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Inactivation of the Mouse Adenylyl Cyclase 3 Gene Disrupts Male Fertility and Spermatozoon Function

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Mammalian spermatids and spermatozoa express functional G protein-coupled receptors. However, bicarbonate-regulated soluble adenylyl cyclase (AC), the major AC present in these cells, is not directly coupled to G proteins. To understand how G protein-coupled receptors signal in spermatozoa, we investigated whether a conventional transmembrane cyclase is present and biologically active in these cells. Here, we provide evidence for expression of type 3 AC (AC3) in male germ cells and describe the effects of disruption of the AC3 gene on fertility and function of mouse spermatozoa. As previously reported in rat, AC3 mRNA is expressed in mouse testes and localized, together with soluble AC mRNA, mainly in postmeiotic germ cells. AC3 protein was detected by immunolocalization in round and elongating spermatids in a region corresponding to the developing acrosome and was retained in the mature spermatozoa of the epididymis. Forskolin caused a small increase in cAMP production in mouse spermatozoa, but this increase could not be detected in the AC3$^{-/-}$ mice. Inactivation of the AC3 gene did not have overt effects on spermatogenesis; however, AC3$^{-/-}$ males were subfertile with only three litters generated by 11 males over a period of 6 months. When used in *in vitro* fertilization, spermatozoa from these AC3$^{-/-}$ mice produced few embryos, but their fertilizing ability was restored after removal of the zona pellucida. Despite an apparently normal structure, these spermatozoa had decreased motility and showed an increase in spontaneous acrosome reactions. These data support the hypothesis that AC3 is required for normal spermatid or spermatozoa function and male fertility. (*Molecular Endocrinology* 19: 1277–1290, 2005)

CYCLIC AMP IS a second messenger essential for the maturation and capacitation of spermatozoa (1, 2). Despite this critical function, the exact mechanism regulating the levels of this second messenger in germ cells is still unclear. There is general consensus that a bicarbonate-sensitive adenylyl cyclase (AC) with unique properties is present in spermatids and spermatozoa from different species (3–5). The cloning of the soluble AC (sAC) (6) has established that the enzyme expressed in the testis and the bicarbonate-sensitive cyclase is the same entity (7). This sAC is a major component of the cAMP-producing machinery in sperm and is essential for sperm function because sAC null male mice are sterile (8). However, evidence has been reported that several G protein-coupled receptors (GPCRs) are active in spermatozoa, including the receptors for angiotensin (9), adenosine (10, 11), and calcitonin (12), suggesting that G proteins may be involved in the control of cAMP levels in these cells. In the same vein, it has been proposed recently that an olfactory receptor expressed on human spermatozoa is coupled to an AC and may play a role in sperm chemotaxis (13). Given the fact that the soluble and bicarbonate-stimulated cyclase is not directly coupled to G proteins, a major unsolved issue is how GPCRs regulate cAMP and motility in these cells.

A possible explanation for the effects of GPCR in spermatozoa is that conventional G protein-coupled transmembrane (TM) ACs coexist with sAC in male germ cells. However, evidence that TM cyclases coupled to G proteins contribute to cAMP accumulation in spermatozoa is marred by conflicting observations. Several publications suggest that forskolin stimulates sperm cyclase (12, 14, 15) and that the zona component, ZP3, stimulates cAMP through a G protein-mediated mechanism (16, 17), whereas other reports have refuted this possibility (18, 19). Very recently, Fraser and Adeoya-Osiguwa (11) provided evidence that agents influencing membrane-associated AC, but not sAC, modulate cAMP production in mouse spermatozoa and that forskolin significantly stimulates cAMP accumulation in these

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Abbreviations: AC, Adenylyl cyclase; CASA, computer-assisted spermatozoa analysis; COC, cumulus-oocyte complex; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; IVF, *in vitro* fertilization; KO, knockout; mKSOM, modified potassium simplex optimized medium; ORF, open reading frame; PFA, paraformaldehyde; RACE, rapid amplication of cDNA ends; sAC, soluble AC; TM transmembrane; Tr-AC3, truncated AC3.

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cells. In addition, the presence of AC2, AC3, and AC8 in the acrosomal and flagellar regions, and of AC1 and AC4 at lower levels in the midpiece and acrosomal cap regions, has been reported (15, 20). However, Western blot analysis investigating the presence of these enzymes in sperm extracts has yielded results that are inconsistent with the presence of full-length active enzymes.

In olfactory neurons, AC3 is a 130-kDa glycosylated protein involved in the cascade required for detection of odorants (21). In these cells, this cyclase is activated mainly through the G protein G_{olf} expressed in the olfactory epithelium. In a strikingly similar fashion, it has been reported that the olfactory type cyclase (AC3) and G_{olf} are expressed in the male germ cells of the rodent testis (22, 23). In rat, AC3 mRNA is expressed in rat spermatids with the protein localized in the acrosomal region of spermatids (22, 23). These findings demonstrate that olfactory receptors, G protein, and cyclase involved in olfactory signaling are expressed in spermatids and are possibly retained in spermatozoa, suggesting that an olfactory-like signaling pathway including AC3 may have a key role in spermatogenesis and spermatozoa functions. The involvement of a TM cyclase in chemotaxis has been implied by pharmacological manipulation in human spermatozoa (13).

The AC3 knockout (KO) mouse was developed to investigate olfactory-dependent behaviors (24). These mice display anosmia as suggested by the loss of electroolfactogram responses induced by odorants with consequent high neonatal mortality. Because of this loss of olfaction, AC3 null pups are not able to locate the nipple and nurse. These authors reported that homozygous mating was unproductive even though $AC3^{-/-}$ males exhibited normal anogenital sniffing and mounting behavior with females. Mating is thought to be dependent on the function of the vomeronasal organ, and AC3 is not expressed in this organ (25–27). Thus, impairment of male fertility due to defects outside the brain may be present in these mice.

With the present report, we have reinvestigated the expression of AC3 in mouse testes and spermatozoa. Furthermore, we investigated the reproductive phenotype of the $AC3^{-/-}$ males and demonstrated that the *in vivo* and *in vitro* fertilizing ability of $AC3^{-/-}$ spermatozoa is compromised.

RESULTS

Expression and Localization of AC3 mRNA in Mouse Testis

RT-PCR performed on mouse testis total RNA with primers specific for the region of the AC3 messenger coding for the second TM domain (oligo pairs *a* in Table 1) of this cyclase showed specific products of the expected size (570 bp; Fig. 1A). By using testis RNA from adult rat (data not shown) or from human, the same amplification product could be detected. No PCR products were detected in the minus reverse transcriptase control. Therefore, the AC3 messenger is present in the testis of rodents and humans.

When AC3 mRNA levels were evaluated by semiquantitative RT-PCR, this transcript was expressed in the testis at a level that was one fourth of that measured in the brain (Fig. 1B). These levels of expression were confirmed with two different sets of AC3 primers (see Table 1). Sequencing of the fragment obtained with both sets of primers confirmed that the AC3 sequence was amplified.

In situ hybridization of mouse testes with an AC3 antisense probe detected a specific signal in male germ cells. The signal was minimal at the base of the tubule, intense in the area corresponding to spermatocytes, and was still detected in the inner portion of the tubule where round spermatids are located (Fig. 2). The AC3 sense probe was used as a negative control, and only diffuse background signal was observed throughout the section (data not shown). A probe for sAC used as a positive control

Table 1. Primers Used for the Characterization of the AC3 Transcripts

Gene	Orientation	Size (bp)	Sequence (5′ → 3′)	T hyb (C)
AC3a	S	570	AGATTCATGGACCCAGAGATGGA	55
	AS		CGGGATCCTTGTGGTCGTATTCATCAAA	
AC3b	S	300	CTCTGCTTGCAGTGCCCAGGAGCT	68
	AS		ATACTGCCAGCGCGCTTCTGGCCCAG	
AC3c	S	450	GCCTCCTTGCCCAACTTTGCTGAC	68
	AS		TGTGGATTCCATCCTGCTGGCCAC	
TP1	S	220	GTCGACCAGCCGCAAGCTAAAGAC	62
	AS		CATGCTCCTGCCCCGTGTTGTTTG	
sAC	S	1540	CTCGAATTCAAATCCTGACCACTGTCC	55
	AS		GATCTTAGTCAGTGCAATAGC	
β-Actin	S	505	AAGAGAGGTATCCTGACCCT	52
	AS		GGCCATCTCTTGCTCGAAGT	

S, Sense; AS, antisense; T hyb (C), melting temperature.

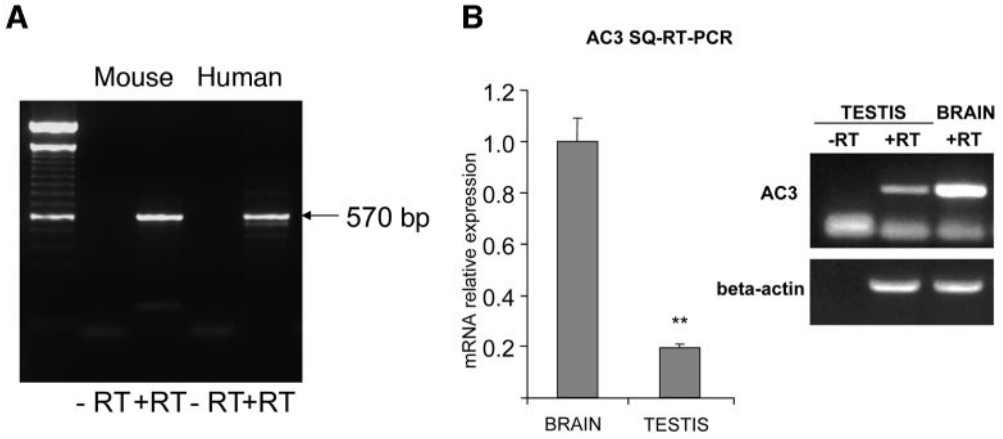

Fig. 1. AC3 mRNA Expression in Mouse Testis and Brain.

RNA isolation was performed with Trizol reagent (Invitrogen) according to the manufacturer's protocol. To avoid contamination from genomic DNA, RNA was treated with deoxyribonuclease. Reverse transcriptase reactions were performed with 5 μg of RNA and oligo-dT primers. For semiquantitative assessment of mRNA expression, different cycle numbers were used for the amplification, and data were derived from the exponential phase of amplification. PCR was performed with specific primers for AC3, and for β-actin and TP1 as controls. Primer sequences, the size of the amplified bands, and the annealing temperatures are reported in Table 1. To confirm amplification of AC3 mRNA, several of PCR products were sequenced. AC3 mRNA was also detected in human testis using commercially available RNA preparations. RT, Reverse transcriptase; SQ-RT-RCR, semiquantitative RT-PCR.

showed a similar pattern of expression at the same stages of spermatogenesis (Fig. 2). Thus, sAC and AC3 most likely coexist in spermatocytes and spermatids. Mouse epididymi also were used for *in situ* hybridization with AC3 but no signal could be detected in this tissue (data not shown).

Immunolocalization of AC3 Protein in Mouse Testis

To confirm that the AC3 protein is present in male germ cells, immunohistochemistry and immunofluorescence studies were conducted on mouse testis using a specific AC3 antibody recognizing the C-terminal part of the cyclase (23, 24). Both methods yielded a signal mainly in round and elongating spermatids (Fig. 3, A–C). When immunofluorescence was used, the AC3 protein was more clearly localized in the acrosomal area of these cells (Fig. 3, A and B). In elongated spermatids, AC3 immunofluorescence was detected on the acrosomal cap (Fig. 3B). The signal was retained in elongating spermatids, which sometimes showed a clear localization along the acrosomal membrane (Fig. 3, B and C). As a control of the specificity of the antibody, immunofluorescence was performed on COS7 cells transfected with a construct encoding for rat AC3 (Fig. 3D). Transfected cells showed a strong signal in Golgi and plasma membrane, whereas cells transfected with the antisense construct showed no signal. To further confirm the specificity of the signal observed in testis, the AC3 antibody used for immunohistochemistry was preincubated with a 500-fold excess of the AC3 peptide. The signal was greatly reduced with only faint staining seen in round spermatids (data not shown). The immunolocalization of AC3 in mouse testis is in agreement with previously published data in rat (22, 23).

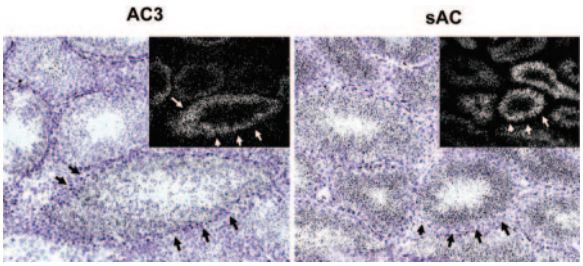

Fig. 2. Overlapping Expression of AC3 and sAC mRNAs in the Mouse Testis

Testes from adult mice were rapidly frozen in OCT compound, and sections were postfixed with PFA. Complementary RNA probes for AC3 and sAC were prepared as previously described and used for hybridization. As a control, sense cRNAs were used. Expression of both sAC and AC3 was observed in the inner layers of cells of the seminiferous tubules (*arrows*). Grain distribution at higher magnification indicated expression of both AC3 and sAC mRNA in late pachytene spermatocytes (stage 12), a major accumulation in round spermatids, and a decrease in elongating spermatids. Background signal was observed with sense probes.

Several attempts to determine the expression of AC3 in spermatids and spermatozoa by Western blot analysis have not been successful because of the high background and the low level of expression of this enzyme (see below) or possibly due to its recovery in detergent-resistant fractions. The presence of AC3 has been reported by two independent groups by Western blot in rat male germ cells and in mouse

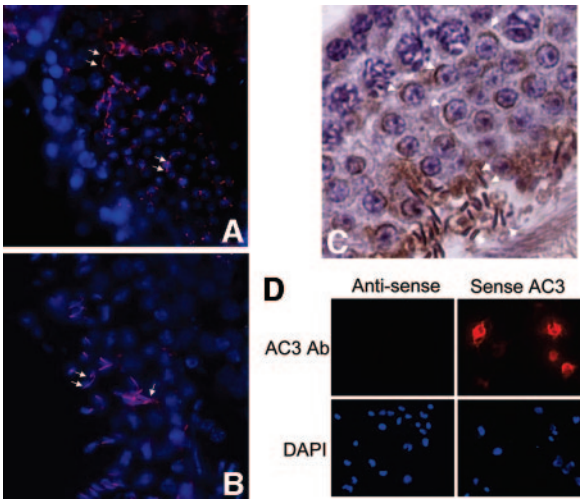

Fig. 3. Immunolocalization of AC3 during Spermatogenesis and after Overexpression in COS7 Cells

Sections of adult testes were used for either immunofluorescence (A and B) or immunohistochemistry (C). Using immunofluorescence, it was observed that predominant staining was localized as a thin line in the acrosomal region of round spermatids and more diffusely in elongating spermatids (panels A and B, *arrows*). Immunohistochemistry shows *brown* staining on the acrosomal cap of round spermatids and in elongating spermatids (*arrowheads* in panel C). As a control for specificity of the antibody, COS7 cells were transfected with AC3 sense and antisense cDNA, and immunofluorescence was determined. Positive cells were observed only with the sense AC3 transfection (panel D). Ab, Antibody; DAPI, 4′,6-diamidino-2-phenylindole.

spermatozoa; however, the molecular weight reported was not consistent with those of a full-length AC3 (15, 20).

Inactivation of the Mouse AC3 Gene and Transcript Expression in Testis

Inactivation of the AC3 gene in mouse has been previously carried out by homologous recombination targeting the first exon that codes for the amino terminus and part of the first set of TM domains (24). Surprisingly, when RT-PCR was used to evaluate the effect of homologous recombination, an increase in AC3 mRNA steady state was observed in the testis. To further determine the nature of these transcripts, pairs of primers were designed to determine the presence of exon 1 and the C1 C2 coding regions of the AC3 open reading frame (ORF) (Fig. 4A). This analysis (Fig. 4B) showed that the transcript detected in testis, brain, and kidney lacked exon 1, thus confirming that the homologous recombination had effectively removed this exon. However, a transcript could be detected when primers that encompass the catalytic domain of AC3 (primers AC3b for exon 4–6 and AC3c for exon 17–20) were used, and their expression was significantly increased in testis from AC3$^{-/-}$ mice when assessed by semiquantitative RT-PCR (Fig. 4, B and C).

The cause of an increased expression of this incomplete ORF in the testis was investigated by 5′-rapid amplication of cDNA ends (RACE) using reverse primers corresponding to exon 2 or 3 of the sequences. *Adcy3* resides on mouse chromosome 12 and spans approximately 80 kb of genomic sequence (Chr 12:3,999,118–4,079,139; UCSC Genome Browser). 5′-RACE using testis RNA yielded a product that corresponds to the exon 1/exon 2 of *adcy3* with the correct splicing, confirming that full-length AC3 mRNA is expressed in the mouse testis. However, additional products were retrieved corresponding to a transcript where exon 2 of *adcy3* is spliced to three additional exons (sequences Chr 12: 3,969,099–3,968,145; 3,971,732–3,971,974 and 3,972,089–3,972,288). These exons are part of a gene (Chr12 3,947,542–3,975,533) located immediately upstream of *adcy3*. This gene codes for a protein similar to Rab-related GTP-binding protein. However, these Rab-related exons were not in frame with the AC3 ORF. The significance of this finding was not pursued further. Finally, 5′-RACE using RNA from the AC3$^{-/-}$ testes consistently yielded a sequence where exon 2 of *adcy3* is fused to the sequence of the targeting vector used for homologous recombination (Fig. 4D). Thus, we surmise that the increased level of expression of this transcript is probably attributable to the murine phosphoglycerate kinase promoter that drives the Neo resistance gene (24), and that this promoter active in germ cells directs the expression of a truncated AC3 (Tr-AC3).

The expression of this Tr-AC3 in human embryonic kidney (HEK)293 cells caused the accumulation of a protein of approximately 100 kDa detected with either a V5- or AC3-selective antibody (Fig. 5); however, no AC activity was associated with this expressed protein (Fig. 5). Conversely, expression of a full-length AC3 cDNA in the same cells led to a significant increase in both basal as well as forskolin-stimulated activity (Fig. 5). These data strongly indicate that the mRNA originating from the recombined AC3 locus codes for a truncated protein with no detectable AC activity. Thus, the homologous recombination of the AC3 gene indeed produces a catalytically inactivated AC3. However, we cannot exclude the possibility that the truncated protein encoded by the recombined allele has additional unknown functions.

When spermatozoa from the AC3 KO mice were used for immunofluorescence analysis with the AC3 antibody, a signal was present in the AC3 KO, but the pattern of expression was different from the wild-type control (Fig. 6). The staining was no longer localized only to the acrosome cap but appeared more diffuse as a rim surrounding the acrosome and the nucleus of the spermatozoon. The whole of these data indicate that, although accumulation of AC3 transcript and proteins is not prevented *in vivo*, AC3 homologous re-

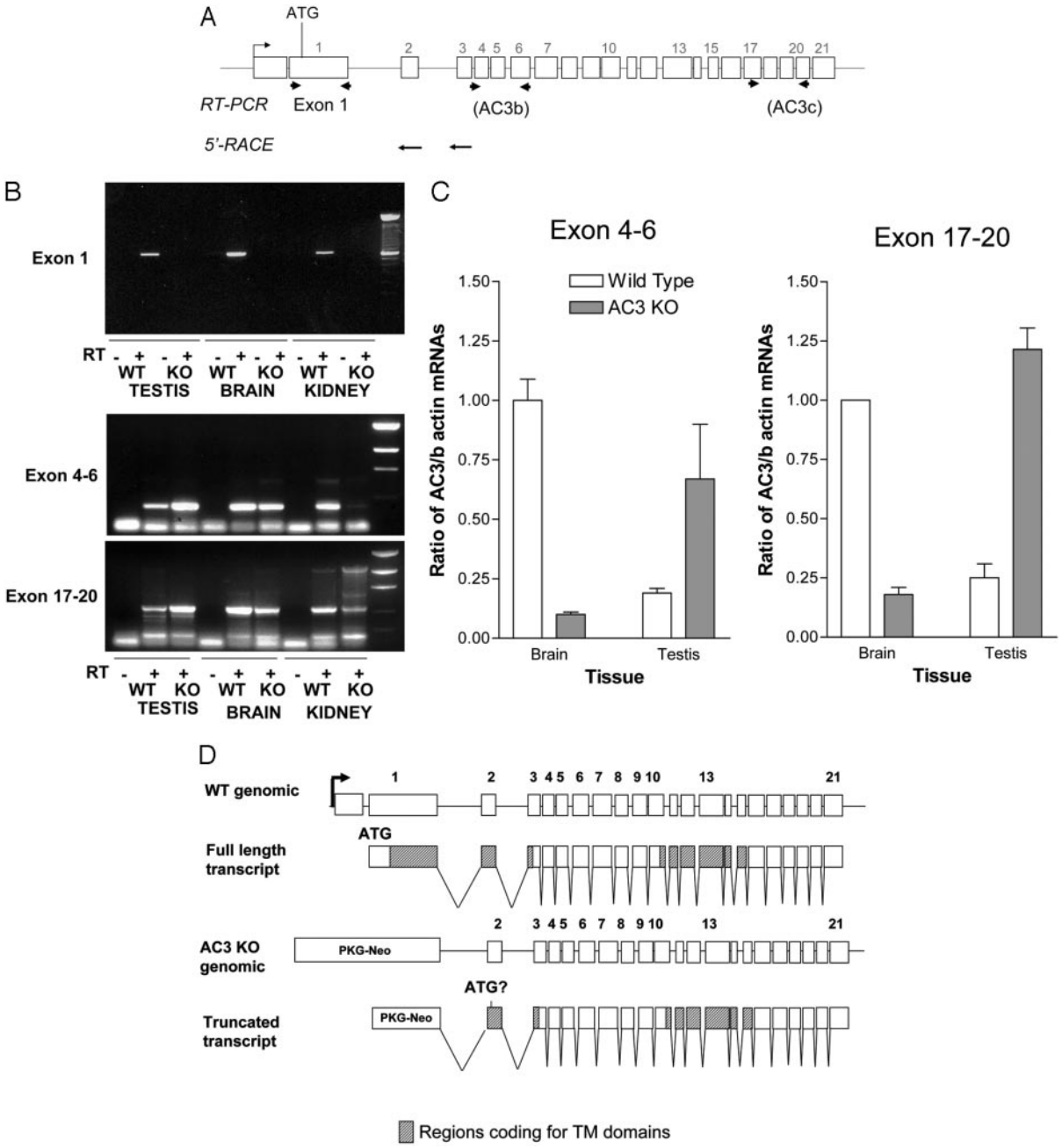

Fig. 4. Expression of Tr-AC3 mRNA in the Testis of AC3 Null Mice

A, Location of the primers used. B, Representative amplification of different regions of the AC3 transcript in different organs from wild-type and AC3$^{-/-}$ mice. C, Semiquantitative RT-PCR of AC3 transcripts in brain and testis from wild-type and AC3$^{-/-}$ mice. D, Schematic representation of the transcripts identified by 5′-RACE derived from wild-type and AC3$^{-/-}$ mice. Experiments were performed as detailed in *Materials and Methods.* RT, Reverse transcriptase; WT, wild type.

combination yields a catalytically inactive and mislocalized protein. Attempts to verify the presence of the shorter protein in spermatozoa of the AC3$^{-/-}$ mice by Western blot were unsuccessful.

cAMP Measurements in Spermatozoa

To determine whether forskolin-sensitive TM cyclase is detectable in spermatozoa, we measured cAMP production under different conditions (Fig. 7). Spermatozoa were isolated from the cauda epididymis, and their contamination was assessed by microscopic examination before incubation with different agents. cAMP production was measured in the absence or presence of forskolin, a known stimulator of the TM-AC, or in the presence of bicarbonate known to stimulate sAC. In AC3$^{+/+}$ spermatozoa, a small but significant increase in cAMP levels was observed in the

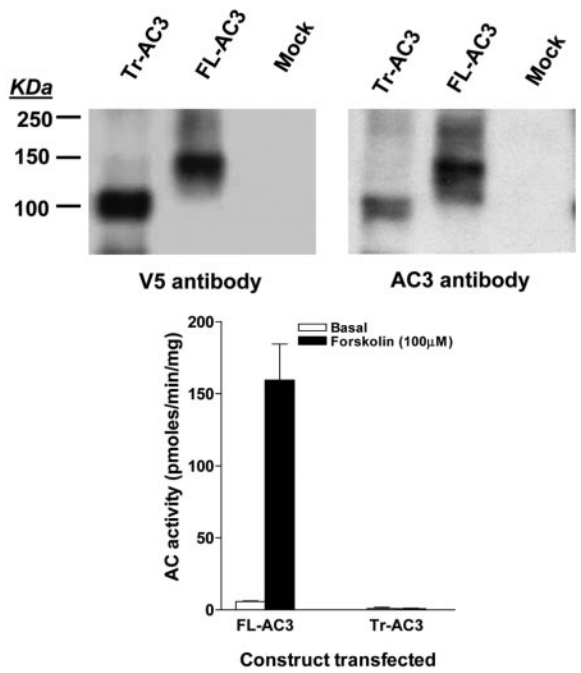

Fig. 5. Properties of the Protein Encoded by the Tr-AC3 ORF Identified in the AC3$^{-/-}$ Testis

cDNAs including the complete AC3 ORF [full-length AC3 (FL-AC3)] or lacking exon 1(Tr-AC3) were inserted into pCDNA3.1/V5-His-TOPO expression vector in frame with a V5 tag. Plasmids were transfected in HEK293 cells. After 48 h, cells were harvested and AC activity was measured in the cell homogenate in the absence or presence of 100 μM forskolin. An aliquot of the cell extract was used for Western blot analysis with either a V5 or the AC3-specific antibody. The experiment was repeated twice with identical results.

presence of forskolin. This stimulation was not always detectable and varied considerably among different sperm preparations from different mice (Fig. 7). Conversely, bicarbonate stimulation was always present (data not shown). When AC3$^{-/-}$ spermatozoa were used, basal cAMP accumulation was not significantly different from that of wild-type controls. Incubation of AC3$^{-/-}$ spermatozoa with forskolin did not produce a significant effect. Conversely, bicarbonate stimulated cAMP accumulation in AC3$^{-/-}$ spermatozoa in a manner similar to wild-type controls (data not shown). These findings suggest that a forskolin-sensitive cyclase activity is present in mouse spermatozoa but is only a minor component of the overall sperm cAMP synthesizing capacity.

Fertility Is Impaired in AC3 KO Males

To determine the physiological relevance of AC3 expression in testis, we investigated in detail the fertility of male AC3$^{-/-}$ mice (Table 2). Both AC3$^{-/-}$ and AC3$^{+/-}$ males were mated with AC3$^{+/+}$ or AC3$^{+/-}$ females. Whereas AC3$^{+/-}$ males were fully fertile (nine of nine), AC3$^{-/-}$ males produced few or no litters; only three males of the 11 tested had offspring (Table 2). These data are consistent with the reported finding by Wong *et al.* (24) that AC3$^{-/-}$ mating is unproductive. Furthermore, AC3$^{+/+}$ or AC3$^{+/-}$ female mice housed overnight with AC3$^{-/-}$ males showed vaginal plugs the following morning. Therefore, infertility of AC3$^{-/-}$ males is not due to a compromised reproductive behavior after disruption of olfaction.

When the number of litters per month per male was calculated, there was a major reduction observed with the AC3$^{-/-}$ mice. Indeed, the only three AC3$^{-/-}$ males that produced offspring produced only one litter each, even after mating with several females for more than 6 months (Table 2). Thus, the AC3 inactivation greatly reduced male fecundity.

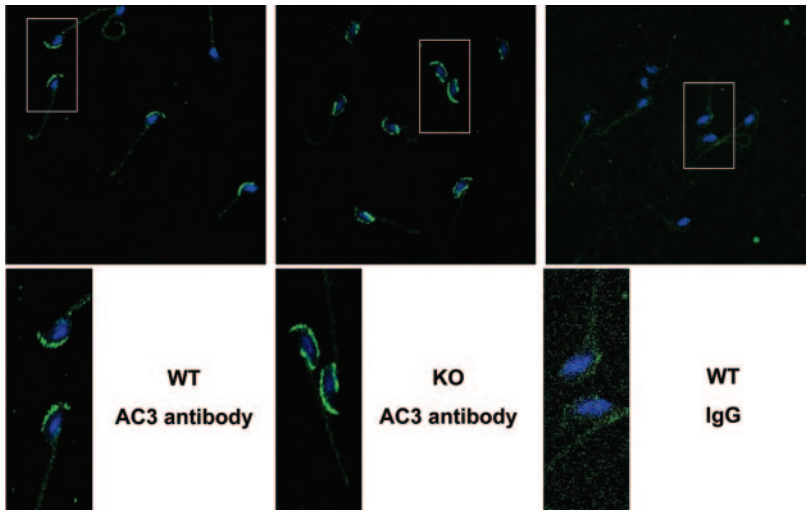

Fig. 6. Immunofluorescence Localization of AC3 in Wild-Type and AC3 Null Spermatozoa

Immunofluorescence was observed in the acrosomal region of epididymal spermatozoa whereas no signal was observed with control IgG. Notice the diffuse staining in AC3 null spermatozoa. WT, Wild type.

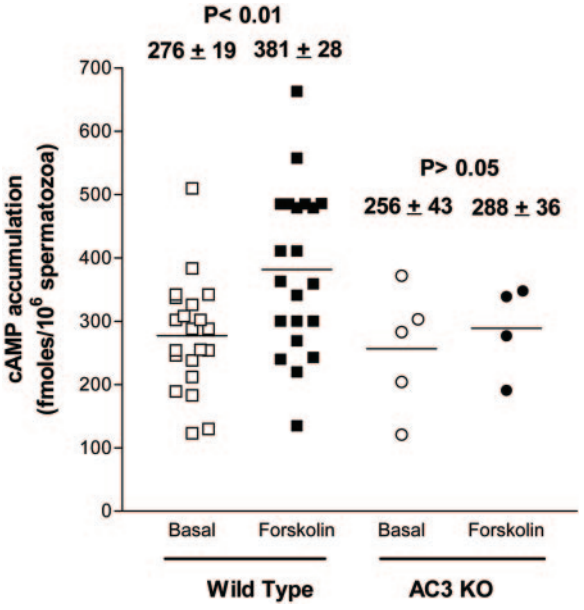

Fig. 7. Forskolin Stimulation of cAMP in Wild-Type and AC3$^{-/-}$ Spermatozoa

Spermatozoa from wild-type and AC3$^{-/-}$ mice were incubated for 15 min in the absence or presence of 100 μM forskolin. At the end of the incubation, trichloroacetic acid/ETOH solution was added. cAMP was measured by RIA after extraction. Each *point* represents spermatozoa from each individual mouse and is the average of measurements in two to three different spermatozoa samples.

Morphology of the Testis and Epididymis of the AC3 KO

To understand the cause of this subfertility, we studied the morphology of the reproductive tract of the AC3$^{-/-}$ males in greater detail. No gross anomalies could be seen along the reproductive tract; testis and accessory glands had a normal shape and size. When histological examinations of the AC3$^{-/-}$ reproductive tract were performed, all spermatogenic stages could be detected in the testis with a normal proportion of the different germ cells (Fig. 8A). Similarly, the epididymis showed no gross abnormality, and the lumen was filled with spermatozoa. Lastly, the number of spermatozoa retrieved from the epididymis of AC3$^{+/+}$ and AC3$^{-/-}$ animals was evaluated. AC3$^{-/-}$ males produced slightly less spermatozoa than AC3$^{+/+}$ mice, but this decrease was not statistically significant (data not shown). The AC3$^{-/-}$ spermatozoa did not display structural anomalies (head location, flagellar folding) in comparison with the AC3$^{+/+}$ spermatozoa at light microscopic level (Fig. 8, B and C). Neither could structural defects be detected at the electron microscopic level (Fig. 8, D–H). All the structural components were present and appeared to be organized correctly.

As a further control of spermatogenesis, we determined the effect of AC3 inactivation on the expression of two other male germ cell-specific genes, sAC and Tp1. When the expression of these two genes was measured by semiquantitative RT-PCR relative to β-actin, no difference between AC3$^{+/+}$ and AC3$^{-/-}$ testes was detected (data not shown). The brain was used as a negative control where no expression could be detected (data not shown).

AC3 Inactivation Perturbs Sperm Motility

Because the infertility of the AC3$^{-/-}$ males is not associated with an overt perturbation of spermatogenesis or of any testicular function, the motility of AC3$^{-/-}$ spermatozoa was assessed. The percentage of motile spermatozoa (showing flagellar beat) was significantly decreased ($P < 0.001$) in the AC3$^{-/-}$ sperm population (Fig. 9). This decrease in motility was confirmed by computer-assisted spermatozoa analysis (CASA) (data not shown). In addition, the percentage of AC3$^{-/-}$ spermatozoa recovered from the swimming-up procedure was consistently reduced by 80% compared with wild-type controls ($P < 0.01$).

AC3 Inactivation Impairs Ability of Spermatozoa to Fertilize Eggs *in Vitro*

In view of reduced motility of the AC3$^{-/-}$ spermatozoa, the function of these spermatozoa was further demonstrated by *in vitro* fertilization. When oocytes from wild-type females were used, the AC3$^{-/-}$ spermatozoa from the four mice assayed independently showed a very low fertility rate as assessed by the low number of two-cell embryos obtained (<20%) (Fig. 10A). Conversely, AC3$^{+/+}$ spermatozoa used in the same conditions and in similar numbers showed a high *in vitro* fertilization (IVF) success rate (Fig. 10A). This finding conclusively demonstrates that the low fecundity of the AC3$^{-/-}$ males comes from a defect at the spermatozoon level. It is important to notice that although decreased fertilization was observed when AC3$^{-/-}$ spermatozoa were incubated with the cumulus-oocyte complex (COC), we noticed binding of many spermatozoa to the oocytes, suggesting that the initial sperm egg interactions are normal. By using a live/dead staining on AC3$^{-/-}$ and AC3$^{+/+}$ spermatozoa, more than 95% of spermatozoa were alive with no difference between the two genotypes. Therefore, the deficiency in fertilization cannot be attributed to a lower survival rate of the AC3$^{-/-}$ spermatozoa.

Table 2. Mating of Heterozygous and Homozygous Null AC3 Males over a Period of 6 Months

Males	N	No. of Litters	Litters per Month	Litter Size
+/−	9	29	1.13 ± 0.12	6.48 ± 0.46
−/−	11	3	0.07 ± 0.04[a]	4.33 ± 1.86[b]

[a] $P < 0.001$; [b] $P < 0.10$ using Student's *t* test.

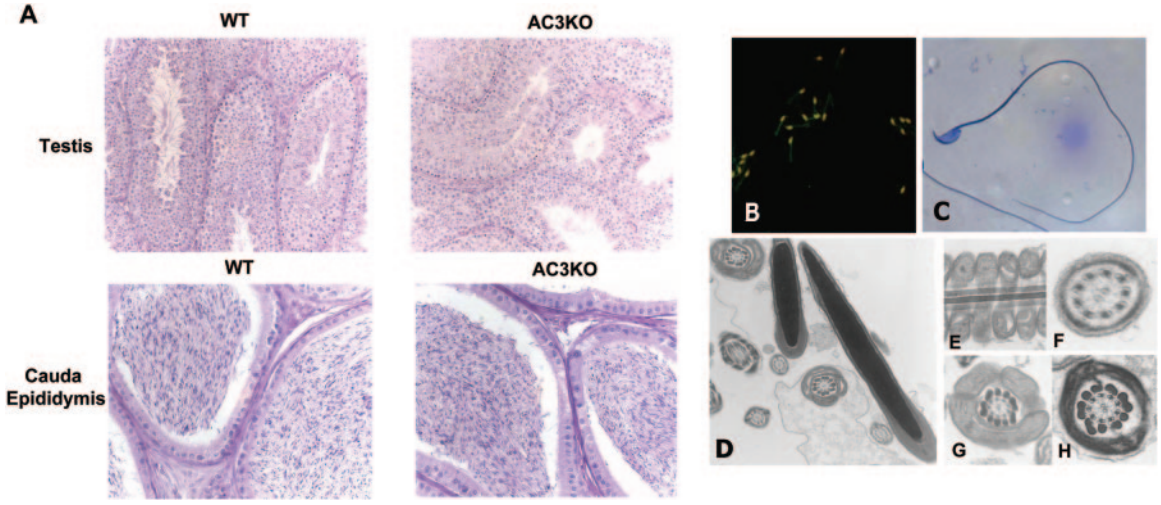

Fig. 8. Morphology of the Testis, Epididymis, and Spermatozoa from AC3$^{-/-}$ Mice

A, Wild-type and AC3$^{-/-}$ testes and epididymis were processed for histology and hematoxylin/eosin staining. A representative testis and epididymis section is pictured. No major differences were observed in the architecture of the testis or epididymis. Seminiferous tubules appear normal, and all the stages of spermatogenesis are represented. Numerous spermatozoa are present in the lumen of the epididymis. Magnification, ×200. B, Live/dead staining of spermatozoa from AC3$^{-/-}$ mice. Light (C) and electron microscopy (D–H) of spermatozoa from the AC3$^{-/-}$ mice. All the subcellular structures were present in AC3$^{-/-}$ spermatozoa and appear to be normal (E–H); no significant structural defect could be identified using this analysis. WT, Wild type.

To determine whether the fertility defect of AC3$^{-/-}$ spermatozoa could be reverted, IVF was set up with zona-drilled oocytes (Fig. 10B). Using these oocytes with acrosome-reacted AC3$^{+/+}$ and AC3$^{-/-}$ spermatozoa, we obtained a comparable number of embryos with both sperm populations. Therefore, it is possible to restore a normal fertility rate with AC3$^{-/-}$ spermatozoa. These data indicate that AC3 inactivation produces subtle changes in the spermatozoon, including deficits in motility and zona-induced acrosome reaction.

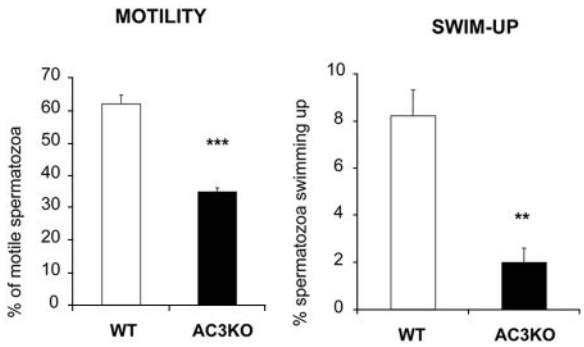

Fig. 9. Spermatozoa Motility of Wild-Type and AC3$^{-/-}$ Spermatozoa

After a brief incubation in complete medium, the spermatozoa were diluted in PBS, and motile and total spermatozoa were counted. For each mouse, at least 200 spermatozoa were scored. The decrease in motility was confirmed using CASA. For the swim-up assay, concentrated spermatozoa were carefully transferred to the bottom of an Eppendorf tube containing capacitating medium BSA and glucose. Tubes were incubated 1 h at 37 C in 5% CO_2 in air. At the end of the incubation, 50 μl of medium at the top of the tube was collected and the number of spermatozoa recovered was counted in a hemocytometric chamber. The percentage of spermatozoa swimming up was reduced by 4-fold as this parameter mainly represented the forward motility of capacitated spermatozoa. WT, Wild type.

Finally, we measured the percentage of acrosome-reacted spermatozoa untreated or treated with 1 μM or 10 μM concentrations of the calcium ionophore (Fig. 11). Interestingly, in the untreated AC3$^{-/-}$ spermatozoon group, the percentage of spermatozoa undergoing spontaneous acrosome reaction was significantly higher than in the AC3$^{+/+}$ spermatozoa. When spermatozoa was treated with calcium ionophore to artificially induce the acrosome reaction, no significant difference could be observed between the AC3$^{+/+}$ and AC3$^{-/-}$ spermatozoa. Altogether, these data indicate that both the motility and the acrosome reaction may be affected by AC3 inactivation.

DISCUSSION

Although it is firmly established that the second messenger cAMP plays a critical role in the control of sperm function, the mechanism regulating cAMP concentration in these cells has been elusive and often controversial, most likely because of the unusual arrangement of this signaling pathway in the male gamete. The molecular characterization of the

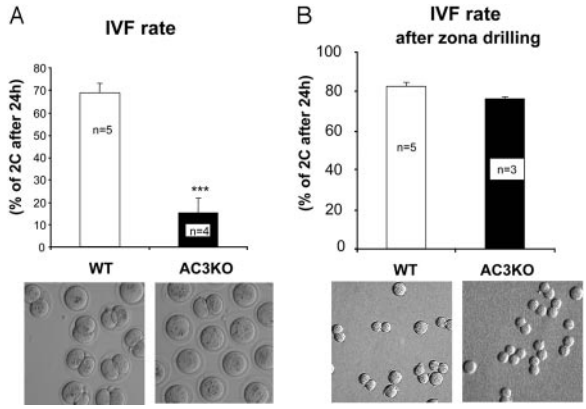

Fig. 10. *In Vitro* Fertilization Using Wild-Type and AC3$^{-/-}$ Mice

Highly motile spermatozoa (2–5 $\times$ 10^5/ml) from the swim up were added to each fertilization drop containing the COCs and incubated for 4 h. Oocytes were then washed twice in 50 μl mKSOM under oil and finally transferred to droplets of the same medium. The dishes were incubated at 37 C in 5% CO_2 in a humidified incubator. Fertilization was assessed by recording the number of two-cell embryos 24 h after fertilization. Some of the dishes were further kept in culture to ensure that the two-cell embryos could progress to further developmental stages. Embryos were observed at $\times$400 magnification on the warmed stage (37 C) of an inverted microscope. Zona drilling was performed on mouse oocytes by treatment with hyaluronidase in M2 medium at 37 C and exposure for 1–2 sec to Tyrode's solution. The oocytes thus treated were washed thoroughly several times before being transferred to the IVF drop. Acrosome reaction in spermatozoa was induced by 10 μM A23187 ionophore. The spermatozoa used for IVF were derived from different mice tested on different days. The total number of mice used is reported in the *bar*. WT, Wild type.

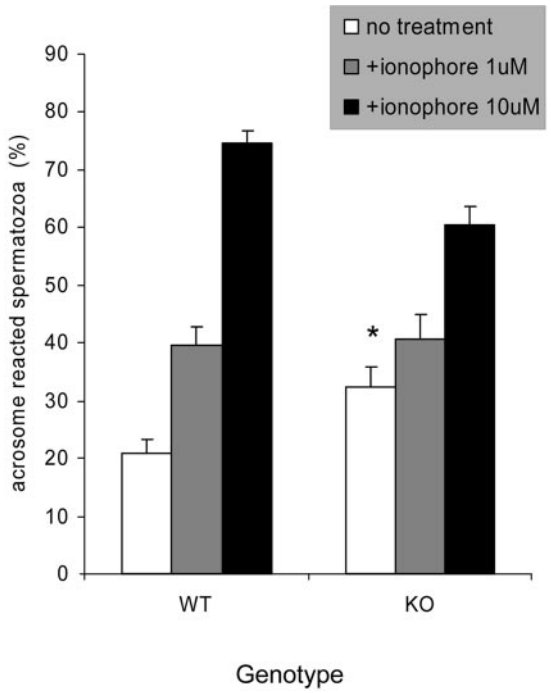

Fig. 11. Acrosome Reaction in Wild-Type and AC3 Null Spermatozoa

After incubation in capacitating conditions, spermatozoa preparations were incubated for 30 min in the absence or presence of 1 or 10 μM concentrations of calcium ionophore (A23187) and then fixed in 4% PFA for 10 min at room temperature and stained with G-250 Coomassie blue (4% in 3.5% perchloric acid). The acrosome-reacted cells were counted using $\times$1000 magnification. Acrosome reaction causes the loss of intense staining on the antero/superior portion of the spermatozoa head. Data were expressed as a percentage of acrosome reacted. A minimum of 200 cells were counted for each *point*. At least five mice for each group were used to score the acrosome reaction. WT, Wild type.

bicarbonate-sensitive sAC (6) and the phenotype that follows its inactivation (8) has established that this is a critical component of the machinery regulating cAMP and motility, and that its unique features account for many of the properties of cAMP signaling in sperm. Nevertheless, some aspects of cAMP regulation in the male gamete are inconsistent with the properties and regulation of sAC. For instance, it remains to be determined how ligands that act through GPCRs impact cAMP levels and sperm function because sAC is not directly regulated by G proteins (6, 28). A possibility to be investigated is that one or more conventional TM cyclases, together with sAC, control cAMP concentration in spermatozoa. Although the exact lesion of the AC3 null spermatozoa could not be identified, we provide evidence that a TM cyclase may play a role in the overall control of cAMP and of spermatid or sperm functions that are critical for fertilization.

Expression of AC3 in mouse spermatids is suggested by both the detection of the mRNA in germ cells and the immunocytochemical data. *In situ* hybridization clearly shows that spermatogenic cells expressing sAC also express AC3 mRNA. In addition, forskolin-sensitive cyclase activity is readily detected in seminiferous tubules and in enriched spermatid preparations, even though somatic cell ACs may contribute to this activity. That AC3 is retained in epididymal spermatozoa has been reported in several studies (15, 20). In agreement with these findings, we show that mouse epididymal spermatozoa are immunoreactive to AC3 antibodies with staining confined to the acrosomal region. A caveat of our studies and those of others is that the localization of AC3 relies on the use of a single antibody. Unlike previous reports, specific staining in the mid or principal piece of the flagellum was minimal. Surprisingly, staining in the head of sperm is still present in spermatozoa from the AC3 null mice. Because this staining is not only in acrosomal cap, and in view of the presence of Tr-AC3 mRNA in the testis, our interpretation of the data is that a protein lacking a critical membrane-anchoring do-

main is produced in the AC3 null mice and is retained in spermatozoa. On the basis of the expression in a heterologous system, this protein is catalytically inactive. This latter conclusion is consistent with the cAMP measurements even though it has been difficult to detect forskolin-stimulated cyclase activity in spermatozoa. Several previous reports have indicated the presence of forskolin-regulated cyclase in sperm from different species, even though other reports have refuted this possibility (16–19).

Ablation of AC3 does not produce an overt disruption of spermatogenesis differentiation. Testis weight, histological appearance of seminiferous tubules, expression of key mRNAs, and ultrastructure of spermatozoon AC3 null testis are undistinguishable from control littermates. The number of sperm produced in the AC3 mice is also normal. Thus, ablation of AC3 in spermatids does not appear to have a major impact on qualitative or quantitative differentiation of these cells. Given the fact that sAC is active in production of cAMP in spermatids (29), it is possible that cAMP produced by this cyclase is sufficient to compensate for the loss of AC3 in spermatids. In addition, one should consider the possibility that additional TM cyclases are expressed in spermatids. Indeed mRNAs for AC9 are readily detectable in mouse testes (data not shown).

Despite apparently normal spermatogenesis, $AC3^{-/-}$ males are largely infertile, a finding consistent with the initial report of difficulty in producing offspring with homozygous null mating (24). The results of the homozygous mating are complicated by the fact that females also exhibit greatly reduced fertility. It is unlikely, however, that this infertility is due to the altered behavior of mice with disruption of the olfactory system. Mounting behavior is present, and vaginal plugs can be detected in wild-type females housed with AC3 null males. The finding that sexual behavior is unaffected in these mice is consistent with the notion that the vomeronasal organ, not affected by AC3 ablation, is more relevant for this function (27). By probing the function of sperm produced by the AC3 null mice, we have determined that AC3 spermatozoa display a greatly reduced ability to fertilize an egg *in vitro*. The yield of embryos using sperm preparations from five different $AC3^{-/-}$ mice was reduced by 90%. Because the fertilizing ability can be restored by dispersion of the cumulus matrix and removal of the zona, we concluded that AC3 null sperm are unable to efficiently penetrate the oocyte vestments. This defect may be caused by a subtle disruption in flagellar movements, by altered or untimely acrosome reaction, or both. Although a defect in motility was uncovered with the swim-up procedure, this may only indirectly cause inefficient fertilization, because the same number of motile spermatozoa was used in the IVF experiment.

In view of the difficulty of detecting the expression of AC3 activity in spermatozoa, we cannot formally exclude that the effect of AC3 disruption on fertility is indirect and caused by a loss of function early during spermatid differentiation. This latter possibility, however, is inconsistent with the normal morphology of the testis and spermatozoa and the normal expression of key genes involved in spermatid differentiation. Although the *adcy3* heterozygous mice had no fertility phenotype, we cannot exclude the possibility that the truncated protein derived from the recombined allele has biological effects other than cAMP production in spermatids or spermatozoa, perhaps functioning as a dominant-negative allele.

It is remarkable that many of the components of the transduction pathway used for sensing olfactory cues are expressed in spermatids and spermatozoa. Olfactory receptor mRNAs and, in some instances, proteins have been detected in germ cells from different species (30, 31). The olfactory G_{olf} protein is expressed in spermatids and retained in spermatozoa (22). As shown here, the major olfactory cyclase AC3 is expressed together with the phosphodiesterases PDE1 and PDE4A, all expressed at high levels in the olfactory system. Cyclic nucleotide-gated channels also are shared by spermatozoa and olfactory cells (32). To explain this remarkable similarity, it has been proposed that an olfactory-like signaling pathway may be used by the male gamete to sense the external chemical environment (31, 33). In agreement with this hypothesis, it has been reported recently that an olfactory receptor expressed in human spermatozoa is involved in sperm chemotaxis (13). Given the pharmacological data suggesting that this olfactory receptor is coupled to a cyclase with the properties of a TM-AC (13, 34), it is tempting to speculate that the olfactory cyclase AC3 functions downstream of this receptor. If this hypothesis is correct, AC3 null spermatozoa may carry defects in the chemosensory system required to recognize and penetrate an egg. Although no obvious deficit in sperm binding to the egg was observed during IVF, this possibility needs to be explored further.

In summary, our data indicate that the AC3 TM cyclase is directly or indirectly necessary for sperm function and fertility. Even though the exact biochemical lesion disrupting AC3 fertility remains to be determined, these findings suggest a complex interplay between bicarbonate-sensitive sAC and other ACs and GPCRs. In view of the probable localization of these cyclases in different spermatids and spermatozoa compartments, the site of cAMP production may be an important determinant of the biochemical functions of these different components of cAMP signaling. Thus, spermatozoa provide a unique model in which to study this signaling compartmentalization.

MATERIALS AND METHODS

Culture Media and Reagents

All reagents, except where stated otherwise, were of the highest quality from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (La Jolla, CA).

Experimental Animals

Wild-type mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animal procedures were in accordance with accepted standards of humane animal care and were approved by the Animal Care and Use Committee at Stanford University.

Analysis of the Genotype and Mating the AC3-Deficient Mice

$AC3^{+/-}$ mice provided by Daniel Storm from the University of Washington Animal Facility were used to generate $AC3^{-/-}$ mice to study male fertility (24). Due to the poor efficiency of this mating, some $AC3^{+/-}$ males were also mated with $AC3^{-/-}$ females to improve the yield of $AC3^{-/-}$ pups. To reduce litter size and increase the survival rate, pups were transferred to foster mothers as soon as possible after birth. To genotype the mice, PCR was performed on genomic DNA extracted from tails of the offspring. The primers 5′-cctgtgctctagtagcttacgg-3′ and 5′-ctgtgaagtaggttcctacctg-3′ identified the mutant allele, and 5′-ctggtgaagtggcttgacct-3′ and 5′-gttatgaagaaggagaagaca-3′ corresponded to the wild-type allele.

$AC3^{+/-}$ and $AC3^{-/-}$ males between 2 and 6 months of age were mated (two females per male) with $AC3^{+/+}$ or $AC3^{+/-}$ females. No difference was observed in the fertility of $AC3^{+/-}$ or $AC3^{+/+}$ females. The presence of vaginal plugs was recorded on many occasions for both $AC3^{+/-}$ and $AC3^{-/-}$ males. When pregnancy was detected by an increased weight, females were removed from the mating cage and housed separately until delivery. Males siring at least one litter were defined as fertile, and the number of litters per month obtained by one male was used as the index of fecundity.

Histology and Electron Microscopy

Testes and epididymi of $AC3^{-/-}$and $AC3^{+/+}$ mice were fixed in Bouin's solution for 6 h at room temperature and then dehydrated and stained with eosin. After embedding in Paraplast, 5-μm sections were cut, deparaffinized, rehydrated, and then stained with hematoxylin and eosin. Slides were cleared with xylene, mounted, and analyzed by light microscopy with a Zeiss microscope (Carl Zeiss, Thornwood, NY) fitted with AxioCam (Zeiss, Oberkochen, Germany).

For electron microscopy, spermatozoa suspensions were fixed in 2% glutaraldehyde in PBS on ice. Next, samples were washed several times in PBS, dehydrated through an ethanol series, and embedded. Samples were visualized on a Philips CM12 transmission electron microscope.

Immunohistochemistry and Immunofluorescence

Detection of AC3 protein in mouse testis and spermatozoa was performed as previously described (35). Briefly, testis were fixed in Bouin's solution or 4% paraformaldehyde (PFA) and then either embedded in Paraplast or frozen in optimal cutting temperature compound (Tissue-Tek, Torrance, CA). Sections (5–10 μm) were cut. For immunohistochemistry, the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) was used. Paraplast sections were rehydrated and then incubated 1 h at room temperature with 10% goat serum, followed by antibodies overnight incubation with anti-AC3 antibodies (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a humidified chamber at 4 C. The distribution of the primary antibody was revealed with a biotinylated goat antirabbit secondary antibody, and the avidin-biotin-peroxidase complex was visualized with 3,3′-diaminobenzidine. For immunofluorescence, frozen sections were treated similarly but incubated with AC3 antibodies diluted 1:1000, and the primary antibody was visualized with a Texas Red goat antirabbit secondary antibody (Vector Laboratories).

Fresh spermatozoa were fixed in 4% PFA (30 min at 4 C), washed in PBS, and dried on slides coated with poly-L-lysine. If the slides were not to be used immediately, they were stored at 4 C.

The specificity of staining was monitored by replacing the first antibody with nonimmune IgG or by blocking the signal with an excess of peptide. As a further positive control for the antibody, COS7 cells were transfected (Effectene transfection kit; QIAGEN, Chatsworth, CA) with 2 μg of constructs encoding the rat sense or antisense AC3 cDNA (22). Cells were fixed 24 h later in 4% PFA (15 min at 4 C) and then rinsed in PBS and stored at 4 C before staining.

In Situ Hybridization

In situ hybridization was carried out as previously described (35). Briefly, a partial segment of 387 bp (3301–3687 bp) of rat AC3 cDNA (M55075) subcloned in pcDNA3.1/V5-His-TOPO vector was used as template for the synthesis of radioactive ^{35}S-labeled RNA probes.

Mouse testes were fixed in 4% PFA for 6 h and incubated in 0.5 M sucrose overnight at 4 C. Testes were embedded in OCT (Tissue-Tek), cut into 10-μm sections, and mounted. Slides were postfixed in PFA and treated as previously described. Radioactive hybridized sections were exposed in NTB2 Emulsion (Eastman Kodak, Rochester, NY) for 7 d, developed photographically, counterstained with Gill's hematoxylin and eosin (0.25% wt/vol in ethanol), cleared with xylene, and mounted with Permount (Fisher Scientific Co., Pittsburgh, PA). Testes were visualized and photographed with AxioCam.

IVF: Preparation of Spermatozoa

Males of ages ranging between 2.5 and 4 months were caged alone at least 5 d before the IVF was performed. Mice were euthanized by CO_2 narcosis and hypoxia. The cauda epididymi and a portion of the vas deferens were removed and quickly transferred into a culture dish containing 2 ml of M2 medium and then transferred into another dish with 200 μl of M2 medium. Four incisions were made in the cauda epididymis to allow spermatozoa to swim out. After 10 min of incubation at 37 C, tissue fragments were discarded, and the spermatozoa suspension was collected. Cells were counted and, if necessary, diluted to obtain similar concentrations in $AC3^{+/+}$ and $AC3^{-/-}$ preparations. Some of the spermatozoa suspension (50 μl) was transferred to the bottom of a 1.5-ml Eppendorf tube containing 200–450 μl of KSOM [potassium simplex optimized medium (Specialty Media, Phillipsburg, NJ), supplemented with 4 mM glucose and 4% BSA (later referred to as modified-KSOM, mKSOM) (36)]. Capacitation was allowed to proceed for 1 h at 37 C, in a 5% CO_2 incubator and then 50 μl was collected from the upper fraction of the spermatozoa suspension, which represents a population of highly motile capacitated spermatozoa, and the concentration of the spermatozoa in the fraction was determined and, if necessary, adjusted to obtain similar concentrations of $AC3^{+/+}$ and $AC3^{-/-}$ sperm. Addi-

tionally, live/dead staining was performed with SYBR 14 dye (Live/Dead Spermatozoa Viability Kit; Molecular Probes, Inc., Eugene, OR) according to the recommendations of the manufacturer.

IVF: Preparation of Oocytes

C57BL/6 female mice (25-d old) (Charles Rivers Laboratories) were induced to superovulate by ip injection of 5 IU pregnant mare's serum gonadotropin (Calbiochem) followed by 5 IU human chorionic gonadotropin (Goldline Laboratories, Fort Lauderdale, FL) 48 h later. Animals were killed by CO_2 14 h after chorionic gonadotropin injection. Oviducts were collected in a 35-mm dish containing 2 ml M2 medium. The COCs were recovered by gentle dissection of the oviducts. Each COC was transferred to a 20-μl droplet of capacitated spermatozoa suspension in mKSOM and covered with mineral oil.

IVF Procedure

Highly motile spermatozoa (2–5 $\times$ 10^5/ml) from the swim-up fraction were added to each fertilization droplet with the COCs and placed in an incubator for 4 h. Oocytes then were washed twice in 50 μl mKSOM under oil and finally transferred to droplets of the same medium. The dishes were incubated at 37 C in 5% CO_2 in a humidified incubator. Fertilization was assessed by recording the number of two-cell embryos 24 h after fertilization. Some of the dishes were maintained in culture to monitor two-cell embryo progression to later stages of development. Embryos were observed at $\times$400 magnification on the warmed stage (37 C) of an inverted microscope.

In a set of experiments, zona drilling was performed on mouse oocytes, and motile spermatozoa from the swim-up fraction were artificially acrosome reacted. First the oocytes from the COC were denuded by treatment with 0.03% hyaluronidase in M2 medium at 37 C. The denuded oocytes then were treated for 1–2 sec with acidic Tyrode's solution until almost complete disappearance of the zona and then washed with several rinses of medium before being transferred to the IVF drop. Spermatozoa were treated with calcium ionophore (10 μM A23187) for 30 min to induce a synchronous acrosome reaction. The ionophore was then quenched with a high concentration of BSA (20 mg/ml), and the spermatozoa suspension was diluted 10- to 20-fold before being added to the oocytes for a 2-h incubation. Oocytes were then washed twice as described above.

Measurement of Spermatozoa Motility

Motility of the spermatozoa was assessed subjectively using an inverted microscope. After a brief incubation into mKSOM, spermatozoa were diluted in PBS, and motile and total spermatozoa were counted. For each point, at least 200 spermatozoa were evaluated. Whenever possible, these data were confirmed by CASA (integrated visual optical system from Hamilton Thorne Biosciences, Inc., Beverly, MA).

Measurement of cAMP Production

The spermatozoa were collected from cauda epididymi and allowed to swim out into BWW medium (95 mM NaCl; 4.8 mM KCl; 1.3 mM $CaCl_2$; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 20 mM sodium lactate; 5 mM glucose; 0.25 mM sodium pyruvate; 25 mM $NaHCO_3$, pH 7.4) supplemented with 10 mM HEPES (pH 7.4) and 3% BSA (37). Spermatozoa were counted and normalized to 20 million cells per ml and pretreated for 10 min with 0.5 mM isobutylmethylxanthine. Of this suspension, 50 μl was added to 50 μl of the medium with or without 25 mM bicarbonate or 100 μM forskolin and incubated for 15 min at 37 C. To stop the reaction, 1 ml of ice-cold 0.1% trichloroacetic acid in ethanol was added. Samples were then centrifuged at 3000 rpm for 30 min at 4 C, and the supernatant was evaporated while centrifuging under vacuum and reconstituted with 500 μl of PBS. cAMP was measured by the RIA method of Harper and Brooker (38).

Measurement of Acrosome Reaction

After capacitation, spermatozoa preparations were incubated for 30 min with or without various concentrations of calcium ionophore (A23187) and then fixed in 4% PFA for 10 min at room temperature. Spermatozoa suspensions were then centrifuged at 1000 $\times$ *g* for 10 min and resuspended with 500 μl of 0.15 M ammonium acetate (pH 9). Spermatozoa suspensions were centrifuged one more time and resuspended in 50 μl PBS, and then spotted and dried on slides coated with 0.1% poly-L-lysine. G-250 Coomassie blue (4% in 3.5% perchloric acid) was added onto the slide for 2–3 min. The slides then were washed four times, 5 min each, in water. The acrosome-reacted cells were counted with $\times$1000 magnification. Acrosome reaction caused the loss of intense staining on the anterior aspect of the spermatozoa head. Data were expressed in percentage of acrosome-reacted cells compared with the total number of cells. At least 200 cells were counted for each point.

RT-PCR and 5′-RACE

Testes were quickly removed from the animals, washed in PBS, and frozen at −80 C. RNA isolation was performed with 1 ml of Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. To avoid contamination with genomic DNA, RNA was treated with deoxyribonuclease. Reverse transcriptase reactions were performed with 5 μg of RNA and oligo-dT primers. PCR with specific primer for AC3, sAC, TP1, and β-actin was performed. The primers sequences, the size of the amplified bands, and the annealing temperatures are given in Table 1. To ensure that we specifically amplified the type 3 AC, and no other similar AC, the PCR products were sequenced. Semiquantitative RT-PCR also was performed, and the expression of these genes was compared with the β-actin expression. This protocol assures that all measurements were taken during the exponential phase of the PCR.

5′-RACE was performed on brain and testis RNAs using commercially available kits (5′ RACE System for Rapid Amplication of cDNA Ends, Invitrogen; Marathon-Ready cDNA, CLONTECH Laboratories, Inc.) following the manufacturer's instructions. The products of the reaction were sequenced and sequences were analyzed using the BLAT or Browser utilities of the UCSC Genome Bioinformatics web site (http://genome.ucsc.edu/index.html?org=Mouse).

Expression of the Recombinant Proteins and AC Activity

Full-length AC3 (FL-AC3) and truncated Tr-AC3 (Tr-AC3) tagged with V5 were transfected in HEK293 cells using Mirus transfection reagents following the manufacturer's directions. Cells were harvested 48 h after transfection and protein was extracted and measured for AC activity as described earlier (29). An aliquot of the extract was separated on 8% SDS-PAGE, transferred on immobilon membrane, and immunoblotted with AC3 or V5 antibodies.

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