The adenine nucleotide translocases (Ant) facilitate the transport of ADP and ATP by an antiport mechanism across the inner mitochondrial membrane, thus playing an essential role in cellular energy metabolism. We recently identified a novel member of the Ant family in mouse, Ant4, of which gene configuration as well as amino acid homology is well conserved among mammals. The conservation of Ant4 in mammals, along with the absence of Ant4 in nonmammalian species, suggests a unique and indispensable role for this ADP/ATP carrier in mammalian development. Of interest, in contrast to its paralog in nonmammalian species, Ant4 appears to be constitutively expressed in all tissues and seems to be an integral component of the mitochondrial permeability transition pore (9–11), although this function is still in question (12).

The Ant proteins are the most abundant proteins of the mitochondrial inner membrane and are comprised of 300–320 amino acid residues that form six transmembrane helices. The functional unit is likely a homodimer acting as a gated pore that channels single molecules of ADP and ATP (13). Until recently, it has been believed that humans possess three members of the ANT family of genes: ANT1 (SLC25A4), which is expressed primarily in the heart and skeletal muscle; ANT2 (SLC25A5), which is expressed in rapidly growing cells and is inducible; and ANT3 (SLC25A6), which appears to be constitutively expressed in all tissues (14, 15). In contrast, rodents were believed to possess only two members of the Ant family: Ant1, which is expressed at high levels in heart, skeletal muscle, and brain; and Ant2, which is expressed in all tissues but skeletal muscle (2). Mouse Ant2 is the ortholog of human ANT2 and seems to combine the functions of human ANT2 and ANT3 (16, 17). Genetic inactivation of Ant1 resulted in viable mice (3). However, these animals developed mitochondrial myopathy and severe exercise intolerance along with hypertrophic cardiomyopathy (3, 7). In humans, there is a clinical manifestation known as autosomal dominant progressive external ophthalmoplegia that is associated with ANT1 as well as TWINKLE and POLY mutation (18). This disorder is characterized molecularly by the accumulation of numerous mitochondrial DNA mutations and clinically by the appearance of external ophthalmoplegia, ptosis, and progressive skeletal muscle weakness (19). In the cases of ANT1 mutation, A114P, L98P, A90D, D104G, and V289M substitutions have been reported to be associated with autosomal dominant progressive external ophthalmoplegia (20). Gene disruption of Ant2 in mice appears to result in embryonic or perinatal lethality, although a detailed phenotype has not yet been published (www.patentdebate.com/PATAPP/20050091704). There

deoxynucleotidyltransferase-mediated dUTP nick end labeling: X-gal, 5-bromo-4-chloro-3-indoly]-β-D-galactopyranoside; DAB, diaminobenzidine.

Evolutionarily Conserved Mammalian Adenine Nucleotide Translocase 4 Is Essential for Spermatogenesis

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The adenine nucleotide translocase/translocator (Ant), also called ADP/ATP carrier, belongs to the mitochondrial solute carrier family, which supports a variety of transport activities across the mitochondrial inner membrane (1–8). The Ant proteins facilitate the exchange of ADP/ATP by an antiport mechanism across the inner membrane of the mitochondria, (1, 3, 4) and thus are considered to be essential for the utilization of ATP produced by oxidative respiration (1–4, 7). The Ant proteins are also thought to be an integral component of the mitochondrial permeability transition pore (9–11), although this function is still in question (12).

The Ant proteins are the most abundant proteins of the mitochondrial inner membrane and are comprised of 300–320 amino acid residues that form six transmembrane helices. The functional unit is likely a homodimer acting as a gated pore that channels single molecules of ADP and ATP (13). Until recently, it has been believed that humans possess three members of the ANT family of genes: ANT1 (SLC25A4), which is expressed primarily in the heart and skeletal muscle; ANT2 (SLC25A5), which is expressed in rapidly growing cells and is inducible; and ANT3 (SLC25A6), which appears to be constitutively expressed in all tissues (14, 15). In contrast, rodents were believed to possess only two members of the Ant family: Ant1, which is expressed at high levels in heart, skeletal muscle, and brain; and Ant2, which is expressed in all tissues but skeletal muscle (2). Mouse Ant2 is the ortholog of human ANT2 and seems to combine the functions of human ANT2 and ANT3 (16, 17). Genetic inactivation of Ant1 resulted in viable mice (3). However, these animals developed mitochondrial myopathy and severe exercise intolerance along with hypertrophic cardiomyopathy (3, 7). In humans, there is a clinical manifestation known as autosomal dominant progressive external ophthalmoplegia that is associated with ANT1 as well as TWINKLE and POLY mutation (18). This disorder is characterized molecularly by the accumulation of numerous mitochondrial DNA mutations and clinically by the appearance of external ophthalmoplegia, ptosis, and progressive skeletal muscle weakness (19). In the cases of ANT1 mutation, A114P, L98P, A90D, D104G, and V289M substitutions have been reported to be associated with autosomal dominant progressive external ophthalmoplegia (20). Gene disruption of Ant2 in mice appears to result in embryonic or perinatal lethality, although a detailed phenotype has not yet been published (www.patentdebate.com/PATAPP/20050091704). There

deoxynucleotidyltransferase-mediated dUTP nick end labeling: X-gal, 5-bromo-4-chloro-3-indoly]-β-D-galactopyranoside; DAB, diaminobenzidine.
have been no reports regarding ANT2 or ANT3 mutations in human.

Utilizing various approaches, we and others recently identified a novel member of the Ant family, Ant4, both in mouse and human (4, 5, 21). The mouse Ant4 gene was predicted to encode a 320-amino acid protein and shared amino acid sequence homology with the other mouse Ant proteins previously identified (70.1 and 69.1% overall amino acid identity to Ant1 and Ant2, respectively). The Ant4 gene also contained three tandem repeats of a domain of ~100 residues, each domain containing two transmembrane regions, a characteristic shared by all members of the Ant family (22). Dolce et al. (4) demonstrated that human ANT4 indeed localizes to mitochondria in cells and can actively exchange ADP for ATP by an electrogenic antipor mechanism in vitro. Of interest, the Ant4 gene is expressed selectively in the testis, both in mouse and human (5, 21). However, neither the exact expression pattern in testis nor the specific function of Ant4 has been determined. The present study demonstrates an indispensable role for Ant4 in male germ cell development in mice and discusses the molecular evolution of Ants in mammals.

**EXPERIMENTAL PROCEDURES**

**Immunostaining**—Testes were harvested from 6-week-old wild type or mutant male mice. All of the mice have been maintained under standard specific pathogen-free conditions, and the procedures performed on the mice were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee. The tissues were then fixed in a mild fixative (10% formalin) overnight with rocking. Following fixation the tissues were dehydrated using an organic solvent (PBS-Citasol). The tissues were then imbedded in paraffin and sectioned. Formalin-fixed, paraffin-embedded human testis tissue was obtained through the University of Florida Department of Pathology tissue bank. Use of human tissue was performed in accordance with IRB-approved protocols at the University of Florida. The tissues were rehydrated with organic solvent (PBS-Citasol) of decreasing concentrations. Deparaffinized and rehydrated 5-μm tissue sections were stained with rabbit polyclonal antibodies against mouse Ant4 (5) or a cleaved Caspase-3 (Cell Signaling Technologies, Danvers, MA). The slides were blocked for endogenous peroxidase activity and then unmasked in target retrieval solution (Dako, Carpenteria, CA). Antibody was applied at 1:600 (Ant4) or 1:200 (Caspase-3) for 1 h at room temperature prior to identification using the diaminobenzidine (DAB) Envision kit (Dako). An isotype and concentration matched negative control section was included for each tissue. The slides were counterstained with hematoxylin. For immunostaining, of human ANT4, rabbit polyclonal antibodies were raised against the N-terminal human ANT4 peptide (REPAK-KKAERKLFDCC) and purified through an affinity column using the same peptide (Sigma Genosys, The Woodlands, TX).

**Preparation of Stage-specific Spermatogenic Cells**—Stage-specific spermatogenic cells were prepared from neonatal, prepubertal, and adult CD-1 mice by sedimentation through a 2–4% bovine serum albumin “Sta Put” gradient at unit gravity as described previously (24, 44). Specifically, primitive type A spermatogonia were recovered from testis of male mice at 6 days postpartum. Similarly, type A and type B spermatogonia were recovered from males at 8 days postpartum; preleptotene, leptotene + zygotene, and juvenile pachytene spermatocytes were recovered from males at 18 days postpartum; and adult pachytene spermatocytes, round spermatids, and residual cytoplasmic bodies were recovered from males at 60+ days postpartum. The purities of recovered cell types were assessed on the basis of morphological characteristics when viewed under phase optics and were ≥85% for prospermatogonia, spermatogonia, and juvenile spermatocytes (preleptotene, leptotene plus zygotene, and juvenile pachytene) and ≥95% for adult pachytene spermatocytes and round spermatids (44).

**Real Time PCR**—Total RNA was extracted using the RNA aqueous kit (Ambion). cDNA was synthesized using the HiCapacity cDNA archive kit using random primers (Applied Biosystems, Foster City, CA). Real time PCR was performed using the TaqMan gene expression assay (Applied Biosystems) according to the manufacturer’s instructions. Each 20-μl reaction consisted of 10 μl of TaqMan Universal PCR Master Mix, No AmpErase-UNG, 1 μl of TaqMan gene expression assay mix, for β-actin (VIC-labeled), Ant4 (FAM-labeled), or Ant2 (FAM-labeled), and 9 μl of cDNA (50 ng) (primer sequences available upon request). The reactions were performed using Applied Biosystems 7900HT Fast Real Time PCR instrument. Gene expression analysis was performed using the comparative CT method using β-actin for normalization.

**Targeting Vector Construction**—The targeting vector was designed to replace exons 2–4 of the mouse Ant4 gene with an SV40 splicing donor/acceptor signal-IRES (internal ribosomal entry site)-βgal-and PGKneo (neomycin-resistant gene cassette driven by the PGK promoter) cassette of the pNf-SBN targeting vector. The targeting construct was generated by sequential subcloning of the 5’ homology arm, 3’ homology arm, and diphtheria toxin gene into the pNf-SBN vector. A 2.0-kb fragment containing exon 1 and a 5.3-kb fragment containing exons 5–6 was amplified from mouse embryonic stem (ES) cell (R1, 129/SvJ strain) genomic DNA and used as the source of 5’ and 3’ homologous arms for the targeting constructs, respectively. Targeting arms were amplified by LA Taq PCR system (Takara, Madison, WI) with the following primers Ant4–5′f (5’-CCGCTCGAGCTCTCATTTTGAATCTGGATCATG), Ant4–5′r (5’-GCGTGTCGACTGCGCCCTTGCATTCTCTCAAAACACC), Ant4–3′f (5’-CCGCTCGAGTAATTTGTAGCTTTAAAGTGG), and Ant4–3′r (5’-GCGTGTCGACTGCGCATATATTAAAAATTGGACTCTCGG). The homologous arms were cloned into pCR 2.1-TOPO vector (Invitrogen). Following excision from pCR2.1-TOPO vectors, the 5’ homologous arm was ligated into the Xhol site, and the 3’ homologous arm was ligated into the Sall site in the pNf-SBN targeting vector. To increase selection efficiency of positive clones, we inserted the negative selection gene (diphtheria toxin) into the Xhol site.

**Generation of Ant4−/− Mice**—The targeting vector was linearized with Sall digestion and transfected into J1 ES cells by electroporation as we previously described (23). Genomic DNA from ~450 G418-resistant colonies was screened, and homologous recombination in ES cells was confirmed by genomic Southern blotting as previously described (23). Upon initial Southern blot screening with a 5’ external probe followed by
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| TABLE 1 | Chromosomal location, number of exons, and deduced amino acid identities of the Ant1, 2, 3, and 4 genes |
|----------|-----------------------------------------------------------------------------------|
| Species  | Chromosome location | Exon number | Amino acid identities | Ensembl gene ID |
| **Ant1 (slc25a4), skeletal muscle and heart** | | | | |
| Homo sapiens | 4 | 4 | 100 | ENSG00000151729 |
| Macaca mulatta | 5 | 4 | 100 | ENSMMUG0000015472 |
| Mus musculus | 8 | 4 | 95 | ENSMUG0000013633 |
| Rattus norvegicus | 16 | 4 | 95 | ENSRNOG0000010830 |
| Bos taurus | 27 | 4 | 95 | ENSTAG0000013208 |
| Monodelphis domestica | 5 | 4 | 94 | ENSMODG0000004639 |
| Canis familiaris | 16 | 4 | 92 | ENSCAFG00000007596 |
| Danio rerio | 1 | 4 | 88 | ENSDARG00000027235 |
| Xenopus tropicalis | NA | 4 | 89 | ENSXETG00000009435 |
| **Ant2 (slc25a5), somatic cells** | | | | |
| H. sapiens | X | 4 | 100 | ENSG0000005022 |
| M. musculus | X | 4 | 98 | ENSMUG0000016139 |
| R. norvegicus | X | 4 | 98 | ENSRNOG00000039980 |
| B. taurus | X | 4 | 97 | ENSTAG00000020245 |
| C. familiaris | X | 4 | 97 | ENSCAFG00000018384 |
| D. rerio | X | 4 | 92 | ENSDARG00000053529 |
| X. tropicalis | NA | 4 | 91 | ENSXETG00000025466 |
| **Ant3 (slc25a6), somatic cells** | | | | |
| H. sapiens | X, Y<sup>a</sup> | 4 | 100 (92)<sup>a</sup> | ENSG000000169100 |
| B. taurus | X, Y | 4 | 97 (92) | ENSTAG00000013487 |
| **Ant4 (slc25a31), male germ cells** | | | | |
| H. sapiens | 4 | 6 | 100 | ENSG00000151475 |
| Pan troglodytes | 4 | 6 | 99 | ENSPTRG00000016432 |
| M. mulatta | 5 | 6 | 99 | ENSMUG00000015243 |
| M. musculus | 3 | 6 | 88 | ENSMUG00000069041 |
| B. taurus | 17 | 6 | 91 | ENSTAG00000012826 |
| C. familiaris | 19 | 6 | 90 | ENSCAFG00000003924 |
| M. domestica | 5 | 6 | 88 | ENSMODG00000012128 |

<sup>a</sup> NA, not available.

<sup>b</sup> Ant3 is on a pseudautosomal region of X and Y chromosomes.

<sup>c</sup> Amino acid identities are compared with the human ortholog.

<sup>d</sup> Ant3 sequence was also compared with human Ant2 paralog.

confirmation with a 3’ internal probe, three successfully targeted ES clones were identified. ES cells from one positive clone were injected into blastocysts of the C57BL/6 (B6) strain. Chimeric male mice were mated with females on a B6 background.

**Immunoblotting**—We used testis and heart samples from 6-week-old mice for Western blotting. For testis and heart, the tissues were frozen using liquid nitrogen and mechanically minced with a razor blade. We then lysed the cells in radiomimmune precipitation assay buffer, and 35 μg of total protein was separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following were used as primary antibodies: the rabbit polyclonal antibodies against Ant4 as we previously described (5), anti-Ant1 and Ant2 antibodies provided by Douglas C. Wallace (UC Irvine); actin (sc-1615 Santa Cruz, Santa Cruz, CA); and glyceraldehyde-3-phosphate dehydrogenase (RDI-TRK5G4–6C5 Research Diagnostics, Flanders, NJ). Peroxidase-conjugated immunoglobulin G (Santa Cruz) was used as the secondary antibody, followed by enhanced ECL detection (Amersham Biosciences).

**RT-PCR Analysis**—We isolated total RNA from testes of wild type, heterozygous, and homozygous mutant 6-week-old mice using the RNA aqueous kit (Ambion, Austin, TX). cDNA was synthesized using a SuperScript II first strand synthesis system with oligo(dT) (Invitrogen). PCR was performed using Taq DNA polymerase (Eppendorf, Westbury, NY). For each gene, the DNA primers were designed on different exons to ensure that the PCR product represented the mRNA and not genomic DNA (primer sequences available upon request).

**TUNEL Assay**—Paraformaldehyde fixed, paraffin-embedded sections (5 μm) were deparaffinized and rehydrated through a graded series of ethanol and water. The slides were then placed in 0.1 M citrate buffer, pH 6.0, and permeabilized by exposure to 6 min of microwave irradiation (350 W). Staining was performed using and in situ cell death detection kit (Roche Applied Science) following the manufacturer’s instructions. TUNEL reaction mixture containing TdT and fluorescein-dUTP was incubated on the slides for 1 h at 37 °C, with negative control slides receiving labeling mixture devoid of TdT enzyme. After three washes in 1 × PBS, slides were coverslipped using Vectorshield with 4’,6’-diamino-2-phenylindole (Vectorlabs, Burlingame, CA). In some experiments, fluorescein-dUTP was visualized using anti-fluorescein antibody conjugated with alkaline phosphatase.

**X-Gal Staining**—Testes were harvested from 6-week-old wild type (+/+), heterozygous (+/-), and homozygous (-/-) mutant male mice and dissected into two equal halves. The tissues were then fixed in a mild fixative for ~10–15 min. Following brief fixation, X-gal staining was carried out overnight with rocking, to prevent misshaping of the organ. The samples then underwent post fixation to further ensure the integrity of the tissue. Following post fixation the tissues were dehydrated using an organic solvent (PBS-Citrasol). The tissues were then imbedded in paraffin and sectioned. Following paraffin imbedding, the tissues were rehydrated with organic solvent (PBS-Citrasol) of decreasing concentrations. The slides were counterstained with hematoxylin.
RESULTS

The Autosomal Ant4 Gene Is Conserved in Mammals—The deduced amino acid sequence of Ant4 is well conserved among mammals (around or over 90%) (Table 1); however, a phylogram indicates that Ant4 is relatively distant from the other mammalian Ant family peptides, Ant1, 2, and 3 (Fig. 1). Indeed, the amino acid identity between Ant4 and other Ants is ~70%. Of interest, the gene configuration of Ant4 is also well conserved among mammals but different from that of other Ant members. The Ant4 gene always consists of six exons, whereas the other Ants have four exons in all mammalian species investigated. Another distinguishing characteristic of mammalian Ants is in their chromosomal location. The Ant1 gene, which is predominantly expressed in skeletal muscle and heart, is on an autosome. The Ant2 gene, which is ubiquitously expressed in somatic organs, is encoded by the X chromosome and the Ant3 gene, which has been identified in only a portion of mammalian species so far, including human, cow, and dog, is also located on the X chromosome. Rodents apparently do not have the Ant3 ortholog, based on a search of the published genome data bases. Ant3 has the highest homology with Ant2 and is ubiquitously expressed in somatic organs like Ant2. It should be noted that the human ANT3 gene is localized to the tip of the short arm (Xp22) of the X chromosome, which is known as pseudautosomal region 1. In contrast to Ant2 and Ant3, the Ant4 gene is always encoded by an autosome. Moreover, in contrast to Ant1 and Ant2, of which orthologs are found in other species including amphibians and fish, the Ant4 gene apparently exists only in mammals including the marsupials (Table 1). Ant4 is found in both eutherian and metatherian species, suggesting the presence of Ant4 in their common therian ancestor. The eutherian radiation event representing the divergence of eutherian and metatherian lineages occurred ~150 million years ago, suggesting that the emergence of Ant4 occurred at least 150 million years ago (24, 25), relatively close to the origin of mammals (~200 million years ago).

Ant4 Expression Is Highest in Spermatocytes—The exact expression profile of Ant4 in testis had not been determined. We demonstrated in our previous paper that Ant4 protein was expressed in testicular germ cells of mice (5); however, because of the limited resolution we obtained during immunostaining of cryopreserved tissues, we were unable to further define the exact expression profile of the protein within the testis. Here, paraffin-embedded formalin-fixed tissues were utilized to obtain a more precise expression pattern of Ant4 in mouse testis. Of interest, it appears that the Ant4 protein expression is highest in spermatocytes among testicular germ cells, based upon nuclear morphology and position within the seminiferous tubules (Fig. 2A). We also examined the expression pattern of Ant4 in human testis samples using polyclonal antibodies raised against human ANT4 and were able to more clearly distinguish the cell types within which ANT4 was expressed (Fig. 2B). The human immunohistochemistry data provided us with evidence that primary spermatocytes express the highest levels of the ANT4 protein, whereas spermatogonial cells express a lower level. Importantly, Sertoli cells or other somatic interstitial cells did not express ANT4. To further define stage-specific expression of Ant4 in male germ cells, we analyzed Ant4 mRNA expression in separated spermatogenic cell types of mouse using real time RT-PCR analysis (Fig. 2C). Ant4 transcript levels began to increase upon transition of premeiotic type B spermatogonia into the early stages of meiosis as represented by preleptotene spermatocytes. The transcript level of Ant4 continued to increase through the leptotene and zygotene spermatocyte stages, peaking in early pachytene spermatocytes. Ant4 transcript levels then began to decrease in late pachytene spermatocytes and in later round spermatids. Thus, high levels of Ant4 expression are likely associated with entry of the male germ cells into meiosis. In contrast, the fraction enriching Sertoli cells expressed a very low level of Ant4. We also confirmed here, by real time RT-PCR, that the Ant4 transcript is very low or undetectable in somatic organs and ovary. It should be noted here that, in contrast to a previous observation using a cryopreserved specimen (5), developing oocytes did not show any detectable Ant4 expression in paraffin-embedded formalin-fixed tissues (supplemental Fig. S1).

Using the same RNA samples prepared for the study above, we also investigated the expression pattern of the Ant2 gene in various organs and spermatogenic cell types (Fig. 2C). Of interest, the expression profile of Ant2 in mice was reciprocal to that of Ant4. The Ant2 transcript was high in somatic organs, but relatively low in whole testis, and almost completely undetectable in testicular germ cells except primitive spermatogonia.
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FIGURE 2. Ant4 expression is highest in spermatocytes. A, immunohistochemical analysis of Ant4 expression in mouse testis. Formalin-fixed, paraffin-embedded sections of mouse testis from wild type 6-week-old mice were incubated with a rabbit polyclonal antibody against mouse Ant4. Ant4 staining was visualized using DAB (brown), and slides were counterstained with hematoxylin. For control (top left), rabbit IgG was used as a primary antibody. Scale bars, 40 μm. B, immunohistochemical analysis of ANT4 expression in human testis: Formalin-fixed, paraffin-embedded sections of human testis from a 32-year-old male were incubated with a rabbit polyclonal antibody raised against human ANT4. ANT4 staining was visualized using DAB (brown), and the slides were counterstained with hematoxylin. Arrows, arrowheads, and asterisks indicate spermatogonia, Sertoli cells, and spermatocytes, respectively. Scale bars, 50 μm. C, TaqMan real-time PCR analysis of Ant4 and Ant2 transcript levels in purified mouse spermatogenic cell types (PA, primitive type A spermatogonia; A, type A spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocytes; L+Z, leptotene + zygotene spermatocytes; EP, early pachytene spermatocytes; LP, late pachytene spermatocytes; RS, round spermatids; JS, juvenile Sertoli cells) and various other tissues (whole testis, heart, liver, brain, kidney, ovary, and embryonic stem cells) (6-week-old mice). The relative transcript levels are shown in each graph when the transcript level of Ant4 in heart was set to 1.

FIGURE 3. Gene targeting of Ant4. A, strategy used for targeted disruption of the Ant4 gene. B, Southern blot analysis of BamHI-digested genomic DNA extracted from tails of wild type (+/+), heterozygous (+/-) and homozygous (-/-) mutant mice. DNA was hybridized with the probe shown (5' external probe). C, PCR analysis using allele-specific primers of genomic DNA of the indicated genotypes. Arrowheads in A denote the primers used for PCR amplification. D, Western blot analysis of Ant4 peptide expression as well as Ant1 and Ant2 in both testis and heart.

Generation of Mice with a Targeted Disruption of Ant4—To investigate the in vivo function of Ant4, we generated Ant4-deficient mice by homologous recombination in ES cells. The targeted disruption deleted exons 2–4, which encode amino acid residues 79–212 (Fig. 3A). An IRES-β-gal cassette was inserted with a splicing acceptor site to allow for examination of the activity of the Ant4 promoter. Disruption of the Ant4 gene in mice was confirmed by Southern blot analysis and genomic PCR amplification (Fig. 3, B and C). Immunoblotting was used to confirm the absence of Ant4 protein expression in the Ant4-deficient mice as well as to analyze the levels of Ant1 and Ant2 (Fig. 3D). The relative protein levels of Ant2 in the Ant4⁻/⁻ testsis, when normalized by total protein amount, were increased in comparison with controls, whereas Ant2 levels were unaffected in heart. The levels of Ant1 protein expression, which were high in heart and undetectable in testis, were unaffected by Ant4 disruption. The Ant4 promoter-driven β-galactosidase expression from our targeted allele also enabled us to examine the Ant4 expression profiles in mice. As expected, X-gal staining in Ant4⁺/⁻ mice was observed only in the testis but not in any other organs (data not shown). X-Gal staining of the Ant4⁻/⁻ testis demonstrated that the β-galactosidase activity became detectable when male germ cells transitioned into spermatocytes (Fig. 4), consistent with the immunohistochemistry data above.

Ant4-deficient Mice Exhibit Impaired Spermatogenesis and Infertility—The Ant4⁻/⁻ mice were viable and exhibited apparently normal development. The interbreeding of Ant4⁻/⁻ mice produced offspring of normal litter size and conformed to the Mendelian ratios of Ant4⁺/+ , Ant4⁻/⁻ , and Ant4⁻/⁻ inheritance, 9, 27, and 13, respectively. In contrast to the similar body sizes between the wild type and mutant mice (data not shown), the testes of Ant4⁻/⁻ adults were smaller than...
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FIGURE 4. Ant4 promoter-driven β-galactosidase expression pattern in testes. X-Gal staining of wild type (+/+, left panels), and heterozygous (+/−, right panels) testes, with low (top panels) and high (bottom panels) power magnification. Ant4 promoter-driven β-galactosidase was not detected in spermatogonia or Sertoli cells but was seen in primary spermatocytes and the subsequent cell types of spermatogenesis in heterozygous testes. The slides were counterstained with hematoxylin. Scale bars, 50 μm.

FIGURE 5. Defective spermatogenesis in Ant4-deficient mice. A, gross morphology of testes from 6-week-old mice. B, weight comparison of testis of the indicated genotypes (7–49 days old and 5 months old). C, histological analysis of testis (6-week-old) by hematoxylin and eosin staining. Scale bars, 50 μm. D, RT-PCR gene expression analysis in testis (6 weeks old).

Close examination of testicular development revealed similar growth patterns of the testis until ~17 days after birth, suggesting normal growth at earlier spermatogenic stages (Fig. 5B) (26). Subsequent development was impaired in Ant4−/− testis. Histological analysis of Ant4-deficient testis demonstrated clear morphological aberrations in the process of spermatogenesis as evident by the severe reduction of spermatocytes (Fig. 5C). Furthermore, mating of Ant4-deficient males with wild-type females did not produce any offspring. In contrast, Ant4−/− females were fertile and did not show any apparent ovarian abnormalities.

Ant4−/− Germ Cells Undergo Meiotic Arrest—To determine the stage at which Ant4−/− germ cells undergo arrest, RT-PCR analysis of transcripts present in different specific spermatogenic cell types was carried out (Fig. 5D). Dazl, which is expressed throughout spermatogenesis, was similarly expressed in the Ant4+/+, Ant4+/−, and Ant4−/− testis. The DNA mismatch repair gene Dmc1, which is expressed before the pachytene spermatocyte stage (27, 28), did not exhibit significantly different expression patterns either. The expression of A-Myb, which is a transcription factor of the Myb family that is expressed in type B spermatogonia and leptotene to pachytene spermatocytes (29), decreased in the Ant4−/− testis. The expression of Dvl3, which has been shown to be present from primitive type A spermatogonia through pachytene spermatocytes (30), also decreased in the Ant4−/− testis. Synaptonemal complex protein 3 (Sycp3), which is restricted to the zygotene to diplotene spermatocytes (31), was markedly decreased in the Ant4−/− testis. Transcripts normally present in pachytene spermatocytes and at later stages, such as HoxA4 and CyclinA1 (32, 33), were not detected in the Ant4−/− testis. In addition, Dvl1, which is expressed in round, elongating, and elongated spermatids (30), was also undetectable in the Ant4−/− testis. These data indicate a decrease in meiotic, specifically at the stage of pachytene and beyond, and an absence of the postmeiotic germ cells in the Ant4−/− testis.

Ant4-deficient Male Mice Exhibit Increased Levels of Apoptosis within the Testis—TUNEL labeling and caspase-3 staining were utilized to analyze the apoptotic profile of adult (6 weeks) Ant4−/− testis in comparison with controls (Fig. 6). The testis of Ant4-deficient mice exhibited increased levels of TUNEL-positive cells within the seminiferous tubules as compared with controls (Fig. 6A). Upon closer examination, the majority of the TUNEL-positive cells within the seminiferous tubules of Ant4−/− mice appeared to be spermatocytes based upon cellular morphology and position within the seminiferous epithelium (Fig. 6A, bottom panel). We also utilized caspase-3 staining within the testis to confirm the differential apoptotic profiles present between Ant4-deficient testis and controls (Fig. 6B). Taken together, these results suggest that the Ant4−/− testis contains a significantly higher number of apoptotic cells than controls, and the majority of these cells appear to be early spermatocytes. To investigate further the testicular development of Ant4−/− mice in comparison with controls, we utilized the synchronous nature of the first spermatogenic cycle in postnatal testes.
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Spermatogenesis is the process by which self-renewing testicular precursors undergo proliferation, differentiation, and maturation to produce viable spermatozoa. This process of spermatogenesis is one of the most elegant and complex examples of cellular growth and differentiation present within the mammalian system. Thus, there are many stages at which aberrations in spermatogenesis may lead to infertility. During the complex and energy demanding process of spermatogenesis, the proliferating and differentiating spermatogenic cells rely on the production and availability of ATP from the mitochondria. Classically, aberrant mitochondrial function has been connected with deficient sperm motility. Reduced sperm motility has been reported in patients with mitochondrial diseases (34, 35), and pathogenic mutant mitochondrial DNA has also been identified in semen samples of patients with fertility problems (36–38). However, a recent study revealed that the accumulation of mutant mitochondrial DNA in mice induced male infertility caused by oligospermia and asthenozoospermia (39). Further, spermatogenic cells carrying >75–80% mutant mitochondrial DNA demonstrated meiotic arrest and displayed enhanced apoptosis, indicating that male germ cell meiosis as well as sperm motility are particularly susceptible to mitochondrial dysfunction (39).

The present study has identified an essential role for the Ant4 gene in mammalian spermatogenesis. The Ant4 gene is expressed exclusively during spermatogenesis both in mice and humans, whereas other Ants are utilized in somatic cells. Thus, Ant4 likely serves as the sole mitochondrial ADP/ATP carrier during spermatogenesis. Furthermore, without a functional ADP/ATP carrier protein, ATP would not be efficiently transported into the cytosol; thus Ant4 is considered to be critical for normal spermatogenesis. Indeed, the disruption of the Ant4 gene resulted in meiotic arrest in mice, as evidenced by the loss of meiotic and postmeiotic germ cells in the Ant4-deficient testis. The phenotype was similar to that seen in mice with aberrant mitochondrial DNA (39). Further, this loss appeared to result from an increase in apoptosis in early spermatocytes. Although exact Ant4 function in male germ cell mitochondria remains to be determined, the current study supports the idea that the ATP supply through normal oxidative respiration is critical for the processes of male germ cell meiosis.

The chromosomal locations of the Ant family genes are unique and conserved among mammalian species. The Ant2 gene, which is ubiquitously expressed in somatic cells, is encoded by the X chromosome in all of the mammalian species investigated. In mammalian males, the X and Y chromosomes are known to undergo a heterochromatic transformation upon entry into meiosis, during prophase I, because of a lack of a homologous pairing partner (40–46). This transformation, known as meiotic sex chromosome inactivation, confers transcriptional repression upon the X and Y chromosomes as demonstrated by RNA polymerase II exclusion (44, 45). On the other hand, the Ant4 gene, which apparently exists only in mammals, is always encoded on autosomes. These implicate a hypothesis that Ant4 may have originally arisen to compensate for the loss of Ant2 function during male meiosis. Female mammals have two X chromosomes and do not undergo meiotic sex chromosome inactivation (44, 45), which is consistent with the fact that Ant4-deficient female mice exhibit no observable decrease in fertility. Indeed, the Ant2 and Ant4 expression pro-

**DISCUSSION**

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**FIGURE 6. Increased apoptosis within Ant4-deficient testis.** A, TUNEL analysis of testis from 6-week-old heterozygous mice (left panel), homozygous mutant mice (right panel). Cells having DNA breaks were labeled using TdT and fluorescein-dUTP. The nuclei were counterstained with 4',6'-diamino-2-phenylindole. Scale bars, 50 μm. B, immunohistochemical analysis of cleaved caspase-3 expression in testis from 6-week-old heterozygous mice (left) and homozygous mutant mice (right). Cleaved caspase-3 staining was visualized using DAB (brown), and the slides were counterstained with hematoxylin. Scale bars, 50 μm.
files were mutually reciprocal in the mice, and the Ant2 expression was particularly low during spermatogenesis (Fig. 2C). Of interest, the expression of Ant2 is very low not only in male meiotic germ cells but throughout spermatogenesis within the testis. Although the classical examples of meiotic sex chromosome inactivation show the repression of the genes after the pachytene stage, it is known that almost half of the X chromosome-linked genes are not expressed throughout spermatogenesis as like the Ant2 gene (45). After the emergence of Ant4 in mammals, the expression of Ant2 may have undergone further modifications to reduce transcription of the gene. In contrast to the low Ant2 transcript levels in testicular germ cells, the overall Ant2 expression, both protein and mRNA, were more detectable in the whole testis preparation (Figs. 2C and 3D). This discrepancy may be due to a predominant expression of Ant2 in somatic cells of the testis such as interstitial Leydig cells and vascular cells. However, we are currently unable to test this assumption because antibodies we had raised against Ant2 as well as any other available Ant2 antibodies do not work for immunohistochemistry.

Mammals have evolved a mechanism to compensate for the loss of gene expression from the sex chromosomes during male meiosis (25, 41, 43). Multiple autosomal retrogenes of X chromosome origin have been reported as candidates potentially compensating for the absence of essential sex-linked gene expression during male meiosis, as exemplified by the Pgk1/Pgk2 gene family (47–50). Although such retrogenes are considered to positively support male meiosis, there has been only one report so far (Utp14b) to clearly demonstrate the absolute necessity of such retrogenes in male meiosis (51, 52). It seems that even Pgk2 null mice demonstrate minimal male infertility (depending on genetic background) mainly because of a sperm motility defect (53). In contrast, the present study demonstrates that the Ant4 gene is essential for male meiosis. Indeed, the Ant4 gene likely arose before the divergence of eutherian and metatherian lineages, around the time when meiotic sex chromosome inactivation may have initiated (46). Thus, the Ant family of genes may be among the most essential to be compensated for during male meiosis. Interestingly, the Ant4 gene is not a retrogene in contrast to all the other known potential autosomal “compensation” genes. This suggests that Ant4 may have been generated by a standard gene duplication event in mammalian ancestors. It should be noted here that certain mammalian species including human, cow, and dog but not rodents have another Ant, Ant3 on the tip of the X chromosome (Table 1) (40, 54). Human ANT3 is encoded on Xp22 within pseudoautosomal region 1 (40). This region is highly conserved between X and Y chromosomes and is known to escape from sex chromosome inactivation during male meiosis (42, 44, 54). Thus, it would be plausible that some mammalian species may have evolved an additional protective mechanism to secure male meiosis. However, the role of ANT3 in human spermatogenesis is questionable, considering the fact that ANT3 expression is very low, just as ANT2 expression, in human testis (symatlas.gnf.org/SymAtlas/).

An alternative hypothesis, not entirely exclusive of the above theory, is that the specification of the Ant4 gene may have occurred to better support the process of spermatogenesis. Indeed, Ant4 has distinctive N- and C-terminal regions that are conserved across mammalian species, which could potentially be adapted to a specific energy-requiring process during male meiosis or subsequent sperm function. In addition, Ant4 has been recently isolated from the fibrous sheath of the human sperm flagellar principal piece using mass spectrometry proteomics and was shown to colocalize with glycolytic enzymes (21). Ant4 may have obtained an additional function that is advantageous for mammalian fertility regarding sperm function as well.
Ant4 Is Essential for Spermatogenesis

In summary, the present data demonstrate an essential role for Ant4 in murine spermatogenesis, more particularly in the survival of meiotic male germ cells. Additionally, the study implies that the Ant4 gene may have arisen in mammalian ancestors and been conserved in mammals to serve as the sole and essential mitochondrial ADP/ATP carrier during spermatogenesis where the sex chromosome-linked Ant2 gene is inactivated. Experiments that will reveal the redundant and/or specific function of Ant4 and Ant2 need to be conducted to provide us with critical insights regarding the evolution of mammalian spermatogenesis as well as the molecular evolution of the Ant family of genes. To date there are no known clinical male fertility deficiencies associated with ANT4. Future studies may elucidate a link between abnormal ANT4 and certain cases of male infertility in humans. In addition, developing specific ANT4 inhibitors for their potential use as male contraceptives would be interesting.

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