Molecular Cloning and Characterization of a Novel Gene Specifically Expressed in Gonad

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Abstract: *We identified a novel gene, termed GSE (gonad-specific expression gene). Nucleotide sequence analysis of GSE cDNA revealed that the open reading frame of 745-bp encodes a protein of 247 amino acids with a predicted molecular mass of 27.6 kDa. The deduced amino acid sequence indicated that GSE protein might be a soluble protein in the cytoplasm without a signal peptide. Northern blot analysis showed that this gene was abundantly expressed in mouse testis and slightly expressed in the mouse ovary. RT-PCR analyses indicated that the GSE mRNA in the testis was first detected at Day 14 postpartum, when spermatocytes at mid-pachytene are likely to appear. In situ hybridization confirmed its expression at this stage of spermatogenesis. On the other hand, the GSE mRNA in the ovary was already present at birth, when germ cells are in meiosis. These observations suggest that GSE may be associated with meiosis during gametogenesis.*

Key words: *cDNA, Gametogenesis, Mouse*

In mammals, the germ cells develop from primordial diploid cells to haploid gametes through a complex developmental process. The differentiation of germ cells in morphological and biochemical properties is determined by changes of gene expression. It requires a strict program of stage- and cell-specific gene expression in germ cells as well as in surrounding somatic cells. Therefore, it is necessary to study the stage- and cell-specific expression of genes in order to understand the molecular mechanisms of spermatogenesis and oogenesis. Some stage- and cell-specific genes that exert specific rules during gametogenesis have been cloned. A protooncogene c-kit, a member of the tyrosine kinase receptor family, is directly involved in the mitotic cell cycle of spermatogonia, which is associated with the survival and proliferation of differentiating type A spermatogonia [1, 2]. LRTP, a member of the family of leucine-rich repeat proteins, was abundantly expressed in late pachytene and diplotene cells and significantly declined after the first meiotic division, suggesting a functional association of this protein with the prophase of meiosis [3]. Haspin is specifically expressed in haploid round spermatids, and it is believed that haspin plays a role in cell cycle regulation after meiosis in haploid germ cells [4, 5]. On the other hand, meiosis-specific protein SCP3 is suggested to be linked to inherited aneuploidy in the female germ cell [6]. More recently, two genes, fragilis and Pgc7/stella, were reported to be involved in initiating germ cell competence and specification in proximal epiblast cells by the extra-embryonic ectoderm [7, 8].

Recently, the mouse full-length cDNA encyclopedia was reported [9]. This is extremely powerful for analyzing how certain genes cause a given phenotype. In this report, we focus on a novel gonad-specific gene and investigate its expression during gametogenesis in the mouse testis and ovary.

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Materials and Methods

Cloning of mouse GSE cDNA

We recently identified a novel gonad-specific partial cDNA (*GSE*, gonad-specific expression gene) in a previous experiment [10]. This gene showed 100% similarity with mouse cDNA clone (AK005861 in GenBank™, 1700011E24 in RIKEN full-length enriched mouse cDNA library). So, we isolated and cloned the full-sequence of this cDNA by RT-PCR from mouse testis. For cDNA cloning of mouse *GSE* gene, forward primer (5'-GGCACCAATATTGTTTTAT-3') and reverse primer (5'-GGCAAGAGGAGGTCGCAATG-3') were designed on the basis of the sequence of adult testis cDNA clone (AK005861). These primers were used for reverse transcriptase-PCR (94°C for 30 second, 55°C for 30 second, 72°C for 1 minute for 40 cycles) with EX Taq™ (Takara) and mouse testis mRNA prepared as described in the Quick Prep™ Micro mRNA Purification Kit (Amersham). The RT-PCR product of the expected size (~800 base pairs) was cloned into pGEM-T Easy Vector (Promega) and sequenced. The nucleotide sequence was determined by using a model 310 DNA sequencer (ABI) with ABI PRSIN BigDye™ Primer Cycle Sequencing Ready Reaction Kit (ABI). The nucleotide sequence and the deduced amino acid sequence were analyzed and determined through a homology search with GenBank™, EB, SWISSPROT, and PIR data banks.

Northern blot analysis

Total RNA was extracted from 9 different mouse tissues by the Trizol / phenol / chloroform extraction method. Poly(A)$^+$ mRNA was purified by Olgotex (Takara). Two micrograms of mRNA was separated by electrophoresis on 1% agarose gel containing formaldehyde and transferred onto Hybond-N$^+$ nylon membranes (Amersham). The blot was hybridized with random-primed ^{32}P-radiolabelled GSE cDNA probe (a 763-bp fragment) according to the manufacturer's instructions of Express-Hybrid™ buffer (Amersham) and detected using BAS-2500 Bio-Image Analyzer (Fuji Film).

In situ hybridization

The paraffin-embedded mouse testes were cut into 5–6 μm sections and attached to silan-treated slides. The sections were routinely dewaxed and hydrated. After each treatment with 0.3% Triton-X, 0.2% HCl, and 20 μg/ml Proteinase K, sections were refixed in 4% paraformaldehyde, treated with 0.2% glycine and then 50% formamide in 2X SSC. *GSE* sense and antisense RNA probes were synthesized from GSE cDNA (a 763-bp fragment) cloned into pGEM-T Easy Vector with digoxigenin-labeled UTP according to the manufacturer's protocol (Boehringer Mannheim). The hybridization mixture consisted of 50% deionized formamide, 5X SSC, 10% dextran, 1X Denhardt's solution, 100 μg/ml sheared and denatured salmon sperm DNA, 100 μg/ml E. coli tRNA, 10 mM dithiothreitol, and denatured probe. After hybridization overnight at 50°C, the slides were washed in 4X SSC at 42°C for 20 minutes and then treated with 20 μg/ml RNase A (37°C, 30 minutes) to remove the nonhybridized riboprobe. The slides were washed in 2X SSC at 68°C for 1 hour and then incubated in 0.2X SSC at 68°C for 1 hour. The hybrids were reacted with 1.5 U/ml anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, and then they were developed with 5-bromo-4-chloro-3-indolyl-phosphate-nitroblue tetrazolium (BCIP/NBT) as substrate.

RT-PCR analysis

Messenger RNA (0.5 μg each) extracted and purified from mouse testes and ovaries on different postnatal weeks were reverse transcribed using AMV reverse transcriptase (Takara) and random 9-mer primers in a 40 μl reaction mixture. The cDNA from the testis was amplified using specific primers (5'-AGCTCCCTCTTGTGCTGAGA-3', 5'-TTCAGCTCATAGTCACCTGG-3') with parameters consisting of 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds for 40 cycles. To detect *GSE* gene expression in the mouse ovary, the nested PCR was performed as follows. First PCR was done with specific primers (5'-GAAGGATGGTGGAGGCTCAC-3', 5'-GGTTGAAGACTGCTTGGGGA-3') under cycling conditions consisting of 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds for 40 cycles. One microliter of reaction mixture of the first PCR products was put into tubes with a reaction containing the inner set of primers (5'-AGCTCCCTCTTGTGCTGAGA-3', 5'-TTCAGCTCATAGTCACCTGG-3'). Cycling conditions were 40 cycles of 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds. All amplified products were separated on a 2% agarose gel by electrophoresis. RT-PCR for mouse *G3PDH* (glyceraldehyde 3-phosphate dehydrogenase) gene was performed using Clontech primers. Three independent experiments were done in this study.

Southern blot analysis of GSE gene

Ten micrograms of genomic DNA from mouse testes was digested with either *BamHI*, *EcoRI*, *HindIII*, *SacI*, or

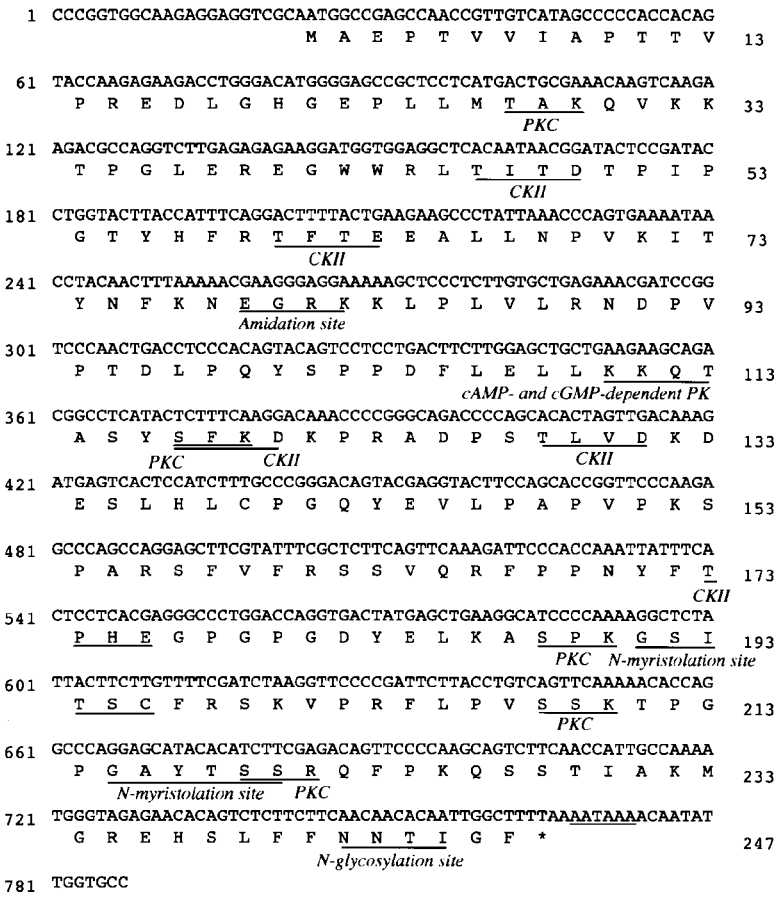

Fig. 1. Nucleotides and deduced amino acids of mouse GSE. The nucleotides and deduced amino acids are numbered on the left and right, respectively. The putative polyadenylation sequence is underlined. The stop codon is marked with an asterisk. cAMP- and cGMP-dependent PK: cAMP- and cGMP-dependent protein kinase phosphorylation site. PKC: protein kinase C phosphorylation site. CKII: casein kinase II phosphorylation site.

XbaI, separated on 1% agarose gel by electrophoresis and transferred to a nylon filter (Hybond N^+, Amersham). Hybridization and detection were performed with ECL gene detection procedures (Amersham) according to the manufacturer's recommended procedure.

Results

Molecular cloning and structural features of mouse GSE

The nucleotide sequences of mouse *GSE* cDNA and the deduced amino acid sequences are shown in Fig. 1. The open reading frame of mouse *GSE* extends 745-bp and encodes a protein of 247 amino acids residues with a predicted molecular mass of 27.6 kDa. The N-terminal region of amino acid sequences of mouse GSE showed no similarity to known proteins on the data base, whereas the C-terminal region (amino acids 107–235) of GSE showed similarity with possible alpha collagen-related protein (accession number Q9NE64). Computer-assisted sequence analysis showed that GSE contained one potential glycosylation and several protein kinase C, cAMP- and cGMP-dependent, and casein kinase-II phosphorylation sites. It is also highly basic (pI=9.84; the content of basic residues is 27.3%) with no signal peptide, indicating that it might be a soluble protein in the cytoplasm.

Gonad-specific expression of mouse GSE

The tissue distribution of GSE gene expression was examined by Northern blot analysis of poly(A)$^+$ RNAs

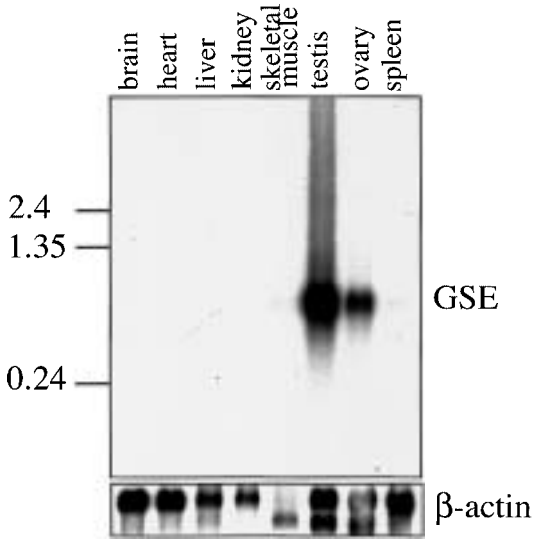

Fig. 2. Expression of *GSE* mRNA in mouse tissues. Two micrograms of Poly(A)$^+$ RNA from various tissues of adult mice were subjected to Northern blot analysis using the mouse GSE cDNA probe.

Fig. 3. Developmental changes of *GSE* mRNA expression in mouse testes and ovaries. mRNA (0.5 μg each) from mouse testes and ovaries on different postnatal weeks (W) were analyzed by RT-PCR as described under "Material and Methods".

from various tissues of adult mice using the full-length of GSE cDNA as a probe. One transcript of about 0.8-kbp was exclusively detected in the testis as shown in Fig. 2. Furthermore, there was GSE mRNA in the mouse ovary that showed a very weak signal with this probe (Fig. 2). No transcript was observed in somatic tissues such as the heart, liver, kidney, lung, brain, skeletal muscle, and spleen. The blots were stripped and reprobed with β-actin cDNA to ensure equivalent mRNA loading.

Expression pattern of mouse GSE during testis and ovary development

RT-PCR analysis of mRNA from 0-day-old to adult mouse testes (49-day-old) was performed to confirm the developmental change of *GSE* mRNA expression in the mouse testis and ovary. As shown in Fig. 3, *GSE* mRNA in the mouse testis was expressed initially at Day 14 after birth and also expressed thereafter, corresponding to the appearance of spermatocytes at mid-pachytene. On the other hand, a substantial increase of GSE gene expression in the ovary was observed from newborn, prepubertal, and sexually mature mice.

Stage-specific distribution of mouse GSE

To investigate further whether the *GSE* gene is specifically expressed in germ cells and the localization of *GSE* mRNA in the testis, we examined the *GSE* mRNA localization in the mouse testis by in situ hybridization using mouse *GSE* cDNA as a probe. The *GSE* mRNA was distributed within the cytoplasm of spermatocytes I (pachytene), round spermatids, and elongated spermatids, either cell of which was observed in stage VI-VII or VII-VIII tubules (Fig. 4B). No significant signal was detected in hybridization using a sense probe (Fig. 4A).

Genomic southern blot analysis of mouse GSE

To determine if the mouse genome contains more than one *GSE* gene, we did Southern blot analysis. The sizes of the resulting fragments corresponded to the restriction fragments of the database sequence (AC084383), which includes *GSE* genomic DNA (Fig. 5). These results indicate that the *GSE* gene is a single copy in the mouse genome and not an intronless gene like the *haspin* gene [4, 5]. The latter result is consistent with the preliminary result indicated by genomic PCR with specific primers for *GSE* cDNA (data not shown).

Discussion

We report here the cloning and characterization of a novel mouse cDNA that is expressed in the testis and ovary but is not expressed in the somatic tissues in mice. It is therefore referred to here as GSE (gonad-specific expression gene). This *GSE* gene has one homology region; the C-terminal region displays homology to the possible alpha collagen-related protein. Furthermore, GSE contains many potential target sites for protein kinases, i. e. cAMP- and cGMP-dependent protein kinase phosphorylation sites, protein kinase C phosphorylation site, and casein kinase-II phosphorylation site. These suggest that GSE might have biological activity in

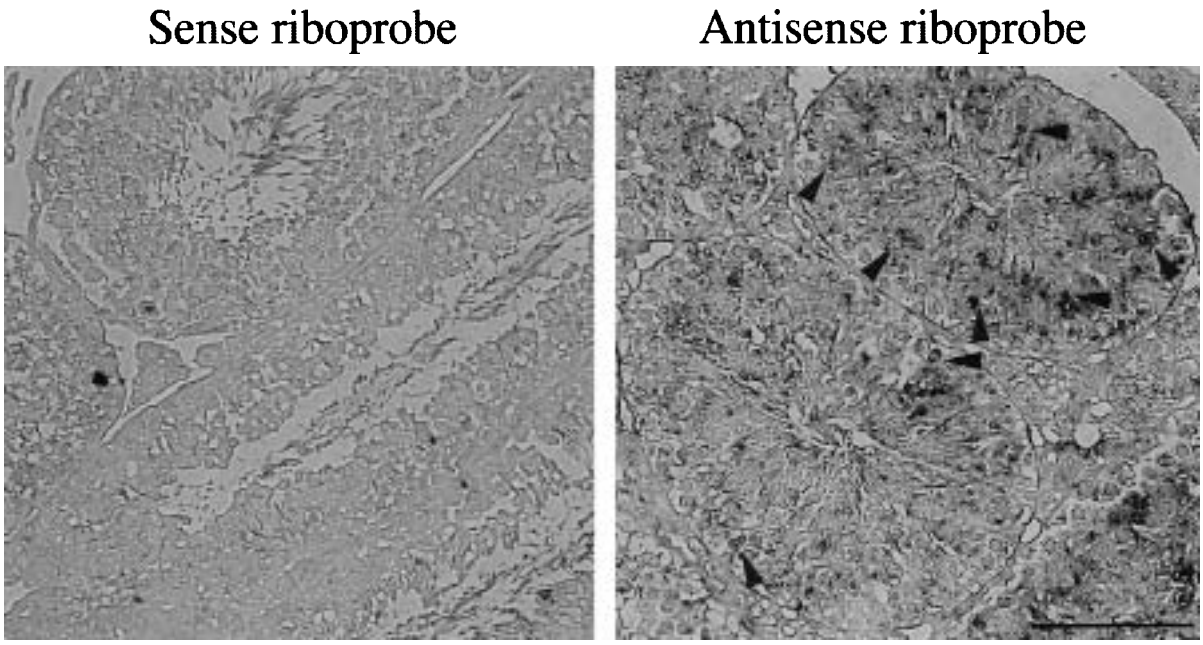

Fig. 4. The distribution of *GSE* mRNA in mouse testis. A riboprobe was transcribed from mouse full (787-bp) *GSE* cDNA and used for in situ hybridization. Signals are visible in the cytoplasm of spermatocytes I (pachytene), round spermatids, and elongated spermatids (arrowheads). Scale bar is 100 μm.

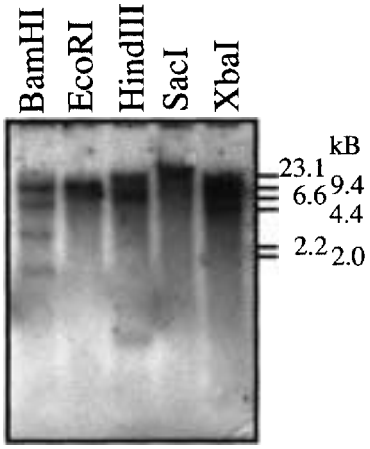

Fig. 5. Southern blot analysis of *GSE* gene. Mouse genomic DNA (10 μg) was digested with either *BamHI*, *EcoRI*, *HindIII*, *SacI*, or *XbaI*, fractionated on a 1% agarose gel, then transferred to a nylon membrane. The membrane was probed with mouse *GSE* cDNA probe.

phosphorylated or dephosphorylated form. Protein phosphatases are involved in the regulation of cell proliferation and differentiation by co-operation with protein kinases [11]. Protein phosphorylation seems to be involved as an important molecular mechanism in the regulation of spermatogenesis because some studies showed that several protein kinases and phosphatases are expressed specifically in the testis. Recently, Varmnza S. et al. (1999) reported that target disruption of the protein phosphatase *PPLR* gene, which encodes an isoform of type-1 serine protein phosphatase, induces meiotic defects in the testis [12]. This finding suggests that the protein phosphatase is involved in the stage-specific regulation of spermatogenesis. Further study to determine the GSE protein function in gametogenesis will be necessary.

Northern blot analysis revealed that the mouse *GSE* gene is expressed exclusively in both the testis and ovary. Although only a faint band of mRNA was detectable in the ovary, *GSE* gene expression in the testis was much higher than its expression in the ovary. Thus, it is possible that GSE has physiologically important role in the testis. Spermatogenesis is a complex differential process which involves changes in gene expression. Generally, in order to satisfy the developmental demands of the tissue, the differentiation of male gametes requires the expression of many germ cell-specific proteins at specific stages of development [13–15]. The expression patterns of many genes that are expressed in the testis are cell type-specific or

stage-specific [16, 17]. In our present study, we demonstrated the developmentally regulated pattern of expression of the *GSE* mRNA in the mouse testis, which exemplifies such a pattern. In the mouse seminiferous tubule, spermatogenetic cells of the same development stage form a concentric layer of cells. Mitotic proliferation of stem cells, which are seen in the initial phase of spermatogenesis, occurs in the basal compartment. Successively, meiosis and spermiogenesis occur toward the inside of the seminiferous tubules. Therefore, it is generally possible to specify the developmental stage of germ cells on the basis of their sizes, shapes, and spatial distribution [18]. Our in situ hybridization analysis in the mouse testis demonstrated that the *GSE* mRNA expresses in spermatocytes I (pachytene), round spermatids, and elongated spermatids. RT-PCR analysis of testes from 0 to 49-day-old mice indicated that the *GSE* mRNA became detectable around 14 days after birth, which almost paralleled with the stage of spermatogenesis, when mouse spermatocytes undergo meiosis and differentiation into haploid round spermatids. Therefore, the characteristic distribution and developmental expression of mouse *GSE* gene indicates that the regulation of GSE is involved in the meiotic division.

Although the expression of the *GSE* gene was detected in the adult mouse ovary, only a low level of *GSE* mRNA was seen. RT-PCR analysis of ovaries from 0 to 49-day-old mice indicated that the *GSE* mRNA was already detectable just after birth, when germ cells are in meiosis. However, further investigation will be needed to confirm if the *GSE* gene can regulate meiosis of mammalian female germ cells.

In summary, GSE is a novel gene that is specifically expressed in the mouse gonad, and abundantly expressed in the testis, suggesting an important role for the GSE gene in meiosis of mammalian germ cells.

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