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Animal Models of Physiologic Markers of Male Reproduction: Genetically Defined Infertile Mice

by Curtis Chubb*

The present report focuses on novel animal models of male infertility: genetically defined mice bearing single-gene mutations that induce infertility. The primary goal of our investigations was to identify the reproductive defects in these mutant mice. The phenotypic effects of the gene mutations were deciphered by comparing the mutant mice to their normal siblings. Initially testicular steroidogenesis and spermatogenesis were investigated. The physiologic markers for testicular steroidogenesis were steroid secretion by testes perifused *in vitro*, seminal vesicle weight, and Leydig cell histology. Spermatogenesis was evaluated by the enumeration of homogenization-resistant sperm/spermatids in testes and by morphometric analyses of germ cells in the seminiferous epithelium. If testicular function appeared normal, we investigated the sexual behavior of the mice. The parameters of male sexual behavior that were quantified included mount patency, mount frequency, intromission latency, thrusts per intromission, ejaculation latency, and ejaculation duration. Females of pairs breeding under normal circumstances were monitored for the presence of vaginal plugs and pregnancies. The patency of the ejaculatory process was determined by quantifying sperm in the female reproductive tract after sexual behavior tests. Sperm function was studied by quantitatively determining sperm motility during videomicroscopic observation. Also, the ability of epididymal sperm to function within the uterine environment was analyzed by determining sperm capacity to initiate pregnancy after artificial insemination. Together, the experimental results permitted the grouping of the gene mutations into three general categories. We propose that the same biological markers used in the reported studies can be implemented in the assessment of the impact that environmental toxins may have on male reproduction.

Introduction

The end product of successful male reproduction is the deposition of spermatozoa capable of fertilization into the female reproductive tract. To achieve this end, the synchronization of a cascade of events is required. These events include the release of gonadotropin from the pituitary, testicular testosterone biosynthesis, production of functional spermatozoa, and the complex behavior of ejaculation (*1–5*). The aim of our research program is to elucidate the genetic control of the individual events that constitute male reproduction.

Genetically defined mice are a major aspect of our experimental approach. Mice were chosen as the animal model because they are mammals that exhibit reproductive characteristics approximating those of men (*6*) and because of the voluminous knowledge of mouse genetics (*7–9*). The original working hypothesis was that mutations of single genes controlling male reproduction would be expressed phenotypically as either infertility or reduced fertility. An exhaustive search of the literature revealed that mutations at 40 genetic loci had been reported to induce male infertility. The paucity of information about the mice bearing the gene mutations was striking.

The goal of the described studies was to elucidate the phenotypic effects of the single-gene mutations that induce male infertility in mice. This goal was accomplished by implementing a spectrum of biological markers of male reproduction. In this report, we present an overview of our experimental results while emphasizing the biological markers. We propose that the same biological markers can be used to assess the impact that environmental toxins have on male reproduction.

Experimental Approach

The power of the experimental approach selected for the studies lies in the fact that the phenotypic effects of each gene mutation could be defined by comparing sibling mice: one sibling being normal or an unaffected heterozygote (+/−) and the other sibling being the mu-

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tant. The primary difference between the two mice was the single gene mutation. This paradigm permitted the differences between the two mice to be assigned to the gene mutation. At present, we cannot state in every case if the effect is direct or a result of spurious pleiotropy (*10*).

Genetically Defined Mice

The review of the literature identified the gene mutations reported to cause infertility in male mice. A list of these gene mutations is presented in Table 1. Instead of supplying unabridged references in the table, I have cited primary literature sources that refer to the gene mutation's effect on male fertility. The following reviews provide comprehensive information about the gene mutations: (*7,16–24*). Although I did not include chromosomal variants (*25*), the *T* locus and *t*-haplotypes (*26,27*), or the mutations at the *c* and *p* loci (*7*), all of these have been reported to influence male reproduction. Gene mutations that have been reported recently in the *Mouse News Letter* are not included in Table 1 because they may not be available to all investigators. Finally, the *Mouse News Letter* (*8*) is the most valuable publication for current information about mouse genetics (including recent linkage maps) and for identifying the sources of mutant mice (see *Mouse News Letter* number 76 for the 1986 mouse gene list). During our studies, the mutant and control mice were either obtained directly or derived from breeding pairs generously provided by individual scientists or the Jackson Laboratory.

Biological Markers of Male Reproduction

Biological markers were selected on the basis of their potential to assess the physiological function of the individual events that constitute male reproduction. A brief discussion of biological markers for each event follows. The reader will be referred to publications containing more details about the experimental procedures and alerted to possible difficulties.

Hypothalamus-Pituitary Function

The function of the hypothalamus-pituitary axis was assessed directly by measuring plasma concentrations of three hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL). LH, FSH, and PRL are the primary secretions of the anterior pituitary that modulate the function of Leydig and Sertoli cells (*18*). In our studies, one plasma sample from each animal was analyzed in order to detect significant differences in hormone concentration; however, multiple blood samples from each animal would be necessary to obtain accurate measurements because of the episodic secretion of the hormones (*28*). Assay details can be located in the following references: LH (*29*), FSH (*30*), and PRL (*31*).

Testicular Steroidogenesis

Testosterone is the predominant steroid secreted by mammalian testes and is considered the primary androgen (*32*). Two conventional methods were used to estimate *in vivo* testosterone secretion: radioimmunometric determination of plasma testosterone levels (*33*) and quantitation of the weight of the androgen-dependent seminal vesicles. Possible problems with these markers include the pulsatile release of testosterone (*34*) and the variable response of organs to androgen stimulation (*35*).

The steroidogenic capacity of testes was quantified by perifusing the testes *in vitro* with a defined perifusion medium (*33*). Each testis was maximally stimulated with LH. Initially, the venous effluent from the *in vitro* perifused testes was analyzed for testosterone with a specific antibody (*33*). If testosterone secretion by mutant mouse testes was decreased, we constructed a secretion profile of testosterone biosynthetic intermediates by using a gas chromatograph equipped with capillary columns (*36*). Alterations in specific steroidogenic reaction activities could be detected by comparing the steroid secretion profiles of the mutant and normal mouse testes (*37*).

In vitro perifusion provides several important features for assessing the steroidogenic potential of testes. For example, the complex interrelationship between seminiferous tubules and Leydig cells is maintained, secretory products are removed via the blood vasculature thereby minimizing product inhibition, steroid secretion is continuous and can be maximally stimulated, steroidogenesis results only from endogenous cofactors and substrates, and the controlled conditions approximate those occurring *in vivo*.

The biochemical analysis of Leydig cell function was complemented with microanatomical studies. In these studies, testes were perifusion-fixed, embedded in methacrylate, and stained with toluidine blue (*38*). Morphometric methods (*38*) were used to determine the Leydig cell volume and mass. Concomitantly, Leydig cell morphology was examined for gross alterations.

Spermatogenesis

Two methods were used to assess spermatogenesis: enumeration of homogenization-resistant sperm/spermatids (*39,40*) and germ cell morphometric analysis (*41*). In the first method, phase-contrast microscopic analysis of sperm/spermatid nuclei in testicular homogenates yielded a general estimation of spermatogenesis. This method is usually accurate unless the gene mutation alters the physical properties of spermatid nuclei and renders them less resistant to homogenization. The spermatogenic efficiency can be expressed as sperm/spermatids per milligram testis, thereby negating the

Table 1. Gene mutations causing male infertility in the mouse.

A. Mutations studied in the experiments described.

Gene mutation	Genotype: Mutant	Genotype: Control	Background strain	Source[a]	Reference
Atrichosis	*at/at*	+/−[b]	*a/a d/d at* +/+ *eb* (F33)	Jackson	(*7*)
Blind-sterile	*bs/bs*	+/−	129.AKR-*bs* (N3F5)	Jackson	(*11*)
Ames dwarf	*df/df*	+/−	closed colony	Bartke	(*7*)
Dwarf	*dw/dw*	+/−	closed colony	Bartke	(*7*)
Flipper-arm[c]	*fl/fl*	+/−	*fl* (F8)	Skow	(*7*)
Hypogonadal	*hpg/hpg*	+/−	*hpg*/+	Jackson	(*7*)
Hybrid sterility	*Hst*-1^{s}/*Hst*-1^{wa}	*Hst*−1^{s}/+	*PWK* or *PWD* (F20)	Forejt	(*7*)
Hightail	*Ht*/+	+/+	49PB (N4F8)	Skow	(*7*)
Hemimelic extra toes	*Hx*/+	+/+	B10.D2/nSn−*Hx*/+ (F41)	Jackson	(*7*)
Hypothyroid[d]	*hyt/hyt*	+/−	C.RF−*hyt* (N10F15)	Jackson	(*12*)
Hypothyroid[d]	*hyt/hyt*	+/−	Random bred	Wilson	(*12*)
Limb-deformity[c]	*ld*^{J}/*ld*^{J}	+/−	*ld*^{J} (F61)	Jackson	—[e]
Little	*lit/lit*	+/−	C57BL/6J−*lit* (N4F8)	Jackson	(*13*)
Oligotriche	*olt/olt*	+/−	C3H/He Orl−*olt*	Moutier	(*7*)
Purkinje cell degeneration	*pcd/pcd*	+/−	C57BL/6J−*pcd* (N10F6)	Jackson	(*7*)
Pygmy	*pg/pg*	+/−	C3H/HeNIcrWfp−*pg* (N20F6)	Jackson	—[e]
Quaking	*qk/qk*	+/−	B6C3−*a/a*−*qk* (N6F1)	Jackson	(*7*)
Steel-Dickie	*Sl/Sl*^{d}	*Sl*^{d}/+	WCB6F1/J-Sl/Sl^{d}	Jackson	(*7*)
Stubby	*stb/stb*	+/−	*stb*+ *a*/+ *fi a* (F59)	Jackson	(*7*)
Sex reversed[f]	X/X *Sxr*	X/Y	B6C3-A^{W-J}−Y^{Sxr}	Jackson	(*7,14*)
Testicular feminization	*Tfm/Y*	X/Y	C57BL/6J−A^{W-J}−*Ta* +/+ *Tfm* (N3F26)	Jackson	(*7*)
Viable dominant spotting	*W/W*^{v}	*W*^{v}/+	WBB6F1/J−*W/W*^{v}	Jackson	(*15*)

[a] The sources were: A. Bartke, Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL; J. Forejt, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia; The Jackson Laboratory, Bar Harbor, ME; R. Moutier, Department of Genetics, Centre de Sélection et d'Élevage d'Animaux de Laboratoire, Centre National de la Recherche Scientifique, Orléans Cedex, France; L. Skow, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN (Skow is no longer at ORNL—the contact person is now L. Russell); C. Wilson, Department of Internal Medicine, University of Texas Health Science Center at Dallas.

[b] +/− represents +/+ or unaffected heterozygote.

[c] Significant neonatal mortality.

[d] Initially reported as petite (*pet*).

[e] Personal communication.

[f] Actually a chomosomal translocation; provisional symbol *Tp*(Y)*1Ct*.

B. Mutations *not* studied in the experiments described.

Gene mutation	Symbol	Reason for deletion	Reference
Ataxia	*ax*^{J}	Physical impairment	(*7*)
Calvino	*cv*	Considered extinct	(*16*)
Gyro	*Gy*	Unsuccessful breeding program	(*7*)
Hemolytic anemia	*ha*	Not available	(*7*)
Hotfoot	*ho*^{4J}	Prohibitive cost	(*7*)
Hop-sterile	*hop*	Considered extinct	(*7*)
Hydrocephalic-polydactyl	*hpy*	Not available	(*7*)
Ichthyosis	*ic*	Fertile[a]	(*16*)
Jagged-tail	*jg*	Neonatal mortality	(*7*)
Strong's luxoid	*lst*	Neonatal mortality	(*7*)
Luxoid	*lu*	Prohibitive cost	(*7*)
Luxate	*lx*	Physical impairment	(*7*)
Marcel	*mc*	Considered extinct	(*7*)
Motheaten	*me*	Neonatal mortality	—[a]
Viable-brindled	*Mo*^{vbr}	Unsuccessful breeding program	(*7*)
Cloud-gray	*Sl*^{cg}	Not available	(*7*)
Shaker-short	*st*	Considered extinct	(*7*)
Tremulous	*tm*	Not available	(*7*)
Varitint-waddler	*Va*	Neonatal mortality	(*7*)
Ballantyne's spotting	*W*^{b}	Not available	(*7*)
Wobbler	*wr*	Neonatal mortality	(*7*)

[a] Personal communication.

effect of differences in testis weight. Interestingly, the experimental data provided by our homogenization method revealed that ~200,000 sperm or spermatids/mg testis was the value for normal spermatogenesis regardless of genotype.

If a perturbation in spermatogenesis was indicated by the homogenization data, sections of the perifusion-fixed testes were morphometrically analyzed for the nuclear volume fraction (%) of five germ cell classes: spermatogonia, early spermatocytes (leptotene, zygotene), late spermatocytes (pachytene, diplotene), round spermatids, and elongated spermatids (*42*). More precise determinations of specific stages could be performed when the testis sections were stained with Sidman's acid fuchsin-toluidine blue (*43*).

Function of Male Genital Ducts and Accessory Sex Glands

The genital ducts comprise the epithelium-lined tubes that transport sperm from the testis to the exterior. These ducts also contribute to the maturation and protection of sperm during their transit. The male accessory sex glands, evaginations of the genital ducts, secrete the seminal fluid that mixes with sperm during ejaculation (*44*).

The function of these ducts and glands was investigated by anatomical studies and by indirect assessments of function. The weights of seminal vesicles, preputial glands, epididymides, and penes were determined. Additionally, the morphology of penes was microscopically examined for the presence of key structures including the corpus spongiosum, corpus cavernosum, os penis, and epithelial papillae (*45*). The patency of efferent ductules and epididymal ducts was elucidated by determining the number of sperm/spermatids in epididymal

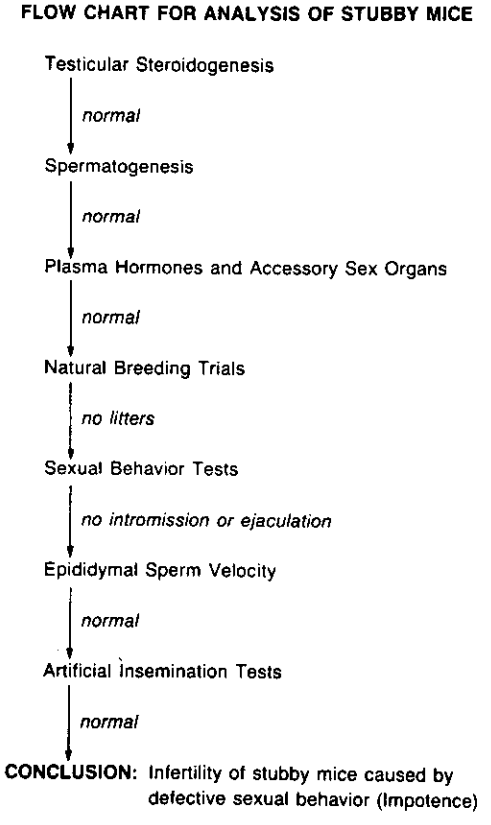

FIGURE 1. Example of how the biological markers of male reproduction were applied in the reported studies.

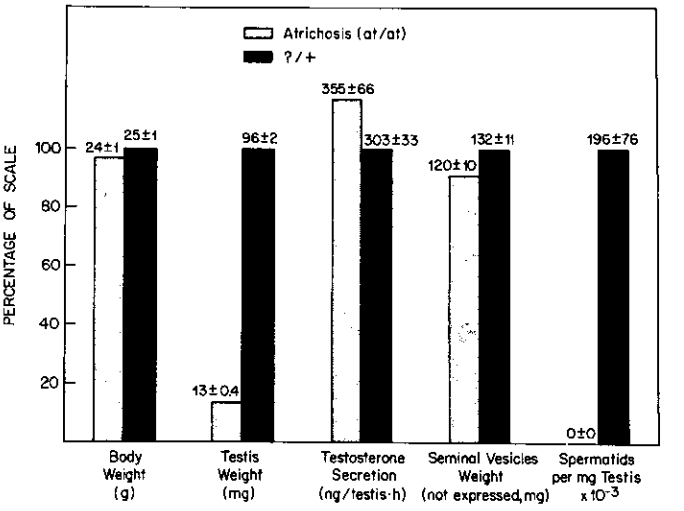

FIGURE 2. Summary of data for atrichosis mice (*at/at*) and their normal siblings (?/+). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from 6–12 mature mice. Testosterone secretion by *in vitro* perifused testes maximally stimulated with LH was determined.

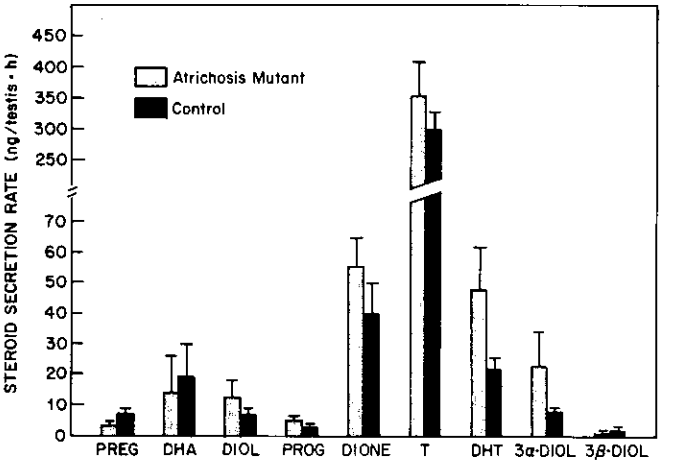

FIGURE 3. Steroid secretion profile for LH-stimulated, *in vitro* perifused testes of atrichosis (*at/at*) and control (+/−) mice. Each value represents the mean ± SE (n = 4–6). Abbreviations are: PREG (pregnenolone), DHA (dehydroepiandrosterone), DIOL (androstenediol), PROG (progesterone), DIONE (androstenedione), T (testosterone), DHT (dihydrotestosterone), 3α-DIOL (3α-androstanediol), and 3β-DIOL (3β-androstanediol). [From Chubb and Nolan (*57*) with permission.]

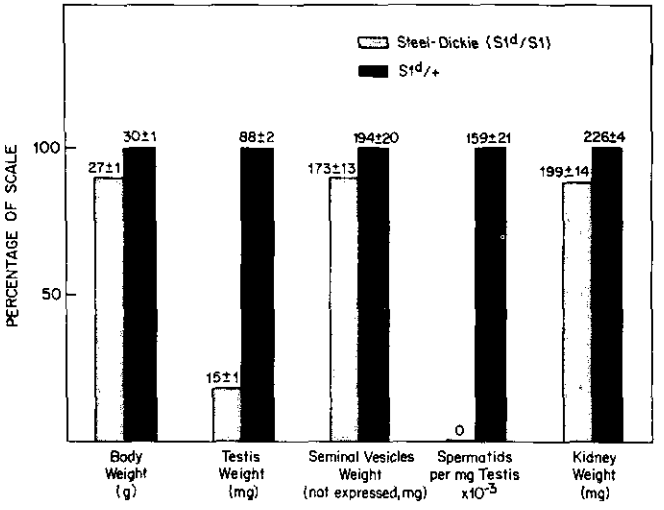

FIGURE 4. Summary of data for Steel-Dickie mice (Sl^d/Sl) and their siblings ($Sl^d/+$). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from seven mature mice. The reduction in seminal vesicle weight can be explained by the corresponding decrease in body weight. Kidney weight was included because kidneys respond to androgenic stimulation.

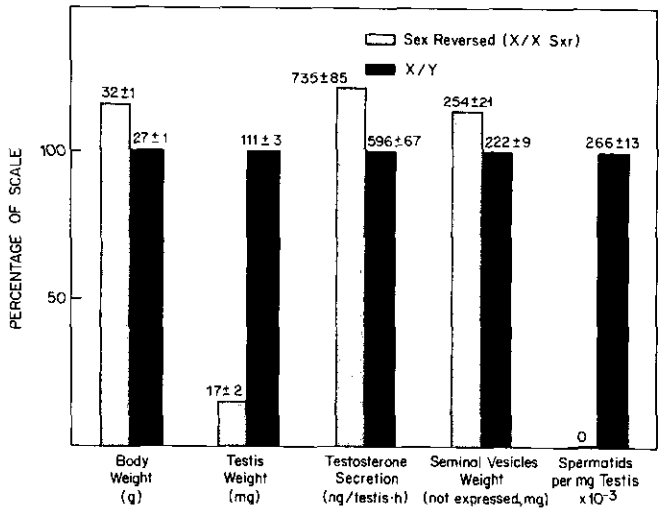

FIGURE 5. Summary of data for sex reversed mice (X/X *Sxr*) and normal males (X/Y). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from six to eight mature mice. Testosterone secretion by *in vitro* perifused testes maximally stimulated with LH was determined.

homogenates with the aid of phase-contrast microscopy (*38*). Finally, the normal function of the entire system of ducts and accessory sex glands was indicated by the presence of a vaginal (copulatory) plug and sperm in the female reproductive tract following ejaculation.

Sperm Function

Sperm function was assessed by *in vivo* and *in vitro* methods. The most critical test was the ability of the mice to sire litters. If the mice did not sire litters, we determined if the sperm could successfully enter the uterus by enumerating the sperm in uterine homogenates 1 hr after ejaculation. Additionally, we quantitatively measured the velocity of epididymal sperm by a videomicroscopic method similar to that reported by Katz and Overstreet (*46*). In our tests, we cut the cauda epididymides at their junction with the vas deferens and expressed the epididymal sperm into either modified Toyoda medium (*47*) or Alvarez medium (*48*). After incubation, the following parameters were measured: net velocity, average path velocity, and linear index (*49*). Also, the video recordings permitted analyses of sperm size and percent motility.

The fertilizability of epididymal sperm under uterine conditions was assessed by artificial insemination of hormonally-primed female mice and the subsequent quantification of live, near-term fetuses. Artificial insemination was performed similar to the vaginal route method reported by Wolfe (*50*) with Depoprovera injections 4 days after insemination (*51*; P. Olds-Clarke, personal communication).

Male Sexual Behavior

Male sexual behavior was analyzed quantitatively by observing male mice paired with females induced into behavioral estrus by hormonal injections (*52*). A series of behavioral parameters were observed and recorded during repetitive tests. These parameters included mount latency, intromission latency, ejaculation latency, number of head mounts, mounts without intromission (time per mount, total number), mounts with intromission (time per mount, total number, total number of intravaginal thrusts), and ejaculation duration (*53,54*). In the intervals between sex behavior tests, female mice paired with male mice were inspected for vaginal plugs. The presence of vaginal plugs was considered as evidence that the male mice had the capacity for male sexual behavior. The results from the sex behavior tests and natural breeding trials were compared to determine if the more stressful conditions of the sex behavior tests inhibited the male mice.

Utilization of Biological Markers

The biological markers of male reproduction were validated for use with mice. However, all of the biological markers were not assessed for each genotype. For example, if mouse testes were proven to be devoid of germ cells, we would not analyze sexual behavior or sperm function. A specific example from our stubby mouse studies (*55*) is cited to demonstrate the sequence in which the biological markers could be used to define the phenotypic effects of a gene mutation on male reproduction (Fig. 1).

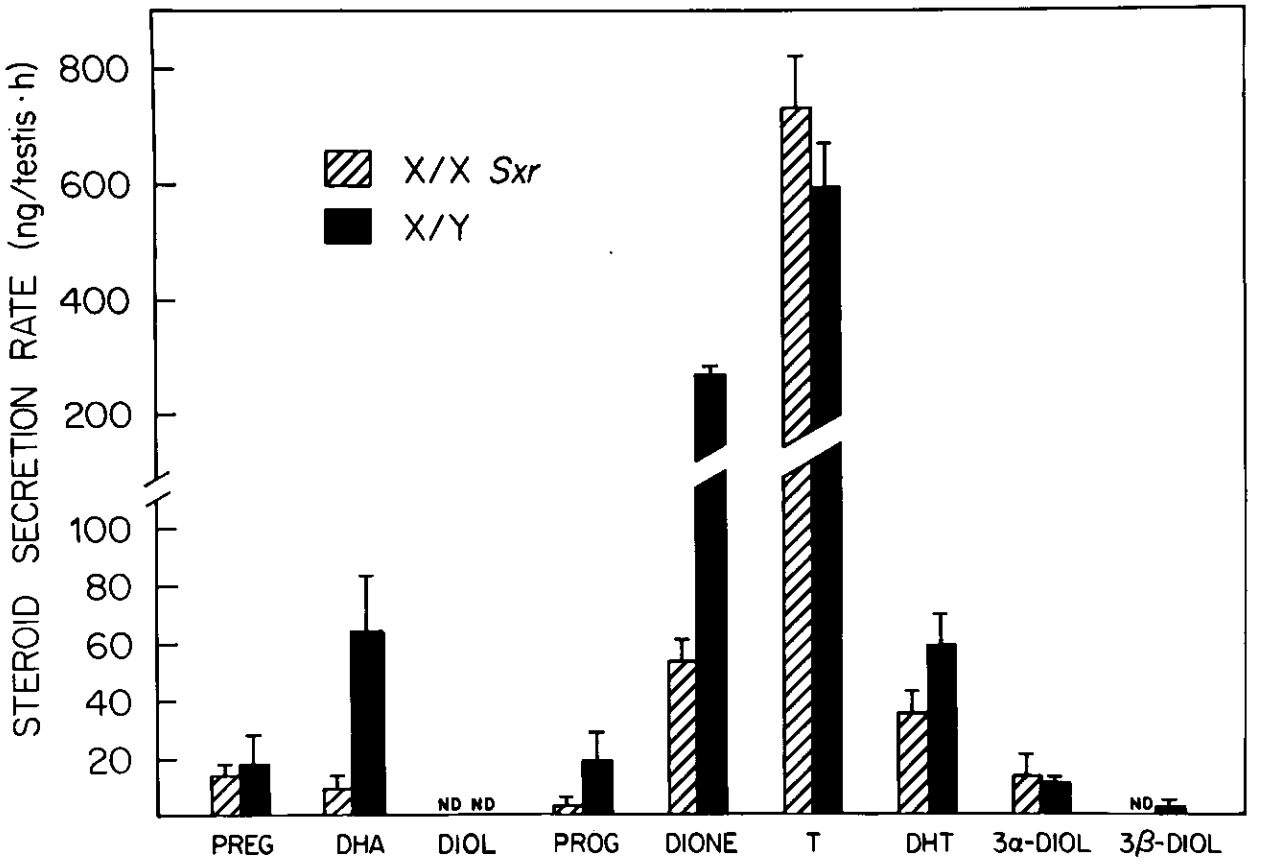

FIGURE 6. Steroid secretion profile for LH-stimulated, *in vitro* perifused testes of sex reversed mice (X/X *Sxr*) and normal males (X/Y). See Fig. 3 legend for explanation of abbreviations. The results are expressed as the mean ± SE ($n=5$). [From Chubb and Nolan (*64*) with permission.]

Animal Models of Male Infertility

The biological markers of male reproduction were used initially to classify gene mutations according to their effect on testicular function. Based on these initial results, certain gene mutations were selected for additional study. A brief description of the reproductive biology of the mutant mice studied in our laboratory is presented in the following paragraphs. Please be aware of two caveats: male infertility may be one of several pleiotropic actions of the gene mutation and this report presents only a brief overview of selected data collected in our laboratory and other laboratories. The cited references provide more information.

Gene Mutations That Affect Spermatogenesis Only (*at, bs, olt, qk, Sl^d, Sxr, W^v*)

***Atrichosis* (at/at).** The atrichosis mutant mouse is an animal model of the Sertoli-cell-only syndrome (*56*). At present, the etiology of the absence of germ cells is unknown. However, our studies (*57*) demonstrated that Leydig cell function was not impaired (Figs. 2 and 3). Both sexes are sterile.

***Blind-Sterile* (bs/bs).** Blind-sterile mouse testes contain seminiferous tubules with either Sertoli cells only or varying degrees of spermatogenesis (*58*). Of interest is the deleterious effect of the gene mutation on the acrosomal development of spermatids (*58*). Testicular steroidogenesis is not affected (unpublished observations). Blind-sterile female mice are fertile (*11*).

***Oligotriche* (olt/olt).** Spermiogenesis is abnormal in oligotriche mice, but testicular steroidogenesis is not affected by the gene mutation (*59,60*). Female oligotriche mice are fertile (*60*).

***Quaking* (qk/qk).** The quaking and oligotriche gene mutations have similar phenotypic effects on spermatogenesis and testicular steroidogenesis: abnormal spermatid flagellum development (*61*) and normal testicular steroidogenesis (*59*). Quaking female mice are fertile (*7*).

***Steel-Dickie* (Sl/Sl$_d$).** Steel-Dickie mice have several deficiencies, including the total absence of testicular and ovarian germ cells (*7*). Our studies (Fig. 4) confirmed the conclusion of Younglai and Chui (*62*) that testicular steroidogenesis is not affected by the Steel-Dickie gene mutation.

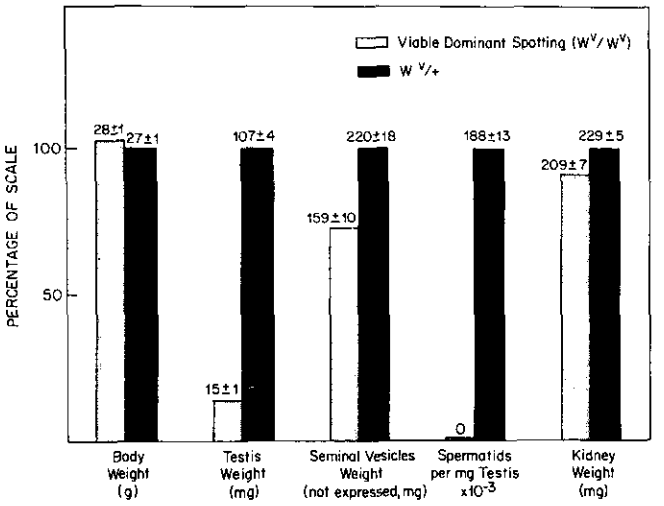

FIGURE 7. Summary of data for viable dominant spotting mice (W/W^v) and their siblings ($W^v/+$). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from 7–16 mature mice.

***Sex Reversed (X/X* Sxr).** Although originally postulated to be a single-gene mutation, the sex-reversing factor (*Sxr*) is now known to be a distal portion of the Y chromosome that is translocated to the X chromosome during meiosis (*14*). Genotypic X/X female mice bearing *Sxr* develop a male phenotype (*63*). We provided evidence that the Sertoli-cell-only testes of X/X *Sxr* mice secrete the same amount of steroids as testes from X/Y controls (*64*) (Figs. 5 and 6).

***Viable dominant spotting (*W/W^v*).** One of the pleiotropic effects of the W/W^v genotype is the failure of primitive germ cells to proliferate and migrate to the embryonic gonads of both sexes (*15*). Few or no spermatogonia can be identified in the testes of W/W^v mice. The small size of the testes prevented their *in vitro* perfusion; however, seminal vesicle weights (Fig. 7) support the finding of Amador and co-workers (*65*) that testicular steroidogenesis is not dramatically affected by the gene mutation.

Gene Mutations That Affect Spermatogenesis and Steroidogenesis (*df, dw, fl, hpg, Hst-1, Tfm*)

***Ames Dwarf (*df/df*).** Both sexes of Ames dwarf mice exhibit infertility that can be reversed by growth hormone, prolactin, or thyroxine replacement (*66,67*). The gene mutation reduces body growth and depresses both spermatogenesis and testicular steroidogenesis (*38*) via its affect on the pituitary (*66*).

***Dwarf (*dw/dw*).** Although the phenotypes of *df/df* and *dw/dw* are similar (*66,67*), the gene loci are on different chromosomes (*df*-mouse chromosome 11; *dw*-mouse chromosome *16*). The dwarf mouse testis is characterized by seminiferous tubules with varying degrees of spermatogenesis and diminished steroidogenesis (*38*) (Figs. 8–10).

***Flipper-Arm (*fl/fl*).** Flipper-arm mice have not been studied extensively because of their high neonatal mortality. Other than initial report of the mutation (*7*), we have provided the only experimental data about the

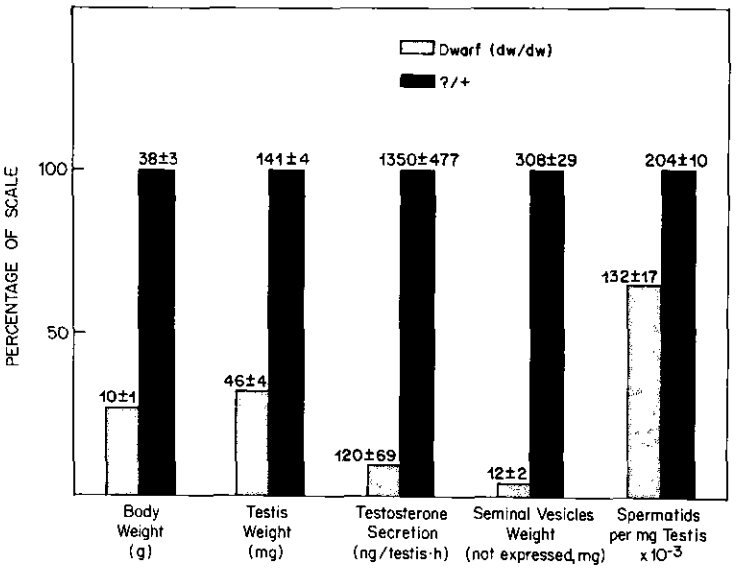

FIGURE 8. Summary of data for dwarf mice (*dw/dw*) and their normal siblings (?/+). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from 8–11 mature mice. Testosterone secretion by *in vitro* perifused testes maximally stimulated with LH was suppressed more than body weight in the mutant mice. [From Chubb and Nolan (*38*) with permission.]

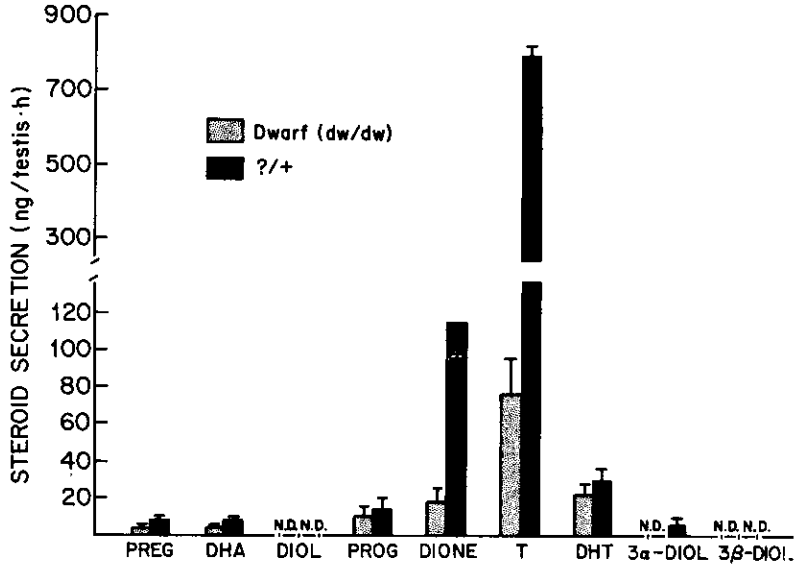

FIGURE 9. Steroid secretion profile for LH-stimulated, *in vitro* perifused testes of dwarf (*dw/dw*) and normal sibling (?/+) mice. See Fig. 3 legend for explanation of abbreviations. Each value represents the mean ± SE ($n = 4$–6). The relative decrease in secretion of all the measured steroids suggests that the mutation is affecting steroidogenesis prior to pregnenolone production. [From Chubb and Nolan (*38*) with permission.]

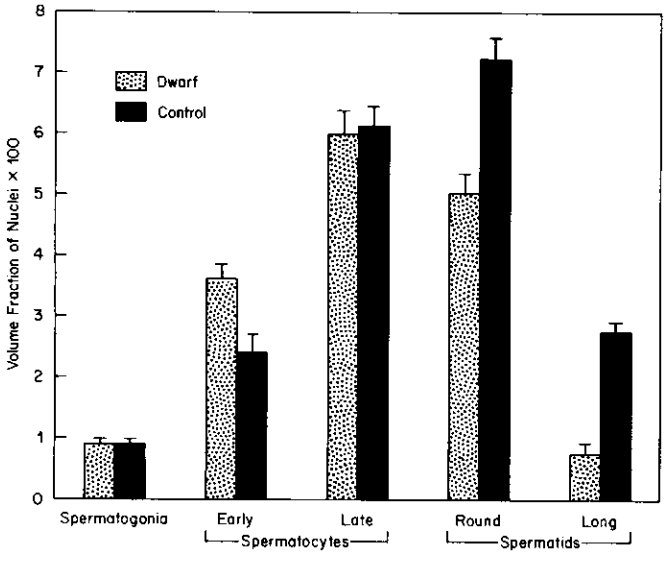

FIGURE 10. Nuclear volume fractions (%) of five germ cell classes in testes of dwarf mice (*dw/dw*) and their normal siblings (?/+). The values represent the mean of data collected from the evaluation of 10,000 test points on sections from testes of two mice for each genotype.

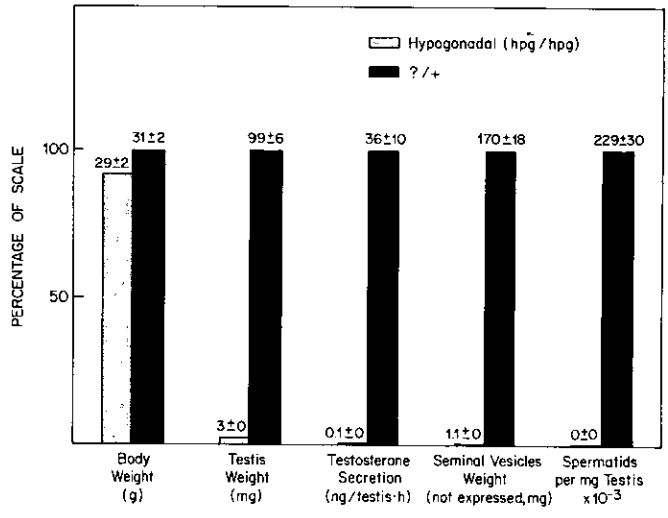

FIGURE 11. Summary of data for hypogonadal mice (*hpg/hpg*) and their normal siblings (?/+). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from four to six mature mice. Testosterone secretion was determined with perifused testes maximally stimulated with LH.

flipper-arm mice (*38*). One male survived to maturity during our breeding program. Steroidogenesis was decreased and mature spermatids were not produced in flipper-arm mouse testes.

***Hypogonadal* (hpg/hpg).** Male hypogonadal mice have immature reproductive tracts and small testes, and spermatogenesis rarely advances past the primary spermatocyte stage (*68*). This mouse has been used in several experiments (*69,70*) that include the description of the molecular defects in the gene coding for luteinizing hormone-releasing hormone (*71*) and the reversal of the mutation effects by gene therapy (*72*). Our experiments confirmed the drastic effect of the hypogonadal gene mutation on male reproduction in the absence of a decrease in body size (Fig. 11). Hypogonadal female mice are infertile (*68*).

***Hybrid Sterility* (Hst-1^{s}/Hst-1^{ws}).** One specific com-

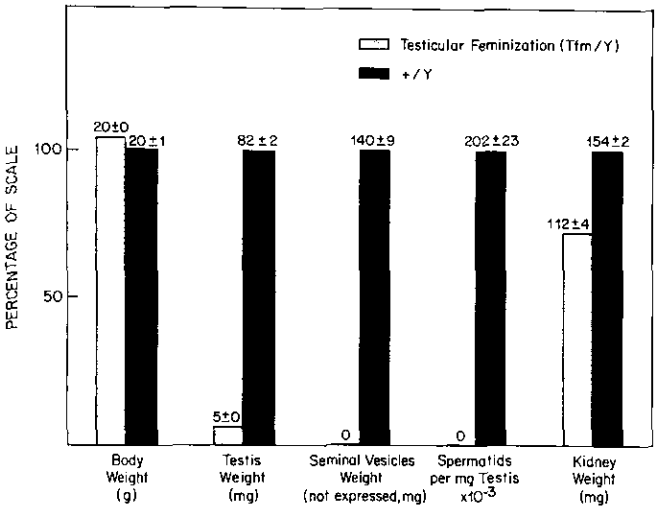

FIGURE 12. Summary of data for testicular feminized mice (*Tfm*/Y) and normal siblings (+/Y). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from four to ten mature mice.

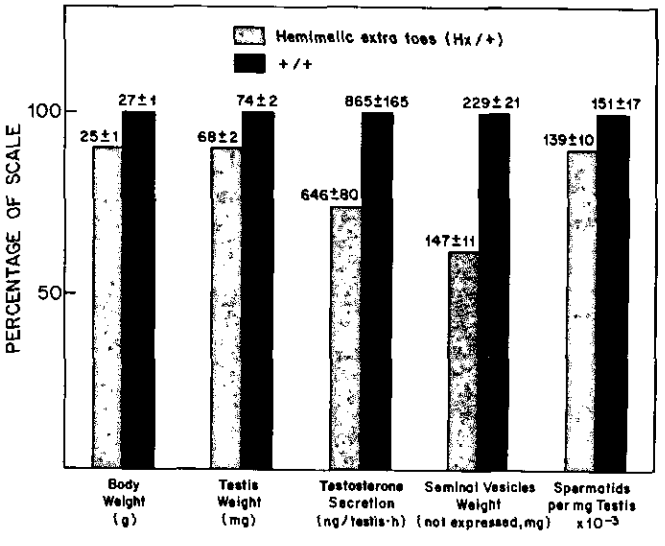

FIGURE 13. Summary of data for hemimelic extra toes mice (*Hx*/+) and their normal siblings (+/+). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from five to eight mature mice. Testosterone secretion by *in vitro* perifused testes maximally stimulated with LH was not significantly different between the two genotypes. Seminal vesicle weights were significantly different ($p < 0.05$).

bination of the alleles at the hybrid sterility gene locus (*Hst-1*^{s} and *Hst-1*^{f} for *Mus musculus domesticus* and *Hst-1*^{ws} and *Hst-1*^{wf} for *Mus musculus musculus*) results in the production of sterile male progeny: male mice bearing the *Hst-1*^{s}/*Hst-1*^{ws} genotype are sterile (*73,74*). Near-total spermatogenic arrest at the pachytene stage causes the sterility. Our studies (*75*) confirmed the spermatogenic arrest and provided evidence that the *Hst-1*^{s}/*Hst-1*^{ws} genotype decreases testicular steroidogenesis. Female hybrids are unaffected.

***Testicular Feminization* (Tfm/Y).** Male mice bearing the testicular feminization mutation resemble females phenotypically but have an XY karyotype (*76*). The primary defect is androgen insensitivity due to the absence of effective androgen receptors (*77*). Testes of *Tfm*/*Y* mice are characterized by spermatogenesis arrested at the spermatocyte stage or earlier (*76*) and defective steroidogenesis (*78*). Our studies of *Tfm*/*Y* mice provided data that were in agreement with the earlier reports (Fig. 12). The testes could not be perfused *in vitro* but the significant decline in seminal vesicle and kidney weight reflects their inability to respond to androgen stimulation.

Gene Mutations That Do Not Affect Either Spermatogenesis or Steroidogenesis (*Ht, Hx, hyt, lit, pg, stb*)

***Hightail* (Ht/+).** Although the initial report of hightail mice (*7*) did not mention their fertility, investigators at Oak Ridge National Laboratory stated that hightail males are infertile while hightail females are unaffected (E. M. Kelly, personal communication). Our preliminary studies (*38*) indicated that hightail mice have normal testicular function. The cause of the reported male infertility is not known.

***Hemimelic Extra Toes* (Hx/+).** The hemimelic extra toes gene mutation causes male-specific infertility (*79*). Our laboratory elucidated the effects of the mutation on testicular function (*36*) (Figs. 13 and 14). We concluded that testicular function is normal in the mutant mouse. One possible etiology of the male infertility is the incapacity to copulate effectively because of physical impairment (*7*).

***Hypothyroid* (hyt/hyt).** Hypothyroid male and female mice have been reported to be infertile (*12*). The gene mutation causes primary hypothyroidism. Thyroxine-supplemented feed reverses the deleterious effects of the gene mutation (*12*). In previous studies (*38*), we demonstrated that testicular steroidogenesis and spermatogenesis of hypothyroid mice were normal and that the defective aspect of the male reproductive process had not been defined. Our most recent studies of the fertility and sexual behavior of hypothyroid male mice suggest that they are fertile, although puberty may be delayed (unpublished observations).

***Little* (lit/lit).** Little male mice have been reported to exhibit a marked incidence of infertility, although little female mice are fertile (*13*). The primary defect of little mice is isolated growth hormone deficiency partly due to the altered function of somatotroph receptors for growth hormone-releasing factor (*80*). Our investigations revealed that testicular steroidogenesis and spermatogenesis are normal in little mice (*38*) (Fig. 15). In addition, videomicroscopic analyses of sperm motility and sperm length (Table 2) suggested that sperm function and morphology were normal. Sex behavior tests

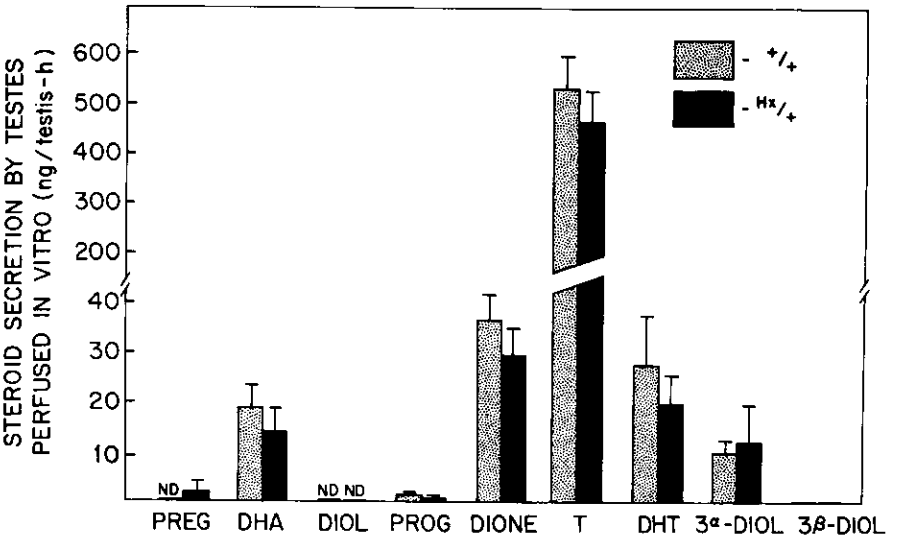

FIGURE 14. Steroid secretion profile for LH-stimulated, *in vitro* perifused testes of hemimelic extra toes (*Hx*/+) and normal sibling (+/+) mice. See Fig. 3 legend for explanation of abbreviations. The results are expressed as the mean ± SE of six determinations. While the testosterone secretion rate in Fig. 13 was determined radioimmunometrically using venous effluent collected during the third hour of perifusion, the values in this figure were derived by gas chromatographic analysis of venous effluent collected during the third and fourth hours of perifusion. The difference in sampling may explain the different values. [From Chubb and Nolan (*36*) with permission.]

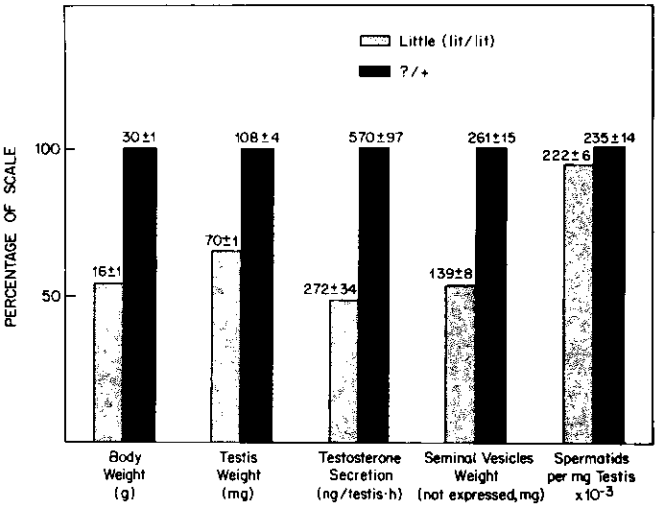

FIGURE 15. Summary of data for little mice (*lit/lit*) and their normal siblings (?/+). Data for mutant mice are displayed as a percentage of control values: absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from seven to eight mature mice. Testosterone secretion by LH-stimulated, *in vitro* perifused testes was determined. Organ weights and testosterone secretion determinations are allometrically correct. [From Chubb and Nolan (*38*) with permission.]

and natural breeding trials provided evidence that little mice were more accurately described as subfertile in contrast to infertile (unpublished observations). Together, the experimental data indicated that the primary etiological factor of the subfertility of little male mice is their diminutive size.

Table 2. Motility and length of sperm from little mice and their control siblings.

	n^a	*lit/lit*	+/−
Sperm length, μm	84	117.9 ± 0.8[b]	119.5 ± 0.5
Percent motility	480	58.8 ± 5.6	69.2 ± 2.2
Net velocity, μm/sec	300	56.3 ± 9.3	55.5 ± 12.8
Average path velocity, μm/sec	300	62.5 ± 9.7	63.9 ± 14.0
Linear index	300	0.90 ± 0.02	0.89 ± 0.01

[a] Number of sperm observed for each value; the epididymal sperm were obtained from 3–4 mice for each genotype.
[b] Mean ± SE.

***Pygmy* (pg/pg).** The miniature size of pygmy mice results from tissue unresponsiveness to growth hormone (*81*). Although King (*82*) originally reported that both sexes of pygmy mice were infertile, it is now known that the pygmy gene mutation on genetic backgrounds favoring large body size may not induce infertility (*7*). The latter observation agrees with our experimental data demonstrating that spermatogenesis and testicular steroidogenesis were not significantly affected by the pygmy gene mutation (*38*). Consequently, the defect in male reproduction of pygmy mice may be due either to a physical barrier to copulation related to their small body size or to an insufficient quantity of sperm in the ejaculate due to their small testes.

***Stubby* (stb/stb).** Stubby male mice were originally reported as infertile by Lane and Dickie (*83*). Female mice are unaffected by the gene mutation (*83*). We examined the testicular function of stubby mice and found that testicular steroidogenesis and spermatogenesis were normal (*38*) (Fig. 16). Subsequent studies dem-

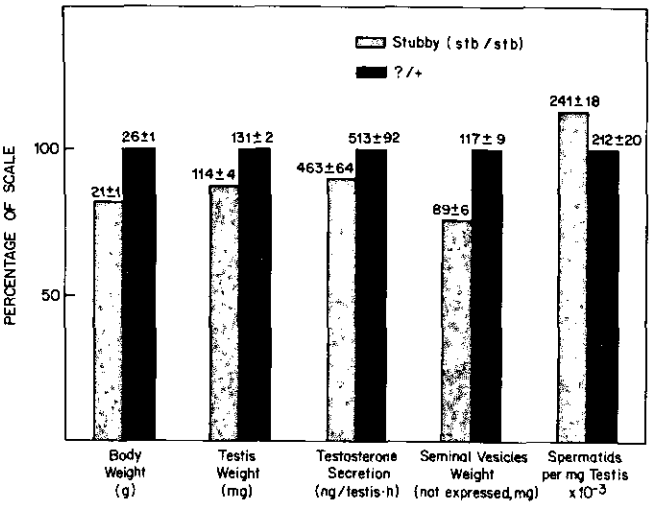

FIGURE 16. Summary of data for stubby mice (*stb/stb*) and their normal siblings (?/+). Data for mutant mice are displayed as a percentage of control values; absolute values are presented at the top of the columns. Each value represents the mean ± SE of results from six to eight mice. Testosterone secretion by LH-stimulated, *in vitro* perifused testes was determined. [From Chubb and Nolan (*38*) with permission.]

Table 3. Quantitative analysis of the male sexual behavior of stubby mice and their normal siblings.[a]

	stb/stb	+/−
Mount latency, sec	169.2 ± 28.9[b]	90.7 ± 18.1
Intromission latency, sec	—	168.9 ± 32.4
Mounts without intromission:		
Frequency, mounts/min	0.92 ± 0.06[b]	0.39 ± 0.07
Time per mount, sec	5.7 ± 0.4	4.8 ± 0.5
Mounts with intromission:		
Frequency, mounts/min	—	0.71 ± 0.06
Time per mount, sec	—	27.5 ± 2.1
Total number of thrusts	—	166.3 ± 24.3
Interval between mounts, sec	—	43.8 ± 6.1
Total mounts per min	0.92 ± 0.06	1.1 ± 0.05
Mounts before intromission	—	1.8 ± 0.2
Mounts with intromission before ejaculation	—	7.2 ± 1.3
Head mounts per mouse	1.0 ± 0.3[b]	0.1 ± 0.1
Ejaculation latency, sec	—	585.5 ± 85.6
Ejaculation duration, sec	—	20.0 ± 0.5
Test period duration, min	45 ± 0[b]	9.8 ± 1.4
Ejaculated sperm, $\times 10^{-6}$	0	27.3 ± 1.2
Female receptivity score	4 ± 0.1	4 ± 0.2
Number of observations[c]	43	53

[a] Values were derived by determining the mean of the included tests of each mouse and calculating the $\bar{x}$ ± SE of the means for all 12 mice of each genotype.

[b] Significant difference ($p < 0.05$, Wilcoxon Rank Sum Test).

[c] Tests were not included in data analyses if more than one female was used or if the receptivity score for the female was < 3 since the effects of these situations on male sexual behavior could not be considered comparable to those of acceptable tests.

onstrated that stubby mice were infertile during natural breeding trials, and furthermore, that they did not intromit or ejaculate during sex behavior tests (*55*) (Table 3). Next, we elucidated sperm motility by quantitative videomicroscopy and tested sperm function by artificial insemination. Both of these tests supported the normal function of stubby mouse sperm (unpublished observations). Together, the experimental observations provided unequivocal evidence that stubby mice are infertile because they are impotent. The etiology of the impotence may involve the central nervous system.

Conclusions and Applications

Our studies have provided the first comprehensive assessment of the effects that the described gene mutations have on male reproduction in mice. The phenotypic effects were elucidated by the application of several biological markers of male reproduction. We propose that these same biological markers would be effective in defining the action of environmental toxins on male reproduction. Figures 17*A* and *B* contain our suggestions concerning the sequence in which the biological markers could be implemented.

Two hypothetical situations will be discussed. In the first situation, an environmental chemical has been determined to cause male infertility. The flow diagrams in Figures 17*A* and *B* could aid in pinpointing the deleterious action of the toxin. In the second scenario, the goal is to assess the reproductive toxicology of a chemical. Details about the dosage, time schedules, and protocols will not be discussed (*84–87*). Initially, the experimental animals would be paired with behaviorally-primed females. Since the end point is the presence of a copulatory plug at a defined time, one person could monitor a large number of animals for reproductive defects. Also, the same male mice could be tested repetitively. The male mice could be examined for sperm output by quantifying the number of ejaculated sperm; again, this involves simple analysis and repetitive observations of the same mice. After the initial noninvasive markers were used, the more time-consuming, invasive biological markers could be assessed to identify specific causes of male infertility.

Inbred mice offer important advantages in the animal bioassays of reproductive risks. The advantages include genetic uniformity, availability of different inbred strains, and the control of conditions affecting male reproduction (age, social experience, environment, disease, genotype). Additionally, mice have short generation times and are reproductively active under diverse conditions (*88*).

We have described biological markers that primarily reflect physiological functions. The markers could be used as screening tests for the selection of more complex analyses. These biochemical and molecular analyses could focus on *in vitro* fertilization (*89*), postimplantation development (*90*), testicular autoimmunity (*91*), sperm capacitation (*92*), Sertoli cell function (*93*), sperm

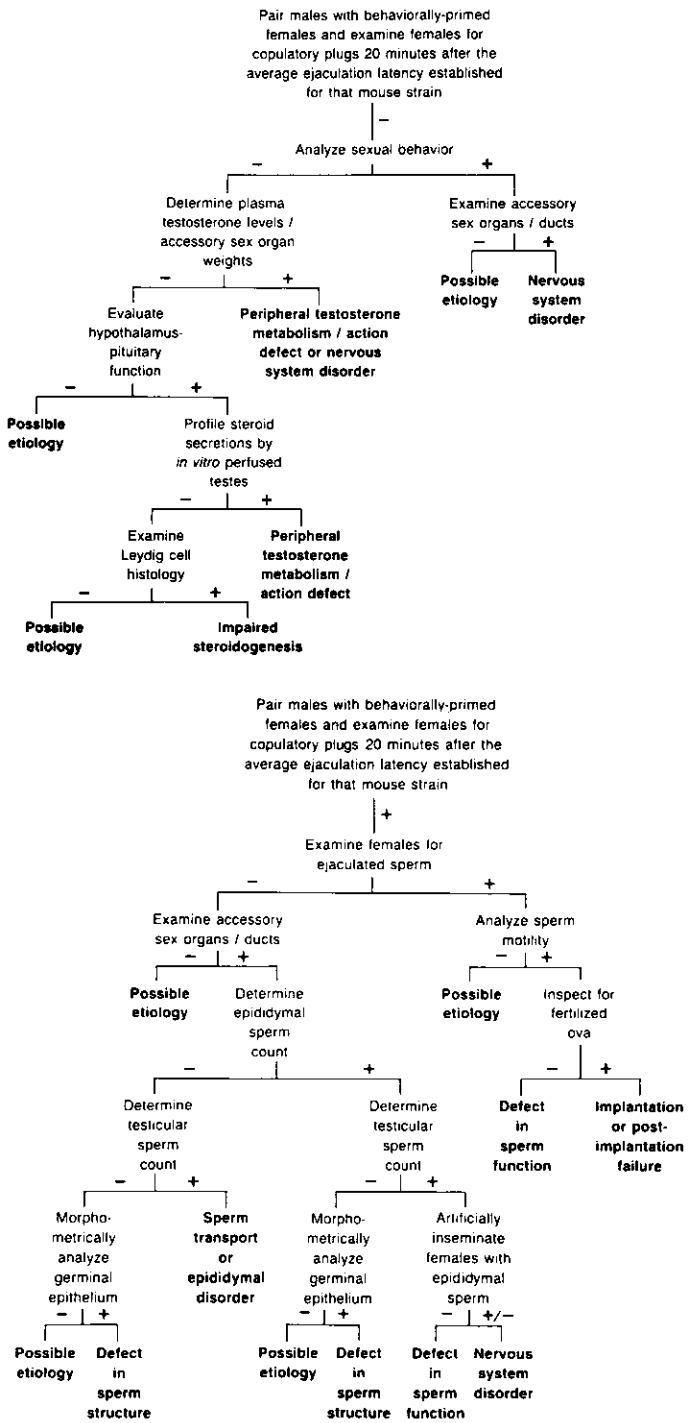

FIGURE 17. Suggested sequences for applying the biological markers of male reproduction. The '+' and '−' indicate results that are either similar or different, respectively, to those of control mice. Possible etiologies of the reproductive defects are displayed in bold letters.

antigens (*94*), germ cell cytogenetics (*85*), or Leydig cell ultrastructure (*95*).

Future Directions

The mutant mice are novel animal models that may assist in the development of accurate diagnostic parameters and effective therapies for male infertility. Furthermore, the mouse models represent a spectrum of reproductive defects and, as such, provide experimental tools for discovering new biological markers of male reproduction that could be evaluated noninvasively and be more sensitive than current markers. Future analysis of the gene mutations will be directed to deciphering

the biochemical events that are nearer to the primary gene action.

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