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Stage-Specific Expression of Testis-Specific Protein Kinase 1 (TESK1) in Rat Spermatogenic Cells

Jiro Toshima,* Takehiko Koji,† and Kensaku Mizuno*,[1]

**Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-8581, Japan; and †Department of Histology and Cell Biology, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan*

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TESK1 (testis-specific protein kinase 1) is a serine/threonine kinase, with a unique structure composed of an N-terminal protein kinase domain and a C-terminal proline-rich domain. Northern blot analysis revealed that TESK1 mRNA is predominantly expressed in testicular germ cells. We present here evidence that expression of TESK1 mRNA and protein in the rat testes is developmentally regulated and increases after 20-22 postnatal days. To identify cells which express TESK1 mRNA and protein during male germ cell differentiation, *in situ* hybridization and immunohistochemistry were done using frozen sections of adult rat testes. Prominent expression of TESK1 mRNA and protein was detected in testicular germ cells at stages of late pachytene spermatocytes to round spermatids, but not in somatic cells such as Sertoli and Leydig cells. Expression of TESK1 mRNA and protein at specific stages of testicular germ cells suggests a role for this kinase in spermatogenesis, particularly at stages of meiosis and/or early spermiogenesis.

Spermatogenesis is a highly coordinated process consisting of three major successively occurring events; spermatogonial mitotic renewal and differentiation into spermatocytes, meiotic division of spermatocytes into haploid spermatids, and spermiogenesis, a morphological change of spermatids into highly differentiated spermatozoa (1-4). These events are synchronized in each seminiferous tubule and are thought to be regulated by numerous gene products which are expressed at each stage of germ cell differentiation (5). Recent studies on gene-disrupted mice demonstrated that various gene products, including secretary factors, transcription factors and DNA repair enzymes, expressed in testicular germ cells or somatic cells, are essential for male germ cell differentiation (6). However, little is known about molecular mechanisms which control spermatogenesis.

We earlier identified rat and human cDNA clones encoding a novel protein serine/threonine kinase, termed TESK1 (testis-specific protein kinase 1) (7). The genomic organization and chromosomal localization of the mouse TESK1 gene were also elucidated (8). TESK1 has a unique structure composed of an N-terminal protein kinase domain and a C-terminal proline-rich domain. The kinase domain of TESK1 is phylogenetically related to those of LIM-kinase 1 (LIMK1) and LIM-kinase 2 (LIMK2), with about 50% sequence identity (7-11). Northern blot analysis on RNAs from rat tissues revealed that the major and relatively broad band of TESK1 mRNA of about 3.6-kb was almost exclusively expressed in the testis, whereas a 2.5-kb band was faintly detectable in other tissues and cell lines (7). Northern analysis also showed that TESK1 mRNA was detected in germ cell-enriched preparations from rat testes and in purified populations of pachytene spermatocytes and round spermatids of mouse testes. Expression of TESK1 mRNA in mouse testes was detectable only after the 18-20th days of postnatal development (7). As mouse spermatocytes begin to undergo meiosis and to generate haploid round spermatids on 18-20th postnatal days (4), the developmental expression of TESK1 mRNA in the mouse testis appeared to be consistent with the temporal appearance of round spermatids. Based on these findings, we suggested that the TESK1 gene product may play a role at and/or after the meiotic stages of spermatogenesis (7).

In the present study, we examined expression patterns of TESK1 mRNA and protein in the rat testis during postnatal development, using Northern and western blot analyses. We also examined types of cells and at what stages of spermatogenesis TESK1 mRNA and protein are expressed, making use of *in situ* hybridization and immunohistochemistry on frozen sections of adult rat testes. It became clear that TESK1 mRNA

[1] To whom correspondence should be addressed. Fax: +81-92-642-2645. E-mail: kmizuscb@mbox.nc.kyushu-u.ac.jp.

Abbreviations used: HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TESK1, testis-specific protein kinase 1.

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and protein are expressed in testicular germ cells at stages of late pachytene spermatocytes to round spermatids.

MATERIALS AND METHODS

Northern hybridization. Total RNA of rat testes was extracted by the acid guanidine thiocyanate/phenol/chloroform method (12). Total RNA (10 μg each) was denatured with formaldehyde, electrophoresed on 1.0% agarose gels, and transferred onto a Hybond-N nylon membrane. Blots were probed with ^{32}P-labeled full-length rat TESK1 cDNA and analyzed using a BAS1000 Bio-Image Analyzer (Fuji Film), as described (7).

Immunoprecipitation and immunoblotting. Decapsulated rat testicles were homogenized in 10 volumes of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), using a Potter-Elvehjem homogenizer. After centrifugation at 10,000 $\times$ g for 20 min, supernatants were preadsorbed with Protein A-Sepharose (40 μl of 50% slurry) for 1 h at 4°C and centrifuged. The supernatants were incubated overnight at 4°C with anti-TESK1 antiserum and Protein A-Sepharose, and centrifuged. Immunoprecipitates were washed three times with RIPA buffer and used for immunoblot analysis. Immunoblot analysis was performed, as described (7). Rabbit anti-TESK1 antiserum (TK-C21) was raised against the peptide corresponding to the C-terminal 21 amino acid residues (CHRGHHAKPPTPSLQLPGARS) of rat TESK1 protein.

Haptenization of oligo-DNA probes. Oligo-DNA containing sense or antisense sequence of rat TESK1 mRNA (1844-1901) or complementary sequence of rat 28S rRNA (13) with additional 3 and 2 repeats of adenine-thymine-thymine (ATT) at 5′ and 3′ ends respectively, was synthesized and used as the probe for *in situ* hybridization. The probes were haptenized by generating thymine-thymine (T-T) dimers by ultraviolet (UV) light irradiation, as described previously (13, 14). The optimal dose of UV irradiation for T-T dimerization for these oligo-DNA probes was determined to be 12,000 J/m^2 by dot-blot hybridization, as described (15).

In situ hybridization. Procedures used have been described in detail elsewhere (13, 14). Testes from adult male Wistar rats (7-8 weeks old) were cut into small pieces and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 4 h at 4°C. The tissues were then immersed in 30% sucrose containing 0.02% diethylpyrocarbonate for 2 h at 4°C, then were embedded in OCT compound (Miles) and frozen in ethanol/dry ice. The frozen sections (8 μm thickness) were mounted onto silane-coated glass slides. They were baked, rehydrated and treated with 0.2 N HCl for 20 min and proteinase K (1 μg/ml) at 37°C for 10 min. After post-fixation with 4% paraformaldehyde in PBS (pH 7.4), the sections were immersed in 2 mg/ml glycine, and kept in 40% formamide in 4 $\times$ SSC (1 $\times$ SSC: 150 mM NaCl, 15 mM sodium citrate) for 30 min. Hybridization was performed overnight at 37°C with the T-T dimerized oligo-DNA probe (1 μg/ml) in the hybridization solution (10 mM Tris-HCl, pH 7.4, 600 mM NaCl, 1 mM EDTA, 40% formamide, 1 $\times$ Denhardt's solution, 0.25 mg/ml yeast tRNA, 0.125 mg/ml salmon testis DNA, 10% dextran sulfate). After washing with 50% formamide in 2 $\times$ SSC, 0.5 $\times$ SSC, and 0.2 $\times$ SSC, the haptenized probes were detected immunohistochemically using horseradish peroxidase (HRP)-conjugated anti-(T-T dimer) antibody (14). The sections were blocked with PBS containing 5% bovine serum albumin, 0.5 mg/ml normal mouse IgG, 0.1 mg/ml salmon testis DNA, 0.1 mg/ml yeast tRNA and 300 mM NaCl for 1 h and reacted overnight with HRP-conjugated mouse anti-(T-T dimer) antibody placed in the blocking solution. After washing with PBS containing 0.075% Brij 35, HRP sites were visualized using 3,3′-diaminobenzidine and hydrogen peroxide in the presence of nickel and cobalt ions, as described (13, 14). The sections were also counterstained with methyl green, and the stage of germ cells was identified on the basis of nuclear morphology and topographical relationships in the seminiferous tubule, as described (1).

Immunohistochemistry. The frozen sections prepared as above were rehydrated and immersed in 0.3% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase activity. Then the sections were blocked with PBS (pH 7.4) containing 1% bovine serum albumin and 0.5 mg/ml goat IgG for 30 min and reacted overnight with an anti-TESK1 antiserum (TK-C21). After washing with PBS containing 0.075% Brij 35, the sections were reacted for 1 h with HRP-conjugated goat anti-rabbit IgG Fab and visualized with 3,3′-diaminobenzidine and hydrogen peroxide, as described above.

RESULTS

Developmental Changes in Expression of TESK1 mRNA in the Rat Testis

We first examined developmental changes in expression of TESK1 mRNA in rat testis. Total RNA was prepared from testes of prepuberal (7- to 24-day-old) and sexually mature (56-day-old) rats and subjected to Northern blot analysis. The two mRNA species of 3.3-kb and 3.6-kb were detected, which differed in developmental patterns of expression (Fig. 1A). The shorter 3.3-kb mRNA was almost continuously present throughout 7-56th postnatal days. In contrast, the 3.6-kb mRNA was detectable only after the 20th day of postnatal development, and the level of expression gradually increased, the highest level being observed in adults. The expression pattern of the 3.6-kb, but not the 3.3-kb, TESK1 mRNA in the rat testis is similar to that of TESK1 mRNA in the mouse testis previously reported (7).

Specificity of Anti-TESK1 Antiserum and Developmental Changes in Expression of TESK1 Protein in the Rat Testis

To determine if TESK1 mRNA is translated to protein in the testis, immunoblot analysis was performed, using anti-TESK1 antiserum raised against the C-terminal peptide of TESK1 protein. Extracts of adult rat testis were immunoprecipitated with an anti-TESK1 antiserum, separated on SDS-PAGE and analyzed by immunoblotting, using the same antibody. The one major immunoreactive band migrating at around 68-kDa was similar to the molecular mass (67,984 Da) predicted from rat TESK1 sequence (Fig. 1B, lane 1). This band was not seen when testis extracts were immunoprecipitated with preimmune serum (Fig. 1B, lane 2) or with anti-TESK1 antiserum preincubated with the antigenic peptide (Fig. 1B lane 3). These results suggest that the 68-kDa immunoreactive band represents the TESK1 protein endogenously expressed in the rat testis and that this antiserum has specificity suitable for immunohistochemical studies.

Using this antiserum, we examined developmental changes in the expression of TESK1 protein (Fig. 1C).

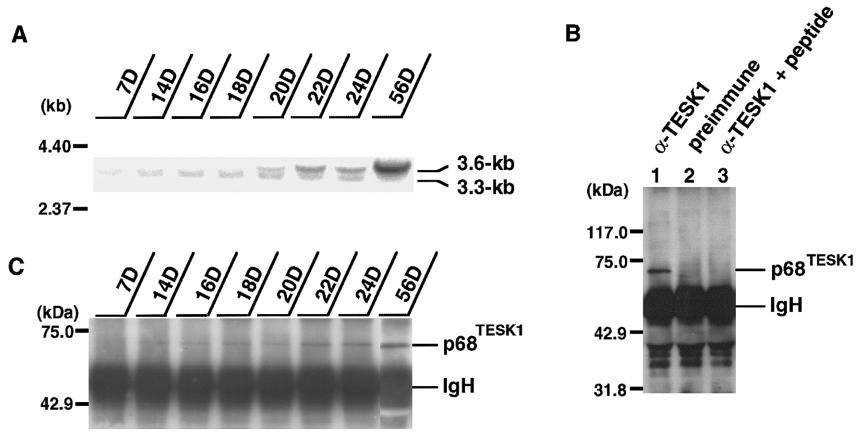

FIG. 1. Developmental changes of expression of TESK1 mRNA and protein in rat testis. (A) Developmental changes of TESK1 mRNA expression in rat testes. Total RNAs (10 μg each) from rat testes on different postnatal days (D) were analyzed, using the rat TESK1 cDNA probe, as described in "Materials and Methods". (B) Immunoprecipitation and immunoblotting of TESK1 protein. Total proteins (8 mg) in extracts from 8-week-old rat testes were immunoprecipitated with anti-TESK1 antiserum (lanes 1 and 3) or preimmune serum (lane 2), and the precipitates were run on SDS-PAGE and immunoblotted with anti-TESK1 antiserum. In lane 3, anti-TESK1 antiserum was pretreated with excess amounts of antigenic peptide prior to immunoprecipitation. Positions of molecular weight marker proteins are indicated on the left. IgH, immunoglobulin heavy chain. (C) Developmental changes of expression of TESK1 protein in rat testes. Homogenates from rat testes on different postnatal days (containing 6.3 mg protein) were immunoprecipitated with anti-TESK1 antiserum and analyzed by immunoblotting, as in (B).

TESK1 protein was faint in extracts of rat testes at 14th-20th postnatal days. The level of TESK1 protein significantly increased at 22-24th postnatal days and the highest level of expression was detected in adult rat testis. The expression pattern of TESK1 protein during postnatal development correlated well with the total amounts of two TESK1 mRNA isoforms.

In situ Localization of TESK1 mRNA in Rat Testis

To identify cells in which TESK1 mRNA is expressed in testis, frozen sections of adult rat testis were *in situ* hybridized with the T-T dimerized antisense oligo-DNA probe complementary to TESK1 mRNA and localization of the probe was immunohistochemically detected using a monoclonal antibody specific to T-T dimer. Intense hybridization signals were detected in testicular germ cells at the stages of late pachytene (stages IX-XII) and diplotene (stage XIII) of meiotic prophase and early round spermatids (stages 1-8) (Figs. 2A and 2D). Weak signals were also detected in early pachytene spermatocytes (stages I-VIII) and spermatids at stages 9-10, but no or little signal in spermatogonia, leptotene and zygotene spermatocytes, late spermatids, and somatic Sertoli and Leydig cells. When the T-T dimerized sense oligo-DNA was used as a control probe, no positive staining was observed (Figs. 2B and 2E). The T-T dimerized oligo-DNA complementary to 28S rRNA was also used as a positive reference (Figs. 2C and 2F). The expression of TESK1 mRNA during spermatogenesis is summarized in Table 1. TESK1 mRNA is predominantly expressed in testicular germ cells at stages of pachytene spermatocytes to early spermatids.

Immunohistochemical Localization of TESK1 Protein in the Rat Testis

To determine the localization of TESK1 protein in the rat testis, immunohistochemical analysis was made using TK-C21 TESK1-specific antiserum. Low-magnification views of testis sections revealed the specific staining of seminiferous tubules with anti-TESK1 antiserum, but not with preimmune serum (Figs. 3A and 3B). Variable staining intensity between tubules seems to reflect differences in stage of seminiferous epithelial cycle of each tubule (Fig. 3A). High-magnification views revealed that TESK1 protein is expressed in testicular germ cells, preferentially at stages of pachytene spermatocytes to round spermatids (Figs. 3C and 3D), findings in accord with results of *in situ* hybridization. Little or no staining was detected in germ cells at other stages, including spermatogonia, leptotene and zygotene spermatocytes. Reactions were nil in Sertoli or Leydig cells. The expression pattern of TESK1 protein during rat spermatogenesis is included in Table 1.

DISCUSSION

When examining localization of TESK1 mRNA and protein in rat testis using *in situ* hybridization and immunohistochemistry, TESK1 mRNA and protein were expressed in testicular germ cells at specific stages of spermatogenesis, particularly from the late pachytene spermatocytes to round spermatids. No positive signals were seen in somatic cells such as Sertoli and Leydig cells. The high level of expression of the

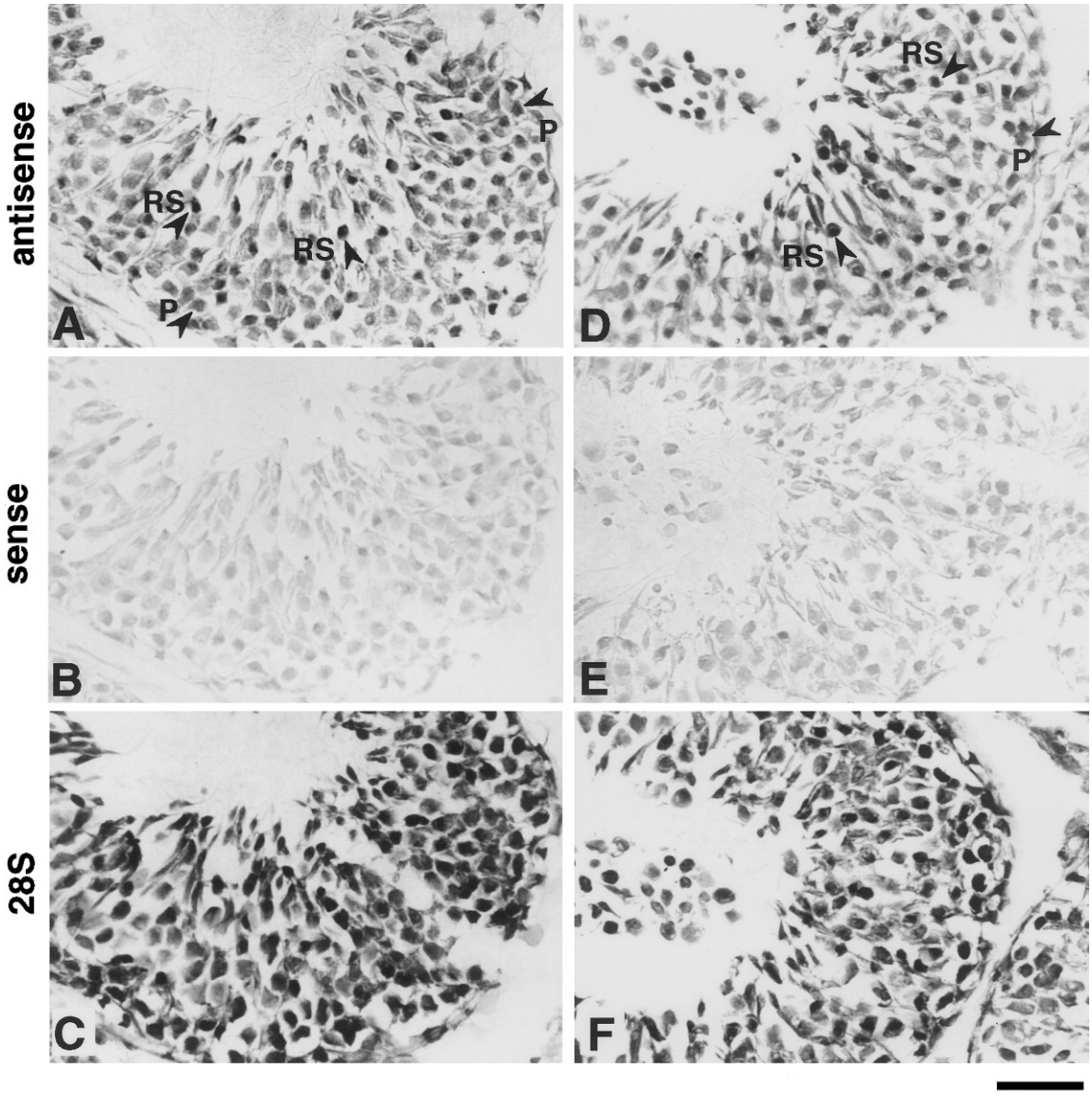

FIG. 2. *In situ* localization of TESK1 mRNA in rat testis. Frozen sections of adult rat testis were hybridized with the T-T dimerized antisense oligo-DNA probe complementary to TESK1 mRNA (A and D), sense oligo-DNA probe as a control (B and E), or 28S rRNA complementary probe as a positive reference (C and F). Serial sections of stages I-III (A-C) and V-VI (D-F) of seminiferous tubules are shown. Arrowheads indicate positive cells. P, pachytene spermatocytes; RS, round spermatids. Bar, 100 μm.

TESK1 gene product in male germ cells at and after meiotic stages suggests that TESK1 protein plays an important role in meiotic cell division and/or early spermiogenesis.

Northern blot analysis revealed two mRNA species of 3.3-kb and 3.6-kb, as expressed in the rat testes. In an earlier study we described the relatively broad band of TESK1 mRNA of about 3.6-kb detected in adult rat testis (7). This broad band is probably composed of 3.6-kb and 3.3-kb mRNA species detected in the present study. As reported earlier (7), we isolated from a rat testis cDNA library two types of clones containing 3.6-kb and 3.3-kb inserts of TESK1 cDNAs; the former contained a 0.6-kb 3′-untranslated region with two polyadenylation signals (AATAAA) and a poly(A) tail, while the latter contained a shorter 0.3-kb 3′-untranslated region with a poly(A) tail downstream of a 5′-side AATAAA signal (7). Based on these observations, two mRNA species detected in the rat testis are probably generated by an alternative use of two polyadenylation signals. There have been reports of plural transcripts of a single gene expressed in the testis and with distinct temporal patterns during postnatal development (16-21). In some cases they are known to be generated by an alternative polyadenylation (16-18), but the molecular mechanisms remain unknown.

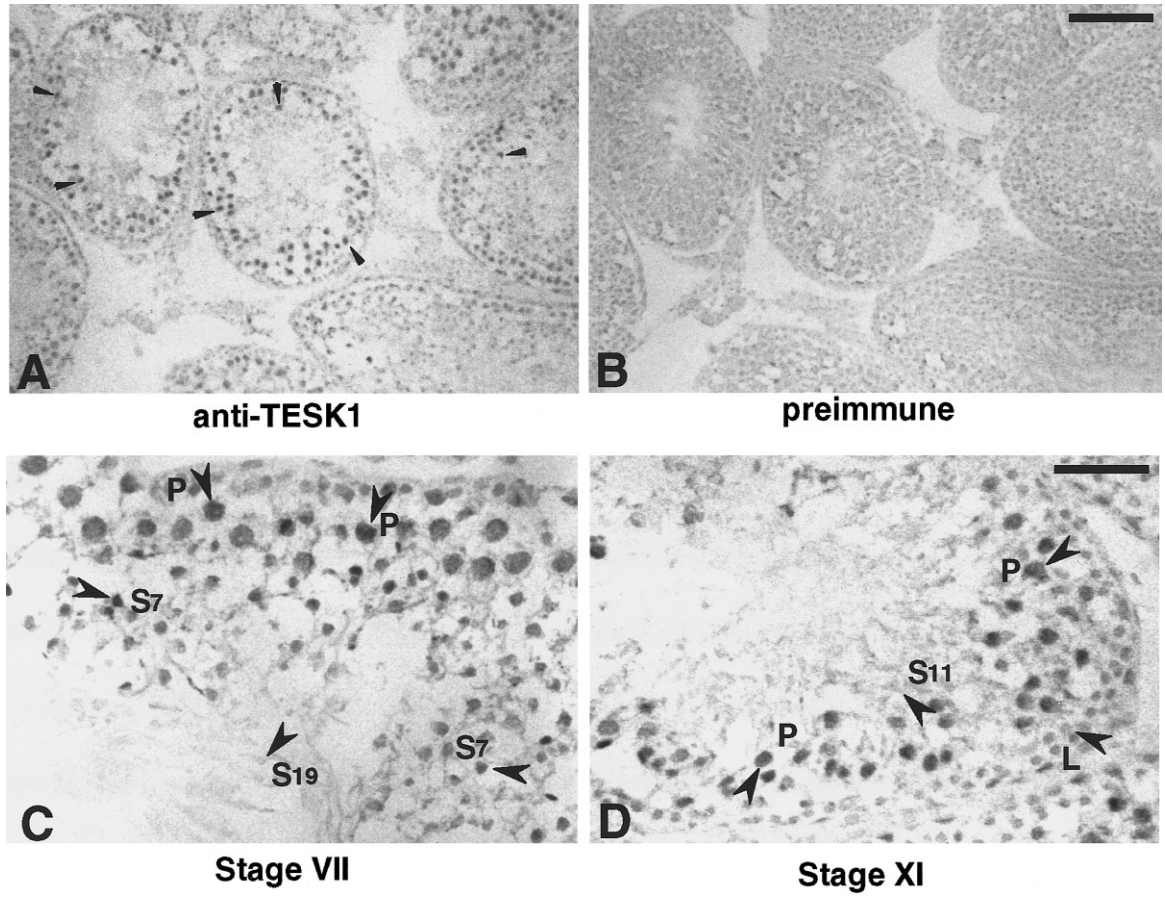

FIG. 3. Immunohistochemical localization of TESK1 protein in the rat testis. The frozen sections of adult rat testis were reacted with anti-TESK1 antiserum (A, C and D) or preimmune serum (B), and visualized with HRP-conjugated goat anti-rabbit IgG. Higher magnification of seminiferous tubules at stage VII (C) and at stage XI (D) shows expression of TESK1 protein in cells at specific stages. Arrowheads in (A) indicate positive cells. Arrowheads in (C) and (D) indicate various cell types. L, leptotene spermatocytes; P, pachytene spermatocytes; S, spermatids (numbers indicate their stages). Bars; 250 μm in (A) and (B), and 100 μm in (C) and (D).

In the mouse testis, the TESK1 mRNA of about 4.0-kb was detected only after the 18-20th postnatal day (7). Developmental expression of 3.6-kb mRNA in the rat testis is similar to that of TESK1 mRNA in the mouse testis, suggesting that their expression is regulated by a similar mechanism. On the other hand, in contrast to the mouse testis, the low-level but discernible expression of 3.3-kb TESK1 mRNA was observed in rat testis throughout the postnatal development. The physiological significance of the existence of 3.3-kb mRNA only in the rat testis is unknown, but detection of TESK1 protein at 14-18th postnatal days by immunoblot analysis indicates that the 3.3-kb mRNA can be translated to the protein product.

Several serine/threonine kinases with expression patterns similar to that of TESK1 have been reported, including Mak (male germ cell-associated protein kinase), Nek2 (NIMA-related kinase 2) and Ayk1 (aurora-family kinase 1), the expression of which was detected at stages of late pachytene spermatocytes to round spermatids (15, 21-23). Similar patterns of expression suggest that the stage-specific gene expression of these genes is regulated in a similar manner, and the functional relationships between these kinases will be investigated.

In the 5′-flanking region of the mouse TESK1 gene, there are several putative binding sites for transcription factors, as predicted by computer analysis (8). Among them, a cAMP-response element (CRE; TGACGTCA) and a Myb binding element (CCGTTTC) are notable, because recent studies on mice lacking genes of the factors (CREMτ and A-Myb) which can bind to these elements revealed that they are involved in expression of several testicular germ cell-specific genes and are essential for spermatogenesis (24-26). The stage-specific expression of TESK1 mRNA in the testicular germ cells suggests that it may possibly be regulated under control of these transcription factors. In addition we noted the CTCAGAA consensus sequence at or near transcription initiation sites of the testis-specific isoforms of three genes, TESK1, calspermin and LIMK2t, which are specifically expressed in testic-

TABLE 1

Summary of TESK1 Expression During Rat Spermatogenesis

Cell type	Stage[a]	Signal intensity	
		mRNA	Protein
Spermatogonia		+/−	+/−
Spermatocytes			
(Leptotene)		+/−	+/−
(Zygotene)		+/−	+/−
(Pachytene)	I-VIII	+	+
	IX-XII	++	++
(Diplotene)	XIII	++	++
Spermatids			
(Round)	1-10	+++	++
(Elongated)	11-19	+/−	+/−

[a] According to Perey et al (1).

ular germ cells (8, 27, 28). This sequence motif may also have some role in germ cell-specific transcription of these genes.

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REFERENCES

1. Perey, B., Clermont, Y., and Leblond, C. P. (1961) *Am. J. Anat.* **108,** 47–77.
2. Monesi, V. (1964) *J. Cell Biol.* **22,** 521–532.
3. Bellve, A. R., Millette, C. F., Bhatnagar, Y. M., and O'Brien, D. A. (1977) *J. Histochem. Cytochem.* **25,** 480–494.
4. Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M., and Dym, M. (1977) *J. Cell Biol.* **74,** 68–85.
5. Willison, K., and Ashworth, A. (1987) *Trends Genet.* **3,** 351–355.
6. Sassone-Corsi, P. (1997) *Cell* **88,** 163–166.
7. Toshima, J., Ohashi, K., Okano, I., Nunoue, K., Kishioka, M., Kuma, K., Miyata, T., Hirai, M., Baba, T., and Mizuno, K. (1995) *J. Biol. Chem.* **270,** 31331–31337.
8. Toshima, J., Nakagawara, K., Mori, M., Noda, T., and Mizuno, K. (1998) *Gene* **206,** 237–245.
9. Mizuno, K., Okano, I., Ohashi, K., Nunoue, K., Kuma, K., Miyata, T., and Nakamura, T. (1994) *Oncogene* **9,** 1605–1612.
10. Nunoue, K., Ohashi, K., Okano, I., and Mizuno, K. (1995) *Oncogene* **11,** 701–710.
11. Okano, I., Hiraoka, J., Otera, H., Nunoue, K., Ohashi, K., Iwashita, S., Hirai, M., and Mizuno, K. (1995) *J. Biol. Chem.* **270,** 31321–31330.
12. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
13. Koji, T., and Nakane, P. K. (1996) *J. Electron Microsc.* **45,** 119–127.
14. Koji, T., and Nakane, P. K. (1990) *Acta Pathol. Jpn.* **40,** 793–807.
15. Koji, T., Jinno, A., Matsushime, H., Shibuya, M., and Nakane, P. K. (1992) *Cell Biochem. Funct.* **10,** 273–279.
16. Meijer, D., Hermans, A., von Lindernm, M., van Agthoven, T., de Klein, A., Mackenbach, P., Grootegoed, A., Tararico, D., Valle, G. D., and Grosveld, G. (1987) *EMBO J.* **6,** 4041–4048.
17. Oyen, O., Myklebust, F., Scott, J. D., Cadd, G. G., McKnight, G. S., Hansson, V., and Jahnsen, T. (1990) *Biol. Reprod.* **43,** 46–54.
18. Mishima, K., Price, S. R., Nightingale, M. S., Kousvelari, E., Moss, J., and Vaughan, M. (1992) *J. Biol. Chem.* **267,** 24109–24116.
19. Ruppert, S., Cole, T. J., Boshart, M., Schmid, E., and Schütz, G. (1992) *EMBO J.* **11,** 1503–1512.
20. Foulkes, N. S., Mellström, B., Benusiglio, E., and Sassone-Corsi, P. (1992) *Nature* **355,** 80–84.
21. Matsushime, H., Jinno, A., Takagi, N., and Shibuya, M. (1990) *Mol. Cell. Biol.* **10,** 2261–2268.
22. Tanaka, K., Parvinen, M., and Nigg, E. A. (1997) *Exp. Cell Res.* **237,** 264–274.
23. Yanai, A., Arama, E., Kilfin, G., and Motro, B. (1997) *Oncogene* **14,** 2943–2950.
24. Nantel, F., Monaco, L., Foulkes, N. S., Masquilier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M., and Sassone-Corsi, P. (1996) *Nature* **380,** 159–162.
25. Blendy, J. A., Kaestner, K. H., Weinbauer, G. F., Nieschlag, E., and Schütz, G. (1996) *Nature* **380,** 162–165.
26. Toscani, A., Mettus, R. V., Coupland, R., Simpkins, H., Litvin, J., Orth, J., Hatton, K. S., and Reddy, E. P. (1997) *Nature* **386,** 713–717.
27. Sun, Z., Sassone-Corsi, P., and Means, A. R. (1995) *Mol. Cell. Biol.* **15,** 561–571.
28. Ikebe, C., Ohashi, K., and Mizuno, K. (1998) *Biochem. Biophys. Res. Commun.* **246,** 307–312.