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Expression, Immunolocalization and Sperm-Association of A Protein Derived From 24p3 Gene in Mouse Epididymis

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ABSTRACT The cDNA sequence for 24p3 protein in ICR mouse epididymal tissue was determined by PCR using primers designed according to the cDNA sequence derived from 24p3 protein in mouse uterine tissue. In the present study, 24p3 protein was immunolocalized in the epithelial cells and lumen of mouse epididymis. Both immunoblot analysis for protein and northern blot analysis for mRNA level showed a declining gradient of *24p3* expression from the caput to caudal region of the epididymis. The 24p3 protein was undetectable in the testis. These findings suggest that the 24p3 protein is a caput-initiated secretory protein in the mouse epididymis. A postnatal study revealed that *24p3* gene expression occurred in mice at the age of 14 days, before the completion of epididymal differentiation. This expression remained at a constant level until epididymal differentiation was completed. We also found that the secreted 24p3 protein interacted predominantly with the acrosome of caudal spermatozoa. Our findings suggest that the epididymal 24p3 protein is a caput-initiated and sperm-associated gene product and may be important in the reproductive system. *Mol. Reprod. Dev. 57:26–36, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: caput-initiated; epididymis; lipocalin; secretory protein; spermatozoa

INTRODUCTION

Mammalian spermatozoa acquire their motility and the ability to fertilize an oocyte during passage through the epididymis. These maturational events are believed to be dependent on the microenvironment created by the absorptive and secretory functions of the epididymis (Orgebin-Crist and Fournier-Delpech, 1982; Amann et al., 1993; Hinton and Palladino, 1995). The principal components of this environment are specific proteins that are synthesized and secreted in certain regions (caput, corpus or cauda) of the epididymis (Garberi et al., 1979; Brooks, 1981; Devine and Carroll, 1985). These proteins may be important in spermatozoal changes that occur in the different regions of the epididymis during postnatal development and may also be involved in regulating the functional integrity of spermatozoa. Although the regional-specific expression of epididymal proteins has been established for some time, the identity and function of these proteins in spermatozoa have not been elucidated. A number of secretory proteins from epididymal epithelium and the hormonal regulation of their synthesis have been previously studied (Rankin et al., 1992; Lefrancois et al., 1993; Bendahmane and Abou-Haila, 1994), but the biological functions of these proteins remain unclear. While the ontogeny of epididymal protein expression in rats has been studied (Brooks, 1987; Charest et al., 1989; Ueda et al., 1990) and rabbits (Toney and Danzo, 1989), few comparable studies of mice epididymal proteins have been reported.

We previously characterized a 25 kDa mouse uterine glycoprotein named mouse 24p3 protein, which is an estrogen-regulated lipocalin secreted from the uterine epithelium. Its cDNA sequence is identical to that of 24p3-cDNA, which has been cloned from primary cultures of SV40-infected kidneys in mice (Hraba-Renevey et al., 1989; Chu et al., 1997, 1998). Previously, we purified 24p3 protein from mouse uterine luminal fluid and demonstrated by northern blot analysis that the *24p3* gene is normally expressed in the lung, spleen, uterus, vagina and epididymis of mice (Chu et al., 1996). In the reproductive organ of the male mouse, *24p3* gene expression is unique to the epididymis. The protein derived from the *24p3* gene has also been found in lipopolysaccharide-stimulated mouse PU5.1.8 macrophage cells (Meheus et al., 1993) and bFGF-stimulated 3T3-cells (Davis et al., 1991). Liu and Nilson-Hamilton (1995) showed that 24p3 protein is an acute phase protein in liver. The function of 24p3 protein in the reproductive tract has not been reported. In the present study, we found that the epididymal caput is responsible for 24p3 protein secretion and *24p3* gene expression. We also established that the 24p3

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protein associates with epididymal spermatozoa. The 24p3 protein's synthesis and secretion in epididymis and association of 24p3 protein on spermatozoa may concern spermatozoal maturation.

MATERIALS AND METHODS

Materials

Diethylstilbestrol dipropinate (DES) and aprotinin were obtained from Sigma (St. Louis, MO). [α-^{32}P]dATP and ^{125}I-labeled anti-rabbit IgG prepared from donkeys were purchased from Amersham International (Bucks, U.K.). Anti-rabbit IgG-alkaline phosphatase and IgG-fluorescein conjugate prepared from goats were obtained from Sigma (St. Louis, MO). All of the reagents and enzymes used in cDNA preparation, PCR, and the T7 DNA polymerase sequencing system were purchased from Promega (Madison, WI). A Geneclean kit was purchased from BIO101, Inc. (La Jolla, CA). The random primed DNA probes (Prime-A-Gene kit) were obtained from Promega (Madison, WI). All chemicals were of reagent grade.

Animals

Outbred male mice were purchased from Charles River Laboratories (Wilmington, MA) and were maintained and bred in the animal center at the College of Medicine, National Taiwan University. Animals were treated in accordance with the institutional guidelines for the care and use of experimental animals. The animals were killed by cervical dislocation and the epididymis was removed from each animal for further study.

RNA Isolation, cDNA Preparation and Northern Analysis

Total cellular RNA was isolated and double-stranded cDNAs were prepared on the polyadenylated fraction of epididymal RNA by a standard procedure (Sambrook et al., 1989) using AMV reverse transcriptase (Promega, Madison, WI). Total cellular RNA was isolated and electrophoresed in 1% (w/v) agarose gel containing 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (1 mM EDTA, 20 mM MOPS, 5 mM Na-acetate, pH 7.0) and 2.2 M formaldehyde. Transfer to nylon filter (hybond-N$^+$; Amersham) for blotting and northern analysis was performed as previously described (Thomas, 1980). Hybridization to the specific probe was performed overnight at 42°C in a hybridization buffer. The ^{32}P-labeled random-priming kit used a template of a cDNA segment of the mouse *24p3* gene (579 bp) (Hraba-Renevey et al., 1989) or a cDNA segment of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (1.25 kbp) inserted into the pGEM3 vector.

Polymerase Chain Reaction (PCR)

Based on the previously established structure of mouse 24p3 cDNA (Hraba-Renevey et al., 1989), we synthesized one oligonucleotide, CTGGGCCTTGCCCTGCTTGGGGTC, which represents nucleotide 44–67 of 24p3 cDNA and another oligonucleotide, GTTGTCAATGCATTGGTCGGTGGG, which is complementary to nucleotides 599–622 of 24p3 cDNA. These two oligonucleotides were employed as the primer pair for PCR, which amplified the single-stranded cDNAs of epididymis with Taq polymerase for 30 cycles: 94°C for 30 sec; 58°C for 30 sec; 72°C for 1 min. The reaction mixture was subjected to electrophoresis on 2.0% agarose gel. The amplified DNA (579 bp), which was extracted from the gel with a Geneclean kit (BIO101, Inc.), was sequenced according to the sequencing system using both oligonucleotide of the primer pair as the primer for PCR. Each base was determined at least three times. The random-primed DNA probe was prepared using the Prime-A-Gene kit by the method of Feinberg and Vogelstein (1983).

Electrophoresis and Western-Blot Analysis

The luminal fluid was collected by microperfusion of epididymal tubules. Epididymes were removed from the mice and separated into three parts as caput, corpus, and cauda. Then 200 µl phosphate-buffered saline (PBS), containing 10 mM EDTA and 30 µg/ml aprotinin, was added into each part of the intact-tissue and centrifuged at 300 *g* for 5 min to tightly compress the tissues. The supernatant was collected as luminal fluid and the pellet as epididymal fluid-free tissue. The epididymal fluid-free tissue was homogenized in PBS buffer as saline-extracted protein. Epididymal tissues along with or separated from luminal fluid in their ducts were homogenized in PBS/10 mM EDTA in the presence of aprotinin (30 µg/ml) and centrifuged at 100,000 *g* for 20 min in a Beckman airfuge (Beckman, Palo Alto, CA). The concentration of proteins in the clarified supernatant and collected luminal fluid were determined by the modified Lowry method (Lowry et al., 1951) and resolved by SDS-PAGE [15% (w/v) acrylamide] on a gel slab. Proteins were transferred from the gel to a poly(vinylidene difluoride) membrane (Bowen et al., 1980) in PBS at 4°C for 18 hr by diffusion. The transferred proteins were detected with the 1 µg/ml of 24p3 protein-induced antibody, followed by ^{125}I-labeled donkey anti-rabbit IgG diluted to 1:5000 and fluorography. Using the 24p3 protein-induced antibody diluted to 1 µg/ml as the primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase diluted (Sigma, St. Levis, MO) to 1:2000 as the secondary antibody, the sperm-extraction proteins were assayed on the immunoblot. The reactive bands were visualized by enhanced chemiluminescence (ECL) (RPN2132, Amersham Pharmacia Biotech UK Limited) with exposure to X-ray film.

Antibody Preparation

The 24p3 protein was purified from female uterine luminal fluid as previously described (Chu et al., 1996). The 24p3 protein in normal saline (500 µg/ml) was mixed with an equal volume of Freund's complete adjuvant. Each New Zealand white rabbit received a

subcutaneous injection of 1 ml of the mixture. After 4 weeks and 8 weeks, the rabbit was treated in the same way with 250 μg of antigen in a mixture of normal saline and incomplete adjuvant (1:1 by volume). Two weeks after the last boost, blood was collected from the ear vein and the serum fraction was applied to a protein A-sepharose column (Phamarica, Uppsala, Sweden) for IgG isolation. The partially purified antibody was used for immunodetection of 24p3 protein throughout this study. The preimmuserum for control assay was collected from the ear vein by a capillary tube before immunization.

Histology and Immunohistochemistry

Tissues were fixed in freshly prepared Bouin's solution [0.2% picric acid/2% (v/v) formaldehyde in PBS] overnight, dehydrated in ethanol, infiltrated, and embedded in paraffin. Each tissue section (7 μm) was mounted on a slide that had been precoated with Vetabond reagent (Burlingame, CA). Sections were dried at 45°C, paraffin was removed in xylene and the sections were rehydrated through a gradient from alcohol to distilled water. The rehydrated sections were placed in a saturated lead thiocyanate solution and heated as described by von Wasielewski and co-workers (1994). Slides were cooled at room temperature for 15 min, then rinsed in distilled water and PBS each for 15 min. The slides were immersed in a blocking solution (5% nonfat skimmed milk in PBS) in a moisture chamber at 25°C for 1 hr and washed four times with PBST for 15 min each. The epididymal sections on slides were incubated with the 24p3-induced antibody diluted to 1 μg/ml in the blocking solution. Slides were gently agitated in four changes of PBST for 15 min each, then the antibody against 24p3 protein was immunodetected with alkaline phosphatase conjugated anti-rabbit IgG diluted 1:1000 in the blocking solution. The chemical staining was conducted in the presence of 0.033% nitro blue tetrazolium (NBT) and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris-HCl containing 100 mM NaCl and 5 mM $MgCl_2$ at pH 9.0 for 15 min at room temperature. After chemical staining, sections were washed with three changes of PBST for 15 min each.

Epididymal spermatozoa were smeared and air-dried on a glass slide and then immersed in methanol for 20 sec to fix the spermatozoa on the slides. The slides were rinsed twice with PBST and then incubated with blocking solution for 1 hr before histochemical staining. To examine the 24p3 protein associated with spermatozoa, the spermatozoa were incubated with 24p3 protein (4.0 μM) for 60 min and then incubated with 24p3 protein-induced antibody diluted to 1 μg/ml in the blocking buffer for 60 min. After individual incubation, the slides were washed with three changes of PBST for 15 min each. The slides were then incubated with fluorescein-conjugated goat anti-rabbit IgG diluted to 1:400 in blocking solution for 60 min. After incubation, the slides were washed with three changes of PBST for 15 min each. Control slides were allowed to react with both the primary and secondary antibodies without 24p3 protein.

The specimens and spermatozoa on the slides were covered with 50% (v/v) glycerol in PBS and photographed with a microscope equipped with epifluorescence (AH3-RFCA; Olympus, Tokyo, Japan).

The 24p3 Protein Extraction From Spermatozoa

The 24p3 protein was extracted from spermatozoa as described by Rankin and co-workers (1992). Briefly, spermatozoa from caput epididymis (10^6 cells) were suspended in 0.5 ml Tyrode's without BSA containing 1 × Protease Inhibitor Cocktail (PI Cocktail, Cat. No. 1697498, Roche Molecular Biochemicals, Germany) at room temperature and then centrifuged at 80 *g* for 4 min. The spermatozoa suspension was then divided into two equal parts. One part was further washed twice with HM containing PI Cocktail. After each washing, supernatants (low-salt wash) were collected as control samples (C1S and C2S) and the final pellet was resuspended with an equal volume of double-strength Lammli electrophoresis buffer containing 0.125 M Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, and 10% β-mercaptoethanol (SDS extract for control; C3S). The other part of the spermatozoa suspension was resuspended in 200 μl of HM containing 0.5 M NaCl (high-salt wash) for 30 min after low-salt buffer treatment. After high-salt washing, the suspension was collected by centrifugation for 4 min at 500 *g* (HS). The spermatozoa extracted with the high-salt buffer were incubated in 200 μl of HM containing 0.1% Triton X-100 for 30 min on ice and then centrifuged at 500 *g* for 10 min, and the supernatant was collected as Triton-extracted proteins (TS). The Triton-extracted pellet was finally extracted with an equal volume of double-strength Laemmli electrophoresis buffer (SDS extract; SS). All of the SDS extracts were spun for 4 min in a microcentrifuge to collect the extracted proteins for SDS-PAGE. The extracted suspensions were desalted into distilled water and concentrated to volumes of 30 μl by a Microcon-10 device (Amicon Corp., Danver, MA). Ten microliter of each protein extract was added with an equal volume of double-strength Laemmli buffer and subjected to SDS-PAGE and western blotting for analysis.

Spermatozoa Preparation

Tissues were minced gently in a modified Tyrode's solution containing 125 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 4.5 mM glucose, 0.09 mM pyruvate, 25.0 mM lactate, penicillin (100 IU/ml), streptomycin (100 mg/ml), and 5×10^{-4}% phenol red at pH 7.4 (Fraser, 1985). The spermatozoa were extruded out of minced tissues by tearing with a pair of tweezers. The cell suspension was gently filtered through two layers of nylon gauze (400 mesh) and collected by centrifugation at 150 *g* for 10 min. The cells were

washed twice with the medium and resuspended in PBS (10^7 cells/ml) before use.

RESULTS

Sequence Analysis of Mouse Epididymal 24p3 cDNA

We amplified cDNA prepared from the epididymis of mature male mice by PCR using the primer pair (Fig. 1, single underline) designed for amplification of nucleotides 44–622 of 24p3 cDNA (see Materials and Methods section). A major DNA product (~ 579 bp) was found in these nucleotide sequences identical to nucleotides 44–622 of the uterine 24p3 cDNA sequence (Fig. 1). These data reveal that the deduced amino acid sequences of epididymal cDNA are similar to those of mouse uterine 24p3 protein, and further imply that 24p3 mRNA exists in the epididymis of mature male mice. The data strongly support that *24p3* gene expression occurs in the reproductive tract not only in female mice but also in male mice. The deduced amino acid sequence from the nucleotide sequence showed three conserved motifs from members of lipocalin superfamily (Fig. 1, double underline), which have been suggested to be involved in a ligand-binding site. As a result, it is possible for epididymal 24p3 protein to bind a hydrophobic molecule in the reproductive tract. Although the function of mouse uterine 24p3 protein is yet to be determined precisely, it is found that a lipocalin in uterine fluid also exists in epididymis.

Regional Difference of 24p3 Gene Expression in the Epididymis

Using immunoblot analysis, 24p3 protein content intensity was found to be greatest in the caput, less intense in the corpus, and undetectable in the caudal region (Fig. 2A, lane 2–4). Similarly, 24p3 protein was present at higher levels in the luminal fluid of the caput and corpus compared to the caudal fluid (Fig. 2A, lane 5–7). The specific distribution of the transcripts for 24p3 protein in adult mouse epididymis was also determined by northern analysis. Comparisons of mRNA expression level of *24p3* gene were made among the caput, corpus, and caudal regions of mature mouse epididymis. Figure 2B (upper panel) shows the expression of 24p3 mRNA in each part of the epididymis as assayed by northern blot analysis. The *24p3* gene was highly expressed in the caput (Fig. 2B, lane 1), declined sharply in the corpus (Fig. 2B, lane 2) and was almost undetectable in the cauda (Fig. 2B, lane 3). Expression of GAPDH mRNA (internal control) was constant in these regions (Fig. 2B lower panel). The data indicate

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5`--AGACCTAGTAGCTGTGGAAACC
ATG GCC CTG AGT GTC ATG TGT CTG GGC CTT GCC CTG CTT GGG GTC CTG CAG AGC CAG GCC     82
 M   A   L   S   V   M   C   L   G   L   A   L   L   G   V   L   Q   S   Q   A     -1

CAG GAC TCA ACT CAG AAC TTG ATC CCT GCC CCA TCT CTG CTC ACT GTC CCC CTG CAG CCA    142
 Q   D   S   T   Q   N   L   I   P   A   P   S   L   L   T   V   P   L   Q   P     20
+1
GAC TTC CGG AGC GAT CAG TTC CGG GGC AGG TGG TAC GTT GTG GGC CTG GCA GGC AAT GCG    202
 D   F   R   S   D   Q   F   R   G   R   W   Y   V   V   G   L   A   G   N   A     40
                                  motif 1
GTC CAG AAA AAA ACA GAA GGC AGC TTT ACG ATG TAC AGC ACC ATC TAT GAG CTA CAA GAG    262
 V   Q   K   K   T   E   G   S   F   T   M   Y   S   T   I   Y   E   L   Q   E     60

AAC AAT AGC TAC AAT GTC ACC TCC ATC CTG GTC AGG GAC CAG GAC CAG GGC TGT CGC TAC    322
 N   N   S   Y   N   V   T   S   I   L   V   R   D   Q   D   Q   G   C   R   Y     80

TGG ATC AGA ACA TTT GTT CCA AGC TCC AGG GCT GGC CAG TTC ACT CTG GGA AAT ATG CAC    382
 W   I   R   T   F   V   P   S   S   R   A   G   Q   F   T   L   G   N   M   H    100

AGG TAT CCT CAG GTA CAG AGC TAC AAT GTG CAA GTG GCC ACC ACG GAC TAC AAC CAG TTC    442
 S   Y   P   Q   V   Q   S   Y   N   V   Q   V   A   T   T   D   Y   N   Q   F    120
                              motif 2
GCC ATG GTA TTT TTC CGA AAG ACT TCT GAA AAC AAG CAA TAC TTC AAA ATT ACC CTG TAT    502
 A   M   V   F   F   R   K   T   S   E   N   K   Q   Y   F   K   I   T   L   Y    140

GGA AGA ACC AAG GAG CTG TCC CCT GAA CTG AAG GAA CGT TTC ACC CGC TTT GCC AAG TCT    562
 G   R   T   K   E   L   S   P   E   L   K   E   R   F   T   R   F   A   K   S    160
                      motif 3
CTG GGC CTC AAG GAC GAC AAC ATC ATC TTC TCT GTC CCC ACC GAC CAA TGC ATT GAC AAC    622
 L   G   L   K   D   D   N   I   I   F   S   V   P   P   D   Q   C   I   D   N    180

TGA --3                                                                           625
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Fig. 1. The nucleotide sequence of mouse epididymal cDNA. The nucleotide sequence (44–622) was compiled from six independent mouse epididymal cDNA. Underline indicates the primer pairs used for PCR in the epididymal 24p3 gene study. The deduced amino acids are shown by capital letters below the nucleotide sequence. Double underline indicates the three short motifs which are highly conserved between members of the lipocalin protein family (Flower et al., 1991).

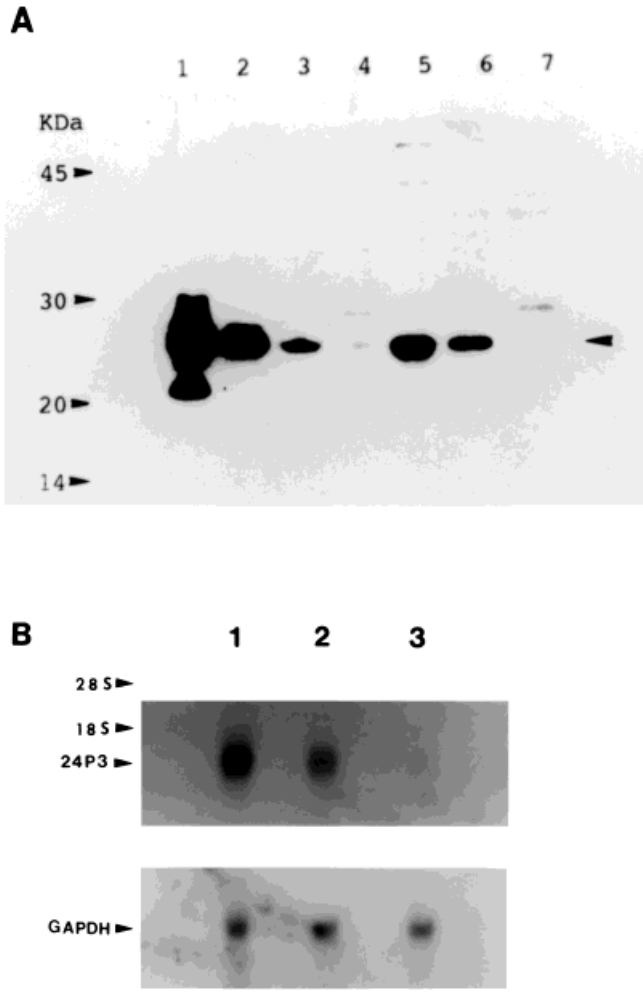

Fig. 2. Immunoblotting and northern blot analysis of mouse epididymal fluid and epididymal homogenate with 24p3 protein-induced antibody and 24p3 cDNA probe. (**A**) Protein extracts (50 μg), prepared from various populations, were separated by SDS/PAGE and processed for the immunoblotting procedure as described in Materials and Methods. Lane 1, crude uterine fluid from mouse uterus; lane 2, the saline extract of the epididymal caput; lane 3, the saline extract of the epididymal corpus; lane 4, the saline extract of the epididymal cauda; lane 5, the fluid of epididymal caput; lane 6, the fluid of epididymal corpus; lane 7, the fluid of epididymal cauda. The right-hand arrow indicates the position of 24p3 protein. (**B**) Northern blot analysis of epididymal RNA from adult mice. Total RNAs (50 μg), prepared from different regions of epididymal homogenate, were run on 1% agarose/formaldehyde gel and transferred to a nylon membrane. The membrane was probed with ^{32}P-labeled random-primed DNA from a cDNA segment of either mouse 24p3 protein (upper) or GAPDH (below). The level of GAPDH mRNA served as an internal control. Lane 1, RNA from epididymal caput; lane 2, RNA from epididymal corpus; lane 3, RNA from epididymal cauda.

that *24p3* gene expression in the caput region was more active than in the corpus epididymis and the caudal epididymis.

Figure 3 shows the results from testis and epididymis using light microscopy to describe the immunolocalization of 24p3 protein. No reactive staining was seen in the basal compartment of the seminiferous epithelium or in the cytoplasmic processes between germ cells. None of the germ cells, including spermatogonia, early spermatocytes, spermatids, and spermatozoa showed immunoreactive staining for 24p3 protein (Fig. 3E). There was a complete absence of reactive staining over the entire testis and epididymis as preimmuserum was used (Fig. 3A–D). Significant immunostaining was found in the epithelial cells of the caput (Fig. 3F, arrowhead E). Reactive stains were also present in luminal spermatozoa of the proximal caput (Fig. 3F, arrowhead S). In the corpus region, principal epithelial cells and luminal spermatozoa were weakly stained (Fig. 3G), and were undetectable in the caudal region (Fig. 3H). The results of northern blot analysis coincided with observations from western analysis and immunohistochemical study, all of which strongly suggest that the expression of *24p3* gene occurs mainly in the caput. These results clearly demonstrate that expression of the male mouse *24p3* gene and the secretion of 24p3 protein initiate in the caput region of the epididymis.

Developmental Profile of 24p3 Protein in Epididymis

It is well-known that the secretory proteins of the epididymis create a microenvironment for spermatozoal maturation during the developmental period (Amann et al., 1993; Hinton and Palladino, 1995). In mice, the pubertal period—defined as the completion of spermatogenesis and the first mating (Jean-Faucher et al., 1978)—occurs between 35 and 40 days of age. We compared the levels of 24p3 protein and the implications of these levels in the epididymis of male mice at different ages determine whether *24p3* gene expression accompanies the development of this reproductive organ. As shown in Fig. 4A, 24p3 protein synthesis was detectable in the epididymis at the age of 2 weeks (lane 2) and thereafter remained at a constant level throughout the maturation process from 3 to 7 weeks of age (lane 3–7). The levels of 24p3 mRNA in epididymis at different ages were also examined. As shown in Fig. 4B, 24p3 mRNA was detectable when mice were 2 weeks old (lane 1). The amount of mRNA in immature mouse was not significantly different from that in mature mice (lane 2–6). The expression of the *24p3* gene in developing epididymis showed a very stable pattern during pre- and post-pubertal development. Immunohistochemical study of 24p3 protein was performed on tissue section from the caput region of the epididymis of 2-, 4-, 6-, and 12-week-old mice. Immunoreactive staining particles existed clearly at 2-weeks of age (Fig. 5E) and remained steadily visible until 12-weeks of age (Fig. 5F–H). No reactive staining was present in control sections (Fig. 5A–D). These results suggest that 24p3 protein may be synthesized by the translation of 24p3 mRNA at a constant level in the epithelium of the epididymis and that the subsequent secretion of 24p3 protein from the epithelial cells results in its accumulation in the lumen. These findings imply that *24p3* gene expression is maintained

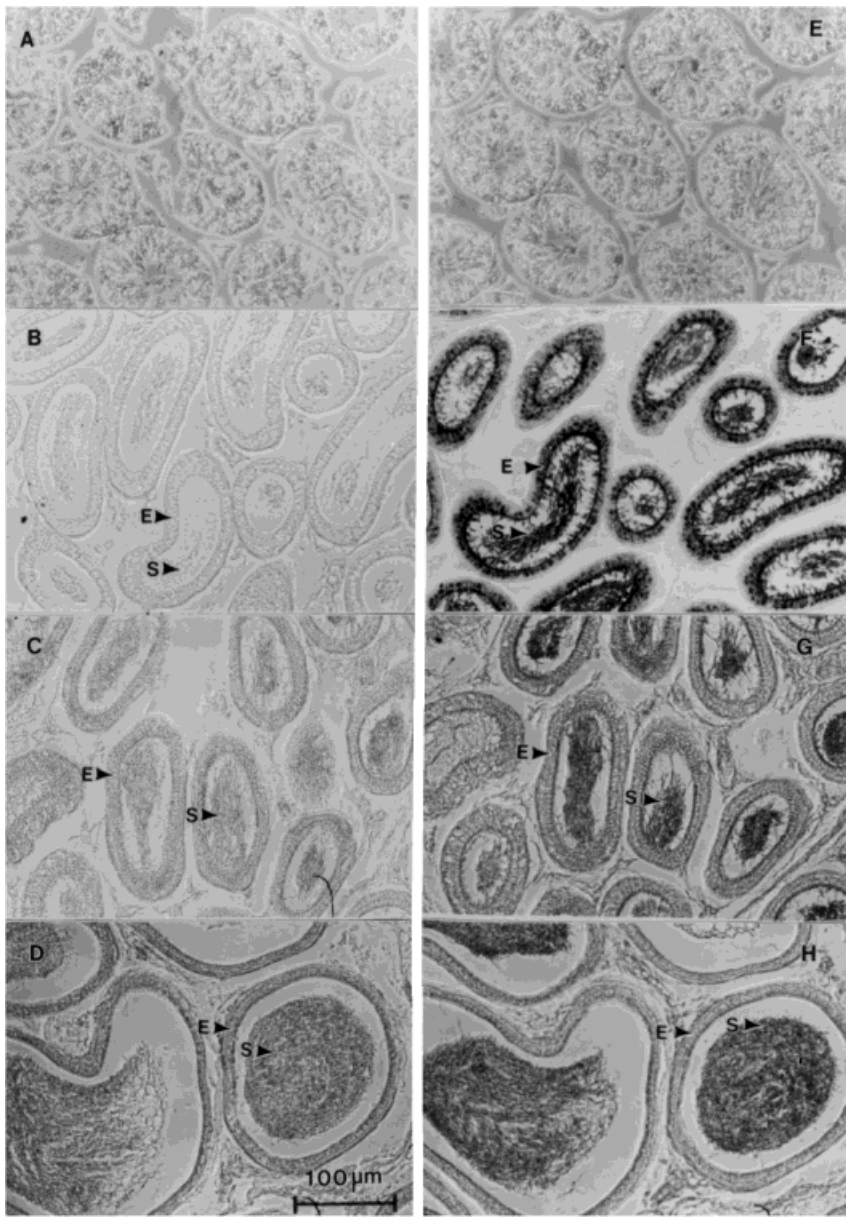

Fig. 3. Immunohistochemical localization of 24p3 protein in mouse testis and epididymal sections. Tissues were fixed in Bouin's solution and thick sections were stained with 24p3 protein-induced antiserum at a concentration of 1 μg/ml as described in Materials and Methods. (**A** and **E**) testis, (**B** and **F**) caput of epididymis, (**C** and **G**) corpus of epididymis, (**D** and **H**) cauda of epididymis. A–D represent the control sections reacted with preimmuserum. E–H indicate the immunoreactive staining of 24p3 protein in tissues. Arrowhead E and arrowhead S indicate the epithelium of epididymis and luminal spermatozoa, respectively. Magnification × 100. Bar = 100 μm.

at a constant level in mice throughout the developmental period.

The 24p3 Protein Interacts With Spermatozoa

The results shown in Fig. 3 imply that the 24p3 protein may associate with spermatozoa. To examine this possibility, spermatozoa from the caput epididymis were sequentially extracted as described in Materials and Methods. Figure 6 shows that 24p3 protein was present in the supernatants of the low-salt and high-salt washes (lane C1S, C2S, and HS), and that a substantial amount of 24p3 protein was present in the Triton X-100 extracts of caput spermatozoa (lane TS) and SDS-extract for Triton-extracted spermatozoa (lane SS). These results indicate that the sperm-associated protein was firmly bound, requiring solubilization of the spermatozoal membrane by Triton X-100 (lane TS) or SDS (lane C3S) to be extracted. To determine the interaction of 24p3 protein with spermatozoa, spermatozoa from the caudal segment of mouse epididymis were extruded as described in Materials and Methods. The control slide had no reactive staining with the FITC-conjugated secondary antibody (Fig. 7A). When the caudal spermatozoa were preincubated

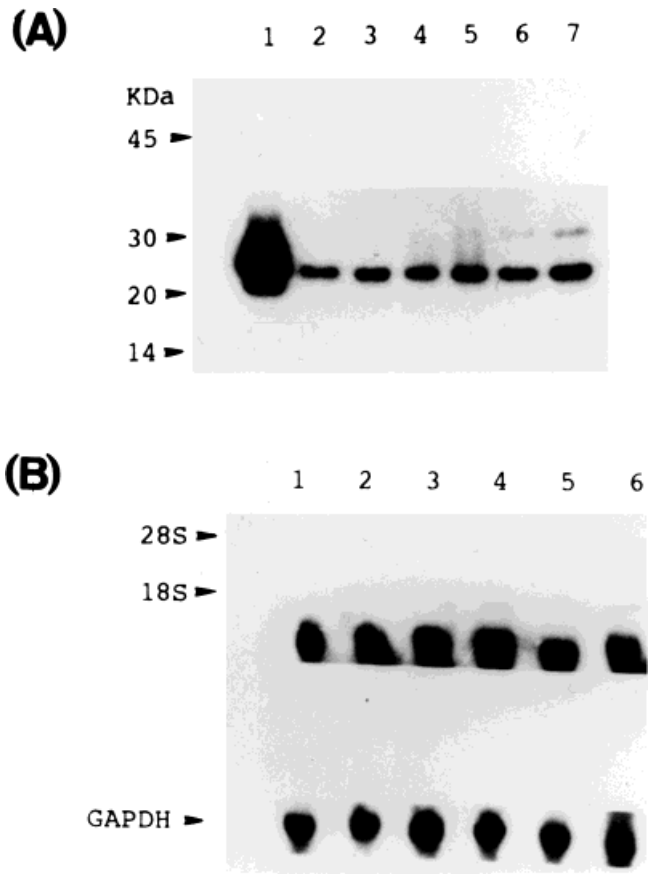

Fig. 4. Ontogeny of 24p3 protein synthesized by mouse epididymis. (**A**) Proteins (50 μg) of epididymal homogenate were resolved by 15% SDS-PAGE and then immunodetected by western blot procedures with antibody to mouse 24p3 protein and followed by ^{125}I-labeled anti-rabbit IgG detection. Lane 1, positive control of crude uterine fluid from female mouse; lanes 2–7, the epididymal protein extract from 2-, 3-, 4-, 5-, 6- and 7-week-old male mice. (**B**) Northern blot analysis of mouse total RNA. Total RNA (50 μg) prepared from epididymal homogenate was run on a 1%-agarose-formaldehyde gel, transferred to a nylon membrane and probed with ^{32}P-labeled random-primed DNA from a cDNA segment of either mouse 24p3 (upper) or GAPDH (below). The level of GAPDH mRNA served as an internal control. Lane 1 to lane 6 indicate the level of 24p3 mRNA from 2–7 week-old mice.

with 24p3 protein before the performance of indirect histochemical staining, a crescent fluorescence zone on the anterior region of the spermatozoal acrosome was visible, implying that this was the 24p3 protein-association site on the acrosomal region (Fig. 7B). Figures 7C and D show the caudal spermatozoa morphology under phase contrast microscopy. These results revealed the association site of the 24p3 protein on the spermatozoal acrosome.

DISCUSSION

Spermatozoa maturational changes occur in the epididymis. The particular importance of epididymal secreted-proteins in promoting these changes has been well-established in several types of animals (Orgelin-Crist and Jahad, 1979; Gonzalez et al., 1984) but not in mouse. Previously, we demonstrated that 24p3 protein, an estrogen-regulated lipocalin, is expressed and secreted by epithelial cells in the uterus (Chu et al., 1996; Huang et al., 1999). In the present study, we found an mRNA sequence that is translatable into 24p3 protein expressed in mouse epididymis. This finding is consistent with a previous report of *24p3* gene expression in male mice (Chu et al., 1996). The 24p3 protein identified in the present study was found in epididymal tissue homogenate and luminal fluid, suggesting that the synthesis and secretion of the protein was most abundant in the caput region. Northern blot and immunohistochemical analysis demonstrated that 24p3 protein's synthesis and tissue localization patterns are different from other epididymal-secreted proteins (Rankin et al., 1992; Cornwall and Hann, 1995). Since spermatozoal maturation occurs early in the proximal epididymis and completes in the cauda (Amann et al., 1993; Hinton and Palladino, 1995), we hypothesized that the caput-initiated secretory 24p3 protein was a good candidate for investigation of spermatozoal maturation in the reproductive tract. Jimenez et al. (1990) and Ghyselinck and Dufaure (1990) reported the immunolocalization of a 24 kDa secretory protein in mouse epididymis that binds to the spermatozoa and shares sequence homology with glutathione peroxidase. Vernet and co-workers (1997) also presented a detailed study of the distribution of the peroxidase protein (GPX5) in mouse epididymis. Rankin and co-workers (1992) reported a 25 kDa secretory protein (MEP9) in the proximal and mid-caput of mouse epididymis whose antibody cross-reacted with a 25 kDa testicular antigen (MTP). Araki and co-workers (1992) characterized MTP as a member of the phospholipid-binding protein family, and suggested that MTP may have a role in lipid metabolism during spermatozoal maturation. However, according to gene sequencing and immunoreactive localization data, 24p3 protein and these other identified proteins are distinct, hence this epididymal caput-synthesized and secreted protein may serve a biological function quite different from other proteins within the epididymis. Our Triton-extracted study indicated an association of 24p3 protein with spermatozoa, and immunocytochemical studies demonstrated that 24p3 protein is associated with the anterior acrosomal membrane of epididymal spermatozoa. We speculate that 24p3 protein existing in uterine luminal fluid (Chu et al., 1996) may associate with spermatozoa in the female genital tract; i.e., that spermatozoal processing occurs during epididymal transit and/or that physiological changes occur in sperm within the female reproductive tract. Further studies are necessary to determine whether the 24p3 protein affects spermatozoal activity.

Sexual hormones appear to be essential for maintaining the synthesis and secretion of reproductive proteins (Ghyselinck et al., 1989; Lefrancois et al., 1993), whereas some secretory proteins are hormone-independent (Defelein et al., 1996). In the present study, we found that 24p3 protein is expressed in

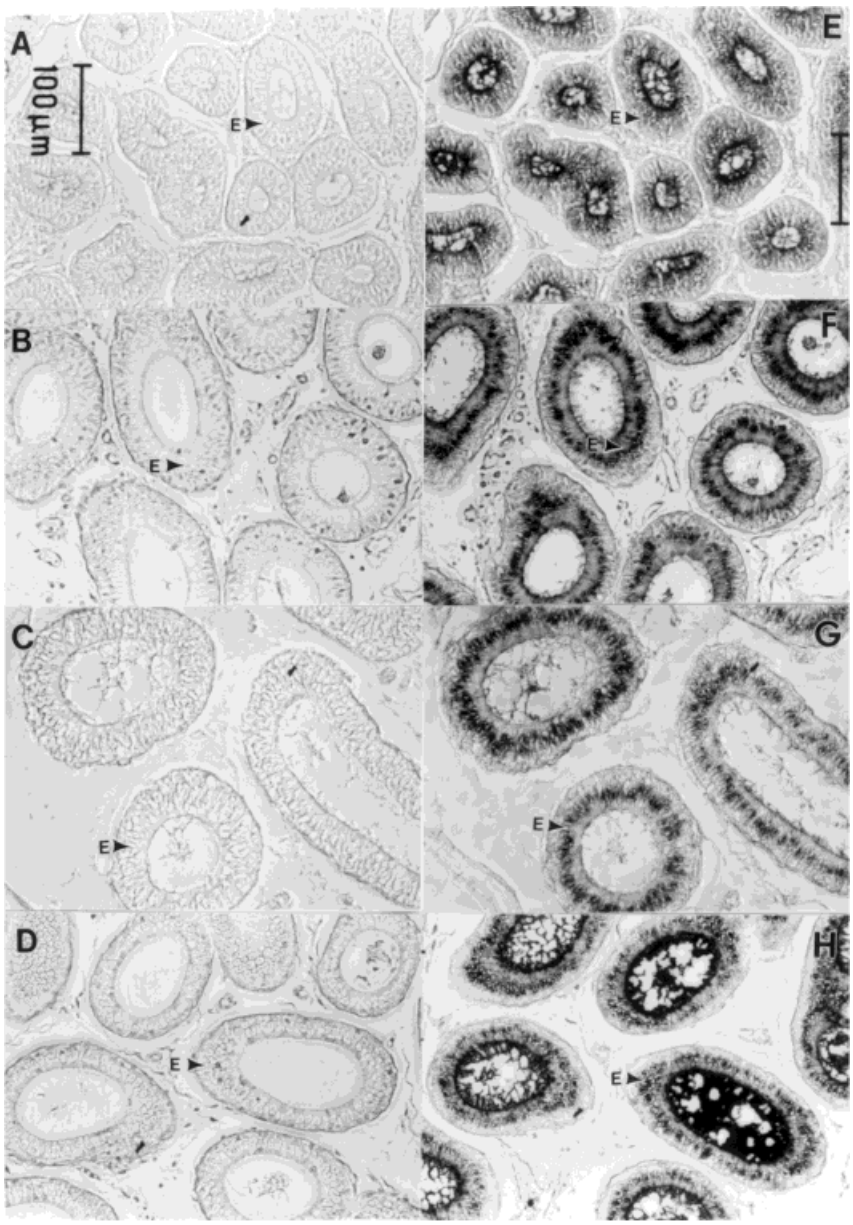

Fig. 5. Epididymal localization of 24p3 protein during postnatal development. Epididymal tissues of mice of different ages were fixed in Bouin's solution and stained with antibody to 24p3 protein at a dilution of 1 μg/ml as described in Materials and Methods. **A–D** represents the control sections of 2-, 4-, 6-, and 12-week-old mouse epididymis. **E–H** shows reactive staining of 2-, 4-, 6-, and 12-week-old mouse epididymis. The arrowhead E indicates the location of 24p3 protein in the epithelium of epididymis. Magnification × 100. Bar = 100 μm.

mouse epididymis from the prepubertal period to adulthood. Our immunohistochemical study showed that 24p3 protein appears in 14-day-old mice prior to differentiation of the epididymis, which is completed at around day 20 (Abou-Haila and Fain-Maurel, 1985) while the androgen content is still low (Jean-Faucher et al., 1985). Indeed, in the mouse epididymis, the content of androgen is age-dependent. Androgen increases progressively during development, and reaches its highest level at 40 days of age and then remains relatively constant until 90 days of age (Jean-Faucher et al., 1985). In the present study, the 24p3 protein and mRNA levels in mouse epididymis were constant from the age of 2–12 weeks. There was no significant correlation between androgen content and the level of 24p3 gene expression in the epididymis during development. Based on these results, 24p3 protein might be considered a constitutive gene product in the luminal environment for spermatozoa from rete testis in male mouse. One way to investigate the influence of sexual hormones on the production of 24p3 protein in vivo would be to examine the effect of injection of short-term sexual hormones into castrated male mice.

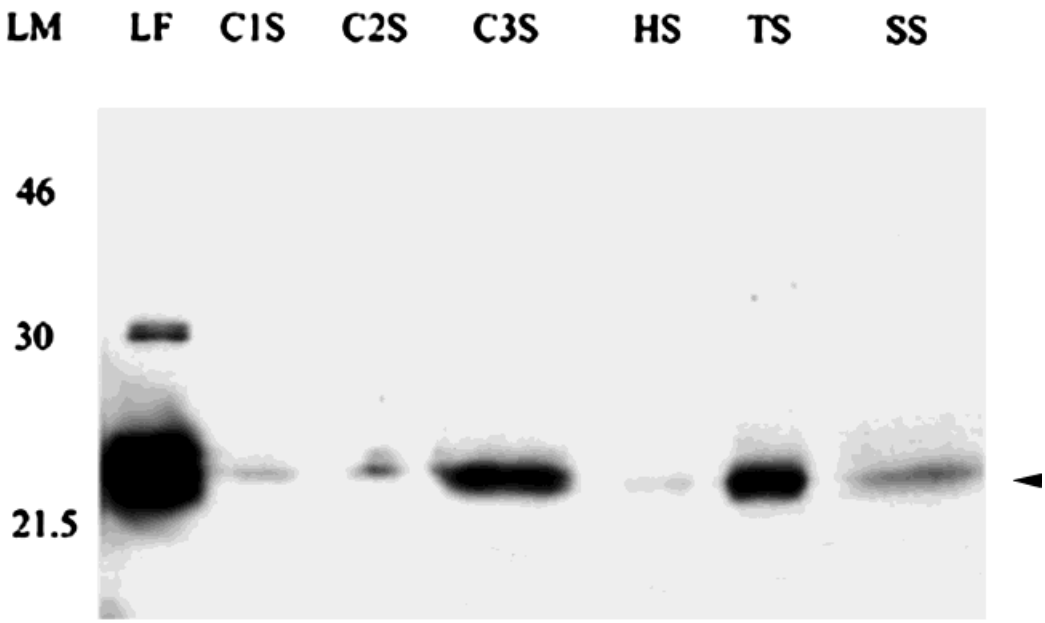

Fig. 6. Sequential extraction of caput spermatozoa. Proteins were extracted as described in Materials and Methods, then resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with 24p3 protein-induced antibody. Lane LF, caput luminal fluid; lane C1S, the first extract with low-salt buffer; lane C2S, the second extract with low-salt buffer; C3S, SDS extracted protein of spermatozoa with twice low-salt wash; HS, 24p3 protein in high-salt extract; TS, the Triton X-100 extractable proteins; SS, SDS extract for Triton-extracted spermatozoa. The arrow indicates the 24p3 protein.

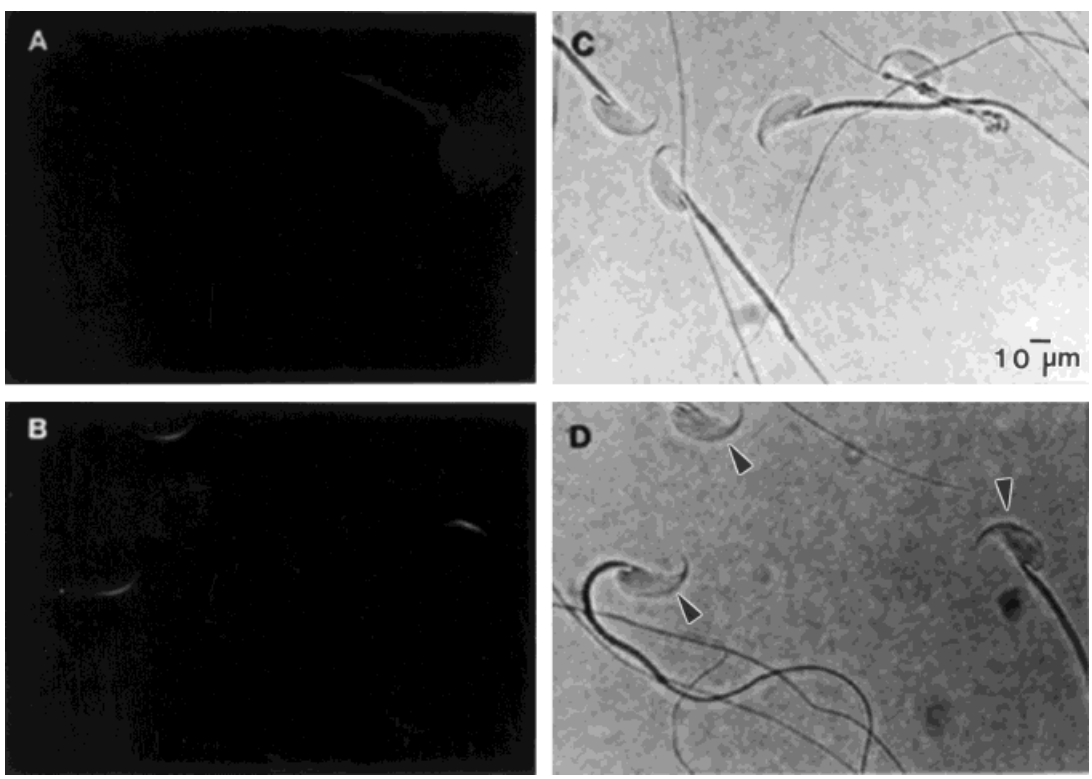

Fig. 7. Indirect immunofluorescence demonstration of the 24p3-protein binding zone on epididymal spermatozoa. Fresh epididymal spermatozoa were smeared and fixed by methanol on glass slides. (**A**) The slide was incubated with 1 μg/ml 24p3 protein-induced antibody and followed with fluorescein-conjugated goat anti-rabbit IgG (1:400 dilution) as control. (**B**) The fresh cells were incubated in blocking buffer in the presence of 40 μM 24p3 protein for 1 hr and allowed to react with 24p3 protein-induced antibody in the blocking buffer (1 μg/ml) for 1 hr, followed by fluorescein-conjugated goat anti-rabbit IgG. A fluorescence microscope was used to observe the slides. (**C**) and (**D**) are as observed under phase contrast microscope under the same conditions as (A) and (B), respectively. The arrows indicate spermatozoal acrosome. Magnification $\times$ 1000. Bar = 10 μm.

The presence of high levels of epididymal retinoic acid–binding protein in the epithelial cell and lumen of the epididymis was thought to indicate that this protein was a retinoid transporter and hence a factor necessary for spermatozoal maturation (Sundaram et al., 1998; Lareyre et al., 1998). A sperm coating lizard epididymal secretory protein (LESP), also a lipocalin of epididymal secretory protein, was suggested to be a hydrophobic molecule transporter during spermatozoal maturation (Laurent et al., 1993). The definitive roles of retinoic acid and hydrophobic molecules in spermatozoal maturation are not yet clear, but they are thought to be important. We previously described 24p3 protein as a lipocalin in uterus, and that the protein showed an ability to bind small hydrophobic ligands such as fatty acids, cholesteryl ester, and retinoids (Chu et al., 1998). It seems reasonable to speculate that the association of 24p3 protein on spermatozoal acrosome may serve as a hydrophobic molecule transfer protein in the epididymal lumen and be involved in spermatozoal maturation.

Although the exact function of mouse 24p3 protein remains unknown, the observations that this protein is synthesized early in epididymal caput, associates with spermatozoa, and is secreted from uterus suggest that 24p3 protein plays an important role both in spermatozoal processing and fertilization. Such a possibility warrants further investigation.

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