Overexpressing Sperm Surface
\(\beta1,4\)-Galactosyltransferase in Transgenic Mice Affects Multiple Aspects of Sperm–Egg Interactions

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Abstract. Sperm surface \(\beta1,4\)-galactosyltransferase (GalTase) mediates fertilization in mice by binding to specific \(O\)-linked oligosaccharide ligands on the egg coat glycoprotein ZP3. Before binding the egg, sperm GalTase is masked by epididymally derived glycosides that are shed from the sperm surface during capacitation. After binding the egg, sperm-bound oligosaccharides on ZP3 induce the acrosome reaction by receptor aggregation, presumably involving GalTase. In this study, we asked how increasing the levels of sperm surface GalTase would affect sperm–egg interactions using transgenic mice that overexpress GalTase under the control of a heterologous promoter. GalTase expression was elevated in many tissues in adult transgenic animals, including testis. Sperm from transgenic males had approximately six times the wild-type level of surface GalTase protein, which was localized appropriately on the sperm head as revealed by indirect immunofluorescence. As expected, sperm from transgenic mice bound more radiolabeled ZP3 than did wild-type sperm. However, sperm from transgenic animals were relatively unable to bind eggs, as compared to sperm from wild-type animals. The mechanistic basis for the reduced egg-binding ability of transgenic sperm was attributed to alterations in two GalTase-dependent events. First, transgenic sperm that overexpress surface GalTase bound more epididymal glycoside substrates than did sperm from wild-type mice, thus masking GalTase and preventing it from interacting with its zona pellucida ligand. Second, those sperm from transgenic mice that were able to bind the zona pellucida were hypersensitive to ZP3, such that they underwent precocious acrosome reactions and bound to eggs more tenuously than did wild-type sperm. These results demonstrate that sperm–egg binding requires an optimal, rather than maximal, level of surface GalTase expression, since increasing this level decreases sperm reproductive efficiency both before and after egg binding. Although sperm GalTase is required for fertilization by serving as a receptor for the egg zona pellucida, excess surface GalTase is counterproductive to successful sperm–egg binding.

Mammalian fertilization is initiated when a sperm binds to the zona pellucida matrix surrounding the egg. The initial interaction between sperm and egg is a carbohydrate-mediated process in which a receptor on the sperm surface binds to its glycoside ligand on the zona pellucida (Miller and Ax, 1990). In mouse, the zona pellucida ligand has been identified as a class of oligosaccharides bound in an \(O\)-glycosidic linkage to the ZP3 polypeptide, one of three glycoproteins that constitute the zona pellucida (Florman and Wassarman, 1985). Several sperm proteins have been suggested to function as receptors for ZP3 oligosaccharides (Bleil and Wassarman, 1990; Leyton et al., 1992; Miller et al., 1992). To date, only one candidate receptor, \(\beta1,4\)-galactosyltransferase (GalTase), satisfies all the criteria required of a sperm receptor for the zona pellucida (Lopez et al., 1985; Shur and Neely, 1988; Miller et al., 1992). GalTase is confined to the sperm plasma membrane overlying the acrosome, as expected for an egg receptor. Affinity-purified sperm surface GalTase competitively inhibits sperm–egg binding, as do GalTase-specific perturbants. Sperm GalTase selectively binds oligosaccharides on

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1. Abbreviations used in this paper: DEPC, pyrocarbonic acid diethyl ester; GalTase, \(\beta1,4\)-galactosyltransferase; GlcNAc, N-acetylglucosamine; hGH, human growth hormone; MT-I, metallothionein; NGS, normal goat serum; NOG, \(n\)-octylglucopyranoside; nt, nucleotides; PAA, protein-A-agarose; PIC, protease inhibitor cocktail.
ZP3 that have sperm receptor activity; sperm GalTase does not bind irrelevant zona pellucida oligosaccharides. That the GalTase-binding site on ZP3 is required for its sperm receptor activity is shown by the loss of ZP3's biological activity following the specific removal or blockage of its oligosaccharide substrate for sperm GalTase. These and other observations demonstrate that surface GalTase is required for sperm–egg binding. Whether either of two other sperm proteins suggested to function as ZP3 receptors cooperate with GalTase during fertilization remains unclear. There is evidence that one of these proteins (p56) binds ZP3; however, it behaves as a peripheral membrane protein on the sperm surface (Cheng et al., 1994; Bleil and Wassarman, 1990). Another candidate ZP3 receptor (p95) has recently been identified as sperm hexokinase (Kalab et al., 1994).

When sperm are first released from the epididymis, they are unable to bind the zona pellucida. This is due, at least in part, to masking of sperm GalTase by epididymal glycosides. Before reaching the egg, these epididymally derived glycosides are spontaneously shed from the sperm surface, thereby exposing the GalTase-binding site and enabling sperm to bind the zona (Shur and Hall, 1982a). GalTase substrates purified from epididymal fluids inhibit sperm–egg binding by competing for the GalTase-binding site; irrelevant glycoproteins that are not substrates for sperm GalTase have no effect on sperm–egg binding. When sperm arrive at the egg zona pellucida, sperm surface GalTase mediates fertilization by binding N-acetylgalcosamine (GlcNAc)-terminated oligosaccharides on ZP3 (Lopez et al., 1985; Miller et al., 1992). Sperm binding to ZP3 induces the acrosome reaction, which is thought to be initiated by aggregation of sperm receptors by multivalent ZP3 oligosaccharides (Florman et al., 1984; Leyton and Saling, 1989). In this regard, each ZP3 glycoprotein has multiple GalTase-binding oligosaccharides (Miller et al., 1992) and aggregation of surface GalTase using multivalent antibodies is sufficient to initiate the acrosome reaction via a pertussis toxin-sensitive mechanism (Macek et al., 1991; Miller and Shur, unpublished observations). During this process, the plasma membrane overlying the acrosome and the adjacent outer acrosomal membrane are shed, releasing acrosomal proteases and N-acetylgalcosaminidase, which enable sperm to penetrate the zona pellucida (Miller et al., 1993a). Egg activation by the fertilizing sperm is also accompanied by release of N-acetylgalcosaminidase from cortical granules, which removes the GalTase-binding site from ZP3 oligosaccharides, thus mediating the block to polyspermic binding (Miller et al., 1993b).

In light of the central role that sperm GalTase plays in murine fertilization, we explored the effects of overexpressing surface GalTase on the process of sperm–egg binding in transgenic mice. The gene for GalTase encodes two nearly identical proteins: the short GalTase contains 386 amino acids, whereas the long GalTase contains 399 amino acids (Russo et al., 1990). The additional 13 amino acids on the long form are located on the amino–terminal cytoplasmic domain and are required for transporting a portion of GalTase to the cell surface, where it functions as a cell adhesion molecule (Evans et al., 1993; Youakim et al., 1994; Evans and Shur, unpublished observations). Sperm express only the long form of GalTase (Shaper et al., 1990; Pratt and Shur, 1993), and consequently, transgenic animals used in this study were created by injecting a mini-gene specifically encoding long GalTase under the control of a heterologous promoter.

We report here, that contrary to our initial expectations, overexpressing surface GalTase on sperm of two independently derived transgenic mouse lines resulted in decreased binding of sperm to eggs, compared to wild-type sperm. This decreased egg-binding ability of transgenic sperm was due to alterations in two GalTase-dependent events during fertilization. First, sperm from transgenic animals bound more epididymal glycosides than did wild-type sperm, effectively masking sperm surface GalTase from its zona pellucida ligand. Second, those sperm from transgenic mice that were able to bind the zona were hypersensitive to ZP3, such that they underwent acrosome reactions earlier than did wild-type sperm and bound to eggs more tenously. Thus, simply increasing the level of surface GalTase on sperm does not lead to a subsequent increase in sperm binding, but rather, leads to decreased binding, illustrating that successful fertilization requires an optimal, rather than a maximal, level of sperm receptors for the egg.

Materials and Methods

Construction of EV-142 Expression Plasmids Containing Long GalTase

The EV-142 expression vector contains the mouse metallothionein (MT1) promoter fused to a fragment of the human growth hormone gene (hGH) containing the polyadenylation signal (Low et al., 1985). Construction of the plasmid containing long GalTase cDNA was performed as described previously (Evans et al., 1993), with the following modification. The 4.7-kb fragment containing MT1, PDGFL, and the hGH 3' untranslated sequences was excised from the vector by EcoRI digestion, and ligated into EcoRI-cut Bluescript (Stratagene Inc., La Jolla, CA). Intron 4 from the GalTase genomic DNA was inserted into the CDNA as follows: a 5-kb fragment was amplified from a partial genomic GalTase clone, using primers flanking a unique Stul restriction site within exon 3 and a unique BsmI site within exon 5. The polymerase chain reaction product was isolated, and digested with BsmI and Ndel and the appropriate 2-kb fragment containing intron 4 was ligated into the vector containing the GalTase cDNA that was previously digested with Ndel and BsmI. The resulting cDNA/genomic hybrid was sequenced across the ligation junctions and into the intron.

Production and Identification of Transgenic Mice

The EV-142 plasmid containing the long GalTase cDNA/genomic hybrid insert was digested with EcoRI to release the insert, which was then purified. Transgenic mice were created by microinjecting the linearized insert into the pronuclei of mouse zygotes. The zygotes were isolated from B6D2F1 CD1 foster mothers. Offspring were genotyped by Southern blotting of the pronuclei of mouse zygotes. The zygotes were isolated from B6D2F1 males and were introduced into the ampullae of CD1 foster mothers. Offspring were genotyped by Southern blotting of DNA isolated from tails, using a 650-bp fragment of the hGH sequences as probe. Positive founder mice were mated with wild-type littermates to establish the F1 generation. Subsequently, genotyping was done by slot blot analysis of tail DNA, using hGH as probe and a 1-kb fragment of the major urinary protein (MUP) gene (Derman, 1981) as an internal standard for quantitation of the DNA.

GalTase Enzyme Assays

Adult tissues. To assay for inducibility of the transgene, 25 mM ZnSO4 was added to the drinking water 4 d before sacrifice. Tissues were isolated from adult male hemizygous transgenic or wild-type mice between 2 and 4 mo of age. Tissue was homogenized on ice in 1 ml of medium B (140 mM NaCl, 4 mM KCl, 20 mM Hepes; pH 7.2) containing freshly added protease inhibitor cocktail (PIC; 2 µg/ml aprotinin, 0.1% aprotinin, 10 µg/ml benzamidine, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml pepstatin), with a Polytron (Brinkman Instrs., Inc., Westbury, NY) for 6 s at a setting of 8. Homogenates were cleared of debris by centrifugation at 13,000 g for 5 min at 4°C. The supernatant was removed to a fresh tube, and n-octylglyco-
pyranoside (NOG) was added to a final concentration of 30 mM. The samples were incubated on ice for 2 h with frequent titration. Insoluble material was removed by centrifugation for 5 min at 4°C. Protein concentrations were determined (Bradford, 1976). Enzyme assays were conducted for 1 h at 37°C in a total volume of 50 μl, and contained 10 μg protein, 100 μM uridine-5'-diphosphate (UDP)[3H]galactose (UDP[3H]Gal; 220 dpm/μmol; Du- pont-New England Nuclear, Wilmington, DE), 1 mM 5'-AMP, 10 mM MgCI2, and 30 mM GlcNac, in medium B/PIC. The reaction was stopped by addition of 10 μl ice-cold 0.2 M EDTA-Tris-HCl, pH 7.2. 50 μl was subjected to high-voltage borate electrophoresis to separate the 3H-labeled products. The complex was incubated overnight on a rocking plate, washed three times with medium B/PIC, and 5% BSA was incubated for 4 h at 4°C. The supernatant was assayed for protein content, and incubated under these conditions, since sperm are unusual in that all of their GalTase is localized to the plasma membrane; there is no intracellular pool of biosynthetic GalTase in mature spermatozoa (Lopez and Shur, 1987; Shur and Neely, 1988). An aliquot of the last wash was also assayed for activity to insure that all soluble epididymal GalTase had been removed from the washed sperm pellet. The assays were performed three times, each in duplicate.

**Immuno blotting**

Sperm were recovered, washed, and solubilized as described above for immunoprecipitation, except that BSA was omitted from all buffers. 250 μg of protein was electrophoresed on a 10% nonreducing polyacrylamide gel. Proteins were transferred to nitrocellulose by electrophoretic transfer for 4 h at room temperature. The filters were blocked overnight at 4°C in PBT (PBS, pH 7.2, containing 0.2% Tween 20 and 5% BSA). The filters were incubated with primary antibodies (anti-peptide 1 immune and preimmune serum) diluted 1:100 in PBT, and washed three times (20 min/wash) with PBT. [125I]-Labeled goat anti-rabbit IgG (ICN, Irvine, CA) in PBT was added to filters for 3 h at 4°C, and filters were washed as before. The filters were exposed to x-ray film for 14 h to visualize immunoreactive proteins.

**Immunofluorescence**

Sperm were capacitated, washed three times, and dried onto poly-L-lysine-coated glass slides. Dried sperm were fixed for 10 min at −20°C in 15% ethanol/5% glacial acetic acid which also permeabilized sperm to expose the cytoplasmic epitope for anti-peptide 1 antiserum. Sperm were rinsed three times in PBS and once in PBS with 5% normal goat serum (NGS) and incubated in PBS/5% NGS containing a 1:1000 dilution of anti-peptide 1 immune or preimmune serum overnight at 4°C in a humidified atmosphere. After rinsing three times in PBS and once in PBS/5% NGS, sperm were incubated in PBS/5% NGS containing a 1:250 dilution of biotinylated goat anti-rabbit IgG (Vector Labs., Inc., Burlingame, CA) for 45 min at room temperature. Finally, sperm were washed three times in PBS and incubated in PBS containing a 1:100 dilution of fluorescein-labeled avidin (avidin-FITC; Vector Labs., Inc.) for 45 min at room temperature. After being washed in PBS, sperm were mounted in glycerol/PBS (9:1) containing 4% nonyl gallate, coverslipped, and viewed with a microscope equipped for epifluorescence (Dialux EB 22; Leitz, Rockleigh, NJ).

**ZP3 Binding Assay**

Sperm were isolated and capacitated as described above, then adjusted to a concentration of 1 × 10^6 sperm/100 μl in dmKRB/BSA. Pertussis toxin was added to the sperm suspensions to a final concentration of 100 ng/ml to inhibit acrosome reactions. [125I]-Labeled and unlabeled ZP3 were isolated and prepared as described (Miller et al., 1992). One million cpm of [125I]-ZP3 were added to the sperm suspension and incubated at 37°C for 30 min. Sperm were washed three times in dmKRB/BSA, and bound [125I]-ZP3 was counted in a gamma counter. Nonspecific binding was determined by including 10 μg unlabeled ZP3 and subtracted from all assays.

**Sperm–Egg Binding Assays**

Cauda epididymal sperm were collected and capacitated as described above for 1 h in dmKRB. Sperm were counted, and the concentration was adjusted to 3 × 10^6 sperm/ml. Motility was monitored visually with phase-contrast optics. Unfertilized eggs were collected from the oviducts of superovulated CF1 females into dmKRB. Cumulus cells were removed by incubation in 0.2% hyaluronidase at room temperature. Eggs were washed through several drops of fresh dmKRB and added in groups of 20–50 to 20–μl drops of dmKRB under paraffin oil. As controls, two-cell fertilized eggs were flushed from the oviducts of superovulated, mated CF1 females 30 h after coitus. Two-cell eggs were washed through several drops of dmKRB and added to 20–μl dmKRB drops with unfertilized eggs. 20 μl of sperm suspension was added to drops containing eggs, and binding was allowed to proceed for 30 min at 37°C, unless otherwise indicated. Following the binding assay, eggs were washed free of bound or loosely bound sperm by transferring the eggs through fresh 50-μl drops of dmKRB with a wide-bore pipet (at least 3 times the diameter of an egg). Washing was discontinued when only 1–2 sperm remained bound to two-cell embryos. Eggs were then fixed in freshly prepared 4% paraformaldehyde, washed once in dmKRB, transferred to glass slides, and coverslipped. Sperm were counted on a Dialux EB 22 microscope using phase optics at a magnification of 400 X. Each experiment was performed in duplicate, and repeated two or three times.

For some experiments, the following variations to the standard sperm-egg binding assay were made:

**Competitive Binding Assay.** Sperm were capacitated in a modified, low-salt solution (0.3 M sucrose, 10 mM TAPSO, 1.1 mM sodium pyruvate, and 6 mg/ml BSA) containing either 2.5 μM DiQ (di-16-ASQ), 4-[p-dihexadecylaminostyryl]-N-methylquinolinium iodide; Molecular Probes, Inc., Eugene, OR) or 2.5 μM BODIPY (5,7-dimethyl-BODIPY**-**1-hexane carboxylic acid; Molecular Probes, Inc.). Sperm were gently centrifuged (800 g, 20 s) and resuspended in dmKRB at a concentration of 2 × 10^6 sperm/ml. Bound sperm were visualized using epifluorescence optics on a Dialux EB 22 microscope at a magnification of 500X.
Determination of Initial Binding and Effect of Washing Sperm and Eggs After 30 Minutes: Sperm were capacitated in dmKRBT, counted, and adjusted to $2 \times 10^6$ sperm/ml as described above. For each genotype, one-third of the sperm/egg incubations were stopped after 5 min and fixed without washing to determine the number of sperm initially bound. After 30 min, another third of the samples were fixed without washing, while the remaining third were washed and fixed as described above.

Sperm Washing Assay: Sperm were capacitated in dmKRBT as described above and then washed twice by centrifugation at 800 g for 5 min. As controls, an equal volume of sperm were mock washed by centrifugation and resuspension in the same medium. Sperm were counted and adjusted to $2 \times 10^6$ sperm/ml. The number of sperm that initially bound to eggs was determined by fixing the sperm and eggs after a 5-min incubation without washing away loosely bound sperm.

Determination of Acrosome Reactions

Spontaneous: Sperm were isolated and capacitated as described above and then counted and adjusted to $2 \times 10^6$ sperm/ml in dmKRBT. Sperm were then incubated at 37°C. One-fifth of the total volume was removed at time zero and at every hour for 4 h. Sperm were fixed in 100 μl 4% paraformaldehyde for 10 min at room temperature, centrifuged at 1000 g for 4 min, and resuspended in 500 μl 0.1 M ammonium acetate, pH 9.0. Sperm were centrifuged once more, resuspended in 100 μl 0.1 M ammonium acetate, and dried onto glass slides. Dried sperm were stained for 10 min at room temperature in 0.22% Coomassie blue-G250 in 50% methanol-10% glacial acetic acid, rinsed a few seconds in dH₂O, air dried, and mounted in Permoun and cover slipped. Acrosome reactions were determined by counting from 100–300 sperm for each time point. Assays were repeated four times.

Zona Pellucida Induced: Sperm were isolated and capacitated as described above, counted, and adjusted to $2 \times 10^6$ sperm/ml in dmKRBT. Solubilized whole zona pellucida proteins were isolated from ovarian homogenates as previously described (Bleil and Wassatman, 1986; Miller et al., 1992) and adjusted to give a final concentration of 10 μg in dmKRBT. Equal volumes of sperm and zona were mixed together in a final volume of 50 μl. At time zero and at 5, 15, and 30 min, one-fourth of the volume was removed, fixed, washed, and stained as described above for spontaneous acrosome reactions. The assay was repeated six times.

Acrosomal Status of Zona-bound Sperm: Coomassie blue staining was found to be unsuitable for monitoring the acrosomal status of zona-bound sperm, since staining of the zona obscured labeling of the sperm head. Consequently, acrosomal status was determined using the lipophilic dye, DiQ, which did not stain the zona. Sperm were capacitated and labeled with DiQ as described above for competitive binding assays, centrifuged, resuspended in dmKRBT, counted, and adjusted to $2 \times 10^6$ sperm/ml. After a 30-min binding assay, eggs and bound sperm were fixed in 4% paraformaldehyde without washing away loosely bound sperm. Sperm were counted, and their acrosomal status was determined using epifluorescence optics at a magnification of 1,000×. The presence of two clearly visible DiQ-stained sperm heads represented an acrosome-reacted sperm.

Results

Production of Transgenic Mice

Transgenic mice that overexpress the long form of GaTase were created by injecting murine GaTase cDNA into the pronuclei of fertilized mouse eggs. The GaTase cDNA was modified to include intron 4 from GaTase genomic DNA, since it has previously been shown that inclusion of introns frequently increases cDNA expression in transgenic animals (Brinster et al., 1988). The GaTase construct was subcloned into the EV-142 vector (Low et al., 1985), which includes the mouse MT-I promoter and the hGH polyadenylation sequence (Fig. 1). Founder animals were identified by Southern hybridization of EcoRI-digested mouse tail DNA, using the hGH sequences as probe. Of 17 live progeny, six contained the transgene (not shown). All six of these animals transmitted the transgene to their offspring, and six independent lines of transgenic mice were created from the F1 progeny. Two transgenic lines, designated 06 and 09, were chosen for further analysis based on high expression of the transgene (discussed below).

Transgenic Sperm Overexpress Surface GaTase

GaTase activity was elevated in a variety of tissues from adult transgenic mice, including liver, kidney, pancreas, and intestine; the addition of zinc to the drinking water to induce further expression of the MT-I promoter resulted in higher levels of GaTase activity in some tissues (data not shown). For this study, we focused our attention on transgene expression in the testes, which ideally would be taken from males homozygous for the transgene. However, we have been unable to produce homozygous transgenic lines, apparently due to embryonic lethality resulting from markedly elevated GaTase expression (manuscript in preparation). This turns out to be inconsequential, since sperm in hemizygous animals are functionally equivalent due to GaTase sharing among cytoplasminically connected spermatids (Braun et al., 1989; Pratt and Shur, 1993).

GaTase activity was 10–12 times higher in testes from hemizygous transgenic males than in wild-type animals (363 or 465 cpm/μg protein for 06 and 09 transgenics, respectively, versus 38 cpm/μg protein for wild type); induction with zinc increased GaTase activity only slightly (375 or 640 cpm/μg protein for 06 and 09, respectively, versus 50 cpm/μg protein for wild type). The lack of inducibility in the testes was not unexpected, given the high basal level of endogenous metallothionein expression in this tissue (De et al., 1991). Therefore, all subsequent assays were performed in the absence of zinc, and represent constitutive levels of transgene expression.

To confirm that the product of the transgene was long GaTase, RNase protection assays were performed using total RNA isolated from testes of transgenic and wild-type mice. The probe used spans the transcription initiation start sites for both long and short GaTase and allows a distinction between the endogenous long and short GaTase mRNAs, as well as the transgenic GaTase mRNA (Lopez et al., 1991; Fig. 2). Testicular RNA from all genotypes protected two fragments of equal intensity: one of 526 nucleotides (nt), which is the predicted size for the testes-specific long GaTase mRNA (Pratt and Shur, 1993); and another of 500 nt corresponding to long GaTase transcripts detected in all tissues examined (Shaper et al., 1990; Lopez et al., 1991; Pratt and Shur, 1993; Fig. 2). No short GaTase mRNA was detected in testes from any genotype, consistent with previous
reports that testis makes primarily long GalTase transcripts (Shaper et al., 1990; Pratt and Shur, 1993). An intense band corresponding to 296 nt was detected only in transgenic testes and represents the protected fragment of the GalTase RNA transcribed from the transgene (Fig. 2). Thus, the product of the GalTase transgene in mouse testes is an mRNA that contains the first in-frame translation initiation start site and encodes long GalTase.

The overexpression of surface GalTase protein in sperm from transgenic animals was confirmed by several criteria, including GalTase enzyme assays, as well as immunoprecipitation of enzyme activity and Western immunoblotting using antisera specific for long GalTase. Assays of GalTase necessarily measure surface-localized protein, since sperm are unique in that all of their GalTase is confined to the plasma membrane domain overlying the intact acrosome (Lopez and Shur, 1987; Shur and Neely, 1988). There is no intracellular pool of biosynthetic GalTase in mature sperm, nor is there any GalTase present within intracellular organelles, such as the acrosome.

Initially, washed, intact sperm were assayed for surface GalTase activity. Transgenic sperm contained 31.3 times (06/+) or 19.3 times (09/) higher enzyme activity than did wild-type sperm (Fig. 3 a). To confirm that the elevated enzyme activity in transgenic sperm reflected the long GalTase protein, an antibody (anti-peptide 1) produced against a synthetic peptide corresponding to the 13-amino acid residue unique to the cytoplasmic domain of long GalTase (Youakim et al., 1994) was used to immunoprecipitate GalTase activity. Anti-peptide 1 antibody was incubated with solubilized sperm proteins, and precipitated with protein A-agarose. GalTase enzyme activity remaining in the supernatant was determined. In wild-type sperm, 75% of the surface GalTase activity was removed from the supernatant with anti-peptide 1 antibody, compared to controls treated with preimmune serum (Fig. 3 b). Since only long GalTase mRNA is detected in testes, a portion (i.e., 25%) of the long GalTase appears to be refractory to immunoprecipitation, possibly the result of limitations intrinsic to the experimental technique or of postranslational modifications that prevent recognition by anti-peptide 1 antibody. A similar phenomenon has been observed in other cells using antibodies raised against the full-length protein (Hathaway and Shur, 1992). In any event, the anti-peptide 1 antibody was not limiting as shown by the fact that 53-60% of the surface GalTase activity from transgenic sperm was immunoprecipitated, although the absolute amount of immunoprecipitated GalTase activity was more than 30 times higher in the transgenic animals (Fig. 3 b).

The specificity of the antibody was demonstrated by coincubation with the 13-amino acid synthetic peptide, which competitively inhibited the formation of the immunoprecipitation complex, since enzyme activity remained nearly equal (i.e., 83%) control levels (Fig. 3 b).

To approximate the relative increase in surface GalTase protein recognized by anti-peptide 1 antibody, Western immunoblotting was performed on sperm proteins from transgenic and wild-type males. A polypeptide with an apparent molecular mass of 61 kD was specifically detected in all

![Figure 2](image-url)  
**Figure 2.** RNase protection analysis of RNAs from testes of transgenic and wild-type mice. 10 µg of total RNA was hybridized to a [32P-labeled riboprobe as described in Materials and Methods. Following digestion of single-stranded RNA, the protected probe was resolved on an acrylamide/urea gel. Protected fragments represent an endogenous long GalTase transcript common to all cell types examined (500 nt), an endogenous testis-specific transcript (526 nt), as well as a transgene-specific transcript (296 nt). The probe and the expected protected fragments are diagrammed below the figure. Undigested probe runs as a single band of 563 nt (not shown). To the right of the gel are the molecular size markers in nt. On the left are the deduced molecular sizes of the major protected fragments in nt.

![Figure 3](image-url)  
**Figure 3.** Transgenic sperm overexpress surface GalTase. (A) Intact, capacitated, and washed transgenic and wild-type sperm were assayed for surface GalTase activity as described in Materials and Methods. Surface GalTase activity was elevated 31.1 times (06/) or 19.3 times (09/) that of wild type. Data represent the average of four assays. Bars, SEM. (B) Surface GalTase activity was immunoprecipitated from solubilized sperm using the anti-peptide 1 antibody. 75% (range 71-79%) of enzyme activity was immunoprecipitated from wild-type sperm (wild-type sperm lysate produced 253 cpm in the presence of preimmune sera), whereas 53 or 60% (range 51-69%) of GalTase activity was immunoprecipitated from the 06/+ and 09/+ sperm, respectively (13,201 and 14,379 cpm in preimmune sera controls, respectively). Coincubation with the competing 13-amino acid peptide restored enzyme activity to nearly control levels (i.e., 83%, range 70-96%). (C) The relative increase in surface GalTase expression in transgenic sperm was assessed by Western immunoblotting using the anti-peptide 1 antibody. A band of ~61 kD was detected in all sperm (arrowhead); however, the intensity of the immunolabeled protein was 5-7 times greater in transgenic samples compared to wild type, as determined by scanning densitometry. Molecular weight standards are shown on the left.
cases (Fig. 3 c), consistent with the reported size of affinity-purified sperm surface GalTase (Shur and Neely, 1988). Densitometric analysis of the detected bands revealed that sperm from both transgenic lines had five to seven times the levels of surface GalTase protein relative to wild-type sperm. Examination of both shorter and longer exposures of two representative radiograms revealed the same relative difference in intensity between transgenic and wild-type sperm. It is presently unclear why an approximately sixfold increase in protein levels resulted in an ~20–30-fold increase in enzyme activity (see Discussion).

Results described thus far show that surface GalTase expression in transgenic sperm was higher than in sperm from wild-type animals. This elevated expression was constitutive despite the presence of an inducible promoter; little additional expression was observed after induction with zinc. Furthermore, only long GalTase was expressed in these tissues and cells as determined by RNase protection analysis, and confirmed by immunoprecipitation of GalTase activity and Western immunoblotting using anti-peptide 1 antibodies specific for long GalTase.

GalTase Is Localized Properly on Transgenic Sperm

The goal of this study was to determine how overexpression of sperm surface GalTase affects the ability of transgenic sperm to bind eggs. The overexpressed surface GalTase must be localized to its proper domain for this issue to be addressed. Therefore, the localization of GalTase on transgenic sperm was determined by indirect immunofluorescence using anti-peptide 1 antibody. The staining patterns of wild-type and transgenic sperm were indistinguishable. In all cases, immunoreactivity was detected on the plasma membrane overlying the acrosome (Fig. 4). (Immunofluorescence intensity was not quantified due to limitations intrinsic to this analysis.) Of those transgenic sperm labeled at this level of resolution, there appeared to be only one population of equal fluorescence intensity. This was expected due to sharing of GalTase mRNAs between haploid spermatids, which produces a functionally homozygous sperm population (Pratt and Shur, 1993).

Transgenic Sperm Bind More ZP3 Ligand

Sperm GalTase participates in sperm-egg interactions by binding to ZP3 in the egg zona pellucida (Miller et al., 1992). The increased expression of GalTase on the sperm surface would be expected to result in increased binding of radiolabeled ZP3. To determine if this was indeed the case, 125I-labeled ZP3 was gel purified and used in binding experiments with intact sperm. Parallel assays included a 10–20-fold excess of unlabeled ZP3 to represent background binding, which was subtracted from all values. All assays were done in the presence of pertussis toxin to inhibit ZP3-induced acrosome reactions. Sperm from both transgenic lines bound approximately twice the level of radiolabeled ZP3 as did wild-type sperm (Fig. 5). Surprisingly, a sixfold increase in surface GalTase expression resulted in only a two-fold increase in ZP3 binding. This suggests that a portion of GalTase on transgenic sperm was inaccessible to the relatively large ZP3 glycosides, an observation that is addressed below. Nevertheless, transgenic sperm that overexpressed surface GalTase bound more ZP3, and we reasoned, therefore, that transgenic sperm would bind the intact zona pellucida better than would wild-type sperm.

Transgenic Sperm Bind Poorly to Eggs

In vitro assays were used to determine the ability of sperm from transgenic mice to bind eggs. Capacitated sperm were added to drops containing unfertilized eggs. After 30 min, unbound and loosely adherent sperm were removed by washing the eggs through successive drops of medium. Washing conditions that result in background levels of nonspecific binding (i.e., 1–2 sperm/2-cell embryo) have been previously described (Shur and Hall, 1982a,b; Miller et al., 1992). The eggs with adherent sperm were fixed, and bound sperm were quantified. Many more wild-type sperm bound to eggs than did sperm from either transgenic line (Fig. 6a). Binding of transgenic sperm to eggs was 15% of wild type for 06/+ and 30% of wild type for 09/+.

To ensure that differences in egg binding were not due to experimental variation between droplets containing sperm of only one genotype, an assay was devised whereby the binding of both wild-type and transgenic sperm could be assayed in the same droplet. Sperm were labeled during capacitation in either of two different fluorescent lipophilic dyes. Equal numbers of wild-type and transgenic sperm were added to
Figure 5. Transgenic sperm bind higher levels of radiolabeled ZP3 ligand. 

Labelled ZP3 was purified and incubated with intact, capacitated sperm. Unbound ZP3 was removed by centrifugation. Sperm from either the 06 or the 09 line bound approximately two times more ZP3 above background (incubations including excess unlabeled ZP3) than did wild-type sperm. Data represent the average of three assays. Wild-type sperm binding = 4094 cpm 

eggs. The dyes did not affect sperm motility, acrosomal status, or GalTase activity (data not shown). To control for any possible differential effects of the dyes, parallel assays were conducted in which the labeling of genotypes was reversed; duplicate drops were scored in all instances. Using this binding assay, wild-type sperm again bound to eggs in greater numbers than did either 06/+ or 09/+ transgenic sperm present in the same droplet, regardless of the dye used (Fig. 6 b). However, in these assays, the binding of transgenic sperm to eggs was not decreased to the same degree as it was in droplets containing sperm of only one genotype (Fig. 6 a). This most likely reflects the fact that assays containing only one sperm genotype measure the absolute ability of sperm to bind the zona, whereas those assays containing sperm of both genotypes measure their relative ability to compete for zona binding. In any event, both assays demonstrate that transgenic sperm show defective egg-binding activity as compared to wild-type sperm.

To understand the apparent conflict between the expression of high levels of sperm surface GalTase and reduced egg-binding activity, sperm–egg binding was examined in more detail. No obvious physiological defect could account for the decreased binding; sperm from transgenic lines had motility similar to that of wild-type sperm throughout the assay (data not shown). The time dependency of binding was examined to define when transgenic sperm displayed defective binding. Sperm binding was found to be defective at two distinct times after insemination. First, sperm from both transgenic lines failed to bind eggs as well as wild-type sperm immediately after insemination. When eggs were collected within 5 min of insemination and the total number of adherent sperm determined (i.e., no subsequent washing away of loosely adherent sperm), eggs incubated with transgenic sperm had 35% (06/+ ) or 73% (09/+ ) fewer bound sperm than did eggs inseminated with wild-type sperm (Fig. 7 a). This suggests that a portion of sperm from transgenic mice were initially incapable of interacting with the egg zona pellucida. Second, at the end of a standard 30-min binding assay, sperm from transgenic mice were more loosely adherent to the zona pellucida than were sperm from wild-type mice, since a greater proportion of transgenic sperm was removed from eggs by the washing conditions used to reduce nonspecific binding (Fig. 7 b). These results suggest that transgenic sperm contained at least two functionally distinct populations, one that was initially unable to bind eggs, and another that could bind eggs, but did so more tenuously and was, therefore, easily removed by washing. The basis for these binding defects was explored further.

Transgenic Sperm Retain Higher Levels of Competitive Epididymal Glycoside Substrates

Previous work has shown that when sperm are released from the epididymis, sperm GalTase is occupied by epididymal glycosides that must be removed from the sperm surface in order for sperm to bind the zona (Shur and Hall, 1982a). The fact that transgenic sperm initially bound to the egg poorly and also bound lower than expected amounts of soluble ZP3 ligand suggested that GalTase on the surface of transgenic sperm was blocked and, therefore, incapable of...
retaining epididymal glycosides. The extent of spontaneous acrosome reactions in each sperm population was monitored and found not to be significantly different under the conditions used in this assay. As previously reported (Shur and Hall, 1982a), washing wild-type sperm increased their binding to eggs by 1.5-fold (Fig. 9). However, washing transgenic sperm produced a 3.5-fold (06/+ ) or 4.7-fold (09/+ ) increase in binding, compared to mock-washed control sperm (Fig. 9). Therefore, transgenic sperm overexpressing surface GalTase bound more epididymally derived glycoside substrates, thus competitively inhibiting sperm-egg binding.

Transgenic Sperm Undergo Precocious Acrosome Reactions

Besides showing that a population of sperm was deficient in initial binding to eggs, the results in Fig. 7 suggest that overexpressing surface GalTase diminished the binding affinity of those sperm that did bind the zona, since transgenic sperm were more easily removed during the washing procedures normally employed to reduce nonspecific binding. We asked if precocious acrosome reactions might account for the weaker binding demonstrated by sperm from transgenic animals, since acrosome-reacted sperm are believed to have reduced affinity for the zona pellucida in that they are unable to initiate binding to the egg. Since it has been shown previously that cross-linking of sperm plasma membrane components by ZP3 induces the acrosome reaction (Florman et al., 1984; Leyton and Saling, 1989) and that cross-linking of sperm GalTase mimics that effect (Macek et al., 1991), it seemed plausible that the increase in surface GalTase on transgenic sperm could make them more susceptible to cross-linking by ZP3, resulting in higher rates of acrosome reactions and weaker binding to the zona pellucida.

The rate of spontaneous acrosome reactions in the absence of the zona pellucida was determined initially. Capacitated sperm were incubated in medium at 37°C and their acrosomal status monitored at 1-h intervals. A small but statistically significant increase in the percentage of acrosome-reacted sperm was observed for both transgenic lines compared to wild-type sperm (Fig. 10). However, the difference in acrosomal status between wild-type and transgenic sperm was only ~6% after 1 h. Since sperm-egg binding assays are terminated within 30 min, it seemed unlikely that a <6% difference in acrosomal status could be responsible
for the observed differences in egg-binding ability. Therefore, the extent of ZP3-induced acrosome reactions was determined.

Capacitated sperm were incubated with solubilized zona pellucida, and the acrosomal status of sperm from each genotype was determined. Within 5 min, transgenic sperm had 2.0–2.3 times the number of acrosome-reacted sperm as did wild-type sperm (Fig. 11). This hypersensitivity of transgenic sperm to zona-induced acrosome reactions suggested that transgenic sperm already bound to the intact zona pellucida may also undergo precocious acrosome reactions. Therefore, the acrosomal status of zona-bound sperm was determined after a 30-min binding assay using the lipophilic dye, DiO, as described in Materials and Methods.

The frequency of acrosome-reacted sperm on the zona pellucida was two times higher among sperm from both transgenic lines compared to sperm from wild-type animals (Fig. 12). After a standard 30-min binding assay, without any subsequent washing, 55% of the transgenic sperm bound to the zona were acrosome-reacted, whereas only 27% of the wild-type sperm were acrosome-reacted (Fig. 12). Since acrosome-reacted sperm are thought to be more tenuously bound to the zona pellucida, these results suggest that a larger portion of transgenic sperm are lost during washing, relative to wild-type sperm, due to a higher proportion of acrosome-reacted sperm.

Discussion

Mouse sperm surface GalTase mediates fertilization by binding to ZP3 oligosaccharides in the egg zona pellucida. In this study, we examined the consequences of altering surface GalTase expression on sperm–egg binding. Sperm GalTase levels were several times higher in transgenic mice than in wild-type littermates. Consistent with this, sperm from transgenic mice had higher surface GalTase levels and bound more ZP3 than wild-type sperm. We therefore expected that sperm overexpressing surface GalTase would bind to eggs better than wild-type sperm. However, contrary to our expectations, sperm overexpressing surface GalTase were defective in their ability to bind the zona pellucida. The reduced egg-binding ability of transgenic sperm results from the fact that sperm GalTase function during fertilization is multifaceted and carefully regulated. First, transgenic sperm retained higher levels of epididymally derived glycoside substrates, relative to sperm from wild-type animals. As a result, a larger number of sperm from transgenic mice had their egg receptors blocked, and these sperm were unable to bind eggs. Second, the increased expression of surface GalTase resulted in a hypersensitivity to ZP3-induced acrosome reactions. This resulted in a greater frequency of acrosome-reacted sperm bound to eggs, which were more easily removed by standard washing conditions.

In the epididymis, GalTase on mature sperm is blocked by epididymal fluid glycosides that have been characterized previously as lactosaminoglycans (Shur and Hall, 1982a). The physiological function of these glycosides, sometimes referred to as decapacitation factors, is unknown, but may serve to prevent sperm from binding to each other or to epithelial cells of the epididymis. Removal of these sperm-bound glycosides is a prerequisite for sperm to acquire the ability to bind eggs, and normally occurs during capacitation in vitro and presumably within the female reproductive tract in vivo. Since these glycosides are present in vast excess in epididymal fluid, one consequence of overexpressing sperm surface GalTase appears to be that sperm bind more of these glycosides, or bind them with higher affinity, thus masking GalTase and preventing sperm from interacting with eggs. Consistent with this, removing these glycosides from transgenic sperm by washing enhanced sperm binding to eggs much more so than did washing of wild-type sperm. The retention of epididymal glycosides on transgenic sperm also appears to block their ability to bind soluble ZP3 ligand, since a sixfold increase in surface GalTase resulted in only a twofold increase in ZP3 binding.

Sperm binding to ZP3 induces the acrosome reaction, a G-protein–dependent process initiated by aggregation of sperm receptors by multivalent ZP3 oligosaccharides (Endo et al., 1988; Leyton and Saling, 1989; Ward and Kopf, 1993). Cross-linking of sperm GalTase also induces the acrosome reaction by a pertussis toxin-sensitive mechanism.

Youakim et al. Overexpressing GalTase Inhibits Sperm–Egg Binding 1581
(Macek et al., 1991; Miller and Shur, unpublished observations). The fact that ZP3 has multiple binding sites for GalTase suggests that at least one of the physiological inducers of the acrosome reaction involves ZP3-dependent crosslinking of GalTase (Miller et al., 1992). The observation that transgenic sperm bound two times more ZP3 than wild-type sperm, and underwent higher levels and faster rates of acrosome reactions when challenged with soluble ZP3 or with intact eggs, is consistent with this hypothesis. However, the fact that transgenic sperm underwent precocious acrosome reactions (although to a slight degree) even in the absence of zona glycoproteins, suggests that these cells were generally hypersensitive compared to wild-type sperm. There are several possibilities to account for these observations. First, because GalTase can multimerize by binding to its own oligosaccharide chains, overexpressing GalTase on transgenic sperm may cross-link GalTase to itself, thereby inducing the acrosome reaction. Alternatively, increased epididymal glycosides bound to transgenic sperm may cross-link surface GalTase, inducing the acrosome reaction. The slightly higher rate of spontaneous acrosome reactions observed in transgenic sperm is consistent with either of these possibilities. More dramatically, increasing surface GalTase on transgenic sperm sensitized these cells to the zona pellucida, causing them to undergo acrosome reactions before stable interactions with the egg could be established. Consistent with this, transgenic sperm underwent zona-induced acrosome reactions, using either soluble or intact zona, twice as frequently as did wild-type sperm.

It is unclear from this study if the decreased binding of transgenic sperm was due to the presence of two distinct sperm populations, one blocked by epididymal glycosides and the other displaying abnormal acrosome reaction kinetics, or whether the two phenotypes were shared by a single sperm population. However, the fact that there were no significant differences in the level of acrosome reactions between sperm from transgenic and wild-type animals at the time of insemination, even though differences in egg binding were immediately apparent, indicates that, initially, there was a subpopulation of sperm in which GalTase is masked so that it cannot bind to eggs.

Overexpressing surface GalTase on sperm has an unexplained effect on enzyme activity in addition to effects on sperm physiology. Quantitation of surface GalTase expression by Western immunoblotting indicated that transgenic sperm possessed approximately six times the wild-type level of GalTase protein. However, when surface GalTase levels were measured enzymatically, transgenic sperm possessed 20–30 times more activity than wild-type sperm. The reason for this discrepancy between the two assays is not clear, but it suggests that increasing GalTase protein on the sperm surface results in a synergistic effect on enzyme activity, perhaps by multimerizing GalTase.

Although increasing sperm surface GalTase levels resulted in decreased binding to eggs in vitro, there appeared to be no dramatic effect on fertilization in vivo, since these animals have relatively normal fertility. This is because all of the sperm in hemizygous transgenic animals are functionally equivalent due to sharing of GalTase RNAs between cytoplasmically connected spermatids (Braun et al., 1989; Pratt and Shur, 1993). Thus, there are no wild-type sperm in the hemizygous transgenic male that would be physiologically superior to the dysfunctional transgenic sperm. The ability to distinguish only one sperm population in transgenic animals by indirect immunofluorescence is consistent with this. Consequently, although their egg-binding ability may be dramatically compromised relative to sperm from wild-type animals, transgenic sperm are present in sufficient numbers and bind with sufficient affinity (even if only 15–30% the level of wild-type sperm) to fertilize eggs. What is clear, though, is that the level of the zona pellucida receptor on sperm must be carefully regulated, since overexpression proved to be counterproductive for optimal sperm–egg binding. This implies that the normal variation within a sperm population produces sperm that may be selected against because of exceedingly high or low levels of surface GalTase.

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