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Original Article

A cystatin-related gene, *testatin/cresp*, shows male-specific expression in germ and somatic cells from the initial stage of murine gonadal sex-differentiation

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ABSTRACT Sex-differentiation in mammals initiates at mid-gestation when the differentiation of Sertoli cells is triggered by the expression of the testis-determining gene, *Sry*. However, little is known about the succeeding germ-soma interaction that directs the sex-differentiation of germ cells. We carried out subtraction and differential screening between male and female gonads at 13.5 dpc (days post coitum). A novel cystatin-related gene was identified and named *cresp* (cystatin-related expressed in Sertoli and spermatogonia), and has recently been reported independently under the name *testatin* (Töhönen *et al.*, 1998). The presumed amino acid sequence of *testatin/cresp* showed considerable homology to the cystatin family, but it lacked a few critical amino acid residues for the cysteine-protease inhibitory activity. A 0.7 kb RNA was detected by northern blotting specifically in the fetal and adult testes from 11.5 dpc and expression increased between 11.5 dpc and 12.5 dpc. Using RT-PCR analysis, the *testatin/cresp* mRNA was first detectable at 9.5 dpc in both male and female embryos but it was maintained only in the male. In females, the expression became weaker at 11.5 dpc and was undetectable after 12.0 dpc. *In situ* hybridization and immunohistochemical analyses, as well as single cell RT-PCR analysis, showed that the *testatin/cresp* mRNA was localized specifically in both the (pro)spermatogonia and Sertoli cells in the testis from 12.5 dpc to adult. Thus, expression of the *testatin/cresp* gene is upregulated in male gonads but downregulated in females immediately after the initiation of sex-differentiation, suggesting roles in the early developmental cascade of testis such as the germ-soma interaction.

KEY WORDS: *testis development, sex differentiation, testatin/cresp, spermatogonia, Sertoli cells*

Introduction

Mammalian sex differentiation depends on the presence or absence of the Y chromosome. The testis determining gene on the Y chromosome, *Sry*, initiates the embryonic gonads to differentiate into the testis (Koopman *et al.*, 1991). Precisely controlled expression of *Sry* at 10.5-11.5 dpc (days post coitum) in somatic cells of the male indifferent gonad triggers their differentiation into Sertoli cell precursors (Palmer and Burgoyne, 1991). These cells then commence secretion of Müllerian inhibiting substance (MIS), which induces regression of the Müllerian duct (Josso *et al.*, 1998; Lane and Donahoe, 1998). Also, interstitial cells in the testis differentiate into Leydig cells, which produce testosterone and promote Wolffian duct development (Parker *et al.*, 1999). Without the proper expression of *Sry*, gonadal somatic cells differentiate as in the female, that is, into follicle cells and theca cells (Berta *et al.*, 1990; Jager *et al.*, 1990).

Mammalian embryonic germ cells have two different developmental pathways depending on the gonadal sex. Mouse primordial germ cells (PGCs) are first recognizable at 7.0 dpc by surface alkaline phosphatase activity, at a location posterior to the primitive streak in the extraembryonic mesoderm (Ginsburg *et al.*, 1990). Then, they migrate into the embryonic mesoderm, through the hindgut endoderm, along the developing dorsal mesentery, and

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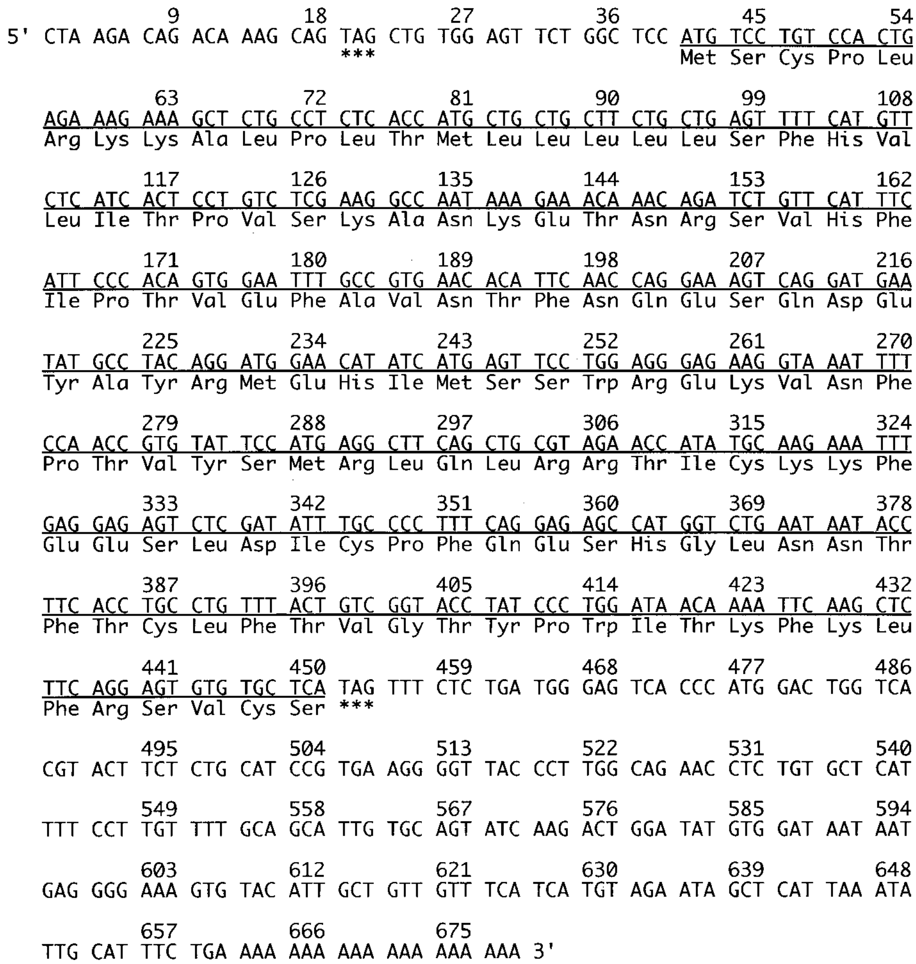
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GAG GAG AGT CTC GAT ATT TGC CCC TTT CAG GAG AGC CAT GGT CTG AAT AAT ACC
Glu Glu Ser Leu Asp Ile Cys Pro Phe Gln Glu Ser His Gly Leu Asn Asn Thr
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B

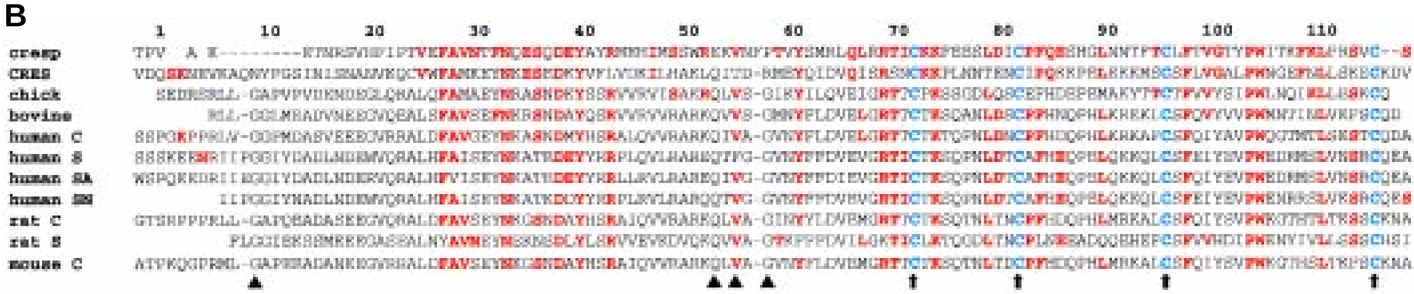

Fig. 1. (A) *Nucleotide sequence and deduced amino acid sequence of testatin/cresp. TAG in-frame stop codons are marked by asterisks. The ORF is underlined.* **(B)** *Comparative analysis of the testatin/cresp protein. Alignment of the derived testatin/cresp amino acid sequence with CRES, chicken cystatin, bovine cystatin, human cystatin S and SN, rat cystatin C and S, and mouse cystatin C. These sequences represent the mature proteins and do not include putative signal peptides. Dashes indicate gaps introduced to optimize the alignment. Numbering is according to the chicken cystatin sequence. Arrows indicate conserved cysteine residues. Arrowheads indicate presumably critical amino acids that are important for the cysteine-protease inhibitory activity.*

finally into the posterior body wall and genital ridge at 11.5 dpc (Wylie *et al.*, 1985). Germ cells continue to proliferate mitotically after arrival at the genital ridge until 12.5 or 13.5 dpc. At 12.5 dpc, genital ridges show the first signs of sexual dimorphism with testicular cord formation in the males. Then, PGCs in the testis are mitotically arrested by 15.5 dpc to become prospermatogonia, while those in the ovary enter into meiosis from 13.5 to 15.5 dpc to become oocytes (McLaren, 1994). Previous studies have indicated that such sex-differentiation of germ cells is directed by the sex of the gonadal somatic cells and not by that of germ cells themselves (McLaren, 1995).

During the early development of fetal testes, it has been reported that several transcription factors, such as *Sry*, *Sox9*, *WT1* and *Ad4BP/SF-1*, play important roles (reviewed in Swain and Lovell-Badge, 1999). However, little is known about genes acting in the developmental cascade under the control of these transcriptional regulators. For example, there is almost no information about the molecules involved in the somatic-germ cell interaction that directs the sex-differentiation of germ cells.

We therefore attempted to isolate such genes involved in the gonadal development and differentiation by using subtraction and differential screening between male and female fetal gonads at 13.5 dpc (Tamura *et al.*, manuscript in preparation). A novel gene found in our screen showed specific expression in the testis from the initial stages of sex-differentiation. It was related to the cystatin family and had the highest similarity with the *CRES* (cystatin-related epididymal specific) gene (Cornwall *et al.*, 1992). After we named it *cresp* and sent the sequence data to the DNA data bank (DDBJ data base, accession no. AB017157), a paper reporting the same gene, named *testatin,* was published (Töhönen *et al.*, 1998).

Our present study reports both confirming and new results regarding the expression of *testatin/cresp*, including data that appear to contradict with the published report.

Results

Identification of a novel gene by subtractive cloning

To identify novel genes that have sex-specific expression patterns during gonad development, an 11.5-13.5 dpc mouse gonadal cDNA library was screened with male-specific subtracted probes. From such screening, a cDNA containing a 678bp insert was isolated and sequenced in both directions. Figure 1A shows the nucleotide sequence and deduced amino acid sequence of *testatin/cresp*. An open reading frame was identified and it had an upstream in-frame TAG stop codon at nucleotides 19-21, an ATG initiator at 40-42, and a TAG stop codon at 451-453.

Nucleotide and protein databases were searched with these nucleotide and amino acid sequences. Although overall sequence homology at the nucleotide level was not found, minimal homology with members of the cystatin family was recognized in several regions of the amino acid sequence. The highest similarity was found with *CRES*, and we named this novel gene *cresp* (cystatin-related expressed in Sertoli and spermatogonia). Figure 1B shows the alignment of the derived amino acid sequences of *testatin/cresp* when compared with sequences of chicken, human, bovine, rat, and mouse cystatin family genes. There were a number of discrete regions conserved between testatin/cresp and cystatins, such as the cysteine residues in the carboxyl-terminal portion (indicated by arrows in Fig. 1B), which were conserved among the cystatin family. Since all the cystatins are secreted proteins, they all possess a hydrophobic signal peptide at the amino-terminal end, which was also present in testatin/cresp. However, the presumed protein product of *testatin/cresp* lacks a few critical amino acids that are thought to be important for the cysteine-protease inhibitory activity. These sites include G at 9, Q at 53, V at 55 and G at 57 as indicated by arrowheads in Figure 1B (Bode *et al.*, 1988).

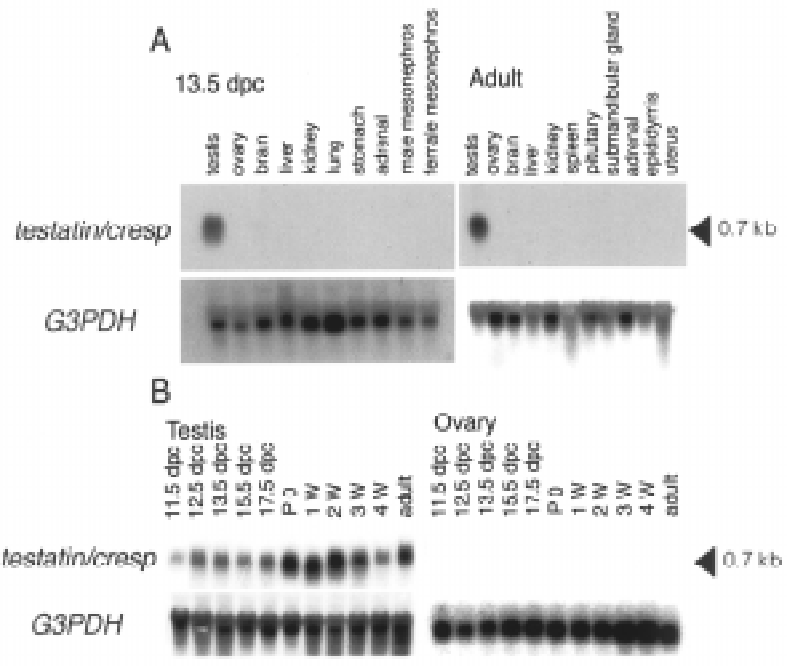

Fig. 2. Northern blotting analysis of *testatin/cresp*. (A) *Tissue-specific expression pattern of testatin/cresp. Total RNA isolated form a variety of male and female tissues of 13.5 dpc fetuses and adults were loaded. A 0.7 kb RNA was detected only in the testis.* **(B)** *Developmental time course of testatin/cresp mRNA expression. Total RNA isolated from the prenatal and postnatal gonads were loaded. The mRNA expression level increased from 11.5 to 12.5 dpc in the male, while no hybridization signal was detected in female samples. The blots were then stripped and rehybridized with the G3PDH probe as a loading control.*

Testatin/cresp mRNA expression

To determine the expression pattern of *testatin/cresp* mRNA among various tissues, Northern blotting was performed using RNAs from a wide variety of fetal and adult tissues. A 0.7 kb transcript was detected specifically in fetal and adult testes (Fig. 2A). To examine expression during gonadal development, RNAs were isolated from fetal and postnatal gonads at various ages (Fig. 2B). Low-level expression of *testatin/cresp* expression was observed in the male urogenital ridge at 11.5 dpc, whereas a higher level of expression was observed in the testis from 12.5 dpc to adult. Expression was not detected in female gonads.

To detect any lower-level expression, we carried out RT-PCR analysis during early embryogenesis (Fig. 3). It revealed that *testatin/cresp* mRNA expression was first detectable at 9.5 dpc in both male and female embryos (Fig. 3A). This expression was maintained in male embryos, while in females, the expression became weaker at 11.5 dpc and was not detectable at 12.5 dpc (Fig. 3A). To determine a more accurate time course for *testatin/cresp* mRNA expression, the developmental stages of embryos at 11.0 to 12.5 dpc was determined by counting the tail somite number. Analysis of these staged samples revealed that the expression level was maintained until the 20-somite stage and decreased to an undetectable level in female gonads by the 24-somite stage, which corresponds to 12.0 dpc (Fig. 3B; Table 1).

Expression analysis using in situ hybridization

Cellular localization of the *testatin/cresp* mRNA was examined further by *in situ* hybridization. Paraffin sections of fetal and postnatal testes were used for the ordinary *in situ* hybridization or double-stained with digoxigenin-labeled antisense *testatin/cresp* RNA and anti-SSEA-1 monoclonal antibody or TRA98 monoclonal antibody.

In adult testes, significant labeling appeared to localize to both Sertoli cells and spermatogonia (Fig. 4A), although Töhönen *et al.* (1998) implied that *testatin* mRNA was expressed only in Sertoli cells. Double-staining with the germ cell-specific TRA98 antibody (Fig. 4C) indicated that not all the spermatogonia were labeled, and that all the labeled spermatogonia were located at the most peripheral region, close to the basal lamina of the seminiferous tubules. We recognized many spermatogonia that showed distinct labeling of both the *testatin/cresp* mRNA and TRA 98 antibody (Fig. 4D). We also detected the *testatin/cresp*-negative spermatogonia (Fig. 4E). Approximately one fourth of the spermatogonia labeled with the TRA98 antibody were double-labeled cells. Negative controls using the sense probe showed no significant labeling (Fig. 4B).

We also labeled fetal gonads at 12.5 dpc. The hybridization signals were observed specifically within the developing testis cord (Fig. 5A). Double labeling with the germ cell-specific anti-SSEA-1

antibody showed that the *testatin/cresp* mRNA was expressed in somatic cells and also in approximately 20-25% of germ cells (Fig. 5A-C). Male gonads at 11.5 dpc also showed similar expression signals on germ and somatic cells (data not shown). There was no significant labeling for the *testatin/cresp* mRNA in female gonads at any stages (data not shown).

Figure 6 shows the expression pattern of *cystatin C*, a member of the cystatin family, in the embryo and adult testes. Its mRNA expression pattern was clearly different from that of the *testatin/cresp* mRNA. *Cystatin C* was expressed not only in the testis cord but also in the mesonephric region at 12.5 dpc (Fig. 6A). It was expressed in most cells inside the seminiferous tubules, including the Sertoli cells and spermatocytes, in adult testis (Fig. 6B). Interestingly, the labeling was stronger on the inner layer containing spermatocytes and much weaker on the peripheral spermatogonia, exhibiting an almost complementary pattern to the *testatin/cresp*.

Expression analysis using single-cell RT-PCR

Single-cell RT-PCR analysis was carried out to confirm the double-labeling results indicating *testatin/cresp* mRNA expression in both the somatic and germ cells in male gonads. We analyzed mRNA obtained from individual germ or somatic cells that had been picked-up from dissociated 12.5 dpc male gonads after vital staining with the anti-SSEA-1 and anti-alkaline phosphatase antibodies (Fig. 7). We detected the *testatin/cresp* amplification product in some of the germ cells that also expressed the germ cell-specific *Oct3/4* mRNA. We performed such RT-PCR experiment three times using 10~20 embryos from two batches and obtained similar results. In total, 3 out of 16 germ cells gave a detectable signal representing *testatin/cresp* mRNA expression, while 6 out of 16 somatic cells were positive for *testatin/cresp*. Such ratio of the *testatin/cresp* positive germ cells was comparable to that in the *in situ* hybridization. The observed expression pattern of WT1 in somatic cells was caused by the fact that these somatic cells included both the Sertoli and interstitial

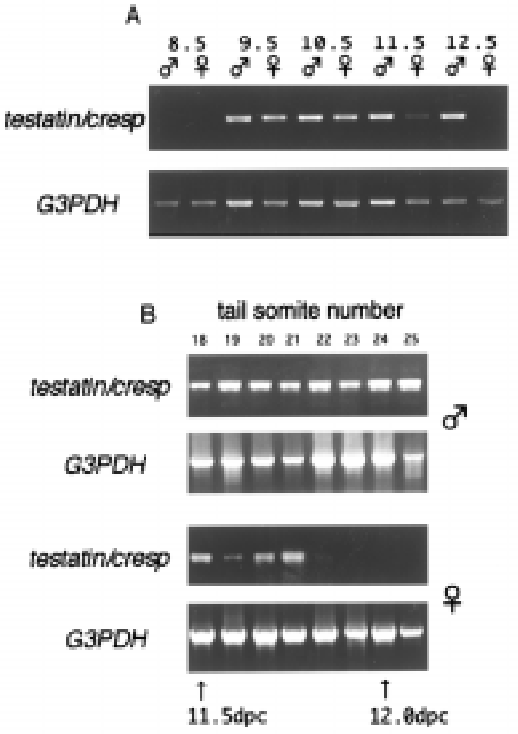

Fig. 3. RT-PCR analysis of the *testatin/cresp* expression. (A) *During embryogenesis, testatin/cresp mRNA expression was first detectable at 9.5 dpc in both male and female embryos. Such expression was maintained in male embryos, while in females, the expression became weaker at 11.5 dpc and was not detectable at 12.5 dpc.* **(B)** *A more accurate staging of the development of embryos from 11.5 to 12.5 dpc was determined by counting the tail somites. Expression in the female gonad decreased to an undetectable level in the middle of these stages. An embryo with 18 tail somites corresponds to the 11.5 dpc, and 24 somites to 12.0 dpc.*

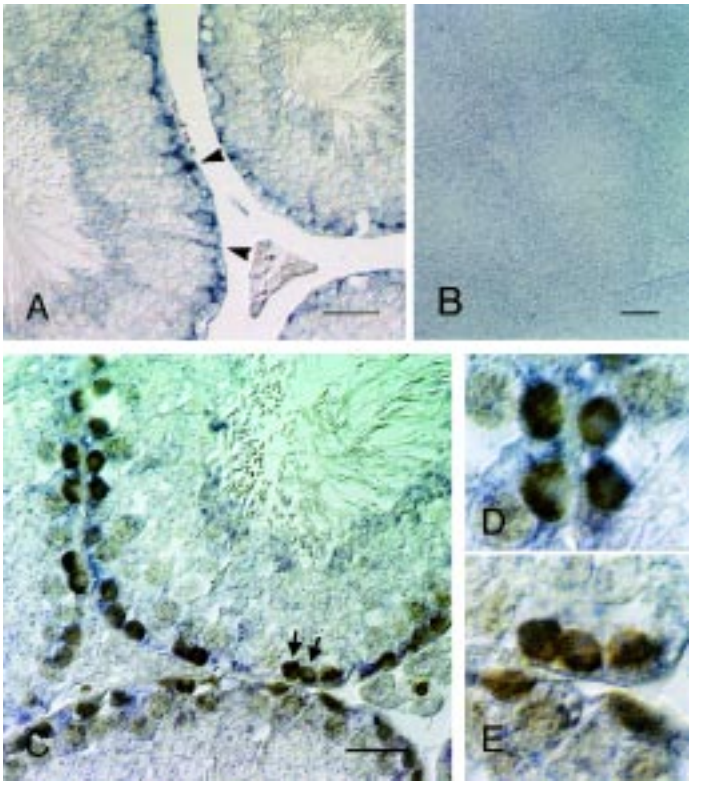

Fig. 4. *In situ* hybridization analysis of the adult testis. (A) *In situ hybridization revealed labeling in the round cells in the extreme periphery of the seminiferous tubules and radially elongated Sertoli cells (arrowheads).* **(B)** *Control labeling with the sense probe.* **(C)** *Double-staining with digoxigenin-labeled anti-sense testatin/cresp RNA and TRA98 monoclonal antibody. Arrows indicate spermatogonia labeled with TRA98 but not with the hybridization signals.* **(D,E)** *Magnified views of the double-labeled spermatogonia in the upper-left part of (C) and spermatogonia indicated by arrows in (C). Bar, 50 μm.*

TABLE 1

RT-PCR ANALYSIS OF *TESTATIN/CRESP* EXPRESSION IN FEMALE GONADS DURING THE EARLY SEX-DIFFERENTIATION PERIOD

number of tail somites	17	18	19	20	21	22	23	24	25
testatin/cresp positive/total sample	3/3	4/4	4/4	4/4	5/6	3/5	1/5	0/5	0/5

Number of *testatin/cresp* positive individuals per total samples are indicated. Embryos with 18 somites and 24 somites correspond to 11.5 dpc and 12.0 dpc, respectively.

cells. Previous studies (Mundlos *et al.,* 1993) reported that *WT1* was expressed in the Sertoli and peripheral cells but not in the germ cells of the embryonic testis. Also, there was no evidence that all the Sertoli cells expressed the *WT1* gene.

Discussion

The *testatin/cresp* gene described in this report showed homology with type II cysteine proteinase inhibitors (cystatins). Four highly conserved cysteine residues showed exact alignment with other member of the cystatin family. These four cysteines govern the overall conformation of the cystatins through formation of two disulfide bonds (Grubb *et al*., 1984). In addition, testatin/cresp has a putative signal peptide and a predicted cleavage site at the same relative position as that of the cystatins. These similarities suggest that the testatin/cresp protein has a conformational resemblance with the cystatins and that it is a secreted protein. Unlike the cystatins, however, testatin/cresp does not contain highly conserved amino acid residues that are presumed to be necessary for the cysteine-protease inhibitory activity. This fact suggests a relatively remote relationship with the known cystatins, and it may represent a member of a new family of cystatins. The testatin/cresp protein therefore, may either inhibit still unknown cysteine proteases or bind to proteins that are not cysteine proteinases.

Cysteine proteases, members of the papain superfamily, comprise a group of proteolytic enzymes that cleave peptide bonds by the use of a catalytic cysteine residue and they are inhibited by the cystatins (Barrett *et al*., 1986). Such cysteine proteases are probably involved in the protein catabolism (Kominami *et al*., 1986) and the proteolytic processing of prohormones and proenzymes (Taugner *et al*., 1985; Marks *et al*., 1986). These proteases are present in most living organisms and are thought to have medical importance (Barrett *et al*., 1986). On the other hand, the cystatins are present in most tissues as well as biological fluids such as saliva, tears, urine, plasma, cerebrospinal fluid and seminal plasma (Abrahamson *et al*., 1986; Tavera *et al*., 1990). In the testis, the cysteine protease cathepsin L (Elicson-Lawrence *et al*., 1991) and its inhibitor cystatin C (Tavera *et al*., 1990) are secreted from Sertoli cells. It has been suggested that cathepsin L and cystatin C have interactive roles in the adherence of germ cells to Sertoli cells and subsequent formation of the intercellular junctions (Murk *et al*., 1997).

In addition to its unusual structure among the cystatins, the expression pattern of *testatin/cresp* was different from any other members of the cystatin family. Northern blotting and RT-PCR analyses demonstrated that expression of the *testatin/cresp* mRNA was almost confined exclusively to the male gonad and expression increased immediately after the initiation of testis differentiation at 11.5-12.5 dpc, while it decreased to an undetectable level in the female gonad during the same period. Our detailed *in situ* hybridization and immunohistochemical analyses demonstrated that the *testatin/cresp* mRNA was localized both in the germ and Sertoli cells in the fetal and adult testes. Such highly restricted expression is in marked contrast to the wider expression of cystatin C and other members of the cystatin family in the testis and other tissues as observed in the present and previous studies (Cole *et al*., 1989).

Töhönen *et al.* (1998) reported expression of *testatin/cresp* in Sertoli cells and its precursors from 11.5 dpc, and they suggested that this gene is located downstream of *Sry* or *Sox9* during early events of the sex-differentiation in testis somatic cells. Our present results indicated expression of the *testatin/cresp* in both the germ and somatic cells in the male gonad from 11.5 dpc, and also in embryos of both sexes at earlier developmental stages. At 9.5 dpc, the urogenital ridge is being formed from the intermediate mesoderm, while the PGCs are migrating through the hindgut region.

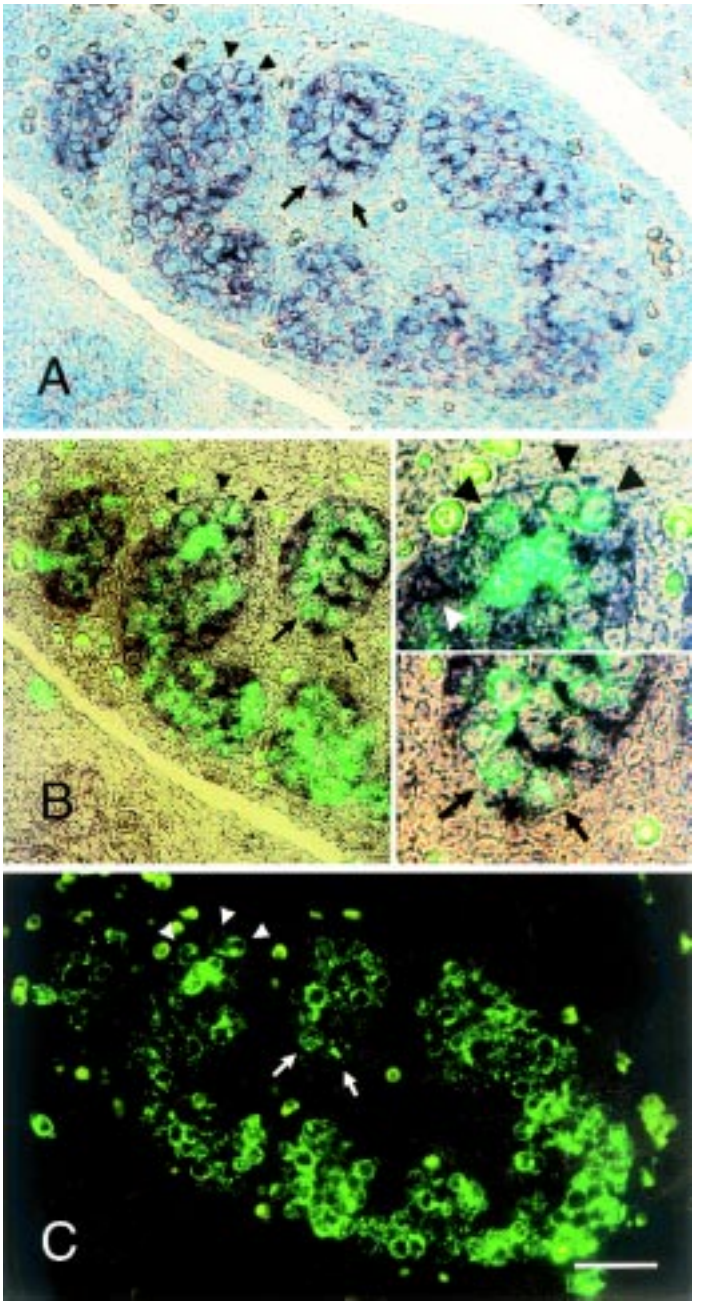

Fig. 5. *In situ* hybridization analysis of the fetal testis. *A section of a male gonad at 12.5 dpc, double-stained with digoxigenin-labeled antisense testatin/cresp RNA and anti-SSEA-1 monoclonal antibody and FITC-labeled second antibody, counter stained with methylgreen. Photographs of* **(A)** *bright-field microscopy,* **(C)** *fluorescence microscopy and* **(B)** *composite images are shown. Arrows indicate germ cells labeled with the anti-SSEA-1 antibody but not with the hybridization signals. Black arrowheads indicate the double-labeled germ cells. In the insets showing magnified views, arrows, black arrowheads and white arrowheads indicate the germ cells labeled only with SSEA-1, the double-labeled germ cells, and somatic cells labeled only with the hybridization signals, respectively. Bar, 50 μm.*

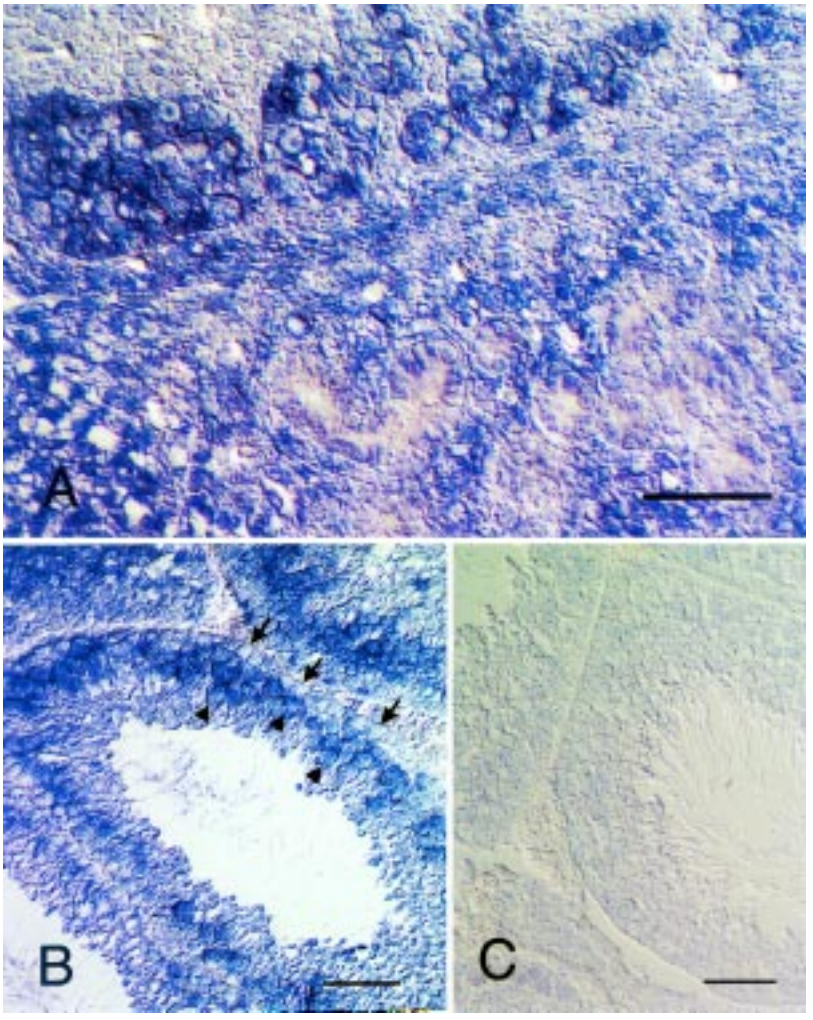

Fig. 6. *In situ* hybridization of the cystatin C mRNA. (A) *In the male embryos at 12.5 dpc, cystatin C mRNA signals were present within the testis cord in the gonad (upper-left part) and the attached mesonephros.* **(B)** *Labeling of the adult testis revealed hybridization signals on most cells inside the seminiferous tubules including the spermatocyte layer (arrowheads) but excluding the spermatogonia (arrows) located at the extreme periphery.* **(C)** *A negative control hybridized with the sense probe. Scale bars indicate 50 μm.*

Although *testatin/cresp* expression was first detected by RT-PCR at 9.5 dpc, it was not clear which cells expressed this gene. In spite of many trials, we could not detect the expression by *in situ* hybridization at these earlier stages, perhaps because of a lower expression level.

Expression of the *testatin/cresp* mRNA was not detected in all the germ cells at 12.5 dpc when analyzed by the single cell RT-PCR method. Also, not all the spermatogonia were labeled by *in situ* hybridization. It is not clear whether such results represent variation in the expression level among cells or presence of heterogeneous sub-populations. More detailed analyses are necessary to clarify these matters.

Expression of *Sox-9* starts at around 10.0 dpc in both the male and female, and it is upregulated in the testis and downregulated in the ovary at 11.5 dpc (Morais da Silva *et al.,* 1996), thus taking a very similar course to *testatin/cresp* expression. Also, expression of *Ad4BP/SF-1*, a transcription regulator important for steroidgenic cell development (Ingraham *et al*., 1994), starts at 9.5 dpc in somatic cells of the gonadal rudiment (Ikeda *et al*., 1994; Hatano *et al*., 1996), and it is sex-dependent during gonad development (Hatano *et al*., 1994). Thus, these two genes may be involved in the transcriptional regulation of *testatin/cresp*.

It has been known that PGCs arriving at the urogenital ridge undergo a major change in cellular phenotype (Donovan *et al*., 1986; Ffrench-constant *et al*., 1991). More recently, it was shown that expression of the mouse *vasa* homolog starts after they arrive at the genital ridges (Fujiwara *et al*., 1994). Germ cells at these stages take different developmental pathways depending on the sex of the gonadal somatic cells, more precisely, Sertoli precursor cells (McLaren, 1995). PGCs in the female gonad enter into meiosis, while those in the male gonad go into mitotic arrest. Thus, interactions of the PGCs and gonadal somatic cells are very important for sex-differentiation of germ cells. The upregulation of *testatin/cresp* expression in the male gonad and its downregulation in the female takes place immediately before such sex-differentiation of the germ cells, suggesting an important function at these stages. Also, since *testatin/cresp* is expressed in both the germ and somatic cells at the same critical period of early testis differentiation, it may be involved in the interaction between the germ and somatic cells. For example, it is possible that the testatin/cresp protein may be involved in the processing of signaling molecules in collaboration with a cysteine protease.

Materials and Methods

Animals and embryos

ICR strain mice were purchased from Japan SLC, Inc. Embryos were obtained from mating of the mice maintained in a room with light/dark periods between 8 am and 8 pm. Noon of the day of appearance of the vaginal plug was designated as 0.5 dpc. For more accurate staging of embryos from 11.5 to 12.5 dpc, the number of tail somites posterior to the hind limb bud was counted as described by Hacker *et al.* (1995). The genetic sex of phenotypically sex-indifferent embryos was determined by genomic PCR of the *Ube-1* gene on the X and Y chromosomes (Chuma and Nakatsuji, submitted for publication). The protocol was designed so that samples from male embryos gave two bands from the X and Y chromosomes and those of females gave a single band from the X chromosome. We isolated genital ridges or gonads at 11.5 dpc or later stages, urogenital ridges at 10.5 dpc, posterior trunk regions between the forelimb bud and tail at 9.5 dpc, or the posterior embryo proper at 8.5 dpc, respectively.

Library screening

Male-specific probes were obtained by subtracting cDNAs of 13.5 dpc male gonads with those of female gonads by using the method as described before (Kaneko-Ishino *et al.,* 1995) with minor modifications. A λZAPII cDNA library of genital ridges and gonads at 11.5-13.5 dpc was differentially screened by using the male-specific and 13.5 dpc female gonadal cDNA probes. Plasmids were rescued using manufacture's protocol (Stratagene). cDNA clones were first examined by dot blot hybridization to remove any false-positives. The subtracted male-specific, testis and ovary cDNAs were spotted and hybridized with inserts of the clones.

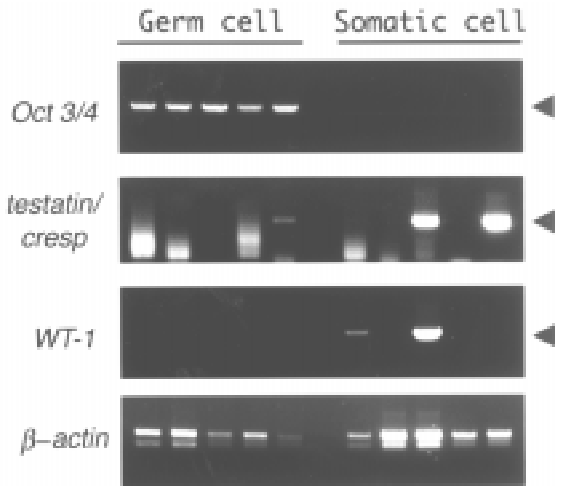

Fig. 7. Single cell RT-PCR analysis of the *testatin/cresp*. *We analyzed mRNAs obtained from individual germ or somatic cells that had been picked-up from dissociated 12.5 dpc male gonads. Besides testatin/cresp, Oct 3/4 and WT1 were also detected as the markers for germ and a subpopulation of somatic cells respectively. Arrowheads indicate the size of specific amplification products.*

DNA sequencing

The nucleotide sequences of clones were determined by using the dye-terminator cycle sequencing kit (Perkin-Elmer), and nucleotide data was analyzed with the Blast program (Altschul *et al.*, 1990).

Northern blotting analysis

Total RNAs were extracted from mouse tissues by ISOGEN following the manufacturers protocol (Nippon Gene). Ten μg of total RNA was fractionated on 1.3% formaldehyde-agarose gels, and transferred to Hybond-N^+ (Amersham). After prehybridization, membranes were hybridized at 65° in Church buffer with about 10^5 cpm ^{32}P-labeled probes. After hybridization, membranes were washed in 2x SSC – 0.1% SDS two times for 10 min at RT, 0.5x SSC – 0.1% SDS for 15 min at 65°, and 0.1xSSC – 0.1% SDS for 15 min at 65°. ^{32}P-labeled probes were prepared using Strip-EZ random priming system (Ambion). The full length *testatin/cresp* insert and *G3PDH* PCR product were used as templates for probes.

RT-PCR

cDNAs were synthesized from total RNAs with oligo $(dT)_{12\text{-}16}$ and Superscript II RNase H^- reverse transcriptase following the manufacturers protocol (Gibco BRL). One μg of total RNAs was used for the reverse transcription reaction in a 20 μl of reverse transcription mixture. One μl of the cDNAs were used as templates for 35 cycles of PCR (94°, 60 sec → (95°, 60 sec; 60°, 60 sec; 72°, 60 sec) → 72°, 60 sec) for *testatin/cresp*, and 25 cycles of (94°, 60 sec → (95°, 60 sec; 60°, 60 sec; 72°, 90 sec) → 72°, 60 sec) for *G3PDH*. The following primers were used for PCR:

testatin/cresp-F, 5'-CACAGTGGAATTTGCCGTGA-3';
testatin/cresp-R, 5'-AAGGTAACCCCTTCACGGGAT-3';
G3PDH-F, 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3';
G3PDH-R, 5'-CATGTAGGCCATGAGGTCCACCAC-3'.

In situ hybridization and immunohistochemistry

Mouse embryos were fixed with 10% formaldehyde in PBS. Five μm paraffin sections were prepared, and mounted on silane-coated glass slides (Matsunami). *In situ* hybridization was performed as described by Saito *et al.* (1996). Then, immunohistochemical staining was carried out by using an anti-SSEA-1 monoclonal antibody (American Research Products) or a TRA98 monoclonal antibody (gift from Dr. Y. Nishimune, Osaka Univ.). The TRA98 antibody labels spermatogonia and primary spermatocytes, although staining of the latter appear less intense than the former, but not any somatic cells in the testis (Nishimune *et al.*, personal communication). FITC-labeled secondary antibody was used for the SSEA-1 staining. Biotinylated secondary antibody and Vectastain ABC Elite kit (Vector laboratories) were used for the TRA98 staining. If necessary, the specimens were counter-stained with methylgreen.

Single cell RT-PCR

Male gonads at 12.5 dpc were dissociated by digestion with a 0.05% trypsin and filtration. Dissociated cells were incubated at 4°C for 30 min in the culture medium containing anti-SSEA-1 and anti-calf intestinal alkaline phosphatase antibodies (Sigma). Then, such cells were washed with PBS- two times, and suspended in the medium with fluorescein-conjugated 2nd antibody. Cell suspensions were observed with a fluorescence microscope. The double-labeled germ cells, or the double negative and adherent somatic cells, were picked-up by using a glass micropipette and a micromanipulator (Eppendorf). mRNAs from each cell were separately purified using the Dynabeads mRNA DIRECT kit (Dynal), followed by solid phase-reverse transcription using Superscript II RNase H^- reverse transcriptase. Since the cDNAs were attached to particles, the same templates were used for several times of PCR amplification serially. Expression of *Testatin/cresp*, *β-actin* (Alonso *et al*., 1986), *Oct* 3/4 and *WT1* was analyzed using the following PCR primers.

Oct 3/4–F, 5'-ATTCTCGAACCTGGCTAAGCT-3';
Oct 3/4–R, 5'-ATGGTGGTCTGGCTGAACACCTTT-3';
WT1–F, 5'GTGCGGCGTGTATCTGGAGTG-3';
WT1–R, 5'-TGAAAGGTGAGTGGGAGGAAT-3'.

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