Deletion of the Parkin coregulated gene causes male sterility in the *quaking*^{viable} mouse mutant

Diego Lorenzetti*†, Colin E. Bishop‡, and Monica J. Justice†§

*Graduate Program in Molecular and Human Genetics, Graduate School of Biomedical Sciences, and Departments of ‡Obstetrics and Gynecology and †Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

Edited by Mary F. Lyon, Medical Research Council, Oxon, United Kingdom, and approved April 16, 2004 (received for review March 15, 2004)

***Quaking*^{viable} (*qk*^{v}) is a recessive neurological mouse mutation with severe dysmyelination of the CNS and spermiogenesis failure. The molecular lesion in the *qk*^{v} mutant is a deletion of ≈1 Mb on mouse chromosome 17 that alters the expression of the *qk* gene in oligodendrocytes. Complementation analysis between the *qk*^{v} mutation and *qk* mutant alleles generated through chemical mutagenesis showed that the male sterility is a distinctive feature of the *qk*^{v} allele. This observation suggested that the sperm differentiation defect in *qk*^{v} is due to the deletion of a gene(s) distinct from *qk*. Here, we demonstrate that the deletion of *Pacrg* is the cause of male sterility in the *qk*^{v} mutant. *Pacrg* is the mouse homologue of the human *PARKIN*-coregulated gene (*PACRG*), which encodes for a protein whose biochemical function remains unclear. We show that *Pacrg* is highly expressed in the testes in both mice and humans. In addition, the expression pattern of *Pacrg* during spermiogenesis suggests that it plays a role in sperm differentiation. In support of this hypothesis, we show that transgenic expression of *Pacrg* in testes restores spermiogenesis and fertility in *qk*^{v} males. This finding provides the first *in vivo* evidence, to our knowledge, for the function of Pacrg in a model organism. Immunolocalization experiments on isolated spermatozoa show that the Pacrg protein is present in mature sperm. Remarkably, the mammalian Pacrg protein shares significant sequence similarities with gene products from flagellated protozoans, suggesting that Pacrg may be necessary for proper flagellar formation in many organisms.**

The mouse mutant *quaking*^{viable} (*qk*^{v}) has been investigated for nearly four decades for its neurological phenotype. Affected animals display severe tremor of voluntary movements, with onset ≈10 days of age (1). The main neuropathological finding in the CNS of *qk*^{v} mice is a severe lack of myelin (1). In addition to the CNS abnormalities, *qk*^{v} homozygous males are sterile due to a severe oligospermia (2). Very few morphologically abnormal immotile spermatozoa are present in the semen of *qk*^{v} males, because spermatids fail to complete differentiation. A recent report (3) showed that sperm from *qk*^{v} semen and testes are unable to fertilize mouse oocytes *in vitro*. However, the intracytoplasmic injection of *qk*^{v} spermatozoa resulted in normal live offspring. These findings indicate that although the *qk*^{v} mutation affects functions necessary for proper sperm differentiation and fertilization, it does not affect other epigenetic factors necessary for syngamy and normal embryonic development (3).

The molecular defect in *qk*^{v} is a large deletion spanning ≈1 Mb of mouse chromosome 17 (4, 5). The *qk* gene, which encodes for RNA binding proteins involved in posttranscriptional mRNA regulation (6, 7), is in close proximity to the proximal deletion breakpoint of *qk*^{v} (8). The *qk*^{v} deficiency affects the region upstream of the *qk* gene to reduce the expression of *qk* mRNAs in oligodendrocytes, resulting in the CNS myelination defect (9, 10). In addition to the spontaneous *qk*^{v} deletion, several *N*-ethyl-*N*-nitrosourea (ENU)-induced alleles of *quaking* have been isolated (11–13). Each of these ENU-induced mutations (*qk*^{e}) selectively complements the male sterility associated with the *qk*^{v} allele. This observation suggests that the spermiogenesis defect in *qk*^{v} is the result of the loss of function of a locus (or loci) distinct from *qk*, mapping within the *qk*^{v} deletion boundaries.

To identify loci involved in the *qk*^{v} spermiogenesis defect, we determined the gene content of the deleted interval. Two large genes map to the *qk*^{v} deletion (14). One of these genes is *Parkin*, the mouse homologue of the human *PARKIN* gene. Loss-of-function mutations in *PARKIN* cause an autosomal recessive form of early-onset Parkinson's disease (15). Although Parkin protein expression is lost in *qk*^{v} homozygous mice (14, 16), *qk*^{v} mutants do not recapitulate neuropathological features of early-onset Parkinson's disease (14). The second gene mapping to the *qk*^{v} interval is the mouse homologue of a human gene termed *PA*RKIN coregulated *g*ene (*PACRG*) and therefore it will be referred to hereafter as *Pacrg* (14, 16).

In this study, we show that the *Pacrg* transcript is highly expressed in mouse testes during spermatogenesis and its gene product is present in mature spermatozoa. Transgenic expression of the mouse *Pacrg* cDNA in testes rescues the sperm differentiation defect and restores normal fertility in *qk*^{v} males. These results demonstrate that deletion of *Pacrg* is the cause of male sterility in *qk*^{v} and validate the hypothesis that the reproductive phenotype in this mutant is due to the loss of function of a gene distinct from *qk*. Furthermore, the involvement of *Pacrg* in sperm differentiation provides evidence of its function *in vivo* in model organisms.

Materials and Methods

Cloning, Expression, and Sequence Analysis of *Pacrg*. The assembly of the bacterial artificial chromosome contig spanning the *qk*^{v} deletion and the sequence analysis were described elsewhere (14). Several cDNA clones corresponding to ESTs matching the AK005771 sequence deposited in GenBank were obtained through the IMAGE consortium, and their inserts were fully sequenced. The IMAGE clone 516103, from a mouse cDNA testis library (dbEST accession no. AA089000), was found to contain the entire mouse *Pacrg* coding region and most of its 5′ and 3′ UTRs. Multiple sequence alignments of metazoan and protozoan Pacrg proteins were generated by using the CLUSTALW algorithm (version 1.8) supported by the Baylor College of Medicine Search Launcher server, which can be accessed at www.hgsc.bcm.tmc.edu/SearchLauncher. Conserved amino acids functional motifs were identified by using the subprograms of the PSORTII algorithm, which can be accessed at http://psort.nibb.ac.jp.

The insert from cDNA clone 516103 was used as a probe for both Northern blot and for *in situ* hybridization experiments on mouse testis. For Northern blot analysis, total RNA was isolated from mouse tissues samples by using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer's

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: dpp, days postpartum.

§To whom correspondence should be addressed at: Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza (S413), Houston, TX 77030. E-mail: mjustice@bcm.tmc.edu.

protocol. Ten micrograms of total RNA per lane was electrophoresed on a 1% 4-morpholinepropanesulfonic acid-formamide agarose gel and was transferred onto a nylon membrane. Hybridization was carried out overnight at 65°C, by using the cDNA inserts radiolabeled with a random primer kit (Prime-It, Stratagene). *In situ* hybridization was carried out by using the *Pacrg* cDNA insert as described by Lu and Bishop (17). For the expression analysis of *PACRG* in human tissues, a FirstChoice Northern human blot 2 membrane (Ambion, Austin, TX) was hybridized at 55°C by using the ULTRAhyb solution (Ambion) according to the manufacturer's protocol. A human *PACRG* testis cDNA insert derived from the IMAGE clone 1292856 (dbEST accession no. AA776722) was used as a probe.

Assembly of the *PGK2-Pacrg* Minigene and Generation of Transgenic Mice. A 1.4-Kb *Sal*I/*Hin*dIII fragment containing the upstream regulatory region of the human phosphoglycerate kinase 2 gene (*PGK2*), derived from the pSVOCAT construct (ref. 18; a kind gift of R. Erickson, University of Arizona, Tucson), was subcloned in the pBluescript-II-KS vector (Stratagene) and was then ligated to a PCR-amplified *Eco*RV/*Not*I fragment containing the *Pacrg* ORF. Subsequently, a *Not*I/*Stu*I fragment from the plasmid vector pIRES-EGFP (Clontech) containing simian virus 40 polyadenylation signal sites was ligated to the 3′ end of the *Pacrg* ORF. For microinjection, the 2.5-kb *PGK-Pacrg* transgene was cut free from the vector backbone with a *Kpn*I/*Afl*I digestion and was purified by using the QIAEX-II Gel extraction kit (Qiagen, Valencia, CA). The pronuclei of C57BL/6J one-cell embryos were injected with the *PGK2-Pacrg* transgene. Six transgenic founder animals were identified by Southern blot analysis on *Eco*RV-digested genomic DNA by using the 1.4-kb *PGK2* promoter region as a probe. Five of the founder animals were crossed to BTBR mice carrying the qk^v deletion, and four transmitted the *PGK2-Pacrg* transgene to their offspring. The qk^v/qk^v animals carrying the transgene were generated through a two-generation breeding scheme and were analyzed for sperm count and fertility.

Testis Histology, Sperm Analysis, and Fertility Tests. Testes were treated for histological analyses essentially as described by Clark *et al.* (19). Sperm was collected from the epididymis and vas deferens of transgenic and control animals in 2 ml of PBS solution. The sperm suspension was incubated at 37°C for 15 min and the concentration was obtained by counting sperm in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA).

To assess fertility of transgenic and wild-type control animals, sexually mature males were placed in a cage with two wild-type BTBR females for 8 weeks.

Antibodies, Western Blotting, and Immunofluorescence. Polyclonal antibodies were raised against the following two peptides derived from the conceptual translation of the AK005771 cDNA nucleotide sequence: RPAKPTTFRKCYERGD (corresponding to amino acid residues 48–64) and YSQQKRENIGDLI (amino acid residues 197–209). The peptides were conjugated to keyhole limpet hemocyanin as carrier and were combined for injection in two rabbits. Peptide synthesis, conjugation, and immunization were carried out by Bethyl Laboratories (Montgomery, TX). Affinity purification of the immune serum was carried out by using the Sulfolink kit (Pierce) following the manufacturer's protocol. Western blot analysis was carried out as described (14). Protein extracts from semen were prepared by collecting sperm by centrifugation (3,000 × *g* for 5 min at 4°C). Pellets were washed twice in PBS, resuspended in 100 μl of lysis buffer (50 mM Hepes, pH 7.5/150 mM NaCl/10 mM EDTA/1% IGEPAL CA-630 (Sigma), containing Complete proteinase inhibitors mixture (Roche, Mannheim, Germany) and incubated on ice for 30 min. Total anti-Pacrg serum or affinity-purified anti-Pacrg antibodies were used for Western blot analysis on testes and semen protein extracts at a 1:2,000 and 1:500 dilutions, respectively.

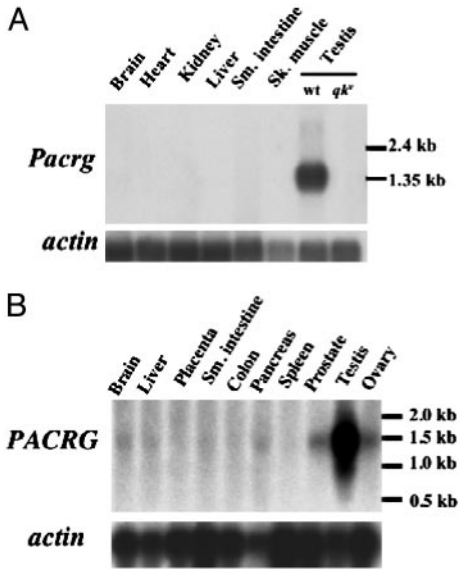

Fig. 1. *Pacrg* transcript is highly expressed in testis in both mouse and humans. (*A*) Northern blot on multiple mouse tissues shows that *Pacrg* mRNA is detected only wild-type testis (testis lane wt). The *Pacrg* mRNA is completely absent in testes from qk^v mutant animals (testis, qk^v/qk^v lane). (*Lower*) Hybridization with an actin probe as loading control. (*B*) Northern blot on human tissues shows that *PACRG* mRNA is most abundant in testis. Lower levels of expression are also detected in brain, liver, small intestine, pancreas, prostate, and ovaries. (*Lower*) Hybridization with an actin probe is shown as a loading control.

Immunolocalization of Pacrg was performed on methanol-fixed isolated spermatozoa essentially as described by Quill *et al.* (20). Anti-Pacrg serum was diluted 1:200 in PBS and 10% normal goat serum and incubated overnight at 4°C. After washing in 1× PBS, slides were incubated with an anti-rabbit Alexa Fluor 488-conjugated antibody (dilution 1:4,000, Molecular Probes). Slides were overlaid with Vectashield mounting medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole before observation under the microscope.

Results

***Pacrg* Transcript Is Highly Expressed in Mouse Testes.** To determine the tissue distribution of *Pacrg* mRNA in the mouse, we performed Northern blot analysis on total RNA from multiple tissues. The *Pacrg* mRNA is most abundant in mouse testes, where it could be detected with a short exposure time (≈5 h) of the Northern blot filter autoradiograph (Fig. 1*A*). In addition, RT-PCR amplification showed expression of the *Pacrg* transcript in brain, skeletal muscle, and kidney (data not shown). The qk^v deletion encompasses the entire *Pacrg* locus, thus, as expected, the *Pacrg* transcript is absent from testes as well as from all other tissues tested in qk^v homozygous animals (Fig. 1*A*, qk^v/qk^v testes lane, and data not shown). To compare the expression pattern of the mouse *Pacrg* with its human counterpart, we performed Northern blot analysis on a panel of human tissues by using a human *PACRG* cDNA probe (Fig. 1*B*). Similar to the mouse, *PACRG* mRNA was most abundant in the testes.

***Pacrg* Is Expressed in Germ Cells During Spermiogenesis.** Given the high levels of *Pacrg* mRNA detected in testes, we performed analyses aimed at determining both the developmental expres-

GENETICS

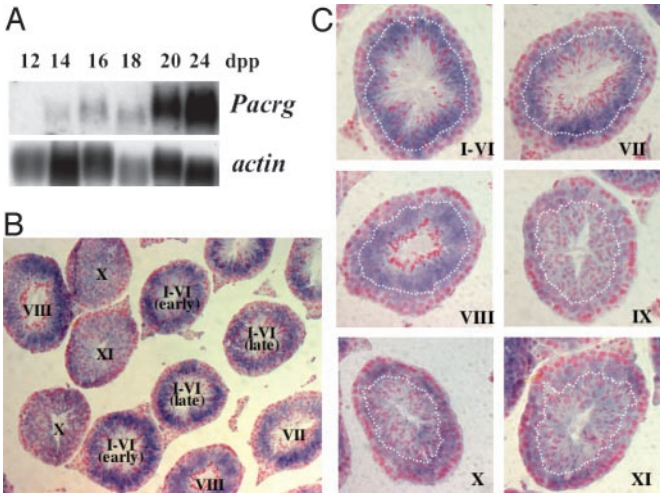

Fig. 2. *Pacrg* developmental and cellular expression during mouse spermatogenesis. (*A*) Northern blot analysis of *Pacrg* in neonatal testes shows the onset of expression of *Pacrg* at 14 dpp. Higher levels of *Pacrg* transcript are detected from 20 dpp onward, when sperm differentiation initiates. (*Lower*) Hybridization with an actin probe as loading control. (*B*) *In situ* hybridization analysis by using a *Pacrg* RNA probe on adult mouse testis shows expression in the seminiferous epithelium. Roman numerals indicate the stage(s) assigned to each seminiferous tubule (×200 magnification). (*C*) Cellular localization of *Pacrg* mRNA by *in situ* hybridization on testis seminiferous tubules at different stages (indicated by Roman numerals in the bottom right corner, ×400 magnification). The dotted curves delineate the approximate boundary between the spermatocyte (toward the basal membrane) and the spermatid cellular layers (toward the lumen) in each of the stages showed.

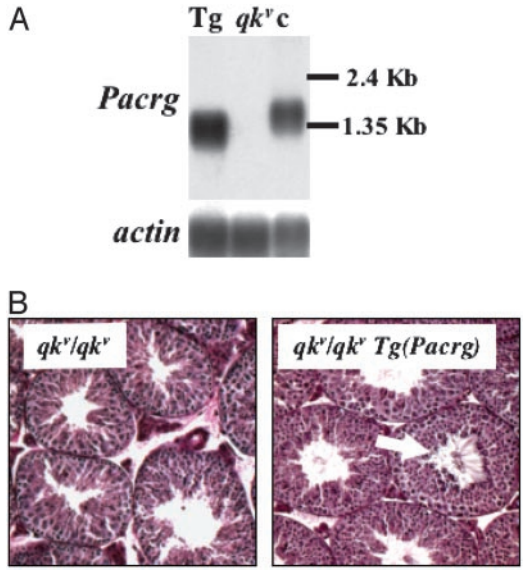

Fig. 3. Transgenic expression of *Pacrg* restores spermiogenesis in qk^v males. (*A*) Northern blot analysis on testis total RNA from qk^v/qk^v *Tg*(*Pacrg*) transgenic (Tg), qk^v/qk^v mutant (qk^v), and unaffected control mice (c). (*Lower*) Hybridization with an actin cDNA probe as a loading control. (*B*) Periodic acid Schiff staining of testes sections from qk^v/qk^v mutant and from a qk^v/qk^v *Tg*(*Pacrg*) transgenic littermate. The arrow points to sperm tails in the lumen of the seminiferous tubules in the qk^v/qk^v *Tg*(*Pacrg*) testes.

sion and cellular distribution of the *Pacrg* transcript in this tissue. The *Pacrg* transcript was absent from the testes of Odd sex mutant males, which completely lack germ cells (21), suggesting that *Pacrg* is predominantly expressed in this cell type (data not shown).

The developmental expression pattern of *Pacrg* in the postnatal period was determined by Northern blot analysis on testes of newborn mice starting at 2 days postpartum (dpp) up to 28 dpp. *Pacrg* mRNA was first detected in 14-dpp testes (Fig. 2*A*). This time point in the mouse developing testis corresponds to the initiation of pachytene phase of meiosis in spermatocytes (22). The level of expression of *Pacrg* appears to reach its maximum at 20 dpp, when spermatids first appear, and then remains constant at later ages (Fig. 2*A* and data not shown). The cellular distribution of the *Pacrg* mRNA in adult testes was determined by *in situ* hybridization experiments. *Pacrg* is expressed only in the spermatogenic cells of the testis and it is not present in interstitial cells or in the epididymis (Fig. 2*B* and data not shown). The highest *Pacrg* expression is detected in seminiferous tubules at stages I–VI (Fig. 2*B*). The *Pacrg* transcript is most abundant in the round spermatids, but it is also present at lower levels in pachytene spermatocytes (Fig. 2*C*, stages I–VI). Expression of *Pacrg* gradually decreases from stage VII onward, as nuclear condensation proceeds in elongating spermatids. *Pacrg* expression appears to be at its lowest level at stage IX, and gradually increases in pachytene spermatocytes at subsequent stages (Fig. 2*C*). The tissue distribution as well as the developmental expression pattern of *Pacrg* is highly consistent with a potential role in the spermiogenesis defect associated with the qk^v mutation.

Transgenic Expression of *Pacrg* Restores Fertility in qk^v Mutant Males. To determine whether the deletion of *Pacrg* is responsible for the spermiogenesis defect in the qk^v mutant, we attempted to restore its function in the testes by transgenic expression in sterile qk^v males. Given the large genomic interval covered by the *Pacrg*-coding region (14), expression of the *Pacrg* transcript cannot be achieved by generating transgenic mouse lines harboring single bacterial artificial chromosome clones. We therefore assembled a transgenic construct containing the *Pacrg* cDNA under the control of the testis-specific promoter of the human phosphoglycerate kinase 2 (*PGK2*) gene. This promoter has been previously used to drive expression of transgenes in the male germ line at developmental time points similar to *Pacrg* (18). Four independent transgenic lines carrying the *PGK2*-driven *Pacrg* minigene: *Tg*(*Pacrg*)*6Jus*, *Tg*(*Pacrg*)*15Jus*, *Tg*(*Pacrg*)*16Jus*, and *Tg*(*Pacrg*)*21Jus*, were established by crossing founder animals to mice heterozygous for the qk^v deletion. Because each of these lines had similar levels of expression of the transgene and produced similar results, they are hereafter referred to collectively as *Tg*(*Pacrg*). The offspring of these matings were then backcrossed to mice carrying the qk^v deletion to generate qk^v homozygous animals carrying the *PGK2-Pacrg* transgene [qk^v/qk^v *Tg*(*Pacrg*)]. Northern blot analysis showed that the *PGK2-Pacrg* minigene is expressed in testes of qk^v mutant mice at similar levels in all four transgenic *Tg*(*Pacrg*) lines (Fig. 3*A* and data not shown). The *PGK2-Pacrg* transgene produces a shorter transcript compared with its endogenous counterpart because the 5′ and 3′ UTRs of the *Pacrg* cDNA were not included in the minigene construct (Fig. 3*A*).

Semen collected from the genital tracts of qk^v/qk^v *Tg*(*Pacrg*) male mice contained numerous motile spermatozoa, whereas only rare abnormal sperm were seen in the semen of nontransgenic qk^v/qk^v males (data not shown). Accordingly, histological analysis on testes from qk^v/qk^v *Tg*(*Pacrg*) males showed several seminiferous tubules with flagella in their lumen, indicating that transgenic expression of *Pacrg* restores spermiogenesis in qk^v/qk^v mutants (Fig. 3*B*). Sperm titers were compared between qk^v/qk^v *Tg*(*Pacrg*) and qk^v heterozygous or wild-type littermates. Sperm counts from qk^v/qk^v *Tg*(*Pacrg*) males showed an ≈30% reduction compared with control littermates ($qk^v/+$ or wild-type: $3.07 \pm 0.25 \times 10^6$ sperm per ml, $n = 13$; qk^v/qk^v *Tg*(*Pacrg*):

Table 1. Fertility test on qk^v/qk^v *Tg* (*Pacrg*) transgenic males

Genotype	Fertile males (tested)	No. of litters	Pups per litter (mean ± SEM)
qk^v/qk^v *Tg* (*Pacrg*)*	10 ($n = 10$)	30	10.85 ± 0.37†
qk^v/qk^v	0 ($n = 7$)	0	0
$qk^v/+$ or wt	11 ($n = 11$)	31	10.57 ± 0.5†

*At least two animals for each of the four *Pacrg* transgenic lines were tested.
†$P > 0.05$, *t* test for independent samples ($t = 0.4628$, df = 17).

$2.14 \pm 0.16 \times 10^6$ sperm per ml, $n = 19$, $P < 0.005$, two-tailed *t* test). This moderate reduction in sperm count in the transgenic animals may be the result of the expression of *Pacrg* under the control of an exogenous human promoter.

To determine whether the *PGK2-Pacrg* transgene could rescue male sterility in qk^v/qk^v mutants, we performed fertility tests on qk^v/qk^v *Tg*(*Pacrg*) transgenic males, qk^v/qk^v males and control nontransgenic littermates. All of the qk^v/qk^v *Tg*(*Pacrg*) males tested were fertile and sired a number of litters similar to the control males (Table 1). In addition, the average litter size did not differ significantly between qk^v/qk^v *Tg*(*Pacrg*) and control fertile males (Table 1). As expected, no offspring were produced from the matings of qk^v/qk^v males.

Pacrg Is Present in Mature Spermatozoa. To gain insight into the cellular function that *Pacrg* plays in spermiogenesis, we raised polyclonal antibody against peptides derived from the mouse Pacrg-predicted amino acid sequence. Western blot analysis using an anti-Pacrg serum on protein extracts of wild-type testis detected a band of ≈28 kDa, corresponding to the predicted molecular weight of Pacrg (Fig. 4*A*, testis). This 28-kDa species was absent in testis protein extracts from qk^v/qk^v mutant animals, indicating that this band corresponds to Pacrg (Fig. 4*A*, testis). A 50-kDa cross-reacting band was also detected in both wild-type and mutant testes (Fig. 4*A*, testis). The presence of this unidentified cross-reacting species hampered attempts to unequivocally determine the localization of the Pacrg protein by immunohistochemistry in testes sections (data not shown). However, when Western blot analysis was performed on sperm extracts from wild-type animals by using anti-Pacrg antiserum, a single 28-kDa band corresponding to Pacrg was detected (Fig. 4*A*, sperm representation). To determine to which sperm structure the Pacrg protein localizes, we performed immunofluorescence on isolated spermatozoa by using anti-Pacrg serum. A signal was detected in the postacrosomal region of the sperm head, directly underlying the nucleus (Fig. 4*B*, anti-Pacrg representation, arrowheads in *Inset*). Also, the anti-Pacrg antibodies stained the midpiece of the spermatozoa flagellum (Fig. 4*B*, anti-Pacrg, arrows in *Inset*). No fluorescent signal in these structures was detected when the anti-Pacrg serum was preabsorbed with the immunizing peptides (Fig. 4*B*, preabsorbed).

Pacrg Is Evolutionarily Conserved in Flagellated Protozoa. The conceptual translation of the mouse *Pacrg* cDNA (GenBank accession no. AK005771) yields a 241-aa protein that does not display any sequence similarities with proteins of known function. Searches of the publicly available databases identified several gene products with significant sequence similarity to *Pacrg* in metazoan species including *Caenorhabditis elegans*, *Drosophila*, Zebrafish, and *Xenopus* (see Fig. 6, which is published as supporting information on the PNAS web site). Sequence alignment of Pacrg-like proteins showed that a putative C-terminal prenylation site previously described in

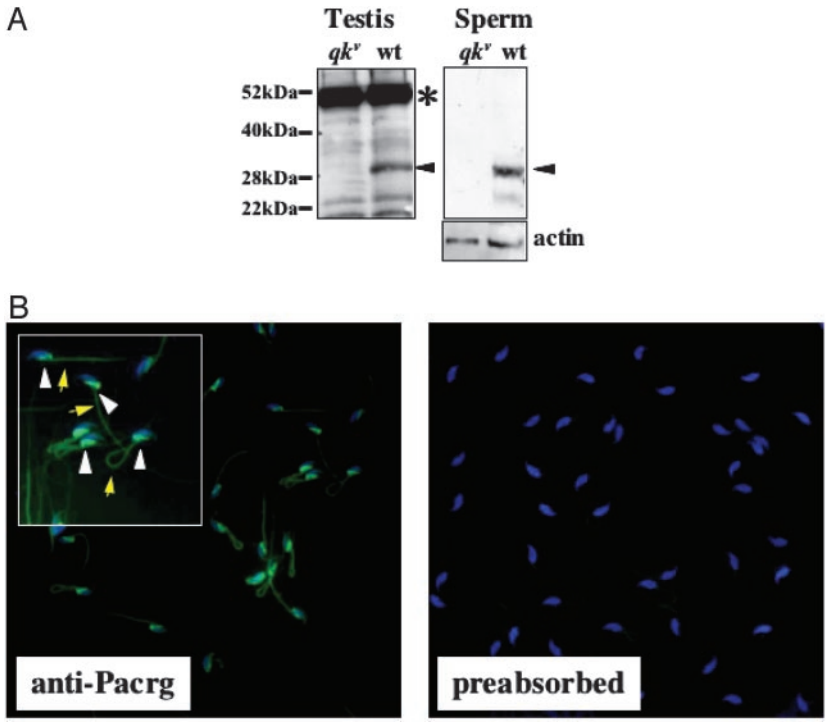

Fig. 4. The Pacrg protein is present in mature spermatozoa. (*A*) Western blot analysis on protein extracts from testis (*Left*) and isolated sperm (*Right*) from mutant (qk^v) or control wild-type (wt) animals. The arrowheads identify the 28-kDa band corresponding to Pacrg in both testes and sperm; the asterisk indicates the cross-reacting band present in the testis extract. (*B*) Immunofluorescence on methanol-fixed epididymal spermatozoa with anti-Pacrg antiserum (*Left*, ×200 magnification; *Inset*, ×400 magnification). White arrowheads and yellow arrows identify the Pacrg staining (green fluorescence) in the postacrosomal region and in the midpiece of the sperm flagellum, respectively. No specific fluorescence is detected in sperm incubated with anti-Pacrg sera preabsorbed with 5 μg/ml of the antigen peptides (*Right*). Sperm were counterstained for nucleic acids with 4′,6-diamidino-2-phenylindole (blue fluorescence).

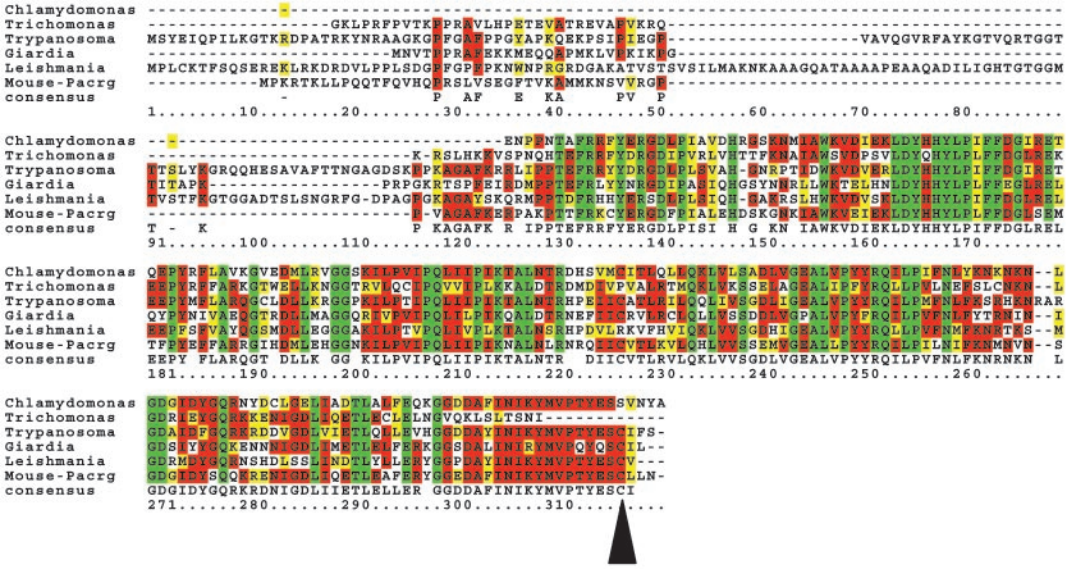

Fig. 5. Pacrg is evolutionarily conserved in protozoan species. Protein sequence alignment of mouse Pacrg protein with gene products from protozoa. Residues conserved in the majority of sequences are highlighted in red, conservative amino acid substitutions are highlighted in yellow, and residues identical in all sequences are highlighted in green. The consensus sequence is shown in the lower line of the alignment. The arrowhead indicates the partially conserved cysteine residue within the putative prenylation motif. The protein sequences were obtained by conceptual translation of DNA GenBank and dbEST database entries from each species, corresponding to the following accession nos.: Mouse, AK005771; Chlamydomonas, BG856901; Trichomonas, CD664326; Trypanosoma, AC091330; *Giardia*, BAB56145; Leishmania, AAF69760.1. Alignment was generated by using CLUSTALW version 1.8 (see *Materials and Methods*).

mammalian Pacrg,[¶] is completely conserved in all metazoan species (see Fig. 6). Remarkably, the mammalian Pacrg also showed sequence similarity with gene products from flagellated protozoa (Fig. 5). All of the protozoan Pacrg-like proteins display at least 50% identity at the amino acid level with the mammalian Pacrg. The C-terminal prenylation motif is conserved in most but not all of the protozoan Pacrg proteins (Fig. 5), presumably as the result of incomplete or inaccurate EST sequences from some of these species. In addition to the prenylation site, other highly conserved domains were detected in Pacrg throughout all species, which include amino acid motifs found in proteins that are targeted to cellular organelles (see Fig. 6).

Discussion

In this study, we show that the deletion of the mouse *Pacrg* locus is the cause of male sterility in the qk^{v} mutant. This result confirms the hypothesis that the mutation of a gene distinct from *qk* is responsible for the spermiogenesis defect associated with the qk^{v} deletion (11). The transgenic expression of *Pacrg* in testis is necessary and sufficient to rescue the severe oligospermia associated with the qk^{v} mutation and restores normal fertility in mutant males. Therefore, our work also implies that the deletion of the *Parkin* gene does not play a role in the infertility of qk^{v} males. In further support of this conclusion, mice lacking *Parkin* have been reported to be fertile (23, 24). In mice as in humans, *Pacrg* and *Parkin* are closely linked and share a bidirectional minimal promoter (25). It has been therefore proposed that *PACRG* and *PARKIN* may be functionally related and may both have role in the pathogenesis of Parkinson's disease (25). However, qk^{v} mutants do not display any Parkinson's disease-like neuropathology (14), suggesting that like *Parkin*, *Pacrg* is not necessary for the survival of dopaminergic neurons in the mouse brain (23, 24).

The *Pacrg* mRNA is highly expressed in the male mouse germ cells and, consistent with its role in sperm differentiation, *Pacrg* is abundant during the postmeiotic phase of spermatogenesis, in the round and elongating spermatids. *Pacrg* is also expressed in several mouse tissues other than testes, although at much lower levels (data not shown). Confirming previous reports (25, 26), our Northern blot analysis detected human *PACRG* mRNA in several tissues, including the brain. However, our study shows that the *PACRG* transcript is most abundant in testes, indicating that this gene may play a role in human spermiogenesis as well.

We show that the mouse Pacrg protein is a component of mature sperm and that it localizes to the postacrosomal region of the sperm head and to the flagellum midpiece. In qk^{v} mutants, morphological abnormalities in both of these structures have been described (2). Abnormally shaped sperm heads are evident in qk^{v} animals in late spermiogenesis at the onset of chromatin condensation. In mutant spermatids condensation proceeds normally, but the membrane structures surrounding the nucleus fail to conform to the changing shape of the chromatin. Also contributing to the abnormal head morphology in qk^{v} spermatids are frequent finger-shaped intrusions of Sertoli cell cytoplasm. Pacrg has been reported to bind to tubulins (26), suggesting that it may be associated with microtubular cytoskeletal elements. All metazoan homologues of Pacrg contain a potential binding site for a lipid moiety as well as other evolutionary conserved amino acid motifs, indicating that Pacrg may associate with cellular organelles (see also Fig. 6). In agreement with both of these observations, Pacrg appears to localize to axonal processes and cytoplasmic vesicles in neuronal cells (26). It is possible that in the testis Pacrg may be necessary for the proper association between the membrane structures (i.e., plasma membrane and nuclear envelope) and the microtubular elements of the spermatid (i.e., manchette and nuclear ring), which are thought to be involved in head shaping during spermiogenesis. Interestingly, mutation of the *Hook1* mouse gene, which encodes for a protein known to interact with both microtubules and cellular organelles, results in sperm head defects that resemble those described in qk^{v} (27).

[¶]Imai, Y., Soda, M., Kataoka, A. & Takahashi, R., 33rd Annual Meeting Society for Neuroscience, Nov. 8–12, 2003, New Orleans, abstr. 78.3.

In addition to the sperm head malformations, qk^v mutants display a marked flagellar defect. In qk^v spermatids the flagellum begins to develop normally but the microtubular filaments fail to maintain their regular 9 plus 2 arrangement at late stages of spermiogenesis. This finding suggests that Pacrg may be also essential for the assembly or the maintenance of the axonemal microtubule array in the developing flagellum. In support of this hypothesis, we report that the Pacrg proteins display significant sequence similarities with gene products in several flagellated protozoan species (Fig. 5). Therefore, the mammalian Pacrg may have evolved from an ancestral protein necessary for flagellar development in monocellular organisms. Structural and functional conservation of flagellar components between protozoa and mammals has been described. For instance, mice lacking Sperm antigen 6, the mammalian homologue of the *Chlamydomonas* axonemal protein PF16, are sterile due to sperm flagellar abnormalities (28). Ultrastructural localization of Pacrg in the developing sperm as well as additional biochemical analyses will be needed to confirm its function(s) in spermiogenesis.

In conclusion, we have shown that *Pacrg* is necessary for spermiogenesis and male fertility in mice. This is the first report, to our knowledge, to describe the function of Pacrg *in vivo* in any model organism. Because it is likely that the role of *Pacrg* in spermiogenesis may be conserved in other mammalian species, including humans, the understanding of its cellular function may also help elucidate the molecular basis for some infertility cases in men. Furthermore, its conservation in monocellular organisms may reveal an essential role for Pacrg in flagellar development throughout evolution.

We thank Cavatina Truong for technical help with the *in situ* experiments and Dr. Marvin Meistrich for assistance with the staging of testes sections and for ongoing discussions. This work was supported by a grant from the Kleberg Foundation and by Public Health Service Grant U01 HD39372 (to M.J.J.).

1. Sidman, R. L., Dickie, M. M. & Appel, S. H. (1964) *Science* **144,** 309–311.
2. Bennett, W. I., Gall, A. M., Southard, J. L. & Sidman, R. L. (1971) *Biol. Reprod.* **5,** 30–58.
3. Yanagimachi, R., Wakayama, T., Kishikawa, H., Fimia, G. M., Monaco, L. & Sassone-Corsi, P. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 1691–1695.
4. Ebersole, T., Rho, O. & Artzt, K. (1992) *Genetics* **131,** 183–190.
5. Cox, R. D., Shedlovsky, A., Hamvas, R., Goldsworthy, M., Whittington, J., Connelly, C. S., Dove, W. F. & Lehrach, H. (1994) *Genomics* **21,** 77–84.
6. Wu, J. I., Reed, R. B., Grabowski, P. J. & Artzt, K. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 4233–4238.
7. Lakiza, O., Villavicencio, E., Walterhouse, D., Goodwin, E. B. & Innacone, P. (2003) *Dev. Biol.* **259,** 522.
8. Ebersole, T. A., Chen, Q., Justice, M. J. & Artzt, K. (1996) *Nat. Genet.* **12,** 260–265.
9. Hardy, R. J., Loushin, C. L., Friedrich, V. L., Jr., Chen, Q., Ebersole, T. A., Lazzarini, R. A. & Artzt, K. (1996) *J. Neurosci.* **16,** 7941–7949.
10. Lu, Z., Zhang, Y., Ku, L., Wang, H., Ahmadian, A. & Feng, Y. (2003) *Nucleic Acids Res.* **31,** 4616–4624.
11. Justice, M. J. & Bode, V. C. (1988) *Genet. Res.* **51,** 95–102.
12. Shedlovsky, A., King, T. R. & Dove, W. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 180–184.
13. Cox, R. D., Hugill, A., Shedlovsky, A., Noveroske, J. K., Best, S., Justice, M. J., Lehrach, H. & Dove, W. F. (1999) *Genomics* **57,** 333–341.
14. Lorenzetti, D., Antalffy, B., Vogel, H., Noveroske, J. K., Armstrong, D. & Justice, M. J. (2004) *Mamm. Genome* **15,** 210–217.
15. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. & Shimizu, N. (1998) *Nature* **392,** 605–608.
16. Lockhart, P., O'Farrell, C. & Farrer, M. (2004) *Movement Disorders* **19,** 101–104.
17. Lu, B. & Bishop, C. E. (2003) *J. Biol. Chem.* **278,** 16289–16296.
18. Robinson, M. O., McCarrey, J. R. & Simon, M. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8437–8441.
19. Clark, A. T., Firozi, K. & Justice, M. J. (2004) *Biol. Reprod.* **70,** 1317–1324.
20. Quill, T. A., Ren, D., Clapham, D. E. & Garbers, D. L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 12527–12531.
21. Bishop, C. E., Whitworth, D. J., Qin, Y., Agoulnik, A. I., Agoulnik, I. U., Harrison, W. R., Behringer, R. R. & Overbeek, P. A. (2000) *Nat. Genet.* **26,** 490–494.
22. McCarrey, J. R. (1993) in *Cell and Molecular Biology of the Testis*, eds. Desjardins, C. & Ewing, L. L. (Oxford Univ. Press, New York), pp. 58–89.
23. Goldberg, M. S., Fleming, S. M., Palacino, J. J., Cepeda, C., Lam, H. A., Bhatnagar, A., Meloni, E. G., Wu, N., Ackerson, L. C., Klapstein, G. J., *et al.* (2003) *J. Biol. Chem.* **278,** 42628–42635.
24. Itier, J. M., Ibanez, P., Mena, M. A., Abbas, N., Cohen-Salmon, C., Bohme, G. A., Laville, M., Pratt, J., Corti, O., Pradier, L., *et al.* (2003) *Hum. Mol. Genet.* **12,** 2277–2291.
25. West, A. B., Lockhart, P. J., O'Farell, C. & Farrer, M. J. (2003) *J. Mol. Biol.* **326,** 11–19.
26. Imai, Y., Soda, M., Murakami, T., Shoji, M., Abe, K. & Takahashi, R. (2003) *J. Biol. Chem.* **278,** 51901–51910.
27. Mendoza-Lujambio, I., Burfeind, P., Dixkens, C., Meinhardt, A., Hoyer-Fender, S., Engel, W. & Neesen, J. (2002) *Hum. Mol. Genet.* **11,** 1647–1658.
28. Sapiro, R., Kostetskii, I., Olds-Clarke, P., Gerton, G. L., Radice, G. L. & Strauss, I. J. (2002) *Mol. Cell. Biol.* **22,** 6298–6305.