RESEARCH COMMUNICATION

Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes

Yael Gur and Haim Breitbart[1]

Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

It is widely accepted that spermatozoa are translationally silent. The present study demonstrates, for the first time, incorporation of labeled amino acids into polypeptides during sperm capacitation, which was completely inhibited by mitochondrial translation inhibitors but not by the cytoplasmic translation inhibitor. Unlike 80S cytoplasmic ribosomes, 55S mitochondrial ribosomes were present in polysomal fractions, indicating that these ribosomes are actively involved in protein translation in spermatozoa. Inhibition of protein translation significantly reduced sperm motility, capacitation and in vitro fertilization rate. Thus, contrary to the accepted dogma, nuclear genes are expressed as proteins in sperm during their residence in the female reproductive tract until fertilization.

It is generally accepted that nuclear-encoded protein translation is unlikely to occur in mature sperm cells. However, indications to the contrary include the findings that mammalian spermatozoa contain nuclear-encoded mRNAs (Wykes et al. 1997; Miller et al. 1999; Ostermeier et al. 2002) in addition to their ability to synthesize mitochondrial-encoded RNA (Premkumar and Bhargava 1972; Hecht and Williams 1978; Alcivar et al. 1989) and proteins (Premkumar and Bhargava 1972; Bragg and Handel 1979). It is interesting that those papers, in spite of being published in respectable journals, did not disprove the generally accepted axiom that spermatozoa are translationally and transcriptionally incapable. In the 1970s it was believed that there was no physiological need for protein replacement before fertilization.

Mammalian spermatozoa reside in the female genital tract for several hours between ejaculation and fertilization, first as sperm reservoir (Suarez 2002) and later on for capacitation. Sperm capacitation includes a cascade of biochemical changes that must occur before spermatozoa can effectively interact with an oocyte, as PKA-dependent protein tyrosine phosphorylation, cholesterol efflux, actin polymerization, increases in $[Ca^{2+}]i$ and

changes in sperm motility (Breitbart 2003). Binding to the oocyte zona pellucida (ZP) induces the sperm cell to undergo the acrosomal reaction and to penetrate the oocyte (Primakoff and Myles 2002).

We hypothesized that new proteins are synthesized in spermatozoa to (1) replace proteins that degrade in the delay before fertilization and (2) produce capacitation-specific proteins. The present study demonstrates for the first time that protein translation does occur in mammalian spermatozoa prior to fertilization.

Results and Discussion

Sperm incubated under capacitation conditions incorporate [35S]Methionine (Met)–[35S]Cysteine (Cys) into newly synthesized polypeptides (Fig. 1A). Novel protein translation was demonstrated in human, mouse, bovine, and rat sperm, indicating the widespread nature of this phenomenon. Labeled amino acid incorporation was completely blocked by the mitochondrial translation inhibitors D-chloramphenicol (CP) (Fig. 1A) tetracycline and gentamycin (Supplementary Fig. S1), but not by the cytoplasmic 80S ribosomal inhibitor cycloheximide (CH) (Fig. 1A), indicating that protein translation in sperm involves mitochondrial but not cytoplasmic ribosomes.

The incorporation was also completely inhibited by the mitochondrial uncoupler FCCP (carbonylcyanide p-trifluoromethoxyphenyl hydrazone) (Supplementary Fig. S1b,c), indicating the dependence of the protein translation on mitochondrial synthesized ATP and/or mitochondrial $\Delta\Psi$.

To visualize the cellular site of the nascent translated proteins within sperm cells, we used BODIPY-lysine-tRNA[Lys] that labels newly made polypeptides in permeabilized capacitating spermatozoa in which the mitochondria are intact. High fluorescence was detected mainly in the sperm midpiece, and relatively low signal was detected in the principal part of the tail (Fig. 1B, panels 2,4). The labeling was inhibited in 98% of the cells by CP (Fig. 1B, panel 3), but not by CH (data not shown).

Both the 35S-amino acid and BODIPY-lysine-tRNA[Lys] labeling techniques revealed a plateau after 1 h of incubation under capacitation conditions (Fig. 1C; Supplementary Fig. S2), showing the consistency of the two techniques.

To identify specific proteins that are translated during sperm capacitation, the levels of sperm-related proteins were measured by Western blot (Supplementary Table S1). CP reduced the levels of 22 proteins including sperm-specific proteins. For example, CP treatment reduced the level of protein kinase C (PKC) (Breitbart and Naor 1999), angiotensin II type I receptor (AT_1-R) (Gur et al. 1998), epidermal growth factor receptor (EGFR) (Lax et al. 1994), and progesterone receptor (PR) (Meizel and Turner 1991), which are involved in sperm capacitation, acrosome reaction, and fertilization. CP treatment also reduced the sperm-specific proteins CatSper (Ren et al. 2001), Na-K-ATPase αIV (Woo et al. 2000), the catalytic subunit of protein kinase A (PKA-Cs) (San Agustin and Witman 2001), and AKAP 110 (Vijayaraghavan et al. 1999). In addition, CP reduced cytochrome C, which is an intramitochondrial protein. On the other hand, CP did not reduce the expression of 4 of the 17 protein ki-
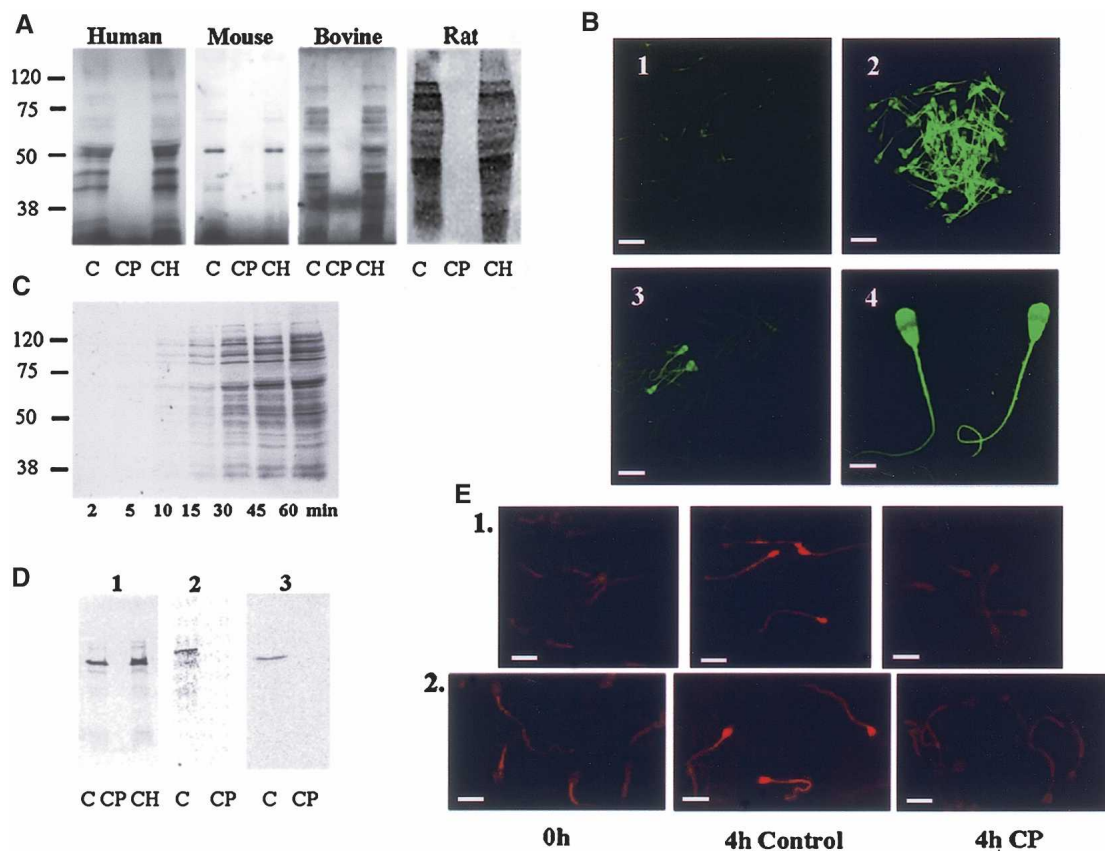
Figure 1. Protein synthesis in spermatozoa. (*A*) Autoradiogram of an electrophoretic gel with [^{35}S]Met–[^{35}S]Cys-labeled polypeptides from human, bovine, mouse, and rat sperm incubated for 6, 4, 3, and 3 h, respectively. Sperm were incubated under capacitation conditions with no additional treatment (C) or after treatment with CP (0.1 mg/mL) or CH (1 mg/mL). (*B*) Fluorescence photograph of bovine sperm with BODIPY-lysine-tRNA^{Lys}-labeled polypeptides photographed at 0 h (panel *1*), or after 1 h capacitation without (panels *2,4*) or with (panel *3*) CP treatment. Bar: panels *1–3*, 40 μm; panel *4*, 10 μm. (*C*) Time course of [^{35}S]Met–[^{35}S]Cys incorporation into polypeptides in bovine sperm under capacitation conditions. (*D*) Immunoprecipitation of [^{35}S]Met–[^{35}S]Cys-labeled: angiotensin II type I receptor (AT$_1$-R) (lanes *1*), progesterone receptor (PR) (lanes *2*), and PKCα after 4 h under capacitation conditions with no additional treatment (C) or after treatment with CP (0.1 mg/mL) or CH (1 mg/mL) (lanes *3*). (*E*) Immunocytochemistry of AT$_1$-R (row *1*) and PR (row *2*) in bovine sperm at 0 h and at 4 h of capacitation ± CP (0.1 mg/mL). Bar, 20 μm.

nases examined, indicating that those kinases are relatively stable during sperm capacitation (Supplementary Table S1). We assume that some of the degraded proteins must be replaced by newly synthesized proteins.

Identification of ^{35}S-amino acids labeled AT$_1$-R, PR, and PKCα was performed by immunoprecipitation. The addition of CP to the cells completely blocked their synthesis while CH had no effect (Fig. 1D). Similarly, there was a significant increase in the amount of AT$_1$-R and PR during capacitation as measured by immunocytochemistry, a phenomenon that was blocked by CP application (Fig. 1E).

It is likely that mRNA is present in sperm cells to serve as a template for the translation of new proteins. Indeed, RNA transcripts for the sperm-specific CatSper and CatSper II, Na-K ATPase αIV, PKA-Cs, and AKAP 110 proteins were detected in sperm from several species, using RT–PCR (Fig. 2A).

Possible sources of mRNA in sperm might be newly transcribed mRNA molecules or long-lasting mRNA molecules transcribed during spermatogenesis. [^{35}S]Met–[^{35}S]Cys incorporation into proteins was unaffected by the transcription inhibitor actinomycin D (Supplementary Fig. S1b,c), suggesting that the source of mRNA for protein translation is long-lasting mRNAs.

The localization of mRNAs and proteins at a specific cellular site may be an indication of the protein translation site. Thus, the cellular localization of bovine AT$_1$-R, mouse PKA-Cs, and mouse CatSper mRNA and proteins was determined. In situ hybridization of digoxigenin (DIG)-conjugated CatSper antisense PCR fragments with permeabilized sperm cells revealed high fluorescence in the sperm midpiece and head (Fig. 2B). This suggests that protein translation might occur in the midpiece mitochondria and/or in the head.

To further support this suggestion, specific mRNAs were localized at the organelle level using DIG-conjugated antisense visualized by electron microscopy. Bovine AT$_1$-R, mouse PKA-Cs, and mouse CatSper mRNAs were found predominantly inside the sperm mitochondria (Fig. 2C) as well as in the nucleus (data not shown).

Bovine AT$_1$-R, mouse PKA-Cs, and CatSper proteins were also localized at the cell organelle level using specific antibodies detected by secondary gold-conjugated antibody. All three proteins were localized inside the mitochondria (Fig. 2C), as well as in their active sites outside the mitochondria (data not shown). AT$_1$-R protein was also localized to the sperm head (Supplementary Fig. S3).

The demonstration of the presence of activated ribo-
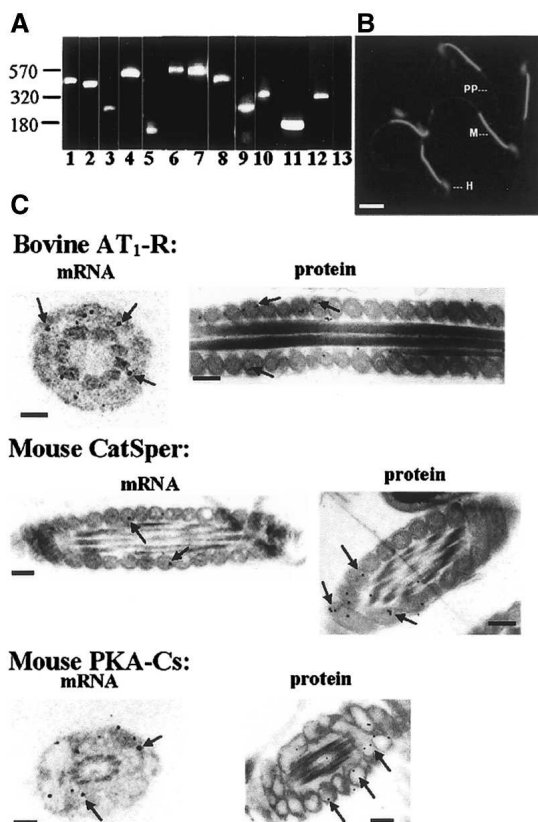
Figure 2. Identification and localization of mRNAs and proteins in sperm. (*A*) RT–PCR of sperm-related mRNA. Sperm RNA was purified and amplified by RT–PCR using specific primers (Supplementary Table S2). PCR products are mouse PKA-Cs (lane *1*), bovine PKA-Cs (lane *2*), human PKCα (lane *3*), bovine AKAP 110 (lane *4*), human PKCβI (lane *5*), mouse CatSper (lane *6*), human CatSper (lane *7*), mouse CatSper II (lane *8*), human ATPase α4 (lane *9*), rat ATPase α4 (lane *10*), human AT$_1$-R (lane *11*), bovine AT$_1$-R (lane *12*), and control—PCR with mRNA but without RT (lane *13*). (*B*) Immunostaining of CatSper mRNA in mouse sperm by in situ hybridization. The head (H), midpiece (M), and principal piece (PP) regions of the sperm are indicated. (*C*) Immunogold localization of bovine AT1-R (*top* row), mouse Catsper (*middle* row), and mouse PKA-Cs (*bottom* row) mRNAs and proteins by electron microscopy. Cross-section is through the midpiece of bovine and mouse sperm, and the arrows indicate gold particles localized inside the mitochondria. Bar, 0.2 μm.

somes in capacitating sperm cells would further support the contention of protein translation in sperm. Sperm ribosomes were analyzed using a sucrose gradient and RT–PCR. The configurations of mitochondrial (55S) and cytoplasmic (80S) ribosomes were evaluated. The 12S rRNA, a component of the 28S small subunit of the 55S mitochondrial ribosome (Sharma et al. 2003), was found in all fractions (Fig. 3A). The presence of 12S rRNA in the polysomal fractions indicates that the 55S ribosomes are actively involved in sperm protein translation. On the other hand, the 18S rRNA, a component of the 40S small subunit of the 80S cytoplasmic ribosome, was identified only as subunits or monosomes (around fractions 2–8), however not in the polysomal fractions (Fig. 3B), indicating that 80S ribosomes are present but not active in spermatozoa.

In addition, AKAP 110 mRNA was present in the poly-

somal fractions (Fig. 3C), indicating that this sperm-specific, nuclear-encoded protein is likely to be translated in sperm by 55S mitoribosome. To our knowledge, this is the first indication for the translation of nuclear encoded mRNA on mitochondrial ribosomes. At this point it is not clear how the initiation of translation of the nuclear mRNA on the mitoribosome is operating. In bacteria, the initiation (5′)AUG is guided to its correct position by the Shine-Dalgarno sequence in the mRNA. In eukaryotes the initiation (5′)AUG is located within the mRNA not by its proximity to a Shine-Dalgarno-like sequence but by a scan of the mRNA from the 5′ end until the first AUG is encountered. A complex of initiation factors called eIF4F is probably involved in the scanning process. It seems that mitochondrial mRNA does not have the Shine-Dalgarno sequence (Denslow et al. 1989). This point will need further investigation in the future.

mRNAs and their translated proteins were localized both inside and outside of the mitochondria, supporting the notion that protein translation of nuclear-encoded proteins in sperm occurs either in the mitochondria and/or by mitochondrial-type ribosomes located outside the mitochondria. These two possibilities raise a number of questions about how nuclear-encoded mRNAs are transported into the mitochondrion and how they are translated by mitochondrial ribosomes.

Experimental findings supporting the import of RNA into the mitochondria including tRNA in yeast, plants, and protozoans (Entelis et al. 2001) and 5S rRNA in mammalian cells (Doersen et al. 1985). On the other hand, experimental findings that mitochondrial-type ribosomes are present outside of the mitochondria in the germ plasm of *Drosophila* embryos (Amikura et al. 2001; Marc et al. 2002) and in a discrete region of egg cytoplasm in ascidian and sea urchin embryos (Ogawa et al.
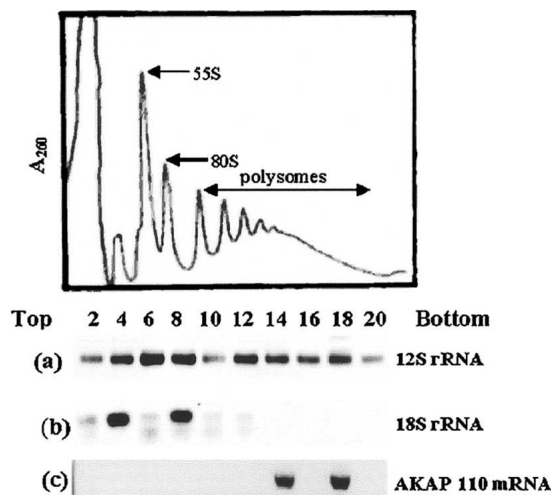
Figure 3. Identification of active sperm ribosomes. Sperm ribosomal fraction was allowed to sediment using sucrose gradient, then the optical density tracing at 260 nm was determined, 20 fractions were collected, and the absorbance measured at 260 nm in each fraction was measured again. RNA was isolated from each fraction and treated with DNase. All fractions were detected for the presence of rRNAs. 12S rRNA of the 55S mitochondrial ribosome (*a*) and 18S rRNA of the 80S cytoplasmic ribosomes (*b*) were identified by RT–PCR at the linear phase of the PCR. (*c*) mRNA of AKAP 110 was identified by RT–PCR. The S values were determined using standards for 55S mitoribosomes and 80S cytoplasmic ribosomes from HeLa cells.

1999; Oka et al. 1999) support the possibility that atypi-
cal translation may occur in mitochondrial ribosomes
outside of the mitochondria. Similarly, particles resem-
bling ribosomes are associated with spermatid acrosomal
membranes in spermatozoa (Mollenhauer and Morre
1978).

Additionally, 16S mitochondrial rRNA are localized in
the nucleus of mouse spermatogenic cells and human
spermatozoa (Villegas et al. 2002) indicating that these
rRNAs may form ribosomes in the nucleus. Protein
translation takes place in the nuclei of HeLa cells (Iborra
et al. 2001) via 80S ribosomes, which raises the possibil-
ity that unexpected cellular loci may control atypical
translation processes.

Thus, one possible explanation of the present findings
is that protein translation occurs in the sperm nucleus
via mitochondrial-type ribosomes that are sensitive to
CP. Indeed, in situ hybridization assays demonstrated
that AT_1-R was localized to the sperm nucleus (Supple-
mentary Fig. S3), suggesting that this protein may be
translated by nuclear 55S ribosomes or translocated to
the nucleus after extranuclear translation.

The physiological importance of protein synthesis in
sperm was evaluated by testing sperm motility, capaci-
tation (actin polymerization and acrosome reaction)
(Brener et al. 2003; Cohen et al. 2004), and in vitro fer-
tilization.

All functional measures were reduced by CP treat-
ment in a time-dependent manner (Fig. 4). A 6-h incuba-
tion with CP reduced sperm motility by 60%, the acro-
some reaction rate by 80%, almost completely blocked
actin polymerization (90%), and inhibited in vitro fertil-
ization by 45% (Fig. 4). The in vitro fertilization rate was
reduced by 61% after 12 h of CP treatment. During treat-
ment with CP, the ongoing translation of a variety of
proteins is blocked; however, the cell is still able to func-
tion using existing proteins until they are degraded. The
functional inhibition by CP treatment was time-depen-
dent, indicating that it may be related to the rate of pro-
tein degradation. It is likely that spermatozoa synthesize
new proteins needed for capacitation and replace pro-
teins that have degraded during the spermatozoa's sub-
stantial sojourn in the female genital tract before fertil-
ization. As described in the introduction, previous works
failed to undoubtedly prove that translation occurs in

Figure 4. Inhibition of sperm functions by CP. Bovine sperm were
incubated in capacitation medium for the specified times with (gray
columns) or without (white column) 0.1 mg/mL CP. At the end of
the incubation, sperm motility, actin polymerization, acrosome re-
action, and in vitro fertilization rates were determined. Data are
expressed as percentage of control ± SD from four independent ex-
periments.

spermatozoa. In the 1970s, capacitation was not well de-
fined; thus scientists failed to incubate cells under opti-
mal conditions and to induce capacitation. Moreover, as
of today, more accurate techniques, such as RT–PCR,
have been developed, and thousands of mRNA species
have been identified in sperm, indicating the optional
use of these mRNAs for protein synthesis.

In summary, the data in the present study indicate
that 55S mitochondrial ribosomes conduct nuclear-en-
coded protein translation during sperm capacitation.
Protein translation is essential for sperm functions that
contribute to fertilization, such as motility, actin poly-
merization, and the acrosome reaction. Thus, the ability
of spermatozoa to synthesize proteins, including
nuclear-encoded proteins, by the 55S ribosomal machin-
ery is critical for the final maturation step leading to
successful fertilization.

Materials and methods

In the methods below all chemicals were obtained from Sigma unless
otherwise stated.

Sperm preparation and capacitation
Human semen was obtained from healthy donors. After liquefaction, the
semen was loaded on a percoll gradient (95%, 65%, 40%, and 20%) and
centrifuged for 30 min at 9000 rpm at room temperature. The lower layer
containing the sperm was collected and washed twice in HAM F-10, then
spun again and incubated for "swim-up" for 30 min at 37°C. The motile
cells were collected without the pellet and resuspended in capacitation
medium.

Ejaculated bovine semen was collected by artificial vagina. The semen
was washed twice in buffer containing 110 mM NaCl, 5 mM KCl, and 10
mM MOPS (pH 7.4), and left for swim-up before the third wash, then
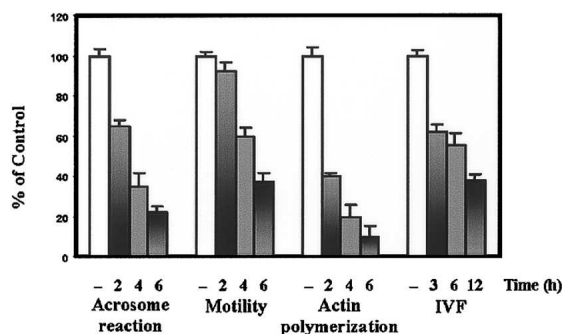resuspended in capacitation medium.

Sexually mature male mice (Balb/c) and rats (Sprague-Dawley) were
sacrificed by CO_2 asphyxiation. The pair of cauda epididymides and part
of the vas-deferens were rapidly removed and minced in 0.5 mL HM
medium (modified Krebs-Ringer bicarbonate medium; Visconti et al.
1995). The sperm were released from the epididymal lumen for 10 min at
37°C. The medium was carefully collected and the cells were washed
twice at the same medium, and then left for swim-up for 20 min at 37°C.
The motile fraction was carefully collected and resuspended in capaci-
tation medium.

Sperm from all sources were carefully separated from somatic cells or
microorganisms by swim-up, and were checked for the absence of round
and somatic cells by light microscopy. For bacterial contamination de-
tection, samples of sperm suspension were smeared on Heart and Brain
agar plates and left overnight at 37°C to verify the absence of microor-
ganisms' colonies.

Sperm were capacitated by incubation in capacitation media that were
supplemented with Penicillin (200 units/mL), cephalexin (200 µg/mL)
(for penicillin-resistant microorganisms), and a mixture of amino acids.
Human sperm (30×10^6/mL) were incubated for 6 h at 37°C in HAM F-10
(Biological Industries) containing 0.5 mM MgCl, 15 mM HEPES (pH 7.4),
and 3 mg/mL HSA. Bovine sperm (10^8/mL) were incubated for 4 h at 39°C
in m-TALP (modified Tyrode's medium) containing 100 mM NaCl, 3.1
mM KCl, 1.5 mM $MgCl_2$, 0.24 mM KH_2PO_4, 25 mM $NaHCO_3$, 40 mM
HEPES, 0.1 mM pyruvic acid, 21.6 mM Na lactate, 10 mg/mL heparin,
0.2 M $CaCl_2$, and 3 mg/mL BSA. Mouse or rat epididymal sperm (10^7/mL)
were incubated for 3 h at 37°C in HMB (Visconti et al. 1995).

Protein labeling by [35S]Met–[35S]Cys incorporation
Sperm were incubated in capacitation media as follows: human sperm
from healthy donors in HAM F-10 (Biological Industries) containing 0.5
mM $MgCl_2$, 15 mM HEPES (pH 7.4), and 3 mg/mL HAS; bovine sperm in
m-TALP (modified Tyrode's medium) (Parrish et al. 1989) containing 3
mg/mL BSA; and mouse (Balb/c) and rat (Sprague-Dawley) epididymal
sperm in HMB (Visconti et al. 1995). All media contained penicillin (200
units/mL), cephalexin (200 µg/mL) (for penicillin-resistant microorgan-
isms) (Teva), a mixture of unlabeled amino acids, and [35S]Met–[35S]Cys

(30 μCi/mL)(^{35}S–^{35}S-protein labeling mix; NEN). Sperm proteins were separated on SDS-PAGE and exposed to film for 2 wk.

Protein labeling by lysyl-transfer RNA tagged with BODIPY

Partially permeabilized bovine sperm were incubated with FluoroTect Green$_{Lys}$ [4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propanoyl-lysyl-tRNA$_{Lys}$] (Promega). Aliquots were spread on coverslips, allowed to dry in the dark, and examined using a confocal microscope (MRC1024, Bio-Rad). The fluorescent intensity was quantitatively analyzed using "Image J" (NIH Image) software.

Detection methods for specific proteins of interest

For immunoprecipitation, sperm were capacitated for 4 h in the presence of [^{35}S]Met–[^{35}S]Cys-labeled amino acids, lysed, and immunoprecipitated with anti-AT$_1$-R, anti-PKCα, or anti-PR antibodies (Santa Cruz Biotechnology).

For immunostaining, capacitated and uncapacitated sperm were incubated with anti-AT$_1$-R or anti-PR followed by rhodamine-conjugated anti-rabbit secondary antibody.

For immuno-electron microscopy, anti-CatSper (a gift from D.E. Clapham, Howard Hughes Medical Institute, Children's Hospital, Boston, MA), anti-PKA-Cs (a gift from G.B. Witman, Department of Cell Biology, University of Massachussetts Medical School, Worcester, MA), or anti-AT$_1$-R antibodies were added to 100-Å sections of embedded sperm cells and then incubated with 15 nm gold-conjugated secondary antibody (Zymed). Control experiments produced only background level signals.

mRNA detection methods

Total RNA was extracted from spermatozoa by RNAqueous-4PCR kit (Ambion). RT–PCR was performed with specific primers (see Supplementary Table S2). The sequences were cut with the appropriate restriction enzyme to verify the expected sequence.

mRNA localization of CatSper was detected in permeabilized sperm by hybridization with a DIG-labeled DNA fragment (see sequence in Supplementary Table S2) and anti-DIG rhodamine-conjugated antibody according to the manufacturer's instructions (Roche Applied Science).

Immunogold localization of mRNAs was performed on sperm sections using DIG-labeled antisenses. After hybridization, the grids were probed with 0.8-nm gold-labeled anti-DIG antibody and enhanced with silver at a ratio of 1:1 (silver:gold) for 4 min (Amersham). Controls for the specificity of the hybridization, including hybridization with antisense to transcripts not found in sperm (DIG-labeled enhanced green fluorescent protein [EGFP] and DIG-labeled SL, which represent splice leader subunit in Trypanosome mRNAs), secondary antibody without antisense, and prehybridization treatment with RNase. Control experiments produced only background level signals.

Analysis of ribosomes profile

Ejaculated bovine sperm cells were incubated in capacitation medium for 1 h, then the cells were lysed in buffer A (100 mM NH$_4$Cl, 10 mM MgCl$_2$, 20 mM Tris-Cl at pH 7.5), containing 150 mM KCl, 0.1% SDS, 1.6% Triton, and proteases inhibitors. Cell debris were pelleted, and the lysate containing ribosomes was layered on continuous 10%–30% (w/v) sucrose gradient in buffer A containing 150 mM KCl and centrifuged at 35,000 rpm for 3 h at 4°C. Twenty fractions were collected and measured at A$_{260}$ for total RNA. The RNA was extracted by ethanol/sodium-acetate and purified by RNaquose-4–PCR and DNase treatment. rRNAs and mRNA were identified by RT–PCR.

Sperm functional measurements

Sperm analyzed by functional assays: Motility was assessed by a "Sperm Motility Analyzer" (Bartoov et al. 1991), actin polymerization, and acrosome reaction using FITC-phalloidin and pissum-sativum-aglutinin staining, respectively (Mendoza et al. 1992), and in vitro fertilization with BALB-C mice by standard protocol (Eppig et al. 1996).

Acknowledgments

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Yael Gur and Haim Breitbart

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