Insertional mutation in a transgenic mouse allelic with Purkinje cell degeneration

(neurodegeneration/spermatogenesis)

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ABSTRACT Purkinje cell degeneration (pcd) is an autosomal recessive mutation which maps to chromosome 13 in the mouse. The pcd mutation causes loss of cerebellar Purkinje cells, retinal photoreceptor cells, and olfactory bulb mitral cells, as well as abnormalities of spermatogenesis. pcd is among a number of autosomal recessive mutations in mice and humans that affect neurologic function and male fertility. The cloning of one or more of these loci would contribute significantly to our understanding of the genetic control of development and maintenance of affected cell types. We report here identification of a transgenic mouse line with an insertion mutation that is allelic with pcd and manifests histopathologic features indistinguishable from those of the spontaneous mutation. The creation of an allele of pcd by transgene insertion should make possible the cloning of the pcd locus.

In both mice and humans, an unmistakable but poorly explained connection exists between development of cells within the nervous system and within male reproductive organs. This relationship is made apparent by several unlinked autosomal mutations which affect both systems. In the mouse, the hotfoot (ho) mutation, located on chromosome 6 (1), causes a severe motor disorder, and the mice with the first alleles recognized at this locus were male sterile (1). The quaking (qk) mutation, located on mouse chromosome 17, causes abnormalities of myelination, and it disrupts spermatogenesis at the spermatid stage (2). A third unlinked mutation of this category is Purkinje cell degeneration (pcd), located on chromosome 13. Homozygotes for this mutation exhibit a dramatic and almost complete loss of cerebellar Purkinje cells, and in addition, severely reduced numbers of retinal photoreceptor cells and olfactory bulb mitral cells (1, 3, 5). Affected animals of both sexes are afflicted with an obvious motor ataxia, and males are often infertile, with sperm that lack motility (3, 5). pcd is particularly interesting because of its clearly identifiable histopathologic manifestations and because of its resemblance to a condition of familial cerebellar degeneration and male hypogonadism reported in humans (6).

The existence of multiple independent genes which affect both nervous system and male reproductive function suggests that the mechanism(s) by which development is controlled in these areas are similar. An obvious approach to understanding this control is to clone one or more of the aforementioned loci. Unfortunately, no obvious means has existed to isolate any of these genes.

The introduction of DNA into the mouse germ line by retroviral infection (7) or microinjection (8) can result in insertional disruption of genes with important roles in development. At the same time, foreign DNA insertion provides an approach to the cloning of disrupted host loci. By using the introduced DNA as a probe to screen genomic libraries from mutant animals it has been possible in a few instances to isolate clones which contain DNA flanking the exogenous integrated material and, thus, include portions of the interrupted gene. The α1(1) collagen gene, disrupted by retroviral infection (9), has been cloned by this approach, and subsequent analysis has led to a detailed characterization of the mechanism by which proviral DNA insertion resulted in host gene inactivation (9–12). Other groups have reported transgenic insertional mutant pedigrees that manifest embryonic lethality (13, 14), limb deformities (15, 16), dystonia musculorum (17), or abnormal sperm function (18). In none of these latter instances has the interrupted gene been well characterized, though allelism between some insertional mutations and previously identified host loci (15, 17) indicates that production of mutations by gene transfer has significant potential for elucidating the mechanism of action of many developmentally important genes.

With this potential in mind, we herein report identification and initial characterization of an insertional mutation at the pcd locus.

MATERIALS AND METHODS

Plasmids. The plasmid pFR400 (19) is a 4400-base-pair (bp) molecule which contains the cDNA of a mutant dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADPH oxidoreductase, EC 1.5.1.3) and the simian virus 40 early promoter (base pairs 1–550).

Production and Identification of the Transgenic Mutant Line. A series of transgenic mice carrying pFR400 was produced by the method of Gordon et al. (8), and the number of copies of the foreign gene insert in each line was determined by Southern blot hybridization (20) using known amounts of pFR400 as a standard. The line studied here, P432, was established on an outbred background by microinjecting embryos derived from CD-1 females crossed with B6D2F1 (C57BL/6J × DBA/2J) males, and it carried approximately 400 copies of the plasmid integrated at a single site in the genome (21).

Tissue Sections. Mutant mice were deeply anesthetized and perfused with a fixative solution of 4% (wt/vol) formaldehyde, 5% (wt/vol) acetic acid, and 60% (vol/vol) ethanol. Paraffin-embedded neural tissues were sectioned at 6–8 μm and stained with hematoxylin and eosin. Testes were fixed in 3% glutaraldehyde in sodium cacodylate buffer, refixed in 2% OsO4, dehydrated in ethanol, and embedded in Spurr resin. Sections 1–2 μm in thickness were stained with toluidine blue. Epididymides were prepared by fixation in Bouin’s fixative, embedded in paraffin, sectioned at a thickness of 10 μm, and stained with hematoxylin and eosin.

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Table 1. Progeny testing of the P432 transgenic line

<table>
<thead>
<tr>
<th>Cross</th>
<th>Litters</th>
<th>Pups born</th>
<th>Pups affected</th>
<th>% carriers among non-affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>H × N</td>
<td>2</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N × H</td>
<td>7</td>
<td>84</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H × H</td>
<td>20</td>
<td>156</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>A × N</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A × H</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

N, normal mouse; H, hemizygous transgenic mouse; A, affected transgenic mouse; NT, not tested.

Library Construction and Screening. DNA from a mutant animal was partially digested with Mbo I and fragments 10–20 kilobases (kb) long were electroeluted from agarose. These fragments were used to construct a genomic library in the bacteriophage EMBL3 (22). One million recombinant bacteriophage were screened with pFR400. Positive recombinants were then rescreened with pBR322, which has homology to pFR400 (19), to exclude cloning of the endogenous dihydrofolate reductase gene. Two positive clones carrying flanking mouse DNA were identified by digestion of bacteriophage minipreparations (4) with EcoRI. Since neither EMBL3 cloning arms nor pFR400 has EcoRI sites (19, 22), recombinant clones with such sites were identified as containing mouse genomic DNA. A 2.4-kb EcoRI fragment of one clone was shown to lack both pFR400 and mouse repeated elements by failure to hybridize with radiolabeled pFR400 or mouse genomic DNA.

Transgene Dosage Study. A Southern blot of Sac I-digested spleen DNA from one normal mouse, four obligate hemizygous transgenic mice, and five mutant transgenic mice was hybridized simultaneously with a mouse reference probe consisting of the 2.4-kb EcoRI fragment cloned from the region flanking the transgene, and with pFR400. The reference probe was added to the hybridization solution at a concentration of 10 ng/ml, and radiolabeled pFR400 was added at 0.1 ng/ml. Probes were radiolabeled by random priming using a kit obtained from International Biotechnologies (Prime Time). After exposure of the Southern blot to x-ray film, the relative intensities of plasmid and endogenous genomic bands were determined by scanning densitometry (E-C Apparatus).

Allelic Testing. Hemizygous P432 transgenic males were crossed to pcd<sup>-2</sup>/pcd<sup>-2</sup> females. Animals were examined for phenotypic anomalies characteristic of pcd. Normal and affected animals were then studied by Southern hybridization to determine if they carried pFR400.

RESULTS

Identification of a Mutant Phenotype in P432 Mice. All 17 transgenic lines (21) carrying pFR400 were bred to homozygosity to screen for recessive insertion mutations. In one line, P432, offspring from such crosses displayed abnormal neurologic and reproductive function. Affected P432 animals were normal until approximately 20 days after birth, when they developed a progressively ataxic gait. Eight of 22 affected males were sterile as evidenced by failure to sire progeny after at least three documented matings to normal females. When carrier P432 transgenic mice were mated to normal (nontransgenic) mice, all pups were normal (Table 1). When hemizygous males and females were interbred, 24% of offspring were affected; all of the affected and 60% of the nonaffected offspring were later shown to be transgenic ($\chi^2 = 24.11, P << 0.0001$). A fertile mutant female, when mated

![Fig. 1.](image-url) Photomicrographs of neural tissues from homozygous P432 transgenic mouse and an age-matched control. (Original magnification, ×250; printed at ×400.) In wild-type cerebellar cortex several Purkinje cells are indicated by arrows (a). These large neurons are absent within the cerebellar cortex of adult mutants (b). In wild-type retina the outer nuclear layer (ONL) contains the perikarya of rod and cone photoreceptors (c). In mutant retina, the ONL is thinned due to loss of photoreceptors (d). In wild-type olfactory bulb several mitral cells are indicated by arrows (e). In mutant olfactory bulb (f), a rare surviving mitral cell is indicated.
to a carrier male, produced three transgenic offspring, all of which exhibited the mutant phenotype (Table 1). Thus, genetic analysis indicated that these abnormalities followed a recessive pattern of Mendelian inheritance, with linkage of the phenotype to the transgene. Indeed, no recombination between the transgene and the locus associated with the neurologic trait has been found among 156 animals tested (Table 1).

Histologic Abnormalities in P432 Mutant Mice. Figs. 1 and 2 show the associated histopathologic findings in affected animals compared with normal mouse tissues. Purkinje cells are totally absent from affected adult mice (Fig. 1b). Degeneration of photoreceptor cells is evident from the progressive thinning of the outer nuclear layer of the retina (Fig. 1d). Examination of the olfactory bulb of a 10-month-old affected mouse revealed loss of almost all mitral cells (Fig. 1f). The sterile mutant males were found to be azoospermic. Sections of their reproductive organs revealed testes devoid of late spermatids, and epididymides which contained no mature sperm (Fig. 2). All mutant female mice tested were fertile, but they produced small litters (Table 1).

Southern Blot Analysis of Mutant Animals Indicated Homozygosity for the pFR400 Insertion. Restriction mapping was performed on recombinant bacteriophage to subclone fragments which could be used to test at the molecular level whether mutant animals were homozygous transgenics. Fig. 3 shows that map of one such phage studied in detail. A 2.4-kb EcoRI fragment was subcloned from the mouse genomic DNA of this bacteriophage and used as a reference probe for the amount of pFR400 present in affected and unaffected animals. As demonstrated in Fig. 3, digestion with Sac I would not be expected to generate a restriction fragment length polymorphism associated with transgene insertion, because a Sac I restriction site lies between the 2.4-kb probe and the pFR400 component of the recombinant phage. This probe identified a 15-kb fragment within the mouse genome after Sac I digestion (Fig. 4). Radiolabeled pFR400 added simultaneously to the hybridization detected a 4.4-kb band in mutant and hemizygous transgenic mice (Fig. 4, arrow). This band is the size expected for the core of the concatameric transgene insert, because the 4.4-kb pFR400 molecule contains a single Sac I site and because the low quantity of radiolabeled plasmid included in the hybridization (0.1 ng/ml) would not be expected to detect minor plasmid-derived bands. When the intensities of the plasmid band relative to the band identified by the reference probe were compared by scanning densitometry, it was clear that all mutant animals contained approximately twice as much pFR400 DNA per diploid genome as hemizygous animals did (Fig. 4).

Allelic Testing Indicates Insertion of pFR400 into the pcd Locus in P432 Transgenic Mice. Because of the remarkable
 similarities between P432 homozygous transgenic mice and pcd homozygotes, allelism with pcd was tested by mating hemizygous P432 males to pcd<sup>fl</sup>/pcd<sup>fl</sup> females. In two litters, 7/10 animals were affected with the ataxic disorder. Non-complementation of such closely matched traits suggests allelism. If the trait in the transgenic pedigree is due to insertional mutagenesis, then all mutant animals in these crosses would be predicted to be transgenic mice, while all phenotypically normal animals would not be expected to carry the transgene. Dot blotting of DNA from these offspring showed that all affected mice were hemizygous for pFR400 and all phenotypically normal animals were not transgenic (Fig. 5). That transgene segregation would follow a pattern of allelism with pcd as a chance event is highly unlikely ($\chi^2 = 10, P << 0.005$). These DNA analyses strengthen the phenotypic analyses which indicated allelism between the pFR400 insertion site and the pcd locus. The observed recombination frequency between the trait and the transgene is 0%. The upper 95% confidence limit is 17% when only the 156 repulsion intercross (Table 1) and 10 backcross offspring are considered. Thus the breeding data confirm that the pFR400 insertion site is most likely to be between 0 and 17 centimorgans from the pcd locus.

**DISCUSSION**

In our mouse colony a transgenic line established by micro-injection of the recombinant plasmid pFR400 exhibits a severe motor disorder and male infertility when male and female hemizygotes are crossed. Genetic analysis indicates that the mutant phenotype probably results from recessive insertional mutagenesis. Southern analyses further support this conclusion, showing that affected animals carry twice the amount of pFR400 per diploid genome that unaffected members of the line do.

Although persuasive, the above data do not formally differentiate insertional mutants from those which might arise from transgene expression. Hemizygous transgenic mice might express pFR400 at a level not sufficient to cause abnormalities, while additive expression of two transgenes in homozygous mice could exceed a threshold of expression beyond which developmental abnormalities are induced. This possibility was addressed by testing the P432 mutation for allelism with spontaneous mouse mutations known to affect Purkinje cell development. The behavioral and histologic features of P432 mutants bore striking similarities to those of pcd/pcd mice, with the same three cell types of the central nervous system affected, and with severe impairment of spermatogenesis. These findings encouraged us to test this transgene insertion site for allelism with pcd. The finding that double heterozygotes for the transgene and the pcd allele manifest the mutant phenotype constitutes a positive allelism test and provides compelling evidence that the disorder in the transgenic pedigree resulted from insertion into the pcd locus. This pcd mutation has accordingly received the allele designation pcd<sup>fl</sup>. It is not surprising that some mutant pcd<sup>fl</sup> males were sterile (Fig. 2) while others were fertile. Whereas pcd mutant males are infertile, pcd<sup>fl</sup> mutant males are not. Since pcd<sup>fl</sup> was established on a hybrid background (21), breeding may result in cosegregation of the transgene with modifying alleles which alter the effect of the insertional mutation upon male fertility.

As noted previously, we have cloned material flanking pFR400 in P432 mice. Initial tests for RNA homologous to the cloned material have been negative. However, as with several other insertional mutations, continued chromosome walking should lead to isolation of coding sequences of the pcd gene. Moreover, because the mutations of many independent genes affect both neurologic and reproductive tissues, it is reasonable to hypothesize that these genes are members of a family, and as such, share sequence homology. If this is the case, cloning of the pcd locus may lead to the isolation of a number of related genes with similar functions. Even if this proves not to be the case, continued analysis of pcd<sup>fl</sup> should lead to isolation of the pcd gene and thereby contribute significantly to our understanding of development and maintenance of the mammalian nervous system and to the relationship between those processes and spermatogenesis.

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