Insertional Mutation of the Murine Kisimo Locus Caused a Defect in Spermatogenesis*

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Spermatogenesis is a developmental process that occurs in several phases and is regulated by a large number of gene products. An insertional transgenic mouse mutant (termed kisimo mouse) has been isolated that results in abnormal germ-cell development, showing abnormal elongated spermatids in the lumina of seminiferous tubules. We cloned the disrupted locus of kisimo and identified a novel testis-specific gene, THEG, which is specifically expressed in spermatids and was disrupted in the transgenic mouse. The yeast two-hybrid screening method revealed that THEG protein strongly interacts with chaperonin containing t-complex polypeptide-1, suggesting that THEG protein functions as a regulatory factor in protein assembly. Our findings indicate that the kisimo locus is essential for the maintenance of spermiogenesis and that a gene expression disorder may be involved in male infertility.

The integration of foreign DNA into the mouse germ line by retroviral infection or microinjection can result in insertional disruption of endogenous genes with important roles in development (1–3). Foreign DNA insertion provides an approach to the cloning of disrupted host loci. By using the introduced DNA as a probe to screen genomic libraries from mutant animals, it has been possible in a few instances to isolate clones that contain DNA flanking the exogenous integrated material and, thus, include portions of the interrupted gene (4, 5). We have produced a series of 12 transgenic mouse lines with the human phosphodiesterase 5A (PDE5A)† gene (6). As the mice were bred, it became evident that many males of one line were sterile and that the sterility arose from a defect in spermatogenesis (we named this mutant kisimo for a Japanese goddess of easy delivery). Because the sterility segregated with the hemizygous transgene and occurred in the absence of the detectable expression of the transgene, we concluded that the abnormal phenotype was due to mutation by insertion of the transgene. In this study, to clone the junctions between the inserted transgene and adjoining cellular DNA, we used thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (7) and defined a genomic locus important for spermatogenesis.

The process of spermatogenesis in the mouse has been well characterized at the morphological level. The spermatogenic process can be subdivided into three main phases. Spermatogonia, the germinal stem cells, undergo mitosis to produce additional spermatogonia, a portion of which develop into primary spermatocytes. The spermatocytes enter meiosis and proceed through two cell divisions to give rise to haploid round spermatids. These, in turn, undergo a complex morphological transformation involving nuclear condensation and elongation resulting in the production of mature spermatozoa. However, at the molecular level, relatively little is known about the control of cellular differentiation and the architectural changes during spermatogenesis.

In this study, we found that an insertion of foreign DNA results in abnormal germ-cell development, showing abnormal elongated spermatids in the lumina of seminiferous tubules with severely abnormal or absent flagella, and that a novel testis-specific gene was disrupted in the transgenic mouse. This novel mouse autosomal recessive mutant exhibited a phenotype similar to asthenoospermia and provides us an approach to understand the mechanisms underlying the formation of flagella during spermiogenesis.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). 293 cells were from Dainippon Pharmaceutical Co. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were obtained from Life Technologies, Inc. [γ-32P]ATP was from Amersham Pharmacia Biotech. The MATCHMAKER II two-hybrid system and the mouse testis MATCHMAKER cDNA library were obtained from CLONTECH.

Transgenic Mice—A DNA fragment carrying the full-length human PDE5A cDNA downstream of cytomegalovirus promoter was generated by digestion with PvuI. The DNA was used to inject the male pronuclei of (C3H x C3H)F1 embryos, by standard techniques. The transgenic mice were selected after screening tail DNA by dot or Southern blot analyses with 32P-labeled human PDE5A cDNA.

Historical and Electron-microscopic Examinations—Five male ki/ki mice aged 12 weeks were examined histologically together with age- and sex-matched +/+ mice. Organs were excised, fixed in Bouin’s solution, embedded in paraffin, sectioned in 4-μm slices, and stained with hematoxylin/eosin. The testes were also stained with periodic acid-Schiff, and quantitative evaluation of spermatogenic cells was performed using the simplified morphological method (8). For electron-microscopic examination, testes pieces of ki/ki and +/+ mice were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 and post-fixed in buffered 1% osmium. The fixed samples were dehydrated and embedded in an epoxy resin. Ultrathin sections were prepared and stained with lead citrate and uranyl acetate. The specimen was examined by a transmission electron microscope (JEM-100CX, JEOL).

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‡ The abbreviations used are: PDE, phosphodiesterase; THEG, testicular haploid expressed gene; TCP, t-complex polypeptide; CCT, chaperonin containing TCP-1; TAIL-PCR, thermal asymmetric interlaced polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; ki, kisimo; FISH, fluorescence in situ hybridization; kb, kilobase; CREM, cAMP-responsive element modulator.
Mouse cDNAs were cloned from mouse testis cDNA library (CLONTECH) by screening 5×10^5 plaques with 32P-labeled mouse genome DNA obtained by TAIL-PCR as a probe according to the protocol originally developed by Liu and Whittier for chromosomal walking (7). Four relatively short arbitrary degenerate primers were designed according to the Kisimo Locus Required for Spermatogenesis (ki/ki) and Crem (crem) testis-specific probes corresponding to protamine 2 (pm2) or Crem (crem) full-length mouse cDNA as a probe. Library screening was performed as described previously (11). Digoxigenin-labeled cRNA probes (antisense and sense) were made by in vitro transcription using cDNAs subcloned into pGEM-T vector (Promega) as templates in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals) according to the manufacturer's instructions. Yeast Two-hybrid Screening—The yeast two-hybrid screening was performed as described by the manufacturer. A cDNA encoding full-length mouse THEG was subcloned into the yeast expression vector pAS2-1 (CLONTECH) fused in frame with the DNA binding domain of yeast transcriptional activator GAL4, which generated pAS2-1-mTHEG1b. The yeast strain Y190 was co-transformed with pAS2-1-mTHEG1b and the mouse testis cDNA libraries in pGADT7 using the lithium acetate method. Transformants were selected on synthetic dropout agar plates lacking tryptophan, leucine, and histidine but including 25 μg/ml 3-amino-triazole. Yeast colonies were transferred onto nylon membrane and processed by the β-galactosidase filter assay. Plasmid DNA was isolated from positive colony and re-transformed into the yeast strain Y190 with either pAS2-1 empty vector or pAS2-1-mTHEG1b. The β-galactosidase assay was again conducted to ensure the THEG-dependent activity.

In Vivo Binding Analysis—293 cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO2. The full-length mouse THEG cDNA in the expression vector pFLAG-CMV-2 (Eastman Kodak Co.) and/or the full-length mouse Crem cDNA in pDNA3.1/HisA (Invitrogen) were transiently expressed in 293 cells using LipofectAMINE PLUS reagent as described by the manufacturer (Life Technologies, Inc.). Cells were washed with ice-cold phosphate-buffered saline 24 h after transfection and scraped in an ice-cold TNE buffer (10 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 10 mg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM dithiothreitol). Cell extracts were centrifuged at 16,000 × g for 15 min at 4 °C to remove cellular debris and immunoprecipitated with anti-Xpress polyclonal antibody (Invitrogen) with protein G-Sepharose. The beads were washed five times with TNE buffer, and immune complexes were further analyzed by immunoblotting with anti-FLAG M5 monoclonal antibody (Kodak) as described previously (12).

RESULTS AND DISCUSSION

All twelve independent transgenic lines carrying the human PDE5A gene (6) were bred to homozgyosity to screen for recessive insertion mutations. One of these mice transmitted the transgene to its progeny, but when they were intercrossed the resulting homozygotes (ki/ki mice) were infertile. The average testis weight of ki/ki mice (46.9 ± 8.2 mg; n = 5) was 60% that of wild-type littermates (78.0 ± 6.3 mg; n = 5) at 12 weeks of age. In wild and heterozygous mice, none of the spermatogenic cell types at stages II-III, V, VII, and XI showed either pathological or quantitative changes. On the contrary, ki/ki mice had virtually no spermatozoa in the lumina of seminiferous and epididymal tubules. As shown in Fig. 1B, elongated spermatids exposed to x-ray film at −70 °C for 2 days. In situ hybridization using digoxigenin-labeled probes was performed as described previously (11). Digoxigenin-labeled cRNA probes (antisense and sense) were made by in vitro transcription using cDNAs subcloned into pGEM-T vector (Promega) as templates in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals) according to the manufacturer's instructions. Yeast Two-hybrid Screening—The yeast two-hybrid screening was performed as described by the manufacturer. A cDNA encoding full-length mouse THEG was subcloned into the yeast expression vector pAS2-1 (CLONTECH) fused in frame with the DNA binding domain of yeast transcriptional activator GAL4, which generated pAS2-1-mTHEG1b. The yeast strain Y190 was co-transformed with pAS2-1-mTHEG1b and the mouse testis cDNA libraries in pGADT7 using the lithium acetate method. Transformants were selected on synthetic dropout agar plates lacking tryptophan, leucine, and histidine but including 25 μg/ml 3-amino-triazole. Yeast colonies were transferred onto nylon membrane and processed by the β-galactosidase filter assay. Plasmid DNA was isolated from positive colony and re-transformed into the yeast strain Y190 with either pAS2-1 empty vector or pAS2-1-mTHEG1b. The β-galactosidase assay was again conducted to ensure the THEG-dependent activity.

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in the vicinity of the lumina of seminiferous tubules showed vacuolation and were occasionally phagocytosed by Sertoli cells. Several studies on male mice that were sterile because of blocked spermatogenesis have demonstrated the appearance of multinucleated giant cells and numerous spermatocytes undergoing apoptotic cell death (13, 14). However, analysis of cross-sections of these testes by Tdt-mediated dUTP-biotin nick-end labeling assay showed the absence of apoptotic cells in the lumina of seminiferous tubules from ki/ki mice (data not shown). Further characterization of spermatids by electron microscopy revealed the elongated spermatids to have abnormal or completely nonexistent flagella. In the elongated spermatids of wild-type mice, the axoneme was composed of microtubules emanating straight from the centriole at the base of the spermatid nucleus (Fig. 1C). On the contrary, the microtubules and coarse fibers were arranged in a whir, the nuclei were misshapen, and the cytoplasmic electron density was increased in elongated spermatids of ki/ki mice (Fig. 1D). Intracytoplasmic vacuoles and autophagolysosomes were increased in elongated and some round spermatids. In addition, in the quantitative evaluation of spermatogenic cells in seminiferous tubules, elongated spermatids were significantly decreased at all stages (stage VII is shown in Fig. 1E). Neither the number of spermatogonia nor that of spermatocytes exhibited significant differences. Furthermore, we analyzed testis RNAs derived from wild-type and ki/ki mice for markers of testis development, post-meiotic-specific cAMP-responsive element modulator (CREM), and haploid-specific protamine 2 transcripts. Neither CREM nor protamine 2 RNA levels were significantly affected by the insertional mutation (Fig. 2C), also indicating that spermatogenesis was not suppressed in the proliferative and meiotic phases.

Several copies of the transgene were integrated into the ki/ki genome (data not shown). The chromosomal localization of the transgene insertion site was determined by FISH using the whole transgene as a probe, which resulted in a single pair of symmetrical signals mapped to the C1 region of chromosome 10, showing multiple copies of the transgene to be integrated in tandem at a single locus (Fig. 2B). To clone the junctions between the inserted transgene and adjoining cellular DNA, we employed TAIL-PCR by using degenerated primers and specific primers corresponding to the nucleotide sequence of the transgene. One junction fragment containing DNA flanking the transgene insertion site was identified. A wild-type mouse genomic phage library was screened using the flanking DNA fragment as a probe. These isolated phage clones covered about 25 kb of the mouse genome, showing that the integration of the transgene produced a deletion of about 20 kb in the genomic locus (Fig. 2A).

To identify exons in the genomic locus, Northern blot analysis was performed using several DNA fragments (3–6 kb) obtained by endonuclease digestion, covering the deleted region, as a probe. When using 5 kb of the KpnI/XhoI fragment as a probe, a 1.6-kb transcript was detected in the testes from wild-type mice. However, Northern blot analysis showing no signal in testis RNA from ki/ki mice revealed ki/ki mice to be a null strain for this transcript (Fig. 2C). Next, a mouse testis cDNA library was screened with the 5-kb KpnI/XhoI fragment to isolate the corresponding cDNA. We cloned three cDNAs generated by alternative splicing. The proteins predicted from these nucleotide sequences are 313, 351, and 375 amino acids long (Fig. 3A). In vitro transcription/translation analysis demonstrated that the mouse cDNAs encode corresponding proteins (data not shown). To isolate a human counterpart, a human testis cDNA library was screened at a reduced stringency using the mouse full-length cDNA as a probe. The isolated human cDNA encoded a 380-amino acid protein and showed 59.6% identity with the mouse protein (Fig. 3B). A search of the database using the nucleotide and amino acid sequences revealed that the isolated cDNA is identical to a novel TEG transcript (15). Expression of the TEG is highly specific to the testes of mice. To verify this in situ hybridization with a cDNA probe (Fig. 4B). In testis, the transcripts were detectable only in round and elongated spermatids, consistent with the abnormal spermiogenic process of ki/ki mice.

To investigate the potential functions of TEG protein and to determine the mechanism by which these functions are carried out, we employed the yeast two-hybrid system using TEG as bait and a mouse testis MATCHMAKER cDNA library. We found a single positive clone, CCE, that could interact with the TEG protein (Fig. 5A). To confirm this interaction between TEG protein and CCE in intact cells, we co-expressed a FLAG epitope-tagged TEG (FLAG-mTEG) with an Xpress epitope-tagged CCE (Xpress-CCE) in 293 cells. The ability of antibody against the Xpress epitope to precipitate a complex of TEG and CCE suggests that the two proteins interact in the cytoplasm (Fig. 5B). The TCP-1 gene is located in the mouse t-complex on chromosome 17 (16, 17). A
Fig. 4. Expression of THEG. A, Northern blot analysis probed with full-length human THEG cDNA. Each lane contained 2 μg of poly(A)+ RNA (human MTN blot, CLONTECH). B, In situ hybridization analysis of mouse THEG mRNA expression in testis. Antisense probe gave no signal in testis from ki/ki mice (data not shown).

Fig. 5. A, two-hybrid analysis of THEG and CCTe constructs, and cell extracts were immunoprecipitated (IP) with antibody specific to Xpress (IP: Xpress) and immunoblotted with anti-FLAG (Blot: FLAG). Total cell extracts were blotted with anti-Xpress or anti-FLAG antibody.

mouse t-complex mutation was discovered to produce a phenotype with a tail-less sperm and, to date, TCP-1 identical to the Kisimo Locus Required for Spermatogenesis.

References
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