Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein

Tomoko Nakanishi[a], Masahito Ikawa[a], Shuichi Yamada[b], Martti Parvinen[c], Tadashi Baba[d], Yoshitake Nishimune[e], Masaru Okabe[a,*]

[a] *Genome Information Research Center, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan*
[b] *Program for Promotion of Basic Research Activities for Innovative Biosciences, Tokyo, Japan*
[c] *Department of Anatomy, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland*
[d] *Institute of Applied Biochemistry and Tsukuba Advanced Research Alliance, University of Tsukuba, and the National Institute for Advanced Interdisciplinary Research, Tsukuba Science City, Ibaraki 305-8572, Japan*
[e] *Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

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Abstract We produced transgenic mouse lines that accumulate mutated green fluorescent protein (EGFP) in sperm acrosome, a membrane limited organelle overlying the nucleus. The sperm showed normal fertilizing ability and the integrity of their acrosome was easily examined in a non-invasive manner by tracing the GFP in individual 'live' sperm with fluorescent microscopy. The time required for the dispersal of acrosomal contents was demonstrated to be approximately 3 s after the onset of acrosome reaction.

Key words: Sperm; Acrosome reaction; Fertilization; Green fluorescent protein

1. Introduction

The acrosome is an organelle analogous to a lysosome and is found almost universally in sperm. Upon the discovery of acrosomal disruption before fertilization in sea urchin and starfish, this exocytotic event was named the acrosome reaction and it is regarded as an essential step for fertilization. In mammals the mechanism of the acrosome reaction has been studied extensively using the mouse as a model species. However, the acrosome of the mouse is thin, making it difficult to observe the acrosome reaction under a microscope. Therefore, electron microscopy (EM), specific antibodies [1], lectins [2] and chlortetracycline (CTC) [3] have been utilized to assess the integrity of the acrosome.

Previous studies indicate that the acrosome reaction involves multiple fusions between the outer acrosomal membrane and the overlying plasma membrane, enabling acrosomal contents to escape through the fenestrated membranes. The duration of the acrosome reaction was estimated by measuring the half-time of the acrosome reaction: 2 min in mouse sperm using CTC [4] and around 3 min for human using EM [5].

However, these techniques have two major common problems. (i) When sperm are fixed before examining (e.g. in the case of CTC and EM), it is impossible to trace changes in a single sperm. It is also not possible to evaluate the viability or motility of the specific sperm before fixation. Even when the antibodies are applied to live sperm, it is possible that the bound probes may alter sperm function (e.g. induce the acrosome reaction [6–8], or hinder sperm/egg fusion [1]). (ii) These techniques indicate the number of sperm that have undergone the acrosome reaction by the time of observation, but do not suggest the duration of the acrosome reaction in individual sperm.

Recently, Chalfie et al. [9] cloned the green fluorescent protein (GFP) gene from jellyfish *Aequorea victoria* and reported that the gene would serve as a useful marker. Unlike other markers, GFP requires neither substrate nor pretreatment for detection.

We previously indicated that the GFP could be expressed in the cytosol of almost all kinds of cells and that mice carrying the transgene become fluorescent green throughout the body [10]. 'Green mice' that we produced ovulated green eggs but sperm were not green because a majority of the cytosol had been discarded as residual bodies during spermiogenesis. In the present paper, we designed the GFP transgene to be expressed in the sperm acrosome and produced transgenic mouse lines. Using sperm from these mutant mice, we observed the moment of the acrosome reaction non-invasively and on a real-time basis.

2. Materials and methods

2.1. Construction of transgenes

To prepare acrosin/EGFP transgenes (Acr-EGFPs) shown in Fig. 1, the protein-coding region of EGFP (Clontech Laboratories, Palo Alto, CA, USA) was amplified by PCR using oligonucleotide primers as described before [10,11]. The amplified DNA fragment was digested by *Eco*RI and introduced into pBluescript II SK$^+$ at the *Eco*RI site. A 538 bp DNA fragment containing bovine growth hormone polyadenylation signal (bGH polyA) was inserted downstream of the EGFP cDNA in the pBluescript II SK$^+$ at the *Eco*RV and *Hin*dIII sites to provide RNA processing. A 2.4 kb mouse genomic region carrying the acrosin gene [12] with or without the sequences of a signal peptide and an N-terminal peptide was also PCR-amplified using 5′ primer and four kinds of 3′ primers (primers 1–4) to make various transgenes, acr1-EGFP to acr4-EGFP (5′ primer: GGATCCTCTTACTTTTAGTTAGG, primer 1 for Acr1-EGFP: CTGCAGACTCCTGCCCTAACCTGGC, primer 2 for Acr2-EGFP: CTGCAGGGCAACCACGGACACTGCCAAG, primer 3 for Acr3-EGFP: CTGCAGCGTGGTGTTATCCTTGGCA, primer 4 for Acr4-EGFP: CTGCAGCGTGGTCTGATCCTTGGCA). After digestion with *Bam*HI and *Pst*I, the DNA fragments were introduced into pUC19. The resulting plasmids were cut by *Pst*I and *Hin*dIII, and inserted by the DNA fragment encoding EGFP and bGH polyA derived from the above

*Corresponding author. Fax: (81) (6) 6879-8376.
E-mail: okabe@gen-info.osaka-u.ac.jp

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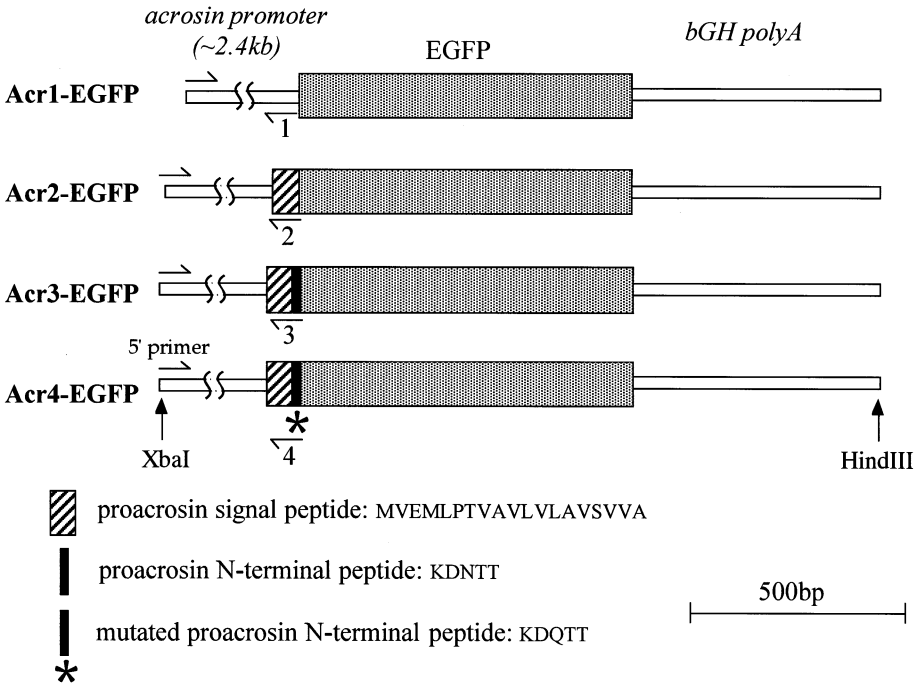

Fig. 1. Schematic maps of the transgenes used in the experiment. Acrosin promoter and acrosin sequences were PCR amplified using various primers (see Section 2).

pBluescript II SK$^+$ plasmid. The transgenes were excised by *Xba*I and *Hin*dIII, and purified by agarose gel electrophoresis followed by a Qiaex II kit (Qiagen, Chatsworth, CA, USA).

2.2. Production of transgenic mice

The transgenic mouse lines were produced by injecting the purified DNA fragments into B6C3F1×B6C3F1 fertilized eggs. The incorporation of the transgene was examined by PCR analysis using DNA extracted from the tail. The founder mice were mated with B6C3F1 mice and both the transmittance of the transgene and the expression of the protein were measured by PCR and by observation of sperm under a fluorescent microscope.

2.3. Testicular cell preparation

The testes from 6-week-old transgenic males were decapsulated, and the isolated seminiferous tubules were subjected to transillumination under a stereomicroscope. Different stages of the seminiferous epithelial cycle were recognized and small segments (ca. 0.5 mm) from selected stages were carefully squashed and analyzed under a phase contrast microscope [13]. The exact stage of the cycle was identified using Oakberg's criteria [14]. Digital phase contrast and fluorescent images were then combined from the same fields using a PXL KAF1400-G2 digital camera (Photometrics Co. Ltd.), and pseudo-colored.

2.4. Sperm preparation

The medium used was TYH (modified KRB supplemented with pyruvic acid and bovine serum albumin) [15]. Sperm were collected from the caudae epididymides of 10-week-old transgenic mice into a 400 μl drop of TYH under paraffin oil and dispersed. For fluorescent microscopic observation, sperm were fixed with 4% paraformaldehyde for 5 min at room temperature, photographed with a PXL KAF1400-G2 digital camera (Photometrics Co. Ltd.), and pseudo-colored. For the experiments in Figs. 2–5 and Table 2, a transgenic mouse line B6C3F1 TgN (acr3-EGFP) Osb17 was used.

2.5. In vitro fertilization

Eight-week-old females (B6C3F1) were superovulated by injection of 5 IU of pregnant mare serum gonadotropin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotropin (HCG). Ovulated egg masses were collected from the oviducts 16 h after HCG injection. Eggs were placed in a 400 μl TYH drop covered with mineral oil. Sperm preincubated for 90 min were added to the drop containing the eggs at a final concentration of $1.0–1.5\times10^5$ sperm/ml. After 15, 30, 60, and 360 min of incubation, eggs were treated with hyaluronidase (300 U/ml) to remove cumulus cells and stained with lacmoid. Fertility of the sperm was assessed by observing sperm heads that had penetrated through the zona pellucida and fused to eggs at 15, 30 and 60 min, and by pronuclear formation at 360 min.

2.6. Flow cytometry

Without pretreatment, an aliquot of freshly prepared sperm suspension was subjected to analysis with a FACSCalibur flow cytometer (Becton Dickinson Co. Ltd.). The only reagent added to the sperm suspension was propidium iodide (final 10 μg/ml) to measure the sperm viability. The response of sperm to Ca^{2+} ionophore was analyzed 5, 10 and 20 min after A23187 (10 μM) treatment. A 515–545 nm band path filter and 650 nm long path filter were used for GFP and propidium iodide, respectively.

2.7. Sequential photograph

Eggs collected from the oviducts were treated with 300 U/ml hyaluronidase (Sigma; IV-S) to remove the cumulus cells. Sperm were preincubated for 90 min in TYH medium to cause capacitation and mixed with cumulus-free eggs for 3 min at a concentration of $1.0–1.5\times10^4$ sperm/ml. The eggs were then pipetted to remove lightly

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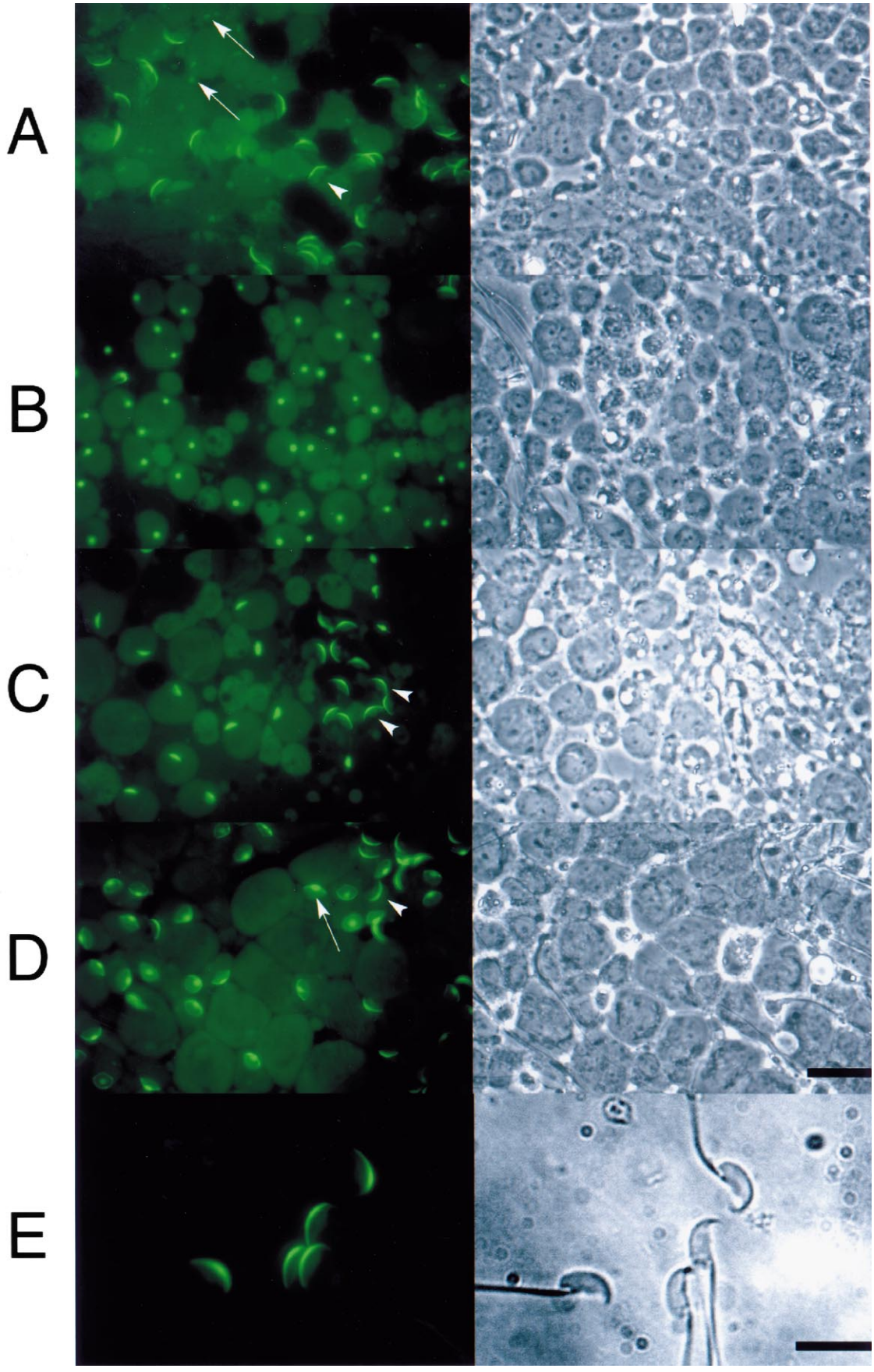
Fig. 2. Localization of the GFP-acrosin fusion protein in male germ cells of transgenic mice determined by fluorescent microscopy. A: Early spermatids corresponding to step 2 spermatids showing first evidence of distinct proacrosomal granules (see arrows), and step 14 spermatids (arrow head). B: Step 3 spermatid with a single large proacrosomal granule before flattening out over the surface of the nucleus. C: Step 5 spermatid with the acrosome flattened over on the surface contacting the nucleus with a spreading angle of 40–95°. The arrowheads indicate step 15 spermatids. D: Step 6 spermatid with the acrosome spreading angle of about 120°. The localization of an acrosomal granule (see arrow) was clearly seen. The arrowhead indicates step 15 spermatids. E: Bright green fluorescence was observed in the acrosomal cap region of sperm, while the equatorial segment was slightly fluorescent. Bars: A–D, 25 μm; E, 20 μm.


A
B
C
D
E

attached sperm on the zona pellucida. Finally, the eggs were transferred to TYH medium containing 10 μM A23187 and the time was set to zero at this point. The fluorescent microscopic images of the sperm were photographed sequentially with exposure of 1 s, followed by a 1-s interval, using a PXL KAF1400-G2 digital camera (Photometrics Co. Ltd.). Fluorescent intensity from sperm acrosome in each image was calculated by IPLab Spectrum (Signal Analytics Corporation, Virginia, USA) and was plotted in Fig. 5.

Table 1
Expression of GFP in the acrosome of transgenic mice

Construct	Number of transgenic founders	Number of lines with green acrosome
Acr1-EGFP	3	0/3 (0%)
Acr2-EGFP	4	1/4 (25%)
Acr3-EGFP	9	6/6 (100%)
Acr4-EGFP	7	6/6 (100%)

3. Results

3.1. Production of transgenic mouse lines

We constructed chimeric cDNAs encoding EGFP fusion polypeptide with (i) no extra peptide (Acr1-EGFP), (ii) proacrosin signal peptide (Acr2-EGFP), (iii) signal peptide and proacrosin N-terminal peptide (Acr3-EGFP), and (iv) signal peptide with mutated proacrosin N-terminal peptide (Acr4-EGFP) at the amino-terminus of EGFP (Fig. 1).

From these transgenes, we have produced 23 transgenic mouse lines. Among 19 lines tested (three female transgenic founders were abandoned), only 13 lines had sperm with green acrosome (Table 1). Even in the six lines with no 'green sperm', the spermatids were green (four from Acr1-EGFP and three out of four from Acr2-EGFP). In these transgenic mouse lines, the GFP was not localized in their acrosome and the cytosolic GFP was discarded from sperm during spermiogenesis. We are not aware of the reason why three out of four Acr2-EGFP transgenic mice failed to transport the GFP into their acrosome. In spite of the fact that the proacrosin signal peptide (MVEMLPTVAVLVLAVSVVA) was insufficient to localize GFP in the acrosome, the addition of proacrosin N-terminal peptide (KDNTT) facilitated the localization of GFP in the acrosome without exception (Acr3-EGFP). The asparagine residue included in the N-terminal peptide is a putative glycosylation site and is supposed to play a role in transporting proacrosin into the acrosome. However, the introduction of a mutation (Asn-3-Gln) did not affect the transportation of GFP to the acrosome (Acr4-EGFP).

3.2. Acrosomal formation during spermiogenesis

In transgenic mouse lines in which GFP was localized in the acrosome, an accumulation of GFP was evident from a very early stage of spermatid formation. The green fluorescence was visible as early as in step 2 spermatids as tiny dots corresponding to proacrosomal granules (Fig. 2A). These merged together to form one large proacrosomal granule in step 3 spermatids (Fig. 2B) and then deformed as spermiogenesis proceeded and became a characteristic cap shape in step 6 (Fig. 2D). The green fluorescence was mostly localized in the acrosomal cap region and a weak fluorescence was also observed in the equatorial segment in mature sperm (Fig. 2E). All the live sperm had a green fluorescent acrosome just after collection. However, a fraction of the live sperm lost fluorescence during incubation, probably due to a spontaneous acrosome reaction.

3.3. Fertilizing ability of 'green sperm'

All the founder mice tested (19 lines) were fertile and the transgenes were transmitted from the founders to their offspring. The litter size of the transgenic lines was an average of 11–12. The number of transgenic mice obtained from hemi-

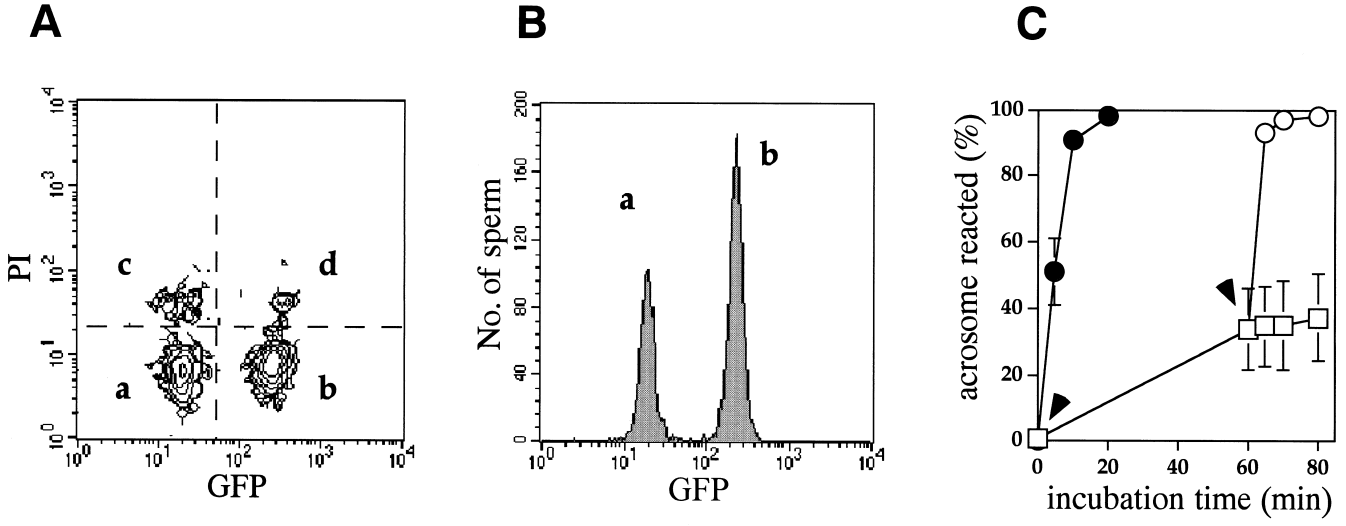


Fig. 3. Analysis of green fluorescence from sperm acrosome by a flow cytometer. A: Sperm were divided into four populations according to GFP^+ (acrosome intact; b and d) and GFP^- (acrosome reacted; a and c), and propidium iodide stained (dead; c and d) and not stained (live; a and b). B: A histogram of green fluorescence from live sperm. Propidium iodide negative sperm were gated. The peaks of 'green sperm' (b) and 'non-green sperm' (a) were clearly distinguished from each other. This figure was obtained by incubating sperm in TYH medium for 90 min. The ratio of 'green' and 'non-green' sperm was 64:36 in this figure, indicating that the spontaneous acrosome reacted sperm at this moment was 36%. C: The appearance of acrosome reacted sperm responding to ionophore A23187 (final 10 μM) was measured by flow cytometer and was plotted over time. The data indicate mean ± S.E.M. of three experiments. Arrowheads indicate the addition of A23187. □, no addition; ●, 0 min; ○, 60 min.

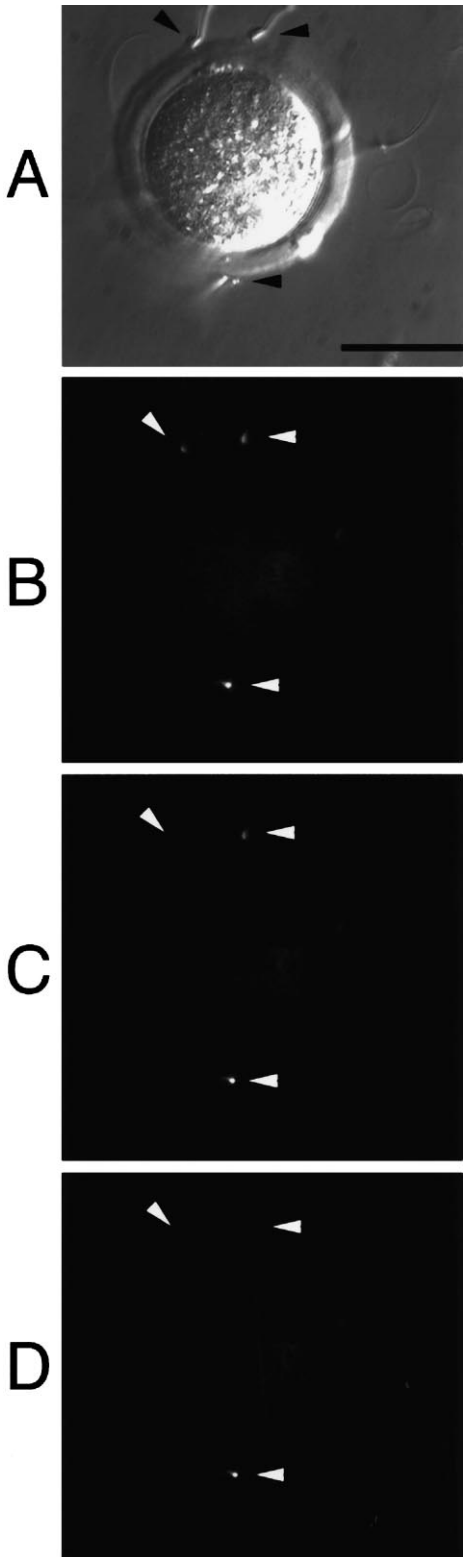


Fig. 4. Sequentially taken photos showing a moment of sperm exocytosis (acrosome reaction) on the zona pellucida. Under normal lighting, three zona bound sperm heads were focused in a plane (A). After the addition of A23187, at time 0, all these sperm had 'green' acrosome (B). After 102 s, the top left sperm lost its fluorescence (C); the top middle sperm lost the fluorescence at 158 s (D). (A Quicktime movie sequence from the experiment is available at FTP://ftp.gen-info.osaka-u.ac.jp/ARmovie/) Bar: A–D, 50 μm.

zygous F1 mice followed the Mendelian ratio (tg+/tg− = 24/25 from line Acr3-EGFP #17 F1 male).

In vitro fertilization was also performed and the time required for 'green sperm' to fertilize the eggs was examined. As shown in Table 2, there was no difference between 'green sperm' and wild-type sperm in the timing of zona penetration and sperm/egg fusion. There was also no difference in the number of eggs that formed pronucleus. It was obvious that 'green sperm' have normal fertilizing ability in spite of the existence of GFP in the acrosome.

3.4. Analysis of acrosome reaction by a flow cytometer

The 'green sperm' were analyzed on a flow cytometer after adding propidium iodide to the sperm suspension. The sperm were separated into four categories (a, b, c, and d) as shown in Fig. 3A. In area 'a', live (propidium iodide negative) and acrosome reacted (GFP negative) sperm were distributed while live and acrosome intact sperm were separated in 'b'. The numbers of sperm in 'a' and 'b' were measured using histograms as shown in Fig. 3B. In this case, sperm in 'a' (acrosome reacted) increased gradually to 36% during 90 min incubation due to a 'spontaneous' acrosome reaction. When the acrosome reaction inducing agent A23187 was added to the sperm, the population of 'a' increased to near 100% within 5 min of incubation (Fig. 3C). The same phenomenon was observed in the sperm from separately prepared transgenic mice with a C57BL/6 genetic background.

3.5. Time course of exocytosis

Usage of green sperm in the study of the acrosome reaction facilitates observation of acrosomal integrity of individual sperm non-invasively and continuously. Sperm bound to the zona pellucida were challenged to induce the acrosome reaction with A23187 and the moment of acrosome reaction (proven by the disappearance of GFP) was demonstrated by sequential photos (Fig. 4).

Fig. 5A,B shows an example of the time course for loss of fluorescence intensity during the acrosome reaction. The exocytosis starts with a rapid decrease in fluorescence, followed by a slower decrease. Within 3 s of the onset of acrosomal exocytosis, $61 \pm 5\%$ ($n = 6$) of initial intensity was lost. The decrease continued even after 3 s at a much slower pace and the remaining fluorescence after the initial burst decreased at the rate of $0.92 \pm 0.14\%$/s ($n = 6$) in the following 60 s. This was not a quenching effect because when sperm GFP was fixed to prevent the diffusion before measurement, the average decrease observed was 10 times smaller than the slow decrease after the acrosome reaction (Fig. 5C). These results indicate

Table 2
Time required for sperm penetration in vitro by 'green sperm' and wild-type sperm

Time after insemination (min)	Number of eggs fertilized[a]/number of eggs examined (%)	
	'green sperm'	wild-type sperm
15	31/88 (35.2)	34/92 (37.0)
30	67/79 (84.8)	52/62 (83.9)
60	61/67 (91.0)	46/51 (90.2)
360	125/136 (91.9)	141/148 (95.3)

[a]Eggs were observed for zona penetrated sperm or fused sperm with oolemma at 15, 30 and 60 min after insemination. Pronucleus formation was observed at 360 min after insemination.

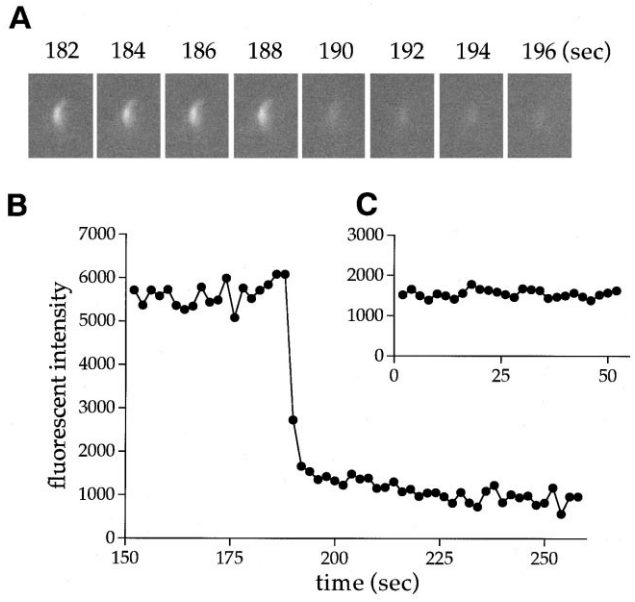


Fig. 5. Real time observation of exocytosis of GFP from live sperm. A: Photos were taken sequentially after the addition of ionophore A23187 (at 0 s). The numbers indicate the seconds after the ionophore addition. A sudden drop of fluorescence was observed between 188 and 192 s in this particular sperm. B: Time required for the GFP to disperse from individual acrosome. A sharp drop (from 188 to 192 s) of the onset of acrosomal exocytosis was followed by a gradual decrease (from 192 to 260 s). When the fluorescent intensity at 188 s was set at 100, 73% of the GFP was discharged within the following 3 s (the average of six independent observations was $61 \pm 5\%$). From 192 to 260 s, 46% of the remaining fluorescence disappeared at a rate of 0.77%/s in the following 60 s (the average of six independent observations was $0.92 \pm 0.14\%$/s in 60 s). C: 'Green sperm' were fixed with 4% paraformaldehyde and observed under the fluorescent microscope to measure the quenching effect. Sperm that show a fluorescent intensity varying from 1556 to 7530 (average 4480) were chosen to observe the quenching. The decrease was consistent ($0.09 \pm 0.02\%$/s ($n = 8$) in 60 s) in all the sperm observed. An example of sperm that had about the same fluorescent strength is shown in the figure. A Quicktime movie sequence from the experiment in A and B is available (see legend to Fig. 4).

that the release of acrosomal contents may not be a simple exocytosis following the membrane fusion, but a more complicated process.

4. Discussion

4.1. Localization of GFP inside acrosome

The addition of the proacrosin signal peptide (MVEMLPTVAVLVLAVSVVA) was necessary for GFP to be distributed inside the acrosome. However, for some unknown reason, the signal peptide was not always effective enough to make GFP localized inside the acrosome. Three out of four transgenic lines showed a non-green acrosome despite the fact that they were green at their spermatid stage. When we added proacrosin N-terminal peptide (KDNTT), the GFP localized to the acrosome without exception. Since the KDNTT sequence has a putative glycosylation site that is thought to be effective in localizing acrosin inside the acrosome, we introduced a mutation, changing the asparagine to glutamine. However, the mutated N-terminal peptide was also effective in transporting the GFP into the acrosome, indicating that the putative glycosylation site in the proacrosin N-terminal peptide was not essential for GFP to be localized in the acrosome (Table 1). We do not know why the signal peptide alone failed to permit GFP to be distributed in the acrosome. If one looks for differences in the structure of the transgenes, it should be pointed out that in transgenes Acr3-EGFP and Acr4-EGFP, the signal peptides are directly followed by proacrosin N-terminal sequence, while in Acr2-EGFP, an artificial peptide (LQEFAT) follows the signal peptide. Therefore, there remain two possibilities: (i) the proacrosin N-terminal peptide per se has some role in protein trafficking, and (ii) the function of the signal peptide was disturbed by the following artificial peptide.

4.2. Fertilizing ability

The retardation of zona penetration was reported in acrosin knockout mice [16,17]. Since the mouse acrosin promoter was used for the production of EGFP, it was conceivable that the endogenous acrosin was diminished. However, the transcript of endogenous acrosin in the transgenic mice was as much as that of wild-type mice (data not shown). We also considered that even if 'green sperm' had sufficient acrosin, GFP accumulation might have some detrimental effect on the sperm fertilizing ability. However, neither the timing of zona penetration nor sperm/egg fusion was affected by the accumulation of GFP in acrosome (Table 2). The sperm obtained from the hemizygous transgenic mice were all fluorescent green, despite the fact that only half of the sperm carried the transgene. This was probably due to the cytoplasmic bridges enabling spermatids to share molecules [18].

4.3. Release of acrosomal contents

In guinea pig, the acrosome can be differentiated into three different compartments (M1, M2 and M3) [19] and different acrosomal proteins are segregated into soluble and matrix compartments [20]. These results indicate that the release of acrosomal content might be multiphasic. To reinforce this notion, it is reported that one of the acrosomal enzymes, acrosin, is not released immediately after the acrosome reaction, but remains on the sperm probably because acrosin interacts with the acrosomal membrane [21]. Furthermore, in mouse, it was also indicated that the release of some acrosomal antigens was shown to be regulated by acrosin [22]. These results suggest that there might be some mechanism regulating the acrosomal content to be released or to be held in the acrosomal matrix after the acrosome reaction. In the present paper, it was demonstrated that the release of acrosomal contents was not a simple diffusion phenomenon. The quick dispersal of the initial burst may reflect a very water-soluble nature of GFP. The following slow release may indicate the existence of some mechanisms to hold GFP on sperm. This also indicates that some other acrosomal contents are held on sperm even after the acrosome reaction (Fig. 5).

4.4. Observation of acrosome reaction

It has been reported that virtually all viable spermatozoa collected from the human cervical mucus had an intact acrosome even 3 days after artificial insemination [23] and all viable sperm in the oviductal isthmus have an intact acrosome in hamster [24]. Thus, the sperm acrosome is likely to be maintained intact in vivo until sperm encounter the zona pellucida in the female reproductive tract. However, the exact

moment of the acrosome reaction is not known in relation to sperm penetration into zona pellucida. How does the acrosomal enzyme participate in this penetration? To answer this, we tried to observe the moment of the acrosome reaction on zona pellucida using 'green sperm'.

Although there is extensive evidence that ZP3 induces the acrosome reaction, little is known about the timing of the acrosome reaction in relation to sperm binding and penetration of the zona pellucida. When we added green sperm to cumulus-free eggs for 5–10 min and washed the eggs thoroughly, the sperm still remaining on the zona pellucida were regarded as 'bound' sperm. Almost all of these bound sperm were acrosome intact and we failed to observe the moment of acrosome reaction by continuously observing these 'bound' sperm. This indicates that zona induced acrosome reaction does not take place in the majority of the zona 'bound' sperm.

In the next experiment, we tried to induce the acrosome reaction to sperm on the zona pellucida with Ca^{2+} ionophore. By introducing A23187, the acrosome reaction was successfully induced in the majority of the sperm on the zona pellucida. However, sperm penetration was not observed in these A23187 induced acrosome reacted sperm. This may partly be due to the loss of vigorous movement of the sperm tail after A23187 treatment.

As far as we observed the acrosome reaction induced by A23187 treatment on a real-time basis, the membrane fusion seemed to occur as a matter of probability. The time duration of individual acrosomal burst was accomplished within 3 s as shown in Fig. 5B. However, the time required for the sperm to reach the bursting point was very different (Fig. 4). A contingent close encounter and remodeling of lipid bilayer membrane [25] may regulate the last part of the acrosome reaction.

As shown in the present paper, we produced 'green sperm' with normal fertilizing ability and showed that the sperm could be used to study exocytosis non-invasively. The 'green sperm' will provide further clues for solving the mechanism of fertilization in the future.

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