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# Isolation and characterization of cDNA clones specifically expressed in testicular germ cells

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**Abstract** We have cloned cDNAs involved in germ cell-specific expression. For this, a subtracted cDNA library was generated by subtracting cDNAs derived from supporting cells of mutant testis from wild-type testis cDNAs. Detailed analyses of mRNA expression revealed that the genes corresponding to the cloned cDNAs were exclusively expressed in testes and were developmentally controlled.

*Key words:* Testis; Mouse; Protamin; cDNA library; Subtraction; W/W$^v$ mutant; Actin capping protein

## 1. Introduction

Mouse spermatogenesis is an excellent model system to study regulation of gene expression during differentiation. Postnatal development of the mouse seminiferous epithelium is a complicated process which finally generates a tissue able to produce functional spermatozoa. The whole process can be subdivided into three parts: (i) a premeiotic phase characterized by an increase in cell number due to mitotic divisions of diploid spermatogonia; (ii) a meiotic prophase, which leads to the formation of haploid round spermatids; and (iii) a post-meiotic phase, which includes the morphogenetic events required for spermatozoa formation (spermiogenesis) [1,2]. Specific cells derived from the stem cells in seminiferous epithelium of the adult testis undergo these processes and continuously provide the mature sperms. The precise regulation of such germ cell differentiation requires a strict program of stage- and cell-specific gene expression in germ cells as well as in surrounding somatic cell types [3]. To understand the mechanism of testicular germ cell differentiation, it is of great interest to isolate specific genes and characterize their functions as well as their regulation.

## 2. Materials and methods

### 2.1. Preparation of cDNA libraries carrying directional inserts

Total RNA followed by purification of poly $(A)^+$ RNA was extracted by the guanidine thiocyanate/CsTFA method from the testes of adult wild-type B6 mice and 4-month-old W/W$^v$ mutant mice [4]. Their cDNA libraries were prepared as described by Gubler and Hoffmann with some modifications (Kobori et al., manuscript in preparation) [5]. Briefly, cDNA was synthesized in a reaction mixture including $^5$Me-dCTP with reverse transcriptase (Superscript II) from 2–5 μg of mouse testis poly$(A)^+$ RNA and 1.6 μg of oligo (dT) primer carrying a *Not*I site. The reaction mixture was treated with RNase H, followed by reaction with DNA polymerase I. Each end was blunt-ended with T4 DNA polymerase and ligated to an unphosphorylated *Bgl*II–*Sma*I adaptor. After digestion with *Not*I, small DNA fragments of less than 300 bp were removed by a CROMA spin-400 column (Clontech, USA). The cDNA fragments were directionally inserted between the *Not*I (dephosphorylated) and *Bgl*II sites of vector pAP3*neo* (H. Nojima, unpublished). The ligation mixture was electroporated into MC1061A cells as described [6]. The complexities of the cDNA libraries used here were $6.0 \times 10^6$ colony forming units (cfu) for the B6 wild-type mouse and $2.8 \times 10^6$ cfu for the W/W$^v$ mutant mouse.

### 2.2. Preparation of a subtracted cDNA library

The strategy for preparation of the subtracted cDNA library is depicted in Fig. 1. To prepare single-stranded plasmid DNA, the plasmid DNA prepared from the cDNA library of wild-type testes was introduced into *E. coli* DH12S cells by electroporation. After 1 h of culture in rich medium (2× TY), transformed cells were infected with R408 helper phages. Then, single-stranded DNA was purified from the supernatant of the overnight culture [7]. Biotinylated RNA drivers were isolated from the testicular cDNA library of the mutant mouse (W/W$^v$) as follows: 5 μg of the *Not*I-digested plamid DNA prepared from the mutant cDNA library was used as the template for the T7 RNA polymerase reaction (Stratagene, USA) and 30 μg of synthesized RNA was labeled by photobiotin (Vector Lab., Burlingame). 1 μg of single-stranded DNA prepared from the cDNA library of wild-type testes was hybridized with 15 μg of biotinylated RNA in 25 μl hybridization buffer including 80% formamide, 50 mM HEPES (pH 7.5), 2 mM EDTA, 0.2% SDS and 1 μg of oligo-poly(rA). After hybridization, the mixture was added to 400 μl of the hybridization buffer without SDS, and a further 10 μg of streptavidin was added. The mixture was incubated at room temperature for 5 min and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The organic phase was back-extracted with 100 μl TE (10 mM Tris-HCl, pH. 7.5, 1 mM EDTA). The aqueous phase was pooled. Streptavidin binding and phenol treatment were repeated once more. The recovered DNA was subtracted with biotinylated RNA one more time. The single-stranded DNA obtained was subjected to BcaBEST DNA polymerase (Takara Shuzo, Japan) reaction at 65°C for 30 min to obtain the double-stranded cDNA. The DNA was dissolved in 10 μl TE buffer and 1 μl aliquots were introduced into *E. coli* DH12S cells by electroporation [6].

### 2.3. Screening of B6 specific clones by dot blots

Plasmid DNA of each clone randomly selected from the subtracted cDNA library was bound to a nitrocellulose filter by slow vacuum filtration, and was subjected to cDNA dot blot analysis (The Convertible Filtration Manifold System; BRL). As a probe to select cDNA clones specifically expressed in testicular germ cells, RNAs labeled by the hapten digoxigenin (DIG) were generated by T7 RNA polymerase reaction from testicular cDNA library of wild-type and mutant mice. The DIG hybridization method was utilized according to the instructions recommended by the manufacturer (DIG Luminescent Detection Kit).

### 2.4. Northern blots and DNA sequencing

RNA samples containing 2.2 M formaldehyde were subjected to electrophoresis in 1.0% agarose gels containing 0.66 M formaldehyde [7]. The RNA was transferred to a nitrocellulose filter in 20× SSC. Hybridization was performed with $^{32}$P-labeled cDNA by the random

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priming method in a solution containing 4× SSC, 5× Denhardt's, 0.2% SDS, 12 μg/ml denatured salmon sperm DNA and 50% formamide at 65°C for 16 h. Filters were washed twice in 0.1% SSC and 0.1% SDS at 55°C. Signals of the bands were detected by an Image analyzer (Fuji Film, Japan).

Dideoxy-chain termination sequencing reactions [8] were performed with fluorescent dye-labeled primers and thermal cycle sequencing kits purchased from Applied Biosystems. The reaction products were analysed by GENESCAN-373A (Applied Biosystems, USA).

## 3. Results

### 3.1. Preparation and characterization of a subtracted cDNA library

Systematic cloning of testis-specific cDNA was conducted by the following strategy: the wild type (+/+) mouse cDNA library was subtracted by that of mutant ($W/W^v$) mRNA generated from the mutant cDNA library. Since all of the germ cells were absent in the mutant ($W/W^v$) mouse testis at 4 months of age, the testis contained only the supporting cells [9]. On the other hand, the testis of the wild-type mouse contained mRNA derived from both germ and supporting cells. Thus, the subtraction process described above was expected to yield the cDNA clones specifically expressed in testicular germ cells.

Approximately 100 cDNA clones were isolated from the subtracted library and each clone was analyzed by dot blot hybridization (Fig. 2). The RNA probes generated from the testicular cDNA library of both wild-type and mutant mouse library were used for the dot blotting analysis. Dot nos. 1–6 were clones isolated from the cDNA library of wild-type mRNA before subtraction. No.96 was vector pAP3*neo* without a cDNA insert. These clones were used as a controls. All these clones isolated without subtraction were hybridized equally

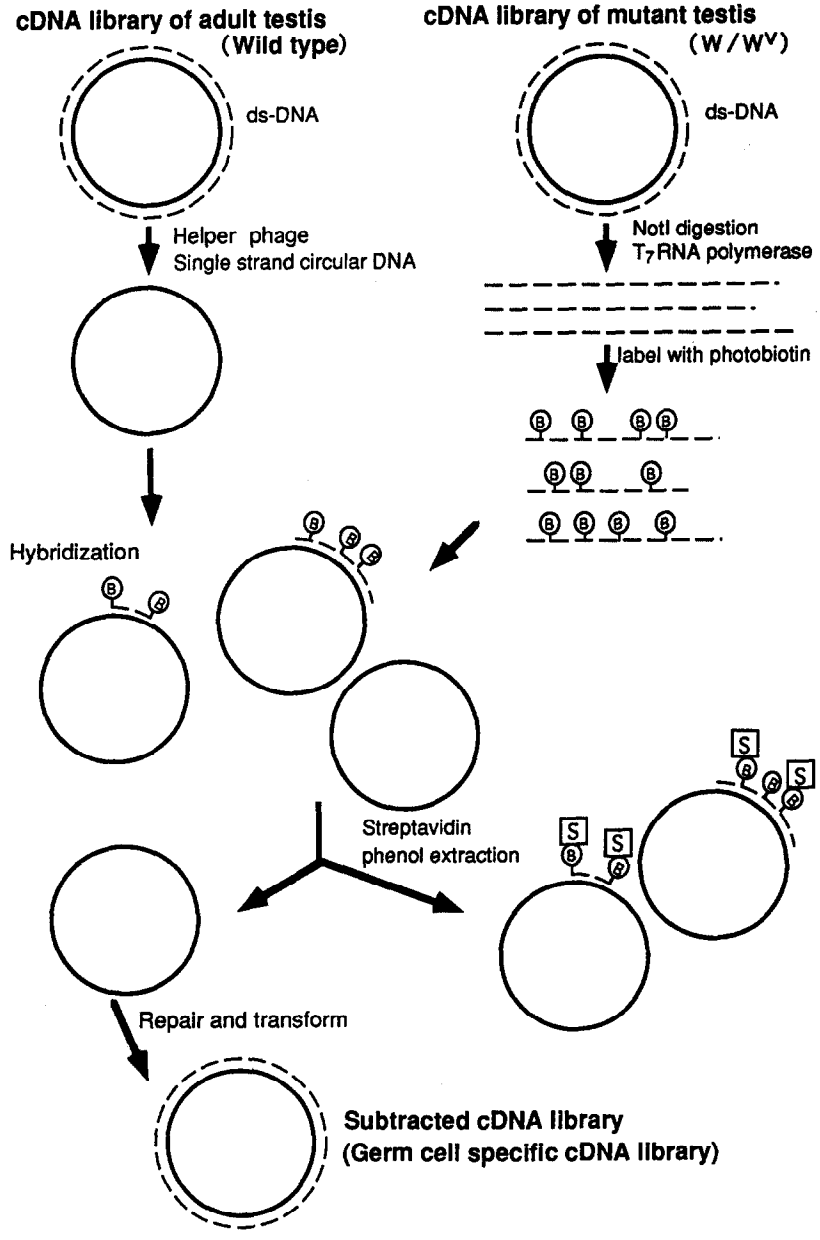


Fig. 1. A schematic presentation of the strategy for subtraction used to isolate cDNA clones specifically expressed in testicular germ cells.

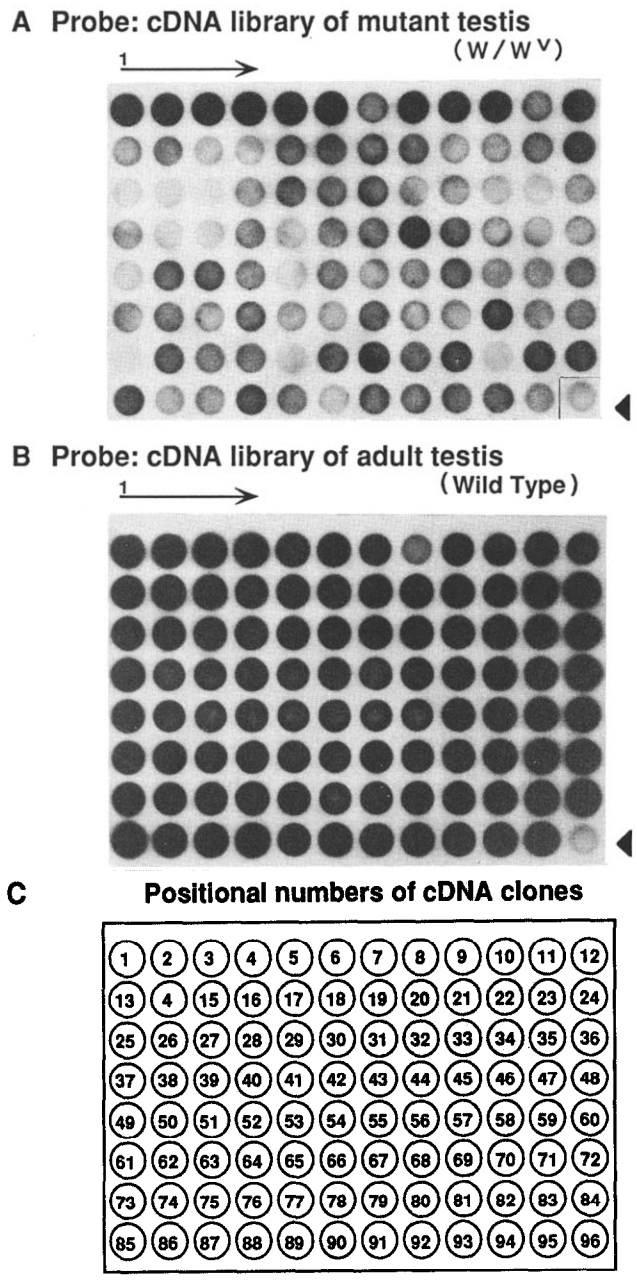


Fig. 2. Dot blot analysis of 96 cDNA clones randomly selected from the subtracted cDNA library. Five $\mu$g of plasmid DNA from each clone obtained from the subtracted cDNA library were applied onto a nylon membrane using the convertible filtration manifold system apparatus (BRL) and hybridized with DIG labeled RNA synthesized in vitro from the mutant (A) and wild-type (B) testicular library, respectively. As controls, nos. 1–6 were cDNA clones randomly picked from the non-subtracted wild-type testicular library and no. 96 was a dot of pAP3*neo* vector without an insert as a negative control. For details see section 2.2.

with the mRNA probes derived from both wild-type and mutant cDNA libraries. On the other hand, as shown in Fig. 2A, almost all of the clones isolated after subtraction showed weak signals with the mutant library probes as compared to those with the wild-type probes (Fig. 2B).

The clones isolated from the subtracted library were divided into three groups by the pattern of hybridization with the wild-type mRNA probes compared to the hybridization with the mutant probes: hybridization signal was (i) equal, (ii) weaker with mutant than with wild-type probes: and (iii) no or almost no signal was detected with the mutant mRNA probes. Approximately 10% of the clones belonged to group (i). These clones may have escaped from the subtraction processes. However, 80% of the clones showed stronger signals with the wild-type probes than those with the mutant testis mRNA probes (group (ii)). Furthermore, the remaining 10% of the clones showed little or no signal with the mutant mRNA probes (group (iii)). Eight clones belonging to group (iii) were picked up and further analyzed. These cloned cDNAs were sequenced partially from the 5′ end. Their cDNA sequences were subjected to computer-based analyses with the published nucleotide sequences. The results showed that 5 cDNA clones (nos. 26, 34, 35, 53 and 82) were outer dense fiber protein [10], protamin 2 [11] and three of protamin 1 [12], respectively. These genes had already been identified and known to be expressed specifically in testicular germ cells. They all hybridized very weakly with the probe of mutant testis but strongly with that of wild type testis (Fig. 2). These results implied that the subtraction cloning method used here worked successfully to isolate the germ cell specific cDNA clones as we expected. The remaining 3 cDNA clones, belonging to group (iii) (dot nos. 7, 27 and 39), were subjected to further analysis. They were specifically expressed

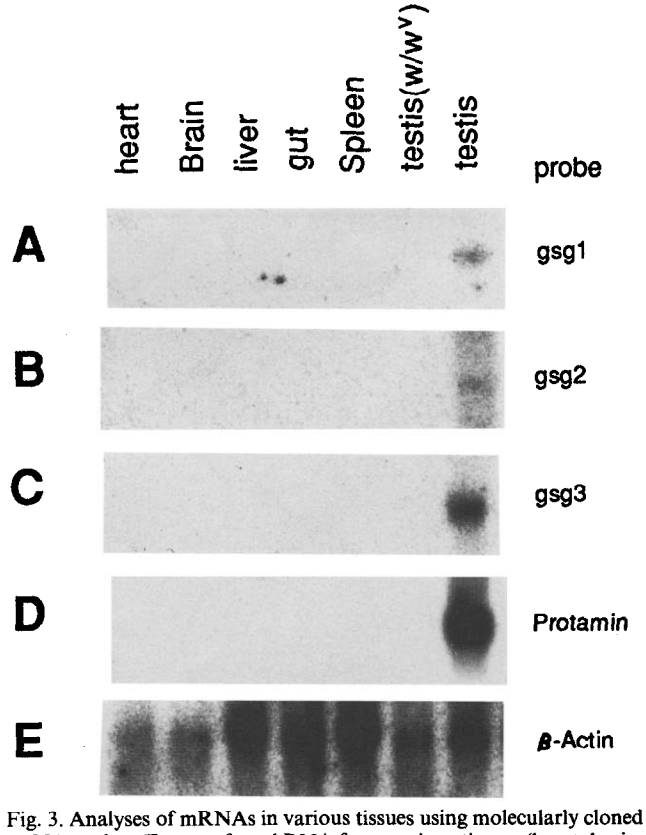


Fig. 3. Analyses of mRNAs in various tissues using molecularly cloned cDNA probes. Ten $\mu$g of total RNA from various tissues (heart, brain, liver, gut, spleen, and testis) were each electrophoresed, transferred to nylon membranes and hybridized with cloned cDNA probes. The cDNAs were labeled by using a BcaBest random primer kit (Takara, Japan). After autoradiography, the same filters were rehybridized with the following probes: (A) *gsg*1, (B) *gsg*2, (C) *gsg*3, (D), protamin 1, and (E) $\beta$-actin.

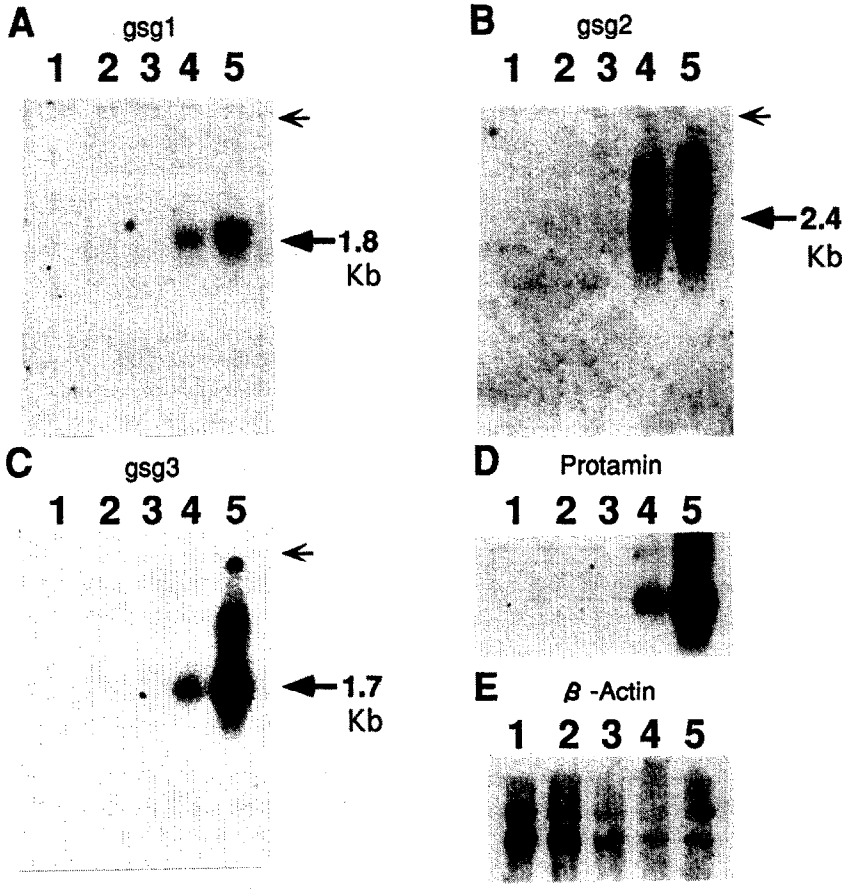


Fig. 4. Developmental expression of germ cell-specific genes (*gsg*) in prepubertal mouse testes. Ten μg of total RNA from prepubertal mouse testes (lane 1: 4 day old; 2, 10 day old; 3, 16 day old; 4, 24 day old; and 5, adult testis) were used for Northern blotting analysis. The probes used were as follows: (A) *gsg*1, (B) *gsg*2, (C) *gsg*3, (D) protamin 1, and (E) β-actin. The large and small arrows indicate the size of the transcripts and the origin of the gel, respectively.

in germ cells, and these three newly isolated clones were designated as *gsg* (germ cell-specific gene) 1, 2, and 3.

### 3.2. *Body mapping and developmental expression of newly isolated cDNA clones*

To further characterize molecularly cloned cDNAs, we have examined the transcription patterns in various mouse tissues. Total RNAs were extracted from the testis, heart, spleen, liver, gut and brain, and were analyzed by Northern hybridization with each cDNA probe. In all cases a strong band was observed only with the testis but not with other tissues (Fig. 3). The result indicated that all of these clones were transcribed exclusively in the testis.

To investigate the developmental expression pattern in the mouse testis, total RNA was isolated from the prepubertal mouse testis at 4, 10, 16 and 24 days of age. A 4-day-old mouse testis contains only gonocytes or undifferentiated spermatogonia. In a 10-day-old mouse testis, the majority of germ cells are spermatogonia both differentiated and undifferentiated. Spermatocytes and spermatids become predominant in 16- and 24-day-old mouse testis, respectively. All three clones, germ cell-specific genes (*gsg*) 1–3, were expressed in both 24-day-old and adult testis but not in 4-, 10- and 16-day-old testis. These results indicate that they were expressed in spermatids in germ cell development (Fig. 4). The mRNA sizes of these cDNA clones were 1.8 kb (*gsg*1), 2.4 kb (*gsg*2), and 1.7 kb (*gsg*3), judging from the results obtained by Northern blot analysis. Semiquantitative analysis of mRNA blots of these clones showed approximately 0.04 fold (*gsg*1), 0.04 fold (*gsg*2), and 0.5 fold expression (*gsg*3) as compared with the expression level of protamin 2 (Fig. 4).

### 3.3. *Sequencing of three gsg cDNAs and comparision of their DNA sequences with those of the data base*

The above three cDNAs *gsg*1–3 were examined for similarities by using the Genbank, EMBL, and NBRF nucleic acid data base with VAX computer-mediated UWGCG systems. All cDNA clones analysed here identified no significant homologue in these databases. Moreover, *gsg*1 and *gsg*2 had no significant motif. Only *gsg*3 (1.7 kb) showed a weak cDNA sequence homology with actin-capping protein (ACP) [13–15]. The complete nucleotide sequence and the deduced amino acid sequence are shown in Fig. 5. Since a stop codon is located upstream of the ATG codon (at nucleotide residues 1–3) in the same reading frame (data not shown), the ATG is very likely the translation intiation codon for *gsg*3. The open reading frame of the cDNA would begin with the sequence CTGGCCATGT, which agrees with the consensus sequence for translation start sites in eukaryotic mRNAs. The presence of a purine (G) at position −3 is the most important feature of

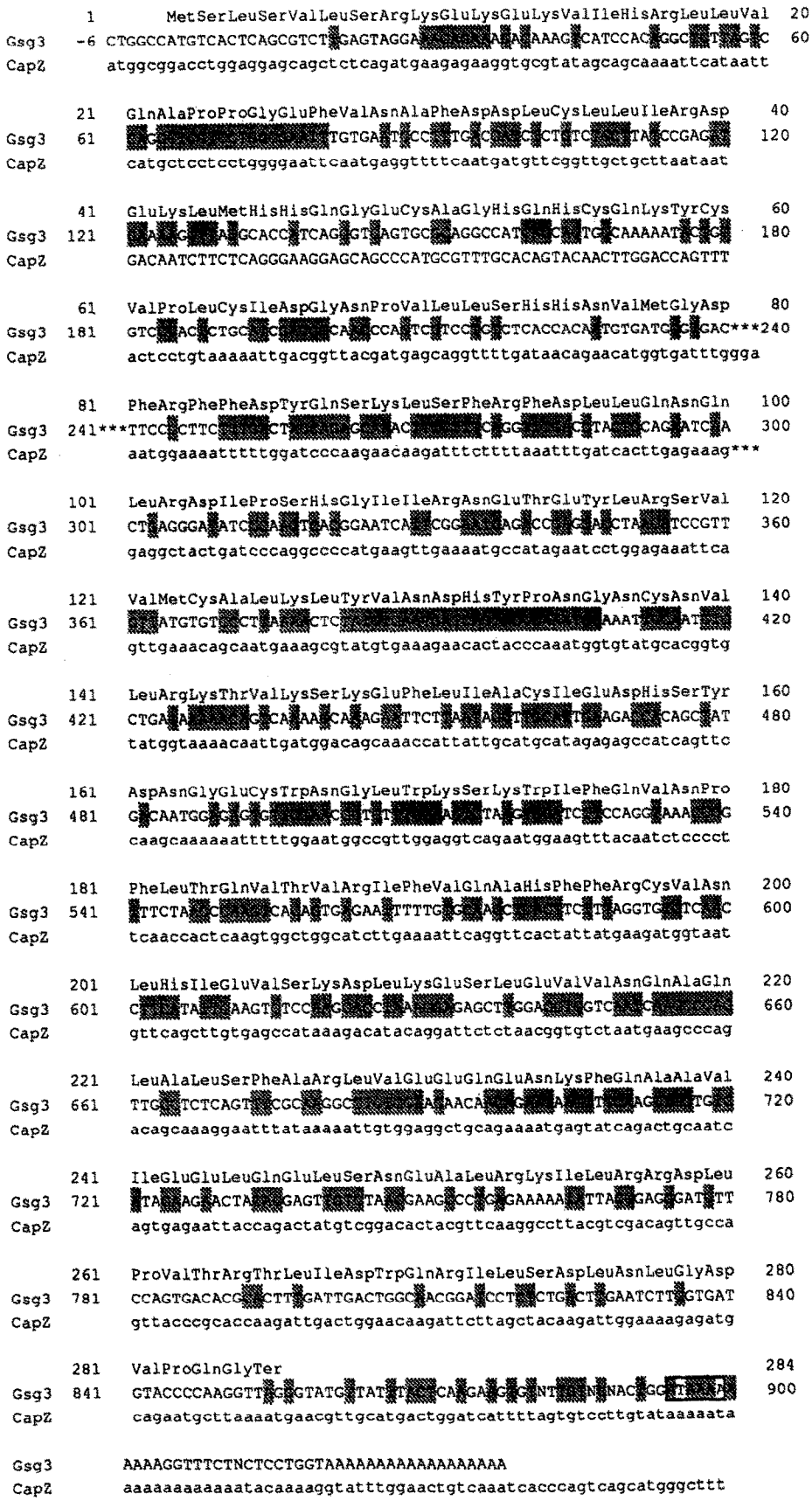


Fig. 5. The nucleotide and deduced amino acid sequences of *gsg3*. The predicted nucleotide sequence of the putative coding region of Gsg3 is compared with CapZ [15]. Shadowed sequences indicate the sequences identical to CapZ. The potential polyadenylation signal is indicated by an open box. The sequence of CapZ shows the open reading frame from the initiation codon. Asterisks denote nucleotide deletions introduced into regions so as to maximize homology.

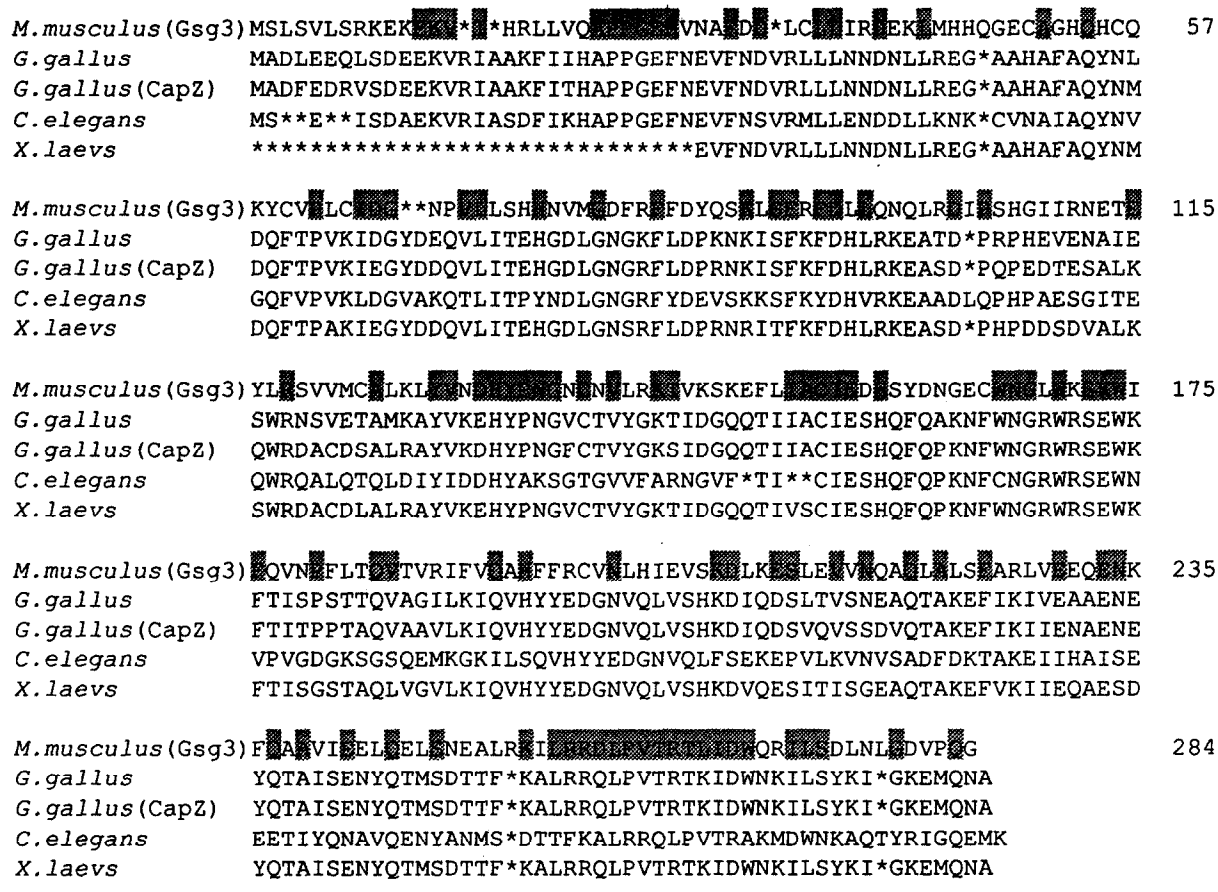


Fig. 6. Comparison of the deduced amino acid sequence of Gsg3 with other actin-capping proteins. The deduced amino acid sequence of Gsg3 was compared with those of the actin-capping proteins of *G. gallus* [13,15], *C elegans* (EMBL accession number: S31326) , and *X.laevis* [14]. Shadowed amino acids shared identical residues with more than two of the other sequences. Asterisks denote amino acid deletions introduced into regions so as to maximize homology.

the consensus [16]. It includes 852 bases of cDNA and codes for the Gsg3 protein with 284 amino acid residues having a moleculer weight of 33 kDa. Homologous regions of the Gsg3 with ACP were depicted in Fig. 6. Gsg3 displayed about 40% homology with ACP as a whole molecule. Some highly homologous regions were also noted (Fig. 6).

## 4. Discussion

Mammalian spermatogenesis occurs in a precise and coordinated manner in the seminiferous tubules [1,2]; and all of the stages of spermatogenesis from spermatogonial stem cells to the mature spermatozoa are present in the adult testis. One attempt to understand the morphological events of spermatogenesis at the molecular level has been to study testicular expression of genes required for spermatogenesis [17–19]. For this, the availability of mutant strains of mice [9,20,21], experimentally induced cryptorchidism and orchidopexy or surgical reversal [22], age-dependent progression of germ cell differentiation in juvenile testes [23] and cell-separation techniques [24,25], made the studying of the mouse spermatogenesis system particularly amenable to biochemical and molecular analyses. By using the various techniques and methods, the specific expression of some genes has been observed in testes, and some interesting information has been obtained by the isolation of cDNA clones specifically expressed in testes [3]. Furthermore, more systematic screening methods have been designed, leading to the isolation of new cDNA probes which recognized mRNAs with testis- and cell type-specific expression [26–29]. The analyses revealed that regulation at the level of transcription, resulting in the stage-specific activation of specific genes, was clearly occurring to many of the genes [27–33], and that the translational regulation apparently played a significant role in coordinating gene expression in the testis [34] since some of the mRNAs were not actively translated until meiosis had completed [34,35].

The genes that are expressed during spermatogenesis can be separated into the following 3 categories based on their characteristic expression patterns. There are genes that are expressed exclusively (or almost exclusively) during spermatogenesis, such as protamin [12]. The next group includes the genes that are expressed in several tissues, but that exhibit quantitative or qualitative differences in the expression pattern during the male germ cell development, for example, the protooncogene c-*abl* [36]. Finally, there are genes which represent a testis-specific isotype of a more generally expressed genes, such as PGK-2 [37].

The aim of this study was to systematically isolate and characterize the cDNA clones specifically expressed in testicular germ cell differentiation. It has been shown that 90% of randomly selected cDNA clones from a testicular cDNA library turned out to be previously known 'housekeeping' genes [26]. In contrast, our strategy of subtraction cloning of the wild-type

testicular cDNA library by the mutant is a powerful tool for isolating the germ cell-specific cDNAs.

The three novel genes *gsg*1 to *gsg*3 we isolated here were found to be expressed exclusively during spermatogenesis (Fig. 4). One of them, *gsg*3, however, may play a pivotal role(s) in morphogenesis of spermatid since Gsg3 had some structural similarities to the actin-capping protein [13–15]. We have also isolated many clones which were expressed in both germ cells and other tissues (data not shown). So far, we have not isolated any clone that belongs to the third group, i.e. the genes representing a germ cell-specific isotype of generally expressed genes such as PGK-2 [37].

Although the number of novel clones isolated here is limited, the strategy we employed seems to be powerful for isolation of germ cell specific cDNA clones, especially when it is systematically applied on a large scale. The cDNA clones specifically expressed in a certain stage of germ cell differentiation would be useful tools to elucidate the molecular mechanism of spermatogenesis.

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