Biology of Reproduction (2016.9) 95(3):50.

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BIOLOGY OF REPRODUCTION (2016) **95**(3):50, 1–11
Published online before print 14 July 2016.
DOI 10.1095/biolreprod.116.140400

The Behavior and Acrosomal Status of Mouse Spermatozoa In Vitro, and Within the Oviduct During Fertilization after Natural Mating[1]

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ABSTRACT

Although 90%–100% of mouse oocytes can be fertilized in vitro with capacitated spermatozoa within 1 h after insemination, oocytes within the oviduct are fertilized one by one over a period of several hours. In vitro experiments showed that both acrosome-intact and acrosome-reacted spermatozoa entered the cumulus oophorus, but that acrosome-reacted spermatozoa reached the surface of oocytes more readily than acrosome-intact spermatozoa. During the period of fertilization within the oviduct, acrosome-reacted spermatozoa were seen throughout the isthmus, but with higher incidence in the upper than in the mid- and lower segments of the isthmus. Very few spermatozoa were present in the ampulla, and almost all were acrosome reacted. Although the cumulus oophorus and zona pellucida are known to be able to induce or facilitate the acrosome reaction of spermatozoa, this picture makes it likely that almost all fertilizing mouse spermatozoa within the oviduct begin to react before ascending from the isthmus to the ampulla. We witnessed a reacted spermatozoon that stayed on the zona pellucida of a fertilized oocyte for a while; it then moved out of the cumulus before reaching the zona pellucida of the nearby unfertilized oocyte. We noted that only a few spermatozoa migrate from the isthmus to the ampulla during the progression of fertilization, and this must be one of the reasons why we do not see many spermatozoa swarming around a single oocyte during in vivo fertilization.

acrosome reaction, cumulus oophorus, fertilization, hyperactivated motility, mouse, oviduct, sperm, zona pellucida

INTRODUCTION

Mammalian spermatozoa become competent to fertilize during their ascent through the female tract [1, 2], a phenomenon commonly referred to as capacitation [3, 4]. Capacitated spermatozoa become vigorously motile and undergo the acrosome reaction before effecting fertilization [5, 6]. Some years ago it was thought that fertilizing mouse spermatozoa undergo the acrosome reaction only after contact with an oocyte's zona pellucida, and that spermatozoa reacting before their contact with the zona lose their ability to penetrate it [7–9]. Although the zona pellucida of the mouse and many other species certainly has the ability to induce or accelerate the acrosome reaction [9–13], Jin et al. [14] discovered that, at least in the mouse, most fertilizing spermatozoa that actually penetrated oocytes in vitro were ''acrosome reacted'' before reaching the zona: only less than 2% of fertilizing spermatozoa underwent the reaction after reaching the zona surface. During the past 50 yr, researchers have chiefly used in vitro systems to analyze the process of mammalian fertilization. Since what can happen in vitro is not necessarily what happens in vivo, we re-examined the behavior of spermatozoa within the female reproductive tract before and during fertilization. To this end, we used transgenic (TG) mice [15], the spermatozoa of which express a green fluorescence in the acrosome and a red fluorescence in the midpiece mitochondria. This allowed us to track the state of live spermatozoa within the oviduct through its wall.

While we were preparing the manuscript of this paper, La Spina et al. [16] and Muro et al. [17] reported that fertilizing mouse spermatozoa begin their acrosome reaction in the upper isthmus of the oviduct. While these authors collected all quantitative data from the females induced to ovulate by gonadotropin treatments, we collected mostly data from naturally cyclic females without any hormone treatment. We examined not only the motility and acrosomes of spermatozoa in the oviducts, but also the behavior of spermatozoa toward unfertilized and fertilized oocytes in the oviductal ampulla during progression of fertilization. In some experiments, we used an in vitro system to help understand what we saw after examination of spermatozoa and oocytes within oviducts.

MATERIALS AND METHODS

Animals

The TG mice [15], the spermatozoa of which express green fluorescent protein (GFP) in their acrosome and red fluorescent protein in their midpiece region (mitochondria; B6D2-Tg [CAG/Su9-DsRed2Acr3-EGFP] RBGS002Osb [RBRC03743]) were provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. Hybrid B6D2F1 mice were purchased from

[1]Supported by Grant in Aid for Innovative Research in Life Science from Asahikawa Medical University to T.H., a Ministry of Education, Culture, Sports, Science, and Technology of Japan grant to M.O., and a University of Hawaii Research Supporting Fund grant to R.Y.
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Received: 29 March 2016.
First decision: 10 May 2016.
Accepted: 6 July 2016.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

the Charles River Co. (Kanagawa, Japan). All mice were subjected to a 14L:10D regime (light from 05:00 to 19:00; dark from 19:00 to 05:00), maintained at 23 ± 2°C and 45 ± 5% humidity with ad libitum access to food and water, and used when they were 8–16 wk old. Most animals were estimated to mate between 20:00 and 01:00 [18, 19] and to ovulate around 03:00 [20]. In two series of experiments, females were induced to ovulate by injections of eCG (7 IU) and hCG (7 IU) 48 h apart. This was in order to repeat some of the experiments performed by Muro et al. [17] and La Spina et al. [16]. At first, females were allowed to mate starting immediately after hCG injection. About 16 h later (about 4 h after ovulation), spermatozoa within the oviducts were examined. Each female was then caged with a male 12 h after hCG injection (about the time of ovulation). Spermatozoa within oviducts were examined 2 h after confirmation of copulation plug, which was found by 30 min after females were put into the males' cage. All experimental procedures conformed to the Guidelines for Animal Experiments of Asahikawa Medical University.

Reagents and Media

Organic and inorganic reagents to prepare culture media were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated. The medium for sperm incubation was TYH medium [21]. which consisted of 119.37 mM NaCl, 4.79 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 5.56 mM glucose, 1.00 mM sodium pyruvate, 4 mg/ml BSA (AlbuMax; GibcoBRL, Auckland, New Zealand), 50 μg/ml streptomycin sulfate, and 75 μg/ml penicillin G potassium, pH 7.4 when equilibrated with 5% (v/v) CO_2 in air. Ca^{2+} ionophore A23187 was purchased from Merck (Darmstadt, Germany). TYH containing 3.42 mM Ca^{2+} was called 2× Ca^{2+} TYH and was used for the induction of the acrosome reaction by Ca^{2+} ionophore.

Sperm Preparation

A small, dense sperm mass, collected from the cauda epididymidis of TG heterozygous (+/–) male, was suspended in a drop (200 μl) of TYH, allowing spermatozoa to disperse into the medium. The concentration of spermatozoa at this stage was about 2×10^7/ml. The suspension was kept for 2 h at 37°C under 5% CO_2 in air to capacitate the spermatozoa. Acrosome-reacted spermatozoa were obtained according to Tateno [22]. Briefly, epididymal spermatozoa were incubated in TYH for 5 min or 2 h, treated with 20 μM Ca^{2+} ionophore (A23187) in 2× Ca^{2+} TYH for 10 min, centrifuged, and allowed to swim out of the sperm pellet into 500 μl prewarmed TYH. Of the swim-up spermatozoa thus obtained, nearly 80% and almost 100% were acrosome reacted after preincubation in TYH for 5 min and 2 h, respectively. According to a previous study by Tateno et al. [23], the proportion of live acrosome-reacted spermatozoa at the end of Ca^{2+} ionophore treatment is about 50%.

Spermatozoa used for insemination were: 1) incubated in TYH for 5 min (designated as fresh spermatozoa); 2) incubated in TYH for 5 min, then treated with Ca^{2+} ionophore before swimming up in TYH for 30 min (designated as ionophore-treated spermatozoa with 5 min preincubation); and 3) incubated in TYH for 2 h, then treated with Ca^{2+} ionophore, and finally swim up in TYH for 30 min (designated as ionophore-treated spermatozoa with 2 h preincubation).

Examination of Sperm Motility and Acrosomal Status

The movement pattern of live spermatozoa in TYH was recorded with a digital camera. The proportions of progressively motile or hyperactivated spermatozoa were determined by slowing down movie images.

The presence or absence of acrosomal caps in TG spermatozoa was assessed by examining them in vivo or in vitro under a fluorescence microscope [24]; however, in some instances, spermatozoa were fixed by adding 2 μl of 10% (v/v) formalin to 20 μl of sperm suspension before examination.

Determination of Fertile Life of Acrosome-Reacted Spermatozoa

To determine how long spermatozoa are able to fertilize cumulus-intact oocytes after the acrosome reaction, we prepared ~100% acrosome-reacted spermatozoa as previously described and incubated them in TYH for up to 6 h. Cumulus-oocyte complexes, collected from oviducts 15 h after hCG injection, and 1–2 μl of sperm suspension were added to 100 μl TYH after 2–6 h of incubation of acrosome-reacted spermatozoa. The final concentration of spermatozoa was approximately 100/μl. Oocytes were examined for evidence of fertilization (the presence of the second polar body and two pronuclei) 6–8 h after insemination.

Electron Microscopy of Spermatozoa

Spermatozoa were fixed for 2 h in 2% paraformaldehyde and 2% glutaraldehyde in 30 mM Hepes buffer containing 100 mM NaCl and 2 mM $CaCl_2$ (pH 7.4) for 1 h at room temperature before postfixation in an aldehyde-OsO_4 mixture (1% OsO_4, 1.25% glutaraldehyde, 1% paraformaldehyde, and 0.32% $K_3[Fe\{CN\}_6]$ in 30 mM Hepes buffer [pH 7.4]) for 1 h. Fixed spermatozoa were washed three times with Milli Q water (Milli Q Integral; Merck Millipore, Tokyo, Japan) and suspended in a small droplet of 2% agar. They were stained en bloc with EM stainer (Nisshin EM, Tokyo, Japan), washed with 50% ethyl alcohol for 1 h, dehydrated in an ethanol series, and embedded in Quetol 812 (Nisshin EM). Sections were examined using a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan).

In Vitro Insemination and Fluorescence Imaging of Spermatozoa

B6D2F1 females were superovulated by intraperitoneal injections of eCG and hCG 48 h apart. Aggregated cumulus-oocyte complexes, collected from oviducts 15 h after hCG injection, were pipetted in TYH to obtain individual cumulus-oocyte complexes. Some were treated with 5 mM $SrCl_2$ in Ca^{2+}-free TYH for 1 h to activate oocytes within the complex. Two or three cumulus-oocyte complexes containing either activated or nonactivated oocytes were placed close together at the center of four dots of vaseline-paraffin mixture (9:1) previously applied to a glass slide. After the preparation was covered with a coverslip (24 × 32 mm), a group of cumulus-oocyte complexes were lightly compressed under the coverslip. The space between the slide and coverslip was filled with TYH. The preparation was kept at 37°C using the Thermo Plate (Mats-55SF; TOKAI HIT, Shizuoka, Japan) on a microscope stage (BX51; Olympus, Tokyo, Japan) very gently blowing 100% humidified, warm 5% CO_2 in air. Cumulus-oocyte complexes under the coverslip were inseminated by adding 10–20 μl of a sperm suspension to one side of the coverslip and drawing it with a piece of filter paper applied to the opposite side of the coverslip. The sperm suspension added to the cumulus-oocyte complexes from the side was diluted 10–20 times immediately before use to avoid exposure of the cumulus to excessive numbers of spermatozoa. Every precaution was taken to avoid insemination with an excessive number of spermatozoa. Videography was started shortly before spermatozoa contacted the cumulus-oocyte complex and was terminated within 10 min. A highly sensitive (up to ISO 409600) digital camera (ILCE-7S; Sony, Tokyo, Japan) and a mercury lamp equipped with neutral density filters (ND6 and ND25) were utilized to reduce UV damage to spermatozoa. We used three fluorescent mirror units: WIG, NIBA, and WIB. The first was for mitochondrial DsRed, the second for acrosomal GFP, and the third for both DsRed and GFP.

Examination of Spermatozoa and Oocytes Within Oviducts

Pre-estrous B6D2F1 females (judged by the appearance of vagina) during their natural cycle were caged overnight with a TG homozygous (+/+) male and examined for the presence of a copulation (vaginal) plug the next morning. A pair of oviducts, isolated from a mated female, was placed on a slide and viewed through a UV microscope, as already described. Video recording of oocytes and spermatozoa within the oviduct was started immediately and stopped within 40 min. This 40-min limit was based on our preliminary observations that cumulus-oocyte complexes in isolated oviducts under the coverslip began to show definite signs of degeneration (e.g., granulation of oocyte cytoplasm) in 60 min. An oocyte was considered fertilized when a sperm midpiece (red fluorescent) was seen within the perivitelline space. After video recording, all oocytes were recovered from oviducts and re-examined for fertilization.

Statistical Analysis

To assess the statistical significance of proportions of GFP-negative spermatozoa in the lower, mid-, and upper isthmus, one-way ANOVA and the Tukey-Kramer method were used after percentile data were transformed into arcsine values.

RESULTS

Motility and Acrosomes of Spermatozoa In Vitro Before and after Ionophore Treatment

More than 90% of fresh epididymal spermatozoa in TYH were progressively motile. The sperm movement pattern

TABLE 1. Motility and the acrosome reaction of spermatozoa after Ca^{2+} ionophore treatment and 30 min swim-up.

Sperm preincubation time (min) in TYH prior to ionophore treatment	Ionophore treatment	Percentage of sperm at 30 min after treatment (range)[a]	
		Actively motile	Acrosome reacted
5	−	92.7 ± 3.6 (87–99)	0
5	+	95.2 ± 2.3 (91–98)	78.3 ± 7.5 (64–89)
120	+	94.0 ± 2.6 (89–98)	99.4 ± 0.6 (98–100)

[a] Experiments were repeated three times, and 70–200 spermatozoa were examined for each determination.

changed from progressive to nonprogressive (hyperactivated) starting from about 30 min of incubation in TYH. Virtually all motile spermatozoa from the cauda epididymis had an intact acrosome, as evidenced by the presence of GFP within the acrosomal cap. A spontaneous acrosome reaction began about 30 min after incubation [23] and, by 2 h, about 20% were acrosome reacted. When treated with Ca^{2+} ionophore, a high proportion of the epididymal spermatozoa quickly acrosome reacted, regardless of preincubation time in TYH (Table 1; [23]). Figure 1 shows fluorescence and electron micrographs of spermatozoa before and after Ca^{2+} ionophore treatment, and illustrates that the loss of GFP from the acrosomal cap region of live sperm head is coincidental with the loss of acrosomal cap via the exocytotic acrosome reaction.

Behavior of Spermatozoa Toward Cumulus-Oocyte Complex In Vitro

Each oocyte is surrounded by two layers of cumulus cells: the outer loose layer and the inner compact layer (Fig. 2A). In the outer layer, cumulus cells were less densely arranged within a gelatinous matrix, whereas, in the inner layer, many cumulus cells were tightly packed within the matrix. A radial arrangement of cumulus cells around each oocyte, which is commonly referred to as ''corona radiata,'' was evident when a cumulus mass was compressed under the coverslip (Fig. 2, B and C). When a cumulus-oocyte complex was inseminated in vitro with fresh epididymal spermatozoa with intact acrosomes, many of the spermatozoa passed through the outer layer of the cumulus, but most failed to advance into the inner layer (Fig. 2D; Supplemental Movie S1; Supplemental Data are available online at www.biolreprod.org). In contrast, ionophore-treated spermatozoa with 5 min preincubation (~80% of which were acrosome reacted) readily penetrated both the outer and inner layers of the cumulus to reach the oocyte's zona pellucida, regardless of their acrosomal status (Fig. 2E; Supplemental Movie S2). Spermatozoa treated with ionophore after 2-h preincubation in TYH were nearly 100% acrosome reacted, and they readily reached the zona pellucida (Fig. 2F; Supplemental Movie S3). We performed a series of experiments to determine how long spermatozoa remain fertile after the acrosome reaction, and found that they are able to fertilize cumulus-intact oocytes for up to 4 h after the reaction, at least under the in vitro conditions we employed (Table 2).

Behavior of Spermatozoa Within the Oviduct Around the Time of Fertilization

We estimated that females that we used ovulated at around 03:00 following the evening of natural mating (see *Materials and Methods*). Fertilization was in progress between 06:00 and 09:00 (Table 3). The sperm:oocyte ratio within the ampulla region of oviduct was less than 2.0 before all oocytes were fertilized. Figure 3, A and B, shows isolated oviducts photographed under ordinary and fluorescent lights. We arbitrarily divided the oviduct into five regions: the uterotubal junction, the lower, mid-, and upper isthmus, and the ampulla. Figure 3, C and D, and Supplemental Movies S4 and S5, show GFP-positive and -negative spermatozoa seen in the lower and midisthmus at about 08:00. Motile GFP-negative spermatozoa (yellow arrowheads) are considered live and acrosome reacted.

We noted that the numbers and proportions of motile and immotile spermatozoa within the lower, mid-, and upper isthmus varied considerably among different animals, even if they were examined at the same time of day following mating. Enormous interanimal variations in the number of spermatozoa within the isthmus after natural mating or artificial insemination have been reported in hamsters [25] and cattle [26].

Table 4 summarizes proportions of GFP-negative spermatozoa that we could see clearly through the wall of various segments of oviductal isthmus during progression of in vivo fertilization. These included both motile and immotile spermatozoa. As far as motile, GFP-negative spermatozoa (considered acrosome reacted) are concerned, it is clear that the incidence of acrosome-reacted spermatozoa is much higher in the upper than the lower isthmus.

We tried, but were unable, to determine exact numbers of spermatozoa within the lower and midisthmus because of enormous interanimal variations as to the numbers of spermatozoa attached to the epithelium and those free in the lumen. Constant wafting of spermatozoa due to peristaltic contractions of the isthmus made examination of individual spermatozoa difficult. Nonetheless, it was clear that only a very small number of spermatozoa in the isthmus moved into the ampulla [19], and almost all of them were GFP negative (acrosome reacted) and moving actively (Table 5; Supplemental Movie S6).

Some females were treated with eCG and hCG 48 h apart and caged with males immediately after hCG injection. When examined 16 h after (about 4 h after ovulation), only 4%–16% of motile spermatozoa within lower and midisthmus were GFP negative (acrosome reacted), whereas most of the motile spermatozoa in the upper isthmus were acrosome reacted (Supplemental Table S1). When eCG- and hCG-treated females were caged with males at about the time of ovulation (12 h after hCG injection) and their oviducts were examined about 2 h later, none or very few (0.6%) motile spermatozoa in the lower and midisthmus were GFP negative (acrosome reacted), whereas 37% of those within the upper isthmus were acrosome reacted (Supplemental Table S2, Supplemental Movie S7).

Behavior of Spermatozoa Toward Activated and Unactivated Oocytes In Vitro and In Vivo

When spermatozoa were preincubated in TYH for 2 h before insemination, we witnessed one spermatozoon passing through the zona about 15 min after contacting with it. Figure 4, A and B, and Supplemental Movie S3 show a cumulus-oocyte complex inseminated in vitro with nearly 100% acrosome-reacted spermatozoa; a spermatozoon attached to and detached from the zona pellucida of an activated oocyte.

The behavior of an acrosome-reacted spermatozoon within the ampulla after natural mating is shown in Figure 4, C–E; Supplemental Movie S8 shows this spermatozoon. We could not observe spermatozoa within the oviduct passing through

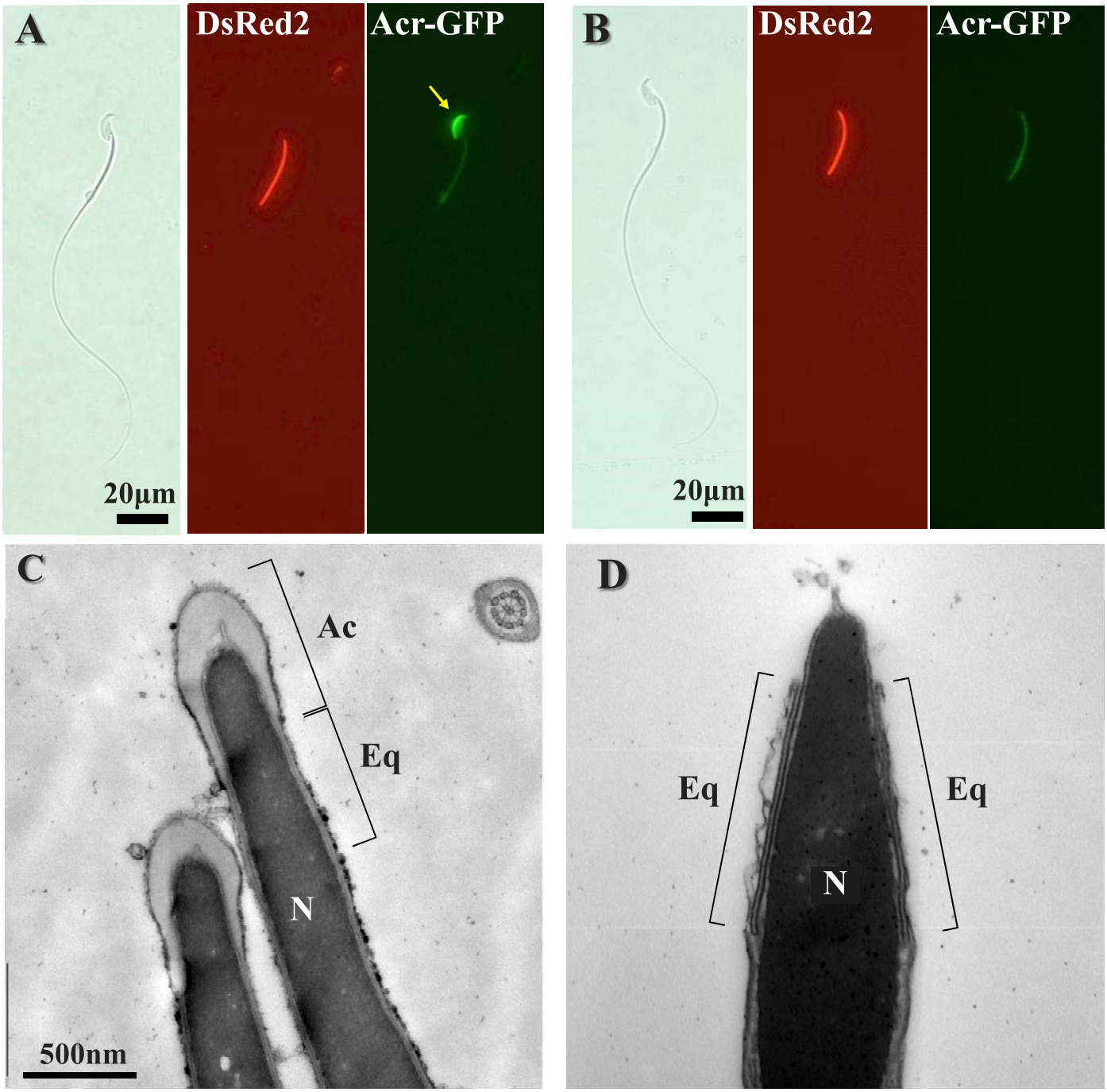

FIG. 1. Light and electron micrographs of TG spermatozoa. **A**) A fresh epididymal spermatozoon before Ca^{2+} ionophore treatment. See red fluorescence in midpiece (DsRed2) and green fluorescence in acrosomal cap (Acr-GFP). **B**) An ionophore-treated spermatozoon with red midpiece, but no green acrosomal cap. **C**) An electron micrograph of sperm head before ionophore treatment. Ac, acrosomal cap; Eq, equatorial segment of acrosome. **D**) Sperm head after ionophore treatment. Note that the acrosomal cap was lost as the result of the acrosome reaction.

the zona pellucida of an oocyte, but we did see an acrosome-reacted spermatozoon that entered an oocyte in vitro. Figure 5, A and B, shows the tracks of two acrosome-reacted spermatozoa moving in and out of cumuli of activated and unactivated oocytes after in vitro insemination. One of the spermatozoa passed through the zona pellucida of an oocyte at between 11 and 15 min after insemination (Fig. 5, C–F; Supplemental Movies S9 and S10). Fertilization was confirmed by the presence of a spermatozoon within ooplasm and extrusion of a second polar body.

DISCUSSION

Fertilization In Vivo after Natural Mating

Although 90%–100% of mouse oocytes can be fertilized in vitro within 1 h after insemination [e.g., 27, 28], those within oviducts are fertilized one by one over several hours after mating. This extended process of fertilization within the mouse oviduct was first reported by Braden and Austin [20], and confirmed by Muro et al. [17] and in the present study (Table 3). The slow progression of fertilization in vivo is obviously

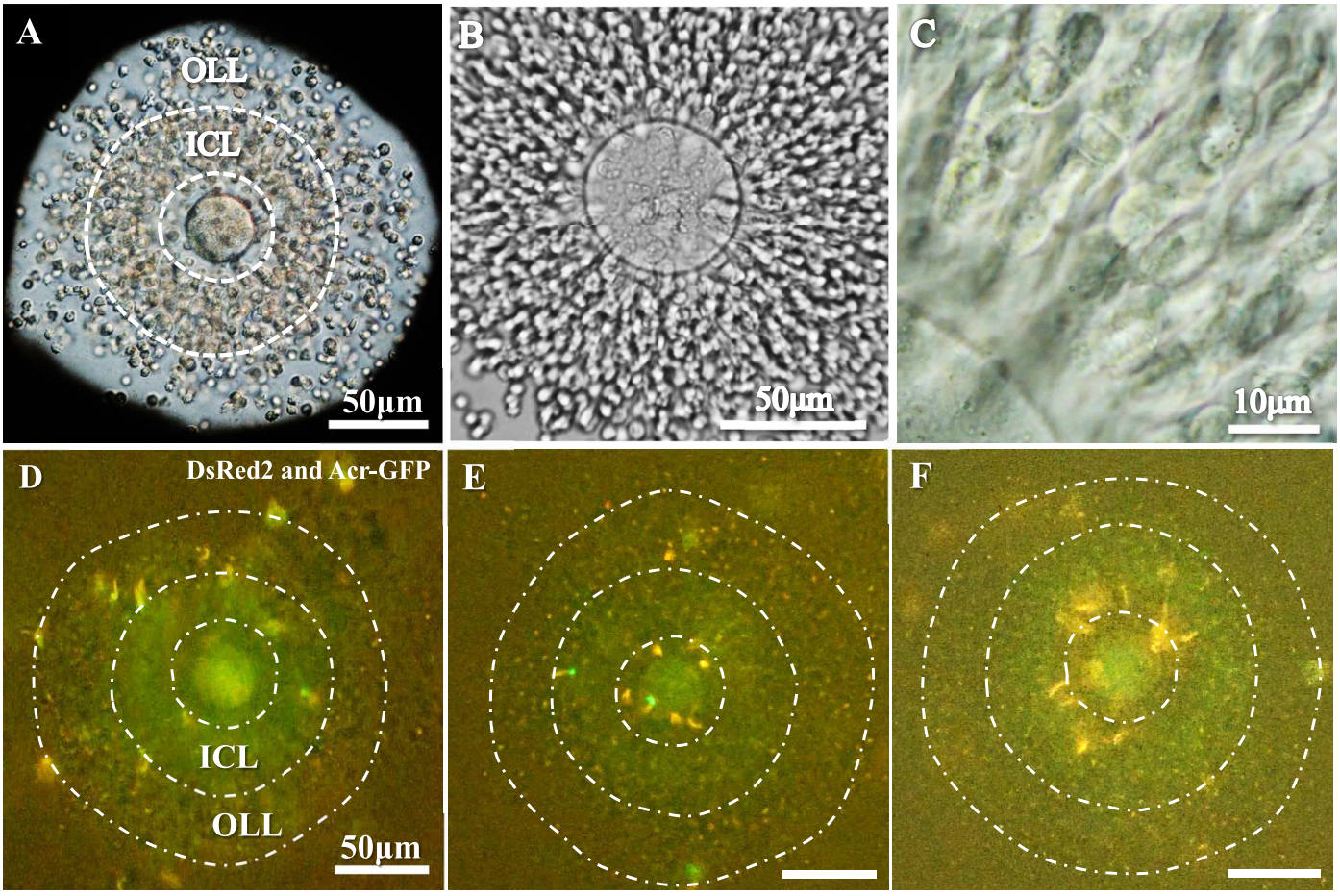

FIG. 2. Sperm penetration into cumulus oophorus. **A**) Cumulus oophorus consists of the outer loose layer (OLL) and the inner compact layer (ICL). Dark background is a medium containing India ink. **B** and **C**) A cumulus oophorus compressed under a coverslip, showing a radial arrangement of cumulus cells around an oocyte. **D**) A cumulus-oocyte complex inseminated with fresh epididymal spermatozoa. This photograph was taken from Supplemental Movie S1. Many spermatozoa entered the OLL, but few entered the ICL. **E**) A cumulus-oocyte complex after insemination using Ca^{2+} ionophore-treated spermatozoa. Spermatozoa were not preincubated in TYH before ionophore treatment; many spermatozoa passed through both OLL and ICL to reach the oocyte. This frame is from Supplemental Movie S2. **F**) A cumulus-oocyte complex after insemination using spermatozoa treated with Ca^{2+} ionophore. Spermatozoa were preincubated for 2 h before ionophore treatment. Numerous (nearly 100% acrosome reacted) spermatozoa reached the surface of an oocyte. This photograph was taken from Supplemental Movie S3. Images in **D**–**F** are of cumulus-oocyte complexes 3–5 min after insemination.

due, in part, to a temporal arrest of fertilizing spermatozoa in the lower segment of oviduct (isthmus) [19], followed by a slow release of spermatozoa from this region, allowing further ascent of spermatozoa a few at a time. Even though mammalian oocytes seem to have several different mechanisms to prevent polyspermy (e.g., the zona reaction) [6], individual oocytes within the oviduct are not swarmed by numerous spermatozoa as almost always occurs after in vitro insemination. The number of spermatozoa in the ampulla during in vivo fertilization is often less than that of the oocytes there, at least in laboratory rodents [29]. The oviduct isthmus certainly plays a crucial role in restricting the number of spermatozoa ascending the oviduct, as shown experimentally by Hunter and Leglise in the pig [30], but the mechanism by which few spermatozoa are released from the isthmus at a time is not fully understood [31]. Smith and Yanagimachi [32] and Suarez [33] proposed that uncapacitated spermatozoa remain attached to the isthmus wall, and those that are ''capacitated and hyperactivated'' leave there for further ascent. However, this does not fully explain why so few spermatozoa reach the ampulla during fertilization in vivo.

TABLE 2. Fertile life of acrosome-reacted spermatozoa.

Sperm incubation time after inophore treatment (h)[a]	No. of oocytes inseminated[b]	No. of fertilized oocytes (%)
2	38	30 (78.9)
4	30	4 (13.3)
6	29	0 (0)

[a] Include 30-min swim-up time after ionophore treatment.
[b] Sperm concentration was approximately 100/μl.

TABLE 3. Sperm:oocyte ratio within oviductal ampulla during progression of fertilization in naturally mated females.

Time of day	No. of females examined	No. of sperm[a]	No. of oocytes	No. of fertilized oocytes (%)	Sperm:oocyte ratio
06:00	4	10	35	7 (20.0)	0.3
07:00	4	61	35	27 (77.1)	1.7
08:00	4	72	37	25 (67.6)	1.9
09:00	4	84	35	35 (100)	2.1

[a] Include those in the perivitelline space and cytoplasm of oocytes.

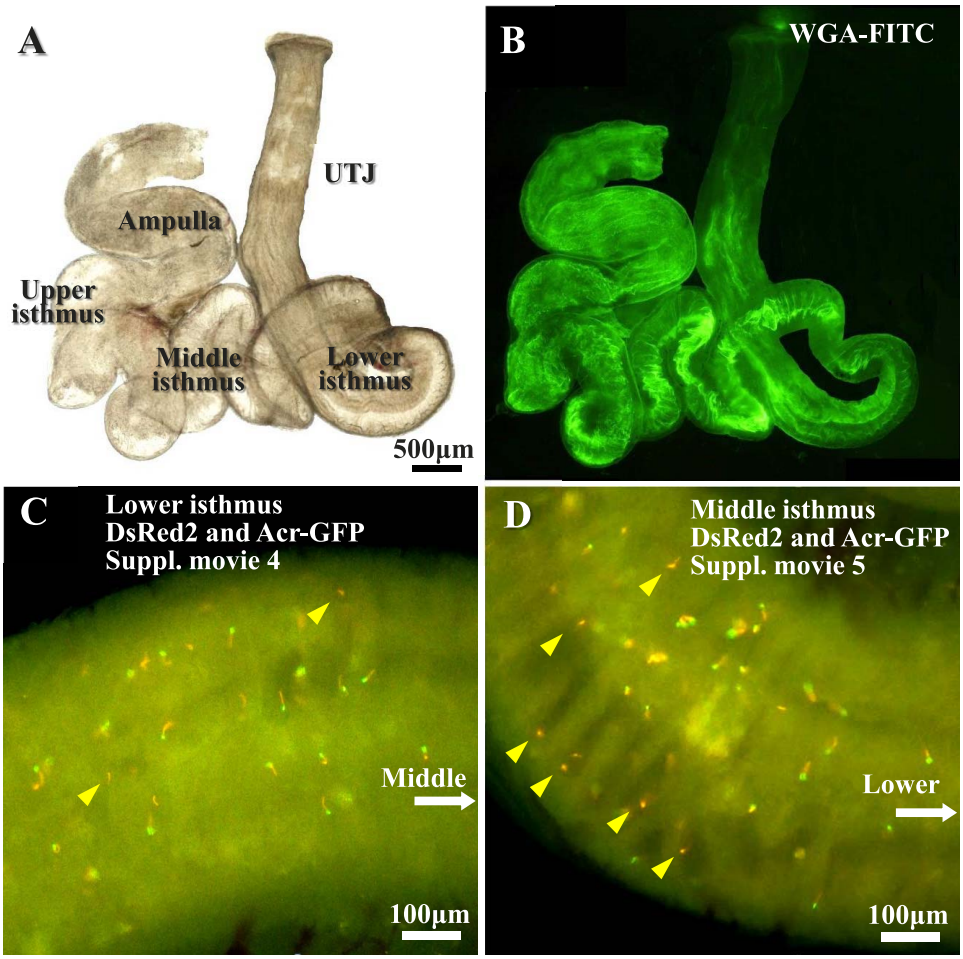

FIG. 3. **A**) Isolated mouse oviduct with five regions; the uterotubal junction (UTJ), the lower, mid-, and upper isthmus, and the ampulla. **B**) An oviduct flushed with TYH medium containing WGA-FITC, showing many grooves created by mucosal folds. **C**) A frame captured from Supplemental Movie S4 showing a motile, GFP-negative spermatozoon (arrowheads) in the lower isthmus. **D**) A frame captured from Supplemental Movie S5, showing a motile, GFP-negative spermatozoon (arrow heads) in the midisthmus.

Initiation of the Acrosome Reaction of Spermatozoa in the Oviduct

In all mammals, the acrosome reaction is essential for sperm passage through the oocyte's zona pellucida [6]. It has long been believed that, in the mouse, and perhaps in many other mammals, the reaction takes place on the zona pellucida, and that the spermatozoa reacted before their contact with the zona cannot penetrate it. This conclusion was based on experiments using cumulus-intact or cumulus-free oocytes inseminated in vitro, with the acrosomal status examined by staining spermatozoa with chlorotetracycline [7], Coomasie blue, or plant lectins [9, 34]. Although zona pellucida glycoproteins of the mouse and many other species are certainly able to induce

TABLE 4. Rates of GFP-negative spermatozoa in the oviduct isthmus during progression of fertilization.

Time of examination	No. of females	Examined spermatozoa	Percentage of GFP-negative spermatozoa ± SEM (range)*		
			Lower isthmus	Midisthmus	Upper isthmus
06:00	6	Motile only†	13.7 ± 4.8 (0–28)[a]	15.0 ± 6.2 (0–35)[a]	100 ± 0 (100)[b]
		Motile and immotile	41.9 ± 8.7 (20–73)[a]	36.9 ± 8.9 (0–62.5)[a]	96.7 ± 3.3 (80–100)[b]
07:00	6	Motile only†	23.8 ± 8.1 (0–50)[a]	17.6 ± 6.4 (0–40)[a]	100 ± 0 (100)[b]
		Motile and immotile	41.6 ± 3.7 (33–56)[a]	41.8 ± 4.1 (32–58)[a]	93.8 ± 6.3 (75–100)[b]
08:00	8	Motile only†	20.5 ± 5.8 (0–50)[a]	22.6 ± 6.6 (0–50)[a]	97.1 ± 2.9 (86–100)[b]
		Motile and immotile	34.8 ± 4.6 (20–57)[a]	43.4 ± 9.2 (9–83)[a]	94.1 ± 4.7 (67–100)[b]
09:00	6	Motile only†	13.2 ± 6.8 (0–50)[a]	24.9 ± 7.4 (0–56)[a]	62.5 ± 23.9 (0–100)[a]
		Motile and immotile	35.5 ± 3.3 (25–45)[a]	49.9 ± 5.7 (33–67)[a]	50.5 ± 18.3(0–10)[a]

* A total of 11–89 of motile and immotile spermatozoa within lower and midisthmus were examined for each determination, while 1–11 motile and immotile spermatozoa within upper isthmus were examined for each determination, except for four determinations; in three determinations (two at 07:00 and one at 08:00), none of the spermatozoa was observed, and in one determination (at 09:00), 34 motile and immotile spermatozoa were observed and all of them were examined.
† Motile GFP-negative spermatozoa are considered acrosome reacted physiologically.
[a,b] Values with different superscripts in the same row are significantly different ($P < 0.05$).

TABLE 5. Rate of GFP-negative (acrosome-reacted) spermatozoa in the ampulla during progression of fertilization.

Time of examination	No. of females used	No. of spermatozoa identified in the ampulla[a]	No. of GFP-negative spermatozoa (%)
06:00	4	3	3 (100)
07:00	4	33	33 (100)
08:00	4	47	47 (100)
09:00	4	48	47 (98)

[a] Almost all were actively motile.

or accelerate the acrosome reaction [e.g., 9–12, 35–39], we found many acrosome-reacted mouse spermatozoa in oviductal isthmus and ampulla before they met oocytes (Tables 4 and 5). Esponda and Moreno [40] examined mouse spermatozoa in the isthmus 0–6 h before ovulation by using transmission electron microscopy, and found many spermatozoa with modified (swollen) acrosomes. According to La Spina et al. [16] and Muro et al. [17], mouse spermatozoa begin their reactions after reaching the upper region of the isthmus. Here, we found motile, acrosome-reacted spermatozoa in both the mid- and

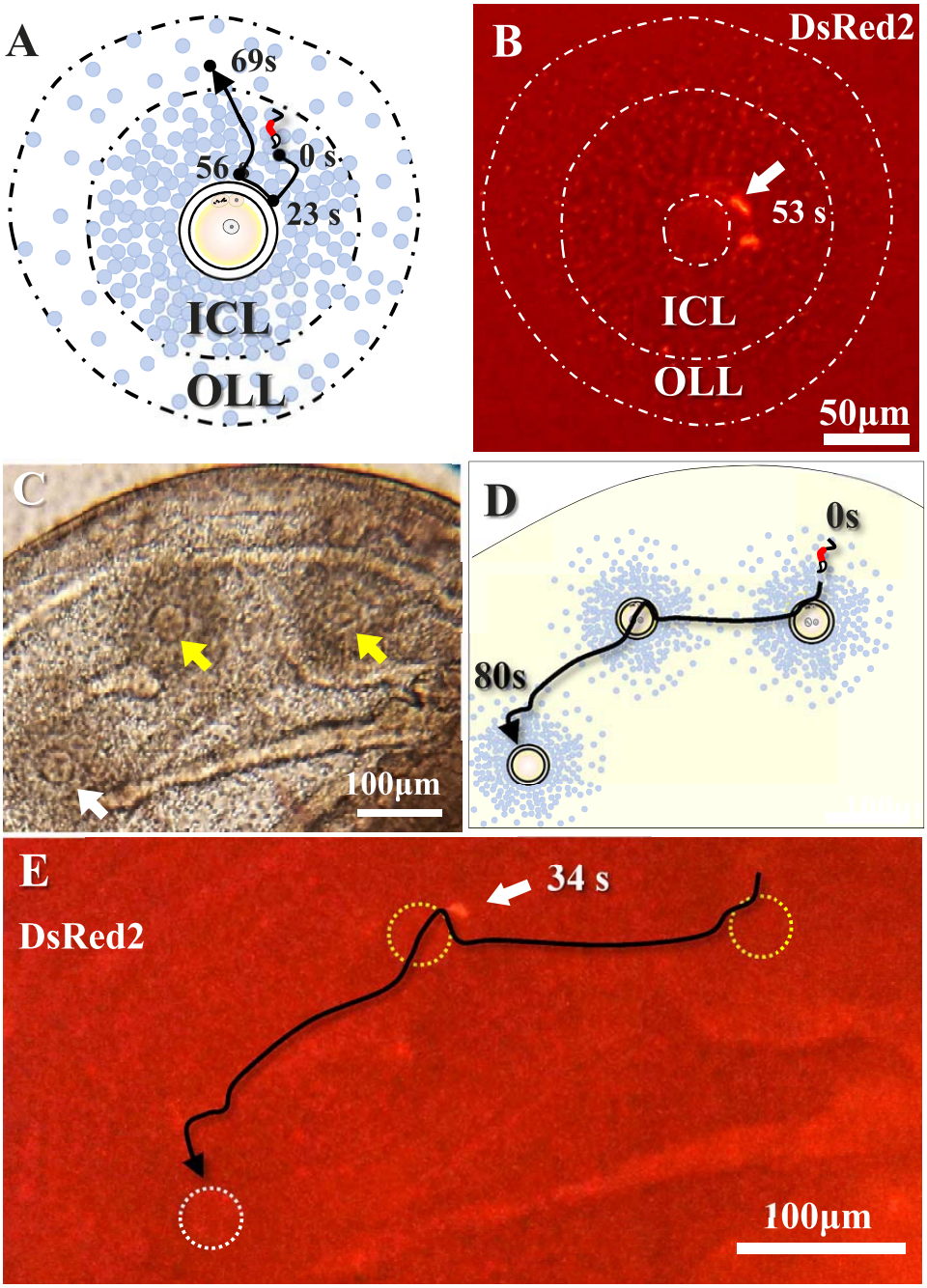

FIG. 4. Tracks of spermatozoa in and out of cumulus oophorus. **A**) A spermatozoon moving within the cumulus oophorus surrounding an activated (pronuclear) oocyte in vitro. This spermatozoon stayed on the zona pellucida for about 30 sec before moving out of the cumulus. **B**) A frame captured from Supplemental Movie S11 showing a spermatozoon that moved out of the cumulus after contacting an oocyte's zona pellucida about 5 min after insemination in vitro. **C**) Two fertilized oocytes (yellow arrows) and one unfertilized oocyte (white arrow) within the oviductal ampulla. **D**) Track of a spermatozoon swimming in and out of cumuli of oocytes shown in **C**. **E**) A combined frame captured from Supplemental Movie S8 showing a spermatozoon colliding with two fertilized oocytes before meeting an unfertilized oocyte.

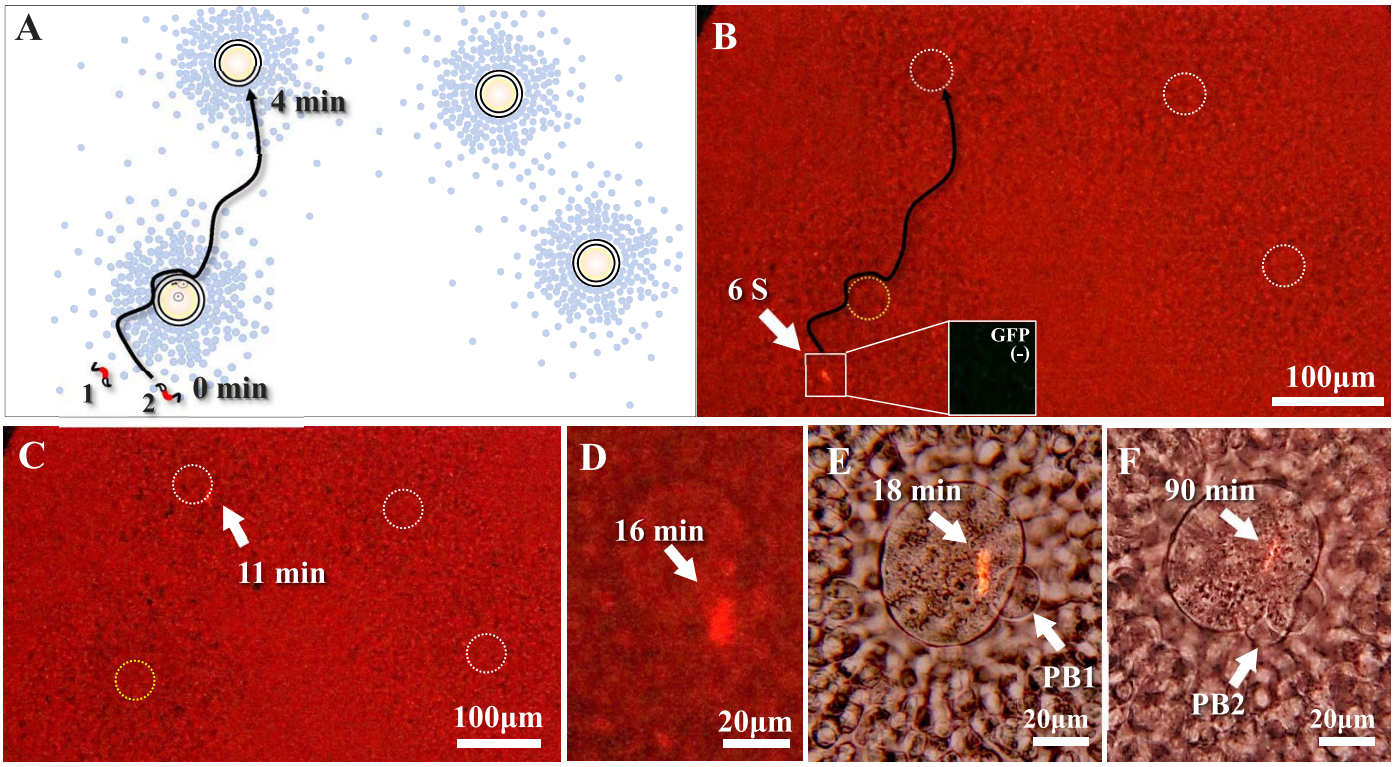

FIG. 5. Tracks of two spermatozoa moving in and out of cumulus in vitro. **A**) One artificially activated (pronuclear) oocyte, at bottom left, and three unfertilized oocytes are shown. One of two spermatozoa (1) moved around an activated oocyte before swimming away from it. The other spermatozoon (2) contacted the activated oocyte and moved toward a nearby unfertilized oocyte and penetrated it. **B**–**D**) Frames captured from Supplemental Movie S9 showing that the spermatozoon (2) was GFP negative (acrosome reacted), reaching the zona pellucida of an unfertilized egg by 4 min after start of recording. **E** and **F**) Frames captured from Supplemental Movie S10. This spermatozoon passed through the zona between 11 and 15 min after insemination, and the oocyte extruded the second polar body (PB2) by 90 min after insemination.

lower isthmus (Table 4). The reason that we did, and La Spina et al. and Muro et al. did not, find reacted spermatozoa in the lower isthmus could be due, partly, to the way we handled animals. In the majority of experiments that they conducted, female mice were induced to ovulate by gonadotropins and mated about the time of ovulation. In contrast, we allowed females to mate naturally without any hormone treatments, starting from early evening. Although we did not determine the exact time of copulation of individual females, it probably took place between midnight and 03:00 [18, 19]. This means that the spermatozoa that we saw in the isthmus between 06:00 and 09:00 (Table 4) were in the oviducts for 5–11 h. All the spermatozoa that Muro et al. examined [17], and almost all the spermatozoa that La Spina et al. examined [16], were in the isthmus for less than 4 h. In fact, when we mated females at about the time of ovulation after gonadotropin injections and examined oviducts about 2 h later, almost all spermatozoa in the lower isthmus were acrosome intact (Supplemental Table 2 and Supplemental Movie S7). According to La Spina et al. [16], over 95% of spermatozoa in the lower isthmus were acrosome intact, regardless of whether mating occurred before or during ovulation. Unfortunately, no quantitative data were available to substantiate their statement. It is possible that ovulation-inducing gonadotropins impact the ability of the oviduct to induce the acrosome reaction, because we found fewer motile acrosome-reacted spermatozoa in the lower and midisthmus after induction of ovulation by gonadotropins than after natural ovulation (compare Table 4 and Supplemental Table S1).

According to Suarez [19, 33], mouse spermatozoa in the isthmus attach to and detach from the epithelium repeatedly while ascending toward the ampulla. Here, we saw both acrosome-intact and -reacted spermatozoa, both on the isthmus epithelium and in the lumen. It is generally thought that acrosome-reacted spermatozoa have a short life, but, at least in the mouse spermatozoa, seem to remain fertile for about 4 h after the reaction (Table 2). The fertile lifespan of guinea pig spermatozoa after the reaction is known to be about 3 h [41]. According to Cummins and Yanagimachi [42], hamster spermatozoa that recently acrosome reacted in vitro can readily enter the cumulus, whereas those reacted hours before meeting the cumulus seldom enter it. We do not know how long mouse spermatozoa in vivo remain fertile after the acrosome reaction, but those reacted in vitro remain fertile for up to 4 h (Table 2). Thus, the mouse spermatozoon that we saw moving in and out of cumuli within the oviductal ampulla (Fig. 4E) could be one of the spermatozoa that reacted within 4 h before being videographed.

It should be noted that we found few acrosome-intact mouse spermatozoa within the ampulla (Table 5). This was expected, as in many species other than the mouse, both acrosome ''intact'' and ''modified'' spermatozoa were found near or within cumulus oophorus after natural mating or artificial insemination [29, 43–49]. Some fertilizing spermatozoa in vivo may complete their acrosome reaction within the cumulus and after reaching the zona pellucida. For mouse spermatozoa that remain fertile for some hours after the acrosome reaction (Table 2), the site where they react may not be of critical importance [50].

What induces the acrosome reaction of mouse spermatozoa within the isthmus is an open question. It is tempting to speculate that progesterone or some unknown substances

secreted by the cumulus-oocyte complex diffuses from the ampulla down to the isthmus to induce the acrosome reaction of spermatozoa and ''attract'' spermatozoa chemotactically to the ampulla [51, 52]. However, it is important to note that the oviduct exhibits an active, adovarian, peristaltic movement during periovulatory and ovulatory periods, which can transports its contents (e.g., a bolus of India ink injected into the isthmus) from the isthmus toward the ampulla. Thus far, this oviduct peristalsis is documented only in the hamster [53], but it must occur in other species as well.

What induces the acrosome reaction within the oviduct isthmus is also an open question. The possibility that ovulation products (follicular fluid, oocytes, and cumulus oophorus) and substances secreted by oocytes, cumulus cells, and ampulla epithelium diffuse down to the isthmus is unlikely in view of an adovarian movement of the oviduct during the periovulatory period, as mentioned above. According to Bray et al. [54], acetylcholine induces the acrosome reaction of human spermatozoa in vitro. Certain forms of the choline-acetyltransferase are predominantly present in the isthmus (and infundibulum) of pig oviduct. We tested if acetylcholine can induce the acrosome reaction of mouse spermatozoa in vitro, with negative results (see Supplemental Table S3). Neurotensin, another neurotransmitter, was proposed as a possible inducer of capacitation and the acrosome reaction of mouse spermatozoa within the oviduct [55]. However, at present, we know virtually nothing of the factors or substances that induce the acrosome reaction of spermatozoa within the oviduct isthmus.

Although most mouse oocytes in vivo seem to be fertilized by spermatozoa that have completed their acrosome reaction before entering the cumulus oophorus, the ability of cumulus to induce the acrosome reaction should not be underestimated. Some spermatozoa that reach the ampulla may have ''intact'' acrosomes and undergo the reaction on or in the cumulus. The ability of cumulus oophorus to induce the acrosome reaction or potentiate fertilizing ability of spermatozoa is well known [6, 14, 56, 57]. What is clear is that the zona pellucida is not the sole or intrinsic acrosome reaction-inducing substance in the mouse, and perhaps in some other species as well.

Sperm Ascent in the Oviduct

Similarly, the mechanism by which spermatozoa ascend from the isthmus to the ampulla is also not fully understood. Many spermatozoa entering the isthmus are known to attach with their heads to the isthmus epithelium. They detach from and reattach to the epithelium a few at a time [19, 58]. Although mouse spermatozoa seem to be able to ascend the oviduct without oviduct contractions [59], adovarian peristalsis of the oviduct, as reported by Battalia and Yanagimachi [53], may assist efficient sperm ascent [6]. Guidobaldi et al. [60] are of the opinion that, at least in the rabbit, both the oviduct movement and the ovulation products (including cumulus-oocyte complex) within the ampulla contribute to the sperm ascent. The concepts that spermatozoa 1) sense temperature difference between the isthmus and ampulla and swim toward the ampulla by thermotaxis [61, 62] or 2) swim against a fluid flow from the ampulla to the isthmus rheotaxically [63] must be reviewed critically. At least in the mouse, the uterotubal junction closes tightly shortly after mating [64] or by the time of natural ovulation [19], and, therefore, a constant downflow of the oviduct fluid from the ampulla to the isthmus is very unlikely to occur. Various substances (including progesterone) have been suggested by several investigators as potential chemoattractants to guide spermatozoa from isthmus to ampulla [51, 65], but concrete evidence to substantiate this notion is absent.

Sperm:Oocyte Ratio During Progression of In Vivo Fertilization

Oocytes are usually surrounded by numerous spermatozoa when inseminated in vitro, but not in vivo. In the rat, mouse, and hamster, for example, the number of spermatozoa present in the ampulla during progression of fertilization is less than or nearly the same as the number of oocytes in the same region of the oviduct [17, 29, 64, 66]. Our video recording of spermatozoa within oviducts (Fig. 4, B and E; Supplemental Movie S8) revealed that an acrosome-reacted spermatozoon does not spend much time in the cumulus. Spermatozoa commonly entered the cumulus vertically (along radially arranged cumulus cells), and quickly reached the oocyte's zona. When the oocyte within the cumulus was already fertilized, the spermatozoon soon swam away from the oocyte to enter the cumulus of a nearby oocyte. It must be the inner acrosomal membrane or the plasma membrane of the equatorial segment of the acrosome that recognized the differences between zonae pellucidae of fertilized and unfertilized oocytes. This assumption is based on observations by Yanagimachi and Phillips [46], who reported that fertilizing hamster spermatozoa attach to the zona using either the inner acrosomal membrane or the plasma membrane over the equatorial segment of the acrosome. These membranes must ''recognize'' chemical differences of the zonae pellucidae of fertilized and unfertilized oocytes. It is known that a proteinase released from cortical granules upon the fusion of a fertilizing spermatozoon with an oocyte partially hydrolyzes a zona glycoprotein, ZP2 [67]. The fact that a fertilizing spermatozoon does not ''waste'' much time on the zona of fertilized oocyte explains why oocytes within the ampulla are fertilized one by one by amazingly small numbers of spermatozoa during the progression of normal fertilization.

The spermatozoon shown in Figure 4E and Supplemental Movie S8 did not enter the zona before video recording was terminated due to a 40-min limit (see *Materials and Methods*). According to Sato and Blandau [68], who examined oocytes recovered from oviducts of mated mice, a spermatozoon takes an average of 20 min (15–26 min) to cross the zona. According to Jin et al. [14], mouse spermatozoa in vivo can pass through the zona in 15 min. However, the time between a spermatozoon's initial attachment to the zona and its complete passage through the zona may vary considerably [14]. The spermatozoon within an isolated oviduct shown in Figure 4E and Supplemental Movie S8 did not pass through the zona during recording, but the one of two spermatozoa that were swimming in and around cumulus-oocyte complexes in vitro passed through the zona sometime between 11 and 15 min after insemination (Fig. 5; Supplemental Movies S9 and S10).

ACKNOWLEDGMENT

The authors thank Dr. J. Michel Bedford (Cornell University) for his critical reading of the original manuscript and for his invaluable help in the preparation of the manuscript.

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