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Cloning of Novel Temperature-Related Expressed Sequence Tags in Rat Testis during Spermatogenesis

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Spermatogenesis needs the relatively cool environment of the scrotum in most mammals, it would be arrested when the testis was exposed to abdominal temperature. In this study, we have used a differential display PCR technique (DD-PCR) to screen temperature-related ESTs during spermatogenesis (TRS) in scrotal testes through a unilateral cryptorchid rat model after *in situ* analysis of testis cell DNA fragmentation. We reported here the cloning and sequencing of three such ESTs: TRS1, TRS3, and TRS4. Northern blot analysis confirmed that they were expressed specifically in scrotal testes. *In situ* hybridization showed that TRS1 was mainly expressed in the spermatocytes and the round spermatids in scrotal testis. Homology searches revealed that TRS1 and TRS3 were unknown cDNA sequences, and TRS4 was identical to a known EST whose function had not been reported. TRS1, TRS2, and TRS3 were first found to be temperature-related during spermatogenesis.

Key Words: **cryptorchidism; testis; temperature; ESTs; DD-PCR.**

Cryptorchidism could cause human male infertility by disrupting spermatogenesis (1). The cease of spermatogenesis is most likely due to the mild hyperthermia environment of the abdomen, since heat stress has been shown to induce germ cell apoptosis (2). When cryptorchid testis was removed surgically back to the scrotum within a certain span, spermatogenesis would be restored (3). The change of a few degrees from the abdomen to the scrotum is believed to maintain an optimal environment for testicular function (4). However, the molecular mechanisms responsible for temperature-induced germ cell apoptosis are still unclear.

Spermatogenesis is a complicated process of germ cell differentiation, involving programmatic expression of diverse cell-type and developmental-stage specific genes (5). Disruption of spermatogenesis and germ cell apoptosis in cryptorchid testes probably result from abnormal expression of temperature-related genes during spermatogenesis. Because experimentally induced unilateral cryptorchid testis is under the same circulating hormonal condition with the scrotal testis, the only difference between them is enviromental temperature (6). Therefore unilateral cryptorchidism could provide a useful model for study of temperature regulation of testicular germ cell apoptosis (6). To elucidate the molecular mechanisms of temperature regulation of spermatogenesis, by using unilateral cryptorchid rat model and DD-PCR technique as well as in situ analysis of testis cell DNA fragmentation, we have identified three temperature-related ESTs in testes.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation. Male Sprague-Dawley (SD) rats (200g-250g) were provided by Animal Facility of the Institute of Zoology, Chinese Academy of Sciences. To induce unilateral cryptorchidism, animals were anesthetized and a small incision was made in the abdomen. The gubernaculum was cut on right side to displace the testis into the abdomen. Testis decent was prevented by closure of the inguinal canal on right side by suturing. The animals were killed by cervical dislocation on day 3 and 5 after surgery. The testes were removed, decapsulated and snap-frozen in liquid nitrogen and stored at −70°C for RNA analysis.

In situ analysis of testis cell DNA fragmentation. Testis samples were fixed in Bouin's solution and embedded in paraffin, 6μm thick sections were mounted on gelatin coated slides. After deparaffinization and rehydration, tissue sections were incubated with proteinase K (10μg/ml) for 15 min at 37°C and washed in PBS. The labeling and signal conversion of apoptotic cells were performed using In Situ Cell Death Detection kit (AP) according to the manufacturer's instructions (Boehringer Mannheim). To detect apoptotic signals, the sections were incubated with a mixture of BCIP and NBT (containing 1mM Levamisole).

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Abbreviations used: ESTs, expressed sequence tags; DIG, digoxigenin; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; PBS, phosphate-buffered saline.

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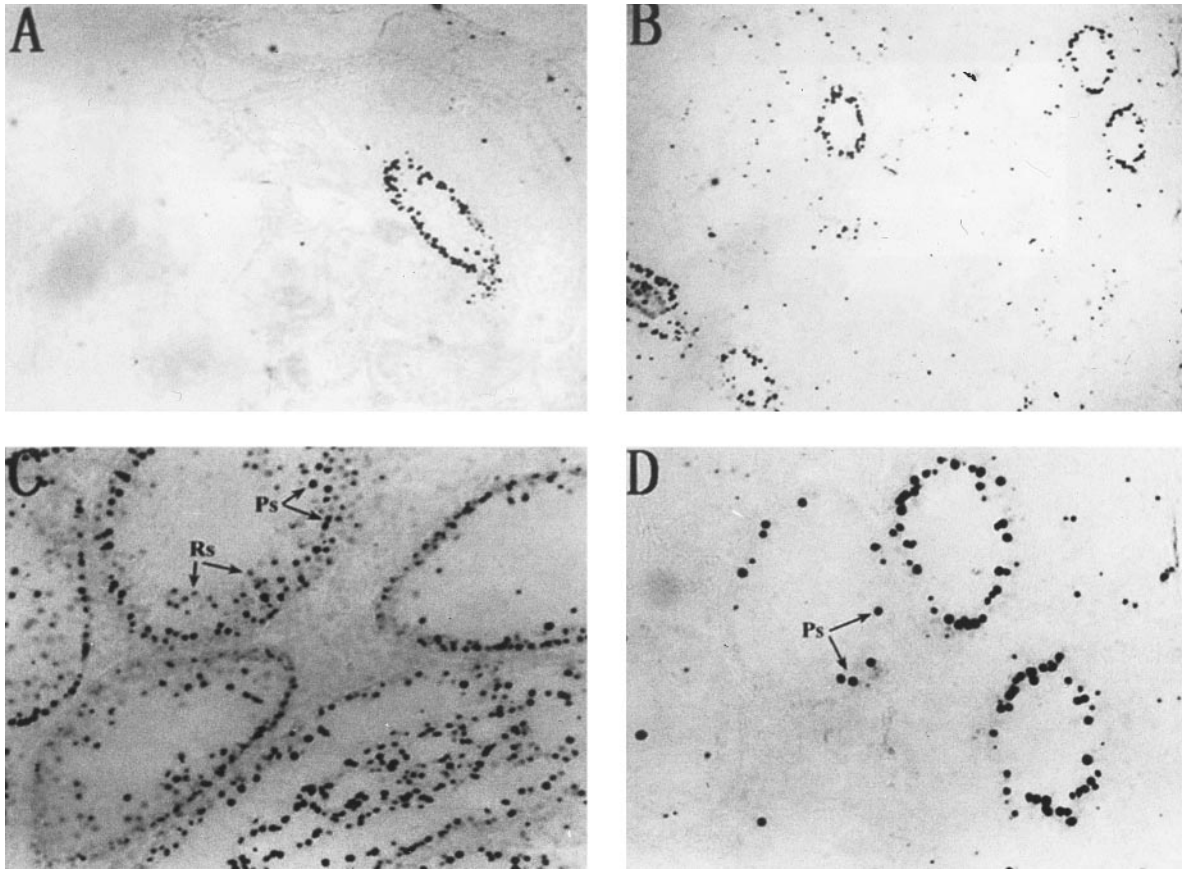

FIG. 1. *In situ* analysis of DNA fragmentation in scrotal and cryptorchid testis. A: contralateral scrotal testis. ×40; B: cryptorchid testis on day 3. ×40; C: cryptorchid testis on day 5. ×100; D: cryptorchid testis on day 3. ×100; Ps: primary spermatocytes; Rs: round spermatids.

RNA isolation and reverse transcription. Total RNA from cryptorchid and scrotal testes were extracted by the single-step method (7) and treated with DNase I to remove DNA contamination. The reaction containing 20μg total RNA, 10U RNase-free DNase I (Gibco/BRL) and 10U RNasin (Promega) was carried out at 37°C for 30 min and extracted with phenol and chloroform.

Reverse transcription (RT) was carried out using $T_{11}CA$ and $GCT_{11}G$ primers, respectively. For each reaction, 0.2μg denatured DNA-free RNA, 1μM primer, 20μM dNTPs and 200U reverse transcriptase (Superscript II, Gibco/BRL),10U RNasin were mixed and incubated at 37°C for 60 min. The reaction was stopped by heating at 95°C for 10 min and the cDNA pool was stored at −20°C until further use.

DD-PCR and isolation of differentially expressed cDNA clones. Random 10mers primers were used as upstream primers, and the RT primers were used as downstream primers. In a total 20μl reaction mixture containing 0.5μM upstream primer, 1μM downstream primer, 2μM dNTPs, 2μl RT mix from reverse transcription reaction, 1μl [α-^{32}P]-dCTP(3000Ci/mmol, 10mCi/ml) and 1.2U Taq polymerase (Promega), amplification was done as following: 95°C for 5 min; and then 30 cycles with 94°C for 30 sec, 41°C for 2 min, 72°C for 30 sec; with an additional 5 min extension at 72°C. After PCR amplification, the radiolabelled cDNAs were electrophoresed in 6% denaturing polyacrylamide gels. The wet gels were then exposed to X-ray films overnight. The differentially expressed cDNAs were excised and the gel slices were soaked in 100μl TE buffer for 10 min and then boiled for 15 min. The supernatant was transferred to a microtube and precipitated with ethanol and glycogen (Boehringer Mannheim). After washing with 70% alcohol, the pellet was dissolved in 10μl water. 3μl of the eluted cDNA were used for reamplification. The reaction was carried out in 40μl solution using identical conditions to DD-PCR except 20μM dNTPs instead of 2μM and no isotope labeled dCTP was added. The reamplified PCR fragments were gel-purified using Advantage PCR-Pure kit (Clontech).

Cloning and DNA sequencing. The candidate cDNAs were cloned into PGEM-T vector using the TA cloning system according to the manufacturer's instructions (Promega). Plasmids with inserts were mini-prepared and sequenced using an automated DNA sequencer (ABI 373A). Comparison of DNA homology with Genebank databases was performed using the BLAST program.

Northern blotting analysis. Cloned cDNAs were used as probes for northern blot analysis. 25μg total RNA were run on 1.2% agarose-formaldehyde gels and transferred onto nylon membranes (Bio-Rad). After cross-linking RNA to the membranes, the membranes were hybridized with [α-^{32}P]-labeled probes at 65°C in a solution containing 1mM EDTA/0.5M Na_2HPO_4, PH7.2/7% SDS(W/V)/15% formamide. The membranes were then exposed to X-ray films at −70°C.

In situ hybridization analysis. The tissue sections were prepared as above. Sections were deparaffinized, rehydrated, and pretreated with proteinase K (10μg/ml) for 15 min at 37°C. Postfixation was done in 4% paraformaldehyde in PBS for 5 min, then sections were dehydrated and dried. Sections were prehybridized at room temperature for 2 h in prehybridization buffer (2×SSC/50% formamide). They were then incubated with antisense and sense DIG-cRNA probes in hybridization buffer (2×SSC/50% formamide/10mM Tris, PH7.5/250μg/ml yeast tRNA/0.5%SDS/1×Denhardt's) at 48°C for 18 h. The sections were washed sequentially with 2×SSC, 1×SSC, 0.1×SSC (2×15 min in each concentration, 42°C). The detection of Dig-labeled probes were carried

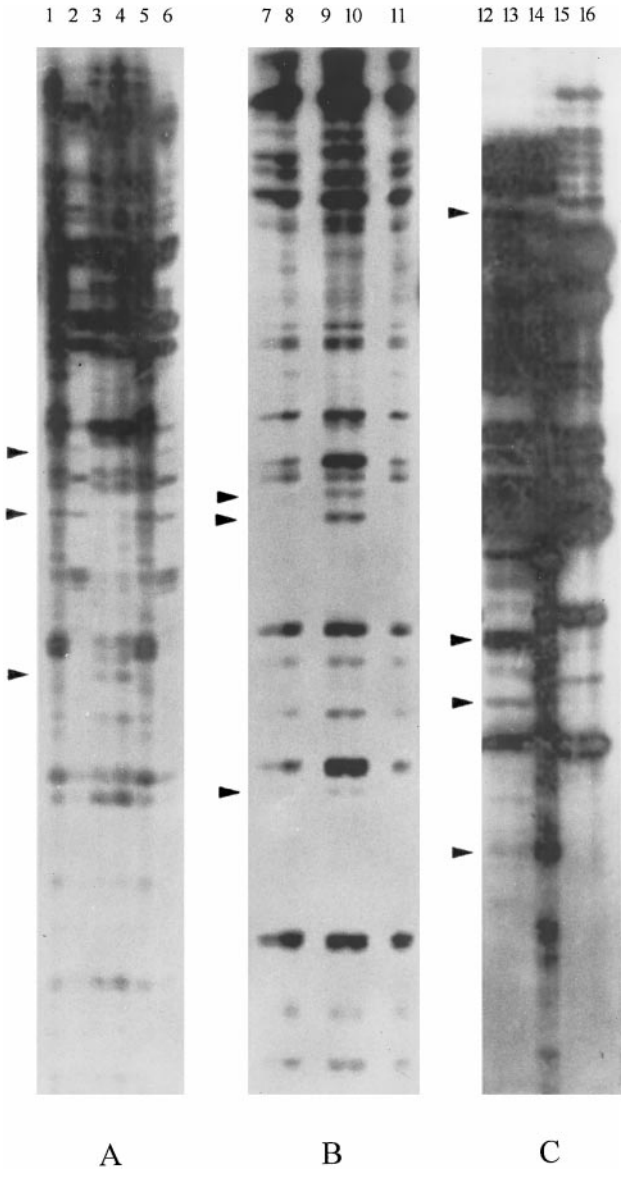

FIG. 2. Gel analysis of differentially displayed mRNAs from cryptorchid and scrotal testes. RNA were reverse transcribed and the reactions were run on 6% DNA sequencing gels as described in Experimental Procedures. Arrowheads show some differentially expressed bands. Lanes 1, 5, 7, 8, 11, and 14 represent the cryptorchid testes on day 3; Lanes 2, 6, 12, and 13 represent the cryptorchid testes on day 5; Lanes 3, 4, 9, 10, 15, and 16 represent the contralateral scrotal testes. The upstream and downstream primers used in A, and B, and C were $GCT_{11}G$ and OPC02, $T_{11}CA$ and S68, $GCT_{11}G$ and OPH06, respectively.

out using alkaline-phosphatase-conjugated anti-DIG Fab and a mixture of BCIP and NBT (containing 1mM Levamisole) according to manufacture's instructions (Boehringer Mannheim).

RESULTS AND DISCUSSION

In Situ Analysis of Testis Cell DNA Fragmentation

Despite the fact that germ cells undergo apoptosis in cryptorchid testes, the reports on the onset time of germ cell apoptosis are discrepant, with Shikone finding that apoptosis was visible on day 2 (6) whereas Yin found it could not be observed until day 6 (2, 4) after the induction of unilateral cryptorchidism. In this study, in situ 3′ end-labeling of DNA technique was used to quantitate differences in DNA fragmentation between the unilateral cryptorchid and scrotal testes. Apoptosis occurred at a quite low level in the sections of the scrotal testes (Fig. 1A). However, the amount of apoptotic cells increased in the cryptorchid testes on day 3, with some seminiferous tubules showing obvious apoptotic signals (Fig. 1B,D), and the affected cells were mainly the primary spermatocytes delineating the periphery of the tubules. In the cryptorchid testes on day 5, the number of seminiferous tubules containing apoptotic germ cells increased more (Fig. 1C). The incidence of apoptotic cells was more significant in the cryptorchid testes on day 7.5 (results not shown). Our

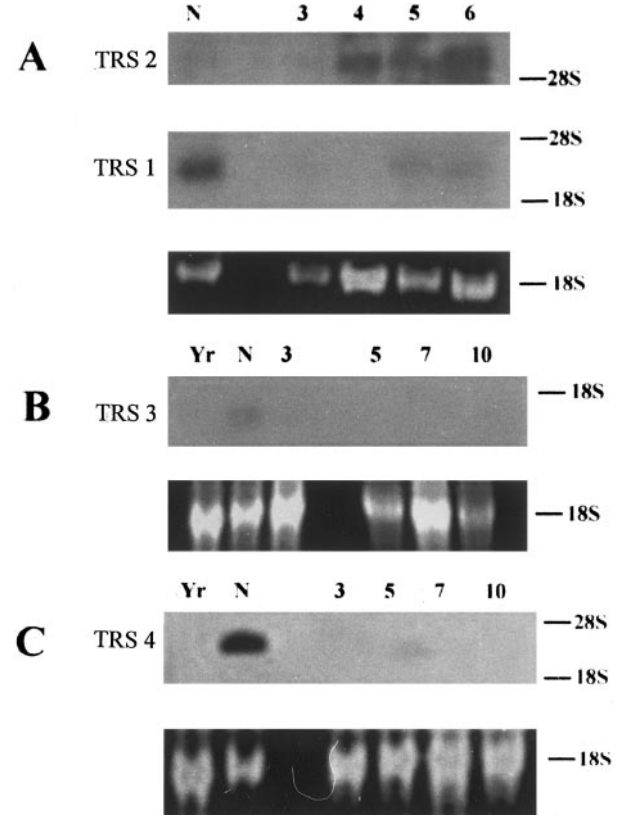

FIG. 3. Northern blot analysis of TRS1, TRS2, TRS3, and TRS4. Total RNA from cryptorchid and scrotal testes were run on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. The membranes were probed with [^{32}p]-labeled cloned differentially displayed cDNAs and exposed to X-ray films at −70°C for 3 days (TRS1), one week (TRS2, TRS3) or 10 days (TRS4), respectively. TRS2 was mainly expressed in the cryptorchid testes; TRS1, TRS3, and TRS4 were mainly expressed in the scrotal testes. Sample loading was indicated by 18S rRNA bands. The same membrane was used for TRS1 and TRS2. N: scrotal testes; Yr: prepuberty rat testes; 3, 4, 5, 6, 7, and 10 correspond to the cryptorchid testes on day 3, 4, 5, 6, 7, and 10, respectively.

TRS1:

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  1 CTCACCGTCC CATGACCAGA CTGTTGCAAC CATGACATCT GAAGAAAAGA
 51 AGGAGCGACC CATAAGTATG ATAAATGAAG CTTCCAATTA CAACATGGCT
101 TCAGACTATA CTGTGCATCC TATGAGCCCC GTGGGCGGGA CATCACGGGC
151 CTCCAAAAAG GTTCATAATT TTGGGAAAAG GTCCAATTCA ATTAAAAGGA
201 ACCCTAATGC CCCTGTGGTC AGGCGCGGCT GGCTTAGCCT TACAGGCTGT
251 TAGTCCTCAG AGCCTCCACG GCAGGACGGT GAG
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TRS3:

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  1 GTGAGGCGTC ATCAAAACTT AACCTTTTAC CTTCACAGAT TTATTCAATG
 51 AACTCCCAGG GCCTCCATGC AAGAAACACA GCACTGCCAT TTAGTACCTG
101 GCATCAGTGA CTTTCTGGAC CTTTGATGGA AGCCCATATA ACTTTGTAGC
151 CCTTGCATTT TGCATGCCTG CAAAGCCAAT CACTGTCGTC GGTTCTGCTC
201 TGGCCCCTTT CACCACAACT GTAAAATCCT CTGCTACCTT GATGGTATGC
251 CAGTGAAAAC GGATACCTGC AAAAAAAAAA AGC
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TRS4:

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  1 ACGCATCGCA CGCCATGAAA CAAGGCAAAC AGTGGAAATG TCACGTTACA
 51 TGCTTCCTCC GGGGAAGTGT TGAGATTCTG TAAGAAGCAT AAACTTGATA
101 AATCAACTGT CCACTGCTTC TCAATAAAGT AAGCAATAAC TCTGACTCCA
151 AAAAAAAAAA GC
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FIG. 4. Nucleotide sequence of TRS1, TRS3, and TRS4 cDNA fragments. Numbering of the nucleotide sequences is shown on the left. The sequences corresponding to the upstream and downstream primers and the putative polyadenylation are underlined.

results indicate testicular germ cell apoptosis is relatively evident on day 3, the apoptotic signals increased dramatically with the lapse of time within 7.5 days after cryptorchid surgery.

Identification of Temperature-Related ESTs in Normal Testis

The enviromental temperature was the only difference between cryptorchid and scrotal testes in unilateral cryptorchid rats (6). This model allowed evaluation of temperature effects while keeping hormonal and other systemic factors constant. Because apoptosis of germ cells might induce secondary changes in Leydig cells, cryptorchid testes at late stage should be avoided to use. In our study, germ cells apoptosis was relatively obvious on day 3 and distinct on day 5 after the induction of cryptorchidism. Some reports showed that no changes were found in Leydig cells LH receptor content and serum testosterone level in 7 days after cryptorchid surgery (6, 8, 9) and our results also proved serum testosterone level unchanged in 7 days (unpublished). So unilateral cryptorchid and their scrotal testes on day 3 and 5 were selected to screen temperature-related genes during spermatogenesis.

By using three sets of primers: $GCT_{11}G$ and OPC02 (GTGAGGCGTC), $T_{11}CA$ and S68 (CTCACCGTCC), $GCT_{11}G$ and OPH06 (ACGCATCGCA), several differentially expressed cDNAs were identified (Fig. 2). The individual bands were cut out and reamplified by PCR and further analyzed using agarose gel. The PCR products were cloned and the plasmids containing inserts were used as probes for northern analysis. Figure 3 shows northern blotting data: TRS1, TRS3 and TRS4 were expressed mainly in the scrotal testes; TRS2 was expressed in the cryptorchid testes. The results were consistent with that of differential display. To search temperature-related genes during normal spermatogenesis, three cDNA fragments (TRS1, TRS3, and TRS4) which were expressed in scrotal testes were chosen for further analysis.

Sequence Analysis

The nucleotide sequences of the three ESTs were shown in Fig. 4. The first 10 bases and the last 14 bases of TRS3 (283bp) and TRS4 (162bp) were identical to the upstream and downstream primers, respectively. Using the BLAST program to search GeneBank databases, revealed that TRS4 was identical to a known EST (accession number AA964632) whose function had not been reported; whereas TRS1 and TRS3 were not found in databases. Computer analysis also revealed that there are five EST sequences sharing a partial homology to TRS1. One EST (accession number AA944701) sharing 98% identity with TRS1 in 195bp overlap was the highest-scoring sequence, it was derived from rat embryo, the significance of this homology is unknown. These results indicate that TRS1 and TRS3 are novel cDNA sequences, TRS1 and TRS3 and

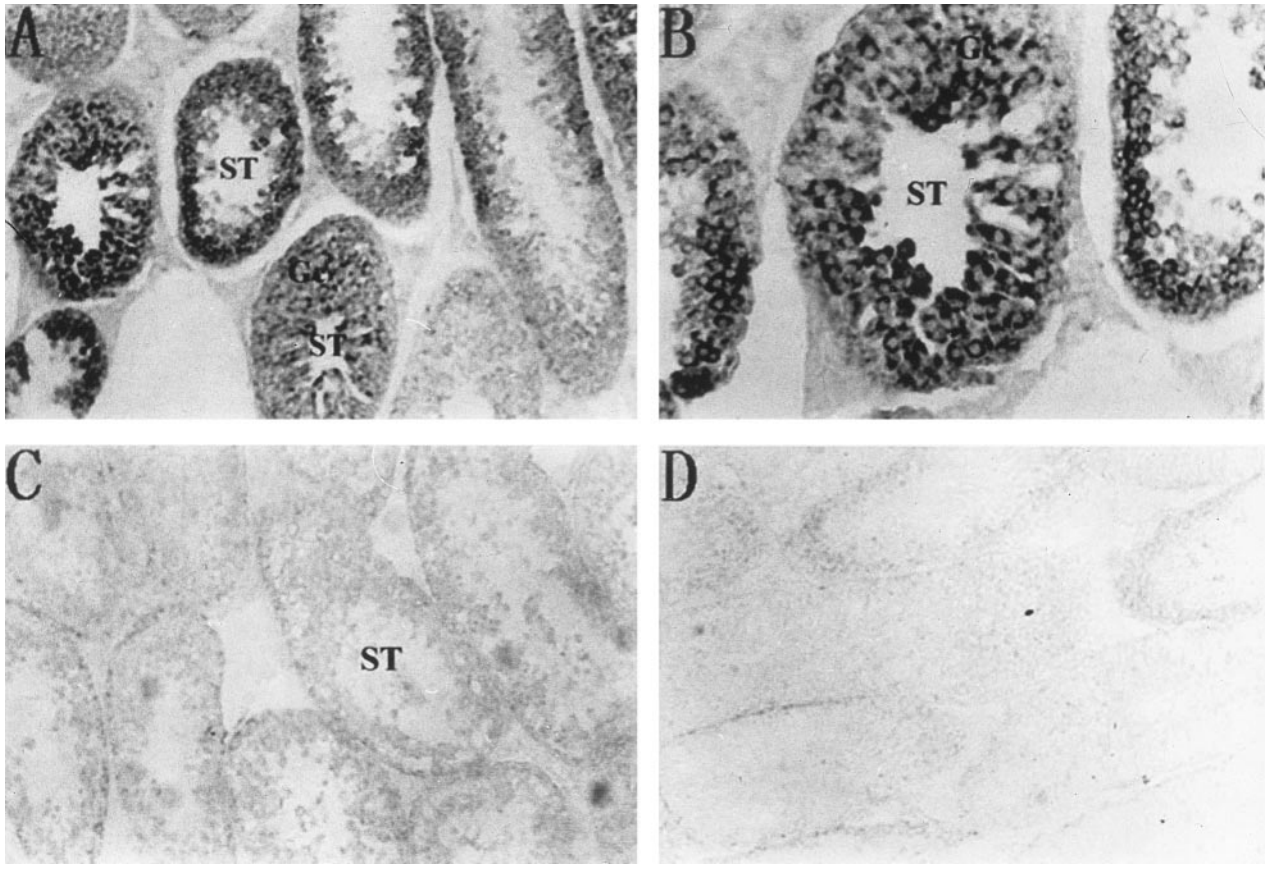

FIG. 5. Expression of TRS1 in cryptorchid and scrotal testes on day 3 after surgery. TRS1 was expressed specifically in germ cells, mainly in the spermatocytes and the round spermatids in scrotal testes. A: scrotal testis, ×100; B: scrotal testis, ×200; C: cryptorchid testis, ×100; D: sense probe control, ×100; Gc: germ cell; ST: seminiferous tubules.

TRS4 are first found to be temperature-related during spermatogenesis.

In Situ Hybridization Assay

To gain more insight in the functions of the novel temperature-related ESTs during spermatogenesis, the cellular localization of TRS1 and TRS3 mRNA in rat testis was examined using in situ hybridization assay. As shown in Fig. 5, TRS1 was expressed in the germ cells of scrotal testes, mainly in the spermatocytes and the round spermatids, and the expression pattern varied with the seminiferous cycle. The expression level of TRS1 was very low in the cryptorchid testes. It has been known that spermatocytes and round spermatids are the most sensitive germ cells to local heat stress in scrotal testes (10). The expression pattern suggests TRS1 may play an important role in normal spermatogenesis as well as temperature-induced germ cell apoptosis in cryptorchid testes. The expression level of TRS3 mRNA was too low to detect using in situ hybridization assay.

In conclusion, we had applied DD-PCR technique to search temperature-related genes during spermatogenesis. By comparing unilateral cryptorchid testes on day 3 and 5 and their scrotal testes after surgery, three temperature-related ESTs had been identified after northern blot analysis. Homology searches revealed TRS1 and TRS3 were novel cDNA fragments. TRS1, TRS3 and TRS4 were first found to be temperature-related during spermatogenesis. The findings will shed light on the temperature regulation network of spermatogenesis.

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