

Fig. 2. (A) Structure of the 5' flanking region of mouse ER β . Arrows indicate putative *cis*-acting motifs (only consensus motifs are displayed). Numbers indicate positions relative to the transcription starting site (exon 1). (B) Successive 5'-deletion analysis of mouse ER β promoter activity. Luciferase reporters containing fragments of 5' flanking regions of ER β were transiently transfected into an ER β -positive rat prostate cancer cell line DT3. Values are relative to that with the null-luc-reporter (mean \pm S.E.M., $n = 3$).

Table 1
LacZ and ER β mRNA in mER β -5'f-lacZ-TG mice

	Ovary	Epididymis	Lung	Ventral prostate	Testis	Liver	Thym	Kidney	Spleen
LacZ									
Line 420	–	0.2	0.5	0.0	238	0.2	n.d.	n.d.	n.d.
Line 481	0.25	35.5	4.9	4.3	1329	5.9	2.1	3	3.5
Line 561	0.16	28.5	15.5	1.4	890	3.8	0.9	0.4	0.4
ERβ									
wt	20.0	9.2	3.2	1.9	0.4	0.0	0.1	0.0	0.0

Values are mRNA levels divided by β -actin mRNA levels (mol/mol β -actin). Eleven-week-old F1 mER β -5'f-lacZ-transgenic mice were sacrificed. Total RNA was isolated from tissues and lacZ mRNA levels were measured by real-time PCR. The numbers of transgene copies were 2, 5–6, and 4 per genome in lines 420, 481 and 561, respectively.

561, lacZ mRNA expression in the epididymis was higher than that in clearly negative tissues.

3.4. LacZ staining in mER β -5'f-lacZ-transgenic mice

LacZ activity was detected in *in situ* frozen tissue sections of epididymis, lung, ventral prostate, testis, ovary and liver in 10-week-old F1 transgenic mice. Positive staining was observed only in the testis (Fig. 3). Localization of staining in the outer line of seminiferous tubules, as well as the triangular shape of the stained cells, suggested that the stained cells were sertoli cells. In addition, a part of the spermatogonia may be lacZ-positive. The partial staining of Leydig cells is probably artifact, since it was also observed in the wild-type testis.

4. Discussion

The 5' flanking region of the ER β gene has been cloned and proved to have promoter activity in mice as well as humans

in cell culture studies [14,12]. A region containing 692 bp from the transcription start site of mouse ER β was sequenced and found to contain several putative *cis*-acting elements. Successive 5'-deletion analysis with luciferase reporters suggested that the promoter for basal transcriptional activity was located near the transcriptional start site, while the other elements may contribute to modulating the transcription. Our cloning and *in vitro* promoter analysis of the 5' flanking region of rat ER β gave very similar results [13]. In the present study, the region of the mouse ER β gene extending to –2139 bp was further cloned and luciferase reporter assay was carried out in a prostate cell line, DT3. The results were in line with those of previous reports, since the reporters containing 2.1, 1.3, 0.7 and 0.15 kbp of the 5' flanking region of mER β showed similar transcriptional activity, while a –60 bp reporter showed no activity. Comparison of the 5' flanking regions revealed a high degree of similarity between the mouse and rat sequences, while the human sequence shows only partial homology with those of rodents and contains different *cis*-acting elements (AML-1a, Nkx-2.5 and AP-1). Recently, a second isoform of human ER β mRNA containing different untranslated 5'-ends was

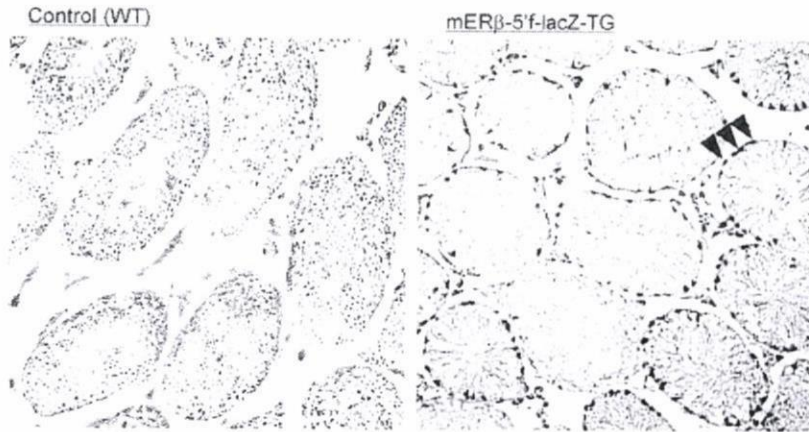


Fig. 3. LacZ staining in testis in WT and ER β -5'f-TG mice. Sertoli cells as well as a part of the spermatogonia are lacZ-positive (arrowheads). Staining of Leydig cells is an artifact (also present in control).

reported to be specifically expressed in ejaculated spermatozoa and peripheral leukocytes, indicating the existence of two distinct promoter structures for the human ER β gene [11].

Significant amounts of ER α are present in a variety of sites including pituitary, bone, liver and skeletal muscle, as well as reproductive tissues, such as ovary, uterus and mammary gland, while ER β expression is restricted mainly to lung, thymus, testis, epididymis, prostate and ovary [1,20,21]. In addition, the tissue distribution of ER β seems differ among species [22,23]. In humans, higher levels of ER β mRNA were detected in the thymus, testis and ovary, while the prostate is the major ER β -positive tissue in rats. On the other hand, in mice, ER β mRNA is expressed in the epididymis, testis, prostate, ovary and lung. Previous *in vitro* studies have showed that the 5'f region of ER β is responsible for cell-specific expression, i.e., higher luciferase reporter activities were noted in ER β -positive cell lines than in ER β -negative lines [13,14,12]. However, *in vivo* functional analysis remains necessary to determine whether the identified promoter regions control the tissue-specific ER β expression.

In the present study, we successfully generated transgenic mice containing a mER β -5'f-lacZ reporter. Three lines were obtained and showed similar tissue distribution patterns of lacZ mRNA expression, which suggested that the expression results from the transgene itself, not from specific insertion sites of the transgene. Data clearly demonstrated a testis-specific expression of lacZ, though a low level of lacZ expression may be present in the epididymis. The differences of mRNA levels among the lines seem to depend on the number of copies of the transgene. In testis, expression of lacZ was localized in sertoli cells, as well as a part of the spermatogonia, in agreement with previous findings based on immunostaining of ER β expression in the mouse testis [24]. Sertoli cell-specific expression of ER β has been confirmed by immunostaining, as well by *in situ* hybridization, in rat testis [25,26]. In addition, a recent investigation suggested that 17 β -estradiol and 5 α -androstane-3 β (3 β -diol),

a possible physiological ER β ligand converted from 5 α -dihydrotestosterone in the tissue, may modulate sertoli cell function through ER β [27]. Although expression of ER β in sertoli cells of the testis is evident in rats as well as mice, it remains controversial whether it is expressed in human testis [28].

ER α expression is regulated by multiple untranslated first exons and promoters. At least four promoters have been postulated for the mouse ER α gene [5,8]. Two proximal promoters, called A and B, are responsible for the expression in most ER α -positive organs, including mammary gland, uterus and ovary, while the other two distal promoters seem to determine liver- and testis-specific expression. An *in vivo* functional analysis of the 5'f region of mouse ER α gene using transgenic mice demonstrated that a proximal 5'f region (promoter A) is sufficient for expression in a variety of organs, including mammary gland and uterus [29]. In contrast, our results indicate that the promoter activity of the proximal 5'f region of mER β gene is able to define only the expression in the testis. Quantitative measurements of lacZ revealed virtually no expression in other ER β -positive organs, such as lung, ovary or prostate, though a very low level of lacZ mRNA was detected in the epididymis. Other enhancer elements may control other aspects of tissue-specific expression. Intronic enhancers would be one possibility, since they have been reported to be essential for some types of tissue-specific gene expression [30,31].

Complex promoter organization involving multiple promoters seems to be a common feature among the nuclear hormone receptor gene family [8]. It is noteworthy that, despite a number of reports suggesting promoter activity in the upstream region of the nuclear hormone receptors based on *in vitro* assay, *in vivo* function of these promoters has not been proven yet, except for a proximal promoter of mouse ER α . The present study of ER β promoter, which indicates that the 5'f region alone possesses very limited promoter activity for tissue-specific expression, provides further insight into promoter function in the nuclear receptor family.

Acknowledgments

This work was supported in part by a Grant-in-Aid (H16-Seikatsu) from the Ministry of Health, Labor and Welfare, Japan and a Grant-in-Aid (#17510046) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Identification of prostatic-secreted proteins in mice by mass spectrometric analysis and evaluation of lobe-specific and androgen-dependent mRNA expression

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Abstract

Rats and guinea pigs have frequently been used to study the development of the prostate and the mechanism of androgen action, but the mouse prostate has also become an attractive model for prostate research, because an enormous range of genetically altered mice is now available. However, the secretion of proteins in the mouse prostate has not yet been thoroughly investigated. In the present study, major secreted proteins from the ventral prostate (VP), dorso-lateral prostate (DLP), and anterior prostate (AP) of mice were identified by means of 2D-gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. A quantitative reverse transcriptase-PCR method was further employed to examine the androgen-dependent transcriptional regulation of the identified proteins. Proteome analysis revealed that the VP secretes spermine-binding protein, serine protease inhibitor

Kazal type-3, and a 91 kDa hypothetical scavenger receptor (AK035662). DLP and AP secrete a protein similar to immunoglobulin-binding protein, immunoglobulin-binding protein-like protein, and one of the experimental autoimmune prostatitis antigen proteins (EAPA2). Peroxiredoxin-6, glucose-regulated protein 78, zinc- α 2-glycoprotein, and phospholipase C α are also secreted. Castration of animals led to a decrease in the mRNAs of these secreted proteins, although the extents of changes varied greatly among different lobes. We present here an outlined view of mouse prostate secretion, which should contribute to an understanding of the biological functions of the prostate gland, as well as the androgen dependency of prostate secretion.

Journal of Endocrinology (2006) **190**, 793–803

Introduction

Rat models have been widely used to study prostate morphology, development, and pathology, as well as androgen-regulated gene expression, in order to understand the basic functions and pathology of this male accessory sex gland (Cunha *et al.* 1987). Mice have generally not been used because the small size of the gland makes morphological studies difficult, and because the mouse prostate is less susceptible to carcinogenesis (Shirai *et al.* 2000). Recently, however, a huge range of transgenic and knockout mice has become available with considerable potential for studies of the prostate (Abate-Shen & Shen 2002, Klein 2005). Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice has been used to study the progression and chemoprevention of prostate cancer (Greenberg *et al.* 1995). Prolactin transgenic mice have been used to investigate the effect of prolactin on prostate growth (Wennbo *et al.* 1997). Estrogen receptor

knockout mice (α ERKO and β ERKO) have been used to examine the role of estrogen in prostate development (Weihua *et al.* 2001, Omoto *et al.* 2005). Aromatase knockout, prolactin receptor knockout, and conditional deletion of Rb mice have been used to study the involvement of those genes in prostate carcinogenesis (McPherson *et al.* 2001, Robertson *et al.* 2003, Maddison *et al.* 2004). However, despite these recent developments, the basic biological function of the prostate, prostatic secretion, is still poorly understood in the mouse. Identification of the secreted proteins will be helpful in understanding prostate development and pathology.

The rodent prostate consists of the ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP), and anterior prostate (AP or coagulating gland). It is well known that rat prostatic secretory proteins, such as prostatein and cystatin-related protein, are mainly produced in the VP, and other proteins, such as prostatic secretory protein of 94 aa (PSP94), probasin,

and seminal vesicle secretion 2 (SVS2) are abundant in the LP and DP (Cunha *et al.* 1987). An early study revealed that spermine-binding protein (SBP) and serine protease inhibitor Kazal type-3 (SPI-KT3) are abundant in the mouse VP (Mills *et al.* 1987a, 1987b). Proteins secreted from the dorso-lateral prostate (DLP) and AP have not yet been identified, although Cunha's group developed a specific polyclonal antibody for major DLP protein(s) to be used as a differentiation marker (Donjacour *et al.* 1990).

In the present study, the major proteins secreted from the VP, DLP, and AP were identified by means of 2D-gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. Further, a quantitative reverse transcriptase (RT)-PCR method was employed to examine the androgen dependence of the transcriptional regulation of the secretory proteins.

Materials and Methods

Animals

Animal experiments were conducted in accordance with *A Guide for the Care and Use of Laboratory Animals of Hiroshima University*. The male C57BL mice were purchased from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. For proteome analysis, three of the 11-week-old mice were killed under ether anesthesia and the prostate and seminal vesicle (SV) were carefully dissected out. In addition, four animals were used for evaluating the sample preparation method. For the study of age-dependent mRNA expression, animals were killed at 1, 2, 4, 6, and 11 weeks (four animals per group), and each of the prostate lobes was dissected under a microscope and immediately fixed in RNA Later solution (Ambion, Inc., Austin, TX, USA). For the castration and hormone-replacement study, animals were divided into three groups, the castrated, castrated plus testosterone injected, and intact. Surgical castration was made at 10 weeks of age and animals were allowed to recover for 1 week. Testosterone propionate (Wako Junyaku KK, Osaka, Japan) was dissolved in the vehicle oil, Panacete 810 (Nippon Oils and Fats Co., Ltd, Tokyo, Japan), and the solution was

administered *i.p.* at a dose of 5 mg/kg body weight. Animals were killed under ether anesthesia 24 h after testosterone injection, and the prostate lobes were collected for RNA extraction.

Preparation of secretion samples

Preparation of secretion samples was performed based on the previously reported method (Donjacour *et al.* 1990). Each dissected prostate lobe from an 11-week-old mouse was rinsed well in saline and placed on a 35 mm culture dish with 100 μ l saline containing 1% protease inhibitor mixture (Sigma). Each lobe was cut into four or five pieces, left to stand for 5 min, and transferred to a 1.5 ml microcentrifuge tube. After centrifugation at 10 000 *g* for 5 min at room temperature, the supernatant was collected as the secretion sample. The incubation time of 5 min was chosen because inner cellular contamination (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) was confirmed to be below by 5 min (Fig. 1). For the SV secretion sample, the content of the vesicle was collected and suspended in saline with protease inhibitors. The protein concentration of each sample solution was determined with a Protein Assay kit (Bio-Rad Lab.). For de-glycosidation, samples were incubated with PNGase F (50 U/ μ g protein; New England Biolabs, Ipswich, MA, USA) at 37 °C for 1.5 h.

Electrophoresis (1D and 2D-PAGE)

For SDS-PAGE gel electrophoresis, 15 μ g total protein of each sample were mixed with the SDS-PAGE buffer containing 2-mercaptoethanol, and applied to a 5–20% gradient PAGE gel (10 \times 10 cm² SuperSep pre-cast gel; Wako Junyaku) with molecular weight markers, Precision Plus (Bio-Rad Lab.). The electrophoresis was carried out at a constant current of 20 mA. The gel was fixed and stained with 45% methanol and 10% acetic acid containing 0.2% Coomassie Brilliant Blue, followed by de-staining with 7% methanol and 7% acetic acid.

For 2D-gel electrophoresis, 1D isoelectric focusing with immobilized pH gradients was performed with Immobiline

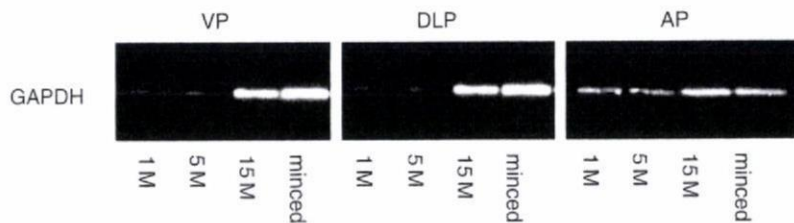


Figure 1 Intracellular contamination in secretion samples. Each prostatic lobe was cut into four or five pieces, and secretion out of the prostatic canals was allowed for 1, 5, and 15 min. Then the lobes were centrifuged and the supernatant was collected as the secretion sample. The samples were applied to the SDS-PAGE (5–20%) at 0.3 μ g protein/lane, transferred to a piece of PVDF membrane and immunostained with antibody to GAPDH, an inner cellular marker (36 kDa band). The intracellular contamination was lesser in the samples incubated for 1 and 5 min (1, 5 M) than in those incubated for 15 min (15 M) or prepared from minced tissues (minced). The incubation time of 5 min was chosen for sample preparation in the present study.

DryStrip (Amersham) and the Ettan IPGphor system (Amersham) according to the manufacturer's protocol. For analytical 2D-PAGE, 10 µg de-glycosidated protein was applied in a 7 cm Immobiline DryStrip (pI 3–11, nonlinear gradient). After rehydration, the strip was isoelectrofocussed (15 kVh). The Immobiline gel was then treated with SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) containing 10 mg/ml DTT for 15 min, followed by the same buffer containing 25 mg/ml iodoacetamide. The Immobiline gel was then placed on the second SDS-PAGE slab gel with 5–20% gradient (SuperSep pre-cast gel, Wako Jyunyaku) and overlaid with hot agarose solution to connect the two gels. The second electrophoresis was run at a constant current of 20 mA. The gel was fixed with 50% methanol and 7% acetic acid, stained overnight in Sypro Ruby (Invitrogen), and de-stained with 10% methanol and 7% acetic acid. Stained gels were scanned with a Molecular Imager FX Pro (Bio-Rad Lab.), with excitation at 532 nm. In the case of preparative 2D-PAGE for mass spectrometric analysis, 60 µg total protein were subjected to electrophoresis as described previously and then stained with silver nitrate by incubation with 0.2 g/l Na₂S₂O₃ for 1 min followed by 1 g/l AgNO₃ for 20 min on ice, and washed with 20 g/l Na₂CO₃ containing 0.1% HCHO. A 24 cm Immobiline DryStrip (pI, 3–11) was also used for preparation. It was rehydrated with 200 µg secreted protein and isoelectrofocussed (25 kVh), then placed on 12.5% SDS-PAGE gel and overlaid with hot agarose solution. The electrophoresis was performed at a constant current of 400 mA. The gel was stained with silver nitrate as described earlier. Three sets of secretion samples from different animals were applied to 2D-electrophoresis and analyzed.

Western blotting

Total proteins, 0.3 µg of each preparation of prostate secretion, were applied to SDS-PAGE (5–20% gel). Proteins were then transferred to a piece of Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham). The membrane was incubated with a monoclonal antibody to GAPDH (Ambion) at 1 µg/ml followed by a peroxidase-conjugated antibody to mouse IgG (MBL Co., Nagoya, Japan) at a dilution of 1:1000. Protein bands were detected using the ECL system (Amersham).

Mass spectrometry (MS)

The protein spots were excised from the polyacrylamide gel and silver nitrate was removed with 15 mM K₃[Fe(CN)₆] and 50 mM Na₂S₂O₃. The gel pieces were incubated in distilled water for 1 h, incubated with CH₃CN for 10 min, and dried in a centrifuge-vacuum concentration system. Each gel piece was incubated with a 20 µl aliquot of 10 µg/ml trypsin solution (sequence grade, Sigma) for 30 min on ice. Excess trypsin solution was removed, and the gel piece was incubated overnight at 35 °C. To extract the digested peptides, 10 µl of 70% CH₃CN containing 0.1% trifluoroacetic acid were added

to each gel piece. An aliquot of 0.5 µl of the extract solution was spotted onto a target plate for an UltraFlex mass spectrometer (Bruker Daltonics, Bremen, Germany) along with 0.5 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid (MS grade, Nacalai tesque Co., Kyoto, Japan). MS was performed using an accelerating voltage of 20 kV, with data acquisition between 1000 and 4000 Da. Some of the fragment peaks were further analyzed by MS/MS. The MS and MS/MS data were evaluated with Biotools software (Bruker Daltonics) in combination with a peptide mass fingerprinting analysis system, MASCOT version 2.1 (Matrix Science, London, UK). The peptide mass fingerprinting was performed based on mass spectroscopy protein sequence database (MSDB; Imperial College London, UK) and the nr database at the National Centre for Biotechnology Information (NCBI; Bethesda, MD, USA) with terminal modifications of peptides set as fixed carbamidomethyl and flexible oxidation ends. The peptide mass tolerance was set to 0.3%.

Quantification of mRNAs by real-time RT-PCR

Total RNA was prepared from each lobe of the prostate with an RNA Isolation kit (Promega), and 2 µg total RNA were reverse transcribed as described previously (Fujimoto *et al.* 2004). An ABI Prism 7700 (Perkin-Elmer Life Sciences, Boston, MA, USA) was employed for quantitative measurement of cDNA using a QuantiTect Sybr Green PCR kit (Qiagen). Specific primer sets with a *T_m* of about 59 °C were designed for each mRNA (Table 1). Prior to quantitative analysis, PCR products were prepared separately and purified by gel electrophoresis. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (Perkin-Elmer Life Sciences). Extracted fragments were used as standards for quantification. The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 s at 94 °C, 30 s at 58 °C, and 40 s at 72 °C. All mRNA contents were normalized with reference to β-actin mRNA.

Serum testosterone levels

Serum testosterone levels were measured with an ELISA kit, purchased from Neogen Corp. (Lexington, KY, USA).

Statistical analysis

Statistical comparisons were made using Student's *t*-test.

Results

1D-PAGE analysis

The secretory proteins from the VP, DLP, AP, and SV were treated with a de-glycosidation enzyme, PNGase F, and analyzed with SDS-PAGE (Fig. 2). In the VP, a broad band at 20–25 kDa was evidently the major band, and a 10 kDa band

Table 1 Quantitative PCR primers for mouse genes

Genes	GenBank accession #	5'-Primer (5' → 3')	3'-Primer (5' → 3')
91 kDa protein	AK035662	GGACCTCCACAAGCGAACAT	GCACCTCTCCAGGTGTTCTCTC
AGR2	M_011783	TTCATCACTTGGACGAATGCC	ACGTAAGTGGCCATCAGGAGAA
Calr	NM_007591	ACCGTGAAGCATGAGCAGAAT	TGTTGATCAGCACATTCTTGCC
EAPA2	AY528666	CCAGACAGGCAGAATTGGGTT	CTCCTCGGAATCTATATTGGCG
GRP78	NM_022310	TCTTGCCATTCAAGGTGGTTG	TTCTTTCCAAATACGCCTCAG
IgBPLP	XM_620455	CTGTGAGTTGCCCGAGCCT	CACAATGGAGAACGCCTCCT
PDI	MUSPDIA	CGCAACAACCTTTGAGGGTGA	TTGGGCAGGAACAGCAGAAT
PLC α	M73329	ATTGCACTGCCAACACAAACA	AACTGAAGCTGGTCTGCTTG
Prdx6	BC013489	AGGACGCCTAACACATGCCTG	GTGCCTGTCAGCTGGAGAGAG
Probasin	AF005204	ACACTGCATGTGCTAGGCGT	TCCCACACAAAATGTGACGG
PSP94	U89840	CCAACGCTACTAGGCCTTTGA	GCCCACACGAAGCACATTTAC
SBP	NM_011321	TGGAACCCGGTCAGATAACTTT	TGCACCCCTTCTAACACAAA
SPI-KT3	BC086887	AGAGGCTAGTTGCCATGATGC	GGACAGGCTCTATGCGTTTCC
SVS2	NM_009300	CAGAGCAGCTCCTCAGAGGG	TCTGGGTCATGTCACCACCA
ZnG	AF281658	CCCACAGGACATAGACCCCTT	CTCATGTCAGGCAGAGGGTA
β -Actin	X03765	CTGTCCTGTATGCCTCTGGTC	TGAGGTAGTCCGTGAGTCCC

seemed to be secondary. PNGase F treatment shifted the major band to a sharper 19 kDa band, while other bands were unaffected. The main band in the DLP appeared to be a broad band at 80–100 kDa, together with bands at 17 and 13 kDa.

PNGase F digested the major band into two sharper bands of approximately 80 and 90 kDa, which showed lower staining intensities. The mobility of other bands was not changed much by PNGase F treatment, although some smear-like

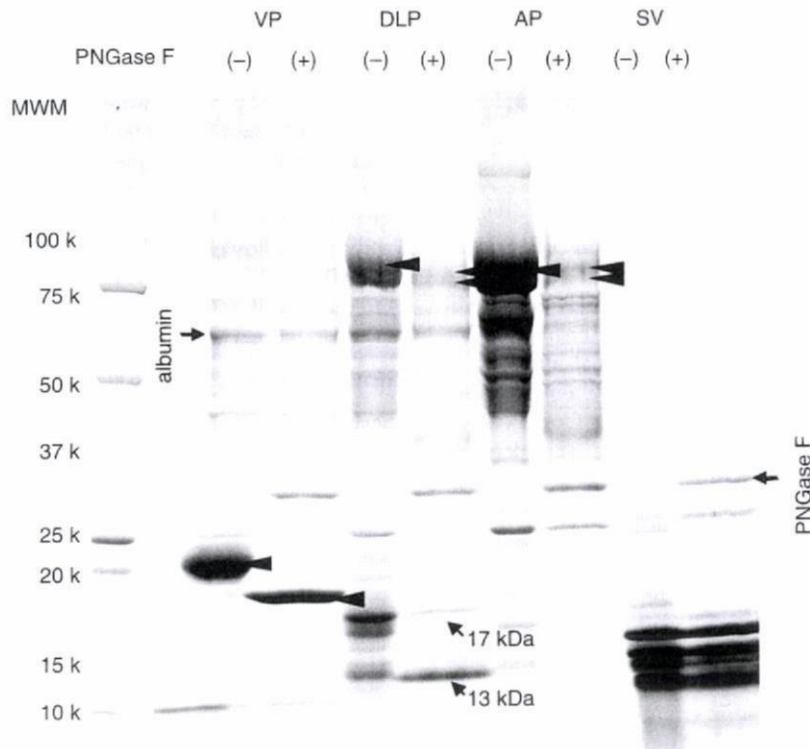


Figure 2 1D-SDS-PAGE analysis of mouse prostate secretory proteins. Secretion was prepared from the VP, DLP, and AP, as well as the SV. Each sample was incubated with (+) or without (-) PNGase F and applied to a 5–20% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue. Arrows indicate major shifted bands by PNGase F.

staining disappeared. When the AP was compared with the DLP, the patterns of bands larger than 25 kDa were similar, as was the effect of PNGase digestion. However, several DLP-specific bands were present in the molecular weight range below 25 kDa. A band of albumin, 68 kDa, representing contamination from serum, was present in the preparations of prostatic secretion, especially in the VP and DLP. The pattern of SV protein bands was completely different from those of prostatic proteins. The major SV bands were observed between 10 and 16 kDa.

2D-PAGE and identified proteins

Secretory proteins from all the lobes were treated with PNGase F and subjected to 2D-PAGE analysis. Owing to the limitation in the pore size of the immobilized pH gradient gel for isoelectric focusing, proteins with a molecular mass of over 100 kDa could not be analyzed in the 2D-PAGE. Gels

were stained and the major spots were picked up for MS analysis (Fig. 3). The analysis of three sets of prostate secretions from independent control mice provided identical patterns. The spots were successfully identified and the results were summarized in Table 2. SBP and SPI-KT3 were major proteins in the VP. In addition, a 91 kDa protein, predicted from urinary bladder cDNA data (AK035662) was identified in the VP, along with glucose-regulated protein 78 (GRP78 or heat-shock 70 kDa protein 5) and peroxiredoxin 6 (Prdx6). Two higher molecular weight proteins in the DLP and AP were identified as experimental autoimmune prostatitis antigen 2 (EAPA2) and a predicted protein similar to immunoglobulin-binding protein (immunoglobulin binding protein-like protein; IgBPLP). Zn- α 2-glycoprotein (ZnG), a mammalian homologue of *Xenopus* anterior gradient 2 (AGR2), as well as PSP94 and probasin, were detected in the DLP secretion. Phospholipase C α (PLC α), calreticulin (Calr), and protein disulfide isomerase were also identified in both DLP and AP secretions. SVS2, 4, 5, and 6 were identified in the SV fluid.

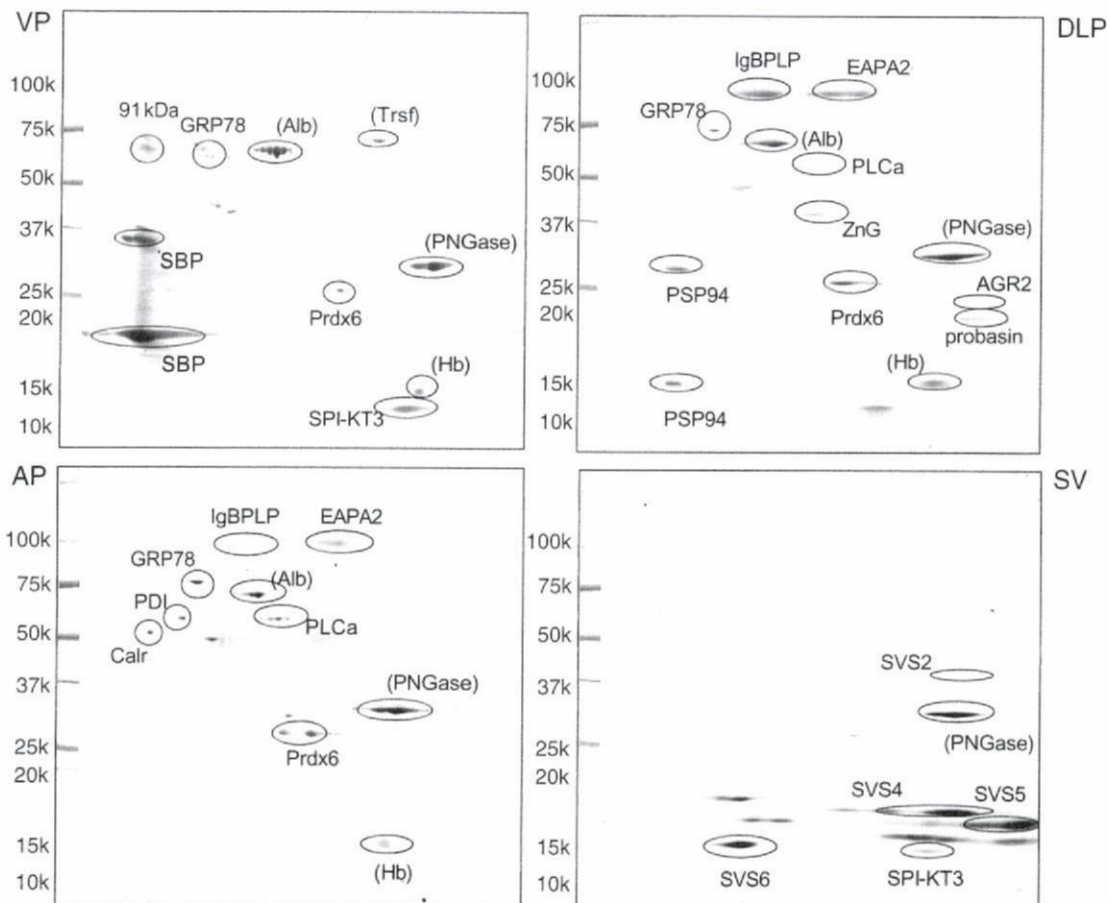


Figure 3 2D-PAGE analysis of mouse prostate secretory proteins. Each sample from the VP, DLP, AP, and SV was treated with PNGase F and applied to an immobilized pH gradient gel, followed by a second SDS-PAGE. Gels were stained with Sypro Ruby. The identified spots are indicated in the figure. Serum albumin (Alb), transferrin (Trsf), and hemoglobin (Hb) were considered to be due to serum contamination.

Table 2 Identified mouse prostatic secretory proteins

	Protein name	Accession #	Observed MW/pl	Theoretical MW/pl	P	Sequence coverage (%)
Abbreviation						
SBP	Spermine-binding protein	NP_035451	18,35/4.5	22/4.6	0.999	46
91 kDa	Protein predicted from cDNA AK035662	Q8BZE1	80/4.5	93/4.8	0.973	13
GRP78	Glucose-regulated protein 78 kDa	A37048	70/5.0	72/5.0	1.000	39
Prdx6	Peroxiredoxin 6	O08709	25/6.0, 6.5	25/5.6	1.000	57
SPI-KT3	Serine protease inhibitor, KT3	NP_033284	10/7.5	9/8.0	1.000	40
PSP94	Prostatic-secretory protein 94	NP_065622	13,28/5.0	13/5.5	1.000	76
IgBPLP	IgG-binding protein-like protein	XP_620455	100/ 5.5	210/5.5	1.000	28
EAPA2	Experimental auto-immune prostatitis antigen 2	NP_98193	100/6.5	103/6.2	1.000	42
PLC α	Phospholipase C α	AAA39944	55/6.0	57/6.0	1.000	29
ZnG	Zinc- α -glyco-protein	Q64726	35/6.0	34/5.8	0.992	28
AGR2	Homolog of <i>Xenopus</i> anterior gradient 2	BAB25181	20/9.5	20/9.5	1.000	56
Probasin	Probasin	AAC01954	22/9.5	21/10.1	1.000	56
Calr	Calreticulin	NP_031617	50/4.5	48/4.2	1.000	35
PDI	Protein disulfide isomerase	AAA39906	60/5.0	57/4.6	0.972	35

Lobe-specific mRNA expression of identified secreted proteins

Expression of identified proteins in the prostate was further confirmed by examining the mRNA levels; the results are summarized in Table 3. Lobe-specific expression of secreted proteins was evident. Both SBP and SPI-KT3 mRNAs were extremely abundant in the VP but virtually undetectable in the other lobes. The mRNA expression of the 91 kDa protein was also VP-specific. Abundant expression of IgBPLP and EAPA2 mRNA was detected in the DLP and AP. Probasin expression was specific to the DLP/AP, while PSP94 mRNA was specific

to the VP/DLP. The level of ZnG mRNA was highest in the DLP. The other protein mRNAs were expressed uniformly among the prostatic lobes.

Androgen dependency in mRNA expression of identified secreted proteins

Transcriptional regulation of identified proteins by androgen was examined by comparing mRNA levels among castrated, castrated plus testosterone-treated, and intact animals (Table 4). Serum

Table 3 mRNA levels of identified proteins in each prostatic lobe in 11-week-old mice

	SBP	SPIKT3	91 kDa	PSP94	ZnG	GRP78	AGR2
VP	511 ± 58.9	184 ± 36.0	3.6 ± 0.8	2.6 ± 0.6	0.9 ± 0.1	1.7 ± 0.5	0.14 ± 0.03
DLP	0	0	0	5.8 ± 1.5	12.3 ± 1.1	6.1 ± 1.7	0.38 ± 0.04
AP	0	0	0	0	1.4 ± 0.3	2.7 ± 0.2	0.54 ± 0.04
	PLCa	Calr	PDI	Prdx6	Probasin	EAPA2	IgBPLP
VP	0.17 ± 0.021	0.7 ± 0.1	15.0 ± 2.1	2.5 ± 0.3	0.06 ± 0.02	0	0.0 ± 0.0
DLP	0.36 ± 0.085	1.2 ± 0.3	8.3 ± 2.1	21.7 ± 2.6	2.15 ± 0.23	18.9 ± 1.5	67.9 ± 10.3
AP	0.60 ± 0.088	1.1 ± 0.3	4.0 ± 0.8	29.0 ± 5.6	0.78 ± 0.05	5.5 ± 0.8	53.0 ± 0.3

Means ± S.E.M. (n = 5). Values are mRNA levels divided by β -actin mRNA levels (mol/mol β -actin). 11-week-old male C57BL mice were killed and total RNA was isolated from each prostate lobe. mRNA levels were measured by real-time RT-PCR.

Table 4 Androgen regulation of mRNA levels of identified proteins

	SBP	SPIK13	91 kDa	PSP94	ZnG	GRP78	AGR2
VP							
Cast	8.2 ± 0.91 ^a	0.24 ± 0.08	0.09 ± 0.02	0.011 ± 0.004	0.38 ± 0.032	0.57 ± 0.36	0.03 ± 0.004
Cast+T	48 ± 7.9 (5.9) ^b	9.3 ± 3.2 (3.9)	0.8 ± 0.23 (8.4)	0.4 ± 0.008 (38)	0.56 ± 0.10 (1.5)	1.02 ± 0.14 (1.8)	0.14 ± 0.03 (4.9)
Intact	511 ± 58.9 (62)	184 ± 36 (769)	3.6 ± 0.79 (40)	2.6 ± 0.55 (236)	0.92 ± 0.09 (2.4)	1.7 ± 0.47 (3.0)	0.12 ± 0.02 (4.3)
DLP							
Cast	—	—	—	0.002 ± 0.001	0.64 ± 0.11	0.55 ± 0.11	0.01 ± 0.001
Cast+T	—	—	—	0.08 ± 0.046 (38)	2.0 ± 0.48 (3.1)	3.4 ± 0.73 (1.8)	0.06 ± 0.02 (6.9)
Intact	—	—	—	5.8 ± 1.48 (2633)	12.3 ± 1.1 (19)	6.1 ± 1.7 (11)	0.38 ± 0.04 (45)
AP							
Cast	—	—	—	—	0.06 ± 0.01	0.33 ± 0.59	0.01 ± 0.002
Cast+T	—	—	—	—	0.21 ± 0.05 (3.6)	1.33 ± 0.19 (4.1)	0.08 ± 0.013 (9.5)
Intact	—	—	—	—	1.4 ± 0.3 (24)	2.7 ± 0.22 (8.3)	0.54 ± 0.04 (64)
PLCa		Calr	PDI	Prdx6	Probasin	EAPA2	IgBPLP
VP							
Cast	0.14 ± 0.081	0.2 ± 0.03	5.6 ± 1.2	3.7 ± 0.73	—	—	—
Cast+T	0.23 ± 0.03 (1.6)	0.7 ± 0.1 (3.3)	11.7 ± 2.1 (2.1)	3.8 ± 1.41 (1.0)	—	—	—
Intact	0.17 ± 0.021 (1.2)	0.7 ± 0.09 (3.5)	15.0 ± 2.1 (2.7)	2.5 ± 0.3 (0.7)	—	—	—
DLP							
Cast	0.09 ± 0.014	0.2 ± 0.05	1.7 ± 0.32	1.5 ± 0.41	0.02 ± 0.003	0.19 ± 0.072	1.2 ± 0.29
Cast+T	0.15 ± 0.02 (1.7)	0.3 ± 0.07 (1.5)	4.5 ± 1.0 (2.7)	2.1 ± 0.22 (1.4)	0.05 ± 0.002 (2.3)	2.4 ± 0.55 (13)	1.6 ± 0.6 (1.4)
Intact	0.36 ± 0.085 (4.0)	1.2 ± 0.3 (6.4)	8.3 ± 2.1 (5.0)	21.7 ± 2.6 (14)	2.2 ± 0.23 (124)	18.9 ± 1.5 (102)	67.9 ± 10.3 (58)
AP							
Cast	0.20 ± 0.03	0.4 ± 0.06	1.4 ± 0.3	1.2 ± 0.15	0.02 ± 0.004	0.17 ± 0.52	0.25 ± 0.18
Cast+T	0.34 ± 0.085 (1.7)	0.7 ± 0.05 (1.8)	2.8 ± 0.5 (2.0)	4.7 ± 0.93 (4.0)	0.04 ± 0.008 (2.6)	4.8 ± 0.9 (29)	1.1 ± 0.8 (4.4)
Intact	0.60 ± 0.088 (3.0)	1.1 ± 0.3 (2.8)	4.0 ± 0.8 (2.8)	29.0 ± 5.6 (25)	0.78 ± 0.051 (49)	5.5 ± 0.8 (33)	53.0 ± 0.31 (215)

^aMean ± S.E.M. (n=5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin).

^bValues in parenthesis are fold change in mRNA over the castrated, 10-week-old male C57BL mice were castrated and maintained for a week (cast). They were killed 24 h after testosterone administration at 5 mg/kg bw, ip (Cast+T). Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCR.

testosterone levels were 0.52 ± 0.15 , and 1.5 ± 0.15 ng/ml in castrated, castrated plus testosterone injected, and intact groups respectively. Serum testosterone levels reached 38 ng/ml, 1 h after a testosterone injection. The mRNA levels of identified secreted proteins decreased 1 week after castration, although the extent of the decrease differed among protein species. For instance, SPI-KT3 mRNA in the VP was greatly decreased in castrated animals to only 1/769 of the intact control level, while castration reduced SBP expression to 1/62 of the control. The extent of change in mRNA expression also varied between lobes. GRP78 mRNA in the DLP, for instance, was decreased to 1/11 of the control by castration but only to about 1/3 of the control in the VP. The mRNA levels were normalized by β -actin levels, which were not affected by castration and testosterone treatment. The β -actin levels in the VP were 3.5 ± 0.36 , 3.8 ± 0.30 , and 3.5 ± 0.34 fg/ng total RNA in the castrated, the castrated plus testosterone and intact groups respectively. The values were 4.8 ± 0.74 , 5.3 ± 0.99 , and 4.3 ± 0.40 for the DLP, and 3.9 ± 0.24 , 4.2 ± 0.60 , and 4.7 ± 0.26 for the AP.

Ontogeny in mRNA expression of identified secreted proteins

The expression of identified secreted protein mRNAs was examined in each lobe of the prostate at ages 1, 2, 4, 6, and 11 weeks (Table 5). Low levels of mRNA expression were noted at 1 week. Significant increases of SBP and EAPA2 mRNAs began at 2 weeks and continued thereafter. Increases in other secretory protein mRNAs, including 91 kDa protein, PSP94 and IgGBPLP mRNAs, were apparent at 4 weeks.

Discussion

In the present study, the major secretory proteins of the mouse VP, DLP, and AP were identified by mass spectrometric analysis after 2D-gel electrophoresis (Table 6). IgGBPLP and EAPA2 were major proteins in the DLP/AP. A 91 kDa protein predicted from a mouse urinary bladder cDNA (AK035662), Prdx6 and PLC α were also found in the prostatic secretion for the first time, in addition to previously reported prostatic proteins, including SBP (Mills *et al.* 1987b), SPI-KT3 (Mills *et al.* 1987a), PSP94 (Xuan *et al.* 1999), and probasin (Johnson *et al.* 2000). The mRNAs for these proteins were expressed in a lobe-specific manner and were regulated by androgen. Our study has delineated the main mouse prostatic secretion pattern for the first time. The data will be useful for studying androgen-dependent gene regulation in the prostate, and may also provide markers for studying functional differentiation of prostate tissue.

Production and secretion of prostatic proteins are the main physiological functions of the prostate gland. Prostatic secretory proteins have been studied in rats as well as in humans, especially from the viewpoint of androgen-dependent regulation of expression and to identify possible markers of prostate cancer. The major human prostatic-secreted proteins are PSA (prostate-specific antigen), PSP94, and prostatic acid phosphatase (Lee *et al.* 1986). In rat, the composition of prostatic proteins is different; only PSP94 is common with the human case, and the production of each protein varies among lobes. In the VP, prostatic-binding protein or prostatein is the major secreted protein, while cystatin-related protein and kallikreins are also produced abundantly (Heyns 1990). The LP and DP secrete probasin,

Table 5 Ontogeny of mRNA levels of identified proteins in the prostate

	SBP	91 K	PSP94	EAPA2	IgBPLP
<i>VP</i>					
1W	0.1 ± 0.04	0.1 ± 0.02	0.1 ± 0.05	–	–
2W	$17.9 \pm 3.8^*$	0.1 ± 0.01	0.2 ± 0.08	–	–
4W	$78.5 \pm 15.3^*$	$0.8 \pm 0.07^{\dagger}$	$2.6 \pm 0.23^{\dagger}$	–	–
6W	$503.0 \pm 106^*$	$3.6 \pm 0.23^{\dagger}$	$16.0 \pm 3.9^{\dagger}$	–	–
11W	$474.9 \pm 77.3^*$	$4.4 \pm 0.50^{\dagger}$	$4.2 \pm 0.6^{\dagger}$	–	–
<i>DLP</i>					
1W	–	–	0.0 ± 0.03	0.18 ± 0.06	0.05 ± 0.02
2W	–	–	0.0 ± 0.01	$0.42 \pm 0.06^*$	0.09 ± 0.02
4W	–	–	$1.1 \pm 0.32^*$	0.88 ± 0.27	0.68 ± 0.21
6W	–	–	$12.7 \pm 1.5^{\dagger}$	$3.95 \pm 0.79^*$	$7.43 \pm 1.5^*$
11W	–	–	$4.0 \pm 0.2^{\dagger}$	$15.3 \pm 2.36^{\dagger}$	$102 \pm 22.0^{\dagger}$
<i>AP</i>					
1W	–	–	–	0.18 ± 0.04	0.04 ± 0.01
2W	–	–	–	$0.74 \pm 0.02^*$	0.1 ± 0.02
4W	–	–	–	1.10 ± 0.4	$1.9 \pm 0.26^{\dagger}$
6W	–	–	–	$3.55 \pm 0.53^{\dagger}$	$30.1 \pm 3.6^{\dagger}$
11W	–	–	–	$5.50 \pm 0.80^{\dagger}$	$53.2 \pm 8.1^{\dagger}$

Means \pm S.E.M. ($n=4$). Values are mRNA levels divided by β -actin mRNA levels (mol/mol β -actin) 1, 2, 4, 6 and 11-week-old (W) male C57BL mice were killed. Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCR. * $P<0.05$ and $^{\dagger}P<0.01$ vs. 1W.

Table 6 Summary: identified mouse prostatic secretory proteins

Abbreviation	Lobe specificity	mRNA decrease by castration	Description
SBP	VP	++	Known prostatic protein (Mills <i>et al.</i> 1987b)
SPI-KT3	VP, (SV)	+++	Known prostatic protein (Mills <i>et al.</i> 1987a)
91 kDa	VP	++	Scavenger receptor cys-rich (SRCR) domains
PSP94	VP, DLP	+++	Known prostatic protein (Xuan <i>et al.</i> 1999)
ZnG	DLP > VP, AP	+	Ribonuclease activity?
GRP78	VP, DLP, AP	+	Heat-shock protein 70 family
AGR2	VP, DLP, AP	+	Human homolog expressed in prostatic cancer cell lines
PLCa	VP, DLP, AP	+	Enzyme involved in phosphatidylinositol metabolism
Calr	VP, DLP, AP	+	Calcium-binding protein
PDI	VP, DLP, AP	+	Enzyme involved in protein folding
Prdx6	DLP, AP > VP	++	Antioxidant protein
Probasin	DLP, AP	+++	Known prostatic protein (Johnson <i>et al.</i> 2002)
EAPA2	DLP, AP	+++	No homology with any known protein
IgBPLP	DLP, AP	+++	IgG binding? Willebrand factor D domains, trypsin inhibitor like

PSP94, and SVS2 (Imasato *et al.* 2001). A kinesin heavy chain-like protein and an IgG-binding protein were recently reported in the secretion of the AP (Esposito *et al.* 2001, Wilhelm *et al.* 2002).

In spite of the morphological similarity of the prostate in mouse and rat, previous studies have suggested a substantial difference in prostatic secretion between the two species (Donjacour *et al.* 1990). Since mouse prostatic proteins are known to be highly glycosylated, we first examined the effects of glycosidase digestion on prostatic proteins. Endo H glycosidase, which cleaves mainly within the chitobiose core of high mannose, did not change the SDS-PAGE pattern (data not shown). On the other hand, PNGase F, which removes all types of N-linked glycosylation, changed the pattern. A broad band of SBP in the VP was converted to a sharp band with smaller molecular weight, and smear-like bands between 40 and 100 kDa in the DLP/AP were also converted to sharper bands, indicating that proteins were de-glycosylated by the enzyme. In spite of highly glycosylated characteristics of mouse prostate proteins, the biological role of glycosylation is not yet understood.

In the mouse prostate, only VP-secreted proteins have been investigated, and two major proteins, SBP and SPI-KT3, were identified (Mills *et al.* 1987a,b). The present study confirmed the secretion of these two proteins and also revealed the presence of other proteins, including 91 kDa protein, Prdx6 and GRP78. The 91 kDa protein is expected to consist of 841 aa with two predicted extracellular (CUB) domains and three scavenger receptor cysteine-rich (SRCR) domains, and is expressed preferentially in the VP. The size of the protein, however, seems to be less than 91 kDa in the gel. Since the sequence coverage of peptide mass fingerprinting is only 13%, the actual reading frame may be shorter than the predicted one. Prdx6 is another new component of the prostatic secretion found in the present study. Since it is an antioxidant enzyme that reduces peroxide and alkyl hydroperoxide to water and

alcohol respectively (Wang *et al.* 2003), it may provide seminal plasma antioxidant capability. GRP78 belongs to the heat-shock protein 70 family, which had been considered as intercellular proteins. However, a recent proteomic analysis of human prostasomes revealed the presence of heat shock proteins in prostatic secretion (Utleg *et al.* 2003). In addition, heat-shock protein 70 has been reported to be secreted from a variety of prostatic cell lines, and to show growth-inhibitory activity (Jones *et al.* 2004, Wang *et al.* 2004). Secreted mouse GRP78 may have a similar activity.

Although mouse DLP proteins had not been biochemically identified, Cunha's group has recognized 110 and 55 kDa bands in SDS-PAGE as major DLP/AP proteins (Donjacour *et al.* 1990). They reported that DLP/AP proteins are highly glycosylated, which was confirmed by the present study. The predicted IgBPLP sequence derived from the cDNA sequence (XM_620455), however, is calculated to contain 1866 aa with a molecular mass of 201 kDa. Because peptide sequencing by the peptide-mass fingerprinting method covered the whole predicted sequence (27% coverage), the 100 kDa spot probably contains a mixture of cleaved fragments derived from the 201 kDa protein, although this remains to be confirmed. Recently, an IgG-binding protein of 115 kDa was reported to be secreted also from the rat AP, suggesting that a rat homolog exists (Wilhelm *et al.* 2002). The predicted cDNA sequence corresponding to this rat protein (XM_620455), which became available more recently, encodes 206 kDa protein (1914 aa) instead of 115 kDa. There is 84% similarity between the mouse and the rat sequences. Secretion of EAPA2, which is one of the antigens found in experimental autoimmune prostatitis, is also a noteworthy finding in the present study. This protein of 914 aa contains no known domain structure and has no homology with any known functional protein. Secretion of both PSP94 and probasin was detected in the DLP, as expected, since both proteins are well characterized in rats and have been reported in mice (Xuan

et al. 1999, Johnson *et al.* 2000). The other identified DLP proteins include GRP78, Prdx6, ZnG, AGR2, Calr, and protein disulfide isomerase (PDI). Prostatic secretion of ZnG has been reported in humans, and ZnG is widely distributed in body fluids and in various epithelia (Lei *et al.* 1998, Hale *et al.* 2001). AGR2 is a mammalian homolog of *Xenopus* AGR2, which was recently reported to be secreted from human prostate under androgen regulation (Zhang *et al.* 2005). It is overexpressed in prostate cancer and the expression level is correlated with pathological grade. Calr is a highly conserved calcium-binding protein involved in a wide variety of cellular processes (Krause & Michalak 1997). Interestingly, the Calr gene was identified as an androgen-inducible gene in the rat VP (Zhu *et al.* 1998). PDI is involved in the maintenance of folding of synthesized proteins. Specific expression of PDI in the prostate was recently reported in humans (Lexander *et al.* 2005). Since both Calr and PDI are considered to be localized in the lumen of endoplasmic reticulum, they may represent contaminants introduced during preparation of the secretion sample. Secretion from the AP is similar to that from the DLP, i.e. the major secretory proteins are IgGBPLP and EAPA2, but little ZnG and no PSP94 are found in the secretion. The results of mass spectrometric identification of SV proteins were generally in agreement with previous reports, i.e. SVS2, 4, 5, and 6, as well as SPI-KT3 (Lai *et al.* 1991, Lundwall *et al.* 1997). Except SPI-KT3, SV proteins are specifically expressed in the SV and not in the prostate gland, which differs from the rat case, where SVS2, for instance, is highly expressed in DLP/AP.

Quantitative determination of mRNA expression revealed a clear transcriptional differentiation of secreted proteins among the lobes. The levels of secretory protein mRNAs were very high, ranging from 1 to 500 times that of the housekeeping gene β -actin, used as an internal control in the present study. The mRNA levels overall correlated with the intensity of protein staining in the gel, although spots of protein with larger molecular weight, such as EAPA2 and IgBPLP show lesser intensity in the 2D-gel, since the 1D-gel used in the present study is only able to hold proteins with molecular weights less than 80–100 kDa. Since all the identified secreted proteins decreased significantly a week after castration of the animal, these protein transcripts are androgen-dependent directly or indirectly through involution of the gland. In rats, various studies have shown a faster response of the VP to androgen action, as compared with other lobes. For instance, castration decreased probasin mRNA expression to 1% of the control level after a week, while the decrease in the DLP was only 50% (Imasato *et al.* 2001). In the mouse, however, large decreases in mRNAs were evident in all the lobes. We examined the effect of a single injection of testosterone on the mRNAs in castrated animals to confirm the androgen dependency of transcription. The serum testosterone level well exceeded the control level within 1 h and was still high 24 h after an injection. Although most mRNA levels increased significantly after an injection,

which clearly demonstrates their androgen inducibility, most of them were not restored to the intact control level. It may suggest that the full activity of androgen-dependent genes in the prostate is involved in both short- and long-term transcriptional regulation mechanisms by androgen.

Although rodent prostate models have been used for investigating the mechanism of prostate carcinogenesis, anatomical differences between rodent and human prostate have led to concerns about the validity of rodents as suitable models for human prostate cancer. Besides, mice are resistant to induction of prostate tumors by chemical carcinogens. However, a number of transgenic or knockout mouse lines have become available in which prostate carcinomas preferentially occur. For instance, the TRAMP transgenic line expresses the SV40 antigen under the control of the rat probasin promoter. The TRAMP mice develop high-grade prostatic intraepithelial neoplasia and prostate cancer within 12 weeks of birth, and ultimately develop metastases to the regional lymph nodes and lung by 30 weeks. In addition, androgen depletion by castration results in decreased tumor incidence. Their futures are similar to the human case, although metastasis to bone, a characteristic feature of human prostate cancer, is rare. The expression pattern of secretion proteins may be related to development of prostate carcinogenesis. Since the present study has revealed mouse prostate secretion, these can now be examined in relation to the development of prostate carcinogenesis as well as androgen-dependent differentiation of the gland. The ontogeny of mRNA expression of secreted proteins indicated that significant expression started 2 weeks after birth, which is consistent with the fact that branching morphogenesis of the mouse prostate is completed in the first 15 days of birth.

The present study has provided an understanding of the major secretory function of the mouse prostate, and identified common aspects of secretory functionality between mouse and human, e.g. for heat-shock proteins, ZnG and peroxiredoxin. The identified secretory proteins should be available as models of androgen-dependent gene regulation and are candidates as markers for prostatic differentiation. Like human PSA or PSP94, some of the identified proteins may be useful as pathological markers associated with prostate disorders; this would facilitate prostate research in mouse models.

Acknowledgements

We thank Ms R. Tai for her expert technical assistance and Dr S. Izumi for his expert suggestions for mass spectrometry. This work was supported in part by a Grant-in-Aid (H16-Seikatsu) from the Ministry of Health, Labor and Welfare, Japan and a Grant-in-Aid (#17510046) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 4 January 2006

Received in final form 24 April 2006

Accepted 26 May 2006

Made available online as an Accepted Preprint

13 June 2006

PSPC1, NONO, and SFPQ Are Expressed in Mouse Sertoli Cells and May Function as Coregulators of Androgen Receptor-Mediated Transcription¹

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ABSTRACT

In Sertoli cells of testis, androgen receptor-regulated gene transcription plays an indispensable role in maintaining spermatogenesis. Androgen receptor activity is modulated by a number of coregulators which are associated with the androgen receptor. Non-POU-domain-containing, octamer binding protein (NONO), a member of the DBHS-containing proteins, complexes with androgen receptor and functions as a coactivator for the receptor. Paraspeckle protein 1 alpha isoform (PSPC1, previously known as PSP1) and Splicing factor, proline- and glutamine-rich (SFPQ, previously known as PSF), other members of the DBHS-containing proteins, are also found in androgen receptor complexes, suggesting that these DBHS-containing proteins may cooperatively regulate androgen receptor-mediated gene transcription. We demonstrated that PSPC1, NONO, and SFPQ are coexpressed in Sertoli cell line TTE3 and interact reciprocally. The effect of the DBHS-containing proteins on the transcriptional activity was assessed using the construct containing androgen-responsive elements followed by a luciferase gene. The results showed that all the DBHS-containing proteins activate androgen receptor-mediated transcription, and PSPC1 is the most effective coactivator among them. Furthermore, we confirmed the presence of PSPC1, NONO, and SFPQ proteins in Sertoli cells of adult mouse testis sections. These observations suggest that PSPC1, NONO, and SFPQ form complexes with each other in Sertoli cells and may regulate androgen receptor-mediated transcriptional activity.

androgen receptor, gene regulation, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is a multistep process leading to the generation of highly specialized spermatozoa. The developmental process begins with spermatogonia that are committed

to further differentiation by undergoing two meiotic divisions, resulting in haploid round spermatids. During spermiogenesis, haploid spermatids undergo drastic morphological changes, including formation of the acrosome and the sperm flagellum, decrease of the nuclear size due to the unique DNA packaging, and exclusion of most of the cytoplasm [1]. The complexity of the differentiation process requires a highly specialized program of gene expression of male germ cells [2, 3]. For example, cAMP responsive element modulator (CREM) has been shown to play an important role in germ cell-specific transcription by binding to CRE sequences [4, 5], and Poly (A) polymerase beta (PAPOLB) is known to adjust the timing of haploid-specific translation by controlling the cytoplasmic mRNA polyadenylation [6].

However, the endogenous gene expression program of male germ cells is not sufficient for spermatogenesis, and support from nearby Sertoli cells is indispensable. Throughout spermatogenesis, Sertoli cells interact directly with germ cells within the seminiferous tubules. Sertoli cells regulate highly organized and precisely synchronized germ cell development by nourishing germ cells via their secretion products [7–9]. These controls by Sertoli cells are also regulated by external stimuli. Androgen and androgen receptor (AR)-mediated gene transcription are important for the Sertoli cell functions [2, 10]. Mice lacking AR in Sertoli cells show spermatogenic arrest, which results in azoospermia and infertility [11, 12]. Thus, AR-mediated transcription in Sertoli cells plays an indispensable role in spermatogenesis.

AR belongs to the nuclear receptor superfamily that includes receptors for thyroid hormone, retinoic acid, estrogen, progesterone, glucocorticoid, and other hormones [13]. AR forms a homodimer and binds to androgen-responsive elements in promoters/enhancers of AR-driven genes. AR is composed of N-terminal transactivation domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD) at the C-terminus. NTD possesses an activation function domain 1 (AF-1) and is involved in making contact with the general transcriptional machinery [14–16]. The transcriptional activity of AR is modulated by coregulators, which include coactivators that enhance AR transactivation and corepressors that suppress AR transactivation [17–19].

Non-POU-domain-containing, octamer binding protein (NONO) is known as one of the coregulators of AR. NONO interacts directly with the AR AF-1 domain and acts as a coactivator [20, 21]. NONO contains a DBHS (Drosophila behavior, human splicing) domain characterized by two tandem RNA recognition motifs (RRMs) and a helix-turn-helix (HTH) DNA binding domain. In mammals, two other DBHS-

¹Supported by grants from the Ministry of Education, Sports, Culture, Science and Technology of Japan (MEXT) to Y.K.

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Received: 23 January 2006.

First decision: 1 March 2006.

Accepted: 20 April 2006.

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ISSN: 0006-3363. <http://www.biolreprod.org>

containing proteins, Paraspeckle protein 1 (PSPC1, previously known as PSP1) and Splicing factor, proline- and glutamine-rich (SFPQ, previously known as PSF), have been reported [22, 23]. PSPC1 has two isoforms, alpha (referred to as PSPC1 in this paper) and beta, produced by alternative splicing. It has been reported that DBHS-containing proteins regulate nuclear receptors, such as progesterone receptor [24] and thyroid hormone receptor [25], and also participate in mRNA regulation in the nucleus, including splicing [26, 27], 3'-end cleavage [27, 28], and nuclear retention of edited RNA [29]. Since DBHS-containing proteins are identified in RNA-transporting granules [30], it is likely that these proteins also participate in RNA metabolism in the cytoplasm. In addition, DBHS-containing proteins are reported to be involved in activation of DNA topoisomerase I [31] and DNA double-strand break rejoining [32]. Therefore, these proteins are regarded as multifunctional proteins involved in various aspects of gene expression. In many cases, NONO and SFPQ are copurified [20, 27–40], suggesting that these proteins may control gene expression as a complex. We have previously confirmed the direct interaction between PSPC1 and NONO, and SFPQ by coimmunoprecipitation experiments and yeast two-hybrid assays [41], indicating that the DBHS-containing proteins interact reciprocally.

We have sought to define expression and function of DBHS-containing proteins in testis, and to elucidate the biological significance of these proteins in spermatogenesis. In this paper, we have shown the expression of the DBHS-containing proteins and reciprocal complex formations in the Sertoli cell line TTE3. These proteins enhanced AR-mediated transactivation, and we confirmed the expression of the DBHS-containing proteins in the Sertoli cells of adult mouse testis. These observations suggest that the DBHS-containing proteins may be involved in spermatogenesis by regulating AR-mediated transcription in the Sertoli cells.

MATERIALS AND METHODS

Plasmids, Cells, and Mice

The expression plasmids for Myc-tagged DBHS-containing proteins (pMyc-CMV-2-Pspc1, pMyc-CMV-2-Nono, and pMyc-CMV-2-Sfpq) were prepared by subcloning mouse *Pspc1alpha*, mouse *Nono*, and mouse *Sfpq* cDNAs (GenBank accession numbers: NM_025682, NM_023144 and NM_023603) into the pMyc-CMV-2 vector (Clontech). A PSPC1 RRM mutant (F118A, F120A, K197A, F199A), which did not bind to RNA, was generated as described previously [42] and cloned into pMyc-CMV-2. By using the same method, the expression plasmids for a NONO RRM mutant (pMyc-CMV-2-Nono RRM mutant, F113A, F115A, K192A, I194A) and a SFPQ RRM mutant (pMyc-CMV-2-Sfpq RRM mutant, F326A, F328A, K405A, I407A) were also generated. The expression vector for androgen receptor pSG5-hAR and p(ARE)₂-luc plasmid containing two consensus androgen-responsive elements were described earlier [43].

COS-1 cells were cultured in Dulbecco modified Eagle medium (DMEM)(Sigma) supplemented with 10% Donor Calf Serum (DCS) (Thermotrace) and maintained at 37°C in an atmosphere of 5% CO₂. TTE3 cells were cultured in DMEM containing 10% DCS on collagen type I pre-coated dishes (Celltight C-1, Sumitomo Bakelite) at the permissive temperature of 33°C or nonpermissive temperature of 37°C in an atmosphere of 5% CO₂.

Nine-week-old male BALB/cAJcl mice were purchased from CLEA Japan. Animal experiments were conducted in accordance with the National Institutes of Health standards established in the Guidelines for the Care and Use of Experimental Animals.

Mouse Monoclonal Antibody Production

Synthetic peptides corresponding to amino acids 505–523 in mouse PSPC1 (CFGRGSQGGNFEGPNKRRR), 453–464 in mouse NONO (CPPAFNR-PAPGAE), and 681–699 in mouse SFPQ (CAGYGRGEEYEGPNKKPRF) were purchased from BIO SYNTHESIS. These peptides, conjugated with KLH (Pierce), were injected twice at a 1-wk interval into BALB/cAJcl mice. Three

days after the second boost, the lymph node cells were fused with the myeloma line P3U1. The culture media were screened by ELISA and following immunoblotting. The cells from the positive wells were cloned by the standard limiting dilution technique. Anti-PSPC1 (clone 1L4), anti-NONO (clone NC5), and anti-SFPQ (clone FC23) mouse monoclonal antibodies were used in this study.

Immunoblotting

Fifteen µg of TTE3 cell lysates prepared as described previously [41] were analyzed on 8% SDS-polyacrylamide gels and blotted onto Immobilon-P membrane (Millipore). After saturation with 5% skim milk (Difco) in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 for 1 h at room temperature, membranes were incubated with a 1:40 dilution of antibodies to either PSPC1, NONO, or SFPQ followed by an incubation with a 1:10000 dilution of HRP-conjugated goat anti-mouse IgG (ZYMED). Enzymatic activities were detected by ECL substrate (Amersham Biosciences).

Immunocytochemistry

TTE3 cells were fixed in 4% paraformaldehyde in PBS for 20 min and for an additional 30 min in methanol. After washing with PBS, coverslips were blocked with 10% goat serum. Cells were incubated with a 1:10 dilution of anti-PSPC1, anti-NONO, or anti-SFPQ antibody respectively for 1.5 h at room temperature. After washing three times with PBS, the cells were incubated with a 1:100 dilution of Alexa Fluor 546 goat anti-mouse IgG (Invitrogen), for 30 min. The expression of proteins was visualized by fluorescence microscopy (Olympus).

Immunoprecipitation

For immunoprecipitation analysis, TTE3 cells (1.8×10^7 cells) were harvested and lysed in 4.8 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40, 1 mM Na₂VO₄, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM DTT) with or without 10 µg/ml RNase A and incubated at 4°C for 30 min followed by centrifugation at 15,000 rpm for 15 min. The resulting supernatants were decanted into fresh tubes and preabsorbed with Protein-A agarose beads (SIGMA) for 30 min followed by incubation with anti-PSPC1, anti-NONO, anti-SFPQ, or anti-Myc (9E10) antibody coupled to Protein A-agarose beads for 2 h. The immunoprecipitates were washed three times with lysis buffer. Immunoprecipitates were immunoblotted with each antibody.

Luciferase Assay

COS-1 cells were transiently transfected by the calcium phosphate precipitate method with 0.2 µg of p(ARE)₂-luc plasmid, 0.1 µg of pSG5-hAR, 0.3 µg of PSPC1, NONO, or SFPQ expression vector. The total amount of vector added to each well was adjusted by adding an empty vector pMyc-CMV-2. Six hours after transfection, the media were replaced with fresh media containing 0.2% DCS. Dihydrotestosterone (DHT, 10⁻⁹ M) ligand was added and cells were incubated for an additional 12 h. Luciferase activities were determined as described previously [44] using a TD-20/20 luminometer (Turner Designs). Each plasmid was assayed in triplicate at least three different times. pRL-tk (Promega) was cotransfected to normalize transfection efficiencies. All the data were analyzed by Student *t*-test using Microsoft Excel. A *P* value of less than 0.05 was considered to be statistically significant.

RT-PCR Analysis

COS-1 cells were transfected and treated with DHT as done in the luciferase assay with the exception that cells were cultured in 90 mm dishes. Cells were harvested, lysed in IP buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 1% Triton-X100, 0.5% NP-40, 250 mM sucrose, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 50 mM NaF, 1 mM Na₂VO₄, 1 mM DTT, 10 U/ml RNase inhibitor (TOYOBO), and incubated at 4°C for 20 min followed by centrifugation at 15,000 rpm for 15 min. The supernatants were decanted into fresh tubes and preabsorbed with Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 30 min followed by incubation with anti-Myc monoclonal antibody (9E10) or anti-HA monoclonal antibody (12CA5) coupled to Protein G-Sepharose 4 Fast Flow for 1 h. After washing the immunoprecipitates five times with IP buffer, RNA was purified with an RNeasy Mini Kit (QIAGEN). Eluted RNA was reverse-transcribed using a Sensiscript RT Kit (QIAGEN) with the luciferase rv primer, (5'-CGAGTGTAGTAAACATTCAAAACCGTGATGG-3'), and amplified

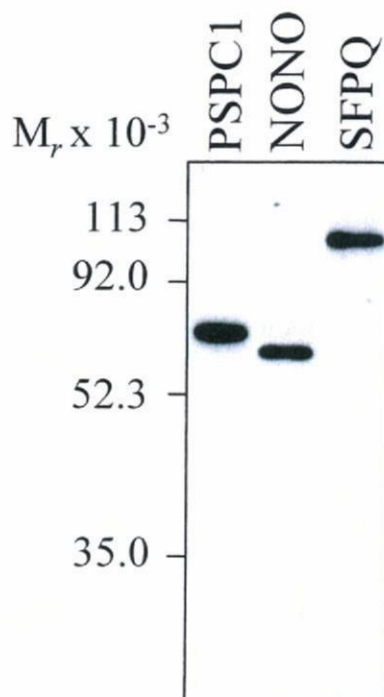


FIG. 1. PSPC1, NONO, and SFPQ expressed in Sertoli cell line TTE3. TTE3 cell extracts were electrophoresed on SDS-PAGE gels, transferred onto polyvinylidene fluoride membranes, and incubated with anti-PSPC1, anti-NONO, or anti-SFPQ antibodies.

using SP-Taq DNA polymerase (Hokkaido System Science) with the luciferase fw primer, (5'-CTAAAACGGATTACCAGGGATTTCAGTCGATG-3'), and the luciferase rv primer.

Immunohistochemistry

Testes were removed from BALB/cAJcl mice deeply anesthetized with Ketalar 50 (Sankyo) and fixed overnight in Bouin fixative. The testes were embedded in paraffin and cut into 8- μ m sections. After deparaffinization and rehydration by xylene and serial dilutions of aqueous ethanol, the slides were immersed in sodium citrate buffer pH 6.0 and heated for 10 min at 121°C for antigen retrieval. The slides were washed in PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After blocking with 10% goat serum and 1% BSA, cells were incubated with a 1:10 dilution of anti-PSPC1, anti-NONO, or anti-SFPQ antibody or a 1:300 dilution of anti-WT1 rabbit polyclonal antibody (Santa Cruz Biotechnology), in 2% goat serum and 1% BSA in PBS overnight at 4°C. Subsequently, slides were washed, incubated with a 1:100 dilution of Alexa Fluor 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (Invitrogen) and goat anti-rabbit IgG (H+L)-TRITC (Zymed) in 10% goat serum and 1% BSA in PBS for 2 h at room temperature, and counterstained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride).

RESULTS

Monoclonal Antibody Production

Previously we reported that rabbit polyclonal antibody raised against bacterially expressed recombinant PSPC1 recognizes both of PSPC1 α and its splicing variant PSPC1 β , and kidney expresses PSPC1 β exclusively [41]. However, mass spectrometric analysis showed that the immunoprecipitates from kidney actually contained one isoform of SEPT4 (previously known as M-Septin) [45], but not PSPC1 β (data not shown). These two proteins have a similar molecular mass (PSPC1 β ; 45 kDa, SEPT4; 44 kDa) and share antigenic determinant EELRRXQE in PSPC1 (369–376) and SEPT4 (359–366). To avoid cross-reaction, we designed a new synthetic peptide specific to PSPC1, immunized BALB/cAJcl with the peptide, and developed a

mouse anti-PSPC1 monoclonal antibody. On Western blotting, this antibody recognized a single band of 59-kDa protein in testis and kidney (see Supplemental Figure 1, available online at www.biolreprod.org). Similarly, we developed anti-NONO and anti-SFPQ mouse monoclonal antibodies. We performed a peptide competition study to verify the specificities, and the results indicated that these antibodies specifically recognized PSPC1, NONO, or SFPQ and had no cross-reactivity with other DBHS-containing proteins (see Supplemental Figure 2, available online at www.biolreprod.org). Moreover we carried out mass spectrometric analysis of the peptides immunoprecipitated from mouse testis extracts by the antibodies. MALDI profiles revealed that anti-PSPC1 antibody immunoprecipitated PSPC1 protein, anti-NONO antibody immunoprecipitated NONO protein, and anti-SFPQ antibody immunoprecipitated SFPQ protein (see Supplemental Table 1, available online at www.biolreprod.org). These data confirmed the antigenic specificities of monoclonal antibodies, and these antibodies were used for the following analysis.

DBHS-Containing Proteins Are Expressed in Sertoli Cell Line TTE3

NONO interacts with AR and enhances transcriptional activity, and PSPC1 and SFPQ, other members of the DBHS-containing proteins, coprecipitate with AR [20]. These data suggest that PSPC1 and SFPQ may also modulate AR activity. Androgen and AR-dependent gene transcription in Sertoli cells are essential for maintaining normal spermatogenesis. We investigated whether DBHS-containing proteins are expressed in Sertoli cell line TTE3, which is a conditionally immortalized testicular Sertoli cell line from transgenic mice bearing the temperature-sensitive simian virus 40 large T antigen gene [46]. Whole-cell extracts from TTE3 cells were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with anti-PSPC1, anti-NONO, or anti-SFPQ antibody. PSPC1, NONO and SFPQ were expressed abundantly in TTE3 cells (Fig. 1). Next, we examined the intracellular distributions of DBHS-containing proteins in TTE3 cells. TTE3 cells were cultured on coverslips, fixed, and immunostained with each antibody. Strong expressions of PSPC1, NONO, and SFPQ were detected in the nucleus while faint signals for PSPC1 and NONO and an intensive signal for SFPQ were also observed in the cytoplasm (Fig. 2).

Endogenous DBHS-Containing Proteins in TTE3 Cells Form Complexes with Each Other

Complex formation among DBHS-containing proteins is a controversial issue. Fox et al. reported that PSPC1-NONO and NONO-SFPQ complexes were observed in HeLa cells, but PSPC1-SFPQ complex was not [42]. In our previous paper, all reciprocal interactions were shown by coimmunoprecipitation assays of overexpressed DBHS-containing proteins, and yeast two-hybrid assays [41]. Therefore, we investigated complex formation among endogenous DBHS-containing proteins in TTE3 cells. DBHS-containing proteins were immunoprecipitated with anti-PSPC1, anti-NONO, or anti-SFPQ antibody from TTE3 cells, and the immunoprecipitants were subjected to Western blotting with appropriate antibodies. PSPC1 coimmunoprecipitated with NONO and SFPQ, and NONO and SFPQ behaved similarly (Fig. 3). Addition of RNase to the cell extracts did not alter the immunoprecipitation, indicating that the interaction between DBHS-containing proteins is a direct protein-protein interaction. DAZAP1, which is abundantly expressed in testis, did not coimmunoprecipitate with DBHS-

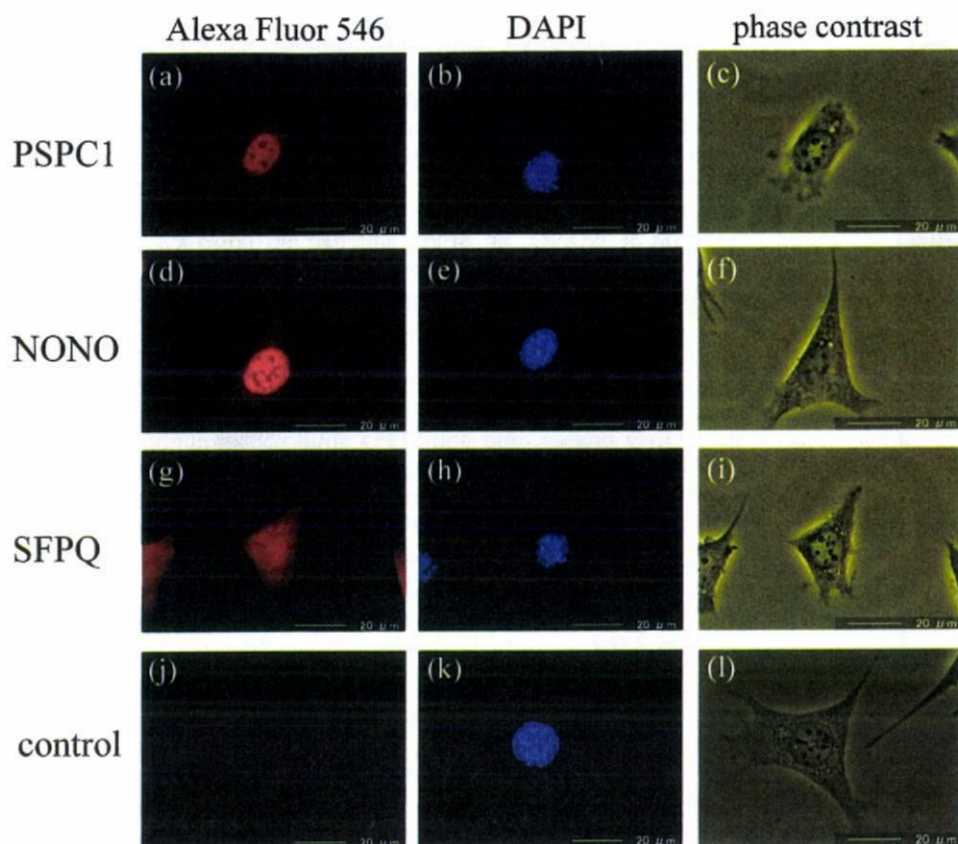


FIG. 2. Localization of the DBHS-containing proteins in TTE3 cells. TTE3 cells were grown on coverslips and labeled for immunofluorescence with antibody to PSPC1 (a), NONO (d), or SFPQ (g). The cells treated only with the secondary antibody were used as negative controls (j). b, e, h, and k) Nuclear staining by DAPI for the same samples in a, d, g, and j, respectively. c, f, i, and l) Phase contrast microscopy for the same samples in a, d, g, and j, respectively.

containing proteins [47, 48], showing that the interactions are significant. Interestingly, the amount of PSPC1 precipitated with anti-PSPC1 antibody was about the same as the amounts of NONO and SFPQ coimmunoprecipitated with PSPC1. Immunoprecipitations by anti-NONO antibody and anti-SFPQ antibody showed similar tendencies.

DBHS-Containing Proteins Regulate AR-Mediated Transcription

To investigate whether DBHS-containing proteins affect AR-mediated transcription, we planned to perform a luciferase assay with TTE3 cells. Although we tried various transfection reagents and transfection protocols repeatedly, transfection efficiency was extremely low. Therefore the luciferase assay with COS-1 cells using a reporter plasmid containing androgen-responsive elements was performed. COS-1 cells were transfected with expression plasmids for AR and DBHS-containing proteins and reporter plasmids, and treated with DHT. NONO enhanced the transactivation function of AR as previously reported [20, 21], and PSPC1 and SFPQ also enhanced the AR function. PSPC1 showed the highest transactivation of the reporter gene while NONO and SFPQ showed equally lower transactivation (Fig. 4a). DBHS-containing proteins are RNA binding proteins that share RNA recognition motifs (RRMs). We tested whether DBHS-containing proteins bind to luciferase mRNA. COS-1 cells were transfected, treated with DHT as done in the luciferase assay, and harvested. Myc-PSPC1, Myc-NONO, or Myc-SFPQ was immunoprecipitated from cell lysates with anti-Myc antibody. RT-PCR was performed for the immunoprecipitant as template with luciferase-specific primers, and weak binding of DBHS-containing proteins to luciferase mRNA was observed (Fig. 4b, lane 4). Next, to address the question

whether the enhancement of luciferase activity is influenced by post-transcriptional regulation, the RRM mutants, in which the RNA binding activity was abolished without disrupting the overall structure of the RRM domain, were constructed according to the procedure of Fox et al. [42], and luciferase assays were performed. These mutants did not show RNA binding activity (Fig. 4b, lane 2) but did show activated AR-mediated transcription as well as the wild type DBHS-containing proteins in the luciferase assay (Fig. 4a). These data demonstrate that the enhancement of luciferase activity is mediated by transcriptional regulation, not by post-transcriptional regulation.

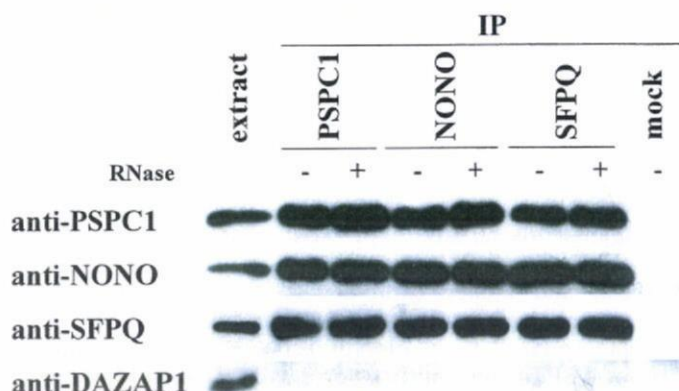
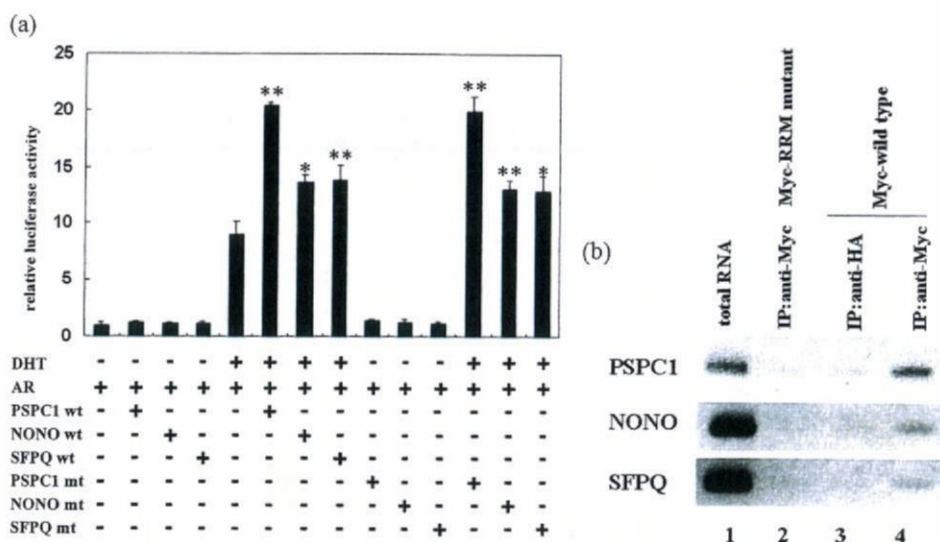


FIG. 3. DBHS-containing proteins interact reciprocally. TTE3 cell lysates treated with RNase or untreated lysates were subjected to immunoprecipitation with anti-PSPC1, anti-NONO, anti-SFPQ, or anti-Myc (9E10, for negative control) antibody. Western blotting was performed with whole extracts and immunocomplexes using an appropriate antibody.

FIG. 4. DBHS-containing proteins regulate androgen receptor-mediated transcription. **a)** COS-1 cells were cotransfected with p(ARE)₂-luc (a luciferase reporter plasmid containing androgen-responsive elements), expression vector containing AR, and wild type (wt) or RRM mutant (mt) DBHS-containing protein expression vector in the absence (-) or presence (+) of dihydrotestosterone (DHT). The bars represent the mean \pm SD. * $P < 0.05$ vs. control experiments without DBHS-containing protein expression vector; ** $P < 0.01$ vs. control experiments without DBHS-containing protein expression vector. **b)** DBHS-containing protein RRM mutants do not bind to luciferase mRNA. COS-1 cells were transfected with Myc-tagged DBHS-containing protein RRM mutant or Myc-tagged DBHS-containing protein wild type expression vectors as luciferase assay. Cell lysates were subjected to immunoprecipitation with anti-Myc or anti-HA antibody. RT-PCR was performed with immunoprecipitants as templates and luciferase-specific primers. Total cell RNA was assayed as a positive control.



DBHS-Containing Proteins Are Expressed in Sertoli Cells of Testis

DBHS-containing proteins were expressed in Sertoli cell line TTE3, along with activated androgen receptor-mediated transcription. We next examined the expression of DBHS-containing proteins in Sertoli cells of testis. Adult mouse testis sections were immunostained with anti-PSPC1, anti-NONO, or anti-SFPQ antibody. Anti-WT1 antibody was used as a marker to identify the Sertoli cells [49]. The expression of each DBHS-containing protein in seminiferous tubules displayed a distinct profile. The germ cells expressed PSPC1 and SFPQ, but not NONO (Fig. 5b, 5l and 5g, respectively). The signals of all DBHS-containing proteins were detected in the cells adjacent to the basal membrane of the seminiferous tubule. These cells also expressed WT1 (Fig. 5a, 5f and 5k), demonstrating that all the DBHS-containing proteins are expressed in the Sertoli cells.

DISCUSSION

In this study, we showed that all of the three DBHS-containing proteins are expressed in mouse Sertoli cell line TTE3, and interact reciprocally. These proteins enhanced AR-mediated transactivation. Expression of the DBHS-containing proteins in Sertoli cells of adult mouse testis suggests that the DBHS-containing proteins may play roles in spermatogenesis by regulating AR-mediated transcription in Sertoli cells.

First, we showed the abundant and equivalent expressions of all the DBHS-containing proteins in Sertoli cell line TTE3. However, in germ cells, PSPC1 and SFPQ were expressed abundantly, but NONO was not detected. In HeLa cells, Fox et al. reported that NONO is expressed at higher levels than that for PSPC1 [42]. Although expression of SFPQ is confirmed in various tissues and cell lines [50, 51], expression levels of NONO are different among tissues and cell lines [52]. PSPC1 is expressed abundantly in mouse testis [41]. These data indicate that at least expression levels of PSPC1 and NONO are different depending on tissues and cell types.

The transcriptional activity of AR is modulated by various coregulators, which include coactivators and corepressors. Luciferase assay using a reporter plasmid containing androgen-

responsive elements indicated that all the DBHS-containing proteins enhance the transcription mediated by AR. PSPC1 showed the highest transactivation of the reporter gene, and NONO and SFPQ showed equal transactivation. These data suggest that DBHS-containing proteins are coactivators of AR. The coregulators can be divided into two major types. Type I coregulators, such as HMGB1 and HMGB2 [53] and CMTM2A [54], function primarily with the nuclear receptor at the target gene promoter to facilitate DNA occupancy, chromatin remodeling, or the recruitment of general transcription factors associated with the RNA polymerase II holo-complex. Type II coregulators, such as FLNC [55] and PAK6 [56], contribute to AR protein stability in the presence of ligands or influence the subcellular distribution of AR [17-19]. Although the molecular mechanism of AR transactivation by the DBHS-containing proteins remains elusive, the DBHS-containing proteins are known to have a DNA binding domain [23, 41], and we confirmed the expression of these proteins in the nucleus of the TTE3 cell line. It was previously reported that NONO enhances the association of transcription factors to their target DNA [57], suggesting that the DBHS-containing proteins enhance the association of AR to their targets, like HMGB1 and HMGB2 enhance the binding of AR, progesterone receptor, and glucocorticoid receptors to their target DNA and enhance the transactivation [53]. Alternatively, DBHS-containing proteins might activate transactivation of AR by stimulating DNA topoisomerase I to relieve torsional strain, and this is supported by the observation that NONO and SFPQ interact with DNA topoisomerase I and activate its enzymatic activity [31]. Thus we speculate that DBHS-containing proteins belong to the Type I coregulators that functions primarily at the target gene promoter site.

DBHS-containing proteins are known not only to regulate transcription but also to bind directly to RNA for post-transcriptional regulation. DBHS-containing proteins are also known to be involved in splicing, polyadenylation, nuclear retention of edited RNA, and transport of mRNA [23, 26-30]. In TTE3 cells, SFPQ was expressed not only in the nucleus but also in the cytoplasm, and weak expression of PSPC1 and NONO was observed in the cytoplasm. We also found weak but significant expression of PSPC1 in the cytoplasm of germ

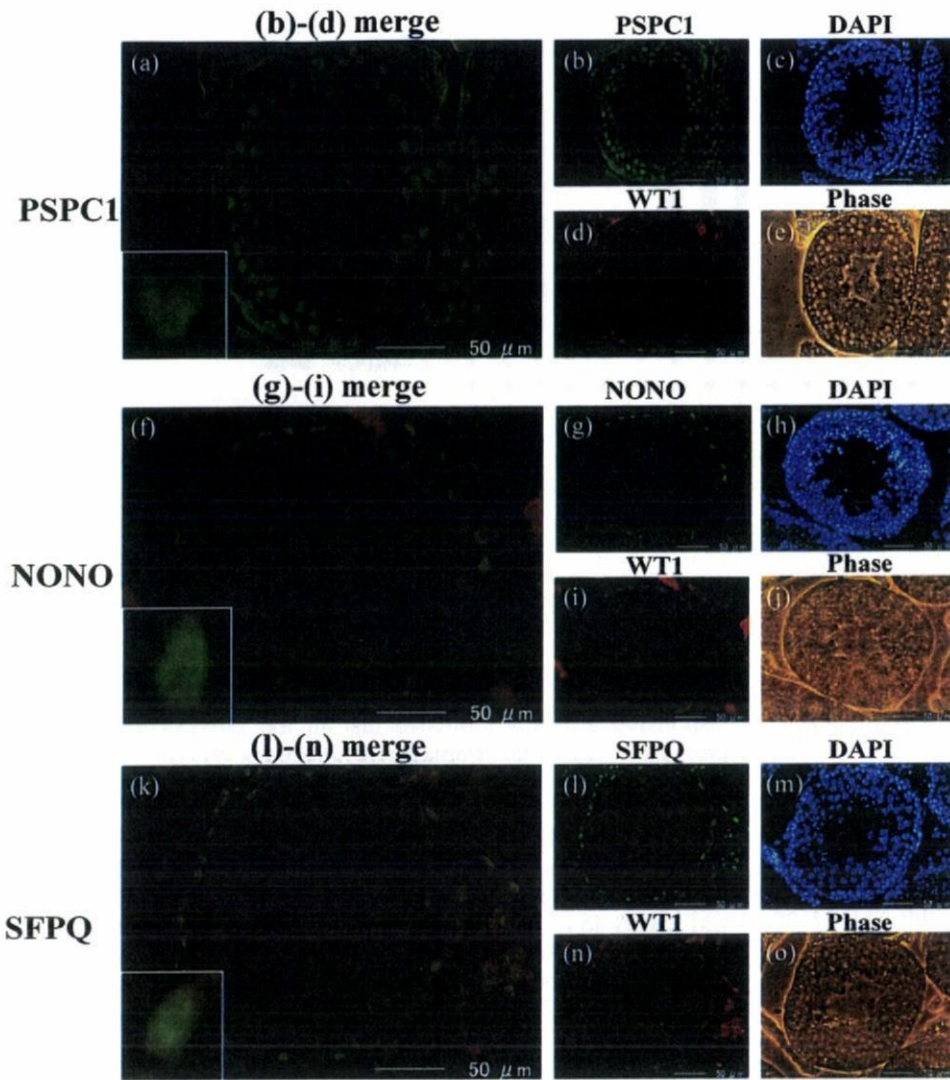


FIG. 5. Expression of DBHS-containing proteins in mouse adult testis. Sections of adult mouse testis were immunostained with anti-PSPC1 (b), anti-NONO (g), or anti-SFPQ (l) antibody. d, i, and n) Immunostaining with anti-WT1 rabbit polyclonal antibody for the same samples in b, g, and l, respectively. c, h, and m) Nuclear staining by DAPI for the same samples in b, g, and l, respectively. e, j, and o) Phase contrast microscopy for the same samples in b, g, and l, respectively. a) Merged image of b-d. f) Merged image of g-i. k) Merged image of i-n. The insets in a, f, and k show high magnifications of Sertoli cells.

cells (data not shown), suggesting that DBHS-containing proteins function also in the cytoplasm. From these findings, we speculate that DBHS-containing proteins may regulate not only transcription but also RNA metabolism such as splicing, mRNA export, and RNA nuclear retention. The DBHS-containing proteins may maintain spermatogenesis by activating AR-mediated transcription and regulating the transcribed mRNA both in the nucleus and cytoplasm of Sertoli cells.

Fox et al. reported that PSPC1 interacts with NONO, and not with SFPQ in HeLa cells [42]. However, we previously showed the interaction between overexpressed PSPC1 and NONO, and also between PSPC1 and SFPQ [41]. In this study, we investigated the complex formation among the endogenous DBHS-containing proteins in TTE3 cells, and observed all combinations of the interactions among PSPC1, NONO, and SFPQ. It has been reported that all the DBHS-containing proteins are found in the protein complex with the AR AF-1 domain [20]. Moreover, our preliminary proteomic analysis demonstrated that anti-PSPC1 antibody immunoprecipitates NONO and SFPQ together with PSPC1 in testis extract, and yeast two-hybrid assays using PSPC1 as a bait protein showed the interaction of PSPC1 with NONO and SFPQ (data not shown). Therefore PSPC1 actually interacts with both NONO and SFPQ. We assume that the inconsistency between the data by Fox et al. and by us may come from the difference in the interacting partner in the cell line used, not only from the

difference in expression levels of the DBHS-containing proteins. If we assume that PSPC1 forms only heterodimers with NONO or SFPQ, PSPC1 should immunoprecipitate most abundantly among these proteins. However, the amount of PSPC1 precipitated with anti-PSPC1 antibody was almost the same as those of NONO and SFPQ coimmunoprecipitated with PSPC1. Immunoprecipitations by anti-NONO antibody and anti-SFPQ antibody also showed similar tendencies. These results raise the possibility that the DBHS-containing proteins may form multimers. It was previously reported that Hrp65 protein, *Chironomus tentans* homolog of the PSPC1/NONO/SFPQ family, can self-associate, and most of the Hrp65 protein are in complexes that consist of three to six Hrp65 protein molecules [58], suggesting that PSPC1, NONO, and SFPQ also form large complexes by interacting reciprocally. DBHS-containing proteins are known not only to regulate transcription but also to be involved in multiple regulations including RNA metabolism [23, 26–30] and DNA metabolism [31, 32]. It is likely that the differences in the composition of the DBHS-containing protein complex bring about functional diversity of DBHS-containing proteins.

We confirmed the expressions of all the DBHS-containing proteins in Sertoli cells of adult mouse testis, and these proteins exhibited enhancement of transactivation of AR. PSPC1 and SFPQ were expressed also in germ cells. Because germ cells do not express AR, PSPC1 and SFPQ expressed in germ cells may