Transgenic mouse eggs with functional hamster sperm receptors in their zona pellucida

ROSS A. KINLOCH, STEVEN MORTILLO and PAUL M. WASSARMAN*

Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110, USA

*Author for correspondence

Summary

Sperm receptors are located in the mammalian egg extracellular coat, or zona pellucida. Mouse and hamster sperm receptor glycoproteins, mZP3 (83 × 10^3 M_r) and hZP3 (56 × 10^3 M_r), respectively, have very similar polypeptides (44 × 10^3 M_r; 81% identical) that are glycosylated to different extents. Purified mZP3 and hZP3 can bind to mouse sperm, prevent them from binding to eggs and induce them to undergo exocytosis, the acrosome reaction, in vitro. A DNA construct that placed the hZP3 gene under the control of mZP3 gene 5' flanking sequence was used in this report to produce two mouse lines that harbored the foreign sperm receptor transgene. In both lines, the transgene was expressed only by growing oocytes, at a level comparable to that of the endogenous mZP3 gene, and the developmental pattern of transgene expression resembled that of the mZP3 gene. In addition to mZP3, transgenic mouse oocytes synthesized and secreted a glycoprotein indistinguishable from hZP3, and incorporated both glycoproteins into a mosaic zona pellucida. Importantly, hZP3 purified from such zonae pellucidae exhibited both sperm receptor and acrosome reaction-inducing activities in vitro and, following fertilization of transgenic mouse eggs, was inactivated. These results demonstrate that a biologically active foreign sperm receptor can be synthesized and secreted by transgenic mouse oocytes, assembled into a mosaic zona pellucida, and inactivated following fertilization as part of the secondary block to polyspermy.

Key words: mammalian fertilization, sperm receptor genes, sperm receptor glycoproteins, transgenic mice, zona pellucida.

Introduction

Fertilization in mammals is initiated by binding of free-swimming sperm to the egg zona pellucida, a thick extracellular coat surrounding mammalian eggs (Gwatkin, 1977; Yanagimachi, 1988; Dietl, 1989). This event is mediated by egg-binding proteins located on the sperm head and sperm receptors located in the egg zona pellucida (Wassarman, 1987a,b, 1990).

In mice, the sperm receptor is mZP3 (83 × 10^3 M_r), one of three zona pellucida glycoproteins (Bleil and Wassarman, 1980a; Wassarman, 1987a,b, 1988, 1990; Kinloch et al., 1991). mZP3 consists of a 44 × 10^3 M_r polypeptide, 3 or 4 asparagine- (N-) linked oligosaccharides and an undetermined number of serine/threonine- (O-) linked oligosaccharides (Wassarman, 1988, 1990). A specific class of O-linked oligosaccharides is responsible for mZP3 sperm receptor activity (Florman and Wassarman, 1985; Wassarman, 1987a,b, 1988, 1989, 1990, 1991). The mZP3 gene has been cloned and its primary structure determined (Kinloch et al., 1988; Ringuette et al., 1988; Kinloch and Wassarman, 1989a). Evidence suggests that mZP3 gene expression is restricted to growing oocytes within ovaries of juvenile and adult female mice (Bleil and Wassarman, 1980b; Salzmann et al., 1983; Philpott et al., 1987; Roller et al., 1989; Kinloch and Wassarman, 1989a). Cis-acting sequences located in the mZP3 gene 5' flanking region, together with oocyte-specific proteins, apparently are responsible for the pattern of expression during development (Lira et al., 1990; Schickler et al., 1992).

In hamsters, the sperm receptor is hZP3 (56 × 10^3 M_r), one of three zona pellucida glycoproteins (Moller et al., 1990). The hZP3 gene has been cloned and its primary structure determined (Kinloch et al., 1990). mZP3 and hZP3 polypeptides are very similar to each other (44 × 10^3 M_r; 81% identical), yet are glycosylated to different extents. Consequently, mature mZP3 (83 × 10^3 M_r) and hZP3 (56 × 10^3 M_r) have significantly different molecular weights. However, either purified mZP3 or purified hZP3 can bind to mouse sperm and induce them to undergo the acrosome reaction in vitro (Moller et al., 1990; Kinloch et al., 1991), suggesting that the two glycoproteins have at least certain O-linked oligosaccharide determinants in common.

To determine whether biologically active foreign sperm receptors could be introduced into the mouse zona pellucida, we produced transgenic mice harboring the hZP3 gene placed under control of the mZP3 gene promoter. These mice enabled us to address several important issues, in-
cluding the following. (i) Whether expression of the hZP3 gene is regulated like the endogenous mZP3 gene during mouse development. (ii) Whether mouse oocytes can discriminate between mZP3 and hZP3 nascent polypeptides, and process the latter properly into a biologically active glycoprotein that resembles the authentic sperm receptor. (iii) Whether hZP3 can associate with mouse zona pellucida glycoproteins and be accommodated together with mZP3 in a mosaic zona pellucida. (iv) Whether, like mZP3, hZP3 is inactivated as part of the secondary block to polyspermy following fertilization of transgenic mouse eggs. Results of experiments reported here provide evidence bearing on these, as well as other aspects of mammalian development.

Materials and methods

Plasmid construction

The plasmid used to generate transgenic mice is depicted schematically in Fig. 1. Briefly, p6.5-5′/hZP3 contains the entire hamster ZP3 (hZP3) gene coding region (nts +28 to +8200; Kinloch et al., 1990), including the polyadenylation and termination signals, placed under control of the mouse ZP3 (mZP3) gene promoter (6.5 kb of the mZP3 gene 5′ flanking sequence and nucleotides 1-10 of mZP3 mRNA; Lira et al., 1990). p6.5-5′/hZP3 was constucted by cloning the mZP3 promoter region, comprising a 6.5 kb SstII fragment, into the SstII site of pPGK/hZP3 (Kinloch et al., 1991). The plasmid was digested with Apol and fractionated over a 10-40% sucrose gradient. The ~11.3 kb m/hZP3 fragment (Fig. 1) was recovered from appropriately pooled fractions by precipitation with ethanol and resuspended at 2.5 µg/ml in injection buffer (10 mM Tris-Cl, pH 7.5; 0.15 mM EDTA).

Production and characterization of transgenic mice

Purified DNA fragment (described above) was microinjected into the male pronucleus of fertilized mouse [(C57BL/6J × DBA/2J)F1; The Jackson Laboratory] eggs. Microinjected eggs were then transferred into oviducts of [(C57BL/6J × CBA/J)].F1; The Jackson Laboratory] foster mothers according to published procedures (Hogan et al., 1986).

Identification of transgenic founders was carried out by polymerase chain reaction (PCR) analysis. A small piece of mouse tail was digested in 50 µl of lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl2, 0.45% NP-40, 0.45% Tween-20, and 60 µg/ml Proteinase K) and incubated overnight at 55°C, followed by 30 minutes at 95°C. Two pairs of oligonucleotide primers were used for PCR analysis: 5′-CTCGGGTCGTCACTGCTGTTGTTAC-3′ and 5′-GAATAGATGGGTCGACGCTGG-3′, specific for the m/hZP3 transgene, and 5′-CAGCTCTACATACCTGCAAGCCAAA-3′ and 5′-CAGCTGGAAGAGACACTCAG -3′, as an internal control specific for the endogenous mZP3 gene. These primers amplify fragments of 406 nt and 511 nt from the hZP3 and mZP3 genes, respectively. A 25 µl PCR reaction mix [50% lysis buffer, 50% PCR buffer (16.6 mM ammonium sulphate, 67 mM Tris-Cl pH8.8, 0.1 mg/ml BSA), 0.2 mM NTPs, 1 unit Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 0.2 µg of each oligonucleotide primer, and 4 µl of DNA sample] (F. Koentgen, personal communication) was overlaid with 25 µl of mineral oil and run in a Perkin Elmer/Cetus DNA Thermal Cycler for 40 cycles of 94°C, 20 sec; 60°C, 30 sec; and 72°C, 30 sec. Ten µl of each sample was subjected to electrophoresis on a 1.5% agarose gel. Positive founder mice were mated to generate transgenic pedigrees.

Reverse-PCR analysis

Total RNA was isolated from tissues using RNAzol B, according to the manufacturer’s instructions (Cima/Biotex Labs, Friendswood, TX). First strand cDNA was prepared in a 20 µl reaction mix (5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM dNTPs, 1 U/ml RNase inhibitor, 2.5 U/µl reverse transcriptase, 2.5 µM random hexamers) as described by Perkin Elmer/Cetus, and run in a Perkin Elmer/Cetus Thermal Cycler for 1 cycle of 20°C, 10 minutes; 42°C, 20 minutes; 99°C, 5 minutes; 5°C, 5 minutes. Three pairs of oligonucleotide primers were used for PCR analyses: 5′-CACCTTTTGGCAGAGCCGACT-3′ and 5′-CTGCGGTCCGGAGAACTACG-3′, specific for mZP3 transcripts; 5′-CCGTTGTGCTCTGCAACACGGC-3′ and 5′-CTGCGGTCCGGAGACCGGAC-3′, specific for hZP3 transcripts; 5′-GTGGGCCGCTTAAGGACCAAAA-3′ and 5′-CTTTTTGATGTACCGACAGATTTGCC-3′, specific for mouse β-actin transcripts (Clontech Labs, Inc., Palo Alto, CA); these primers amplify fragments of 395 nt, 715 nt, and 540 nt, respectively. A 25 µl PCR reaction mix (5 µl cDNA mix, 2 mM MgCl2, 50 mM KCl, 0.2 mM NTPs, 1 unit Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 0.2 µg of each oligonucleotide primer, and 4 µl of DNA sample] (F. Koentgen, personal communication) was overlaid with 25 µl of mineral oil and run in a Perkin Elmer/Cetus DNA Thermal Cycler for 40 cycles of 94°C, 20 sec; 60°C, 30 sec; and 72°C, 30 sec. Ten µl of each sample was subjected to electrophoresis on a 1.5% agarose gel. Positive founder mice were mated to generate transgenic pedigrees.

Fig. 1. Schematic diagram of m/hZP3 transgene and PCR analysis of transgenic mice. Top: Schematic diagram of m/hZP3 transgene. The 5′-flanking region of mZP3 is denoted by a closed box and the hZP3 coding region is denoted by an open box. The numbering represents the nt position of the mZP3 and hZP3 loci with respect to their transcriptional start sites (+1). The hZP3 transgene was excised from plasmid p6.5-5′/hZP3 using restriction endonuclease Apol (A). Bottom: PCR analysis of tail DNA. PCR analysis was performed on tail DNA from all mice born following the microinjection procedures. Shown is a 1.5% agarose gel containing amplified DNA from representative samples. The 511 bp and 406 bp DNA fragments represent the endogenous mZP3 gene and m/hZP3 transgene, respectively. Lanes M and H contain positive control plasmid templates for the mZP3 and hZP3 primer sets, respectively (pPGK/mZP3 and pPGK/hZP3; Kinloch et al., 1991). B, blank lane (H2O control). Sd, size standards (1 kb DNA ladder; BRL).
10 mM Tris-HCl, pH 8.3, 0.625 U Taq Polymerase (Perkin Elmer/Cetus, Norwalk, CT), 0.2 µg of appropriate oligonucleotide primer set) was overlaid with mineral oil and run in a Perkin Elmer/Cetus Thermal Cycler for 40 cycles of 94°C, 20 sec; 60°C, 30 sec; 72°C, 30 sec. Ten µl of each sample was subjected to electrophoresis on 2% agarose gels.

**RNase protection assay**

RNase protection assays were performed essentially as described by Ambion, Inc. (Austin, TX). Total RNA was isolated from oocytes or ovulated eggs using RNeasy B, as described by the supplier (Cinna/Biotex Labs, Friendswood, TX). High specific activity RNA probes were transcribed, using SP6 RNA polymerase, from either EcoRI linearized pGEM-G9/S-A (mZP3 specific probe; Kinloch et al., 1988) or HindIII linearized pGEM-hBR2 (hZP3 specific probe; Kinloch et al., 1990). RNA was mixed with 1 x 10⁶ counts/minute of probe in 20 µl hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 400 mM sodium acetate, pH 6.4, 1 mM EDTA) and incubated overnight at 42°C. Following incubation in the presence of RNase A (0.5 U/ml) and RNase T1 (100 U/ml), protected fragments were analyzed on 6% denaturing polyacrylamide gels.

**Western gel immunoblot analysis**

Ovaries were sonicated in 0.5 ml of lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM iodoacetamide, 0.2 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-Cl, pH 8, 140 mM NaCl, 0.025% sodium azide), and the lysate was cleared by centrifugation at 30,000 g for 30 minutes at 4°C. The supernatant was recovered and adjusted to 2% SDS/10% glycerol/50 mM DTT/0.05% Bromophenol blue (protein sample buffer). Alternatively, isolated ZP were dissolved in protein sample buffer. Aliquots were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. The membrane was processed for western gel immunoblotting by first incubating it in a blocking buffer, then in the presence of a rabbit polyclonal antiserum (Pocono Rabbit Farms, Canadensis, PA) directed against either mZP3 or hZP3 peptides (1:750 dilution), and finally in the presence of a goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3,000 dilution; Bio-Rad, Rockville Centre, NY), and finally processed for silver enhancement using the DAB enhancement kit (Amersham, Arlington Heights, IL), according to manufacturer’s instructions.

**Glycoprotein purification**

Purification of mZP3 and hZP3 from ovaries has been described (Bleil et al., 1988; Moller et al., 1990). hZP3 was purified from transgenic mouse ovaries essentially as follows. Zona pellucidae were isolated from ovaries of 50 transgenic mice as described previously (Bleil et al., 1988). Zonae pellucidae were dissolved in protein sample buffer and glycoproteins were separated on a 10% preparative SDS-PAGE gel. hZP3 was recovered from gel slices using a Centriflutor micro-electroeluter, as described by the manufacturer (Amicon, Beverly, MA). The sample was extensively dialyzed against 8 M urea, then against distilled water, and aliquots were analysed by SDS-PAGE, followed by western immunoblotting and silver-staining to determine purity and concentration.

**Sperm receptor assay**

Assays for sperm receptor activity (“competition assays”) were carried out in vitro essentially as previously described (Bleil and Wassarman, 1980a; Florman and Wassarman, 1985; Moller et al., 1990), using gametes and embryos obtained from randomly bred, Swiss albino mice (CD-1; Charles River Breeding Labs, Wilmington, MA). Assays were carried out in Earle’s modified medium 199 (Gibco B.R.L., Grand Island, NY) containing 25 mM HEPES, pH 7.3, 30 µg/ml pyruvate, and 4 mg/ml BSA (M199-M), at 37°C in a humidified atmosphere of 5% CO₂ in air. Capacitated (M-199M, 4 mM EGTA, 37°C, 1 hour) mouse sperm in 50 µl of medium (5 x 10⁵ sperm/ml, final concentration) were incubated in the presence of either M199-M alone or M199-M containing a purified glycoprotein to be tested. Then 12-15 ovulated mouse eggs and 3-4 two-cell mouse embryos, obtained as previously described (Bleil and Wassarman, 1980a; Florman and Wassarman, 1985; Moller et al., 1990), were added to the cultures and the incubation continued for an additional 30 minutes. Ovulated eggs and two-cell embryos were washed by mouth-pipetting with fresh M-199M, until no sperm remained associated with embryos, and cells were fixed in 1% glutaraldehyde and the number of bound sperm per egg determined by light microscopy. In all competition assays, at least 70% of sperm remained highly motile up to the time of fixation, or the experiment was discarded.

**Acrosome reaction assay**

Capacitated mouse sperm in 25 µl of M199-M (1 x 10⁶ sperm/ml, final concentration) were mixed with 25 µl samples containing M199-M alone, M199-M containing a purified glycoprotein to be tested, or ionophore A23187 (10 µM, final concentration; Sigma, St. Louis, MO), incubated for 1 hour at 37°C, and then fixed and processed, essentially as previously described (Moller et al., 1990). Briefly, treated sperm were fixed with 2% formaldehyde for 30 minutes, washed by centrifugation at 10,000 revs/minute for 2 minutes in 0.15 M ammonium acetate, pH 9, and dried onto gelatin-coated (0.25% gelatin, 0.5% chromium sulphate) glass slides. Sperm were stained with 0.04% Coomassie G-250 (in 3.5% perchorlic acid) for 2.5 minutes, washed with distilled water, and scored for the presence or absence of an acrosome by bright field microscopy.

**Immunohistological analysis**

Ovarian paraffin sections were dewaxed, hydrated and incubated first in a blocking buffer (5% goat serum, 5% fetal calf serum, 0.1% gelatin 4 mg/ml polyvinylpyrrolidone in PBS), then in the presence of a rabbit polyclonal antiserum directed against either mZP3 or hZP3 peptides (1:500 dilution), and finally in the presence of a goat anti-rabbit IgG-alkaline phosphate conjugate (1:3,000 dilution; Bio-Rad, Rockville Centre, NY), and finally processed for silver enhancement using the DAB enhancement kit (Amersham, Arlington Heights, IL), according to manufacturer’s instructions.

Cryosections of ovaries excised from transgenic and control mice were prepared and processed for immunohistological examination using rabbit antisera directed specifically against either mZP3 or hZP3 peptides and goat anti-rabbit IgG coupled to 5 nm colloidal gold (Jansen Biotech, Piscataway, NJ). Sections were prepared and immunostained essentially as previously described (Griffiths et al., 1983; Polak and Varmell, 1984), except that all solutions contained polyvinylpyrrolidone (5 mg/ml; 40 x 10⁶ M₃), all incubations were carried out in a 40°C water bath, and sections were re-embedded in epon prior to examination by transmission electron microscopy (Mortillo and Wassarman, 1991). Sections mounted on copper grids were examined with a Philips 201 electron microscope at 60 kV.
Results

Production of transgenic animals

Seventy-one fertilized mouse eggs were microinjected with the m/hZP3 fragment (Fig. 1) and 50 microinjected eggs were transplanted into oviducts of pseudopregnant recipients. PCR analysis of tail DNA was performed to determine which of the resulting progeny contained the m/hZP3 transgene. Three of 29 newborn animals exhibited a 406 nt DNA fragment that corresponded to amplification of a segment of the m/hZP3 transgene (Fig. 1). In addition, all samples contained a 511 nt fragment that corresponded to amplification of a segment of the endogenous mZP3 gene. Transgenic lines were established from two of the positive animals (designated m/h-6 and m/h-11; a third animal, designated m/h-29, died), more than 180 progeny were analyzed by PCR, and the pattern of amplification was found to be reproducible. Founder animals m/h-6 and m/h-11 distributed the transgene to 47% and 46% of their progeny, respectively. Mice homozygous for the transgene were established for line m/h-11.

Expression of m/hZP3 by transgenic mice

We reported previously that the first 470 nt of mZP3 gene 5′-flanking sequence is sufficient to target expression of the firefly luciferase gene specifically to growing oocytes (Schickler et al., 1992). To determine the tissue specificity of m/hZP3 expression, a combination of reverse transcription followed by PCR amplification was employed. PCR primers, specific for hZP3 mRNA, were used to amplify a region of cDNA that had been reverse transcribed from ovarian RNA samples obtained from littermates of transgenic lines m/h-6 and m/h-11. For both lines, a 720 nt fragment, specific for m/hZP3 mRNA, was amplified from ovarian cDNA prepared from mice shown to carry the transgene. This fragment was absent when littermates not carrying the transgene were analyzed. In all cases, a 395 nt fragment specific for mZP3 mRNA was amplified (Fig. 2). To confirm that the 720 nt fragment did, indeed, correspond to an m/hZP3-specific sequence, aliquots of PCR reactions were digested with SsI, and the 542 nt and 172 nt restriction fragments produced (data not shown) are consistent with SsI digestion of this portion of m/hZP3 cDNA (Kinloch et al., 1990). Reverse-PCR was also carried out with ovarian RNA obtained from founder m/h-29 immediately after it died, and a 720 nt fragment, indicative of m/hZP3 expression, was amplified.

PCR primers, specific for either hZP3 or β-actin mRNA, were used to amplify regions of cDNA reverse transcribed from RNA obtained from a variety of tissues excised from transgenic mice. While a 540 nt DNA fragment, representative of β-actin mRNA, was amplified using cDNA from each tissue tested, the 720 nt fragment, representative of hZP3 mRNA, was amplified only from brain and ovary cDNA (Fig. 2). Expression of the transgene in brain was detected only with line m/h-11 and presumably reflects a position effect.

Results of reverse-PCR analyses indicated that m/hZP3 was expressed only in ovaries. To demonstrate that ovarian expression of m/hZP3 was restricted to growing oocytes, as is the case for mZP3 (Roller et al., 1989; Kinloch and Wassarman, 1989a; Lira et al., 1990; Schickler et al., 1992), and to assess the level of expression, RNase protection assays were carried out. RNA was prepared using oocytes isolated from positive (determined by PCR of tail DNA) littermates of transgenic lines m/h-6, m/h-11, and a negative control littermate, and from ovulated eggs isolated from line m/h-11. One-half of each RNA sample was hybridized with probes corresponding to antisense transcripts of either m/hZP3 or mZP3. A 226 nt m/hZP3-specific fragment was protected in oocyte samples from positive transgenic mice of both lines, but not in the oocyte sample from a negative, control littermate (Fig. 3). In all samples a 233 nt mZP3-specific fragment was protected. Densitometry of the gel revealed that m/hZP3 mRNA accumulated to 48% and 30% of the level of mZP3 mRNA for lines m/h-6 and m/h-11, respectively.

RNase protection experiments were also carried out using RNA prepared from oocytes and eggs isolated from line m/h-11. These experiments revealed that the level of m/hZP3 mRNA fell dramatically in ovulated eggs (Fig. 3), consistent with the behavior of mZP3 mRNA during meiotic maturation of oocytes (Roller et al., 1989; Kinloch and Wassarman, 1989a). The m/hZP3 (226 nt) and mZP3 (233 nt) protected fragments were only detected in oocytes, not in ovulated eggs.

Fig. 2. Reverse PCR analysis of transgenic progeny. Top: PCR analysis was performed on ovarian cDNA samples from progeny of transgenic lines m/h-6 and m/h-11. Amplified DNA fragments were separated on 1.5% agarose gels. Mice harboring the m/hZP3 transgene are denoted + and mice lacking the transgene are denoted −. The 395 bp and 720 bp DNA fragments represent mZP3 and hZP3 mRNAs, respectively. Bottom: PCR analysis of tissue cDNA samples. B, brain; E, epididymis; I, intestine; H, heart; L, liver; M, muscle; S, spleen; T, testis; U, uterus; O, ovary; all from a mouse harboring the m/hZP3 transgene. O−, ovary from a control (non-transgenic) littermate. Amplified DNA fragments were separated on 1.5% agarose gels. The 540 bp and 720 bp DNA fragments represent β-actin and hZP3 mRNAs, respectively. Sd, size standards (1 kb DNA ladder; BRL).
Synthesis of hZP3 glycoprotein by transgenic mice

RNA analyses revealed that the m/hZP3 transgene was expressed by oocytes in mice of both transgenic lines. To demonstrate that hZP3 glycoprotein was present in transgenic mouse ovaries, ovarian lysates were prepared and analyzed on western gel immunoblots using antisera raised against either mZP3 (anti-mZP3p) or hZP3 (anti-hZP3p) peptides (Kinloch et al., 1991). Immunoblots of ovarian lysates of transgenic lines harboring the m/hZP3 gene revealed that, in addition to mZP3 ($83 \times 10^3 \text{Mr}$), the transgenic mice synthesized hZP3 (Fig. 4). hZP3 from transgenic mice migrated as a broad band at $56 \times 10^3 \text{Mr}$, similar to the migration of authentic, purified hamster hZP3 on SDS-PAGE. Lysates of control littersmates, lacking the m/hZP3 transgene, exhibited only mZP3 ($83 \times 10^3 \text{Mr}$) on immunoblots.

Immunohistochemical analyses provided further evidence for hZP3 synthesis by transgenic mouse oocytes. Paraffin sections were prepared from ovaries excised from mice containing the m/hZP3 transgene, as well as from negative control littersmates, and processed for immunohistochemistry using anti-mZP3p and anti-hZP3p. As seen in Figure 5, anti-hZP3p detected hZP3 in zonae pellucidae around oocytes within transgenic mouse ovaries, but not in zonae pellucidae of control mice. As expected, anti-mZP3p detected mZP3 in zonae pellucidae of all mice examined.

Secretion of hZP3 by transgenic mice

The immunohistochemical analyses described above strongly suggest that hZP3 is secreted by oocytes and incorporated into the zona pellucida. To provide additional evidence, two experimental approaches were employed. First, zonae pellucidae were isolated from ovaries of both transgenic lines, as well as from littersmates negative for the m/hZP3 transgene, and were probed on immunoblots using anti-hZP3p and anti-mZP3p. The immunoblots revealed that mice from lines m/h-6 and m/h-11 had hZP3 in their zonae pellucidae, but that control littersmates did not (Fig. 6, top panel). Quantitation of immunoblots by densitometry indicated that, for lines m/h-6 and m/h-11, hZP3 represented 36% and 26%, respectively, of total ZP3 present in zonae pellucidae.

In a second approach, semi-thin cryosections of ovaries excised from transgenic mice and control littersmates were prepared and processed for immunohistology using colloidal gold. As seen in Figure 6 (bottom panel), zonae pellucidae of transgenic mice contained material that was recognized by anti-hZP3p. Zonae pellucidae of control littersmates contained only mZP3 (data not shown). Quantification of gold particles revealed that hZP3 constituted 48% and 14% of total ZP3 contained in zonae pellucidae of transgenic lines m/h-6 and m/h-11, respectively (Table 1). The total amount of ZP3 in zona pellucidae of transgenic mice and in zona pellucidae of control littersmates was very similar. These observations strongly suggest that hZP3 is secreted by transgenic mouse oocytes and incorporated into the zona pellucida.

Biological activities of hZP3 synthesized by transgenic mice

hZP3 was purified from transgenic mouse oocyte zona pel-
lucidae and analyzed on western gel immunoblots using anti-hZP3p and anti-mZP3p. This allowed both an assessment of the effectiveness of the purification procedure and quantitation of hZP3 by comparison to known amounts of authentic, purified hamster egg hZP3.

Two experimental approaches were used to determine whether hZP3 purified from transgenic mouse oocyte zonae pellucidae exhibited biological activity. In the first approach, an in vitro “competition assay” was employed (Bleil and Wassarman, 1980a; Florman and Wassarman, 1985) using mouse gametes. Results of previous experiments demonstrated that purified hZP3 was as effective as

**Table 1. Quantification of antibody binding to transgenic mouse oocyte ZP**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>m/h-6 Transgenic</th>
<th>m/h-11 Transgenic</th>
<th>m/h-6 Non-Transgenic</th>
<th>m/h-11 Non-Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>140±5</td>
<td>–</td>
<td>60±41</td>
<td>–</td>
</tr>
<tr>
<td>Anti-hZP3p</td>
<td>4,320±1,125 (48)</td>
<td>1,175±423 (17)</td>
<td>98±52</td>
<td>249±93</td>
</tr>
<tr>
<td>Anti-mZP3p</td>
<td>4,638±2,006 (52)</td>
<td>5,928±2,295 (83)</td>
<td>8,442±3,586</td>
<td>5,628±1,476</td>
</tr>
</tbody>
</table>

*In these experiments, sections of 5-10 different ovarian oocytes were examined and 10-20 determinations made for each section (i.e., number gold particles/µm²) for each of the 3 antisera. The s.d. values are relatively high due in part to variation in section thickness (45-75 nm). Experimental details are provided under Experimental Procedures.*

*Values in parentheses represent gold particles present per µm² ZP for each of the two antisera expressed as a percentage of total gold particles present per µm² ZP (i.e., anti-hZP3p plus anti-mZP3p).
mZP3 in such an assay (Moller et al., 1990; Kinloch et al., 1991). Capacitated mouse sperm were exposed to culture medium alone or to culture medium containing either purified mouse oocyte mZP3 or hZP3 purified from transgenic mouse oocyte zonae pellucidae. Sperm were then incubated with ovulated eggs and two-cell embryos, and the extent of binding of sperm to eggs was determined. Exposure of sperm to either mZP3 or hZP3 resulted in about 75% inhibition of sperm binding to eggs, as compared with sperm incubated in medium alone (Table 2). These results suggest that hZP3 secreted by transgenic mouse oocytes exhibits sperm receptor activity in vitro.

In a second approach, acrosome reaction-inducing activity of transgenic mouse oocyte hZP3 was assessed. Capacitated mouse sperm were incubated in culture medium alone, or in the presence of ionophore A23187, egg mZP3, or hZP3 purified from transgenic mouse oocyte zonae pellucidae, and were then scored for the presence or absence of an intact acrosome. For each treatment, the percentage of acrosome-reacted sperm ± s.d. was given (3 separate experiments; ∼150 sperm assessed/treatment per experiment). Experimental details are provided under Experimental Procedures.

mZP3 and hZP3 in transgenic mouse eggs are inactivated following fertilization (Waterman et al., 1992). Therefore, like mZP3, hZP3 present in zonae pellucidae of transgenic mouse eggs is inactivated following fertilization.

### Table 2. Sperm receptor and acrosome reaction-inducing activities of transgenic mouse oocyte hZP3

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Number Sperm Bound/Egg</th>
<th>% Sperm Acrosome-Reacted</th>
</tr>
</thead>
<tbody>
<tr>
<td>M199-M</td>
<td>29±4 (100)</td>
<td>27±3</td>
</tr>
<tr>
<td>mZP3</td>
<td>7±3 (24)</td>
<td>66±2</td>
</tr>
<tr>
<td>hZP3 (m/h-6)</td>
<td>8±3 (28)</td>
<td>66±1</td>
</tr>
<tr>
<td>hZP3 (m/h-11)</td>
<td>6±3 (21)</td>
<td>67±3</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>–</td>
<td>80±3</td>
</tr>
</tbody>
</table>

In these experiments, ovulated eggs were incubated in the presence of either untreated sperm (M199-M) or sperm exposed to either purified egg mZP3 (4 ng/µl) or hZP3 purified from transgenic line m/h-6 and m/h-11 ZP (4 ng/µl). The average number of sperm bound per egg ± s.d. is given (3 separate experiments; ∼15 eggs assessed/treatment per experiment). Values in parentheses express binding of treated sperm to ovulated eggs as a percentage of binding of untreated sperm to ovulated eggs (100%).

Experimental details are provided under Experimental Procedures.

In these experiments, sperm were incubated in the presence of M199-M, purified egg mZP3 (4 ng/µl), hZP3 purified from transgenic line m/h-6 and m/h-11 (4 ng/µl), or ionophore A23187, and then scored for the presence or absence of an intact acrosome. For each treatment, the percentage of acrosome-reacted sperm ± s.d. is given (3 separate experiments; ∼200 sperm assessed/treatment per experiment). Experimental details are provided under Experimental Procedures.

Previously, we reported that either 6.5 kb or 0.47 kb of mZP3 gene 5′-flanking sequence can target expression of the firefly luciferase gene exclusively to growing oocytes in transgenic mice (Lira et al., 1990; Schickler et al., 1992). However, reporter mRNA steady-state levels were extremely low in these experiments. In experiments reported here, instead of comparing expression of a cDNA reporter gene with expression of the mZP3 gene, we used a genomic fragment containing all exons and introns of the hZP3 gene (a gene bearing high homology to the mZP3 gene) and a small portion of 3′-flanking sequence (nt +28 to +8,900; Kinloch et al., 1990, 1991) linked to 3.3 kb of mZP3 promoter. Thus, the organization of this transgene (m/hZP3) is very similar to the endogenous mZP3 gene. In this context, it has been shown that genomic constructs, or constructs containing at least the first intron, are expressed far more efficiently than identical constructs lacking introns (Brinster et al., 1988; Palmiter et al., 1991).

mZP3 mRNA was expressed exclusively in growing mouse oocytes, in agreement with previous results that employed reporter gene constructs (Lira et al., 1990; Schickler et al., 1992). Furthermore, mZP3 mRNA was expressed at levels that compare favorably with the high levels of endogenous mZP3 expression (Table 3; Roller et al., 1989; Kinloch and Wassarman, 1990). This suggests that all information required for correct mZP3 expression is contained within the proximal 3.3 kb of mZP3 gene 5′-flanking sequence and, perhaps, within one or more mZP3 introns. Based on Southern blot analysis, mouse lines m/h-6 and m/h-11 contain ~7 and ~3 copies of the m/hZP3 transgene, respectively, at a single unique locus. Therefore, differences in levels of expression of m/hZP3 and mZP3 by transgenic mouse oocytes probably reflect differences in copy number and/or position effects. For instance, locus control regions (LCRs) are required for position-independent, high level expression of transgenes containing regions of the human β-globin complex (Townes and Behringer, 1990). Alternatively, differences in levels of expression could be due to differences in the 5′-untranslated region. m/hZP3 contains the first 10 nt of the 29 nt, 5′-untranslated region of the mZP3 gene; the other 19 nt are vector sequence. For certain genes, sequences around the promoter region, including the 5′-untranslated region, can influence positioning of nucleosomes (Gross and Garrard, 1988; Felsenfeld, 1992). Thus, subtle changes in these sequences may affect transcriptional complex assembly and transcriptional activity.

The fact that m/hZP3 mRNA is present at high steady-state levels in transgenic mouse oocytes permitted assessment of the fate of these transcripts during ovulation. In this respect, once again, the behavior of m/hZP3 mRNA during mouse development mimics that of mZP3 mRNA
mRNAs are translated at comparable rates in oocytes from transgenic mice and that both proteins are stable. Thus, transgenic mice have the potential to serve as a source of relatively large amounts of foreign sperm receptors.

Some of the most significant observations reported here concern glycosylation of nascent sperm receptor polypeptides and its relationship to biological activity. For example, the results strongly suggest that the glycosylation machinery of mouse oocytes distinguishes between nascent mZP3 and hZP3 polypeptides and processes both properly. As a result, hZP3 secreted by oocytes of transgenic mice resembles hamster egg hZP3 (56×10^3 M_r), not mouse egg mZP3 (83×10^3 M_r), and exhibits biological activity in vitro. This occurs despite the fact that mZP3 and hZP3 polypeptides have very similar primary structures (81% identity; Kinloch et al., 1990). These findings are of interest in view of the results of recent experiments with embryonal carcinoma (EC) cell lines stably transfected with either the mZP3 or hZP3 gene (Kinloch et al., 1991). Although cells transfected with mZP3 or hZP3 genes synthesized and secreted glycoproteins that had molecular weights similar to mouse or hamster sperm receptors (EC-mZP3, 83×10^3 M_r; EC-hZP3, 49×10^3 M_r, respectively, only the former glycoprotein exhibited biological activity in vitro. Since O-linked oligosaccharides, not polypeptide, account for sperm receptor activity (Florman and Wassarman, 1985; Wassarman, 1989) and, since the EC cells used were derived from mice, it was possible that faulty oligosaccharide processing of glycoproteins from mammals other than mice could account for the functional differences between EC-mZP3 and EC-hZP3. A similar explanation has been used to account for faulty oligosaccharide processing of other glycoproteins in heterologous systems (Kornfeld and Kornfeld, 1985). Results reported here strongly suggest that this may not be the correct interpretation of the EC cell transfection experiments. Mouse oocytes synthesize hZP3 that is active as a sperm receptor and acrosome reaction-inducer.

The mouse zona pellucida consists of long, interconnected filaments (Greve and Wassarman, 1985; Wassarman, 1988; Wassarman and Mortillo, 1991). Each filament is a polymer constructed of dimers, consisting of mZP2 (120×10^3 M_r) and mZP3, located every 15 nm or so along the filament. The filaments are interconnected by another zona pellucida glycoprotein, mZP1 (200×10^3 M_r). Therefore, in addition to its other roles, mZP3 serves as a structural component of the zona pellucida as it is assembled during oogenesis. In this context, it is of considerable interest that a foreign sperm receptor glycoprotein, hZP3, can interact properly with mZP2 and be incorporated into a mouse zona pellucida. Electron micrographs revealed that hZP3 is an integral part of the zona pellucida and is not simply trapped within the filamentous network. Furthermore, since the amount of hZP3 present in zona pellucida simply reflects the amount synthesized by oocytes of transgenic mice, apparently hZP3 combines with mZP2 to form dimers as readily as does the native glycoprotein, mZP3. These results suggest that the protein and/or carbohydrate determinants on mZP3 that support its interactions with mZP2, are conserved on hZP3. Clearly, the difference in extent of glycosylation of mZP3 and hZP3 polypeptides, does not significantly affect the ability of hZP3 to combine

### Table 3. Estimates of mZP3 and hZP3 mRNA and glycoprotein levels in transgenic mouse oocytes

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>% Total-ZP3 mRNA</th>
<th>% Total-ZP3 glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hZP3</td>
<td>mZP3</td>
</tr>
<tr>
<td>m/h-6</td>
<td>33^a</td>
<td>67</td>
</tr>
<tr>
<td>m/h-11</td>
<td>23^a</td>
<td>77</td>
</tr>
</tbody>
</table>

^a Estimates of mRNA levels were made from densitometric scans of gels on which RNase protection experiments were analyzed (Fig. 3; see Experimental Procedures).

^b These estimates of glycoprotein levels were made from densitometric scans of western immunoblot gels (Fig. 6; see Experimental Procedures).

^c These estimates of glycoprotein levels were made by quantifying immunohistochemical data obtained with ovarian sections (Fig. 6 and Table 1; see Experimental Procedures).
with mZP2 to form zona pellucida filaments. Since the majority of mZP3 oligosaccharides are located on the carboxy-terminal half of the polyepitope (T.K. Rosiere and P.M. Wassarman, unpublished results), it is tempting to speculate that the amino-terminal half of mZP3 (or hZP3) polyepitope interacts with mZP2 to form dimers.

Following fertilization of mouse eggs, mZP3 is inactivated as sperm receptor and acrosome reaction-inducer by enzymes released from egg cortical granules during the cortical reaction (Bleil and Wassarman, 1980a, 1983; Wassarman, 1987a,b, 1990; Ducibella, 1991). There is evidence to suggest that such enzymes modify those mZP3 O-linked oligosaccharides that mediate sperm binding to eggs (J. Bleil and P. Wassarman, unpublished results). Results described here indicate that hZP3 present in mouse egg zona pellucida is also inactivated following fertilization. This suggests that mouse egg cortical granule enzymes, which recognize and modify mZP3 O-linked oligosaccharides, also recognize and modify hZP3 O-linked oligosaccharides. This is consistent with our previous finding that the mouse cortical granule protease that specifically cuts mZP2 following fertilization, also cuts hZP2 (Moller et al., 1990).

By combining the experimental approach described here with site-directed mutagenesis of sperm receptor genes, it should be possible now to identify regions of sperm receptors essential for both zona pellucida assembly and biological activity. Furthermore, this experimental approach could be used to produce eggs with mosaic zona pellucida containing sperm receptors of other mammals. In addition to providing a source for larger amounts of certain sperm receptors (e.g., human sperm receptor) than are currently available, such eggs could provide valuable information about the molecular basis of species-specificity during mammalian fertilization.

We are grateful to Dr Jeff Mann for advice and assistance in producing transgenic mice and Ms. Alice O’Connor for assistance in manuscript preparation. We thank our colleagues for critically reviewing the manuscript.

References

Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. Dev. Biol. 137, 276-286.


(Accepted 1 May 1992)
**Fig. 5.** Immunohistochemistry of ovarian sections from transgenic progeny. Ovarian paraffin sections were prepared and processed for immunohistochemistry using rabbit polyclonal antisera directed against either mZP3 or hZP3, and goat anti-rabbit IgG coupled to horseradish peroxidase, as described in Experimental Procedures. Shown are representative sections from mice harboring the m/ZP3 transgene (panels B, D, and F) and control mice (non-transgenic littermates; panels A, C, and E), probed with either anti-mZP3p (panels C and D) or anti-hZP3p (panels A, B, E, and F). o, oocyte; ZP, zona pellucida; fc, follicle cell.