Spermatogonia-Dependent Expression of Testicular Genes in Mice

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Spermatogenesis is initiated by the interaction of germ cells and somatic cells in seminiferous tubules. We used cDNA microarrays and representational difference analysis to identify genes that are expressed in the testis of the jsd/jsd mutant mouse, which contains only type A spermatogonial germ cells and Sertoli cells, but not in the testis of the W/Wv mutant mouse, where Sertoli cells but few germ cells are present. We isolated 20 known genes and 4 novel genes, including 2 genes encoding lipocalin family members (prostaglandin D synthetase and 24p3) and 2 tumor suppressors (protein tyrosine phosphatase TD14 and Sui1). All 24 of these jsd/jsd-derived genes were highly expressed in the cryptorchid testis as well as in the jsd/jsd testis. This indicates that their selective expression is not directly caused by the as-yet-uncharacterized jsd gene product, but is rather correlated to the cessation of spermatogonial differentiation. In situ hybridization analysis and flow cytometric sorting followed by reverse transcriptase-PCR revealed that these genes are expressed in both the spermatogonial germ cells and the somatic cells in the developing gonads and adult testes. As the mRNAs of these jsd/jsd-derived genes were barely detectable in the W/Wv testis, we propose that early spermatogonial germ cells regulate the expression of a group of testicular genes. © 2002 Elsevier Science (USA)

Key Words: spermatogonia; testis; jsd/jsd mouse; W/Wv mouse; cryptorchid; spermatogenesis; microarray; RDA.

INTRODUCTION

Spermatogenesis is supported by the interaction of spermatogonial germ cells with somatic Sertoli cells that provide them with nutrients and growth factors. A transverse section of seminiferous tubules in a mouse testis reveals a stereotyped array of cells arranged from the basement membrane to the luminal surface, reflecting succeeding waves of spermatogenesis passing along the tubule (Oakberg, 1971; de Rooij, 1973). It is known that during an early stage of spermatogenesis, individual type A spermatogonial stem cells undergo self-renewal near the basal membrane of seminiferous tubules and thereby produce daughter cells that are connected by intercellular bridges (de Rooij, 1998). These daughter cells in turn produce the differentiating spermatogonial cells that eventually become mature spermatozoa. Morphological studies have estimated that, of the 108 total testicular cells in an adult mouse, 35,000 are spermatogonial stem cells (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993). Recent intratesticular engraving studies have also clearly demonstrated the presence of repopulating spermatogonial stem cells in the testis (Brinner and Avarbock, 1994; Brinner and Zimmermann, 1994). More recently, Shinohara et al. (2000b) succeeded in enriching these colonizing spermatogonial stem cells from mouse testis by fluorescent-activated cell sorting (FACS) based on the light scattering property of these cells in combination with their high expression levels of integrin...
α6 and low or no expression of c-kit and αv-integrin. A much higher frequency of spermatogonial stem cells (500 per 10^3 isolated testicular cells) can be generated by using the cryptorchid procedure in mice, which disturbs spermatogonial stem cell differentiation (Shinohara et al., 2000a).

Genetically infertile mice have been used as models to investigate the molecular cues that guide the expansion and differentiation of spermatogonial germ cells. The Dominant white spotting (W) and Steel (Si) loci encode a c-kit tyrosine kinase type of cytokine receptor and its ligand, denoted as steel factor or stem cell factor, respectively (Zsebo et al., 1990; Huang et al., 1990). Mutant mice in either of these loci, namely W/Wv and Si/Si<sup>−</sup> mice, are sterile due to the impairment of proliferation and migration of primordial germ cells during the embryonic stages (Chabot et al., 1988; Geissler et al., 1988). Reciprocal intratesticular transplantation studies revealed that the testicular germ cell deficiency of W/W<sup>v</sup> and Si/Si<sup>−</sup> can be attributed to defects in the spermatogonia and the supportive microenvironment, respectively (Ogawa et al., 2000). This is supported by the observations in the testis of mice, the total number of spermatogonial germ cells is severely reduced, while Sertoli cell numbers are unaffected (see Besmer et al., 1993, for review; De Franca et al., 1994). The mutations in the c-kit/steel factor system affect only the differentiated spermatogonial cells, as proliferation of undifferentiated spermatogonial stem cells appears not to be dependant on this system (Ohta et al., 2000). Other factors involved in the self-renewal of spermatogonial stem cells are also being identified. One such essential factor is glial cell-derived neurotrophic factor (GDNF)<sup>3</sup>, which is produced by Sertoli cells (Meng et al., 2000).

In the testes of juvenile spermatogonial depletion (jsd) mutant and artificial cryptorchid mice, although spermatogonial germ cells are present, unlike what is seen with W/W<sup>v</sup> or Si/Si<sup>−</sup> mutant mice, their further differentiation is blocked (Beamer et al., 1988; de Rooij et al., 1999). In jsd/jsd mice, the first wave of spermatogenesis is completed, but the subsequent spermatogonial differentiation is blocked due to a mutation on chromosome 1 (Beamer et al., 1988). Contributing to this is that jsd/jsd mice have a higher sensitivity to intratesticular testosterone secreted between 4 and 5 weeks of age that causes the rapid deterioration of spermatogenesis (Jean-Faucher et al., 1978). As a result, jsd/jsd mice are sterile. At 8 weeks of age, the seminiferous tubules of these mice consist of Sertoli cells and undifferentiated type A spermatogonia (Kojima et al., 1997).

To identify the genes expressed in the stem cell-containing type A spermatogonia of the adult testis, we compared the testes of the jsd/jsd and W/W<sup>v</sup> mutant mouse strains at a molecular level by using cDNA microarrays and representational difference analysis (RDA). cDNA microarray hybridization assays allow the simultaneous parallel expression analysis of thousands of genes and the rapid identification of differentially expressed genes. However, this analysis is limited to a set of cDNAs immobilized on slide glasses. To compensate for this limitation, we also used RDA, a method originally established to isolate differences between genomic DNA samples (Listtsyn and Wigler, 1993) and later modified to utilize cDNAs to isolate genes that are differentially expressed by two cell or tissue populations (Hubank and Schatz, 1994). We employed both methods in the present study and thereby isolated a group of genes that are highly expressed in the testis of the jsd/jsd but not in the W/W<sup>v</sup> mouse. Intriguingly, most are expressed in both spermatogonial germ cells and somatic cells, suggesting an involvement of spermatogonia in testicular gene regulation.

**MATERIALS AND METHODS**

**Mice**

C57BL/6/jsd/jsd mice, derived from original stocks obtained from the Jackson Laboratory, were maintained at Osaka University. C57BL/6 and WBB6F1-W/W<sup>v</sup> mice were purchased from Nihon SLIC (Hamamatsu, Japan). For the microarray analysis and RDA, RNAs were prepared from the testes of adult mice (>3 months old).

**cDNA Microarray Analysis**

Poly(A)<sup>+</sup> RNAs were isolated by using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). Mouse microarrays carrying approximately 4000 different cDNAs derived from the 17.5 days post coitum (dpc) fetus and adult brain were hybridized with Cy3- or Cy5-labeled cDNA probes prepared from the jsd/jsd testis or W/W<sup>v</sup> testis mRNAs as previously described (Yoshikawa et al., 2000; Maeda et al., 2001). Fluorescent signals were quantified by ScanArray 4000 (GSI Lumonics, Boston, MA), and data were analyzed by using QuantArray software.

**cDNA RDA**

cDNA RDA was performed according to the method described by Hubank and Schatz (1994). The starting materials consisted of 1 μg of jsd/jsd mouse testis mRNA as the tester and 1 μg of W/W<sup>v</sup>...
mouse testis mRNA as the driver, cDNAs were digested with Sau3A I for maximum representation, ligated to R-BgII-12/24 adapters, and then amplified by polymerase chain reaction (PCR) to generate amplicons. The adapters were removed by digestion with Sau3A I followed by cDNA spin column chromatography (Amer sham Pharmacia Biotech). To generate the tester, J-BgII-12/24 adapters were ligated to jsd/jsd mouse testis mRNAs. Subtrac tive hybridization was performed at a driver-tester ratio of 100:1. An aliquot of the hybridization mixture was amplified by PCR for 10 cycles using the J-BgII-24 as a primer. The PCR products were then digested with mung bean nuclease (New England Biolabs) at 30°C for 30 min and further amplified for 20 cycles. Since most of the cDNA fragments encoded prostaglandin D synthetase (PGD-S), a second round of subtractive hybridization was performed at a drive:tester:PGD-S ratio of 100:1:50. The subtracted PCR products were ligated into plasmid vector pGEM-T Easy by using a T/A cloning kit (Promega). Inserts of cdNA clones were recovered by direct PCR amplification of the cultures by using vector primers T7 and SP6. Sequencing of cloned cDNA was determined with a Thermo Sequenase Dye Terminator Cycle Sequencing Pre-Mix Kit (Amer sham Pharmacia Biotech) using an automated DNA se quencer (PE Applied Biosystems). Sequence data were examined for homologies to known sequences by searching public databases with the BLAST and FASTA programs.

In Situ Hybridization

Adult mouse testes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and embedded in paraffin to prepare sections (6-μm thick) for in situ hybridization analysis. Digoxigenin (DIG)-labeled antisense and sense RNA probes were prepared by using the DIG RNA-Labeling Kit (Roche Molecular Biochemicals). Hybridization was carried out at 50°C for 18 h, and bound probes were detected by using alkaline phosphatase (AP)-conjugated anti-DIG antibody and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

RESULTS

Isolation of Differentially Expressed Genes by cDNA Microarray Analysis and RDA

To isolate testicular genes that are specifically expressed in jsd/jsd mice but not in W/W+ mice, we first employed cDNA microarrays containing approximately 4000 distinct mouse cDNAs derived from the brain and the 17.5-dpc fetus. They were hybridized with either Cy3 (or Cy5)- labeled jsd/jsd testis cDNA or Cy5 (or Cy3)-labeled W/W+ testis cDNA. Each image was captured separately and displayed as scatter plots of Cy3/Cy5 or Cy5/Cy3 fluorescence signals (data not shown). Seventeen clones exhibited significantly higher fluorescence ratios of jsd/jsd vs W/W+. To test the reliability of the microarray hybridization results, we analyzed the expression of these clones by Northern blot analysis using various 32P-labeled cDNA probes. We confirmed that the sequences contained in eight of these clones are selectively expressed in the jsd/jsd testis and that they belong to known genes, including those encoding PGD-S, the major histocompatibility complex region class I Q region (MHCI-Q), eIF-2α, ribosomal protein L5, Spi protease inhibitor, and inter alpha inhibitor H2 chain in addition to two uncharacterized expressed sequence tag (EST) clones (Table 1; Fig. 1; data not shown).

To identify additional jsd/jsd-specific genes, we performed a systematic screening by cDNA RDA, where the cDNA of W/W+ is subtracted from jsd/jsd cDNA. After the initial RDA round, 192 cDNA fragments were isolated. Sequence analysis of randomly picked clones revealed that 18 of 20 clones were fragments of PGD-S cDNA. We therefore added PGD-S cDNA as a driver in addition to the W/W+ amplicons in the next RDA experiment. We picked
112 clones from the second RDA product and subjected them to Southern blot analysis using PGD-S cDNA as a probe. The non-PGD-S cDNA clones were sequenced and 30 kinds of cDNAs were finally obtained. Northern blot analysis confirmed that 17 genes were highly expressed in jsd/jsd testis, while they were absent or minimally expressed in the W/W* testis (Fig. 1; data not shown). Of these genes, 15 were known genes, including the genes encoding 24p3, complement component 7, angiotensinogen, kallikrein 24, protein tyrosine phosphatase-TD14, SAM-9, and all the other genes were found to be expressed not only in the jsd/jsd testis but also in the cryptorchid testis (Fig. 2A; data not shown; results are summarized in Table 1). This suggests that the expression of the 24 jsd/jsd-derived genes is related to the stage of spermatogonial germ cell development rather than to the jsd gene defect.

### Temporal and Organ-Specific Expression of jsd/jsd-Derived Genes

We examined the expression of the 24 jsd/jsd-derived genes in the testicles from the wild-type strain at different developmental stages, ranging from fetuses to 8-week-old mice. We found that the 24 genes could be categorized into several groups based on their expression pattern (Table 1). The first group contains 13 genes, represented by PGD-S, Sui1, PTP-TD14, and all the other genes were found to be expressed not only in the jsd/jsd testis but also in the cryptorchid testis (Fig. 2; data not shown; results are summarized in Table 1). This suggests that the expression of the 24 jsd/jsd-derived genes is related to the stage of spermatogonial germ cell development rather than to the jsd gene defect.

### Expression of the jsd/jsd-Derived Genes in Cryptorchid Testes

We used Northern blotting to examine the expression of the 24 jsd/jsd-derived genes in cryptorchid testes that are devoid of differentiated germ cells, similar to what is observed in jsd/jsd testes. mRNAs for PGD-S, Sui1, PTP-TD14, SAM-9, and all the other genes were found to be expressed not only in the jsd/jsd testis but also in the cryptorchid testis (Fig. 2; data not shown; results are summarized in Table 1). This suggests that the expression of the 24 jsd/jsd-derived genes is related to the stage of spermatogonial germ cell development rather than to the jsd gene defect.

### Table 1

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*Not detectable as a band by Northern blot analysis.
fetal testis but becomes undetectable by Northern analysis in the testes of 10-day-old and 3-week-old mice (Fig. 2A). Thereafter, PGD-S mRNA reappears at low levels in the 8-week-old testis and appears to be increased in the 3-month-old adult (Figs. 2A and 2B). The fourth group contains 9 genes, including 24p3, whose expression according to Northern blot analysis is visible in the jsd/jsd or cryptorchid testis but not in the normal mouse testis 8 weeks after birth (data not shown), although their mRNAs were detectable in the testes in normal mouse when RT-PCR was used (see Fig. 4C; data not shown).

When we examined mRNA expression of the jsd/jsd-derived genes in various adult mouse organs, we found that most are not testis-specific genes. For example, PGD-S mRNA was also detected in the brain, and both PTP-TD14 and SAM-9 were expressed in the brain and ovary as well as in the testis (Fig. 2B). In contrast, Sui1 was predominantly expressed only in the testis (Fig. 2B). Expression profiles of some of the other genes according to Northern blot analysis are summarized in Table 2.

mRNAs of the jsd/jsd-Derived Genes Are Localized in the Seminiferous Tubule

We next determined which testicular cells express the jsd/jsd-derived genes by in situ hybridization on sections of seminiferous tubules of a 12-week-old wild type mouse. As shown in Fig. 3, antisense cRNA probes to PTP-TD14, EST-s59, and SAM-9 mainly hybridized to cells located near the basement membrane of the seminiferous tubules. Sense control probes did not give any specific signals. Based on morphological observation, PTP-TD14 is likely expressed in spermatogonia, spermatocytes, and round spermatids, but not in elongating spermatids (Fig. 3A). EST-s59 expression is likely limited to spermatogenic cells in the basal compartment (spermatogonia and early spermatocytes), while SAM-9 is likely expressed in spermatogonia and spermatocytes (Figs. 3C and 3E). Location of mRNAs for 10 other genes (24p3, kallikrein 24, angiotensinogen, PGD-S, MHCI-Q, tetraspanin TM4-A homologue, Sui1, EST-MNCb4625, EST-c39, and inter-alpha inhibitor H2 chain) was as similar as either pattern of PTP-TD14, EST-s59, or
Thus, testicular mRNAs for most of the jsd/jsd-derived genes appear to be produced in the testis by spermatogonial germ cells at an early stage of their development.

Expression of the jsd/jsd-Derived Genes in Germ and Somatic Cell Fractions

To confirm that the jsd/jsd-derived genes are specifically expressed in spermatogonial germ cells, as suggested by the in situ hybridization data, germ cells and somatic cells were fractionated by FACS using antibodies specific for two germ cell antigens, SSEA-1 and EE2. In 8.5- to 14.5-dpc mouse embryos, primordial germ cells (PGCs) are known to express the carbohydrate-differentiation antigen SSEA-1. When we sorted cells from the gonadal regions of 90 male wild type 13.5-dpc mouse embryos with the antibody specific for SSEA-1, we obtained approximately 4 x 10^6 SSEA-1-negative cells and 2 x 10^6 SSEA-1-positive cells (other somatic cells) (Fig. 4A). These were subjected to RT-PCR analysis. Confirming the utility of the SSEA-1 antibody, Oct3/4 mRNA, an authentic marker of PGCs, was detected in the SSEA-1-negative but not the SSEA-1-positive fraction (Fig. 4C) (Scholer et al., 1990), while, in contrast, sulfated glycoprotein (SGP)-2, which is a marker of Sertoli cells (Sylvester, 1993), was only expressed in the SSEA-1-negative fraction (Fig. 4C). However, when we examined the expression of the 24 jsd/jsd-derived genes by RT-PCR, we found that basically all could be detected in both the SSEA-1-negative and SSEA-1-positive fractions (summarized in Table 3). However, transcripts of 24 genes, angiotensinogen, PTP-TD14, SAM-9, spi-proteinase inhibitor, and five other genes appeared to be more abundantly expressed in the SSEA-1-negative fraction (Fig. 4C).

The anti-EE2 antibody is known to recognize a carbohydrate epitope present on type A and B spermatogonia and early meiotic cells in the mouse testis (Koshimizu et al., 1995). Approximately 2 x 10^6 EE2-negative cells and 2 x 10^6 EE2-positive cells were sorted from 2 testes from 3-week-old mice (Fig. 4B) and used for RT-PCR analysis (Fig. 4C). Detection of Oct3/4 and SGP-2 in both fractions indicated that separation of germ cells from somatic cells (mainly Sertoli cells) may not be as clear-cut as when the anti-SSEA-1 antibody was used. This probably reflects the nature of the EE2 antigen. Nevertheless, only the EE2-negative fraction expressed the transketorase-like 1 gene, which was recently isolated as a spermatogonia-specific gene (Wang et al., 2001), suggesting that EE2 antibody was used. This probably reflects the nature of the EE2 antigen. Nevertheless, only the EE2-negative fraction expressed the transketorase-like 1 gene, which was recently isolated as a spermatogonia-specific gene (Wang et al., 2001), suggesting...
that the anti-EE2 antibody is still useful in separating specific cell subsets. RT-PCR analysis of the jsd/jsd-derived genes in the EE2- and EE2+ cell fractions showed that 13 of the 24 jsd/jsd-derived genes were expressed equally in both the germ and somatic cell fractions, while the remaining 11 genes, including PTP-TD14 and spi-proteinase inhibitor, were more abundantly expressed in the testicular somatic cells than in the germ cells (Fig. 4C; Table 3).

**DISCUSSION**

In this study, we have compared, at a molecular level, the testes of the W/Wv and jsd/jsd mutant mice. The testicular development of these two sterile strains differ in that, in the first, very limited number of spermatogonia develop, while in the second, type A spermatogonia develop but are arrested at subsequent differentiation stages. The comparison between the mice was intended to identify the spermatogonial-specific genes or the genes involved in the cessation of spermatogonial development and was made by performing both cDNA microarray analysis and RDA, a PCR-based subtractive hybridization assay. Use of these two methods together proved to be very useful for studying the complex gene expression that characterizes testicular development. These methods also allowed us to demonstrate that spermatogonial cells play a role in regulating testicular gene expression. In total, we identified 24 genes whose mRNAs are upregulated in the jsd/jsd testis and almost undetectable in the W/Wv testis. The use of mouse cDNA microarrays made from the adult brain and whole fetus allowed us to first identify 8 of these genes. RDA then resulted in the isolation of 18 genes with a similar expression pattern, 2 of which had already been identified by the cDNA microarray analysis. One of these 2 genes was PGD-S, which was isolated so frequently in the first round of RDA that we had to perform the second RDA, wherein we added PGD-S cDNA as a driver in addition to the W/Wv ampiclons, thus allowing PGD-S cDNAs to be removed. That only 2 genes, PGD-S and MHCI-Q, were identified by both approaches may be largely due to the limited cDNA coverage on the microarrays we used. It is also possible that some cDNAs may not be recovered by RDA due to the restriction sites used. Given the potential limitations of both methods, this study demonstrates that while future gene expression studies will certainly benefit from the preparation of more cDNA arrays from a variety of mouse tissues and organs, including the testis, combining such microarray analyses with RDA will improve the scope of these studies.

While the jsd gene has not yet been identified, it is known that the jsd mouse mutation causes the second wave of spermatogenesis to arrest, resulting in only type A spermatogonia and Sertoli cells being present in the seminiferous tubules of adult jsd/jsd mice (Beamer et al., 1988; Kojima et al., 1997). Transplantation experiments using spermatogonial cells confirm that the jsd/jsd phenotype is due to a defect in the germ cells themselves rather than in their extra- (Mizunuma et al., 1992) or intratubular (Boettger-Tong et al., 2000; Ohta et al., 2001) environment. A similar testicular cell composition, i.e. consisting largely of type A spermatogonia and Sertoli cells, is also observed in artificial cryptorchid mouse testes (Nishimune et al., 1978; de Rooij et al., 1999), and in fact, the cryptorchid testis contains a higher frequency of spermatogonial stem cells than the normal mouse testis (Shinohara et al., 2000a). Confirming the similarities between the jsd/jsd and the cryptorchid testis, we found that the expression levels of all
FIG. 3. Detection of PTP-TD14, EST-s59, and SAM-9 mRNAs in adult mouse testis by in situ hybridization. Paraffin sections of seminiferous tubules of 12-week-old mice were hybridized with digoxigenin-labeled antisense (A, PTP-TD14; C, EST-s59; E, SAM-9) or sense cRNA probes (B, PTP-TD14; D, EST-s59; F, SAM-9). The original magnification was 200×.
24 of the jsd/jsd-derived genes were also enhanced in the cryptorchid testis. This suggests that the aberrant expression of these genes in the jsd/jsd mice is not directly caused by the jsd gene product, but rather correlates with the cessation of spermatogonial differentiation in the testis. As Sertoli cells are present in the testes of both the jsd/jsd and the W/W' mice further suggests that the mRNA expression of the 24 jsd/jsd-derived genes is dependent on the presence of type A spermatogonial germ cells.

When we examined the expression of the jsd/jsd-derived
genomic expression patterns that have been observed in previous studies. These patterns were then used to identify candidate genes for further analysis.

One gene showed a pattern of expression that distinguished it from the other jsd/jsd-derived genes, namely PGD-S. In normal mouse, this gene was expressed at a high level in the 19.5-dpc testis, but these levels were diminished in the 10-day-old and 3-week-old testes. Later in development, however, PGD-S expression was partially restored, being found at low levels in the 8-week-old and 3-month-old testes. This result is consistent with previous observations made by using in situ hybridization (Hoffmann et al., 1996). These authors also showed that PGD-S expression is age-dependent. Moreover, they found that PGD-S is mainly expressed in Leydig cells, and that the age-dependence of PGD-S expression is due to the Leydig-cell synthesis and secretion of testosterone during testicular differentiation. Supporting these observations is the fact that the concentration of PGD-S in the seminal plasma is significantly lower in oligozoospermia patient group than in normozoospermia control group (Tokugawa et al., 1998). PGD-S activity is not altered in W/W mice (Ika et al., 1984), indicating that the W/W phenotype does not result from a defect that suppresses PGD-S expression in the testis. We observed that PGD-S is not only expressed in the testis, as its mRNA is also found in the brain. This has been previously noted by other investigators, whose work further suggests that the biological role of PGD-S differs between the two organs (Hoffmann et al., 1996). In the brain, PGD-S appears to be critical for regulating sleep-wake rhythms in the central nervous system, while in the testis, PGD-S acts as a member of the lipocalin family that binds and transports small lipophilic ligands, such as retinoic acid and retinol (Pervaiz and Brew, 1987; Chan et al., 1996). Thus, PGD-S could play an important role in the transporting of such metabolites across testis-blood barriers in the func-

### Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>SSEA-1</th>
<th>EE2</th>
<th>Primer sequence sense (5’-3’)</th>
<th>Primer sequence antisense (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PGD-S</td>
<td>++*</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2 24p3</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 complement component 7</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 angiotensinogen</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 kalelikrein 24</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 PTP-TD14</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7 glutathione-S-transferase homologue</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8 GlcNAc-P mutase</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9 SAM-9</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10 alpha-mannosidase</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11 MHC-I-Q</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12 proteasome 26S subunit</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13 translationally regulated transcript</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14 tetraspanin TM4-A homologue</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15 GDP dissociation inhibitor beta</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16 Sui1</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17 EST-c39</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18 EST-e39</td>
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<tr>
<td>19 eukaryotic initiation factor 2 beta</td>
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<tr>
<td>20 ribosomal protein L5</td>
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<td>++</td>
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<tr>
<td>21 EST-MNCb3719</td>
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<td>+</td>
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<td>++</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23 spi proteinase inhibitor</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24 inter alpha inhibitor H2 chain</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* ++*, A higher amount of PCR product was detected.
tionally matured testis. However, whether PGD-S expression is specifically linked to the cessation of spermatogonial differentiation is unclear at this stage. In fact, it is quite possible that, given the dependence of PGD-S expression on testosterone levels (Hoffmann et al., 1996), the upregulated expression of PGD-S in jsd/jsd mice may be simply related to the fact that circulating testosterone levels are unchanged at all ages in these mice (Beamer et al., 1988).

A molecule that shares similar metabolic functions in the testis as PGD-S is 24p3, another member of the lipocalin family (Flower et al., 1991). The gene encoding this protein was identified in the second round of RDA. The expression of the 24p3 gene is visible in the jsd/jsd and cryptorchid testis but not in the normal mouse testis 8 weeks after birth. Recently, it was reported that 24p3 is secreted upon growth-factor deprivation and induces apoptosis in an interleukin-3-dependent leukocytic cell line and primary bone marrow cells (Deveddy et al., 2001). Thus, 24p3 could be involved in the apoptosis of germ cells in the testis. These observations with the two lipocalin genes 24p3 and PGD-S, together with their spermatogonia-dependent expression, suggest that development of early testicular germ cells and the functional maturation of testis are tightly regulated by means of gene expression.

Also detected by RDA were PTP-TD14 and Sui1. PTP-TD14 was observed to be expressed at consistent levels throughout testicular development in wild type mice. Expression was also observed in the brain and ovary (Figs. 2A and 2B). The protein encoded by PTP-TD14 was originally isolated from rat cardiomyocytes and can suppress H-ras-mediated cell transformation (Cao et al., 1998). Furthermore, a hemizygous missense mutation of the human PTP-TD14 gene was recently discovered in a lung cancer cell line (Toyooka et al., 2000). In contrast to PTP-TD14, Sui1 was observed to be predominantly expressed in testis and in no other organs of wild type mice (Figs. 2A and 2B). Similar to PTP-TD14, however, Sui1 appears to be a tumor-suppressor gene as its expression can be suppressed by the hepatitis B X antigen in hepatocellular carcinoma cells (Lian et al., 1999). Furthermore, reintroduction of Sui1 into HepG2 cells inhibited cell growth. As both gene products appear to have tumor-suppressor activity, it is possible that the spermatogonia-mediated induction of PTP-TD14 and Sui1 gene expression could serve as defensive machinery against testicular germ cell tumours during spermatogenesis.

Some of the other jsd/jsd-derived genes may also play roles in testicular development. One of these is the gene encoding GlcNAc-P mutase, which has been previously identified as a gene that is expressed in response to erythropoietin (Epo) and is postulated to regulate Epo-mediated endothelial cell proliferation (Li et al., 2000). As human testicular germ cell tumor cells are known to produce a significant amount of Epo (Reman et al., 1991), it is possible that GlcNAc-P mutase operates downstream of the EPO signal in the testis.

Another jsd/jsd-derived gene is SAM-9, which was originally isolated from differentiated Schwann cell tumors and whose protein possesses a motif that is conserved in the phospholipase D superfamily (Pedersen et al., 1998). Based on the expression spectrum of SAM-9, Pedersen et al. speculate that it is involved in later stages of neurogenesis such as neurotransmission and neuronal survival. We found that SAM-9 is expressed not only in the brain but also in the testis and the ovary (Fig. 2B). The enhanced expression of SAM-9 in the jsd/jsd testis might indicate that the biological function of testicular SAM-9 differs from its role in the brain, similar to what is thought for PGD-S.

Two of the 24 jsd/jsd-derived genes encode proteinase inhibitors, namely the spi-proteinase inhibitor and the inter-alpha inhibitor H2 chain. Peritubular cells, Sertoli cells, and germ cells of the seminiferous tubules are known to synthesize and secrete several proteases and proteinase inhibitors. The complex network of proteolytic enzyme activity and their negative regulation by protease inhibitors are critical for normal male reproduction (Monses et al., 1997). That the expression of the genes encoding the proteinase inhibitor and inter-alpha inhibitor H2 chain may be regulated by spermatogonia indicates that at least these two gene products are utilized in this testicular proteolytic network.

Of the remaining genes that we identified, four are EST clones whose gene products have not yet been characterized. Cloning of the full-length cDNAs of these genes and analysis of their functions are underway in this laboratory.

Spermatogenesis in seminiferous tubules proceeds if an appropriate environment is provided by Sertoli cells (see Clermont, 1972 for review). Sertoli cells are known to provide growth factors, such as the steel factor and GDNF, and support the developing germ cells metabolically. They are also indispensable for inducing meiosis and clearing apoptotic germ cells in the testis (Byers et al., 1993; Rassoulzadegan et al., 1993). In contrast, it appears that germ cells also regulate the biological activities and gene expression of Sertoli cells (Grandjean et al., 1997; Syed et al., 1999; Vidal et al., 2001). For example, in germ-Sertoli cell coculture systems, pachytene spermatocytes were shown to induce Sertoli cells to become phagocytic as well as upregulating their serotonin receptor expression (Syed et al., 1999). Moreover, a gene trap approach successfully identified a number of genes, including fra1 and c-fos, that are specifically induced by adding round spermatids in culture (Vidal et al., 2001). Some of these germ cell-derived factors were revealed to be secreted proteins while others were membrane-associated. It has also been shown that tight junction between Sertoli cells and germ cells is necessary for suppressing the expression of testin, a testosterone-responsive Sertoli cell product (Grima et al., 1998). Furthermore, reporter gene analysis in vivo convincingly demonstrated that transcription of GATA-1 in Sertoli cells was negatively regulated by germ cells in a stage-specific manner (Yomogida et al., 1994). These studies together have established the concept that meiotic or postmeiotic germ cells send molecular messages to Sertoli cells in the testis.
Expanding this notion is the study we have reported here, which demonstrates that spermatogonial germ cells, in early stages of testicular development, send molecular signals to Sertoli cells and other spermatogonial germ cells, and that these signals initiate rather than downregulate the expression of a group of genes. An efficient method to separate spermatogonial germ cells by FACS has recently been established (Shinohara et al., 2000b) and will be of great value in helping establish a coculture system that may reproduce the spermatogonial-dependent gene expression that we have documented here. Other work that is continuing in our laboratory is the identification of putative spermatogonial factors that may govern the expression of the 24 genes we have identified.

In conclusion, by using microarrays and RDA in a complementary strategy, we have isolated 24 genes that are expressed in the testes of jsd/jsd mutant mice and cryptorchid mice but not in the W/W+ tests. We propose that the expression of these genes is regulated by type A spermatogonial germ cells in a novel type of cross-interaction that involves both germ cells and Sertoli cells. These testicular gene products may participate in cell proliferation, differentiation, and the functional maturation of the testis.

ACKNOWLEDGMENTS

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Note added in proof. During the preparation of this manuscript, I. R. Adams and A. McLaren reported the male specific expression of PGD-S in the developing mouse gonad and its possible role as a Sertoli cell differentiation factor (Development, 129, 1155–1164, 2002).

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