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Transgenic Mice with Reduced Numbers of Functional Sperm Receptors on Their Eggs Reproduce Normally

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To initiate fertilization in mice, free-swimming sperm bind to mZP3, an ~83-kDa glycoprotein present in the ovulated egg zona pellucida (ZP). mZP3 is located periodically along the filaments that constitute the ZP. Sperm recognize and bind to specific oligosaccharides linked to one or more of five Ser residues clustered in the carboxy-terminal one-third of the mZP3 polypeptide. When all five Ser residues are converted to nonhydroxy amino acids by site-directed mutagenesis of the mZP3 gene, an inactive form of mZP3, called mZP3[ser], is secreted by embryonal carcinoma cells stably transfected with the mutated gene. Here, seven independent transgenic mouse lines were established that harbor the mutated mZP3 gene. In all lines, the mutant gene is expressed by growing oocytes and mZP3[ser] is synthesized, secreted, and incorporated into the ZP. Purified mZP3[ser] prepared from ovaries of transgenic mice, like mZP3[ser] from transfected embryonal carcinoma cells, is inactive in sperm binding assays in vitro. On the other hand, the presence of mZP3[ser] in the ZP does not significantly affect either the binding of sperm to ovulated eggs in vitro or the reproduction of the mice, i.e., the transgenic mice are fertile, breed at normal intervals, and produce litters of normal sizes. These results indicate that the number of functional sperm receptors in the ZP can be reduced by more than 50% without adversely affecting fertilization of eggs in vivo.

INTRODUCTION

All mammalian eggs are surrounded by a thick extracellular coat called the zona pellucida (ZP) (Gwatkin, 1977; Dietl, 1989). The mouse egg ZP is composed of three glycoproteins, mZP1, mZP2, and mZP3, that are synthesized and secreted exclusively by growing oocytes (Wassarman, 1988a, 1993). mZP2 and mZP3 form heterodimers that polymerize into the long filaments that constitute the ZP; mZP1 apparently serves as the link between filaments (Wassarman, 1988a; Wassarman and Mortillo, 1991).

Paramount among the functions of the ZP is its role in species-specific fertilization of eggs. For the ZP provides the so-called sperm receptors to which free-swimming sperm bind during fertilization (Gwatkin, 1977; Yanagimachi, 1977, 1994; Wassarman, 1988b). Several lines of evidence suggest that mZP3 serves as the sperm receptor (Wassarman *et al.*, 1985, 1989; Wassarman, 1987a,b, 1990; Wassarman and Litscher, 1994).

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mZP3 (~83 kDa) consists of a ~44-kDa polypeptide (402 amino acids) and three to four *N*-linked and an undetermined number of *O*-linked oligosaccharides. Sperm recognize and bind tightly to specific *O*-linked oligosaccharides (~3.9 kDa) present on the carboxy-terminal one-third of mZP3 polypeptide (Wassarman and Litscher, 1994).

Embryonal carcinoma (EC) cells stably transfected with the mZP3 gene, fused to a constitutive promoter, synthesize and secrete a biologically active form of mZP3 (EC-mZP3; Kinloch *et al.*, 1991). However, recently we found that when Ser-329, -331, -332, -333, and -334 were converted to Gly, Val, or Ala by site-directed mutagenesis of the mZP3 gene, an inactive form of mZP3 (mZP3[ser]) was produced by EC cells stably transfected with the mutated gene (Kinloch *et al.*, 1995). These and other results suggest that one or more of these five Ser residues, clustered in a region of polypeptide encoded by exon 7 of the mZP3 gene, carry oligosaccharides essential for biological activity.

In the present investigation, we asked whether a reduction in number of active mZP3 molecules in the

ZP of ovulated eggs would affect fertility. Previous results from our laboratory revealed that foreign sperm receptors could be introduced into the ZP of transgenic mouse eggs to create a mosaic ZP (Kinloch *et al.*, 1992). Accordingly, here we established several independent transgenic mouse lines that harbored the mutated mZP3 gene encoding mZP3[ser]. Eggs from these lines do, indeed, possess a mosaic ZP consisting of differing amounts of wild-type, active mZP3 and mutant, inactive mZP3[ser]. Surprisingly, even when mZP3[ser] represents more than 50% of mZP3 in the ZP, mice are fertile, breed normally, and ovulate eggs to which a normal number of sperm bind in vitro.

MATERIALS AND METHODS

Plasmid Construction and DNA Preparation

Site-directed mutagenesis of the mZP3 gene has been described by Kinloch *et al.* (1995). As seen in Figure 1A, the plasmid P-832/mZP3[ser] was constructed by ligation of the mutated, full-length mZP3 gene (nucleotides (nts) −59 to +8, 900; *Eco*RI/*Xba*I fragment) to the mZP3 promoter region (nts −832 to −59; *Hin*dIII/*Eco*RI fragment). Before microinjection into fertilized eggs, a DNA fragment (832/mZP3[ser]), consisting of 832 bp of promoter, 8504 bp of transcription unit, and 396 bp of transcription termination sequence, was cut from the plasmid vector by digestion with *Cla*I and *Sst*II. This fragment was separated from vector DNA by agarose gel electrophoresis, recovered from a gel slice digested with GelZyme (Invitrogen, San Diego, CA), and dissolved in 2 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, at a concentration of about 2 μg/ml.

Transgenic Mouse Production and Analysis

Transgenic mice were produced by standard procedures (Wassarman and DePamphilis, 1993). Briefly, DNA fragment 832/mZP3[ser] was microinjected into the male pronucleus of fertilized eggs collected from superovulated, female B6CBAF1/J mice (The Jackson Laboratory, Bar Harbor, ME). The eggs were cultured overnight to the two-cell stage of development and transferred into oviducts of pseudopregnant foster mothers according to standard procedures (Wassarman and DePamphilis, 1993).

Identification of transgenic founders was carried out by polymerase chain reaction (PCR) analysis using a small piece of tail taken from offspring born to foster mothers, as previously described (Kinloch *et al.*, 1992). As seen in Figure 1A, a 5′-primer sequence corresponding to exon 6 of the mZP3 gene and a 3′-primer sequence corresponding to the mutated region of exon 7 of the mZP3 gene were used; the former hybridizes to both the wild-type gene and transgene, whereas the latter hybridizes only to the transgene. In addition, a 3′-primer sequence corresponding to intron 7 of the mZP3 gene was used as a control; this primer hybridizes to both the wild-type gene and the transgene. All three primers were added to the PCR reaction and run for 35 cycles of 94°C, 58°C, and 72°C for 30 s each, as previously described (Kinloch *et al.*, 1992). Mice harboring the transgene yielded amplified fragments of 382 bp (wild-type gene) and 236 bp (transgene), whereas nontransgenic mice yielded a single fragment of 382 bp. Positive founder mice were mated to generate transgenic pedigrees.

The transgene copy number was estimated by dot-blot analysis of tail DNA prepared from 12-day-old mice. DNA was denatured with

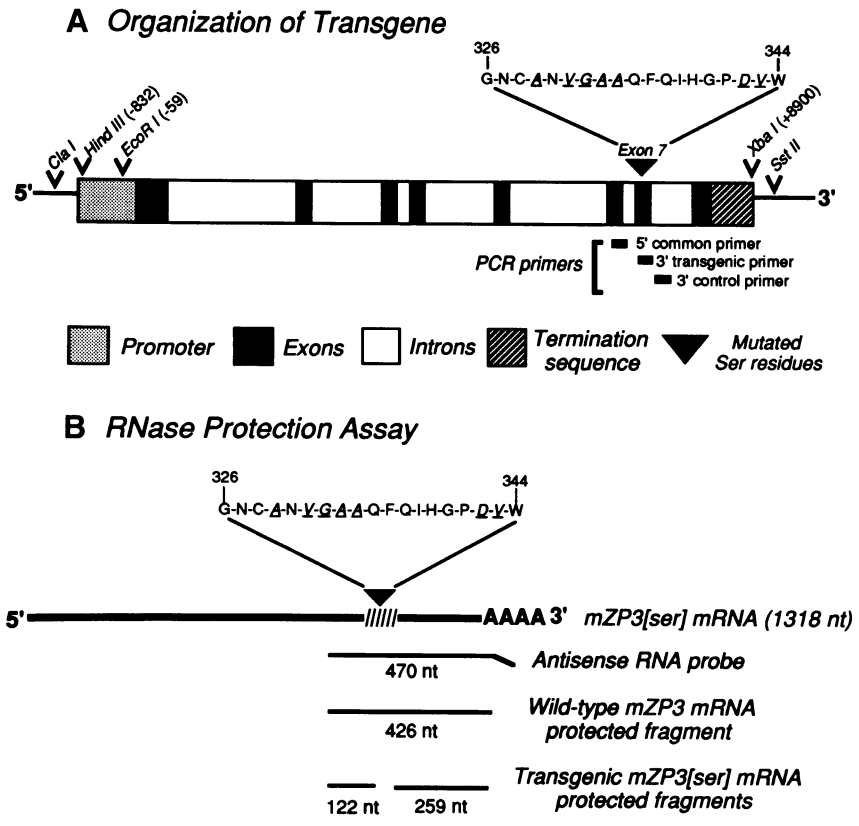

Figure 1. Schematic diagrams of the 832/mZP3[ser] transgene (A) and the RNase protection assay (B). (A) Shown diagrammatically is the organization and location of certain restriction sites of the construct used to produce transgenic mice. The site of mutagenesis in mZP3 gene (exon 7) and changes in amino acid sequence caused by mutagenesis (italicized and underlined) are indicated. Also shown are the relative positions of three PCR primers used to identify transgenic founder mice. (B) Shown diagrammatically is mZP3[ser] mRNA with the location and consequences of site-directed mutagenesis indicated. A 470-nt antisense probe, with 426 nt complementary to mZP3 mRNA and 44 nt of flanking vector sequence, was used in RNase protection assays. This resulted in the generation of a single protected fragment, 426 nt in length, for mZP3 mRNA and in the generation of two protected fragments, 259 nt and 122 nt in length, for mZP3[ser] mRNA.

base (0.3 M NaOH, 65°C, 30 min), neutralized (2 M ammonium acetate, pH 7.0), and then loaded in increasing amounts onto nitrocellulose membrane using a dot-blot apparatus (Life Technologies, Gaithersburg, MD). The membrane was baked and hybridized with an [α-^{32}P]dCTP-labeled mZP3 cDNA (Megaprime DNA Labeling System, Amersham, Arlington Heights, IL), as previously described (Sambrook *et al.*, 1989). To assess variations in DNA recovery and sample loading, a duplicate dot-blot was hybridized with a radiolabeled mZP2 cDNA probe. Radioactivity associated with each sample was quantitated by using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). The slope of a plot of radioactivity versus microgram genomic DNA was used to calculate the number of copies of the mZP3 gene (endogenous plus transgene) per diploid genome.

To identify homozygous transgenic mice, tail DNA was prepared from littermates born to a pair of heterozygous mice and the number of copies of the mZP3 gene per diploid genome was determined by dot-blot analysis, as described above.

RNase Protection Assays

Total RNA was prepared by homogenizing frozen ovaries in RNAzol B (CinnaBiotecx Labs, Houston, TX) using the supplier's protocol. RNase protection assays were performed using RPA II kit (Ambion, Austin, TX), essentially as described by the supplier. Antisense RNA probe was transcribed in vitro in the presence of [α-^{32}P]CTP using *Tha*I-digested mZP3 cDNA as a template. Upon completion of RNase protection assays, protected fragments were subjected to scintillation counting, equal cpm were subjected to electrophoresis on a 6% DNA-sequencing gel, and the gel was processed for autoradiography. To quantitate results, gel slices containing protected RNA fragments were subjected to scintillation counting.

mZP3 and mZP3[ser] Purification

Purification of mZP3 from ovaries has been described (Bleil *et al.*, 1988; Moller *et al.*, 1990). To separate mZP3 from mZP3[ser], the HPLC-purified glycoproteins were resuspended in 10 mM Tris-HCl, pH 7.5, and passed through an affinity column prepared by cross-linking a rabbit polyclonal antibody, anti-mZP3pep (Pocono Rabbit Farms, Canadensis, PA), according to the supplier's protocol (ImmunoPure IgG Orientation kit; Pierce, Rockford, IL). Anti-mZP3pep is directed against a synthetic peptide constructed on the basis of mZP3 primary structure (amino acids 328–343), as previously described (Kinloch *et al.*, 1992). Anti-mZP3pep recognizes mZP3, but not mZP3[ser] because of the amino acid changes introduced by site-directed mutagenesis.

Western Immunoblot and Immunodot-Blot Analyses

Western immunoblotting was performed with purified ZP glycoproteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred to a nitrocellulose membrane. After blocking the membrane with 3% gelatin in Tris-buffered saline (TBS; 137 mM NaCl, 20 mM Tris-HCl, pH 7.6), it was incubated in the presence of either anti-mZP3pep (1:100 dilution; described above) or a rabbit polyclonal antibody directed against mZP3 (anti-mZP3; 1:750 dilution; Pocono Rabbit Farms) in TTBS (137 mM NaCl, 0.05% Tween-20, 20 mM Tris-HCl, pH 7.6) containing 1% gelatin, washed with TTBS, and incubated with ^{125}I-labeled secondary antibody (donkey anti-rabbit Ig; Amersham, Arlington Heights, IL). The membrane was then washed with TTBS and exposed to x-ray film. In some cases, the membrane was cut into pieces and analyzed in a gamma-counter.

For immunodot-blotting, purified mZP3 in electroblotting buffer (20% methanol, 192 mM glycine, 20 mM Tris-HCl, pH 8.3) was loaded onto a nitrocellulose membrane with a dot-blot apparatus (Life Technologies, Gaithersburg, MD). The membrane was incubated with either anti-mZP3 or anti-mZP3pep and then ^{125}I-labeled secondary antibody, as described for Western immunoblotting.

Sperm Binding Assays

Sperm binding assays ("competition assays") were carried out in vitro, essentially as previously described (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Moller *et al.*, 1990; Kinloch *et al.*, 1992). Briefly, gametes were cultured in Earle's modified medium 199 (Life Technologies) containing 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.3, supplemented with 4 mg/ml bovine serum albumin and 30 μg/ml sodium pyruvate (M199-M), at 37°C in a humidified atmosphere of 5% CO_2 in air. Capacitated sperm (M199-M, 37°C, 1 h) were preincubated in the presence of M199-M alone or M199-M containing a purified glycoprotein to be tested, 12–15 ovulated eggs and three to four two-cell embryos were added, and incubations were continued for 30–60 min. Eggs and embryos were washed by mouth-pipetting with fresh M199-M, until only one to two sperm remained associated with embryos, and cells were fixed in 1% glutaraldehyde and the number of sperm bound per egg was determined by light microscopy. In some cases, eggs were lightly stained with 0.05% Coomassie blue G-250, which had no observable effect on the number of sperm bound to the ZP of either wild-type or transgenic eggs.

RESULTS

Generation of Transgenic Animals and Pedigrees

Approximately 600 fertilized mouse eggs were microinjected with 832/mZP3[ser] and, of these, approximately 250 injected eggs were transplanted into oviducts of 14 pseudopregnant B6CBAF$_1$/J mice, as described in MATERIALS AND METHODS. Ten of the 52 resulting offspring (19%) harbored the transgene, as determined by PCR using tail DNA. Seven of these founder mice produced offspring that carried the transgene and these were bred to generate transgenic lines. Dot-blot analyses of genomic DNA revealed that the copy number of 832/mZP3[ser] varied from one to seven copies for the seven different transgenic lines (Table 1). Four of the lines were bred to homozygosity.

Expression of 832/mZP3[ser] by Transgenic Animals

RNase protection assays were used to detect expression of 832/mZP3[ser] in ovaries of transgenic mice, as described in MATERIALS AND METHODS. Ovaries were chosen for the assays because the mZP3 gene is expressed exclusively by growing oocytes in mice (Kinloch and Wassarman, 1989; Kinloch *et al.*, 1993; Chamberlin *et al.*, 1989). A 470 nt antisense RNA, corresponding to the 3'-end of mZP3 mRNA, was used as a probe and, when hybridized with wild-type mZP3 mRNA, yielded a 426 nt protected fragment (smaller than the 470 nt probe because of the presence of 44 nt of flanking vector sequence). On the other hand, when the probe was hybidized with mZP3[ser] mRNA, two protected fragments were produced (259 nt and 122 nt) because of an imperfect match at the site of mutagenesis (Figure 1B). As seen in Figure 2, all

Table 1. Characteristics of transgenic mice harboring the mZP3[ser] gene

Transgenic line no.	Transgenic copy no.	Transgene mRNA (% total)[a]	Litter size	No. litters analyzed
Control	*0*	*0*	*7.8±2.5*	*74*
Heterozygous				
11	4	55	6.8±2.6	42
12	2	41	7.0±2.4	41
14	3	60	7.2±2.6	38
15	5	34	5.7±2.8	22
23	1	35	8.1±2.2	14
31	1	34	7.0±3.2	23
51	7	24	6.3±2.6	34
Homozygous				
11-h	8	62	7.5±2.7	17
12-h	4	50	6.2±2.3	29
14-h	6	65	6.5±2.8	26
23-h	2	61	6.8±2.6	8

[a]Transgene mRNA as a percentage of mZP3 plus mZP3[ser] mRNA, as determined by RNase protection assays (see MATERIALS AND METHODS and Figure 1).

seven transgenic mouse lines expressed 832/mZP3[ser] in the ovary. Quantitation of the levels of mZP3[ser] mRNA revealed that it accounted for 24–65% of the total, i.e., mZP3 mRNA plus mZP3[ser] mRNA, in the seven transgenic lines (Table 1). The highest expression of 832/mZP3[ser] was found in line #14 and the lowest in line #51. As seen in Table 1, the levels of transgene expression do not correlate directly with the number of copies of the transgene present, suggesting that the site of integration probably influences 832/mZP3[ser] expression. A similar situation was found for transgenic mice harboring transgenes consisting of the mZP3 gene 5′-flanking sequence fused to the coding region of the firefly luciferase gene (Lira *et al.*, 1990, 1993).

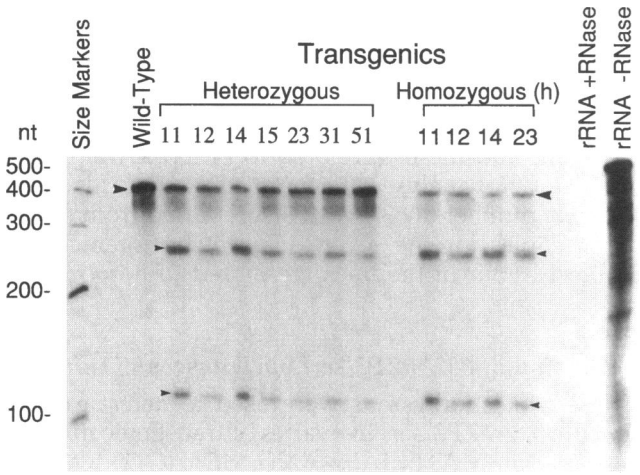

Figure 2. RNase protection assay of ovarian RNA from wild-type and transgenic mice. Assays of seven heterozygous and four homozygous (h) transgenic lines are shown. Ovarian RNA, equivalent to 10% of the RNA purified from one ovary, was hybridized with a ^{32}P-labeled antisense RNA probe complementary to 426 nt at the 3′-end of mZP3 mRNA. After treatment with RNase, each sample was subjected to liquid scintillation counting and equal numbers of cpm were analyzed on 6% DNA-sequencing gels followed by autoradiography. The 426-nt fragment (large arrowhead) represents mZP3 mRNA (endogenous gene) protected by the mZP3-specific antisense probe. The 122-nt and 259-nt fragments (small arrowheads) represent mZP3[ser] mRNA (transgene) protected by the mZP3-specific antisense probe. Also shown are samples of *Escherichia coli* ribosomal-RNA (rRNA) incubated in the presence (+) or absence (−) of RNase and radiolabeled RNA size markers.

Quantitation of mZP3[ser] in Mosaic Zonae Pellucidae

To qualitatively examine the mZP3 present in ZP of transgenic mice, an antibody (anti-mZP3) that recognizes both mZP3 and mZP3[ser] was used, as described in MATERIALS AND METHODS. As seen in the Western immunoblots shown in Figure 3, transgenic mZP3 (i.e., mZP3 plus mZP3[ser]) migrated as a broader band than wild-type mZP3, with significantly more material in the lower molecular weight range. This result was not unexpected because EC-mZP3[ser] also migrates with a lower apparent molecular weight than wild-type mZP3; a property attributed to less extensive *N*-linked glycosylation of the nascent polypeptide (Kinloch *et al.*, 1991, 1995).

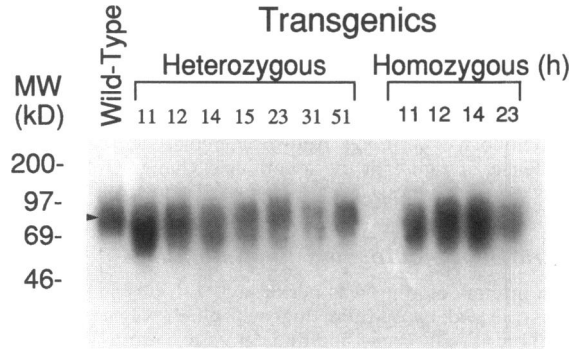

Figure 3. Western gel immunoblot assays of ZP glycoproteins from wild-type and transgenic mice. Assays of seven heterozygous and four homozygous (h) transgenic lines are shown. ZP purified from ovarian homogenates on Percoll gradients (see MATERIALS AND METHODS) were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and an aliquot, equivalent to 25% of ZP glycoprotein from one ovary, was subjected to Western gel immunoblotting followed by autoradiography. Rabbit anti-mZP3 was used as primary antibody and ^{125}I-labeled donkey anti-rabbit Ig (Amersham) as a secondary antibody, as described in MATERIALS AND METHODS. The positions of molecular weight (MW) markers are indicated, as well as the position of wild-type mZP3 (~83 kDa; arrowhead).

To quantitate the amounts of mZP3 and mZP3[ser] in transgenic mouse ZP, immunodot-blots were probed with either anti-mZP3 or anti-mZP3pep as primary antibody and ^{125}I-labeled secondary antibody (Figure 4A), as described in MATERIALS AND METHODS. Anti-mZP3[pep] is directed against the mZP3 polypeptide region that includes amino acids 328–343 (Kinloch *et al.*, 1991) and, consequently, recognizes only mZP3, not mZP3[ser], because of the amino acid changes in mZP3[ser] (see legend to Figure 5). Each sample was analyzed in a gamma-counter and cpm plotted as a function of the amount of glycoprotein loaded onto the nitrocellulose membrane (Figure 4, B and C). The slopes of such plots permit an estimate to be made of the amount of mZP3[ser] present in transgenic mouse ZP. For example, comparisons of the slopes of the lines shown in Figure 4, B and C indicate that ZP of transgenic line #14-h (homozygous) contain 44% mZP3 and 56% mZP3[ser]. It should be noted that, overall, the percentage of mZP3[ser] present in the ZP of transgenic mouse eggs correlates well with the amount of mZP3[ser] mRNA present in the ovary.

Effect of mZP3[ser] on Binding of Sperm to Eggs

Previous experiments revealed that, unlike EC-mZP3, EC-mZP3[ser] synthesized and secreted by transfected EC cells is inactive in an in vitro competition assay (Kinloch *et al.*, 1991, 1995). To determine whether mZP3[ser] present in the ZP of transgenic mouse oocytes is active, purified mZP3[ser] was tested in the competition assay (see MATERIALS AND METHODS). mZP3[ser] was separated from mZP3 by affinity-chromatography using anti-mZP3pep as ligand, as described in MATERIALS AND METHODS. As seen in Figure 5, five passages of mZP3 plus mZP3[ser] (i.e., transgenic ZP) over the affinity column resulted in removal of >90% of the mZP3 from the mixture. When mZP3[ser] (~4–8 ng/μl) was compared with mZP3 at an equivalent concentration for its ability to inhibit binding of sperm to eggs in vitro, it was found that the former had no significant effect on sperm binding (9 ± 8% inhibition; n = 4), as compared with the latter (51 ± 13%; n = 4). Thus, like EC-mZP3[ser] (Kinloch *et al.*, 1995), transgenic mouse mZP3[ser] is inactive in this assay.

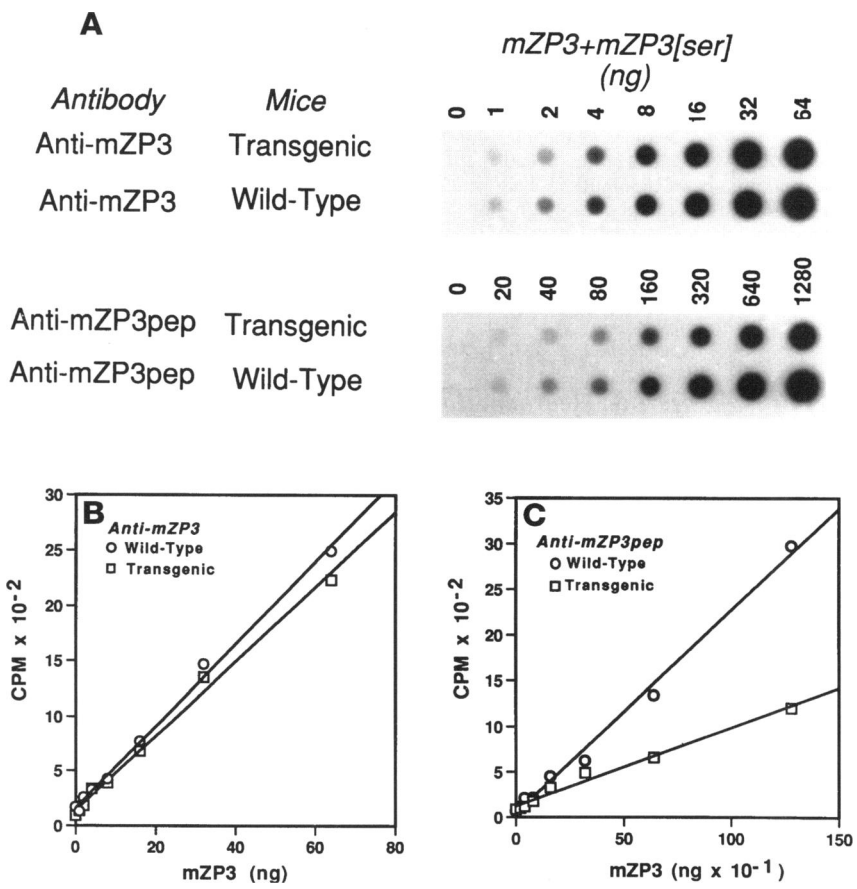

Figure 4. Immunodot-blot analysis of the amount of mZP3[ser] in transgenic mouse egg ZP. (A) mZP3 was purified from ovaries of wild-type mice and mZP3 plus mZP3[ser] was purified from ovaries of transgenic mice, homozygous line #14-h, as described in MATERIALS AND METHODS. Increasing amounts of the purified glycoproteins were loaded onto nitrocellulose membrane using a dot-blot apparatus. The nitrocellulose membrane was incubated first with either anti-mZP3 (recognizes *both* mZP3 and mZP3[[ser]) or anti-mZP3[pep] (recognizes mZP3, but *not* mZP3[ser]), then with ^{125}I-labeled secondary antibody, and was then subjected to autoradiography, as described in MATERIALS AND METHODS. Shown are examples of the resulting autoradiograms with the amount of glycoprotein loaded (ng) indicated. Because anti-mZP3[pep] is a weaker antibody than anti-mZP3, 20 times more glycoprotein was used as antigen with the former than with the latter antibody. (B and C) Each sample was cut from the nitrocellulose membrane and the amount of radioactivity associated with it was determined in a gamma counter. Counts per minute were plotted against ng of glycoprotein and slopes of the plots were used to estimate the amount of mZP3[ser] in transgenic egg ZP. For example, the ratio of slopes (i.e., transgenic/wild type) in panel B (anti-mZP3) is 0.9, whereas the ratio of slopes in panel C (anti-mZP3[pep]) is 0.4. These results indicate that wild-type and mutated mZP3 account for about 44% and 56%, respectively, of the mZP3 in egg ZP from transgenic line #14-h.

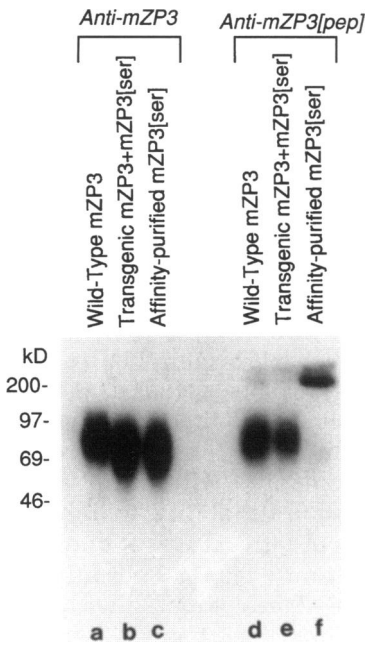

Figure 5. Western gel immunoblot analysis of affinity-purified mZP3[ser]. Total mZP3 (i.e., wild-type mZP3 plus mZP3[ser]), purified from ZP from transgenic line 14-h, was passed five times through an anti-mZP3pep affinity column to remove wild-type mZP3 from the mixture. Unbound mZP3[ser] was collected in the flow-through. Shown is an autoradiogram of a Western gel immunoblot analysis of affinity-purified mZP3[ser] (lanes c and f), the starting mixture of wild-type mZP3 plus mZP3[ser] (lanes b and e), and wild-type mZP3 (lanes a and d) using anti-mZP3 (lanes a–c) and anti-mZP3pep (lanes d–f) as probes. Binding of primary antibody was detected using ^{125}I-labeled secondary Ig, as described in MATERIALS AND METHODS. Because anti-mZP3pep is a weaker antibody than anti-mZP3, lanes a–c and d–f contained ~0.1 μg and ~2 μg protein, respectively. The high molecular weight band (~200 kDa) seen in lane f apparently is antibody leached from the affinity column; the band is not seen as clearly in lane c because ~20 times less protein was loaded on lane c as compared with lane f. The positions of molecular weight markers are indicated.

Effect of mZP3[ser] on Reproduction of Transgenic Animals

During the course of these experiments, reproduction of the transgenic animals was followed closely and compared with control animals. No differences were observed in either the breeding intervals or the litter sizes when transgenic and control mice were compared. The latter data is summarized in Table 1. Therefore, the presence of a mosaic ZP containing 50% or more of inactive mZP3[ser] does not appear to have a significant effect on fertility.

Binding of Sperm to Transgenic Mouse Eggs In Vitro

In view of the ability of mice harboring the transgene to reproduce normally, it must be assumed that adequate numbers of free-swimming sperm bind to eggs ovulated by the transgenic mice. To examine sperm binding directly, ovulated eggs obtained from nontransgenic ("control") and transgenic mice were incubated with sperm in vitro and the number of sperm bound per egg was determined, as described in MATERIALS AND METHODS. Eggs from control and transgenic mice were cultured together and were distinguished from each other by lightly staining one group of eggs with Coomassie blue (Figure 6). When the number of sperm bound per egg (in the largest diameter focal plane) was compared for transgenic (28 ± 10 sperm/egg; n = 4) and control (29 ± 9 sperm/egg; n = 4) mice, no significant difference was found. Therefore, the presence of a mosaic ZP containing 50% or more of inactive mZP3[ser] does not affect binding of sperm to eggs in vitro.

DISCUSSION

EC cells stably transfected with the mZP3 gene fused to a constitutive promoter (PGK/mZP3) synthesize and secrete EC-mZP3 that is active in sperm binding and acrosome reaction-induction (Kinloch *et al.*, 1991). Previously, we reported results of experiments carried out with EC cells stably transfected with PGK/mZP3[ser], a form of the mZP3 gene in which Ser-329, -331, -332, -333, and -334 were converted to Gly, Ala, or Val by site-directed mutagenesis (Kinloch *et al.*, 1995). Oligosaccharides linked to one or more of these five Ser residues have been implicated as playing an essential role in the binding of sperm to mZP3 (Rosiere and Wassarman, 1992; Wassarman and Litscher, 1994). Consistent with this proposal, EC-mZP3[ser] secreted by the transfected EC cells proved to be inactive in in vitro assays of biological activity (Kinloch *et al.*, 1995). Results reported here demonstrate that mZP3[ser] is inactive even when synthesized, secreted, and assembled into the ZP by growing mouse oocytes in vivo, providing additional evidence that one or more of the five Ser residues are located at the mZP3 combining-site for sperm.

The number of binding sites for mZP3 on the sperm-head plasma membrane has been estimated by whole-mount autoradiography (light microscopy) using radiolabeled mZP3 (Bleil and Wassarman, 1986) and by electron microscopy using mZP3 coupled to colloidal gold (Mortillo and Wassarman, 1991). The estimates range from 10^4-10^5 mZP3 binding sites per sperm head (i.e., ~500–5000/μm^2). These binding sites are distributed over the entire sperm-head plasma membrane and are not restricted to a particular portion of the sperm head (Mortillo and Wassarman, 1991). However, because it has been estimated that free-swimming sperm initially adhere to the egg ZP by only ~0.1 μm^2 of the surface area of their head (Baltz and Cardullo, 1989), each sperm could potentially interact with as few as 50 or as many as 500 mZP3 molecules.

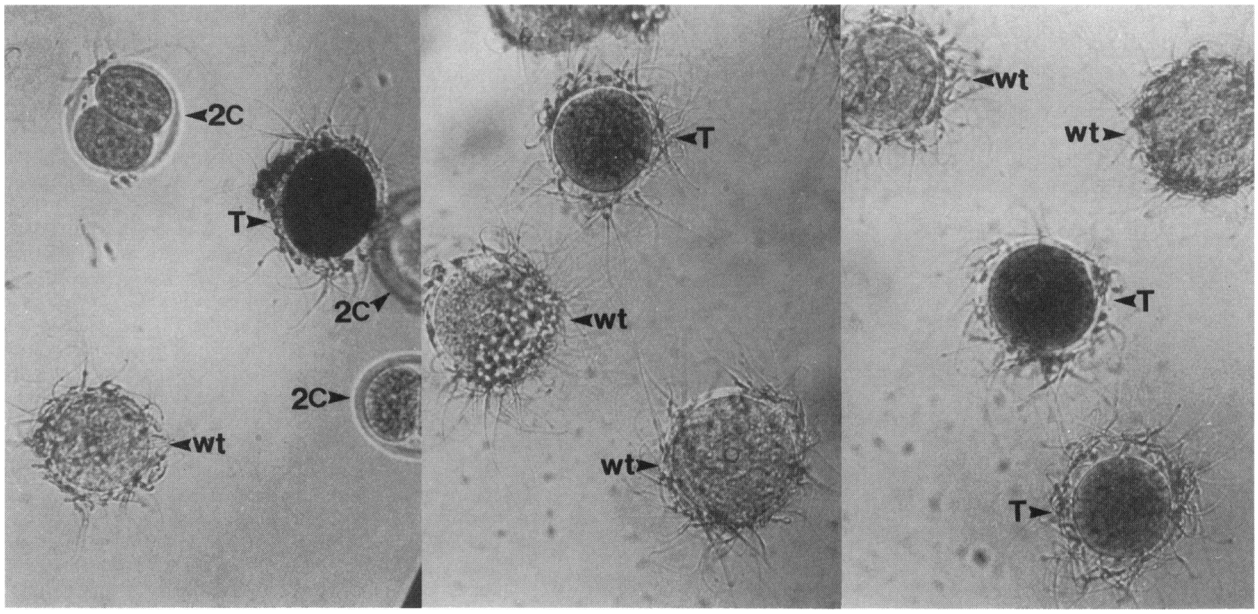

Figure 6. In vitro binding of mouse sperm to unfertilized eggs obtained from wild-type and transgenic mice. Shown are photomicrographs of mouse sperm bound to unfertilized eggs obtained from wild-type (wt) and transgenic (T; line #14-h) mice. Note that sperm do not bind to two-cell embryos (2C) obtained from wild-type mice. Eggs obtained from transgenic mice are stained with Coomassie blue, as described in MATERIALS AND METHODS. Magnification ×~175.

In this context, it should be noted that the affinity of purified mZP3 for the complementary sperm binding-protein is quite strong; e.g., at concentrations in the range of 50–100 nM, mZP3 binds to the sperm head and significantly inhibits binding of sperm to eggs in vitro (Bleil and Wassarman, 1980; Florman and Wassarman, 1985).

There are more than 10^9 copies of mZP3 in the ovulated mouse egg ZP (~7 μm thick). mZP3 is located periodically, every 14–15 nm or so along the interconnected filaments that make up the ZP (Greve and Wassarman, 1985; Wassarman, 1988a; Wassarman and Mortillo, 1991). Because the ZP is a loose extracellular matrix, it is difficult to accurately estimate the number of mZP3 molecules exposed at the ZP surface. However, at very high sperm concentrations, approximately 1500 sperm can bind to an ovulated mouse egg (approximately 1 sperm bound/25 μm^2 of ZP; similar to the maximum number of sperm that can bind to a sea urchin egg; Vacquier and Payne, 1973) and, if each sperm binds 50–500 mZP3 molecules (see above), there must be at least 7.5×10^4 (i.e., 50×1500) to 7.5×10^5 (i.e., 500×1500) copies of mZP3 at the ZP surface. The limitation on the number of sperm that can bind to the ovulated egg ZP apparently is not the number of mZP3 molecules available, but steric constraints having to do with the size (vol) of the sperm head that limit occupancy to about 1500 sperm. For example, free-swimming sperm bind to growing oocytes in vitro (Bleil and Wassarman, 1980), at reduced numbers compared with ovulated eggs, even though the oocytes have an incomplete ZP and, consequently, much less than a full complement of mZP3 (i.e., the ZP increases in thickness as the oocyte increases in diameter; Wassarman and Albertini, 1994). Furthermore, we reported previously that sperm bind to aggregates of transfected EC cell that have EC-mZP3 adventitiously associated with their plasma membrane (Kinloch *et al.*, 1991), as well as to individual silica beads to which mZP3 is covalently linked (Vazquez *et al.*, 1989); in both cases, the number of sperm bound was related to the surface area available.

Experiments described here were carried out to determine whether a significant reduction in the number of active sperm receptors (mZP3) in the mouse egg ZP would affect fertility. As in previous transgenic studies that employed a foreign sperm receptor gene (Kinloch *et al.*, 1992), growing mouse oocytes incorporated mZP3[ser] into ZP filaments and the amount present in the ZP closely parallels the extent of expression of the transgene. Therefore, mutations at the sperm combining–site of mZP3, located in the C-terminal one-third of the molecule, apparently do not interfere with its role as a structural glycoprotein in ZP filaments (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991). Furthermore, reduction of the number of active sperm receptors in the ZP by more than 50% had no observable effect on either binding of sperm to eggs or fertility; this is depicted schematically in Figure 7. In this context, it was recently reported that

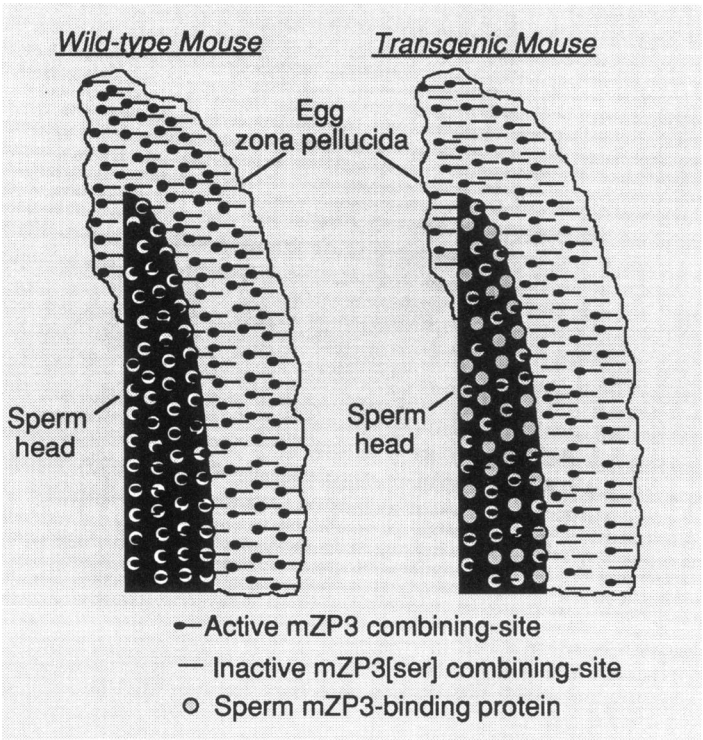

Figure 7. Schematic representation of sperm binding to the ZP of either a wild-type mouse egg (left) or a transgenic mouse egg (right). The wild-type mouse egg ZP has mZP3, with its functional combining site for sperm, is located every 14–15 nm or so along the filaments that make up the ZP ($>10^9$ copies mZP3/ZP). In this diagram (left), every sperm mZP3-binding protein is interacting with a wild-type mouse egg mZP3 combining site. As drawn, filaments of the transgenic mouse egg ZP consist of both mZP3 and mZP3[ser] in approximately equivalent amounts (~1:1). mZP3[ser], the mutated version of mZP3, has a nonfunctional combining site for sperm and, consequently, cannot interact with the sperm mZP3-binding protein. Therefore, filaments that make up the transgenic mouse egg ZP have a functional mZP3 present, on average, every 28–30 nm or so. In this diagram (right), every other sperm mZP3 binding protein is interacting with a transgenic mouse egg mZP3 combining site. It has been estimated that each mouse sperm has ~5–5,000 copies of mZP3-binding protein per μm^2 of sperm-head plasma membrane and that each sperm initially makes contact with ~50–500 copies of the mZP3-binding protein (see DISCUSSION). Here, the section of sperm shown makes approximately twice as many contacts with the wild-type mouse egg ZP than with the transgenic mouse egg ZP. Despite this difference, the sperm makes a sufficient number of interactions with the transgenic mouse egg ZP to support binding and fertilization.

overexpression of a putative ZP3-binding protein β1,4-galactosyltransferase by transgenic mouse sperm resulted in a significant decrease in binding of sperm to eggs (Youakim *et al.*, 1994). In view of these results, it was suggested that binding of mouse sperm to eggs requires an optimal, rather than a maximal level of ZP3-binding protein on the surface of sperm. Results presented here suggest that the number of active mZP3 molecules in the ZP can be altered considerably without altering sperm binding and subsequent steps in the fertilization pathway, including induction of the acrosome reaction (Bleil and Wassarman, 1983; Kopf and Gerton, 1991).

After fertilization or artificial activation of eggs, free-swimming sperm can no longer bind to the ZP (Gwatkin, 1977; Bleil and Wassarman, 1980; Yanagimachi, 1994) because of the modification of ZP components by cortical granule enzymes (Ducibella, 1991; Yanagimachi, 1994). Consistent with this is the finding that mZP3 purified from the embryo ZP does not exhibit activity as a sperm receptor or acrosome reaction–inducer in vitro when compared with equivalent concentrations of unfertilized egg mZP3 (Bleil and Wassarman, 1980). However, when embryo mZP3 is used at concentrations three- to five-times higher than egg mZP3, some activity is observed (Wassarman *et al.*, 1985). There are at least two alternative interpretations of these results. Either all mZP3 molecules are modified such that the activity of each molecule is reduced significantly (i.e., its affinity for the sperm binding–protein decreases) or a large percentage of mZP3 is completely inactivated, but a small amount of active mZP3 remains. In view of the present findings, it is likely that inactivation of 50–65% of mZP3 molecules after fertilization would be insufficient to prevent binding of sperm to eggs and fertilization.

Finally, it should be noted that the experimental approach taken here complements that of gene targeting to produce "knockout" mice. Because mZP3 and mZP2 form the dimers that constitute ZP filaments (Wassarman and Mortillo, 1991), it is likely that mice lacking a functional mZP3 gene will produce oocytes that lack a ZP; of course, this will be dependent on the way in which the mZP3 gene is disrupted by homologous recombination (Wassarman and DePamphilis, 1993; Melton, 1994). Although such "knockout" mice obviously will be extremely useful, a variety of issues concerning mZP3 function during reproduction can be addressed by the alternative approach taken here.

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REFERENCES

Baltz, J.M., and Cardullo, R.A. (1989). On the number and rate of formation of sperm-zona bonds in the mouse. Gamete Res. 24, 1–8.

Bleil, J.D., Greve, J.M., and Wassarman, P.M. (1988). Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev. Biol. *128*, 376–385.

Bleil, J.D., and Wassarman, P.M. (1980). Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell *20*, 873–882.

Bleil, J.D., and Wassarman, P.M. (1983). Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev. Biol. *95*, 317–324.

Bleil, J.D., and Wassarman, P.M. (1986). Autoradiographic visualization of the mouse egg's receptor bound to sperm. J. Cell Biol. *102*, 1363–1371.

Chamberlin, M.E., Ringuette, M.J., Philpott, C.C., Chamow, S.M., and Dean, J. (1989). Molecular genetics of the mouse zona pellucida. In: The Mammalian Egg Coat–Structure and Function, ed. J. Dietl, Berlin, Germany: Springer-Verlag, 1–17.

Dietl, J., (ed.) (1989). The Mammalian Egg Coat–Structure and Function. Berlin: Springer-Verlag, 156.

Ducibella, T. (1991). Mammalian egg cortical granules and the cortical reaction. In: Elements of Mammalian Fertilization, ed. P.M. Wassarman, Boca Raton, FL: CRC Press, 205–232.

Florman, H.M., and Wassarman, P.M. (1985). *O*-Linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell *41*, 313–324.

Greve, J.M., and Wassarman, P.M. (1985). Mouse extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J. Mol. Biol. *181*, 253–264.

Gwatkin, R.B.L. (1977). Fertilization Mechanisms in Man and Mammals. New York: Plenum Press, 161.

Kinloch, R.A., Lira, S.A, Mortillo, S., Schickler, M., Roller, R.J., and Wassarman, P.M. (1993). Regulation of expression of mZP3, the sperm receptor gene, during mouse development. In: Molecular Basis of Morphogenesis, ed. M. Bernfield, New York: Wiley-Liss, 19–33.

Kinloch, R.A., Mortillo, S., Stewart, C.L., and Wassarman, P.M. (1991). Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylate similar polypeptides and secrete active mouse sperm receptor. J. Cell Biol. *115*, 655–664.

Kinloch, R.A., Mortillo, S., and Wassarman, P.M. (1992). Transgenic mouse eggs with functional hamster sperm receptors in their zona pellucida. Development *115*, 937–946.

Kinloch, R.A., Sakai, Y., and Wassarman, P.M. (1995). Mapping the mouse ZP3 combining-site for sperm by exon-swapping and site-directed mutagenesis. Proc. Natl. Acad. Sci. USA *92*, 263–267.

Kinloch, R.A., and Wassarman, P.M. (1989). Profile of a mammalian sperm receptor gene. New Biol. *1*, 232–238.

Kopf, G.S., and Gerton, G.L. (1991). The mammalian sperm acrosome and the acrosome reaction. In: Elements of Fertilization, ed. P.M. Wassarman, Boca Raton, FL: CRC Press, 153–204.

Lira, S.A., Kinloch, R.A., Mortillo, S., and Wassarman, P.M. (1990). An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. Proc. Natl. Acad. Sci. USA *87*, 7215–7219.

Lira, S.A., Schickler, M, and Wassarman, P.M. (1993). *cis*-acting DNA elements involved in oocyte-specific expression of mouse sperm receptor gene *mZP3* are located close to the gene's transcription start site. Mol. Reprod. Dev. *36*, 494–499.

Melton, D.W. (1994). Gene targeting in the mouse. BioEssays *16*, 633–638.

Moller, C.C., Bleil, J.D., Kinloch, R.A., and Wassarman, P.M. (1990). Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. Dev. Biol. *137*, 276–286.

Mortillo, S., and Wassarman, P.M. (1991). Differential binding of gold-labeled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. Development *113*, 141–150.

Rosiere, T.K., and Wassarman, P.M. (1992). Identification of a region of mouse zona pellucida glycoprotein mZP3 that possesses sperm receptor activity. Dev. Biol. *154*, 309–317.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Vacquier, V.D., and Payne, J.E. (1973). Methods for quantitating sea urchin sperm-egg binding. Exp. Cell Res. *82*, 227–235.

Vazquez, M.H., Phillips, D.M., and Wassarman, P.M. (1989). Interaction of mouse sperm with purified sperm receptors covalently linked to silica beads. J. Cell Sci. *92*, 713–722.

Wassarman, P.M. (1987a). The biology and chemistry of fertilization. Science *235*, 553–560.

Wassarman, P.M. (1987b). Early events in mammalian fertilization. Annu. Rev. Cell Biol. *3*, 109–142.

Wassarman, P.M. (1988a). Zona pellucida glycoproteins. Annu. Rev. Biochem. *57*, 415–442.

Wassarman, P.M. (1988b). Fertilization in mammals. Sci. Am. *255*, 78–84.

Wassarman, P.M. (1990). Profile of a mammalian sperm receptor. Development *108*, 1–17.

Wassarman, P.M. (1993). Mammalian eggs, sperm, and fertilization: dissimilar cells with a common goal. Semin. Dev. Biol. *4*, 189–197.

Wassarman, P.M., and Albertini, D.F. (1994). The mammalian ovum. In: The Physiology of Reproduction, ed. E. Knobil and J. Neill, New York: Raven Press, 79–122.

Wassarman, P.M., Bleil, J., Fimiani, C., Florman, H., Greve, J., Kinloch, R., Moller, C., Mortillo, S., Roller, R., Salzmann, G., and Vazquez, M. (1989). The mouse egg receptor for sperm: a multifunctional zona pellucida glycoprotein. In: The Mammalian Egg Coat–Structure and Function, ed. J. Dietl, Berlin, Germany: Springer-Verlag, 18–37.

Wassarman, P.M., Bleil, J., Florman, H., Greve, J., Roller, R., Salzmann, G., and Samuels, F. (1985). The mouse egg's receptor for sperm: what is it and how does it work? Cold Spring Harbor Symp. Quant. Biol. *50*, 11–19.

Wassarman, P.M., and DePamphilis, M.L. (ed.) (1993). Guide to Techniques in Mouse Development. Methods Enzymol. *225*, San Diego, CA: Academic Press, 1021.

Wassarman, P.M., and Litscher, E.S. (1994). Sperm-egg recognition mechanisms in mammals. Curr. Top. Dev. Biol. *30*, 1–19.

Wassarman, P.M., and Mortillo, S. (1991). Structure of the mouse egg extracellular coat, the zona pellucida. Int. Rev. Cytol. *130*, 85–109.

Yanagimachi, R. (1977). Specificity of sperm-egg interaction. In: Immunobiology of Gametes, ed. M. Edidin, and M.H. Johnson, Cambridge, UK: Cambridge University Press, 255–295.

Yanagimachi, R. (1994). Mammalian fertilization. In: The Physiology of Reproduction, ed. E. Knobil and J. Neill, New York: Raven Press, 189–317.

Youakim, A., Hathaway, H.J., Miller, D.J., Gong, X., and Shur, B.D. (1994). Overexpressing sperm surface β1,4-galactosyltransferase in transgenic mice affects multiple aspects of sperm-egg interactions. J. Cell Biol. *126*, 1573–1583.