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A Tetratricopeptide Repeat-Containing Protein Gene, *tpis,* Whose Expression Is Induced with Differentiation of Spermatogenic Cells

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The tetratricopeptide repeat (TPR) is a degenerate 34-amino-acid sequence which forms scaffolds to mediate protein–protein interactions. We have isolated a cDNA named *tpis* from mouse embryonic skin and found that the deduced 529-amino-acid sequence contained 5 TPRs. In addition to skin, the transcript of *tpis* was detected in tissues with stratified squamous epithelium, e.g., tongue, esophagus, and forestomach. *tpis* was most strongly expressed in testis among adult tissues examined. The transcript of *tpis* from testis was longer, encoding 372 additional amino acid residues at the 5′-side with 3 more TPRs. *In situ* hybridization revealed specific expression of *tpis* at a distinct differentiation stage of spermatogenic cells, indicating involvement of *tpis* in spermatogenesis. Chromosomal localization of the *tpis* gene was determined as 18.10 cM of chromosome 15. © 1999 Academic Press

Key Words: **TPR; tetratricopeptide; spermatogenesis; skin; testis; gene.**

Spermatogenesis is a highly specialized process of cell proliferation and differentiation (1, 2). At first, spermatogonia proliferate by mitosis, and then give rise to primary and secondary spermatocytes through the first and second meiotic divisions. The resulting haploid cells called round spermatids undergo extensive remodeling to become spermatozoa, the process being known as spermiogenesis. A number of genes, some which are testis-specific and the others common with somatic cells, have been demonstrated to be involved in the spermatogenesis (3, 4). Targeted disruption of some of those genes including A-myb (5), Atm (6), Bax (7), and Hsp70.2 (8) resulted in male infertility with impaired testicular function and spermatogenesis.

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Among particularly intriguing molecular features of spermatogenesis is that the size of transcripts in testis is often distinct from that found in somatic cells (9). Such transcripts are produced through alternative promoter usage and/or alternative splicing. For example, testis-specific form of angiotensin-converting enzyme gene is transcribed using a promoter that is located within intron 12 of the somatic counterpart (10). Amount of τ-isoform of CREM (cyclic AMP-responsive element modulator) increased abruptly at a specific stage of spermatogenesis through the change of splicing (11).

Protein-protein interaction, whether direct or indirect, is fundamental process of cellular function. Among structural domains involved in the direct protein interactions is tetratricopeptide repeat (TPR) motif (12, 13). TPRs are found in diverse proteins with no common biochemical functions. Thus, proteins with TPR motif include those involved in cell cycle regulation, transcriptional control, mitochondria and peroxisomal protein transport, neurogenesis, and protein kinase inhibition (14).

Here we describe a newly isolated gene, *tpis,* in a screening of genes involved in the morphogenesis of mouse skin. The expression level of *tpis* increased with the proceeding of development. Unexpectedly, *tpis* was expressed in testis as well. The transcript of *tpis* in testis was longer than that observed in other tissues, and the deduced amino acid sequence contained 8 tetratricopeptide repeat domains.

MATERIALS AND METHODS

RNA differential display. RNA differential display for isolating gene fragments was performed using RNAmap kit (GenHunter Corp., Brookline, MA) and described elsewhere (15). Briefly, total RNA samples from dorsal epidermis of mouse embryos from 12.5 dpc (days postcoitus) to 16.5 dpc were reverse-transcribed and amplified. The PCR products were separated and differentially expressed fragments were reamplified, subcloned and sequenced. *tpis* was identified as a gene fragment induced with the progression of development.

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Northern analysis. ICR mice (Nippon SLC, Hamamatsu, Japan) were used throughout the experiments. Embryonic epidermis was separated from dermis by incubating in 10 mM EDTA in PBS, when necessary (15). Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC; 16) using 1 ml of the denaturing solution for 100 mg tissue.

Northern analysis was performed according to conventional conditions (17). Briefly, 20 mg of total RNA was electrophoresed, transferred onto Hybond-N (Amersham Pharmacia Biotech, Uppsala, Sweden), and hybridized to ^{32}P-labeled probes. The filters were finally washed twice with 0.2× SSC, 0.1% SDS, at 43°C for 15 min, and the signals were visualized by exposing to X-ray films. The probe used was 3′ 382 bp or 2,262 bp of *tpis* cDNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and cytokeratin 10 were used as controls.

Preparation and screening of cDNA library. A cDNA library was prepared from 16.5 dpc embryonic mouse skin using lZAP II (Stratagene, La Jolla, CA). After screening ~5×10^5 independent plaques of the cDNA library with ^{32}P-labeled *tpis* probe, positive clones were transformed to pBluescript by *in vivo* excision.

DNA sequencing and sequence analysis. DNA sequencing was performed using an automated sequencer (Amersham Pharmacia Biotech). Sequence homology was searched by BLAST or FASTA method. Possible functional domains were screened in the ProDom protein domain data base and some other data base systems which gave essentially the same results.

Rapid amplification of cDNA end (RACE). 5′-RACE was performed using 5′-Full RACE Core Kit (Takara, Shiga, Japan) essentially under the conditions recommended by the manufacturer. Briefly, 5 mg total RNA from the embryonic skin (16.5 dpc) or testis (10 weeks of age) was reverse transcribed with 5′-phosphorylated primer, ligated and amplified by nested PCR using 2 sets of primers. The primers used were: 5′-phosphorylated primer; 5′-P-GTTTCCTTCTTTGAGG, sense primer 1; 5′-ACCCACAGGAAGTGCAAATGC, sense primer 2; 5′-CATCTTGTATTCAAATAGAGCG, antisense primer 1; 5′-GGACCATCAGCTTCTTCTGA, antisense primer 2; 5′-CTTCTGAATGTTGCCCATG. The PCR products were ligated into pCRII vector (InVitrogen, San Diego, CA) and sequenced.

For 3′-RACE, 3 mg RNA was reverse transcribed with a primer (GAGTCGACTCGAGAATTCTTTTTTTTTTTTTTTTVN), amplified at first with adaptor primer (GAGTCGACTCGAGAATTC) and 3′-sense primer 1 (ATCCACTTTAGAGAAGTGGC), and reamplified with the adaptor primer and 3′-sense primer 2 (GAGAAGTGGCTTACTGTCAC). The resulting DNA fragments were processed as for 5′-RACE.

In situ hybridization. The procedure of *in situ* hybridization was essentially the same as that previously described (15), except for the use of frozen sections. Briefly, a pLITMUS 28 vector (New England Biolabs, Beverly, MA) containing 3′-side 2,262 bp fragment of *tpis* cDNA was used to synthesize anti-sense and sense RNA probes with T7 RNA polymerase (Gibco BRL, Rockville, MD) with digoxigenin labeling (Boehringer Mannheim, Mannheim, Germany). Tissues were frozen in Tissue-Tek O.C.T. Compound (Sakura Fine Technical Co., Tokyo, Japan). Tissue sections were hybridized to the probes partially degradated with alkaline treatment, and specific signals were visualized as alkaline phosphatase activity conjugated to anti-digoxigenin antibody (Boehringer Mannheim).

RESULTS

Isolation of tpis cDNA. The DNA fragment of *tpis* was originally isolated by RNA differential display whose band intensity increased during the morphogenic process of embryonic mouse epidermis from 12.5 dpc to 16.5 dpc. By Northern analysis, the transcript of *tpis* was first detected in epidermis at 14.5 dpc and progressively increased afterwards (Fig. 1A). *tpis* was not expressed in embryonic dermis at any stages observed. Cytokeratin 10 known to be expressed in suprabasal layers of epidermis was used as a control for normal skin development as well as reliable separation of epidermis from dermis.

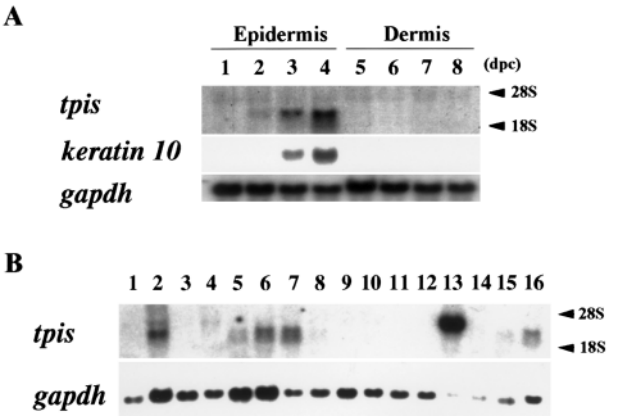

FIG. 1. Expression of *tpis gene* in mouse embryonic skin (A) and various adult tissues (B) as examined by Northern analysis. (A) RNAs were prepared from mouse embryonic epidermis or dermis at 13.5 dpc (lane 1, 5), 14.5 dpc (2, 6), 15.5 dpc (3, 7) or 16.5 dpc (4, 8). Arrowhead shows the positions of ribosomal RNA. (B) RNAs were prepared from cerebrum (lane 1), cerebellum (lane 2), heart (lane 3), Lung (lane 4), tongue (lane 5), esophagus (lane 6), forestomach (lane 7), glandular stomach (lane 8), small intestine (lane 9), large intestine (lane 10), liver (lane 11), kidney (lane 12), testis (lane 13), uterus (lane 14), skin (lane 15), or embryonic skin at 16.5 dpc (lane 16).

In addition to the embryonic mouse skin, *tpis* transcripts were detected in testis, cerebellum, tongue, esophagus, and forestomach among various adult tissues examined (Fig. 1B). The positive results in tongue, esophagus, and forestomach may make sense because the surface of the organs is lined with stratified squamous epithelium like skin. The more intriguing is that the size of transcript in testis was larger than those observed in the other tissues (Fig. 1B).

cDNA and the deduced amino acid sequence of tpis. By screening a cDNA library prepared from mouse embryonic epidermis at 16.5 dpc, 10 independent clones were isolated and sequenced. Based on the ~2.3 kb sequence obtained, 5′- and 3′-RACE were performed using mRNA from embryonic epidermis at 16.5 dpc, giving additional ~30 bp sequence at 5′ side and no additional sequence at 3′ side. Altogether, 2,336 bp cDNA except for polyA$^+$ tail was identified and registered in GenBank (Accession Number: AF181253).

Using mRNA from testis, RT-PCR was performed with primers designed to amplify 5 overlapping regions of the 2.3 kb cDNA. Sizes and sequences of the amplified fragments covering almost entire 2.3 kb sequence showed no difference from those of epidermis, indicating that the variance is present at 5′ side. Actually, 5′-RACE using mRNA from testis gave rise to several

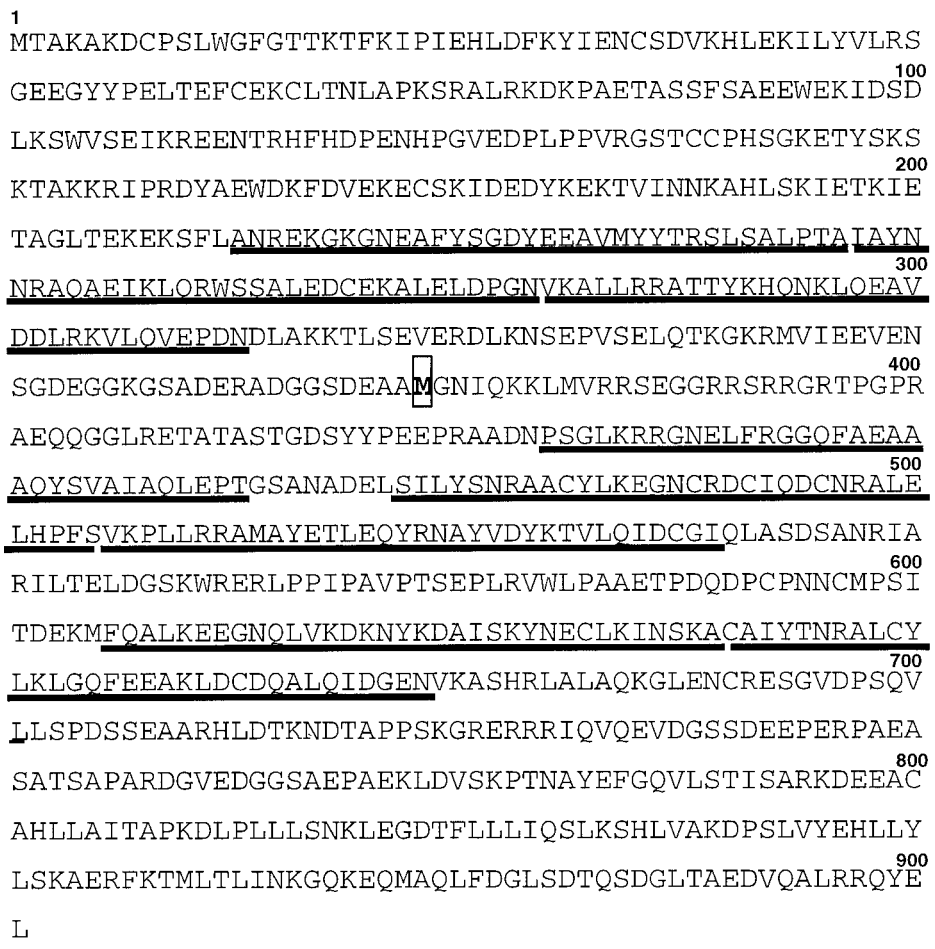

FIG. 2. Deduced amino acid sequence of *tpis* cDNA from testis. Presumptive initiation codon of skin *tpis* cDNA is shown in a square. Eight tetratricopeptide repeats were designated with an underline. Nucleotide sequences of testis and skin cDNA were registered in GenBank under Accession Nos. of AF181252 and AF181253, respectively.

independent clones of ~1.3 kb in size, resulting in determination of 3,644 bp cDNA in case of testis (GenBank accession number: AF181252). The 3′- 2,308 base sequence was common for the two cDNAs. The lengths of 5′ unique sequences were 1,336 and 28 bases in testis and skin, respectively.

The size of both transcripts fits well to those expected from Northern analysis. This and the results of 5′- and 3′-RACE corroboratively indicate that the isolated cDNAs from both organs cover nearly full length of the transcripts. A single reasonable open reading frame (ORF) was found in each of the cDNAs. Figure 2 shows the deduced amino acid sequence of testis *tpis* starting from the 5′-most methionine codon, the surrounding base sequence of which meets Kozak's rule (18). The 5′-most methionine within ORF of skin cDNA is shown in square. The numbers of amino acid residues are 901 and 529 in testis and skin, respectively.

Computer-aided search for functional domain (19) resulted in identification of possible TPR in 8 regions as indicated with underline in Fig. 2. TPR, a degenerate 34-amino acid repeated motif, is known to be involved in protein-protein interaction, particularly with heat shock proteins (13, 14). The amino acid sequence of *tpis* showed limited homology with a number of proteins with diverse structure and function, but containing TPR in common. Amino acid sequence within TPR domains was conserved relatively well. Thus, the fifth and sixth TPRs of *tpis* showed 73% identity with the second and third TPRs of human outer mitochondrial membrane 34 kDa translocase hTOM34 (20). In addition, nucleotide sequence homology search resulted in the identification of human 75 kDa infertility-related sperm protein (21). *tpis* was named after the acronym of *T*PR-containing *p*rotein *i*nvolved in *s*permatogenesis.

Expression of tpis. The expression of *tpis* was examined by *in situ* hybridization. In testis (Fig. 3), the *tpis* transcripts were detected in spermatogenic cells, with stronger signal at the intermediate layer in each seminiferous tubule. Spermatogonia at the outer-most layer of each seminiferous tubule were negative. We

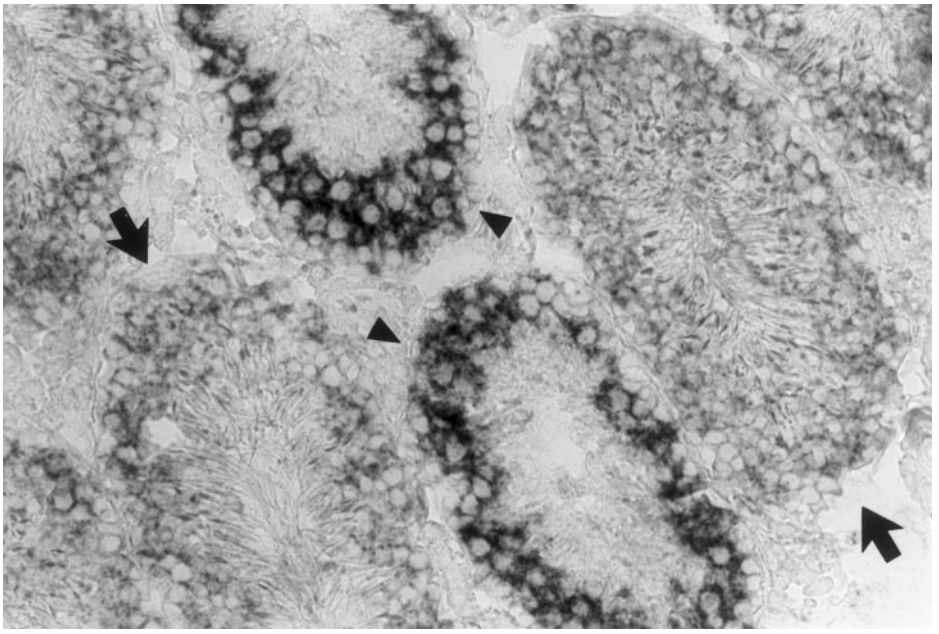

FIG. 3. Expression of *tpis* gene in mouse testis as examined by *in situ* hybridization. The testis was isolated from an ICR mouse at the age of 6 months. The black arrows and arrowheads designate seminiferous tubules with and without spermatozoa in the lemen, respectively.

noted the marked difference of signal intensity among different tubules, with stronger signal in the tubules containing no spermatozoa in lumen. The association of *tpis* expression with the differentiation of spermatogenic cells was corroborated by Northern analysis (Fig. 4), where increasing signal was observed with maturation and functional activation of testis.

Chromosomal locus of tpis. A part of *tpis* sequence was practically identical with D15Ncvs1, a sequence tag site fragment directly submitted to GenBank by Akama *et al.* (Accession No. AB004259; 21). D15Ncvs1 was mapped to mouse chromosome 15, 18.10 cM. At the syntenic site of human chromosome, 8q22, sperm-associated antigen-1 (SPAG1) gene, the byname of human 75 kDa infertility-related sperm protein, was found. SPAG1 was originally isolated with anti-serum from infertile woman that cause sperm agglutination (22). Only part of the cDNA sequence was reported and poorly characterized. Together with the sequence homology of *tpis* and SPAG1 as described above, we concluded that *tpis* is the mouse homologue of SPAG1 and encoded at 18.10 cM of chromosome 15.

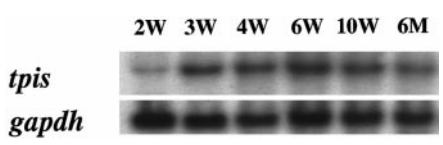

FIG. 4. Expression of *tpis* gene in mouse testis in maturing process. Northern analysis was performed using RNAs from 2, 3, 4, 6, 10 weeks, or 6 months of age.

DISCUSSION

In the present study, we have isolated and characterized a gene, *tpis*, which contains TPR domains and shows a characteristic expression profile, particularly in testis. Together with the observation on human counterpart SPAG1 protein described above (22), it is very likely that *tpis* is involved in the maturation of spermatogenic cells.

tpis has 8 possible TPR domains (Fig. 2). Biological significance of TPR has not fully understood, but it is considered to be involved in protein-protein interactions through either TPR-TPR or TPR-non-TPR interaction (13). In general, amino acid sequence within each TPR domain is poorly conserved. One feature of TPR domain is that the sequences of adjacent TPRs within the same protein show considerable diversification, whereas individual TPRs are extensively conserved among different proteins in evolution in a way that is specific to that individual TPR (23). Thus, the fifth and sixth TPRs of *tpis* showed 73% identity with the corresponding second and third TPRs of human outer mitochondrial membrane 34 kDa translocase, while the TPRs within *tpis* showed at most 38% identity among each others.

The transcript of testis is longer than that of skin, encoding 372 additional amino acid sequence at N-terminal (Fig. 2). Three among the 8 TPRs of *tpis* are present in this extra region, indicating that the testis-type *tpis* may interact with broader spectrum of proteins. Proteins with TPR domain are diverse in their structure, intracellular localization, and therefore pos-

sibly in their biochemical functions (14), while heat shock proteins are often regarded as representative targets of interaction. With this respect, it may be noteworthy that some types of heat shock proteins, Hsp 70-2 (24) and hsc 70t (25), are present at specific stage of differentiating spermatogenic cells, and targeted disruption of Hsp 70-2 gene lead to male infertility due to blockade of spermatogenesis at the pachytene spermatocyte stage (8).

tpis was isolated as a gene whose expression increased during the process of embryonic skin development. In mouse embryos, epidermis is composed of simple layer of epithelial cells at 13.5 dpc, onto which more differentiated layers are consecutively added with the progress of development. Therefore, a given gene whose expression was induced during the period from 12.5 dpc to 16.5 dpc is likely involved in the differentiation of epidermal keratinocytes (26). Since *tpis* was also expressed in tongue and forestomach, *tpis* may play a role in differentiation of keratinizing stratified epithelium.

Reduction of natality and infertility have been a major area of concern in industrially advanced countries. Diverse social, economical and environmental factors are presumably involved, among which environmental endocrine disrupters are of the major recent topics (27). Endocrine disrupters exert deleterious effects on the reproductive systems, particularly on testicular function and spermatogenesis. Since such disrupters comprise synthetic chemicals with broad variety of structures and present at extremely low concentrations, the understanding of molecular mechanisms of spermatogenesis is essential for precise assessment of their biological effects and for establishing sensitive monitoring measures. Clarification of biological functions of *tpis* as well as the nature of its interacting targets may provide a cue to proceed in such a direction.

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REFERENCES

1. Hecht, N. B. (1998) *Bioessays* **20,** 555–561.
2. Guraya, S. S. (1987) *in* Biology of Spermatogenesis and Spermatozoa in Mammals, pp. 7–180, Springer-Verlag, Berlin.
3. Cooke, H. J., Hargreave, M. S., and Elliott, D. J. (1998) *Fertil. Steril.* **69,** 989–995.
4. Eddy, E. M. (1998) *Semin. Cell Dev. Biol.* **9,** 451–457.
5. 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.
6. Xu, Y., Ashley, T., Brainerd, E. E., Bronson, R. T., Meyn, M. S., and Baltimore D. (1996) *Genes Dev.* **10,** 2411–2422.
7. Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., and Korsmeyer, S. J. (1995) *Science* **270,** 96–99.
8. Dix, D. J., Allen, J. W., Collins, B. W., Mori, C., Nakamura, N., Poorman-Allen, P., Goulding, E. H., and Eddy, E. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3264–3268.
9. Okabe, M., Ikawa, M., and Ashkenas, J. (1998) *Am. J. Hum. Genet.* **62,** 1274–1281.
10. Howard, T., Balogh, R., Overbeek, P., and Bernstein, K. E. (1993) *Mol. Cell. Biol.* **13,** 18–27.
11. Foulkes, N. S., Mellstrom, B., Benusiglio, E., and Sassone-Corsi, P. (1992) *Nature* **355,** 80–84.
12. Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990) *Cell* **60,** 319–328.
13. Lamb, J. R., Tugendreich, S., and Hieter, P. (1995) *Trends Biochem. Sci.* **20,** 257–259.
14. Goebl, M., and Yanagida, M. (1991) *ibid.* **16,** 173–177.
15. Takaishi, M., Takata, Y., Kuroki, T., and Huh, N. (1998) *J. Invest. Dermatol.* **111,** 128–132.
16. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
17. Ausubel, F., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (Eds.) (1994) *Current Protocols in Molecular Biology,* Greene Publishing Associates & Wiley Interscience, New York.
18. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148.
19. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5857–5864.
20. Nuttall, S. D., Hanson, B. J., Mori, M., and Hoogenraad, N. J. (1997) *DNA Cell Biol.* **16,** 1067–1074.
21. Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Shibata, H., Akasako, A., Muramatsu, M., Kawai, J., Hirasawa, T., Watanabe, S., Shiroishi, T., Moriwaki, K., Taylor, B. A., Matsuda, Y., Elliot, R. W., Manly, K. F., and Chapman, V. M. (1994) *Genetics* **138,** 1207–1238.
22. Zhang, M. L., Wang, L. F., Miao, S. Y., and Koide, S. S. (1992) *Chin. Med. J.* **105,** 998–1003.
23. Lamb, J. R., Michaud, W. A., Sikorski, R. S., and Hieter, P. A. (1994) *EMBO J.* **13,** 4321–4328.
24. Zakeri, Z. F., Wolgemuth, D. J., and Hunt, C. R. (1988) *Mol. Cell. Biol.* **8,** 2925–2932.
25. Matsumoto, M., and Fujimoto, H. (1990) *Biochem. Biophys. Res. Commun.* **166,** 43–49.
26. Kashiwagi, M., Kuroki, T., and Huh, N. (1997) *Dev. Biol.* **189,** 22–32.
27. Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J. Jr, Jegou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebaek, N. E. (1996) *Environ. Health Perspect.* **104**(Suppl. 4), 741–803.