Symplastic spermatids (*sys*): A recessive insertional mutation in mice causing a defect in spermatogenesis

(transgenic mice/mouse chromosome 14)

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ABSTRACT A line of transgenic mice that carries an insertional mutation in a gene essential for spermatogenesis is described. Males homozygous for the transgenic insert are sterile, while female homozygotes and both male and female heterozygotes exhibit normal fertility. Developing spermatids in homozygous males form prominent abnormal multinucleated syncytia (symplasts) and do not complete maturation. In addition, abnormal cytoplasmic vacuolation is commonly seen in Sertoli cells. One flank of the transgenic integration site within the genome has been cloned and used to show linkage between homozygosity for the transgene and the mutant phenotype. The flank maps to mouse chromosome 14 approximately 4 centimorgans proximal to the gene encoding esterase-10 (*Es-10*). As no other gene that is known to be essential for spermatogenesis has been mapped to this region of the genome and as the mutant phenotype is unique, the transgenic insert appears to affect a previously unidentified gene. We have named the mutation "symplastic spermatids" (*sys*).

The integration of exogenous DNA sequences into the genome of mouse embryos can lead to insertional inactivation of endogenous genes (1–3). Insertional mutations may lead to a variety of phenotypes such as embryonic lethalties (4–8) as well as morphological and neurological disorders (9–13). In such mice, the transgenic insert can provide a molecular tag to facilitate cloning of the genomic sequences of interest.

The process of spermatogenesis in the mouse has been well characterized (14–17). Spermatogonia, the germinal stem cells, undergo mitosis to produce additional spermatogonia, a proportion 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. Throughout this process, the progeny of each spermatogonia remain joined via intercellular bridges, the nuclei sharing a single cytoplasm through which macromolecules can pass (18). Sertoli cells, the somatic cell components within the tubule, play a crucial role in this process, providing a specialized environment in which germinal development occurs.

At the molecular level, relatively little is known about the control of cellular differentiation and the architectural changes that ensue during spermatogenesis. Although a variety of nontransgenic mice with genetic defects in spermatogenesis has previously been identified (16), the mutations have not been characterized at the molecular level. In this regard transgenic mice with such defects are of great value,

as the transgenic insert facilitates maintenance of the mutation and provides a marker to initiate the cloning and molecular characterization of the inactivated gene(s).

While making an evaluation of the *Escherichia coli* gene *lacZ* encoding β -galactosidase (*LacZ*) as a reporter gene in transgenic mice, we identified a unique recessive insertional mutation that leads to sterility in male transgenic mice. Here we present an initial characterization of the mutant phenotype and the chromosomal mapping of the transgene integration site on chromosome 14.

MATERIALS AND METHODS

Production of Transgenic Mice. A DNA fragment containing the Rous sarcoma virus (RSV)-*lacZ* minigene (RSVZ) (Fig. 1A) was generated by digestion of pRSVZ (19) with *Apa* I (which cuts once at the 3' end of the minigene) and partial digestion with *Nde* I (which cuts at the 5' end of the minigene and within the *lacZ* gene). After electrophoresis through low-melting-point agarose, the minigene was purified by phenol extraction, and the DNA was used to inject the male pronuclei of (FVB/N $\times$ C3H)F₁ embryos by standard techniques (20).

Southern Analysis of Tail DNA. Mouse tail DNAs were purified (20), and Southern analysis was performed (19) as described. Filters were washed to a final stringency of 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 65°C and exposed overnight to XAR-5 x-ray film (Kodak) with an intensifying screen at –80°C. Heterozygous and homozygous transgenic mice were distinguished initially on the basis of the intensity of the hybridization signal and later by restriction fragment length polymorphism (RFLP).

Genomic Cloning. Genomic DNA from a mouse homozygous for the RSVZ transgene was digested to completion with *Eco*RI and electrophoresed through a 0.7% low-melting-point agarose gel (FMC). DNA of 6.4 kilobases (kb) $\pm$ 0.3 kb was purified from the agarose and used to construct a genomic library in lambda ZAP (21). Autoexcision of Bluescript from lambda ZAP clones was performed as described (21).

Preparation of Transgenic Mouse Testis for Histology. Fifteen minutes prior to sacrifice, mice (four each of heterozygous and homozygous mice and two nontransgenic littermates) were injected intraperitoneally with heparin (13 units/10 g of body weight) as described (22). Pentobarbital-anesthetized animals were perfused through the heart briefly with 0.9% saline and then with 5% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.4) for 20 min. Testes were excised, weighed, and diced into 1-mm cubes and immersed in iden-

tical fixative for an additional hour. After glutaraldehyde fixation, the tissue was washed in cacodylate buffer at 4°C and post-fixed in 1% osmium tetroxide/1.25% potassium ferrocyanide (23). Tissue blocks were dehydrated in a graded series of ethanol, infiltrated in propylene oxide, and embedded in Araldite (CY212). Semithin sections were cut with a Sorval MT-1 ultramicrotome at a setting of 1 μ m and stained with toluidine blue. The staging scheme developed by Oakberg (24) was used to stage the seminiferous epithelium.

Preparation of Tubule Whole Mounts. Tubule whole mounts were prepared as described (25). Tubules were fixed in Bouin's fixative and stained with hematoxylin.

Generation of Somatic Cell Hybrids. Somatic cell hybrids were derived from the fusion of E36 Chinese hamster cells with peritoneal macrophages or spleen cells from BALB/c strain mice (26). The chromosome content of most hybrids was determined by trypsin-Geimsa banding (27). Hybrids were also typed for specific marker loci. Cellular DNAs were isolated and analyzed by Southern blot hybridization using standard procedures (28).

Generation and Characterization of Interspecies Backcross Mice. NFS/N strain mice were obtained from the Division of Natural Resources, National Institutes of Health. *Mus musculus musculus* mice were obtained from a laboratory colony derived from mice originally trapped in Skive, Denmark, and maintained by M. Potter at Hazelton Laboratories (Rockville, MD). NFS/N females were mated with *M. m. musculus* males, and the F₁ females were backcrossed with *M. m. musculus* males to produce the experimental animals. DNAs were extracted from mouse livers, cleaved with *Eco*RI, electrophoresed through 0.4% agarose gels, and transferred to nylon membranes (Hybond, Amersham). Membranes were hybridized and washed as described (27). Kidney samples from the same mice were typed for inheritance of the markers nucleoside phosphorylase-1 (encoded by *Np-1*) and esterase-10 (encoded by *Es-10*) by histochemical staining after electrophoresis on starch gels (29).

RESULTS

Generation of RSVZ Transgenic Mice. Seven independent founder transgenic mice were generated by microinjection of the RSVZ minigene construct. These mice contained between 2 and 30 copies of the transgene in their genome (data not shown).

Inbreeding and Identification of Male Infertility. Founder transgenic mice were mated to male or female FVB/N animals, and offspring were screened for inheritance of the transgene. To determine whether the integration of the transgene in each founder had disrupted one or more genes essential to some aspect of development, representative mice for each integration site were bred to homozygosity. Male mice homozygous for one site of integration in the 5342 founder mouse were noted to be sterile (Fig. 1B) despite showing normal sexual behavior and copulation. This family has been named OVE3A [full designation Tg(RSV, *dr-g-Adh*, *lacZ*, SV40) OVE3A].

Homozygous OVE3A females are fertile (average litter size was 9.4 from 19 litters). None of the other six lines of mice exhibited a mutant phenotype.

Gross Anatomy and Histology of Reproductive Tissues of Homozygous OVE3A Male Mice. The vasa deferentia of adult homozygous OVE3A male mice were excised, and the contents were examined for the presence of mature spermatozoa. No spermatozoa were observed. The weight of testes from homozygous males (3.6 mg/g of body weight; $n = 4$) was significantly reduced compared with testes isolated from heterozygous (6.8 mg/g of body wt; $n = 4$) and nontransgenic (6.2 mg/g of body wt; $n = 2$) littermates. The testes of adult homozygous, heterozygous, and nontransgenic OVE3A males were fixed and prepared for light and electron micros-

FIG. 1. RSVZ minigene and OVE3 pedigree. (A) The RSVZ (19) minigene is composed of the long terminal repeat element from RSV linked to a *lacZ* (encoding β -galactosidase) fusion gene containing a translational initiation codon (ATG) provided by a 130-base-pair (bp) region of the *Drosophila melanogaster Adh* gene (checkered box) and RNA splice and polyadenylation signals provided by a region of the simian virus 40 (SV40) genome. In addition, there are 50 bp of pBR322-derived sequences at the extreme 5' end of the construct. (B) OVE3 pedigree. The founder animal 5342 (OVE3) was mated to a male FVB mouse, and offspring were examined as to the pattern of inheritance of the RSVZ transgene. Segregation of two integration patterns (arbitrarily named A and B) was observed. OVE3A mice were bred to homozygosity, and females and males were examined for developmental abnormalities. Homozygous males (marked with an asterisk) were sterile.

copy. Light microscopic observation of whole mounts of mutant tubules revealed the presence of multinucleated syncytia (symplasts) of spermatids that often contained 100 or more nuclei (Fig. 2A). Light microscopy on sections of testes confirmed this finding and showed that elongating and mature spermatids were not present in tubules of homozygous males (Fig. 2B and C). Instead, abnormal symplasts of round spermatids were prevalent in the lumina of the tubules. Sections from heterozygote animals appeared normal and similar to nontransgenic littermates (Fig. 2D).

In mutant male testes, spermatid development appeared to be normal in the early phases of spermiogenesis. Although symplasts were rare in the early stages, by stage VI large numbers of nuclei could be seen within one cytoplasmic membrane (Fig. 2B). Nuclear elongation and chromatin condensation were initiated in some symplasts, but these processes were neither normal nor complete.

Defects in spermatocyte maturation were less obvious. Although a relatively large number of spermatocytes appeared to degenerate between stages II–IV, no major differences were found in the numbers of undifferentiated and differentiated spermatogonia and preleptotene spermatocytes between mutant and normal animals.

Abnormal Sertoli cell morphology was also noted in the sections from homozygous animals. The lateral and apical processes of Sertoli cells did not maintain a close relationship with the developing spermatids, and there was extensive intracellular vacuolation (Fig. 2B and C). The Sertoli cells of heterozygous animals appeared normal.

FIG. 2. Light microscopy of histology of adult testes. (A) Whole mount of tubule from homozygous OVE3A testes. The image has been focused in the plane of the lumen of the tubule. Note the symplasts containing spermatid-stage nuclei. ($\times 400$.) (B) Homozygous OVE3A testes: low-power micrograph of the seminiferous epithelium showing several seminiferous tubules. The stage (or range of stages) of each tubule is indicated in Roman numerals. The development of symplasts (arrows) is obvious at midcycle. In all tubules, elongate spermatids are absent. Note the common occurrence of vacuoles (asterisks) within the Sertoli cells. ($\times 185$.) (C) Homozygous OVE3A testes: higher power micrograph of a single tubule. Again, note the presence of symplastic spermatids and the vacuolation within the Sertoli cells (asterisks). No elongate spermatids are seen. ($\times 350$.) (D) Heterozygous OVE3A testes. The heterozygote testes appear normal and indistinguishable from those of age-matched nontransgenic males (not shown). ($\times 350$.)

Cloning of Genomic DNA Flanking the Transgenic Integration Site in OVE3A. Towards identification of the inactivated gene(s) in the OVE3A transgenic mice, genomic DNA sequences flanking the transgenic insert were cloned. Southern analysis of OVE3A genomic DNA identified a 6.4-kb *EcoRI* fusion fragment at the transgenic integration site (data not shown). Consequently, OVE3A genomic DNA was digested to completion with *EcoRI*, size-fractionated, and used to construct a phage λ library. Two hundred and fifty thousand plaques were screened by using the RSV long terminal repeat as a probe, and seven positives were identified. These were further purified, and the inserts were subcloned to generate plasmids via coinfection with helper phage as described (21). One subclone (Zap 1) was identified as having an *EcoRI* insert of the correct size (6.4 kb). A 1700-bp region of the Zap 1 clone was used as a probe in a Southern analysis to test for an *EcoRI* RFLP (Fig. 3). Concordance between homozygosity for the 6.4-kb allele and the spermless phenotype has been confirmed in all 51 male mice tested to date.

Chromosomal Mapping of the Transgene in OVE3A. DNA isolated from Chinese hamster $\times$ mouse somatic cell hybrid cell lines was used for the chromosomal assignment of the genomic sequences adjacent to the transgene integration site in OVE3A. Under stringent hybridization wash conditions

($0.1 \times$ SSC, 65°C), the 1700-bp DNA probe used in the RFLP analysis failed to detect a cross-hybridizing band in Chinese hamster DNA.

Five of 16 hybrids contained the 10.2-kb mouse genomic band. Analysis of the distribution of mouse chromosomes and of the presence or absence of hybridization to the probe is shown in Table 1. The data indicate that the transgenic insert is located on mouse chromosome 14.

Southern analysis of *EcoRI*-digested DNAs from NFS/N and *M. m. musculus* mice showed that the NFS/N DNA produced a cross-reactive band of 19.5 kb and that *M. m. musculus* DNA produced a 10.2-kb band. Analysis of progeny of the backcross [(NFS $\times$ *musculus*) F_1 $\times$ *musculus*] showed that 39 of 96 mice inherited the NFS *sys* allele (Table 2). The segregation pattern of this RFLP was compared with other markers on chromosome 14. This locus is linked to those encoding nucleoside phosphorylase-1 (*Np-1*) and esterase-10 (*Es-10*) with the order *Np-1-sys-Es-10*. The locus is apparently not allelic to previously mapped genes that affect fertility. We have designated the locus symplastic spermatids (*sys*).

DISCUSSION

We have identified a line of transgenic mice (OVE3A) with an insertional mutation that leads to a defect in spermatogenesis.

FIG. 3. Detection of an *EcoRI* RFLP in OVE3A mice. (Upper) A 1700-bp *EcoRI*-*SstI* fragment isolated from the Zap 1 clone was used as a probe in a Southern analysis of *EcoRI*-digested genomic DNA isolated from nontransgenic (□), heterozygous (▨), and homozygous (■) OVE3A male mice. The probe detects a 10-kb DNA fragment in nontransgenic mice and a 6.4-kb fragment in the presence of the transgene. (Lower) Schematic of the probe and the expected hybridizing fragment.

The OVE3A line of mice carries 5–10 copies of the transgenic DNA integrated in a head-to-tail orientation at a single site. Males homozygous for the transgenic insert are sterile, whereas homozygous females and heterozygous animals exhibit normal fertility.

Analysis of the vasa deferentia of homozygous adult OVE3A male mice revealed an absence of spermatozoa. Under light microscopy, both tubular whole-mounts and sections of testes from mutant animals were noted to contain abnormal syncytia (symplasts) containing multiple spermatid-stage nuclei (Fig. 2 A–C). Although symplasts formed by stage VI of the cycle (24), occasionally spermatids that had commenced nuclear condensation and elongation were observed, suggesting that the cells attempt but fail to continue through spermiogenesis. Also, a relatively large number of spermatocytes in the early stages of development (stages II–IV) were noted to degenerate. However, no major differences were found in the numbers of undifferentiated and differentiated spermatogonia and preleptotene spermatocytes between mutant and normal animals.

The defects are not restricted to germ cells. Sertoli cells show a high degree of vacuolation within their cytoplasmic boundaries (Fig. 2C). In addition, they appear unable to maintain a close physical relationship with the developing

Table 1. Analysis of the concordance between specific mouse chromosomes and hybridization to the Zap 1 probe in a series of mouse–hamster somatic cell hybrids

Mouse chromosome	Number of hybrids				Total	% discordancy
	Hybridization/ Chromosome*					
	+/+	-/-	+/-	-/+		
1	1	6	3	7	17	58.8
2	4	5	1	7	17	47.1
3	0	7	1	3	11	36.4
4	1	10	3	3	17	35.3
5	1	11	4	2	18	33.3
6	3	4	2	8	17	58.8
7	4	4	0	8	16	50.0
8	2	10	3	3	18	33.3
9	3	10	2	4	19	31.6
10	0	12	5	0	17	29.4
11	0	12	5	0	17	29.4
12	2	1	0	8	11	72.7
13	3	5	2	7	17	52.9
14	5	12	0	0	17	0.0
15	1	0	0	9	10	90.0
16	1	8	4	3	16	43.8
17	3	4	0	6	13	46.2
18	1	5	4	6	16	62.5
19	2	6	3	5	16	50.0
20	2	7	2	7	18	50.0

The number of discordant observations is the sum of the +/– and –/+ observations.

*Symbols indicate the presence (+/) or absence (–/) of hybridization to the Zap 1 probe and the presence (/+) or absence (–/) of a particular mouse chromosome.

spermatids. In contrast, the intertubular Leydig cells appear to be unaffected.

During maturation, descendants from individual spermatogonia are physically linked via distinctive intercellular bridges that have been shown to permit the passage of cytoplasmic constituents (18). Bridge structures that appear normal are present in the young spermatids of mutant mice (data not shown). However, the intercellular bridges are not maintained throughout spermiogenesis, suggesting that the failure of sperm development may be related to the lack of maintenance of bridges.

Northern analysis of RNA isolated from OVE3A transgenic male testes indicates that the β -galactosidase transgene is transcribed. However, expression is at a level undetectable by a histochemical assay (19) (data not shown). Although it is formally possible that the defect in the mutant mice is due to the expression of the *lacZ* gene, we think this unlikely because the transgene is also expressed in heterozygous animals, which fail to show a similar phenotype.

Table 2. Allelic segregation of *sys*, *Np-1*, and *Es-10* in an interspecies backcross

Mice	Inheritance of the NFS/N allele			Mice, no.	Recombination*		
	<i>Np-1</i>	<i>sys</i>	<i>Es-10</i>		Locus pair	<i>r/n</i>	cM $\pm$ 1 SE
Parental linkage	+	+	+	29	<i>Np-1</i> , <i>sys</i>	17/96	17.7 $\pm$ 3.9
	—	—	—	46	<i>sys</i> , <i>Es-10</i>	4/96	4.2 $\pm$ 2.0
Single recombinants	+	+	—	3	<i>Np-1</i> , <i>Es-10</i>	21/96	21.9 $\pm$ 4.2
	—	—	+	1			
	+	—	—	10			
	—	+	+	7			
Total				96			

*Percent recombination between restriction fragments and the SE were calculated as described by Green (30) from the number of recombinants *r* in sample size *n*. cM, centimorgans.

By using somatic cell hybrid panels, the transgenic site of integration has been mapped to mouse chromosome 14 and, with an interspecies backcross, further localized to a position approximately 4 centimorgans proximal to *Es-10*. We believe that we have identified a mutation in a previously unidentified gene. We propose that the mutation be named symplastic spermatids (*sys*).

To date we have been unsuccessful in isolating the other flank of the transgenic integration site. However, initial results from genomic walking and Southern hybridization studies with the cloned flank indicate that there has been a deletion of at least 10 kb of genomic DNA associated with the integration of the transgene (data not shown). With the genomic DNA clones as probes, Southern analyses performed under reduced stringency failed to detect DNA sequences conserved between diverse animal species, suggesting the absence of exon sequences in this region of the genome (data not shown).

There are several interesting features of the *sys* mutation. One is the apparent absence of pleiotropic effects associated with the *sys* phenotype. Many of the previously documented phenotypes associated with mutations causing murine male sterility include alterations of pigmentation, hematopoiesis, vision, and/or neurological function (16). In contrast, *sys* mice have normal pigmentation and no signs of hematopoietic or neurological defects. Their sexual behavior, capacity to copulate and plug females, accessory sex organs, and secondary sexual characteristics all appear normal.

In addition, the pattern of inheritance of the transgene from heterozygous males suggests that the genetic defect is not haploid specific. The recessive nature of this mutation is in contrast to other previously identified examples of male sterility in transgenic mice, where the defect was inherited in a dominant fashion (31–34).

It is not yet clear whether the primary cell type affected by the gene defect in *sys* mice is the germ cell, the Sertoli cell, or another somatic cell type.

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