The Mouse Tsx Gene Is Expressed in Sertoli Cells of the Adult Testis and Transiently in Premeiotic Germ Cells during Puberty

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INTRODUCTION

Tsx is a gene of unknown function that was previously shown to be expressed specifically in the testis. In order to gain insight into the function of Tsx its pattern of expression was characterized with regard to both timing and cell type in the testis. Northern blot analysis of early postnatal testes showed not only that Tsx message was detectable shortly after birth, but that it increased substantially between 7 and 12 days postpartum (dpp), roughly coincident with the onset of meiosis in the mouse. Alternative Tsx transcripts, detected by RT-PCR, included a spliced form that first appeared at around 12 dpp. In situ hybridization revealed Tsx signal in the somatic Sertoli cells of the adult testis. Consistent with the data from Northern blots, in situ hybridization signal was first detectable in normal pubertal testes at 12 dpp. An anti-Tsx polyclonal antiserum specifically stained premeiotic germ cells in addition to Sertoli cells of pubertal testes at 16, 19, and 27 dpp. Tsx immunostaining in germ cells was nuclear, while Sertoli cells displayed signal throughout the cytoplasm and nucleus. In the adult, Tsx was detected exclusively in Sertoli cells. In contrast, in the adult testis of the oligotriches (olt) mutant, where spermatogenesis is blocked after meiosis, Tsx protein was still present in the spermatogonial nuclei of a subset of tubules. Taken together, these results demonstrate that Tsx expression is induced in both premeiotic germ cells and Sertoli cells during the first wave of spermatogenesis, but that expression is maintained at a detectable level only in Sertoli cells of the normal adult. The persistence of Tsx expression seen in spermatogonia of the adult olt mutant supports the hypothesis that during the first wave of normal spermatogenesis, the advent of a late-stage cell type, either elongating spermatid or spermatozoon, is responsible for extinguishing expression in spermatogonia in normal adult testis. To our knowledge, Tsx is the first gene to show a pattern of germ cell expression that is apparently specific to the pubertal testis. © 1998 Academic Press

INTRODUCTION

Tsx is a gene of unknown function previously shown to be expressed specifically in the testis that was identified in the course of experiments to establish a comprehensive transcription map in the vicinity of the Xist gene on the mouse X chromosome (Simmler et al., 1996; Borsani et al., 1991; Brockdorff et al., 1991). The Tsx gene encoding a 1-kb transcript lies just 35 kb distal to Xist and is currently the closest known gene to Xist (Simmler et al., 1996). Both mouse and rat homologues of Tsx have been identified and these are 72% identical at the level of predicted amino acid sequence, indicating an unusually high rate of sequence divergence. The 794 bp of known mouse Tsx cDNA sequence includes an open reading frame encoding a predicted protein of 144 amino acids that is highly acidic but which shows no significant similarity to any known protein.

The presence of Tsx expression within the testis was of some interest with regard to X inactivation, since chromatin condensation and transcriptional silencing of the male X chromosome occur at the time of X–Y pairing during meiotic prophase in spermatocytes (Monesi, 1965). It is currently thought that this X inactivation is imposed transiently during meiosis and may serve to prevent illegitimate recombination between the paired X and Y chromo-
somes (McKee and Handel, 1993; Hendricksen et al., 1995; Kumari et al., 1996; Moss et al., 1997). If speculation, which has been reinforced by the detection of Xist expression in the testis (Macarrey and Dilworth, 1992; Richler et al., 1992; Salido et al., 1992; but see Marahrens et al., 1997), that female somatic and male germline X inactivation, while fulfilling different functions, share common molecular mechanisms, was to prove true, the proximity of Tsx to Xist on the X chromosome would clearly make Tsx a marker gene of some interest for studies on X chromosome regulation during gametogenesis.

The seminiferous tubule of the mammalian testis displays an unusually complex and dynamic epithelium, with its development and mature function dependent on the action of numerous hormones, growth factors, and intercellular junctions (reviewed in Weinbauer and Nesland, 1993). Within the mature tubule, differentiating diploid spermatogonia, which arise from a population of germline stem cells, give rise to meiotic spermatocytes following several mitotic divisions. Upon completion of meiosis, the resulting haploid spermatids undergo extensive morphological differentiation to produce mature spermatooza. Spermatogonial proliferation and entry into the differentiation pathway occur in cycles that are coordinated with the more prolonged subsequent steps of meiotic prophase and spermiogenesis (Oakberg, 1996; reviewed in De Rooij et al., 1997).

Fetal and postnatal development of the male gonad as well as maintenance of the spermatogenic cycle in the mature testis is regulated and supported to a large degree by Sertoli cells (reviewed in Pelliniemi et al., 1993; Gondos et al., 1993). These highly specialized somatic secretory cells maintain contact with germ cells at all stages of development and mediate hormonal signaling in the testis. In turn, Sertoli cells are themselves dependent on the action of numerous hormones, growth factors, and intercellular junctions (reviewed in Weinbauer and Nilsson, 1993). Within the mature tubule, differentiating diploid spermatogonia, which arise from a population of germline stem cells, give rise to meiotic spermatocytes following several mitotic divisions. Upon completion of meiosis, the resulting haploid spermatids undergo extensive morphological differentiation to produce mature spermatooza. Spermatogonial proliferation and entry into the differentiation pathway occur in cycles that are coordinated with the more prolonged subsequent steps of meiotic prophase and spermiogenesis (Oakberg, 1996; reviewed in De Rooij et al., 1997).

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Here, we show that Tsx expression first appears in the pubertal testis and is present in both premeiotic germ cells and Sertoli cells. However, expression in the germ-line is extinguished with maturation of the testis. This transient expression of Tsx in the germ-line during puberty suggests that it may play a role in the establishment, rather than the maintenance of the spermatogenic cycle. In contrast, since the onset of Tsx expression in Sertoli cells coincides with their final differentiation the constitutive expression seen in the adult testis implies that Tsx also provides a function specific to Sertoli cells in the mature spermatogenic cycle.

**MATERIALS AND METHODS**

**Mouse Strains**

Adult organs and pubertal testes for RNA analysis were from congenic 129/Pgk1a animals. Testes from prepubertal, pubertal, and adult C57BL/6 males were used for in situ hybridization and immunohistochemistry experiments. Sterile interspecies hybrid animals were produced by backcrossing (C57BL/6 × NZB)F1 females with normal NZB males (Matsuda et al., 1991). The animal care was provided by the United Genetic Institute of Mammiferes at the Pasteur Institute, Paris, France.

**RT-PCR**

cDNA was synthesized from 10 μg of total RNA using AMV reverse transcriptase (Promega) as described in Rougeulle and Avner (1996). The PCR primers used for amplification of Tsx were TsxB10 (5’-GCCAGAATTCGAGGATGAAGA-3’) and TsxB11 (5’-CCTGCCTTAAATCAGTTGGGTTC-3’), which produce a 390-bp product spanning Tsx exons I to VII. The amplification program was 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C for the number of cycles specified in a 20-μl volume of standard PCR buffer with 1.5 mM Mg²⁺ and 1 U of Taq polymerase (Cetus). cDNA was amplified using primers Mx10 (5’-CATCACAACAGCAGT-TCTCC-3’) and Mx20 (5’-GTTGATCCCCGGTGATTTA-3’) as described by Kay et al. (1993). Primers for amplification of β-actin cDNA were ActU (5’-GCTGTGCTATGTTGGCTAAG-3’) and Actt (5’-CTCAGTAAACATCCGCTTAG-3’). RT-PCR analysis of alternative transcripts in the 5’ region of Tsx included upper primers RPS (5’-TACCCTAGCTGAGAAGATCT-3’) and RPL (5’-ATGGTGGAGATCCTAACT-3’) each used with lower primer 3.5L (5’-AGCTTGCAAGTGTCCTC-3’).

**Northern Blotting**

Total RNA was isolated by method of Chomczynski and Sacchi (1987). For each sample, 20 μg of RNA was denatured in 1 M glyoxal, 60% dimethyl sulfoxide, electrophoresed on a 1.4% agarose gel, and blotted onto Hybond-N nylon membrane (Amersham) according to the manufacturer’s instructions. Probe DNA was radiolabeled with [α-32P]CTP by random primed elongation using a Megaprime kit (Amersham) according to the manufacturer’s instructions. Probe DNA for Tsx was the PCR product from primers TsxB10 and TsxB11, as described above. Blots were prehybridized in 5% SDS, 0.5M sodium phosphate (pH 7.2), 1 mM EDTA at 65°C for 8 h. Denatured probe DNA was diluted in the same solution to a concentration of 2 × 10⁶ cpn/ml and hybridized at 65°C overnight. Blots were washed in 1× SSC, 0.1% SDS at room temperature for 15 min, followed by 0.1× SSC, 0.1% SDS at 65°C for 2 h, and exposed to autoradiographic film (XAR-5, Kodak) at –80°C.

**Histology**

Histological specimens were prepared as described by Sibony et al. (1994). Briefly, testes were dissected from adult, pubertal, and prepubertal C57BL/6 mice and fixed for 12-24 h in either 4% paraformaldehyde in PBS or 0.1% paraformaldehyde in PBS for in situ hybridization or in Bouin’s fixative for immunohistochemistry experiments. After fixation, the tissues were dehydrated in ethanol and embedded in paraffin and 7-μm sections were mounted on slides treated with TESPA (Sibony et al., 1995) for in situ hybridization and on gelatin-coated slides for immunohistochemistry.
In Situ Hybridization

RNA probes labeled with $^{35}$SUTP$\alpha$S (Amersham) were synthesized from a pGEM-T (Promega) plasmid construct including a 670-bp insert of the Tsx cDNA sequence corresponding to positions 130 to 800 (Simmler et al., 1996; EMBL Accession No. X99796). The plasmid was linearized with EcoRI, gel purified using QIAquick gel extraction kit (Qiagen), and transcribed in vitro with SP6 RNA polymerase to generate the antisense probe. For the sense probe, the plasmid was linearized with NotI and transcribed with T7 RNA polymerase. The probes were radiolabeled using 1 $\mu$g of template DNA, 100 $\mu$Ci of $^{35}$SUTP$\alpha$S, and reagents supplied in the Riboprobe transcription system kit (Promega) according to the protocol supplied by the manufacturer. Prehybridization treatment, probe hybridization, and washing of tissue sections were performed as described by Sibony et al. (1995). Probes were diluted to 5 x $10^4$ cpm/$\mu$l in hybridization solution and 30–50 $\mu$l was used for each section. Hybridization reactions were incubated at 52°C for 16 h. After washing, slides were coated with photographic emulsion (NTB2, Kodak), exposed for 10 to 21 days, photographically developed, and stained with toluidine blue.

Immunohistochemistry

The polyclonal antiserum, Tsx762, was raised in rabbit against an ovalbumin-coupled synthetic peptide, Tsx1773, corresponding to amino acid residues 37 to 52 (PGPSSALDDDTDDQAD) of the predicted Tsx protein (Neosystem Laboratoire, Strasbourg, France). The choice of peptide was based on its relatively high antigenicity index predicted from the computer program PEPTIDESTRUCTURE (Genetics Computer Group, Madison, WI). Polyclonal antiserum 850 was raised in rabbits (Neosystem Laboratoire) against the entire Tsx protein following cloning in a bacterial expression vector and purification as described below.

Rehydrated sections of mouse testes fixed in Bouin's fixative were preincubated with 3% normal goat serum in PBS, followed by incubation with Tsx762 at a dilution of 1:500 in PBS or Tsx850 at a dilution of 1/1000 for 90 min. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase and used at a dilution of 1:200 in PBS. As controls for nonspecific binding of antibodies, consecutive tissue sections were incubated with rabbit preimmune serum as the primary antibody or secondary antibody alone or in the case of Tsx762, with an aliquot of diluted Tsx762 antiserum that had been preincubated with the peptide antigen Tsx1773 at 15 $\mu$g/ml for 1 h on ice. Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Where specified, sections were counterstained with hematoxylin.

Cloning, Expression, and Purification of the Recombinant Tsx Protein

The entire cDNA sequence of the Tsx cDNA (Simmler et al., 1996) was cloned into the bacterial expression vector pQE-40. Purification of the protein from the bacterial strain Escherichia coli M15 (pREP4) was performed under denaturing conditions according to the manufacturer's protocol (Qiagen).

Western Blot Analysis

Protein extracts were separated on 12% acrylamide gels and transferred onto Hybond-C membranes (Amersham). The membranes were blocked with 3% BSA fraction V overnight at 4°C.

RESULTS

Expression of Tsx in Adult Tissues

Northern blot analysis of adult male tissues previously detected Tsx exclusively in the testis. RT-PCR was used to assay with greater sensitivity for the presence of Tsx transcripts in several adult tissues of both male and female (Fig. 1). Tsx product after 30 cycles of amplification was prominent in the testis sample, with only a faint signal in the ovary. A similarly faint signal was seen in other somatic tissues of both the male and the female, with the brain and kidney samples consistently showing evidence of a low level of Tsx message. Amplification of Xist, the gene localized nearest to Tsx, in the same samples as control, produced a strong signal in all of the female tissues and a weak signal exclusively in the testis of the male. Ubiquitous amplification of $\beta$-actin message served to verify the presence of similar levels of cDNA in all samples.
Expression of Tsx in Early Postnatal Testes

The seminiferous epithelium of the mammalian testis undergoes extensive postnatal development in a well-described sequence of germ cell growth and differentiation that continues cyclically throughout adulthood. The chronology of this first wave of spermatogenesis in the mouse includes proliferation of the diploid spermatogonia at 3-4 days postpartum (dpp), the appearance of meiotic spermatocytes at about 10 dpp, the beginning of morphological differentiation of haploid spermatids (spermiogenesis) at about 20 dpp, and the first release of mature spermatozoa at about 35 dpp (Goetz et al., 1984). The onset of gene expression detected during this period can be used to predict a cell type-specific expression pattern in the mature spermatogenic cycle. A Northern blot of total RNA from early postnatal testes probed for Tsx (Fig. 2) showed no signal in the 3-dpp sample, a very faint signal at 7 dpp, and a dramatically increased signal at 12 dpp that remained roughly equivalent in samples from 14 to 20 dpp and the adult. Spermatocytes are absent in the testis at 8 dpp, but by 12 dpp, they constitute about 35% of the cells of the seminiferous epithelium (Bellevé et al., 1977). Thus, the time during which Tsx expression was seen to increase coincides with the onset of meiosis in the pubertal testis.

Alternative Tsx Transcripts

The 5' end of the Tsx transcript has thus far been defined only by 5' RACE (Simmler et al., 1996). The position of the putative transcriptional start site is however supported by the discovery of a similar 5' end on the homologous rat Tsx cDNA (Simmler et al., 1996). Attempts to identify the start site by primer extension have yielded ambiguous results, suggesting that Tsx transcription may originate from multiple start sites. Since many genes, particularly in the testis, employ tissue-specific promoters, we searched for additional Tsx transcripts by RT-PCR using primers upstream of the presumed major start site. Amplification of adult testis cDNA using an upper primer (RPS) positioned 200 bp upstream of the 5' end of the Tsx cDNA and a lower primer within exon IV (3.5L) generated two specific products of 540 and 351 bp (Fig. 3A). Sequencing revealed that the longer product included correctly spliced exons I through IV in addition to the 200 bp of contiguous upstream sequence. The shorter product had the same sequence, except that it lacked a 189-bp segment within the first exon, upstream of the first translation start codon (shown schematically in Fig. 3C). The missing segment was flanked by consensus donor and acceptor splice sites at its extremities; thus it represents a small intron. The level of both the spliced and the unspliced 5' RT-PCR products of adult testis using the primer pair RPS/3.5L was substantially lower than that of the RT-PCR product obtained using primers B10/B11 positioned within the coding region (Fig. 1 vs Fig. 3A and unpublished data). No PCR product was generated from a primer positioned 300 bp upstream of Tsx and the exon IV primer.

Tissues other than testis, tested using the primer pair RPS/3.5L, showed only the longer unspliced product similarly to the earliest stages of the postnatal testis. In cDNA from such early postnatal testes, the longer product was visible at a similar level in all samples from 3 dpp to adult (Fig. 3B). The fully spliced form while absent from the earliest sample became more abundant in the pubertal samples, a pattern paralleling the overall induction of Tsx message during puberty as detected by Northern blotting. Thus, part of Tsx regulation in the pubertal testis appears to occur at the level of RNA splicing.

The sequence of the incompletely spliced and fully spliced 5' RT-PCR products upstream of the presumed Tsx translation start codon included stop codons in all three reading frames and no additional upstream translation start codons. Thus, they possessed no obvious novel coding potential. However, the potential functional significance of this region of the Tsx transcript is underscored by the fact that a comparison of the rat and mouse Tsx cDNA sequences shows 89% identity in the 5'-untranslated region, 84% identity in the coding region, and 67% identity in the 3'-untranslated region (Simmler et al., 1996).

In Situ Hybridization of Tsx

In order to identify specifically which cells express Tsx, a 670-bp Tsx antisense RNA probe was hybridized to sections of adult testis. The highly organized cytoarchitecture and ongoing cycles of spermatogenesis within the seminiferous epithelium of the adult testis allow gene expression to be assigned to precise cell types and phases of differentiation (reviewed in Wolgemuth and Watrin, 1991). On sections of adult testis, signal was found localized to the periphery of the seminiferous tubules and to linear tracks extending...
from the basal lamina toward the lumen, a pattern consistent with Tsx expression in Sertoli cells (Figs. 4A and 4C). These cells are distinguished by large, irregular nuclei positioned adjacent to the basal lamina and by long cytoplasmic processes that project inward between the developing germ cells to the lumen. It was not however possible to exclude the possibility that some Tsx message was present in germ cells. Hybridization of a control probe corresponding to the Tsx sense strand showed only diffuse background hybridization (Figs. 4B and 4D).

The expression of Tsx in mature Sertoli cells was confirmed on testis sections from sterile interspecific hybrid backcross males. In these animals, spermatogenesis is blocked at the end of meiosis (Matsuda et al., 1991). When spermatogenesis arrests, virtually all of the germ cells within the tubule degenerate, leaving mainly Sertoli cells. The Tsx hybridization signal was particularly dense over the tubules depleted of germ cells and was associated with the filamentous cytoplasmic processes of Sertoli cells that remain in the enlarged luminal space (Figs. 4E and 4G). The presence of the Sertoli cell processes within the lumen was apparent on the sections hybridized with the Tsx sense strand probe, where they were not obscured by hybridization signal (Figs. 4F and 4H).

The pattern of in situ hybridization on prepubertal and pubertal testes was consistent with the results from Northern blotting with regard to the developmental timing of Tsx expression. Figure 5 shows the hybridization signal from the Tsx antisense probe on a series of early postnatal testes. At 8 dpp, no Tsx signal above background was detected (Fig. 5A), even though immature Sertoli cells comprise about 73% of the seminiferous epithelium at this stage (Beliveau et al., 1977). The signal above background was visible at 12 dpp, much of it over the cytoplasm in the center of the tubule (Fig. 5B). At 19 dpp, a very dense signal was present over the entire seminiferous epithelium of some tubules.
Tsx Protein Localizes Exclusively to Sertoli Cells of the Adult Testis

In order to define more precisely the cell population expressing Tsx, polyclonal antisera (Tsx762 and Tsx850) were raised in rabbits respectively against a synthetic peptide from the predicted Tsx protein sequence and against the entire Tsx protein sequence itself. Similar immunohistochemistry results were obtained with both antisera when they were used to test adult and pubertal testes. Western blot analysis of both adult and pubertal testes showed the presence of a single specific band which could be competed out by specific absorption (Fig. 6).

When adult testis was examined, the antisera were found to stain the Sertoli cells exclusively (Fig. 7). The intensity of Tsx signal in Sertoli cells was generally uniform in all tubules (Fig. 7A), consistent with the results from in situ hybridization, indicating that its expression was not associated with a specific stage of the spermatogenic cycle. When the Tsx762 antiserum raised against the synthetic Tsx peptide was used, this staining of Sertoli cells could be abolished by preabsorption of the antiserum with the synthetic peptide against which it was raised but not by other irrelevant peptides (Fig. 7B), demonstrating the specificity of the antibody binding to these cells. Reactivity of the antiserum with the flagellae of spermatozoa, developing acrosomes of elongating spermatids, and with interstitial cells was however found to be due to nonspecific antibody binding, as it was not blocked by preincubation of the Tsx762 antiserum with the peptide antigen (Fig. 7B). At high magnification (Fig. 7C), immunostaining was evident both in the Sertoli cell nuclei and throughout the cytoplasmic processes that extend toward the lumen. The various germ cell types were more evident on a section counterstained with hematoxylin (Fig. 7D).

Tsx Protein in Premeiotic Germ Cells during Puberty

As expected from analysis of Tsx message levels, sections of 8-dpp testis showed no Tsx immunostaining (data not shown). On the 12-dpp testis, very little, if any, Tsx signal was apparent (Fig. 8A). This may indicate that translation of Tsx message is slightly delayed, since Northern blot analysis detected abundant transcripts present at 12 dpp. On sections of a 16-dpp testis, the Tsx antiserum produced strong immunostaining of germ cells (Fig. 8C). The signal was most intense in the nuclei and varied considerably between different tubules. As in the adult Sertoli cells, staining using the Tsx762 antiserum was fully blocked by preabsorption of the antiserum with the peptide antigen (Fig. 8D). No immunostaining of Sertoli cells was visible. The identification of germ cell types at this age is hampered by the absence of the full complement of differentiated cells present in the mature tubule that serve as stage-specific hallmarks and are used to infer the identity of precursor cells. Additionally, the characteristic locations of specific germ cells in the adult seminiferous epithelium are not yet established during puberty. In particular, spermatogonia, which in a mature tubule form a single layer of cells at the periphery, can at this age be found clustered toward the center of the compact tubule. Among the readily identifiable cells are pachytene spermatocytes, which display irregular patches of condensed chromatin dispersed throughout a relatively large nucleus. In the 16-dpp testis, they are the most developmentally advanced germ cell type and are positioned centrally in the tubules where they appear (Bellvé et al., 1977). The pachytene spermatocytes showed no Tsx staining. A majority of the immunostained cells were relatively small and round, with granular, densely counterstained chromatin evenly distributed throughout the nucleus, as seen in the control section (Fig. 8D). These features suggest that the cells were type B spermatogonia or preleptotene spermatocytes (Bellvé et al., 1977). A few tubules, generally those that included pachytene spermatocytes, showed densely immunostained germ cell nuclei near the basal lamina at the periphery of the tubule. These relatively large, oval nuclei did not contain condensed chromatin, but displayed a discrete nucleolus, characteristic of type A spermatogonia.

Similar Tsx immunostaining of germ cell nuclei was found at 19 dpp, although overall fewer tubules contained Tsx-positive germ cells than on the 16 dpp (Fig. 8E). Here,
FIG. 5. In situ hybridization analysis of Tsx expression in pubertal testes. Hybridization of the Tsx antisense probe on sections of testes at 8 dpp (A), 12 dpp (B), 19 dpp (C), and 27 dpp (D), shown at high magnification. Bar, 10 μm. Low magnification, dark field view of signal from the antisense probe (E) and sense probe (F) on 19-dpp testis. Dark field view of antisense probe (G) and sense probe (H) on 27-dpp testis. Bar, 100 μm.
the Tsx-positive germ cells were more peripherally positioned than in the 16-dpp testis, as would be expected for spermatogonia that were being sequestered within the basal compartment with the formation of the blood-testis barrier. In addition, Tsx staining was present in both the Sertoli cell nuclei adjacent to the basal lamina and the cytoplasm surrounding spermatogonia and extending a short distance between the unstained spermatocytes toward the center of the tubule. The intensity of staining was again highly variable among tubules, with some showing Tsx signal in both germ cells and Sertoli cells, while adjacent tubules showed virtually no staining above background in any cells. The staining in both Sertoli cells and spermatogonia was absent on sections incubated with antiserum preabsorbed with purified Tsx recombinant protein prior to use. *Tsx-specific bands detected in the cell extracts.

**FIG. 6.** Western blot analysis showing the specificity of the Tsx850 antiserum. Blot A, protein extracts from 14 day postpartum testis (te), adult brain (br), muscle (mu), and spleen (sp). Protein standards (M) are 106, 82, 49, 29, and 19 kDa (Bio-Rad). Blot B, adult testis (te) and ovary (ov), purified Tsx fusion protein (T, see arrow). Blot C, as B but the 850 antiserum was blocked by preincubation with purified Tsx recombinant protein prior to use. *Tsx-specific bands detected in the cell extracts.

**DISCUSSION**

The results obtained in the course of this study confirm that Tsx expression in the adult is confined mainly to the testis (Simmier et al., 1996). Tsx expression does not however appear to be associated with gametogenesis per se as RNA from the adult ovary showed only a very weak Tsx signal by RT-PCR and no signal above background was detected by in situ hybridization on sections of adult ovary (unpublished data). Analysis of the fetal ovary will however be required to determine whether Tsx expression is elevated in either oocytes or supporting follicle cells during the time at which the first meiotic division is initiated (from embryonic day 13 to 16), analogous to that seen in premeiotic germ cells of the testis since the developmental timing of meiotic divisions differs between oogenesis and spermatogenesis.

The results of RT-PCR analysis using primers upstream of the putative major transcriptional start site of Tsx revealed heterogeneity in the 5' end of the Tsx message. In the testis, as in other tissues, the function of such variant transcripts may be to permit cell type-specific gene regulation through specific promoters (Ito et al., 1993; Maiti et al., 1996), to alter the translation efficiency or stability of the message (Yiu et al., 1994; Persengiev et al., 1996), or to produce a functionally distinct protein (Foukes et al., 1992). Although the minor Tsx transcript that we detected, spermatogonia and preleptotene spermatocytes, based on their nuclear morphology and the spermatogenic stage of the tubules (data not shown). In many tubules, the adult pattern of Tsx signal exclusively in Sertoli cells was evident. Control immunostaining with the preabsorbed antiserum showed no signal in Sertoli cells or germ cells, but showed nonspecific antibody binding to interstitial cells and the developing acrosome of spermatids, as in the adult (Fig. 8H).

**Immunolocalization of Tsx Protein in olt Testes**

Since Tsx expression in germ cells was seen only in the immature testis, we determined its distribution in the adult testis of an oligotriche (olt) mutant animal, where spermatogenesis is blocked at a relatively late stage. When homozygous, the recessive olt locus results in a sparse coat in males and females, as well as male specific sterility (Moutier, 1976). Spermatogenesis is disrupted in the postmeiotic phase, where spermatids fail to develop flagella and spermatzoa are absent (Chubb, 1992). Although the seminiferous epithelium appears disorganized and lacks a lumen, Sertoli cells, spermatogonia, and spermatocytes can be identified. On sections of adult olt testis, Tsx staining was found in Sertoli cell nuclei and cytoplasm of most tubules and in spermatogonial nuclei of a subset of tubules (Fig. 9), a pattern analogous to that seen in the normal 27-dpp testis.
FIG. 7. Immunohistological analysis of Tsx protein in normal adult testis. (A) Immunostaining of adult testis by the polyclonal antiserum Tsx762 at 1:500 dilution. (B) Immunostaining of a consecutive section by the antiserum preincubated with the Tsx peptide antigen. Antibody staining of interstitial cells, developing acrosomes in spermatids, and the flagella of spermatozoa in the lumen was not blocked by the peptide, indicating that this signal was nonspecific antibody binding. (C) Tsx distribution in Sertoli cells of the adult seminiferous tubule shown at high magnification. Both the cytoplasm and the nuclei of Sertoli cells were immunostained. Spermatogonia within the basal compartment were negative for Tsx. (D) An adult seminiferous tubule immunostained for Tsx and counterstained with hematoxylin to distinguish germ cell types. Bar, 50 μm.
Tsx Expression during Testis Maturation

originating upstream of the putative major start site, was found in a number of different tissues, the splicing out of the small intron within the 5'-untranslated region was testis specific and linked to the onset of meiosis in the pubertal testis. Such alternative splicing and use of alternative transcriptional start sites has been observed to occur extensively in mammalian germ cells (reviewed by Kleene, 1996). Recently alternative splicing of 5' exons of the DNA methyltransferase Dnmt1 has been shown to occur specifically during spermatogenesis with the onset of pachytene and this is linked to a diminution in the presence of DNMT1 protein (Mertineit et al., 1998). The first appearance of the 5'-spliced form of Tsx during puberty, when the cell type distribution and subcellular localization of the Tsx protein are complex, may reflect the presence of functional variants of the protein that are expressed differentially in germ cells and Sertoli cells. Further characterization of the structure and distribution of alternative Tsx transcripts will be required in order to determine possible variation in their coding potential and translation efficiency and to identify the promoter(s) from which they arise. It should be noted in this context that the previously identified presumed translational start codon for Tsx matches only poorly with the mammalian consensus sequence (Simmler et al., 1996).

Sertoli cells are thought to achieve their ultimate differentiated form over the course of the first spermatogenic wave in puberty (reviewed by Gondos and Berndston, 1993). At the ultrastructural level, pubertal Sertoli cells gradually cease dividing, become anchored to the basal lamina, and establish the Sertoli–Sertoli intercellular tight junctions that constitute the blood-testis barrier. While the expression of some genes is associated with the earliest differentiation of Sertoli cell precursors during embryogenesis, others appear only with final testis development at puberty. For example, Dhh transcription is detected in Sertoli cell precursors in the embryo at 11.5 dpc and continues through adulthood (Bitgood et al., 1996). In contrast, the transcription factors GATA-1 and Per2 are first detected in Sertoli cells at about 9 dpc (Yomogida et al., 1994; Lindsey and Wilkinson, 1996). GATA-1 protein is still found at uniform levels in all seminiferous tubules at 3 weeks of age but by 5 weeks of age, tubules begin to show variation in the levels of GATA-1 signal, and in the adult, expression is restricted to Sertoli cells in tubules at stages VII, VIII, and IX (Yomogida et al., 1994).

The pattern of initially restricted but then increasingly general expression of Tsx in Sertoli cells during maturation of the testis is clearly distinct from the onset of expression of genes such as GATA-1 during the same period. The heterogeneous distribution of Tsx protein in Sertoli cells that we detected during puberty has moreover revealed a new level of complexity in Sertoli cell differentiation at the molecular level. Sertoli cells would not appear, based on these observations, to acquire their fully differentiated pattern of gene expression until an advanced phase of the first spermatogenic cycle. Our results equally suggest that the onset of Tsx expression in Sertoli cells may be dependent in some way on the progress of the initial wave of germ cell differentiation within the tubule. At 19 dpp, for instance, Tsx staining of Sertoli cells was generally found to be strongest in the subset of tubules that also showed positive germ cells, with adjacent tubules showing little or no Tsx signal.

Tsx expression thus provides a unique marker of late Sertoli cell differentiation. Determining Tsx levels in various male sterile mutants, particularly in those blocked at or prior to the onset of meiosis, may help to further characterize the interdependence between the germline and Sertoli cells in the maturation of the seminiferous epithelium. Additionally, Tsx expression as a possible germ cell-dependent marker of Sertoli cell maturation may benefit studies that employ cultures of germ cells and Sertoli cells under controlled conditions in vitro to investigate the hormonal and intercellular signaling pathways that regulate meiosis and germ cell differentiation (Rassoulzadegan et al., 1993).

The transient expression of Tsx in germ cells during puberty was a novel and unexpected finding. The possibility that this immunostaining pattern could be due to nonspecific staining of apoptotic cells was ruled out based on four observations. First, on sections of the adult testis, the patterns of Sertoli cell labeling by in situ hybridization and by immunohistochemistry were consistent supporting the idea that each method specifically detected bona fide Tsx expression. Second, antibody binding to germ cells by the Tsx762 antisera was blocked by preabsorption with the Tsx peptide antigen against which it was raised, but not by a different Tsx peptide. Third, the percentage of pubertal germ cells that was stained was much higher than that observed undergoing apoptosis in other studies (Mori et al., 1997; Rodriguez et al., 1997). Finally, germ cell apoptosis in the pubertal testes has been reported to be elevated in the most developmentally advanced germ cell type characterizing each of the particular developmental stages, initially among spermatogonia but later among spermatocytes and spermatids (Russell et al., 1987). Antibody staining for Tsx was, on the other hand, consistently limited to premeiotic germ cells in testis through 16 to 27 days of age.

What developmental events specific to puberty could account for the transient appearance of Tsx protein in premeiotic germ cells during this period? Three possibilities may be considered: first, the initiation of meiosis in the absence of a mature seminiferous epithelium; second, the regulation of premeiotic germ cell proliferation in order to establish the appropriate number of germ cells that can be sustained by the mature Sertoli cells; and finally, the formation of the blood-testis barrier, along with the positioning of spermatogonia within the basal compartment.

The mature spermatogenic cycle is thought to be coordinated in part by an exchange of signals, possibly mediated by Sertoli cells, between germ cells at different phases of maturation. The question arises whether, in the absence of the postmeiotic germ cells found in the adult seminiferous epithelium, the initial wave of spermatogenesis during
FIG. 9. Immunohistological analysis of Tsx protein in adult olt testis. (A) Immunostaining by the Tsx762 antiserum at 1:500 dilution. (B) Immunostaining of a consecutive section by the antiserum after preincubation with the Tsx peptide antigen. Bar, 100 μm.

FIG. 8. Immunohistological analysis of Tsx protein on sections of pubertal testis. Immunostaining by the Tsx762 antiserum at 1:500 dilution on sections of testis at 12 dpp (A), 16 dpp (C), 19 dpp (E), and 27 dpp (G). Control immunostaining by the same antiserum preabsorbed to the Tsx peptide antigen on sections of testis at 12 dpp (B), 16 dpp (D), 19 dpp (F), and 27 dpp (H). All of the sections were lightly counterstained with hematoxylin to visualize nuclear morphology. Bar in A, C, and E, 100 μm. Bar in G, 50 μm. Similar results were obtained with antiserum Tsx850.

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puberty requires unique regulation and whether Tsx may be involved in this. The expression of Tsx in spermatogonia of the adult olt testis, where spermiogenesis is blocked, was found to be similar to that in the normal 27-dpp testis of control animals suggesting that gene expression in germ cells near the beginning of the spermatogenic cycle may be influenced by postmeiotic cell types which are absent in the adult from the olt mutant testis. Thus, one hypothesis to explain the transient expression of Tsx in premeiotic germ cell nuclei during puberty is that it plays a role in regulating the progression of type B spermatogonia or preleptotene spermatocytes toward the first wave of meiosis in the unique context of the immature tubule and that this function is not required once late spermatids or spermatozoa are present in the mature testis.

Direct contact and communication between Sertoli cells which cease dividing during puberty and therefore represent a finite population in the adult testis appear to be required for the maturation of most germ cell stages (reviewed in Jégou and Sharpe, 1993). Indeed proliferation and differentiation of spermatogonia are almost certainly coordinated to ensure that the number of germ cells entering meiosis does not exceed the capacity of the Sertoli cells to sustain them (Kluin et al., 1984; Orth et al. 1988; Mearchem et al., 1996; reviewed in De Rooij and Van Dissel-Emiliani, 1997). Apoptosis, which occurs mainly in type A spermatogonia and spermatocytes in normal adult testis, represents one mechanism influencing the number of germ cells that reach maturity (reviewed in Dunkel et al., 1997). The transient nuclear expression of Tsx protein in premeiotic germ cells may similarly play a role in modulating the proliferation and differentiation rate of germ cells during early postnatal development to a level that is compatible with the eventual functioning of the mature spermatogenic cycle.

The formation of the blood-testis barrier by Sertoli cells during puberty creates two distinct physiological compartments in the mature seminiferous epithelium (reviewed in Gondos and Berndston, 1993). The basal compartment, containing the diploid spermatogonia and preleptotene spermatocytes, is accessible to circulating components of the interstitial serum across the lamina propria. The adluminal compartment, containing meiotic spermatocytes, haploid spermatids, and mature spermatozoa, is physiologically isolated from the interstitial serum and its composition is determined in large part by Sertoli cell secretion and selective transport. Normal germ cell maturation is thought to require the unique microenvironment of the adluminal compartment. In the mouse, formation of the blood-testis barrier occurs from 10 to 16 dpp (N agano and Suzuki, 1976). Since meiosis commences at 10 dpp, the first wave of differentiating spermatogonia and spermatocytes develops in a microenvironment that is distinct from that of the adult adluminal compartment. The lack of a fully functional blood-testis barrier during puberty may allow an endocrine or paracrine regulatory factor from the serum or peritubular cells to diffuse throughout the immature seminiferous epithelium and incidentally activate Tsx expression in germ cells as well as in the targeted Sertoli cells. Alternatively, Tsx expression in spermatogonia at this time may play a role in directing them into the basal compartment or distinguishing them from spermatocytes during the initial formation of the blood-testis barrier.

In summary, the changing pattern of Tsx expression within the germline and Sertoli cells over the course of testis maturation and the persistence of the immature pattern of expression in the adult olt mutant suggest that it plays more than one role in the establishment and maintenance of the spermatogenic cycle and that its regulation may be influenced by signaling both between germ cell types and between the germline and Sertoli cells. Additional studies of the alternative Tsx transcripts and their distribution in germ cells and Sertoli cells, the expression pattern of Tsx in other male sterile mutants that are blocked at early stages of postnatal testis development, the precise timing of appearance and disappearance of Tsx expression in germ cells, and the phenotype of a Tsx null allele will address these questions. Analysis of Tsx expression during embryogenesis will be required to determine whether its function is indeed specific to spermatogenesis or whether it also plays a role in other developmental pathways.

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