anti-Stat3 immunoprecipitates from cell lysates and analyzed them by protein immunoblotting with anti-Stat3 or antibody to phosphotyrosine. Although similar amounts of Stat3 were immunoprecipitated from both normal and v-Src–transformed cells (Fig. 3A), only the protein from v-Src–transformed cells contained increased amounts of phosphotyrosine. Stat3 was phosphorylated in 3T3-F442A cells stimulated with LIF (8). We did a gel shift assay after incubation of nuclear extracts with various amounts of monoclonal antibody to phosphotyrosine. The SIE-binding activity in v-Src–transformed cells was disrupted by the antibody to phosphotyrosine (Fig. 3B) but not by an irrelevant monoclonal antibody. Together, these results indicate that the DNA-binding complex induced in v-Src–transformed cells contains a tyrosine-phosphorylated protein.

We compared the SIE-binding factors from NIH 3T3 cells stably transformed by (i) normal cellular Src (c-Src) expressed in very high amounts, (ii) c-Src activated by a mutation of the critical regulatory Tyr^{527} to Phe, and (iii) Rous sarcoma virus v-Src (Fig. 4A). The amount of constitutive DNA-binding complex increased with increasing oncogenic potency of the Src proteins (normal c-Src being the least potent, the activated Tyr^{527} mutant being intermediate in potency, and v-Src the most potent) (*15*). Increasing amounts of DNA-binding complex also coincided with elevated levels of tyrosyl phosphorylation of Stat3 (Fig. 4B).

Our results establish that Stat3 or a closely related STAT family member is constitutively activated by the Src tyrosine kinase. Activation of Stat3 by Src may be mediated through direct or indirect mechanisms. Consistent with the possibility that Src kinase directly phosphorylates Stat3, v-Src activates Stat3 in a yeast genetic system (*16*). Alternatively, an indirect mechanism is suggested by the observation that Jak family kinases are activated in Src-transformed cells (*17*). It is possible that STAT proteins may contribute to transformation by diverse oncoproteins. Our finding that constitutive activation of Stat3 correlates with Src transformation raises the possibility that this STAT signaling pathway regulates expression of cellular genes that participate in oncogenesis.

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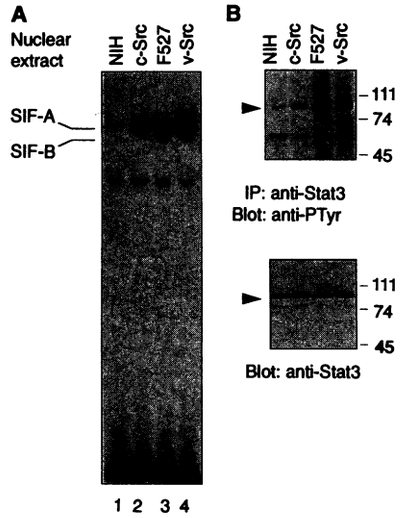

Fig. 4. Comparison of Stat3-related DNA-binding complexes in cells transformed by various Src proteins. (**A**) Nuclear extracts were prepared from normal NIH 3T3 cells (NIH), and NIH 3T3 cells transformed by overexpressed normal c-Src, an activated c-Src mutant with a $Tyr^{527} \rightarrow Phe^{527}$ substitution (Y527F), or Rous sarcoma virus v-Src (*5*) and then analyzed by EMSA with a ^{32}P-labeled high-affinity SIE probe (Fig. 1). (**B**) Cell lysates were prepared from the same cell lines, and proteins were immunoprecipitated with anti-Stat3 (1:125). Immunoprecipitates were subjected to immunoblotting with anti-PTyr or monoclonal antibody to Stat3 as described (Fig. 3). Sizes are indicated to the right in kilodaltons.

Interaction of a Tyrosine Kinase from Human Sperm with the Zona Pellucida at Fertilization

D. J. Burks,* R. Carballada, H. D. M. Moore, P. M. Saling†

A 95-kilodalton mouse sperm protein with characteristics of a protein tyrosine kinase has been identified as a receptor for ZP3, a glycoprotein in the egg's extracellular matrix. The structure of the human homolog was determined by screening an expression library from human testis; a testis-specific complementary DNA was isolated that encodes a protein similar to receptor tyrosine kinases and appears to be expressed only in testicular germ cells. Antibodies against a synthetic peptide from the intracellular domain recognized a 95-kilodalton human sperm protein that contains phosphotyrosine; human ZP3 stimulates the kinase activity of this sperm protein. Synthetic peptides corresponding to regions of the predicted extracellular domain inhibited sperm binding to human zona pellucida. Availability of the primary sequence of a receptor for ZP3 provides a rational starting point for sperm-targeted contraceptive development.

To fertilize an egg, mammalian sperm must first bind to the egg's extracellular matrix, the zona pellucida (ZP). Once bound, sperm undergo the acrosome reaction, an essential exocytotic event that permits a sperm to digest the ZP matrix and contact the egg. In mice, the glycoprotein ZP3 serves as both a ligand for sperm binding and a trigger for acrosomal exocytosis (*1*). Tyrosine phosphorylation in sperm appears to be important for these steps in fertilization, because inhibition of protein tyrosine kinase (PTK) activity prevents acrosomal exocytosis (*2*) and blocks fertilization. Several lines of evidence indicate that the 95-kD ZP3 receptor in mouse sperm is a mem-

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ber of the PTK receptor family. (i) It contains phosphotyrosine, the amount of which increases on exposure to ZP proteins (*3*). (ii) PTK activity is stimulated by direct exposure of the isolated receptor to ligand (*2*). (iii) ZP3 receptor aggregation is an initiating signal in the cascade leading to acrosomal exocytosis (*4*). Given these characteristics, we refer to this 95-kD protein as zona receptor kinase (ZRK).

The evolutionary conservation of receptor PTKs (*5*), as well as the demonstrated similarity between mouse and human ZP3 (*6*), suggests the existence of a ZRK homolog in human sperm. Consistent with our observations in mice (*3*), a 95-kD protein was the major phosphotyrosine-containing protein identified in human sperm, and the amount of phosphotyrosine in this protein increased with capacitation (Fig. 1A), a final maturational process that primes sperm for ZP-triggered acrosome reactions (*7*). A 95-kD human sperm protein, identified with the monoclonal antibody (mAb) 97.25, has been implicated in sperm-ZP interaction (*8*), and we investigated whether the 97.25 antigen was tyrosine-phosphorylated. The mAb 97.25 immunoprecipitated a 95-kD phosphotyrosine-containing protein (Fig. 1B) that was not recognized by an antibody to hexokinase, contrary to a recent suggestion (*9*). The immunoprecipitated protein reacted specifically with the antibody to phosphotyrosine because preincubation of this antibody with 40 mM *o*-phospho-DL-tyrosine eliminated immunoreactivity (Fig. 1B). Staining of live human sperm with mAb 97.25 demonstrated that this antigen was located on the sperm surface in the acrosomal region, appropriate for a role in gamete interaction (Fig. 1C).

We used an antibody to phosphotyrosine (PY20) (*10*) and the mAb 97.25, reagents targeted to intracellular and extracellular domains of ZRK, respectively, as sequential probes in a cloning strategy designed to elucidate the primary structure of a sperm PTK involved in gamete recognition. From a human testis complementary DNA (cDNA) expression library, we isolated a clone, *hu9*, encoding a transmembrane protein reactive with both antibodies. Sequencing of the 2.2-kb insert revealed an open reading frame of 1800 nucleotides, predicting a protein of 600 amino acids (Fig. 2A). Comparison of *hu9* with sequences in the GenBank and Swiss Protein databases revealed that *hu9* encodes a PTK not described previously. The predicted amino acid sequence of the protein Hu9 contains features found in all PTKs (*11*), with a catalytic domain similar (55% identity) to that of c-Eyk, a receptor-like PTK identified as the proto-oncogene of v-*eyk* (v-*ryk*) (*12*). Potential ligands for this receptor PTK subfamily (*13*) have not yet been reported. Binding sites for several Src homology 2–containing proteins are present in the intracellular region of Hu9, including growth factor receptor–bound protein 2 (Grb2), Shc, and Nck (*14*). Comparison of the Hu9 extracellular domain with other sequences in the databases yielded no significant similarities. The putative Hu9 extracellular domain contains several potential glycosylation sites and is cysteine-rich, but the nine cysteine residues present in Hu9 are not arranged in typical clusters, as observed in other receptor PTKs (*15*).

When Northern blots containing RNA from human tissues were probed with the *hu9* cDNA, a single transcript of ~2.2 kb was detected in human testis, suggesting that the cloned insert represents the complete mRNA encoding the Hu9 protein

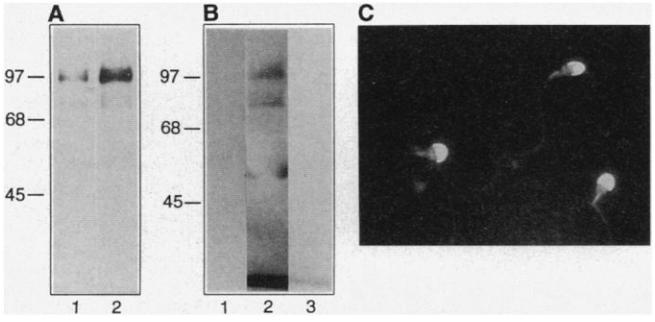

Fig. 1. Tyrosine phosphorylation of a 95-kD human sperm protein during capacitation and its immunoprecipitation by mAb 97.25. (**A**) Proteins from noncapacitated (lane 1) or capacitated (lane 2) sperm were probed with antibodies to phosphotyrosine (*21*). Each lane contained proteins from 2×10^6 sperm. Migration of molecular size markers in kilodaltons are indicated on the left. (**B**) Extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer (*20*) from capacitated human sperm, and proteins were immunoprecipitated with mAb 97.25 (*22*). Blots were probed with either RC20 that had been preincubated with 40 mM *o*-phospho-DL-tyrosine (lane 1), RC20 (lane 2, Transduction Labs, Lexington, Kentucky), or antibody to hexokinase (1 : 10,000 dilution, lane 3). (**C**) Localization of the antigen recognized by mAb 97.25 on live capacitated human sperm. Cells were incubated with affinity-purified mAb 97.25 (10 μg/ml), fixed with formaldehyde, and incubated with fluorescein-labeled rabbit antibody to mouse immunoglobulin G (IgG) and IgM (Dako, High Wycombe, Bucks, United Kingdom).

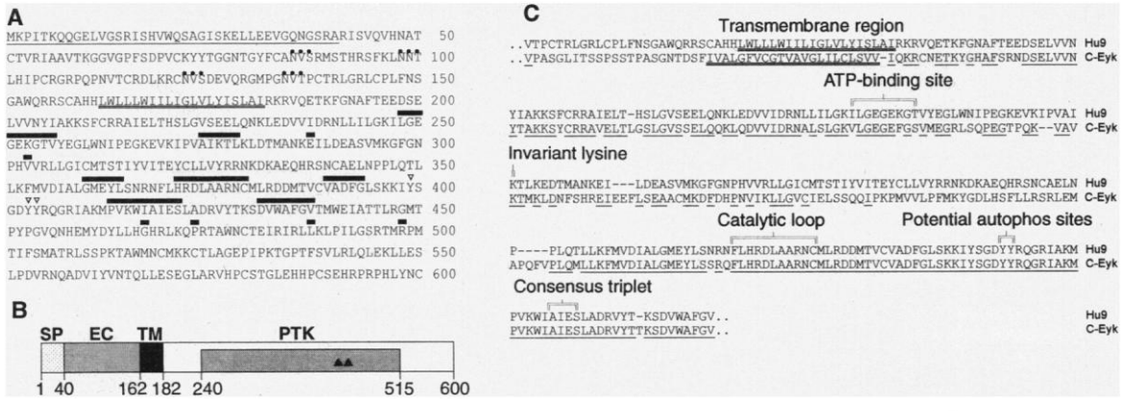

Fig. 2. Amino acid sequence of Hu9 and its comparison to c-Eyk. (**A**) Deduced amino acid sequence for Hu9. The *hu9* cDNA was isolated from a human testis cDNA library, as described (*23*). Features of the predicted amino acid sequence are indicated as follows: single underline, signal sequence (*24*); ●, potential glycosylation sites; double underline, putative transmembrane domain (*25*); overline, invariant (or highly conserved) PTK sequences (*11*); and ▽, potential autophosphorylation sites (*11*). (**B**) Predicted structure of Hu9. SP, signal peptide; EC, extracellular domain; TM, transmembrane region; and ▲, potential autophosphorylation sites. Numbering indicates amino acid positions. (**C**) Similarity of the kinase domains of Hu9 and c-Eyk. The predicted sequence for Hu9 (residues 135 to 437) is compared with that of c-Eyk (residues 473 to 781). The transmembrane regions are double underlined whereas regions of identity are single underlined. Autophos, autophosphorylation. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

(Fig. 3A). Three upstream stop codons (–18, –24, and –54) also suggest that the *hu9* cDNA is full length. The *hu9* cDNA did not hybridize with RNA from other human tissues surveyed. Furthermore, *hu9* hybridized with a single transcript of 2.7 kb in RNA prepared from mouse testis (*16*). In situ hybridization was used to examine the distribution of the *hu9* transcript within the testis (Fig. 3, B and C); localization was confined to postmeiotic germ cells as demonstrated by the grain pattern on cross sections through germinal epithelia, indicating that the *hu9* transcript is specific to germ cells.

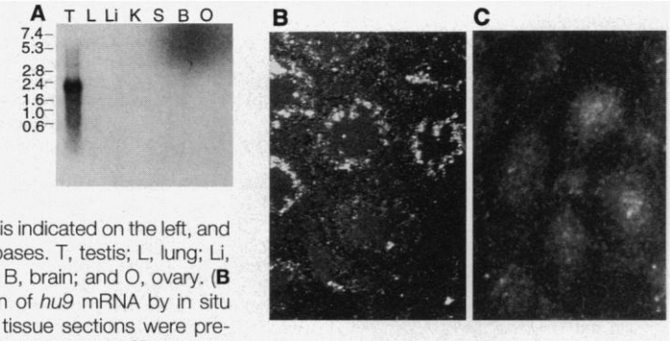

Fig. 3. Expression of *hu9* in spermatogenic cells. (**A**) Tissue distribution of *hu9* expression. A Northern blot containing RNA prepared from various human tissues was probed with ^{32}P-labeled *hu9* cDNA (*26*). The migration of the marker RNA is indicated on the left, and the numbers indicate kilobases. T, testis; L, lung; Li, liver; K, kidney; S, spleen; B, brain; and O, ovary. (**B** and **C**) Cellular localization of *hu9* mRNA by in situ hybridization (*27*). Frozen tissue sections were prepared from mature mouse testes, and ^{35}S-labeled riboprobes were hybridized with tissue sections. In testis sections hybridized with the antisense probe shown in (B), *hu9* RNA transcripts were detected exclusively within the seminiferous tubules; postmeiotic expression was suggested by predominant labeling in the luminal compartment. The control hybridization shown in (C), in which the sense strand *hu9* RNA was used, yielded an even distribution of grains not above background hybridization levels.

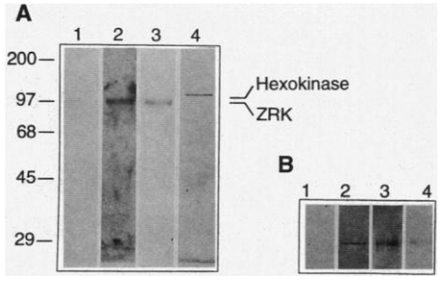

Fig. 4. Immunoprecipitation of a 95-kD tyrosine-phosphorylated protein from human sperm with an antibody to residues 539 to 553 of the deduced amino acid sequence encoded by *hu9*. (**A**) Keyhole limpet hemocyanin (KLH)–conjugated peptide was used for rabbit immunization and resulted in a titer of 1:125,000 against the peptide and a titer of 1:5000 against KLH. Proteins from capacitated human sperm were reduced and separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with preimmune serum (1:200 dilution, lane 1); antiserum to Hu9(539–553) (1:200 dilution, lane 2); anti-phosphotyrosine (RC20, according to manufacturer instructions, lane 3); or antiserum to rat brain hexokinase (1:10,000 dilution, lane 4). Lanes 2 to 4 represent the same nitrocellulose sheet that was stripped between treatments and subsequently reprobed in the order presented. Molecular size markers in kilodaltons are indicated to the left. (**B**) Proteins from capacitated human sperm were immunoprecipitated with mAb 97.25 and probed with either preimmune rabbit serum (1:100 dilution, lane 1); antiserum to Hu9(539–553) (1:100 dilution, lane 2); mAb 97.25 (lane 3); or anti-phosphotyrosine (RC20, lane 4).

To investigate characteristics of the protein encoded by *hu9* in mature sperm, we prepared a polyclonal antibody to a synthetic peptide corresponding to 15 residues in the Hu9 intracellular domain (residues 539 to 553). This antibody recognized a 95-kD human sperm protein that contains phosphotyrosine and is distinct from hexokinase (Fig. 4A). Immunoprecipitation studies indicated that mAb 97.25 and antibody to Hu9(539–553) [anti-Hu9(539–553)] recognized the same antigen (Fig. 4B). The putative receptor function of Hu9 was initially addressed in a competitive sperm-ZP binding assay (*17*). Human ZPs were preincubated with synthetic peptides corresponding to various regions of the predicted Hu9 extracellular domain and were then exposed to sperm to determine if these sequences competed with sperm for ZP binding sites (Fig. 5A). Two of the peptides [pep1 (residues 57 to 71) and pep3 (residues 94 to 105)] caused significant inhibition of sperm interaction with the ZP, inhibiting binding up to 31 and 20% of that in the absence of peptides, respectively. In contrast, a third peptide [pep2 (residues 152 to 166)] had no effect on binding. Furthermore, consistent with current models of cellular activation through receptor PTKs (*18*), anti-Hu9(539–553) immunoprecipitates contain kinase activity that is stimulated by exposure to human ZP3 (*19*), suggesting that the antibody to Hu9 recognized a ZP3-binding protein in human sperm that possesses intrinsic kinase activity (Fig. 5B).

Together with our earlier work on sperm-ZP3 interaction in mice (*2–4*), these data suggest that *hu9* encodes a human sperm ZRK and define a ZP3 receptor containing intrinsic transmembrane signaling potential. These findings imply a critical

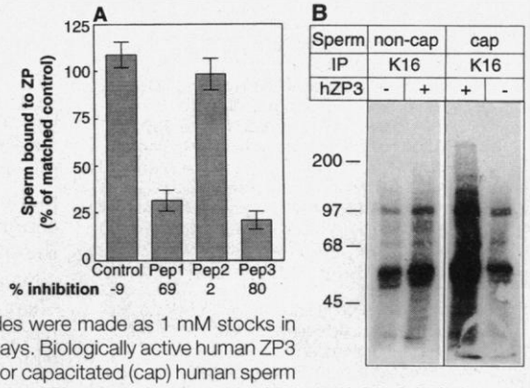

Fig. 5. Functional characterization of Hu9. (**A**) Inhibition of sperm binding to ZP by peptides from Hu9. Unfertilized human eggs were bisected (*28*), and ZP halves were used as matched pairs during all subsequent manipulations of the hemi-zona assay. Half A served as the control hemi-ZP in all cases and was incubated with medium alone, whereas half B was preincubated with medium alone or with medium containing peptides 1, 2, or 3 (pep1, -2, -3) (10 μM). Capacitated human sperm were added to each drop (final concentration, 10^6 cells per milliliter), and after 30 min, sperm binding was evaluated. Each half-ZP was recovered, washed gently to remove loose sperm, and fixed in 2% glutaraldehyde. Samples were examined with phase contrast optics, and the number of sperm bound to each half-ZP was determined. Sperm binding is expressed as percentages, with the number of sperm bound to ZP half B normalized to those bound to ZP half A. Data were analyzed by analysis of variance and paired *t* test with PSPLOT software. Each treatment was evaluated with 9 to 10 independent hemi-ZP pairs. Pep1 and pep3 significantly ($P < 0.001$) interfered with sperm-ZP binding. Pep1 corresponds to residues 57 to 71; pep2, to residues 152 to 166; and pep3, to residues 94 to 105. All peptides were made as 1 mM stocks in Ham's F-12 medium. (**B**) Human ZP3 stimulates the activity of ZRK in immune complex kinase assays. Biologically active human ZP3 was purified from COS cells transfected with a human ZP3 cDNA (*19*). Noncapacitated (non-cap) or capacitated (cap) human sperm (*21*) were solubilized in RIPA buffer (*22*) and used as the starting material for immunoprecipitation (IP) with antibody to Hu9(539–553) (K16) (*29*). Immune complexes were incubated with (+) or without (–) recombinant human ZP3 (hZP3) for 10 min in the presence of [γ-^{32}P]adenosine triphosphate to assess kinase activity (*2*). After three washes with ice-cold kinase buffer (*2*), precipitated proteins were resuspended in SDS sample buffer with 5% β-mercaptoethanol, and analyzed by SDS-PAGE. The basal amount of kinase activity in the noncapacitated sperm immunoprecipitates was stimulated to a small extent by ZP3; however, exposure of the immunoprecipitate from capacitated sperm to ZP3 significantly increased the amount of ^{32}P incorporated into protein.

role for tyrosine phosphorylation in mediating human fertilization. The homology of *hu9* with PTKs provides a basis for evaluating tyrosine kinase signaling paradigms in ZP3-triggered acrosomal exocytosis, mechanisms that may have implications in fertility control strategies.

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21. Human sperm were obtained from fresh ejaculates, collected by centrifugation through a Percoll gradient [F. L. H. Ng, D. Y. Liu, H. W. G. Baker, *Hum. Reprod.* **7**, 261 (1992)], and capacitated by incubation in Ham's F-12 medium, supplemented with 20 mM $NaHCO_3$ and 7.5% serum, at a concentration of 5×10^6 cells per milliliter for 6 hours at 37°C in 5% CO_2. Sperm proteins were solubilized in SDS sample buffer, separated under reducing conditions on 8% polyacrylamide gels [U. K. Laemmli, *Nature* **227**, 680 (1970)], and transferred [H. Towbin, T. H. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4359 (1979)] to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After they were blocked for nonspecific reactivity, the blots were incubated with antibody to phosphotyrosine (PY20; ICN, Irvine, CA) (1 μg/ml). PY20 reactivity was visualized with peroxidase-conjugated goat antibody to mouse IgG (Kierkegaard and Perry Laboratories, Rockville, MD) followed by enhanced chemiluminescence detection (ECL Western Blot Kit, Amersham).
22. Sperm were extracted in RIPA buffer (*20*) in the presence of protease inhibitors, 4 mM dithiothreitol, 500 μM Na_3VO_4, and 10% glycerol. Extracted proteins were separated from insoluble material by centrifugation and incubated overnight at 4°C with mAb 97.25. Immune complexes were recovered with antibody to mouse IgG and IgM coupled to Sepharose (Sigma), washed three times in RIPA, and separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
23. An unamplified λgt11 human testis library (Clontech, Palo Alto, CA) was screened first with PY20 as described [M. Snyder, S. Elledge, D. Sweetser, R. A. Young, R. W. Davis, *Methods Enzymol.* **154**, 107 (1987); R. A. Lindberg and E. B. Pasquale, *ibid.* **200**, 557 (1991); K. Letwin, S.-P. Yee, T. Pawson, *Oncogene* **3**, 621 (1988)]. Positive clones were detected with an ^{125}I-labeled second antibody and then rescreened with mAb 97.25 and detected similarly. Clones were sequenced by the dideoxy method. DNA and amino acid sequences were analyzed with GCG [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)] and deposited in GenBank (accession number L08961).
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26. Total RNA (10 μg) [G. Cathala *et al.*, *DNA* **2**, 329 (1983)] was probed with ^{32}P-labeled *hu9* cDNA [L. G. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986)]. Ethidium bromide was used to ascertain equal loading and transfer of the RNA samples. Final washing of the blots was in 2× saline sodium citrate (SSC) [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)] and 0.1% SDS at 55°C.
27. The ^{35}S-labeled riboprobes were transcribed from *hu9* plasmid DNA with T3 (sense strand) and T7 (antisense strand) polymerase, subsequently hydrolyzed to a final length of 200 base pairs, and hybridized with tissue sections overnight at 55°C. After treatment with ribonuclease A, slides were washed stringently, including a final wash in 0.1× SSC and 10 mM EDTA at 65°C.
28. Excess unfertilized human eggs in Ham's F-12 medium containing 7.5 to 10% human serum, which had not been exposed to sperm and would normally have been discarded, were frozen and maintained at ≤−70°C. At the time of use, vials were thawed rapidly in a 37°C bath, and eggs were removed directly into phosphate-buffered saline containing 2 mM EGTA and 5% serum at 4°C. Oocytes were completely nonviable after this procedure. ZPs were bisected with a micro-scalpel, and matched ZP halves were used subsequently. After bisection, ZPs were washed (three times) and each half-ZP was placed pair-wise in matched drops (40 μl) of Ham's F-12 medium containing 7.5% serum. For each ZP used, one of the halves served as an internal control to which results from the other half were compared.
29. Sperm proteins were incubated for 2 hours at 4°C with anti-Hu9(539–553) (K16). Immune complexes were recovered with antibody to rabbit IgG coupled to Sepharose (Sigma) and washed three times in RIPA.
30. Antiserum to rat brain hexokinase was provided by J. E. Wilson, Michigan State University. The peptides used in this study were synthesized at the Salk Institute (under contract NO1-HD-0-2906 with NIH) and made available by the Contraceptive Development Branch, Center for Population Research, National Institute of Child Health and Human Development. J. Dean (National Institute of Diabetes and Digestive and Kidney Diseases) provided the human ZP3 cDNA. Human ZPs were donated by M. Gerrity and J. Rinehart, Reproductive Medical Associates, Glenview, IL. We appreciate the help of K. Burridge, University of North Carolina at Chapel Hill, and R. Alston and S. Dörre, Duke University, as well as all of the Duke University laboratory members during the writing of this manuscript. Supported by NIH (grants HD 29125 and HD 18201). D.J.B. is grateful for partial support, and R.C. for full support, from A. W. Mellon Foundation fellowships. H.D.M.M. was supported by the Wellcome Trust and the Medical Research Council, United Kingdom.

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Tissue- and Species-Specific Expression of sp56, a Mouse Sperm Fertilization Protein

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Mouse sperm recognize and bind to ZP3, one of three glycoproteins in the egg's zona pellucida. A mouse sperm protein, sp56, was identified that has the characteristics expected of the sperm protein responsible for recognition of ZP3. The complementary DNA encoding sp56 was isolated, and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors. It was shown that sp56 expression is restricted to mouse spermatids and that the presence or absence of sp56 on sperm from different species accounts for species specificity of sperm-egg recognition in mice.

The mouse sperm protein sp56 was identified on the basis of its specific, high affinity for ZP3, the zona pellucida (ZP) protein recognized by sperm (*1*, *2*). sp56 is a homomultimeric peripheral membrane protein confined to the plasma membrane overlying the sperm's acrosome (*3*), and it has a specific affinity for ZP3's functional domain oligosaccharide (FD-oligo), the part of ZP3 recognized by sperm (*3*). Purified sp56, which binds to the ZP of unfertilized mouse eggs but not to fertilized embryos, blocks sperm-egg recognition (Fig. 1). Although a number of proteins have been proposed to function as the mouse sperm's ZP3 recognition protein (*2*–*4*), only sp56 has all of the characteristics required of that protein.

The complementary DNA (cDNA) encoding sp56 was isolated (Fig. 2). Nested polymerase chain reaction (PCR) of reverse-transcribed mouse testes polyadenylated [poly(A)$^+$] RNA was performed with degenerate primers derived from the sp56 NH_2-terminal amino acid sequence and with a primer derived from a fragment of the protein. Sequencing of a 1-kb PCR product confirmed that this polynucleotide encoded

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