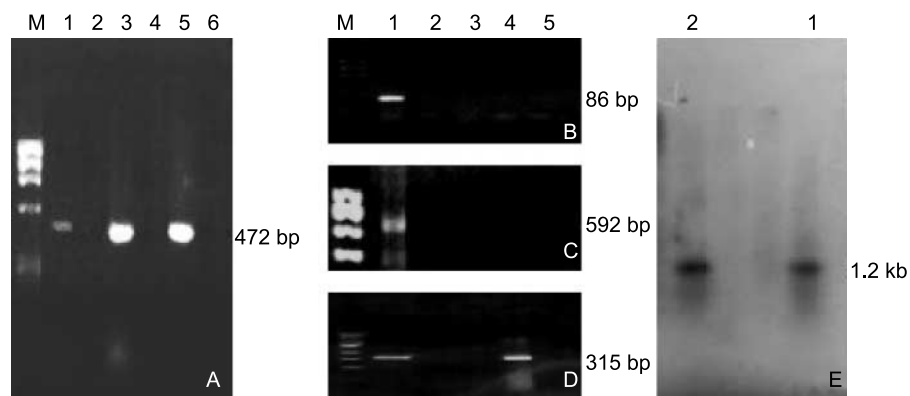
#### RT-PCR

Northern blot results were further verified by RT-PCR. A single band of predicted size was obtained when testicular (Fig. 3A, lane 3) or spermatogenic cell (Fig. 3, lane 5) RNA was used for RT-PCR amplification of SRY. Bands were not detected in the negative control reactions where reverse transcriptase was omitted from the reaction mixture (Fig. 3A, lanes 4 and 6). The specificity of the PCR products was demonstrated by sequencing that showed complete homology to human SRY cDNA (not shown).

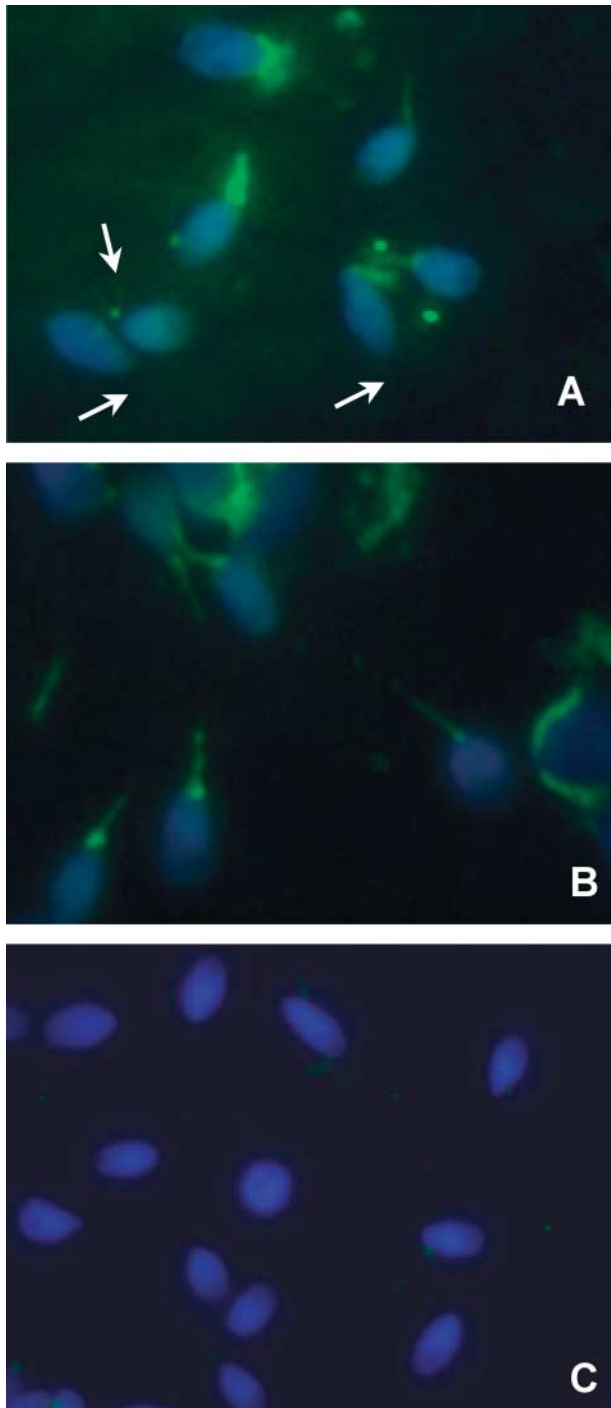
To test the purity of spermatogenic cell preparation, amplification of Sertoli cell marker (FSH receptor, Fig. 3B) and Leydig cell marker (StAR, Fig. 3C) was performed. Bands of predicted sizes were obtained in testicular RNA (Fig. 3B and C, lane 1), but not in spermatogenic cell RNA (Fig. 3B and C, lane 4), indicating that the preparations were free of somatic cell contamination. The integrity of the RNA/cDNA preparation was verified by the presence of 18S RNA in testicular (Fig. 3D, lane 1) and spermatogenic cell (Fig. 3D, lane 5) cDNA preparations. No bands were visible when reverse transcriptase was omitted from the reaction mixture, demonstrating the specificity of the signals (Fig. 3B–D, lanes 2 and 5).

#### Expression of SRY transcripts in the ejaculated spermatozoa

SRY mRNA was detected in RNA from mature spermatozoa by RT-PCR. A single band of predicted size was obtained when RNA from spermatozoa and SRY gene-specific primers were used for RT-PCR (Fig. 3A, lane 1).



**Figure 3** A) Detection of SRY transcripts in testis, spermatogenic cells and spermatozoa. PCR amplification of SRY (472 bp) in spermatozoa (lane 1), testis (lane 3) and spermatogenic cells (lane 5). The respective negative controls (without reverse transcriptase) are shown in lanes 2, 4 and 6. Lane M contains DNA molecular weight marker *Hae*III digested  $\phi$ X 174 fragments. B) Determination of purity of spermatogenic cell preparations by RT-PCR for Sertoli cell markers. With specific primers for follicle-stimulating hormone receptor, a band of 86 bp was detected in testicular cDNA (lane 1; negative control, lane 2), but not in spermatogenic cell cDNA preparations (lane 4; negative control, lane 5). Lane 3 is water control. Lane M: 100 bp DNA ladder. C) Determination of purity of spermatogenic cell preparations by RT-PCR for Leydig cell markers. With specific primers for steroid acute regulatory gene (StAr), a band of 592 bp was detected in testicular cDNA (lane 1; negative control, lane 2), but not in spermatogenic cell cDNA preparation (lane 4; negative control, lane 5). Lane 3 is water control. M DNA molecular weight marker *Hae*III digested  $\phi$ X 174 fragments. D) Expression of 18S RNA (315 bp, positive control) in testis and spermatogenic cells. Testicular cDNA (lane 1; negative control, lane 2) and spermatogenic cell cDNA (lane 4; negative control, lane 5). Lane 3 is water control. Lane M: 100 bp DNA ladder. E) Northern blot for SRY in human testis and isolated spermatogenic cells. A band of  $\sim 1.2$  kb was detected in testicular (lane 1) and spermatogenic cell (lane 2) RNA hybridized with  $^{32}$ P-labeled SRY probe.



**Figure 4** *In situ* localization of SRY and actin mRNA in ejaculated spermatozoa. By fluorescent *in situ* hybridization, SRY (A) and actin (B) mRNA was localized in the midpiece region (green signals). The nuclei are counterstained with DAPI (blue signals). Note the absence of SRY transcripts in some cells (A, arrows). Signals were not detected in the negative controls (C).

Products were not detected when reverse transcriptase was omitted from the reaction mix (Fig. 3A, lane 2), indicating that the band obtained was not due to contaminating genomic DNA. The specificity of the band was

demonstrated by sequencing of the PCR products that shared 100% homology with the human SRY gene (not shown). Although the spermatozoa used for RNA extraction were motile and collected after swim-up procedure to avoid somatic cell contamination, the presence of leukocyte RNA in sperm RNA preparation was also ruled out by the absence of CD44 transcripts (Sachdeva *et al.* 2000), indicating that the SRY transcripts detected in the sperm RNA by RT-PCR were not the result of somatic cell contamination.

To determine the subcellular distribution of SRY in the ejaculated spermatozoa, ISH was performed with SRY gene-specific cDNA probe. After signal amplification, positive signals were detected in the midpiece region of nearly 50% of spermatozoa (Fig. 4A). Interestingly, actin mRNA was detected in almost all spermatozoa (Fig. 4B). No signals were visible in the negative controls (Fig. 4C). These results indicate that SRY transcripts are stored in the midpiece region of presumably Y-bearing spermatozoa.

The results of ISH, RT-PCR and Northern blot studies indicate that the SRY gene is transcribed in the Sertoli cells of the human fetal and the infant testis, postpubertally; SRY transcription is initiated in the spermatogenic cells and also detected in the mature spermatozoa.

## Discussion

The results of the present study reveal that the SRY gene is expressed in the human male gonads well beyond the phase of sex differentiation. SRY mRNA is detectable in the testes of midtrimester fetuses, infants and adults; the transcripts are also detectable in mature spermatozoa. At the cellular level, in the fetal and neonatal stages, the Sertoli cells of the testis express high amounts of SRY mRNA; the germ cells are SRY negative. However, in adulthood, SRY expression is induced in the spermatogonial cells and is also present in the ejaculated spermatozoa. Thus, the ontogeny and the cellular localization of SRY mRNA in the human gonads imply some additional stage-specific roles of the TDF in male reproductive physiology.

In the present study, SRY transcripts were *in situ* localized to the Sertoli cells of the fetal testis studied at 9–22 weeks of gestation. These results contrast with a previous report (Josso *et al.* 1993) that SRY mRNA was not detectable in the testis of any of the fetuses studied at 7–22 weeks of development. Although the reason for this apparent contradiction is unclear, our results are supported by the recent finding that SRY transcripts are detectable by RT-PCR in the testis of a first-trimester human fetus (Olesen *et al.* 2001). SRY protein has also been detected in the testis of a third-trimester human fetus (Salas-Cortes *et al.* 1999, 2001). These results indicate that the SRY gene is expressed in the human testis well beyond the stages of testicular differentiation. In mice, SRY transcripts are only transiently expressed in the XY gonadal primor-

dium at 7.5 dpc; the expression is extinguished after testicular organization (Rossi *et al.* 1993, Hacker *et al.* 1995). Thus, human testicular SRY transcription is less time specific than rodent, and its temporal expression is not restricted to a short period around the onset of gonadal differentiation.

Despite the temporal differences, the cellular localization of SRY transcripts in the fetal testis is similar in mice and man. SRY mRNA has been detected by ISH exclusively in the differentiating Sertoli cells of the rodent and the human XY gonadal primordium (Clepet *et al.* 1993, Hacker *et al.* 1995, Hanley *et al.* 2000). In support of these observations, SRY mRNA was detected in the human Sertoli cells of the developing human testis examined at 9–22 weeks of gestation. SRY protein has also been reported to be present in the testis of a second-trimester human fetuses. However, along with the Sertoli cells, the protein product of SRY has been immunolocalized in the nuclei of the germ cells (Salas-Cortes *et al.* 1999, 2001), whereas the mRNA for SRY was detectable only in the Sertoli cells (present study). There are several explanations for the observed differences between the cellular localization of SRY mRNA and the protein. Firstly, it is possible that the SRY protein detected in the human fetal germ cells is a translocated Sertoli cell product and is not synthesized *de novo* by the germ cells. Secondly, it is probable that in the germ cells the rate of SRY translation may be higher than the rate of transcription, and hence the protein, but not the transcripts, is detectable in the fetal germ cells; sensitive techniques such as *in situ* RT-PCR need to be employed to examine this possibility. Alternatively, it is likely that SRY antibodies may cross-react to other related proteins present in the germ cells, leading to generation of nonspecific signals in immunohistochemical procedure. Indeed, SRY is known to share significant homology with the SOX family of proteins (Haqq & Donahoe 1998, Harley *et al.* 2003). In this context, it is noteworthy that, in one study, SRY protein was not detectable in the germ cells of fetal testis with a monoclonal antibody (Hanley *et al.* 2000), but was found to be localized to the nuclei of germ cells in another study (Salas-Cortes *et al.* 1999, 2001). Nevertheless, as SRY mRNA has not been detected in the fetal germ cells in the testis of a plethora of mammalian and nonmammalian species (Haqq & Donahoe 1998 and references therein), it is tempting to believe that in the fetal testis SRY is exclusively somatic in origin.

The primary function of SRY is testis determination, that is, organization of the gonadal primordium into the testis. *In vitro* approaches have suggested that SRY has biphasic roles in testicular organization. During the earliest phases of gonadal development, SRY is believed to be involved in differentiation of the Sertoli cells in the celomic epithelium; subsequently, it is thought to play an active role in the migration of the Sertoli cells from the mesonephros into the gonad proper (Schmahl *et al.* 2000). Thus, during gonadal differentiation, SRY seems to play a key role in the morphologic organization of the fetal testis. But the

persistence of SRY expression in the Sertoli cells of the human testis beyond the stage of tubular organization is intriguing. Although the seminiferous tubules are thought to be morphologically organized by 10–12 weeks of development in man (Rabionivic & Jaffe 1990), increase in testicular size and volume as a result of Sertoli and germ cell proliferation during infancy has been reported in monkeys (Chemes 2001). Interestingly, investigation with a Sertoli cell-specific marker (müllerian inhibiting substance) showed that tubular organization is morphologically not completed in human fetuses until at least 20 weeks of development (Modi 2001). Thus, it is likely that although initial morphologic differentiation of the testis may be completed in the early first trimester of development, the organization of the gonad may well be extended until puberty in primates. The expression of SRY in the human testis throughout gestation and also in infancy suggests that SRY may be required during this period of organ differentiation.

In the present study, along with the fetal and neonatal testis, SRY gene expression was detected in the adult testis. These results confirm the previously reported detection by RT-PCR of SRY mRNA in the adult testicular RNA in mouse (Capel *et al.* 1993, Rossi *et al.* 1993) and man (Clepet *et al.* 1993, Olesen *et al.* 2001). In the present study, SRY transcripts were detected by ISH in the spermatogenic cells, particularly the spermatogonia, spermatocytes and round spermatids. These observations were also confirmed by RT-PCR, which detected SRY transcripts in spermatogenic cell RNA that was free of Sertoli and Leydig cell contamination. A single band of ~1.2 kb was detected in testicular and spermatogenic cell RNA, further confirming the expression of full-length SRY transcript. To the best of our knowledge, this is the first report demonstrating the expression of SRY mRNA in the germ cell compartment of the human testis. These results suggest that, along with the testicular development and maturation during fetal life and infancy, SRY may have additional roles in spermatogenesis. In this context, it is of interest to note that SRY transcripts are expressed in the germ cells of the mature mouse testis (Rossi *et al.* 1993); however, these transcripts are circular and apparently not translated (Capel *et al.* 1993). In contrast, SRY protein has been detected in the adult human testis by both polyclonal and monoclonal antibodies (Poulat *et al.* 1995, Salas-Cortes *et al.* 1999, 2001), suggesting that in man the germ cell-specific transcripts of SRY may be functional. This switch in the cellular localization further reinforces the concept that SRY may have specific roles beyond sex differentiation, and that the cellular requirements of SRY differ in adulthood from fetal and neonatal life.

In the adult testis, SRY mRNA was detected in the spermatogonia, spermatocytes and round spermatids. The mitotic spermatogonial cells stained strongly positive for SRY mRNA, but the expression was negligible in the meiotic stages. However, after completion of meiotic divisions, strong SRY transcripts were detected in a subset

of spermatocytes and round spermatids. A proportion of elongated spermatids also had detectable SRY expression in the cytoplasm (not shown). Thus, it appears that SRY is required by the germ cells before and after meiosis, while the meiotic stages have minimal requirement of SRY-mediated transcriptional events.

However, the localization of SRY mRNA in the adult testicular cells is predominantly nuclear with some cytoplasmic staining. Such nuclear localization of mRNA, although unusual, is not surprising. According to the concepts of molecular biology, mRNA is transcribed in the nucleus; the pre-mRNA molecule is also processed in the nucleus (for splicing introns, polyadenylation and capping). Only the mature mRNA is translocated to the cytoplasm for translation. Thus, mRNA is always present in the nucleus, and the nuclear transcripts reflect active transcription. In fact, with oligo probes for ISH, the spatial events involved in pre-RNA splicing within the nucleus have been accurately mapped (Zhang *et al.* 1994). Therefore, nuclear mRNA signals in hybridization experiments can be expected. There are several explanations for the predominantly nuclear localization of SRY mRNA in the adult testis. Firstly, it is possible that the SRY transcripts are nonfunctional and may not be translated. These transcripts might be stored in the nucleus and eventually degraded. However, this possibility is less likely, as some spermatogenic cells did show cytoplasmic staining by ISH. Moreover, SRY protein has been detected in the nucleus of spermatogenic cells of adult human testis (Poulat *et al.* 1995, Salas-Cortes *et al.* 1999, 2001). It is also known that the amounts of mRNA in the nucleus and cytoplasm are not always constant; the detection of mRNA either in the nucleus or in the cytoplasm (or both) is dependent upon rates of transcription and translation, abundance and its distribution across the cell. Thus, differential rates of SRY transcription versus its translation may be one of the reasons for the observed nuclear signals.

Apparently, the pattern of SRY mRNA staining in the spermatogenic cells of adult testis was heterogeneous within each seminiferous tubule. In some areas of the tubule, SRY mRNA was strongly expressed in the spermatocytes and the spermatids, while the same cells in adjacent areas of the same tubule had low or negligible SRY mRNA. This pattern of staining suggests that SRY transcription in the adult testis is stage specific. The heterogeneous profile of SRY staining observed in the adult testis is specific and is not owing to partial degradation of RNA, as it was consistently observed in all the samples of the sections examined. Moreover, the expression of beta-actin and tubulin was homogeneous in all the tubules of the samples investigated in this study (Shah *et al.* 2005a). However, the stages at which SRY transcription is switched on or off during spermatogenesis could not be assessed; the pattern of localization implies specific requirements of this transcription factor during different stages of spermatogenesis. Hence, it is tempting to speculate that the spatially and temporally synchronized

gene switching required during spermatogenesis may be controlled by the stage-specific initiation and repression of architectural transcription factors such as SRY. Although stage-specific expression of SRY or any related proteins has not been reported in the testis, our hypothesis is supported by the observation that the expression of other transcription factors, such as estrogen and progesterone receptors, in the human testis is stage specific (Saunders *et al.* 2002, Shah *et al.* 2005a). It will be of interest to determine the stage-specific expression of SRY during spermatogenesis to clarify its role in testicular physiology.

It is now increasingly recognized that mRNA of a specific subset of testis-expressed genes is detectable in spermatozoa (Miller *et al.* 1999, Sachdeva *et al.* 2000, Ostermeier *et al.* 2002, 2004, Dadoune *et al.* 2005, Shah *et al.* 2005b). Transcripts of several genes have been reported to be present in human spermatozoa; a number of these are expressed at high levels in the adult testis (Miller *et al.* 1999, Ostermeier *et al.* 2002). To test whether SRY mRNA expressed in the differentiating germ cells of the adult testis also persists after maturation, RT-PCR for SRY was carried out in RNA isolated from a highly motile population of ejaculated spermatozoa. Somatic cell contamination was excluded by demonstration of the absence of leukocyte antigen transcript CD44 in the sperm RNA preparation, as reported previously (Sachdeva *et al.* 2000, Shah *et al.* 2003). By RT-PCR with SRY gene-specific primers, a single band of expected size that had sequence identical to the published SRY cDNA was detected in sperm RNA. This band was not the result of genomic DNA contamination, as the RNA used was treated with Dnase, and no signal was obtained when reverse transcriptase was omitted from the reaction mixture. Our results contrast with those reported previously (Fiddler *et al.* 1995) in which SRY mRNA was not detected by RT-PCR in the ejaculated human spermatozoa. At present, the reasons for the contrasting results are unclear, but differences in the number of spermatozoa used to isolate total RNA, and PCR conditions and sensitivity may be implicated.

Although we and others have previously demonstrated the existence of transcripts in the ejaculated spermatozoa by RT-PCR, it is likely that these signals are the result of a small fraction of contaminating immature cells. Thus, ISH was performed in the ejaculated spermatozoa to determine the population of ejaculated cells expressing SRY mRNA and its cellular distribution. The results revealed that SRY mRNA is expressed in the midpiece of nearly 50% of the ejaculated spermatozoa. To exclude the possibility that the absence of transcripts in the remaining 50% of cells is a result of RNA degradation, parallel ISH were performed on the same samples for beta-actin mRNA. As expected, almost 95% of cells demonstrated positive signals in the midpiece region. These results corroborate our recent findings in which progesterone receptor mRNA was also detected in the midpiece of ejaculated sperm (Shah

*et al.* 2005b) and suggest that the SRY transcripts detected in spermatozoa may be stored in the midpiece region. Taken together, these results demonstrate that mRNA for some genes is present in most ejaculated sperm cells, and we presume that the 50% of cells positive for SRY mRNA could be the Y-bearing spermatozoa.

At present, the functions of SRY protein and its downstream targets in the testis are unclear, but the presence of these transcripts in postmeiotic germ cells and in the spermatozoa suggests that they may have important functions in germ cell differentiation. Interestingly, transcripts of a number of Y-linked proteins and SOX 13 (a member of HMG family proteins related to SRY) have been reported in ejaculated sperm RNA (Ostermeier *et al.* 2002). However, as sperm is transcriptionally and translationally inactive, there is no apparent requirement for this transcriptional factor in the mature stages. But it is intriguing to note that the transcripts detected in the ejaculated sperm are distinct from those present in the ovum (Ostermeier *et al.* 2002, 2004). This observation suggests that, like the requirement of maternally expressed genes, some paternally derived transcripts may also be essential after fertilization and before activation of the zygotic genome. Indeed, in an *in vitro* fertilization assay, sperm-specific transcripts have been detected in the fertilized ovum, but not in the unfertilized eggs (Ostermeier *et al.* 2004). Thus, the sperm-derived transcripts are physically translocated to the egg at fertilization and may be required after fertilization and before activation of the embryonic genome. In the light of this observation, it can be hypothesized that the accumulation of mRNA for several Y-linked genes, including SRY, in the ejaculated spermatozoa may be a response to the anticipated embryonic requirement for the unique, paternally derived transcriptome after fertilization. Indeed, SRY mRNA has been detected in the XY human and mouse preimplantation embryos (Fiddler *et al.* 1995, Haqq & Donahoe 1998 and references therein), and it is postulated that it may be required to confer the temporal growth advantage that XY embryos have over XX embryos (Haqq & Donahoe 1998). These results further imply some novel roles of SRY not only in the process of gonadal development and gamete differentiation but also in early male zygotic development.

In summary, the results of the present study demonstrate that SRY in man is transcribed in the Sertoli cells of the fetal and infant testis, and in the spermatogenic cells of the adult gonad that also exist in mature ejaculated spermatozoa. This spatial and temporal pattern of SRY expression beyond the stage of testis differentiation in the fetal and adult human testis and in ejaculated spermatozoa suggests that apart from its role in sex differentiation, SRY may have important functions in spermatogenesis, sperm maturation and very early embryonic development. Such developmentally regulated expression of the gene may be of crucial importance in male reproductive physiology.

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