Plasminogen Activator and Mouse Spermatozoa: Urokinase Synthesis in the Male Genital Tract and Binding of the Enzyme to the Sperm Cell Surface

Joaquin Huarte, Dominique Belin,* Domenico Bosco, André-Pascal Sappino,‡ and Jean-Dominique Vassalli

Institute of Histology and Embryology, *Department of Pathology and ‡Division of Oncohematology, University of Geneva Medical School, 1211 Geneva 4, Switzerland

Abstract. When ejaculated mouse spermatozoa were embedded in a plasminogen-containing insoluble protein substrate, a zone of proteolysis developed progressively, centered around the sperm head region. Lysis did not occur in absence of plasminogen or in presence of antibodies against the urokinase-type plasminogen activator (u-PA). Zymographic and immunological analyses confirmed the presence of u-PA in extracts of ejaculated mouse spermatozoa. In contrast, the u-PA activity of sperm cells obtained from testis or from vas deferens was low, although these cells were able to bind added murine u-PA. The sites of u-PA synthesis were identified by measuring u-PA activity and u-PA mRNA content in protein extracts and in total RNA preparations of various portions of the male genital tract. The highest levels of u-PA activity and of u-PA mRNA were found in vas deferens and seminal vesicles. The cells that synthesize u-PA were localized by hybridizing frozen sections of various portions of the genital tract to a u-PA cRNA probe. In all tissues examined, u-PA mRNA was predominantly located in the epithelial layer, and the strongest signal was observed over that of the vas deferens. Hence, the u-PA associated with ejaculated sperm cells is probably acquired from genital tract secretions. Sperm-bound u-PA may participate in the proteolytic events that accompany capacitation and fertilization.

SPERMATOGENESIS occurs in the seminiferous tubules of the testis. Spermatozoa released from the seminiferous epithelium (spermiation) progress towards the epididymis where they become mature and capable of fertilizing ova (Austin, 1985). The epididymal secretions are believed to contain enzymes, nutrients, and hormones, that may be essential for sperm maturation (Bedford, 1975; Hamilton, 1975). Most spermatozoa are stored in the cauda epididymis and vas deferens until ejaculation at coitus; at this time, secretions from accessory glands contribute to the final composition of semen. In rats, mice, and guinea pigs, the semen clots into a hard rubbery mass almost immediately after ejaculation (Williams-Ashman, 1983). Finally, spermatozoa undergo an additional ripening process called capacitation, that occurs normally in the female genital tract but can also be accomplished in vitro (Austin, 1985).

It has been suggested that limited proteolysis could play a role in the apparent loss and/or modifications of sperm surface molecules that occur during epididymal maturation (Eddy et al., 1985) and capacitation (Talbot and Franklin, 1978; Talbot and Chacon, 1981). Furthermore, enzymes of tryptic specificity have been proposed to be involved in the binding of mouse spermatozoa to *zonae pellucidae* (Saling, 1981) and in the acrosome reaction (Meizel and Lui, 1976;

Meizel, 1985). However, besides acrosin, the major tryptic protease of the acrosome, no other characterized protease has been as yet implicated in mammalian sperm maturation, capacitation, or fertilization.

Plasminogen activators (PAs)[1] are highly selective trypsin-like serine proteases that convert plasminogen, an inactive zymogen present in most extracellular fluids, into plasmin, a trypsin-like protease of broad specificity. The two known PAs, urokinase (u-PA) and tissue-type PA (t-PA), are secreted by a large number of cells and they are thought to have multiple functions in the extracellular proteolysis that accompanies tissue remodeling and cell migration (Reich, 1978; Danø et al., 1985). Previous studies have provided evidence for the presence of PAs in the male reproductive tract. Tympanidis and Astrup (1968) showed that rat, rabbit, and human sperm cells express plasminogen-dependent fibrinolytic activity; in addition, both u-PA and t-PA were found in human seminal plasma (Zaneveld et al., 1974; Åstedt et al., 1979). PAs are produced in discrete portions of the seminiferous tubules where two tissue remodeling processes are tak-

---

1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; PA, plasminogen activator; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; WM, modified Whitten medium.

ing place: the detachment of preleptotene spermatocytes from the basal lamina, and spermiation (Lacroix et al., 1979, 1981; Vihko et al., 1984). Recently, Larsson et al. (1984) detected u-PA immunoreactivity in the epithelium of the murine vas deferens. These observations prompted us to reinvestigate the proteolytic activity of spermatozoa with particular attention to PAs.

In this study, we report that washed sperm cells from ejaculates express u-PA activity around the head region of the spermatozoa. We show that u-PA is produced by segments of the male genital tract, and that it binds to the sperm cell membrane at the time of ejaculation. Together with our recent description of t-PA production by mouse oocytes (Huarte et al., 1985), these results point towards a possible implication of PAs in mammalian fertilization.

Materials and Methods

Materials

Adult NMRI mice (Kleintierfarm Madöring, Basel) were used. Diisopropylfluorophosphate (DFP) was from Fluka (Buchs, Switzerland). Culture media of MSV-3T3 (~20 Ploug U/ml; Belin et al., 1984) and A431 (~30 Ploug U/ml; Stoppelli et al., 1986 b) cells were used as sources of murine and human pro-u-PA, respectively. The source of all other products, including antibodies, has been described (Huarte et al., 1985; Busso et al., 1986).

Preparation of Sperm Cells

Superovulated females (Huarte et al., 1985) were used to ensure high frequency of undelayed matings. Females were caged individually with males 13 h after chorionic gonadotropin injection, and matings monitored. Immediately after mating, the females were killed and the vaginal plug dissected from the vagina. Ejaculated spermatozoa were obtained by incubating the recovered plug for 30 min at 37°C in 3 ml of modified Whitten's medium (WM; Hoppe and Pitts, 1973). Sperm suspensions obtained from the different sources described in Results were washed as follows: cell suspensions were brought to 5 ml with WM, centrifuged 5 min at 200 g to eliminate debris and cell aggregates; the supernatants were then centrifuged 5 min at 900 g to recover sperm cells and the pellet was resuspended in 5 ml of WM. After counting, aliquots were again centrifuged and resuspended in appropriate volumes.

Binding studies were achieved by incubating sperm cells, in 50 μl of control or u-PA-containing culture media (MSV-3T3 and A431 culture media were diluted 33-fold and 50-fold respectively), for 45 min at 37°C in a humidified atmosphere of 5% CO_2 in air. Samples were centrifuged 5 min at 12,000 g, and washed twice with 1 ml of WM; after the third centrifugation, sperm cells were extracted as indicated below.

Enzymatic Analyses

The PA plaque assay (Vassalli et al., 1977) was performed in WM medium. Protein extracts of sperm cells were prepared by centrifuging the cells 5 min at 12,000 g and extracting the pellets with 15 μl of NET-TS (0.5 M NaCl, 0.001 M EDTA, 0.05 M Tris-HCl, pH 8.1, 1% Triton X-100, 0.2% SDS). Organ extractions were achieved by homogenizing tissues in a loose fitting Dounce in 0.1 M Tris-HCl, pH 8.1, 0.25% Triton X-100. Immunoprecipitation, gel electrophoresis, and zymography were performed as described (Huarte et al., 1985). Sensitivity to DFP, an inhibitor of serine esterases that irreversibly phosphorylates their active site, was tested by two additions of 10 mM DFP in 0.1 M NaPO₄, pH 7.4, for a total incubation period of 1 h at room temperature; the enzymatic activity was then analyzed by zymography after SDS PAGE. Sperm denudation was performed as described by Brown et al. (1975).

Plasmid Constructions and In Vitro Transcriptions

To obtain a single-stranded probe for mouse u-PA mRNA, the 658-bp Pst I–Hind III fragment (positions 420–1,078) of the mouse u-PA cDNA clone pDB29 (Belin et al., 1985) was subcloned in pSP64 (pSP64-MU) (Melton

et al., 1984). To generate a control probe, the same fragment was also subcloned, in the opposite orientation, in pSP65 (pSP65-MU). To prepare a probe for mouse t-PA mRNA, the 290-bp Pst I–Pvu II fragment (positions 1,389–1,679; Pennica et al., 1983) of the mouse t-PA cDNA clone pUC9-A33 (Rickles, R., and S. Strickland, manuscript in preparation) was subcloned between the Sma I and Pst I sites of pSP65 (pSP65-MT).

In vitro transcription with SP6 RNA polymerase of pSP64-MU and pSP65-MT, both linearized with Pst I, was performed exactly as described (Melton et al., 1984; Busso et al., 1986), and generated cRNA probes complementary to the u-PA and t-PA mRNAs, respectively. Transcription of pSP65-MU, linearized with Hind III, generated a u-PA mRNA fragment. The ^{32}P-labeled cRNA probes were synthesized in the presence of 12.5 μM α^{32}P-labeled GTP (400 Ci/mmol, Amersham, UK). ^{3}H-Labeled probes were synthesized in the presence of 30 μM ^{3}H-labeled UTP (40 Ci/mmol, Amersham, UK). The size of the ^{3}H-labeled RNAs was reduced to 50–100 nt by mild alkaline hydrolysis at 50°C in 50 mM Na₂CO₃ (30–40 min for the u-PA probe and 10–15 min for the t-PA probe). After neutralization with 1 M HOAc, phenol-chlorophorm extraction, and ethanol precipitation, the RNAs were resuspended in 3–5 μl H₂O and diluted in hybridization mixture.

RNA Analysis

RNA extractions and Northern blot hybridizations were performed exactly as described elsewhere (Busso et al., 1986).

Cryopreserved tissue sections were prepared for in situ hybridizations as described by Hafen et al. (1983). Briefly, 5 μm cryostat tissue sections were mounted on gelatinized microscope slides, fixed in 1% paraformaldehyde in PBS for 20 min at room temperature and dehydrated in graded ethanol. Fixed sections were incubated in 0.2 M HCl for 20 min at room temperature, rinsed in water, and immersed for 30 min in 2× SSC (1× SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0) at 70°C. Sections were then treated with 0.25 mg/ml pronase for 10 min at room temperature, washed in PBS containing 2 mg/ml of glycine, postfixed in 4% paraformaldehyde in PBS for 20 min at room temperature, dehydrated in graded ethanol, and air dried. 10–20 ng of ^{3}H-labeled RNAs were applied to each section in 20 μl of hybridization mixture (50% deionized formamide, 0.3 M NaCl, 1× Denhardt's solution, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mg/ml tRNA, and 10% dextran sulfate) (Cox et al., 1984). The sections were covered with coverslips and sealed with rubber cement. Hybridizations were performed in a moist chamber for 14 h at 50°C. Slides were subsequently washed in 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, for 4 h at 50°C and 18 h at 35°C. Sections were dehydrated in 90% ethanol, 0.3 M NH₄Ac, air dried, and immersed in IL-4 emulsion (Ilford Ltd., Basildon, Essex, England). After 3 wk of exposure, they were developed in Kodak D-19 developer, fixed in 30% Na thiosulfate, and counterstained in methylene blue.

Results

Urokinase Is Associated with Spermatozoa

Ejaculated sperm cells were obtained from the vagina of superovulated females immediately after mating. Spermatozoa were washed and analyzed for proteolytic activity by determining their ability to produce lytic zones in a casein substrate (Fig. 1). Proteolytic areas, centered around the head of the sperm cells (Fig. 1, a and e), developed during incubation at 37°C. The proteolysis was completely dependent on the presence of plasminogen in the incubation medium (Fig. 1 c), and was markedly inhibited in the presence of anti–mouse u-PA IgG (Fig. 1 b).

An extract of sperm cells was subjected to SDS PAGE, followed by zymography in the presence of plasminogen (Fig. 2, lane 2). The formation of a single zone of lysis suggested the presence of a single protease migrating with an apparent M_r of 48,000. To characterize this protease, we analyzed equivalent extracts of sperm cells by zymography in the absence of plasminogen, or after immunoadsorption with antibodies directed against the two known types of PAs, u-PA and
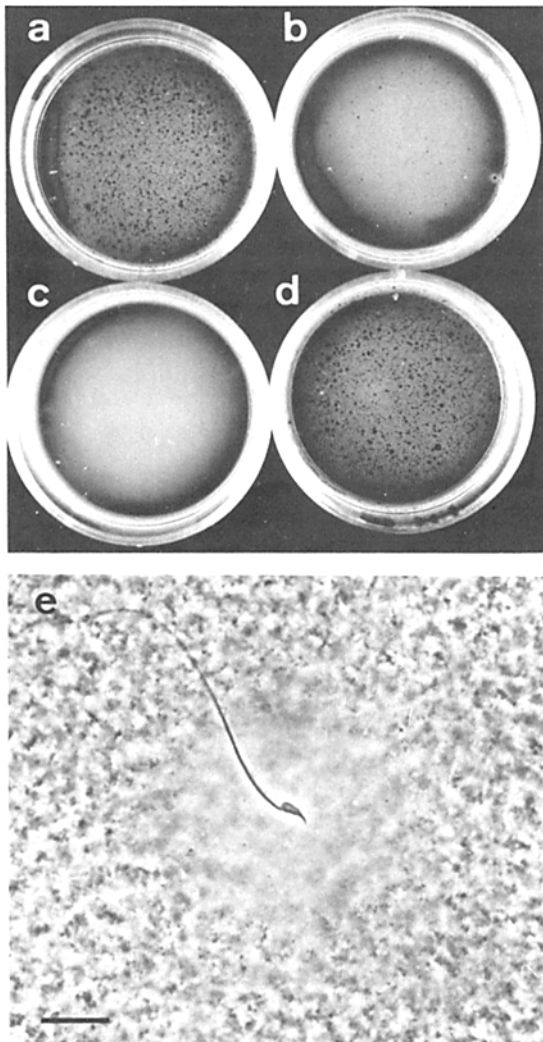
Figure 1. Plasminogen activator plaque assay of ejaculated spermatozoa. 30,000 washed sperm cells were plated on 35-mm petri dishes in 160 μl of agar–casein medium in the presence (*a*, *b*, *d*, and *e*) or absence (*c*) of plasminogen. Anti–murine u-PA IgG (*b*) or irrelevant IgG (*d*) were included at a final concentration of 200 μg/ml. In conditions *a* and *d*, almost all spermatozoa produced proteolytic plaques comparable to that illustrated in *e*. Photographs were taken under dark-field illumination (*a–d*) or by phase-contrast microscopy (*e*) after 3 h of incubation at 37°C. Bar, 20 μm.

t-PA (Fig. 2). No proteolytic activity was observed either in the absence of plasminogen (lane *1*) or after immunoadsorption with anti–u-PA IgG (lane *4*). In contrast, immunoadsorption with anti–t-PA IgG (lane *3*) or irrelevant IgG (lane *5*) did not affect the proteolytic activity of the sperm extract. Finally, the protease was recovered from the immune complexes formed with anti–u-PA IgG (lane *7*).

These results indicated that ejaculated sperm cells express an enzyme electrophoretically and antigenically indistinguishable from u-PA. The spermatozoa-associated u-PA was not affected by addition of DFP to the sperm extract before zymography (Fig. 2, lane *9*), under conditions which led to complete inhibition of active two-chain u-PA (not shown); this suggests that it is the proenzyme, single chain form of u-PA which is associated with ejaculated spermatozoa.
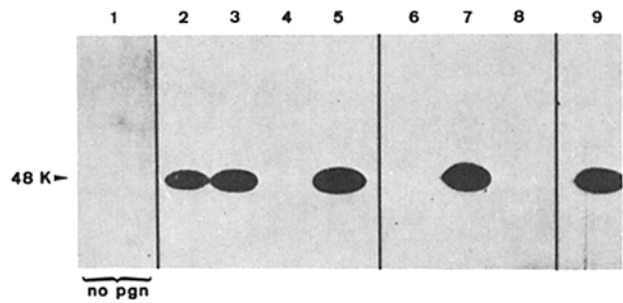

Figure 2. Characterization of the proteolytic activity of spermatozoa by SDS PAGE and zymography. Extracts of 100,000 sperm cells were analyzed in the absence (lane *1*) or presence (lanes *2–9*) of plasminogen; before (lanes *1* and *2*) or after immunoadsorption with anti–human t-PA IgG (lane *3*), anti–murine u-PA IgG (lane *4*), or irrelevant IgG (lane *5*); after treatment with DFP (lane *9*). Eluates of the *S. aureus*-bound immune complexes formed with anti-t-PA IgG (lane *6*), anti–u-PA IgG (lane *7*), and irrelevant IgG (lane *8*) were also analyzed. The photograph was taken after 15 h of incubation at 37°C.

Urokinase Becomes Associated with Spermatozoa after They Have Left the Testis

Spermatozoa were obtained from the testis by squeezing seminiferous tubules with fine forceps, and from dissected vas deferens by allowing for spontaneous draining. No proteolytic activity was detected by SDS PAGE and zymography in the extract of testicular sperm cells (Fig. 3, lane *1*), and only weak u-PA activity was found in the extract of sperm cells that had drained spontaneously from vas deferens (lane *2*). In contrast, when the cells were prepared from vas deferens (lane *3*) or cauda epididymis (not shown) by squeezing these organs with forceps, a common procedure to obtain
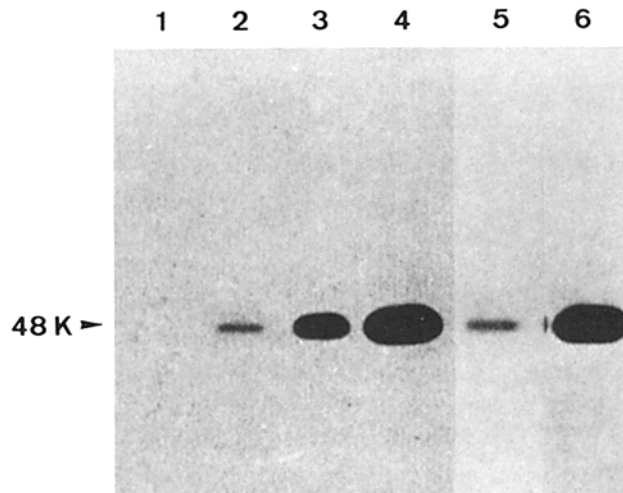

Figure 3. u-PA activity of spermatozoa obtained from the following: testis (lane *1*); spontaneous draining of vas deferens (lane *2*); squeezed vas deferens (lane *3* and *5*); ejaculate after coitus (lane *4*); ejaculate without coitus (lane *6*). Extracts of the same number of cells (100,000) were analyzed in each lane. Lanes *5* and *6* were from a different experiment in which the picture was taken earlier; this accounts for the difference between lanes *3* and *5*. The zymogram of lanes *1–4* was incubated for 12 h at 37°C, and that of lanes *5* and *6* for 6 h at 37°C.
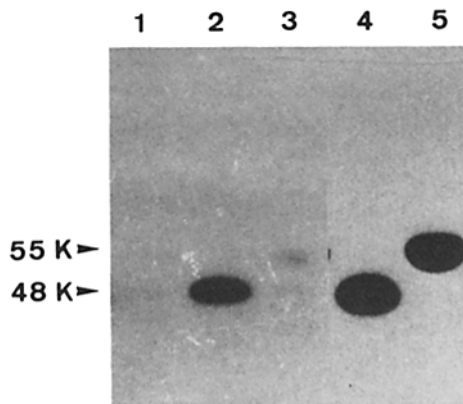
Figure 4. Binding of added u-PA to spermatozoa. Sperm cells from spontaneous draining of vas deferens were incubated in control culture medium (lane *1*), murine pro–u-PA–containing medium (lane *2*), or human pro–u-PA–containing medium (lane *3*). Extracts of 100,000 cells from the different conditions were analyzed by zymography after SDS PAGE. Analyses of incubation media used for binding experiments: murine (lane *4*) and human (lane *5*) pro–u-PA–containing media.

ing of dissected vas deferens were incubated in presence of equivalent catalytic concentrations of murine or human pro-u-PA, extensively washed, and analyzed by zymography after SDS PAGE (Fig. 4). Whereas only very little endogenous u-PA was detected in the extract of these spermatozoa (lane *1*), added mouse pro–u-PA was found to associate with the cells (lane *2*). No significant binding of human pro–u-PA to mouse spermatozoa was detected (lane *3*). Similar results were obtained with testicular spermatozoa (not shown). Thus, the association of exogenous u-PA with spermatozoa from vas deferens is reminiscent of the specific binding of u-PA to human monocytes (Vassalli et al., 1985; Stoppelli, et al., 1985) and to other cell types (Bajpai and Baker, 1985; Stoppelli, et al., 1986*a*; Fibbi et al., 1986). We have recently observed that this binding obeys a characteristic species specificity (see Discussion).

Localization of Proteolytic Activity in Spermatozoa

The results presented above suggest that sperm-associated u-PA is acquired upon binding of the enzyme to the plasma membrane of spermatozoa, and that it is most abundant in the head region of these cells (Fig. 1 *e*). To determine whether u-PA is indeed associated with the plasma membrane, we subjected the cells to a procedure known to result in denudation of mammalian spermatozoa (Brown et al., 1975). Zymographic analysis after SDS PAGE revealed that most of the u-PA was lost upon removal of the plasma membrane (Fig. 5 *a*, lane 2).

Denuded spermatozoa were also analyzed by the casein plaque assay (Fig. 5, *b–d*). The denudation protocol also removes the outer-acrosomal membrane, and thereby exposes components associated with the inner-acrosomal membrane, including the major sperm protease acrosin (Brown et al., 1975). It was thus not surprising to detect proteolysis around the head of such denuded sperm cells (Fig. 5 *c*); as expected, and in contrast to the result obtained with intact ejaculated spermatozoa (Fig. 1), inclusion of anti–u-PA IgG did not decrease the proteolytic activity of denuded spermatozoa (Fig. 5 *d*). Unexpectedly, however, the presence of plasminogen in the substrate was required for the observed proteolysis to develop (Fig. 5 *b*). Similar results were obtained with testicular spermatozoa, which do not have associated u-PA (data not shown).

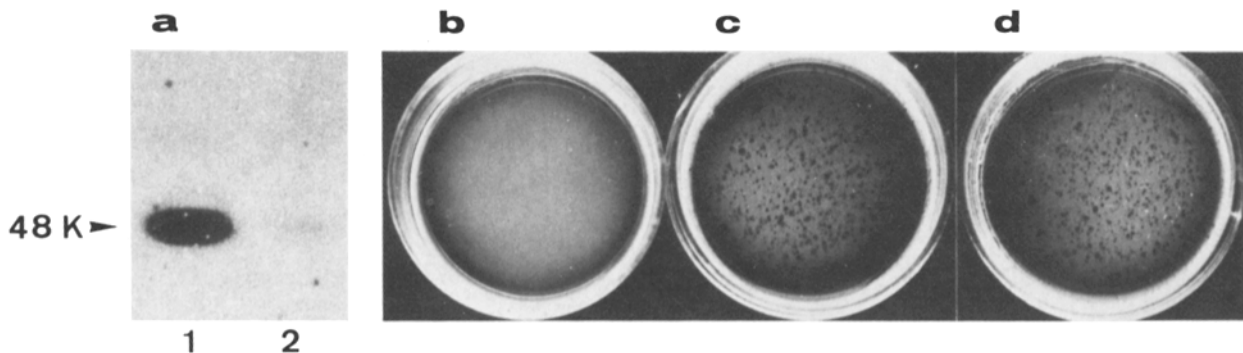Together with the observations of Fig. 1 *e* these results

fertile spermatozoa (Oliphant and Eng, 1981), the zymogram showed an appreciable amount of u-PA activity in the cell extract; this activity was lower than that recovered from ejaculated spermatozoa (lane *4*). We also analyzed sperm cells from a male that had ejaculated at the time of killing by cervical dislocation, their u-PA activity (lane *6*) was also higher than that of sperm cells from vas deferens (lane *5*) and comparable to that of spermatozoa collected after coitus (lane *4*). Thus, the u-PA activity of ejaculated sperm cells isolated after mating is probably not derived from female genital tract secretions.

u-PA Binds to the Sperm Cell Surface

The results presented above indicated that u-PA becomes expressed on spermatozoa at or around the time of ejaculation. In view of the almost complete absence of protein synthesis in posttesticular spermatozoa, this suggests that the enzyme may be secreted by the male genital tract and bind to sperm cells stored in the epididymis and the vas deferens. To explore this hypothesis, cells obtained from spontaneous drain-

Figure 5. Proteolytic activity of denuded spermatozoa. (*a*) Extracts of 100,000 sperm cells were prepared before (lane *1*) or after (lane *2*) denudation, and analyzed by zymography after SDS PAGE in presence of plasminogen. Denuded spermatozoa (30,000 per plate) were analyzed by the plaque assay in the absence (*b*) or presence (*c* and *d*) of plasminogen. In *d*, 200 μg/ml of anti–murine u-PA IgG were included in the medium.

suggest the following: (a) sperm-associated u-PA is predominantly associated with the plasma membrane of the sperm head; and (b) inside the acrosome there is another enzyme (or enzymes, possibly including acrosin) that is detected by the casein overlay assay but not detected by zymography after SDS PAGE (see Discussion). The activity of this unidentified enzyme(s) depends upon or is enhanced by the presence of plasminogen. Whether this enzyme is itself a plasminogen activator, or whether its activity is triggered by the presence of plasminogen or of low levels of contaminating plasmin, cannot be decided at this time.

u-PA Production by the Male Genital Tract

The site(s) of synthesis of the sperm-associated u-PA were explored by assaying for u-PA protein and u-PA mRNA in extracts of segments of the male genital tract. In prepuberal mice (i.e., before the onset of spermatogenesis and therefore in the absence of sperm cells in the genital tract) two PAs were revealed by zymography after SDS PAGE (Fig. 6 a); by immunoprecipitation with specific antibodies, they were shown to be M_r 72,000 t-PA and M_r 48,000 u-PA (data not shown). u-PA specific activity increased from testis (lane 1) to vas deferens (lane 5); high u-PA activity was also found in seminal vesicles (lane 6). In adult organs, t-PA was only detected after longer incubation of the zymogram, and the pattern and level of u-PA activity was similar to that in immature animals, except that the highest activity was found in seminal vesicle and that the specific activity was higher in cauda epididymis than in vas deferens. u-PA in extracts of genital tract organs was not inhibited by exposure to DFP, as shown above for the sperm-associated u-PA (Fig. 2), and was therefore present as the one-chain proenzyme (data not shown).

Preparations of total RNA from various portions of the genital tract from adult males were analyzed by Northern blot hybridizations for the presence of u-PA and t-PA mRNAs (Fig. 7). The highest levels of u-PA mRNA (a) were detected in the vas deferens (lane 5) and in seminal vesicles (lane 6); the u-PA mRNA content was lower in caput (lane 3) than in cauda (lane 4) epididymis, while only weak signals were found in testis (lane 2) and prostate (lane 7). A quantitative estimate of u-PA mRNA content was provided by hybridizing dot-blots of total RNA and counting the amount of hybridized probe. The level of u-PA mRNA in total RNA from the vas deferens was ~1.2 pg/µg of total RNA (comparable to

that in total RNA from whole mouse kidney, lane 1), and 5-, 20-, and 80-fold higher than in seminal vesicles, caput epididymis, and testis, respectively. The levels of t-PA mRNA (b) were comparable in the different organs analyzed, and approximately equivalent to that of u-PA in caput epididymis.

Finally, the cells that contain u-PA mRNA were identified by in situ hybridization. We first analyzed the specificity of u-PA mRNA detection under conditions comparable to those of in situ hybridization (Fig. 8): total RNAs from u-PA– and t-PA–producing cell lines were applied to nitrocellulose and hybridized in parallel to a ^{32}P-labeled u-PA cRNA probe either under the stringent conditions where only near-perfect RNA–RNA duplexes are stable (lanes 1 and 2), or under the conditions that are required for the maintenance of an appropriate morphology of the tissues (lanes 3 and 4); the relative intensities of the signals were comparable under both sets of conditions. Frozen sections of various portions of the male genital tract were then hybridized to a ^{3}H-labeled u-PA cRNA probe, and autoradiographed (Fig. 9). In all tissues, the signal was concentrated over the epithelial layer. The spe-
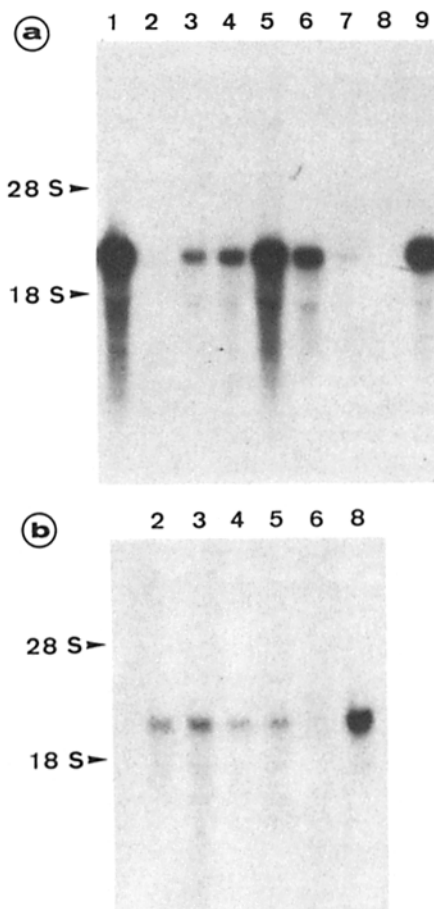
Figure 7. Northern blot analysis of u-PA (a) and t-PA (b) mRNA content in adult organs. Each lane contained 10 µg of total RNA from the following: kidney (lane 1), testis (lane 2), caput (lane 3) and cauda (lane 4) epididymis, vas deferens (lane 5), seminal vesicle (lane 6), and prostate (lane 7). As controls, total RNA from a t-PA–producing cell line (PYS, Marotti et al., 1982; lane 8) and from u-PA–producing murine peritoneal macrophages (Collart et al., 1986; lane 9) were also analyzed.
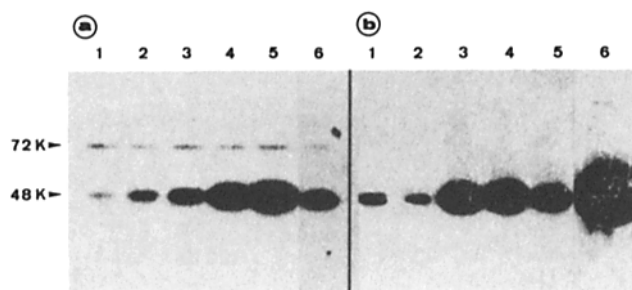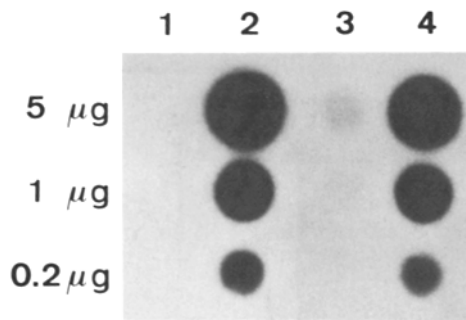
Figure 6. Zymographic analysis after SDS PAGE of organ extracts from immature (a) or adult (b) mice. Testis (lanes 1); caput (lanes 2), corpus (lanes 3), and cauda (lanes 4) epididymis; vas deferens (lanes 5); and seminal vesicles (lanes 6). Lanes in panel a contained 10 µg and lanes in b contained 13 µg of total protein.

Figure 8. Comparison of Northern blot and in situ hybridization conditions. Total RNA from a u-PA– (MSV-3T3, Belin et al., 1984; lanes 2 and 4) and a t-PA– (PYS, Marotti et al., 1982; rows 1 and 3) producing cell line was applied to nitrocellulose (Lanes 1 and 2) Hybridization was at 58°C in 50% formamide, 0.8 M NaCl, and the filter was washed at 65°C in 0.04 M NaCl (Busso et al., 1986). (Lanes 3 and 4) Hybridization and the stringency wash were at 50°C in 50% formamide, 0.3 M NaCl.

cificity of the in situ hybridization was also verified by hybridizing comparable frozen sections with a fragment of u-PA mRNA; only background signal was observed (Fig. 9 *f*).

Thus, together with our detection of u-PA enzymatic activity in protein extracts of these organs, these results are fully compatible with the synthesis of u-PA by the epithelial cells from the vas deferens, the seminal vesicles, and also, to a lesser extent, the epididymis.

Discussion

Proteases in gametes and genital tract secretions are thought to be involved in the cell surface modifications occurring during sperm maturation and capacitation, as well as in fertilization and in postfertilization reactions. However, the enzymatic mechanisms responsible for these events are still only poorly understood. It is thus of interest to further identify the proteases that are associated with or act upon gametes. In this context, the results presented here provide novel information which could be relevant to our understanding of capacitation and fertilization in mammals.

Our zymographic analysis of protein extracts from segments of the male mouse genital tract confirms and extends previous studies by Larsson et al. (1984), which described u-PA immunoreactivity in the epithelium of the vas deferens. In addition, we also found large amounts of u-PA activity in cauda epididymis and seminal vesicles. Enzymatic and immunological assays do not always identify unequivocally the site of synthesis of a given molecule at the cellular level, particularly in the case of secreted proteins: for example, u-PA could be synthesized in the epididymis and accumulate by adsorption and/or endocytosis in the vas deferens. However, our analysis of u-PA mRNA content demonstrates the presence of this mRNA in large amounts in cauda epididymis, vas deferens, and seminal vesicles, and thus strongly suggests that u-PA is synthesized by these organs; in situ hybrid-

ization identified the epithelium as the site where u-PA mRNA was predominant.

We have shown that u-PA is associated with the cell surface of ejaculated spermatozoa. u-PA is bound to sperm cells only at or around the time of ejaculation, perhaps via a specific plasma membrane receptor. A high affinity binding site for u-PA has recently been described on human mononuclear phagocytes. Binding, which involves the noncatalytic A chain, preserves the enzymatic activity of u-PA (Vassalli et al., 1985; Stoppelli et al., 1985), and this interaction is thought to focalize u-PA activity to the close environment of cells which express the receptor. A characteristic species specificity in enzyme–receptor interaction has been observed: human u-PA binds to human but not to murine monocytes–macrophages, and, conversely, murine u-PA binds only to murine monocytes–macrophages (Vassalli, J.-D., A. Estreicher, and A. Wohland manuscript in preparation). A similar species specificity of binding was observed here, since only murine, and not human, u-PA was found to bind to murine spermatozoa. Thus, our data suggest that the interaction of u-PA with murine spermatozoa could also involve binding of u-PA to a receptor analogous to that characterized on mononuclear phagocytes.

Several substances that interact with the cell surface of spermatozoa (sperm-coating antigens) have been described. Synthesized and secreted by various portions of the male genital tract, including the accessory glands, they become associated with spermatozoa upon passage of these cells through the genital tract, or, as we have found to be the case for u-PA, at the time of ejaculation (Lea et al., 1978; Koehler et al., 1980; Dravland and Joshi, 1981; Shivaji et al., 1984; Klinefelter and Hamilton, 1985; Shivaji, 1986). In a u-PA–producing human cell line in which the enzyme is expressed at the cell surface in a receptor-bound form, it has been shown that u-PA is first secreted and subsequently binds to the receptor (Stoppelli et al., 1986a). Thus, it is not surprising that, in the case of sperm cells, u-PA secreted by epithelial cells of portions of the male genital tract can subsequently bind to spermatozoa.

The confluence of the following data suggest that the secretion of u-PA by the vas deferens may be stimulated by ejaculation. (*a*) Spermatozoa stored in the vas deferens possess almost no u-PA immunoreactivity (Larsson et al., 1984). (*b*) Spermatozoa that spontaneously drain from dissected vas deferens have little u-PA activity (Fig. 3, lane 2). (*c*) u-PA immunostaining in the vas deferens was confined to apical granules in the stereociliated cells (Larsson et al., 1984). (*d*) Ejaculated spermatozoa express considerable u-PA activity (Fig. 3, lanes 4 and 6). In this context, it is worth recalling here that sperm cells capable of undergoing capacitation and of fertilizing eggs in vitro can be obtained without ejaculation by squeezing out the contents of the cauda epididymis and the vas deferens (Oliphant and Eng, 1981). It is thus interesting to note that spermatozoa obtained by this method do express u-PA activity (Fig. 3, lane 3).

Although u-PA was the only protease revealed by zymography after SDS PAGE of mouse sperm cell extracts, we do

Figure 9. Detection of u-PA mRNA by in situ hybridization of ³H-labeled u-PA cRNA to frozen sections from cauda epididymis (*a* and *b*), seminal vesicle (*c* and *d*), and vas deferens (*e*). *f* shows the hybridization of a ³H-labeled u-PA mRNA fragment to vas deferens. *a, c, e*, and *f* are bright-field micrographs; corresponding dark-field micrographs of sections *a* and *c* are shown in *b* and *d*, respectively. Bar, 20 µm.
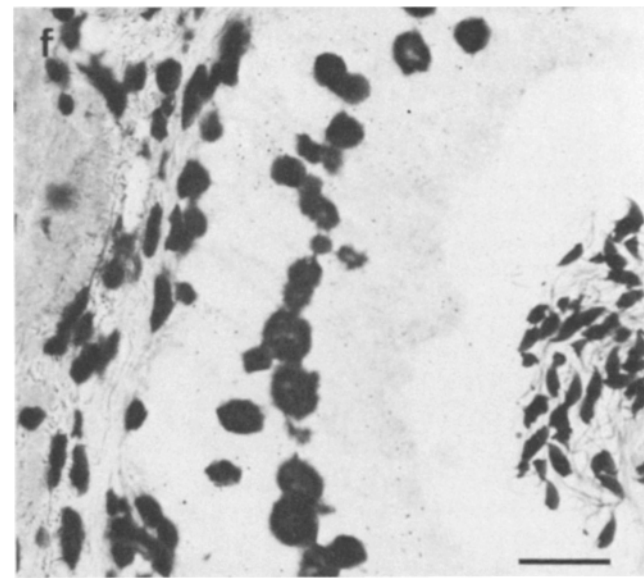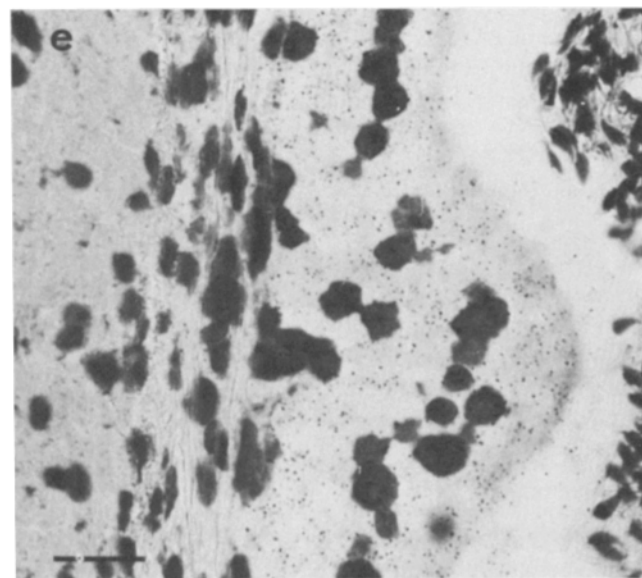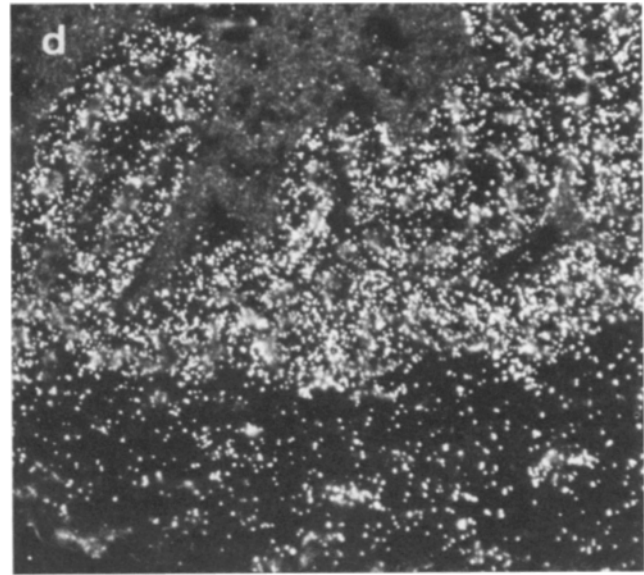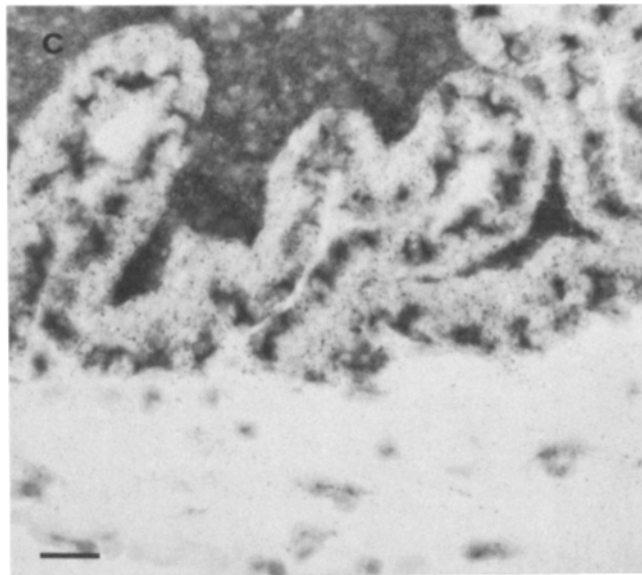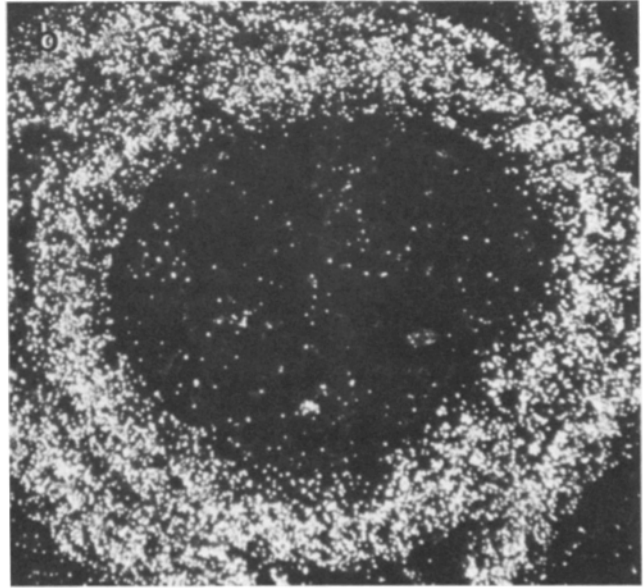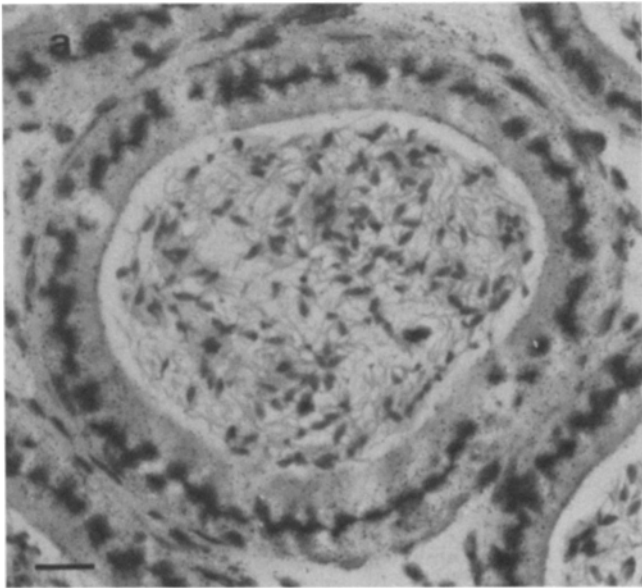
not suggest that it is the major protease in these cells. We have observed an additional proteolytic activity in mouse sperm, which is not related to u-PA, but that also requires the presence of plasminogen for its detection. We have not identified the enzyme responsible for this second activity, but, as has already been suggested (Zaneveld et al., 1975), it could be acrosin (Brown and Hartree, 1976; Bhattacharyya and Zaneveld, 1982). The fact that acrosin was not detectable by zymography after SDS PAGE may be related to the known lability of the murine enzyme (Brown and Hartree, 1976). Interestingly, the occurrence of more than one protease in sperm cells has also been demonstrated in ascidians (Sawada et al., 1984a, b).

The only known macromolecular substrate for u-PA is plasminogen, a zymogen abundant in plasma and other extracellular fluids, including the uterine lumen (Finlay et al., 1983), and possibly the seminal plasma (Zaneveld et al., 1975). Thus, cell surface–localized u-PA may trigger a proteolytic cascade around spermatozoa. It has been suggested that limited proteolysis is necessary for the sperm surface modifications occurring during capacitation (Talbot and Franklin, 1978; Talbot and Chacon, 1981). Plasmin-mediated proteolysis may also prevent the adhesion of spermatozoa to fibrin deposits on the tubal mucosa, facilitating their migration towards the ampulla, where fertilization takes place (Austin and Bishop, 1957). Finally, added trypsin-like proteases can stimulate capacitation (Talbot and Chacon, 1981) and/or the acrosomal reaction (Meizel, 1985), and inhibitors of tryptic enzymes have been shown to prevent the binding of mouse spermatozoa to *zonae pellucidae* (Saling, 1981), and their penetration through the *zona pellucida* (Fraser, 1982; Brown, 1983).

It is intriguing to note that the two characterized sperm proteases (acrosin and u-PA) are present in or on spermatozoa as inactive zymogens. The physiological mechanism of their activation is unknown, but it can be achieved by exposure to proteases, such as plasmin (Skriver et al., 1982). In the proximity of the egg, activation of these zymogens could involve t-PA, an active enzyme which is secreted by the secondary oocyte around the time of fertilization (Huarte et al., 1985). Plasminogen would then represent the confluence of an amplification system that is reminiscent of the proteolytic cascades of blood plasma. It will be of interest to test for a possible role of PAs and plasminogen in mammalian fertilization.

Note Added in Proof: Ejaculated rat spermatozoa also produce lytic zones centered around their head when embedded in a plasminogen-containing insoluble protein substrate. Zymographic analysis reveals the presence of u-PA associated with these cells.

References

Åstedt B., P. Wallen, and B. Aasted. 1979. Occurrence of both urokinase and tissue plasminogen activator in human seminal plasma. *Thromb. Res.* 16:463–472.

Austin, C. R. 1985. Sperm maturation in the male and female genital tracts. *In* Biology of Fertilization, Vol. 2. Biology of the sperm. C. B. Metz and A. Monroy, editors. Academic Press Ltd., London. 121–155.

Austin, C. R., and M. W. H. Bishop. 1957. Fertilization in mammals. *Biol. Rev. Camb. Philos. Soc.* 32:296–349.

Bajpai, A., and J. B. Baker. 1985. Cryptic urokinase binding sites on human foreskin fibroblasts. *Biochem. Biophys. Res. Commun.* 133:475–482.

Bedford, J. M. 1975. Maturation, transport, and fate of spermatozoa in the epididymis. *In* Handbook of Physiology, Section 7: Endocrinology. Vol. 5, Male Reproductive System. D. W. Hamilton and R. O. Greep, editors. American Physiological Society, Washington. 303–317.

Belin, D., F. Godeau, and J.-D. Vassalli. 1984. Tumor promoter PMA stimulates the synthesis and secretion of mouse pro-urokinase in MSV-transformed 3T3 cells: this is mediated by an increase in urokinase mRNA content. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1901–1906.

Belin, D., J.-D. Vassalli, C. Combépine, F. Godeau, Y. Nagamine, E. Reich, H. P. Kocher, and R. M. Duvoisin. 1985. Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. *Eur. J. Biochem.* 148:225–232.

Bhattacharyya, A. K., and L. D. J. Zaneveld. 1982. The sperm head. *In* Biochemistry of Mammalian Reproduction. L. D. J. Zaneveld and R. T. Chatterton, editors. John Wiley & Sons, Inc., New York. 119–152.

Brown, C. R. 1983. Purification of mouse sperm acrosin, its activation from proacrosin and effect on homologous egg investments. *J. Reprod. Fertil.* 69:289–295.

Brown, C. R., Z. Andani, and E. F. Hartree. 1975. Studies on ram acrosin. Isolation from spermatozoa, activation by cations and organic solvents, and influence of cations on its reaction with inhibitors. *Biochem. J.* 149:133–146.

Brown, C. R., and E. F. Hartree. 1976. Identification of acrosin in mouse spermatozoa. *J. Reprod. Fertil.* 46:249–251.

Busso, N., D. Belin, C. Failly-Crépin, and J.-D. Vassalli. 1986. Plasminogen activators and their inhibitors in a human mammary cell line (HBL-100). Modulation by glucocorticoids. *J. Biol. Chem.* 261:9309–9315.

Collart, M. A., D. Belin, J.-D. Vassalli, S. de Kossodo, and P. Vassalli. 1986. Gamma interferon enhances macrophages transcription of the tumor necrosis factor/cachetin, interleukin-1 and urokinase genes, which are controlled by short lived repressors. *J. Exp. Med.* 164:2113–2118.

Cox, K. H., D. V. De Leon, L. M. Angerer, and R. C. Angerer. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* 101:485–502.

Danø, K., P. A. Andreasen, J. Grondahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–266.

Dravland, E., and M. S. Joshi. 1981. Sperm-coating antigens secreted by the epididymis and seminal vesicle of the rat. *Biol. Reprod.* 25:649–658.

Eddy, E. M., R. B. Vernon, C. H. Muller, A. C. Hahnel, and B. A. Fenderson. 1985. Immunodissection of sperm surface modifications during epididymal maturation. *Am. J. Anat.* 174:225–237.

Fibbi, G., G. Dini, F. Pasquali, M. Pucci, and M. del Rosso. 1986. The Mr 17,500 region of the A chain of urokinase is required for interaction with a specific receptor in A431 cells. *Biochim. Biophys. Acta.* 885:301–308.

Finlay, T. H., J. Katz, L. Kirsch, M. Levitz, S. A. Nathoo, and S. Seiler. 1983. Estrogen-stimulated uptake of plasminogen by the mouse uterus. *Endocrinology.* 112:856–861.

Fraser, L. R. 1982. p-Aminobenzamidine, an acrosin inhibitor, inhibits mouse sperm penetration of the zona pellucida but not the acrosome reaction. *J. Reprod. Fertil.* 67:185–194.

Hafen, E., M. Levine, R. L. Garber, and W. J. Gehring. 1983. An improved in situ hybridization method for the detection of cellular RNAs in drosophila tissue sections and its application for localizing transcripts of the homeotic antennapedia gene complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:617–623.

Hamilton, D. W. 1975. Structure and function of the epithelium lining the ductuli efferentes, ductus epididymis, and ductus deferens in the rat. *In* Handbook of Physiology, Section 7: Endocrinology. Vol. 5, Male Reproductive System. D. W. Hamilton and R. O. Greep, editors. American Physiological Society, Washington. 259–301.

Hoppe, P. C., and S. Pitts. 1973. Fertilization in vitro and development of mouse ova. *Biol. Reprod.* 8:420–426.

Huarte, J., D. Belin, and J.-D. Vassalli. 1985. Plasminogen activator in mouse and rat oocytes: induction during meiotic maturation. *Cell.* 43:551–558.

Klinefelter, G. R., and D. W. Hamilton. 1985. Synthesis and secretion of proteins by perifused caput epididymal tubules, and association of secreted proteins with spermatozoa. *Biol. Reprod.* 33:1017–1027.

Koehler, J. K., E. D. Nudelman, and S. Hakomori. 1980. A collagen-binding protein on the surface of ejaculated rabbit spermatozoa. *J. Cell Biol.* 86:529–536.

Lacroix, M., M. Parvinen, and I. B. Fritz. 1981. Localization of testicular plasminogen activator in discrete portions (stages VII and VIII) of the seminiferous tubule. *Biol. Reprod.* 25:143–146.

Lacroix, M., F. Smith, and I. B. Fritz. 1979. The control of plasminogen activator secretion by Sertoli cells in culture, and its possible role in spermatogenesis. *In* Hormones and Cell Culture. *Cold Spring Harbor Conf. Cell Proliferation.* 6:239–248.

Larsson, L. I., L. S. Skriver, L. S. Nielsen, J. Grondahl-Hansen, P. Kristen-

sen, and K. Danø. 1984. Distribution of urokinase-type plasminogen activator immunoreactivity in the mouse. *J. Cell Biol.* 98:894–903.

Lea, O. A., P. Petrusz, and F. S. French. 1978. Purification and localization of acidic epididymal glycoprotein (AEG): a sperm coating protein secreted by the rat epididymis. *Int. J. Androl. Suppl.* 2:592–607.

Marotti, K. R., D. Belin, and S. Strickland. 1982. The production of distinct forms of plasminogen activator by mouse embryonic cells. *Dev. Biol.* 90:154–159.

Meizel, S. 1985. Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. *Am. J. Anat.* 174:285–302.

Meizel, S., and C. W. Lui. 1976. Evidence for the role of a trypsin-like enzyme in the hamster sperm acrosome reaction. *J. Exp. Zool.* 195:137–144.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

Oliphant, G., and L. A. Eng. 1981. Collection of gametes in laboratory animals and preparation of sperm for in vitro fertilization. In Fertilization and Embryonic Development In Vitro. L. Mastroianni, and J. D. Biggers, editors. Plenum Publishing Corp., New York and London. 11–26.

Pennica, D., W. E. Holmes, W. J. Kohr, R. N. Harkins, G. A. Vehar, C. A. Ward, W. F. Bennett, E. Yelverton, P. H. Seeburg, H. L. Heyneker, D. V. Goeddel, and D. Collen. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. Coli. Nature (Lond.)*. 301:214–221.

Reich, E. 1978. Activation of plasminogen: a general mechanism for producing localized extracellular proteolysis. *In* Molecular Basis of Biological Degradative Processes. R. D. Berlin, H. Hermann, I. H. Lepow, and J. M. Tanzer, editors. Academic Press, Inc., New York. 155–169.

Saling, P. M. 1981. Involvement of trypsin-like activity in binding of mouse spermatozoa to zonae pellucidae. *Proc. Natl. Acad. Sci. USA.* 78:6231–6235.

Sawada, H., H. Yokosawa, and S. I. Ishii. 1984*a*. Purification and characterization of two types of trypsin-like enzymes from sperm of ascidian (prochordata) halocynthia roretzi. *J. Biol. Chem.* 259:2900–2904.

Sawada, H., H. Yokosawa, T. Someno, T. Saino, and S. I. Ishii. 1984*b*. Evidence for the participation of two sperm proteases, spermosin and acrosin, in fertilization of the ascidian, halocynthia roretzi: inhibitory effects of leupeptin analogs on enzyme activities and fertilization. *Dev. Biol.* 105:246–249.

Shivaji, S. 1986. Binding of seminalplasmin to the plasma and acrosomal membranes of bovine spermatozoa. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 196:255–258.

Shivaji, S., P. M. Bhargava, and K. H. Scheit. 1984. Immunological identifica-

tion of seminalplasmin in tissue extracts of sex glands of bull. *Biol. Reprod.* 30:1237–1241.

Skriver, L., L. S. Nielsen, R. Stephens, and K. Danø. 1982. Plasminogen activator released as inactive proenzyme from murine cells transformed by sarcoma virus. *Eur. J. Biochem.* 124:409–414.

Stoppelli, M. P., A. Corti, A. Soffientini, G. Cassani, F. Blasi, and R. K. Assoian. 1985. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc. Natl. Acad. Sci. USA.* 82:4939–4943.

Stoppelli, M. P., C. Tacchetti, M. V. Cubellis, A. Corti, V. J. Hearing, G. Cassani, E. Appella, and F. Blasi. 1986*a*. Autocrine saturation of pro-urokinase receptors on human A431 cells. *Cell.* 45:675–684.

Stoppelli, M. P., P. Verde, G. Grimaldi, E. K. Locatelli, and F. Blasi. 1986*b*. Increase in urokinase plasminogen activator mRNA synthesis in human carcinoma cells is a primary effect of the potent tumor promoter, phorbol myristate acetate. *J. Cell Biol.* 102:1235–1241.

Talbot, P., and R. Chacon. 1981. Detection of modifications in the tail of capacitated guinea pig sperm using lectins. *J. Exp. Zool.* 216:435–444.

Talbot, P., and L. E. Franklin. 1978. Trypsinization increases lectin-induced agglutinability of uncapacitated guinea pig sperm. *J. Exp. Zool.* 204:291–297.

Tympanidis, K., and T. Astrup. 1968. Fibrinolytic activity of rat, rabbit, and human sperm cells. *Proc. Soc. Exp. Biol. Med.* 129:179–182.

Vassalli, J.-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the M_r 55,000 form of the human plasminogen activator urokinase. *J. Cell Biol.* 100:86–92.

Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. *Cell.* 11:695–705.

Vihko, K. K., J. J. O. Suominen, and M. Parvinen. 1984. Cellular regulation of plasminogen activator secretion during spermatogenesis. *Biol. Reprod.* 31:383–389.

Williams-Ashman, H. G. 1983. Regulatory features of seminal vesicle development and function. *Curr. Top. Cell. Regul.* 22:201–275.

Zaneveld, L. J. D., K. L. Polakoski, and G. F. B. Schumacher. 1975. The proteolytic enzyme systems of mammalian genital tract secretions and spermatozoa. *In* Proteases and Biological Control. E. Reich, D. B. Rifkin, and E. Shaw, editors. *Cold Spring Harbor Conf. Cell Proliferation.* 2:683–706

Zaneveld, L. J. D., G. F. B. Schumacher, P. F. Tauber, and D. Propping. 1974. Proteinase inhibitors and proteinases of human semen. *In* Proteinase Inhibitors. H. Fritz, H. Tschesche, L. S. Greene, and E. Truscheit, editors. Springer Verlag GmbH & Co., Berlin. 136–146.