*Proc. Natl. Acad. Sci. USA*
Vol. 95, pp. 14208–14213, November 1998
Developmental Biology

# *Testatin*: A cystatin-related gene expressed during early testis development

**(protease inhibitors/mRNA differential display/sex determination/mouse)**

VIRPI TÖHÖNEN, CHRISTINA ÖSTERLUND, AND KATARINA NORDQVIST†

Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, S-171 77 Stockholm, Sweden

*Edited by Mario R. Capecchi, University of Utah, Salt Lake City, UT, and approved September 18, 1998 (received for review August 17, 1998)*

**ABSTRACT** **To isolate genes involved in morphogenic aspects of testis development, and which may act in cell signaling pathways downstream of the testis-determining gene *Sry*, we have developed a modified mRNA differential display method named signal peptide differential display. It was used to target those genes that encode proteins having a signal peptide sequence. By using this method, we isolated a gene named *testatin*. This gene was found to be related to a group of genes that encodes cysteine protease inhibitors known as cystatins. Cystatins and their target proteases have been associated with tumor formation and metastasis, but also are involved in natural tissue remodeling events such as bone resorption and embryo implantation. We show that *testatin* expression is restricted to fetal gonads and adult testis. Furthermore, *testatin* is expressed during testis cord formation in pre-Sertoli cells, believed to be the site of *Sry* action, at a time immediately after the peak of *Sry* expression. This finding suggests that *testatin* might be activated by transcription factors that are known to orchestrate the early testis development pathway. This gene therefore represents one of the putative downstream targets likely to have an essential role in tissue reorganization during early testis development.**

The mammalian indifferent gonad serves as an interesting and unique model system for organogenesis because it has the ability to develop into two different organs, the ovary and the testis (1). The initial choice of developmental pathway depends on the absence or presence of one gene, *Sry* (sex determining region, y gene), located on the Y chromosome (2, 3). *Sry* acts as a gonad developmental switch by encoding the testis determining factor (4). Onset of *Sry* expression induces the indifferent gonad to begin differentiating as a testis, involving processes such as Sertoli and Leydig cell differentiation, cell migration and proliferation, testis cord formation, and vascularization (reviewed in ref. 5). So far, most genes known to be important for gonad development encode potential transcription factors or cofactors. These include *Sry* and the *Sry*-related gene *Sox9*, the Wilms' tumor 1 gene (*WT1*), and the genes for the nuclear receptors Dax1 and SF-1 (reviewed in ref. 5). Two signaling molecules, anti-Müllerian hormone (AMH or MIS) and testosterone/dihydrotestosterone, also have been shown to be important for male differentiation during fetal development (6, 7). Additional signal molecules that are important for morphogenic events during testis development also must exist. These could include cell–cell signaling molecules (Sertoli-Leydig cell cross-talk), proteins involved in cell migration (movements of mesonephric cells into testis), and proteins important for extracellular reorganization (formation of testis cords) or vascularization. We therefore set out to isolate genes active downstream of *Sry*, i.e., genes involved in morphogenic aspects of testis development. To do this a modified version of mRNA differential display (DD) was developed and named signal peptide differential display (SPDD).

By using SPDD, we have isolated a gene, *testatin*, from mouse fetal testis. *Testatin* was shown to be related to cystatins, a family of small secreted protease inhibitors. *Testatin* expression is restricted to pre-Sertoli cells, and onset of expression coincides with early testis development, immediately after the peak of *Sry* expression.

## MATERIALS AND METHODS

**SPDD.** Total RNA from male and female gonads with attached mesonephros was isolated from 13.5 days postcoitum (dpc) mouse embryos and treated with DNase to remove contaminating DNA as described (8). For each mRNA population, reverse transcription (RT) using different decamer primers (5*13, 5′-TAC AGC TCT C-3′; 5*14, 5′-CAG TGA AAG C-3′; 5*15, 5′-CTC TAA CAG G-3′; and 5*16, 5′-CAA GTT CTC T-3′) was done in the following way: reactions were mixed on ice, each reaction containing 0.45 μg total RNA and 1 μl of 3′ primer (2 μM) in a total volume of 14 μl. The RNA was denatured at 70°C for 8 min and transferred back to ice. The following components then were added to each reaction: 5 μl of 5× RT buffer (200 mM Tris, pH 7.5/150 mM KCl/15 mM $MgCl_2$), 2.5 μl of DTT (0.1 M), 2.5 μl of dNTPs (250 μM), 0.3 μl of rRNasin (40 units/μl, Promega), and 0.5 μl of SuperScript II RT (200 units/μl; GIBCO/BRL). Reactions were incubated at 43°C for 70 min and then stored at −20°C. For the PCR step, the following components were mixed on ice in a PCR plate with 96 wells (Techne Laboratories, Princeton): 1.5 μl of RT reaction, 2.5 μl of 10× PCR buffer (500 mM KCl/100 mM Tris, pH 9.0/1% Triton X-100), 2.5 μl of $MgCl_2$ (20 mM), 0.5 μl of [α-$^{33}$P] dATP (10 mCi/ml, DuPont/NEN), 1 μl of signal peptide primer (5′-CTG CTG CTG CTG CTG-3′, 0.4 μM), 1 μl of decamer primer (2 μM), 2.5 μl of dNTP mix (20 μM dCTP, 20 μM dGTP, 20 μM dTTP, 14 μM dATP), and 0.5 μl of *Taq* DNA polymerase (5 units/μl, Promega). The total volume was 25 μl. Reactions were kept on ice until the PCR machine had reached 85°C, and then they were applied to the PCR block, denatured at 92°C for 1 min, and amplified for 28 cycles at 92°C for 50 sec, 40°C for 90 sec, and 72°C for 90 sec. After the amplification, reactions were immediately transferred to −20°C. Gel electrophoresis, detection of cDNA bands, reamplification, and cloning were done as described (8).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: DD, differential display; SPDD, signal peptide differential display; AMH, anti-Müllerian hormone; dpc, days postcoitum; RT, reverse transcription; *CRES*, cystatin-related epididymal specific gene.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. Y18243).
†To whom reprint requests should be addressed. e-mail: Katarina.Nordqvist@cmb.ki.se.

**Screening of Fetal Testis cDNA Library.** The M12 cDNA was used as a probe to screen a fetal testis cDNA library (constructed by the ZAP Express cDNA synthesis kit (Stratagene). The screening procedure was performed according to the manual for the kit. Recombinants ($5 \times 10^5$) were screened, and about 25 positives were found. From these, three different cDNA clones (2A1, 4C2, and 10B3) were isolated.

**DNA Sequencing.** DNA sequencing was carried out by using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer, no. 402079), and the samples then were processed on a 373 A automated DNA sequencer (Applied Biosystems).

**Semiquantitative RT-PCR.** Semiquantitative RT-PCR was performed essentially as described (8). The following primers were used, shown in 5′ to 3′ direction: Sry-5*, GGT TGC AAT CAT AAT TCT TCC; Sry-3*, CAC TCC TCT GTG ACA CTT TAG; Testatin-5*, ATG TTC TCA TCA CTC CTG TC; Testatin-3*, TTC AGA CCA TGG CTC TCC TG; Hprt-5*, CCT GCT GGA TTA CAT TAA AGC ACT G; Hprt-3*, GTC AAG GGC ATA TCC AAC AAC AAA C; Amh-5*, AGG CCC TGT TAG TGC TAT A; Amh-3*, CCG CGT GAA ACA GCG GGA ATC; CRES-5*, CAG TGT GTT TGG TTT GCC; CRES-3*, CAG GTT GAA CTC GCC ATT; CystatinC-5*, TGG AGA TGG GCC GAA CTA C; and CystatinC-3*, CAT GAT CCT TCT AGA CTC AG.

Total RNA (0.5 $\mu$g) was reversed-transcribed as described (8).

One microliter of the RT reactions was mixed on ice in a PCR plate with 96 wells (Techne) together with 2.5 $\mu$l of 10× PCR buffer (500 mM KCl/100 mM Tris, pH 9.0/1% Triton X-100), 2.5 $\mu$l of 20 mM $MgCl_2$, 0.3 $\mu$l of [$\alpha$-$^{32}$P] dATP (3,000 Ci/mmol, DuPont/NEN), 1 $\mu$l of 5′ primer (100 ng/$\mu$l), 1 $\mu$l of 3′ primer (100 ng/$\mu$l), 2.5 $\mu$l of dNTPs (250 $\mu$M), and 0.3 $\mu$l of AmpliTaq DNA polymerase (5 units/$\mu$l, Perkin–Elmer). The total volume was 25 $\mu$l. Reactions were kept on ice until the PCR machine had reached 85°C and then applied onto the PCR block, denatured at 92°C for 2 min, and amplified for 24 cycles at 92°C for 60 sec, 55°C for 60 sec, and 72°C for 60 sec. After the amplification, reactions were transferred immediately to −20°C. PCR samples then were separated by native PAGE as described (8).

***In Situ* Hybridization.** *3βHSD riboprobe.* Digoxigenin-labeled antisense and sense RNA probes were prepared from linearized plasmids as described (8).

*Testatin riboprobe.* Expression of *testatin* was detected by using a modified labeling method. A 280-bp *testatin* fragment was generated by PCR amplification of the total *testatin* cDNA plasmid. This amplification was carried out by using the same protocol as that used for RT-PCR, using 30 cycles. The *testatin* 3′ primer carries a T7 promoter sequence (in bold) (5′-**TGA TTA ATA CGA CTC ACT ATA GGG** TTC AGA CCA TGG CTC TCC TG-3′), which, after template amplificaton, is used to label the antisense riboprobe with T7 RNA polymerase. The PCR fragment was run through a G-50 sephadex column and extracted with phenol, prior labeling described by Wilkinson (32). The sense probe was generated by the same principle, the T7 promoter sequence (in bold) now being attached to the 5′ primer (5′-**TGA TTA ATA CGA CTC ACT ATA GGG** ATG TTC TCA TCA CTC CTG TC-3′).

*In situ* hybridization was performed as described (9) with the following modifications: frozen sections were cut at 10 $\mu$m thickness, mounted on SuperFrost*/Plus (Menzel-Gläser) slides, and stored at −20°C. Before hybridization the slides were treated for 2 min at 50°C, dried for 30 min at room temperature, and treated with chloroform for 5 min, 2% paraformaldehyde/PBS for 7 min, and PBS for 3 min. The sections were circled with a silicone pen (Pap Pen, Daido Sangyo), and then the slides were treated twice in 2× standard saline citrate (SSC) for 5 min.

A 60-min prehybridization (4× SSC, 10% Denhart's solution, 2 mM EDTA, 50% formamide) followed in a humid chamber at 50°C, with 50 $\mu$l of solution per section. The hybridization buffer was replaced before hybridization by the labeled probe and denatured at 80°C for 5 min at an optimal concentration of 200 ng/ml in the hybridization buffer. Hybridization was carried out for 18 hr under the same conditions.

Posthybridization washes were performed at 50°C for 10 min in 2× SSC, followed by 10 min at 37°C in 2× SSC, 2 × 10 min at 37°C in 1× SSC, and 2 × 10 min at 37°C in 0.1× SSC.

The immunological detection was performed at room temperature starting with a 5-min TN wash (100 mM Tris, pH 7.5/150 mM NaCl), 45-min blocking in TN saturated with 2% milk powder and 2% fetal calf serum, and 60-min incubation at room temperature in TN blocking mix + antidigoxigenin (DIG) Fab fragment, diluted 1:500 (Boehringer Mannheim), followed by 2 × 5 min in TN buffer and 1 × 10 min in TNM (100 mM Tris, pH 9.5/100 mM NaCl/50 mM $MgCl_2$). Development of the alkaline-phosphatase-conjugated anti-DIG antibody with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate was carried out for 1–16 hr at room temperature.

The reactions were stopped with PBS before mounting the slides in a 78% glycerol mounting medium, containing 1 mg/ml of para-phenylene diamine, and finally examined in a light microscope.

**Antibody Production.** Rabbit antisera to *testatin* were produced against the entire protein of *testatin*, (without the signal peptide), fused to dihydrofolate reductase and 6-histidine tag in a bacterial expression vector pQE40 (Qiagen) as described (10).

The fusion protein was expressed in *Escherichia coli* and isolated for antibody production in rabbits at the National Veterinary Institute (SVA) in Uppsala, Sweden.

**Immunochemistry.** Embryonic 13.5 dpc gonads were fixed in 1% paraformaldehyde in PBS for 1 hr on ice, then equilibrated in 0.5 M sucrose for 1 hr on ice and embedded in OCT compound (Miles) before freezing and cryosectioning to 10-$\mu$m sections.

Immunofluorescence was performed as described (10). The primary polyclonal *testatin* antibody was diluted 1:500.

## RESULTS

**SPDD.** Since the first description of DD by Liang and Pardee in 1992 (11), this method has been used extensively to compare gene expression in different tissues or differently treated cell lines and to isolate differently expressed cDNAs (12). We previously adapted the DD method to improve its usefulness in situations when the amount of tissue is limited and used it to isolate genes that are expressed during gonad development in mouse (8). We now have further improved the DD method so it can be used for isolating genes that encode proteins with a signal peptide sequence.

The signal peptides normally consist of a positively charged N-terminal region, a highly hydrophobic central stretch, and a neutral, but polar, C-terminal region (13). The signal peptide sequence is not very conserved, but the hydrophobic domain, which is the primary determinant of the signal peptide function, often contains stretches of leucine residues (G. von Heijne, personal communication). The most frequently used codon for leucine in humans, CTG, was used to construct a primer that corresponded to a poly-leucine stretch. This primer was used as the "signal peptide" primer. In the first step of the SPDD, cDNAs were produced from total RNAs using several different decamers instead of the four different oligo(dT) primers used in classic DD. For the PCR step, these decamers were used in combination with the signal peptide primer (Fig. 1*A*). Total RNA from 13.5 dpc female and male mouse

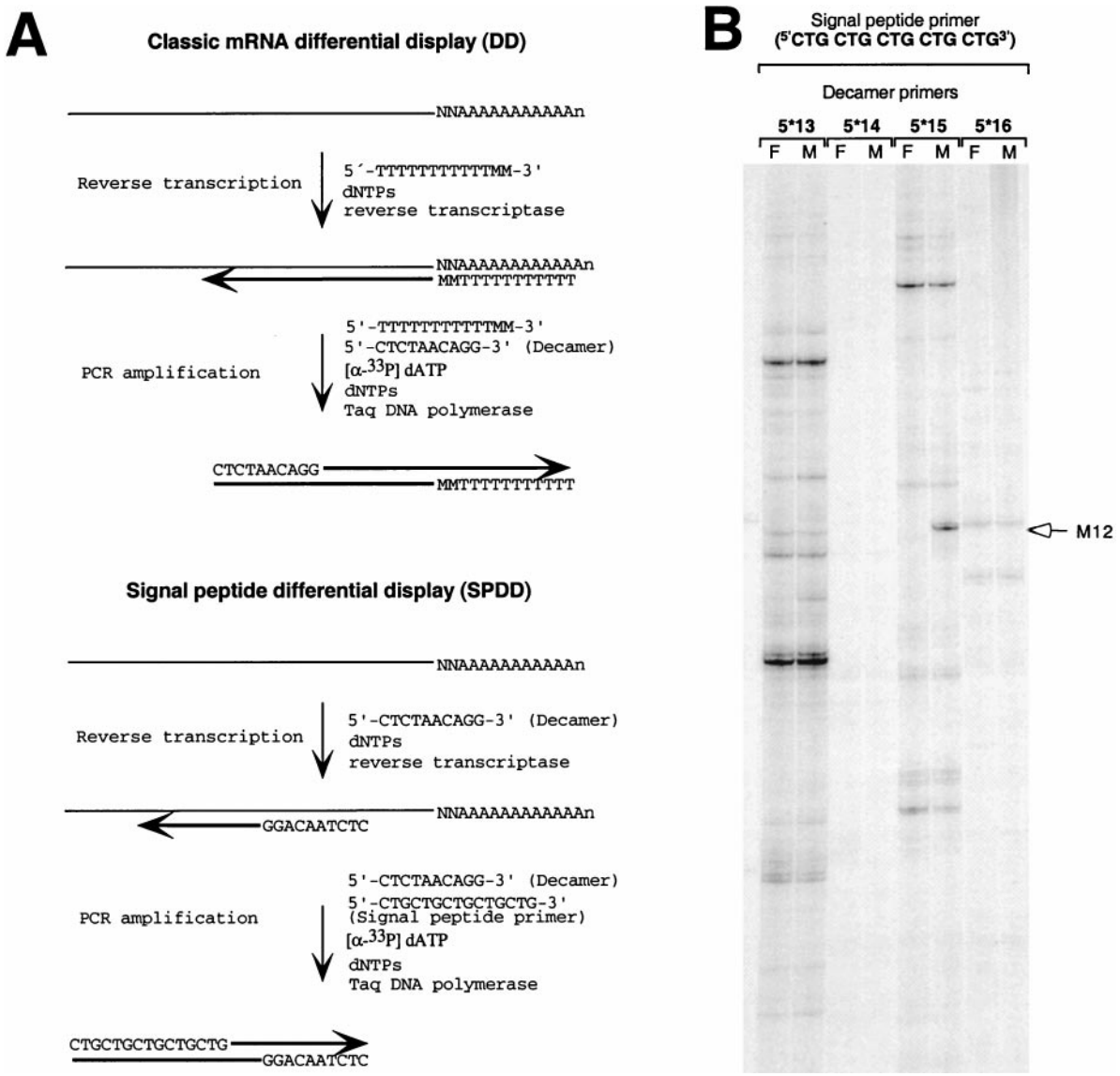


FIG. 1. (*A*) Schematic diagram comparing the SPDD and the classic DD. (*B*) An SPDD screen using total RNA from 13.5 dpc male and female fetal mouse gonads+mesonephros identified a male-specific fragment (M12). RTs were performed by using four different decamer primers 5*13, 5*14, 5*15, and 5*16. By PCR, cDNAs were amplified by using the different decamers in combination with the signal peptide primer in the presence of [$\alpha$-$^{33}$P] dATP for 28 cycles. cDNA bands were resolved on a denaturing polyacrylamide gel and exposed to x-ray film. M12 was isolated, cloned, and sequenced.

gonad+mesonephros was isolated, and by using SPDD, a testis-specific cDNA, M12, was isolated, reamplified, cloned, and sequenced (Fig. 1*B*). No homology was found between the M12 cDNA sequence and other known sequences.

**Isolation and Characterization of the *Testatin* cDNA.** To isolate a full-length cDNA clone, the M12 cDNA was used as a probe to screen a mouse fetal testis cDNA library. Three overlapping clones were isolated (2A1, 4C2, and 10B3), which contained sequences of lengths 670, 665, and 600 bp, respectively (Fig. 2*A*). It was confirmed that these clones represented full-length RNA by Northern blot analysis (data not shown). One ORF was identified that potentially encoded a protein of 137 aa starting from the first methionine codon. Multiple stop codons were present before and after the coding sequence (Fig. 2*A*, marked with ∗), and a putative polyadenylation signal was located 16 bases upstream of the poly(A) stretch. The N-terminal part of the ORF had the properties of a signal peptide with a hydrophobic domain containing multiple leucine residues, and computer predictions indicated a signal peptide cleavage site between Ala (−1) and Asn (+1) (Fig. 2 *A* and *B*; ref. 14). A repetitive CTG sequence was located 42 bp downstream of the first AUG and within the signal peptide sequence of the cDNA. This $(CTG)_n$ sequence corresponded to the binding site for the signal peptide primer and confirmed that this primer was specific when used in the SPDD screening.

The full-length amino acid sequence derived from the ORF displayed similarity to family 2 cystatins, which function as cysteine protease inhibitors (Fig. 2*C*). The family 2 cystatins (cystatin C, D, E, S, SA, and SN) are small secreted proteins of approximately 120-aa residues and have several features in common: a signal peptide, four characteristic cysteines located in the C-terminal domain, and three short conserved amino acid regions that coincide with those forming the inhibitory site of the protein (15). The conserved residues in these three regions include an N-terminal glycine (located as Gly-11 in mouse cystatin C), a Gln-X-Val-X-Gly region (Gln-55–Gly-59) located in a first hairpin loop, and two conserved amino acids (Pro-105–Trp-106) in the second hairpin loop (16, 17). We find that the mouse M12 C-terminus amino acid sequence is very similar to the family 2 cystatins, including the four cysteines in

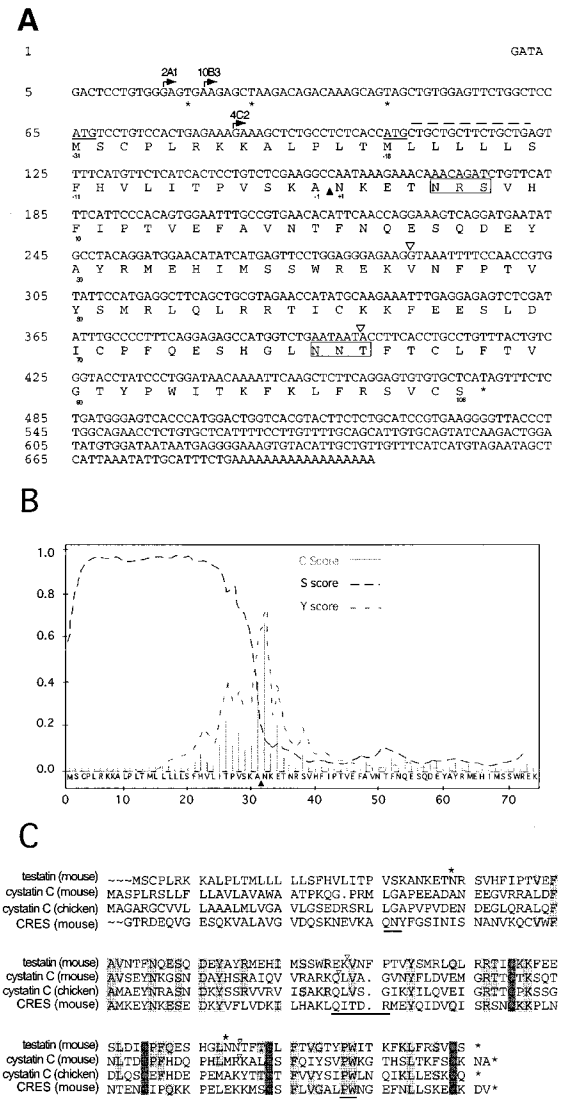


FIG. 2. (*A*) Nucleotide and predicted amino acid sequence of the *testatin* gene. The start sites for the three *testatin* cDNA clones—2A1 (nucleotide 17), 10B3 (nucleotide 22), and 4C2 (nucleotide 85)—are indicated by arrows. The two predicted translation initiation methionine codons, ATG, are underlined (nucleotides 65–57 and 104–106), and stop codons are indicated by ∗. Hatched line shows the potential binding site for the signal peptide primer. The predicted signal peptide cleavage site is indicated by a filled arrow. The boundaries between exons and introns are indicated by open triangles. Potential N-glycosylation sites are marked with open boxes. (*B*) Prediction of the *testatin* signal peptide cleavage site. The amino acid sequence deduced from *testatin* was analyzed to determine the cleavage site between the signal sequence and the mature protein by using the SignalP V1.1 World Wide Web Server (14). The black arrow indicates the most probable cleavage site. (*C*) Alignment of the derived *testatin* amino acid sequence with mouse cystatin C, chicken cystatin C, and *CRES*. Putative signal peptides are indicated in pale gray boxes. The numbering follows the predicted *testatin* sequence. Gray boxes show homologous regions between *testatin* and the cystatins. Dark gray boxes are the aligned cysteine residues. The marked amino acids (∗) indicate possible N-glycosylation sites of the *testatin* protein. The lower black line lies over the three domains thought to be critical to the cystatins for cysteine protease inhibitory activity. The third domain, PW, is common for *testatin*, cystatins, and *CRES*.

exact alignment and Pro-93–Trp-94 in the second hairpin loop. In addition, the genomic M12 sequence has two introns (Fig. 2 *A* and *C*), at almost the same positions as those found in mouse cystatin C and other family 2 cystatins, suggesting that M12 and cystatins evolved from a common ancestor (17). We conclude that the mouse *M12* gene most likely represents an additional member of the family 2 cystatins. Therefore, this gene was named *testatin* (testis-specific cystatin-related gene).

*Testatin* is similar in several ways to the cystatin-related epididymal specific (*CRES*) gene (18). Both genes contain an ORF that lacks the cystatin N-terminal and QXVXG conserved region but share homology with cystatins at the C-terminal end. Both genes are restricted in expression, *testatin* to the testis and *CRES* to the testis/epididymis (see below). We suggest that *testatin* and *CRES* are members of a new subgroup within the family 2 cystatins.

***Testatin* Expression During Testis Development.** We next examined the expression of *testatin* mRNA in adult organs and during mouse gonad development by semiquantitative RT-PCR. *Testatin* displayed a very restricted expression pattern and was detected only in fetal gonads and in the adult testis (Fig. 3*A*). *Testatin* expression was high during the early events of testis development. As shown in Fig. 3*B*, *testatin* mRNA is present in both XX and XY gonads at 11.5 dpc. Expression in XX gonads then decreases, whereas mRNA levels in XY gonads increase between 11.5 and 12.5 dpc. This expression pattern is in contrast to the expression of other members of the cystatin family, *cystatin C* and *CRES*, which are almost uniform throughout gonad development (Fig. 3*B*). mRNA levels for *testatin* were examined in more detail at the time of initiation of testis differentiation, between 11.5 and 12.5 dpc (Fig. 3*C*). *Sry* expression was detected at 11.5 dpc and then almost absent at 12.5 dpc, consistent with previous results from RNase protection assays and PCR (19, 20). *AMH*, which is one of the first pre-Sertoli cell markers known and which has been shown to be activated by SF-1 and WT1 in transient transfection systems (21, 22), was turned on between 11.5 and 11.8 dpc. Accumulation of *testatin* transcripts appears at the same time as *AMH*, between 11.5 and 11.8 dpc, after the peak of *Sry* expression. Thus, *testatin* expression is up-regulated just before the first sign of testis development, formation of the testis cords that appear at 12.0 dpc.

The pre-Sertoli cell is the first cell type to differentiate in the developing gonad, and it is the location for both *Sry* and *Sox9* activity. *WT1*, *SF-1,* and *Dax1* also are expressed in this cell lineage (reviewed in ref. 5). mRNA *in situ* analysis and immunostaining with a *testatin*-specific antibody on sections of fetal testis show that *testatin* mRNA and protein are expressed in pre-Sertoli cells (Fig. 3 *D* and *E*) and up-regulated soon after *Sry* is expressed. *Testatin* also is expressed in Sertoli cells in adult testis (data not shown). Thus, *testatin* might be a target gene for transcription factors known to orchestrate the testis development pathway.

## DISCUSSION

Several potent methods, including DD, have been developed during the past few years for isolating novel genes and genes that are differentially expressed. We have extended the DD method into a method that we call SPDD. Although SPDD was used for developmental biology purposes in this paper, we believe that SPDD can be used in most systems when searching for genes encoding proteins containing a signal peptide.

By using SPDD, we isolated a gene named *testatin,* which showed homology to family 2 cystatins. Cystatins and their target proteases are likely to play important roles in normal processes such as bone resorption (23) and in a variety of pathological conditions, including cancer progression and metastasis, stroke, inflammatory diseases, and infections (reviewed in ref. 24). Little is known about the functions of cystatins and their target proteases during development, although it has been shown that they are important for embryo implantation and placentation (25), a process involving regulated cell invasion and reorganization. The timing of *testatin* expression suggests a role for this gene in early testis devel-

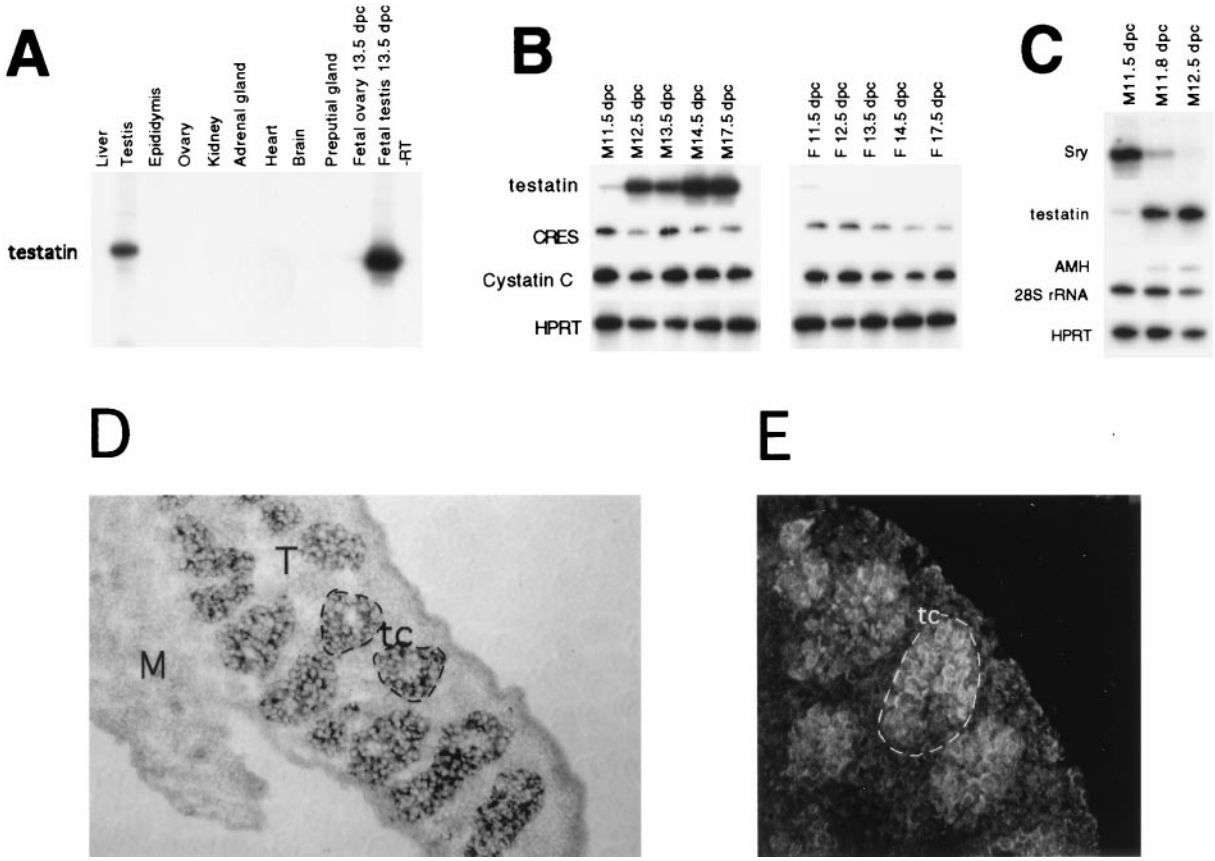


FIG. 3. *Testatin* is expressed specifically in the male gonad soon after *Sry* is expressed and later in adult testis. (*A*) Semiquantitative RT-PCR analysis of *testatin* mRNA expression in adult mouse organs. (*B*) Time-course analysis of *testatin*, *cystatin C*, *CRES,* and *HPRT* expression in developing gonads, from 11.5 to 17 dpc, using semiquantitative RT-PCR. (*C*) Time-course analysis of *testatin*, *Sry*, *AMH,* and *HPRT* expression during the initial phase of testis development, 11.5 to 12.5 dpc, using semiquantitative RT-PCR. (D) *In situ* analysis using a digoxigenin-labeled antisense *testatin* probe on sections from fetal testis (T)+mesonephros (M), 13.5 dpc. *Testatin* expression is located to the Sertoli cells in the testis cords (tc). (*E*) Immunostaining of *testatin* protein in fetal testis, 13.5 dpc, using a *testatin*-specific antibody.

opment, but it remains to be shown whether it acts as an protease inhibitor or has some other function. Cathepsins B, H and L, which are proteases known to be inhibited by family 2 cystatins, are expressed in the mouse fetal testis (K.N., unpublished work). One possible role for *testatin* therefore could be to control the activity of these proteases during the tissue reorganization process, which occurs during early testis development and results in the formation of testis cords. Another possibility involves *testatin* as a regulator of the testis-specific migration of mesonephric cells, which is known to occur around 11.5 dpc (26). Targeted disruption of the *testatin* gene as well as organ culture experiments with recombinant *testatin* will allow us to determine the function of *testatin*.

Genetic analysis of XX-XY chimeras indicated that *Sry* functions within the supporting cell lineage (27), which later on will differentiate into Sertoli cells. *Testatin* is expressed in the same cell lineage immediately after the peak of *Sry* expression; therefore, *Sry* could theoretically induce *testatin* expression. However, *Sry* is certainly not required for its maintenance because *Sry* expression is turned off at 12.5 dpc whereas *testatin* continues to be expressed. Another candidate for regulating *testatin* expression is *Sox9. Sox9* already is expressed in undifferentiated gonads of both sexes at 10.5 dpc. By 11.5 dpc, *Sox9* expression is very abundant in gonads from XY embryos but is absent from those of XX embryos. The timing and location of *Sox9* suggest that this gene might act immediately downstream of *Sry* (28, 29). *Testatin* expression follows *Sox9* expression, with down-regulation of mRNA levels in XX gonads and up-regulation in XY gonads. *Sox9* has been shown to regulate the type II collagen expression during chondrogenesis (30, 31), but so far no gonad specific target gene for *Sox9* action has been found. *AMH*, which also is expressed in pre-Sertoli cells (21), is turned on at the same time as the up-regulation of *testatin* occurs in the XY gonad (Fig. 3C). It recently has been suggested that the *AMH* promoter is regulated by SF-1, WT1, and Dax1 (22). In principle, *testatin* also could be regulated by these factors. Future analysis of the *testatin* promoter will reveal the molecular mechanisms regulating this gene and fit it into the testis developmental pathway. It will be interesting to see whether *testatin* is a link between transcription factors known to regulate testis development and morphogenic events necessary for building a testis.

We thank Gunnar von Heijne, Stockholm University, for help with predicting the signal peptide primer. The fetal testis cDNA library was made by K.N. in Robin Lovell-Badge's laboratory, National Institute for Medical Research. We thank him for help and encouragement. We are grateful to Magnus Abrahamsson, Blanche Capel, Christer Höög, and Anna Wedell for helpful discussions and to George Farrants for critical reading of this manuscript. This work is supported by the Swedish Natural Science Research Council, Jeanssons Foundation, Magn Bergvalls Foundation, and the Karolinska Institutet.

1. Nordqvist, K. & Lovell-Badge, R. (1994) *Nat. Genet.* **7,** 7–9.
2. Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Münsterberg, A., Vivian, N., Goodfellow, P. & Lovell-Badge, R. (1990) *Nature (London)* **346,** 245–250.
3. Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell-Badge, R. & Goodfellow, P. N. (1990) *Nature (London)* **346,** 240–244.
4. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. & Lovell-Badge, R. (1991) *Nature (London)* **351,** 117–121.

5. Capel, B. (1998) *Annu. Rev. Physiol.* **60,** 497–523.
6. Mishina, Y., Rey, R., Finegold, M. J., Matzuk, M. M., Josso, N., Cate, R. L. & Behringer, R. R. (1996) *Genes Dev.* **10,** 2577–2587.
7. Wilson, J. D., Griffin, J. E., George, F. W. & Leshin, M. (1981) *Recent Progr. Horm. Res.* **37,** 1–39.
8. Nordqvist, K. & Töhönen, V. (1997) *Int. J. Dev. Biol.* **41,** 627–638.
9. Dijkman, H. B., Mentzel, S., de Jong, A. S. & Assmann, K. J. (1995) *Biochemica* **2,** 29–33.
10. Starborg, M., Brundell, E., Gell, K., Larsson, C., White, I., Daneholt, B. & Hoog, C. (1995) *J. Cell Sci.* **108,** 927–934.
11. Liang, P. & Pardee, A. B. (1992) *Science* **257,** 967–971.
12. Liang, P. & Pardee, A. B. (1997) *Methods Mol. Biol.* **85,** 3–11.
13. Sakaguchi, M. (1997) *Curr. Opin. Biotechnol.* **8,** 595–601.
14. Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997) *Protein Eng.* **10,** 1–6.
15. Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. & Turk, V. (1988) *EMBO J.* **7,** 2593–2599.
16. Solem, M., Rawson, C., Lindburg, K. & Barnes, D. (1990) *Biochem. Biophys. Res. Commun.* **172,** 945–951.
17. Huh, C., Nagle, J. W., Kozak, C. A., Abrahamson, M. & Karlsson, S. (1995) *Gene* **152,** 221–226.
18. Cornwall, G. A., Orgebin-Crist, M. C. & Hann, S. R. (1992) *Mol. Endocrinol.* **6,** 1653–1664.
19. Hacker, A., Capel, B., Goodfellow, P. & Lovell-Badge, R. (1995) *Development (Cambridge, U.K.)* **121,** 1603–1614.
20. Jeske, Y. W., Bowles, J., Greenfield, A. & Koopman, P. (1995) *Nat. Genet.* **10,** 480–482.
21. Münsterberg, A. & Lovell-Badge, R. (1991) *Development (Cambridge, U.K.)* **113,** 613–624.
22. Nachtigal, M. W., Hirokawa, Y., Enyeartvanhouten, D. L., Flanagan, J. N., Hammer, G. D. & Ingraham, H. A. (1998) *Cell* **93,** 445–454.
23. Lerner, U. H., Johansson, L., Ranjso, M., Rosenquist, J. B., Reinholt, F. P. & Grubb, A. (1997) *Acta Physiol. Scand.* **161,** 81–92.
24. Henskens, Y. M., Veerman, E. C. & Nieuw Amerongen, A. V. (1996) *Biol. Chem. Hoppe-Seyler* **377,** 71–86.
25. Afonso, S., Romagnano, L. & Babiarz, B. (1997) *Development (Cambridge, U.K.)* **124,** 3415–3425.
26. Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R. & Capel, B. (1997) *Curr. Biol.* **7,** 958–968.
27. Palmer, S. J. & Burgoyne, P. S. (1991) *Development (Cambridge, U.K.)* **112,** 265–268.
28. Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A. & Lovell-Badge, R. (1996) *Nat. Genet.* **14,** 62–68.
29. Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H. & Koopman, P. (1996) *Development (Cambridge, U.K.)* **122,** 2813–2822.
30. Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P. & Cheah, K. S. (1997) *Nat. Genet.* **16,** 174–178.
31. Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N. & de Crombrugghe, B. (1997) *Mol. Cell. Biol.* **17,** 2336–2346.
32. Wilkinson, D. G. (1992) in *In Situ Hybridization: A Practical Approach,* ed. Wilkinson, D. G. (IRL, Oxford), pp. 75–83.