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Insertional mutagenesis in transgenic mice generated by the pronuclear microinjection procedure

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ABSTRACT Insertional mutagenesis in transgenic mice is a powerful method to study structure/function relationships between individual genes and complex developmental traits in the whole organism. Unlike spontaneous or chemical-induced mutations, insertional mutations have the advantage that the mutant locus is "tagged" with the transgene and, therefore, readily accessible at the molecular level. Starting with the work on the limb deformity locus, we describe here the characterization of several mouse mutants generated by insertional mutagenesis with the pronuclear microinjection procedure. These transgenic lines have proven to be ideal as models for human disease and for studying the function of novel genes during development. We also describe the unique features of insertional mutations that arise in transgenic mice produced with the pronuclear microinjection procedure and provide recommendations on how to clone and characterize these mutations at the molecular level. Finally, we discuss future prospects for the use of this unique form of germline mutagenesis in the mouse.

KEY WORDS: *mouse mutants, insertional mutants, pronuclear microinjection*

Introduction

The production of transgenic mice with the pronuclear microinjection procedure has become a reliable and powerful procedure for studying mammalian gene function. In a typical experiment, a small volume of DNA is microinjected directly into the pronucleus of the zygote; in some of the mice that develop from these embryos, the exogenously added DNA integrates into a host chromosome to become a stably heritable genetic trait. Integration of the transgene most often occurs at only one or a limited number of sites in the genome of the transgenic founder animal, with each site potentially harboring anywhere from one to hundreds of copies of the microinjected fragment, typically in a head-to-tail configuration. The pronuclear microinjection technique has been utilized primarily for expressing exogenous genes in mice and has emerged as an important tool for developing mouse models for many human diseases, including cancer (Palmiter and Brinster, 1986).

The integration of the transgene into the host genome leads to a disruption in the structure of the chromosomal DNA at the integration site and causes a special class of mutations referred to as "insertional mutations" (Palmiter and Brinster, 1986). Mintz and colleagues were among the first investigators to appreciate the usefulness of insertional mutations arising in transgenic mice (Wagner *et al.*, 1983), and many other investigators have exploited insertional mutations for characterizing mutant mouse lines at the molecular level (Mark *et al.*, 1985; Overbeek *et al.*, 1986; Wilkie and Palmiter, 1987; Kothary *et al.*, 1988; McNeish *et al.*, 1988; Krulewski *et al.*, 1989; Gordon *et al.*, 1990; MacGregor *et al.*, 1990; Ratty *et al.*, 1990; Beier *et al.*, 1991; Crenshaw *et al.*, 1991; Merlino *et al.*, 1991; Tutois *et al.*, 1991; Vogt *et al.*, 1992; Hodgkinson *et al.*, 1993; Keller *et al.*, 1994). Insertional mutations are usually recessive and have the unique property that they are "tagged" at the molecular level with the inserted transgene. In many instances it has been possible to clone the sequences flanking the transgene insertion site utilizing molecular probes corresponding to the transgene. This has been a useful way to achieve access to the genomic region disrupted by the insertion and to identify the gene directly linked with the mutant phenotype (Gridley *et al.*, 1987; Meisler, 1992; Rijkers *et al.*, 1994; Friedman, 1996). The frequency of insertional mutations in transgenic lines appears to be about 5-10% (Palmiter and Brinster, 1986; Meisler, 1992; Rijkers *et al.*, 1994). This estimated frequency is based largely on observable phenotypes and prenatal lethality. This may be an underestimation of the actual frequency of insertional mutation, since not all insertional mutations will be detected with a given screening procedure.

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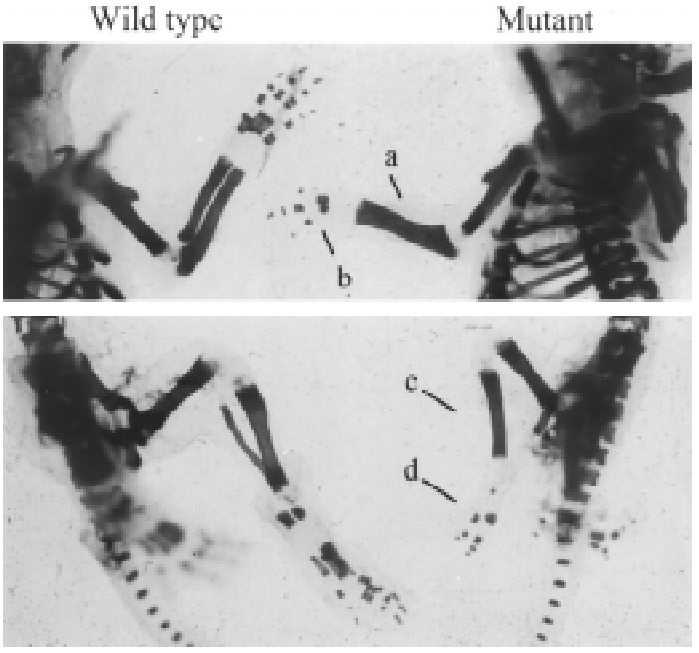

Fig. 1. Skeletal defects in the fore- and hind-limbs of the limb deformity mutant compared to a wild-type littermate. *The skeletal defects are: a, fusion of the radius and ulna, b, oligodactyly and syndactyly of the digits of the fore paw, and fusion of some carpels and metacarpels; c, replacement of the tibia and fibula with a single triangular bone, d, oligodactyly and syndactyly of the digits in the hind paw, and fusion of some tarsals and metatarsals (Woychik et al., 1985).*

Insertional mutations that cause skeletal abnormalities

Many of the insertional mutations arising from the pronuclear microinjection technique cause interesting developmental phenotypes. One of the first insertional mutations that causes a morphogenetic phenotype in mice arose in Dr. Philip Leder's laboratory at Harvard Medical School (Woychik et al., 1985). This new insertional mutation proved to be an allele of an existing recessive mutation called *limb deformity*, which causes a characteristic skeletal defect in the fore- and hind-limbs of the animal (Fig. 1). In the mutant animals that are homozygous for the transgene the radius and ulna are fused, the tibia and fibula are replaced with a single triangular bone, and the carpal and metacarpals are disorganized and reduced (Woychik *et al.*, 1985). Additionally, the animals exhibit kidney agenesis and other renal defects (Maas *et al.*, 1990; Woychik *et al.*, 1990a). The transgene was used as a molecular marker to clone and characterize the mutant locus at the molecular level. In this case, the integration of the transgene also caused a deletion of about 2 kb in the host chromosome at the site of the transgene insertion. Ultimately, a complex gene whose structure and expression is affected by the inserted transgene was identified (Mass *et al.*, 1990; Woychik *et al.*, 1990b).

As a follow-up to the original work on the *limb deformity* mutation, we have more recently been characterizing two different insertional mutations that cause skeletal abnormalities. The first of these is recessive and arose in the *TgN370Rpw* transgenic line. Animals homozygous for this transgene exhibit a kinky-tail defect associated with an undulated spine (Schrick *et al.*, 1995). To characterize the kinky-tail defect in more detail, we prepared alizarin-red stained skeletons from adult mice (day 65+) and systematically analyzed them using an established protocol (Selby, 1987). All of the skeletal structures in the *TgN370Rpw* mutant mice appeared normal except for the distal region of the tail (Schrick *et al.*, 1995). Tails from mutant animals have a disruption in the alignment of the last seven caudal vertebrae (Ca 24-30 in the FVB/N background), which is caused by misshapen development of the posterior portion of individual vertebrae. Consequently, the vertebrae are positioned at right angles to each other rather than in a normal linear arrangement, which causes the visible kink in the tail (Fig. 2). No disruptions of the intervertebral disks or bony fusions were noted in the mutant tails. In mutant neonates, the kinky-tail phenotype first becomes apparent at about nine days after birth.

In order to characterize the mutation in the *TgN370Rpw* line at the molecular level, we cloned and characterized both the mutant locus and the corresponding wild-type region (Schrick *et al.*, 1995). In this case, there were no major structural alterations that could be detected at the cytogenetic or molecular level, other than the insertion of the transgene. In fact, sequence analysis of the mutant and corresponding wild-type regions indicated that a single T nucleotide is duplicated on one side of the transgene, and a duplication of two nucleotides, TA, had occurred on the other end of the transgene (Schrick *et al.*, 1995). Therefore, this mutant line is an example of an insertional mutation where the transgene essentially integrated into a chromosome site without causing any significant structural alterations in the host genomic DNA.

The transgene integration site for the *TgN370Rpw* line was mapped utilizing recombinant inbred strains (Taylor, 1978). In this case the transgene integration site mapped to mouse chromosome 5, closely linked to a previously known mutation called *Thick tail (Tht)* (Green, 1989; Kozak and Stephenson, 1993). *Tht* is a dominant mutation which causes a shortened, thickened tail that is approximately two-thirds of the normal length and develops undulations as the tail grows (Beechy and Searle, 1980). On the basis of the close proximity of *Tht* and the *TgN370Rpw* insertional mutation, we performed a complementation test by generating individual animals that were heterozygous for both mutations. Compound heterozygotes *(Tht/TgN370Rpw)* resemble *Tht/+* mice, except that these mice have even shorter tails (half the average length of wild type) and also show a kink at the end of their tails. The fact that *Tht/TgN370Rpw* mice express a unique phenotype that overall is more severe than either mutation by itself suggests that perhaps *Tht* represents a dominant mutation of the same gene associated with the *TgN370Rpw* insertional mutation. Currently, efforts are underway to clone the gene associated with the *TgN370Rpw* mutation. Identification of this gene will be necessary to understand the molecular basis of the observed phenotype in *TgN370Rpw* and *Tht/TgN370Rpw* mice.

The other skeletal mutation that we are characterizing is recessive and arose in the *TgN737Rpw* transgenic line (Moyer *et al.*, 1994). The most apparent feature of the skeletal phenotype in this line is preaxial polydactyly in both the fore- and hind-limbs of the animals (Fig. 3). Careful analysis of the remaining skeleton revealed a number of other defects, which include disorganized frontal and parietal sutures in the skull, an extra tooth, minor clefting of the palatine process, and an absence of a large

foramen in the periotic capsule surrounding the inner ear (Yoder, Woychik *et al.*, manuscript in preparation).

Molecular characterization of the mutant locus in the *TgN737Rpw* line revealed that the insertion of the transgene did not cause a large deletion of the host chromosome; this ultimately led to the identification of a gene whose structure and expression was interrupted in this line (Moyer *et al.*, 1994). This gene, which was named *Tg737*, encodes a prominent 3.2-kb mRNA and several other transcripts that are normally expressed at very low levels. Analysis of the expression of the *Tg737* gene with the Northern blotting procedure revealed that the gene is expressed with a wide tissue distribution in the adult (Moyer *et al.*, 1994).

The putative protein product of *Tg737* is 824 amino acids in length and represents a novel gene that has not been previously described (Moyer *et al.*, 1994). Analysis of the amino acid sequence and a search with the BLAST network service of the National Center for Biotechnology Information revealed that the protein contains 10 copies of an internally repeated 34-amino acid motif referred to as the tetratricopeptide repeat (TPR). The TPR was first described in lower eukaryotes as a motif associated with several genes involved in cell cycle control (Sikorski *et al.*, 1990; Goebl and Yanagida, 1991). The fact that Tg737 contains a motif common to cell cycle control proteins is consistent with the phenotype observed in the insertional mutant, namely biliary hyperplasia and other cellular changes in the kidney during formation of cystic structures in the collecting duct epithelium.

Mouse models for human disease

We have also been able to use insertional mutagenesis as a powerful tool for generating mouse models for human disease. In addition to the skeletal defects in the *TgN737Rpw* transgenic line, homozygotes also develop bilateral polycystic kidney disease (Moyer *et al.*, 1994; Yoder *et al.*, 1995). Gross examination of mutant kidneys revealed that they were slightly enlarged, pale, and contained numerous cysts, the development of which was concurrent with the destruction of the surrounding parenchyma (Fig. 4A,B). Also, livers in the mutant animals were slightly pale with a prominent reticular pattern (Fig. 4A). Histologically, the lesions in the kidneys and livers of the mutant mice (Fig. 4B,C) were remarkably similar to those seen in humans with autosomal recessive polycystic kidney disease (ARPKD). The cardinal features of both the kidney lesion (collecting tubule ectasia) and the liver lesion (biliary dysplasia and/or portal hepatic fibrosis) typical of human ARPKD (Bernstein and Gardner, 1986; Lippert, 1991) were a constant finding of the disease in the mutant animals. In the kidneys, an initial mild, multifocal, microscopic dilation of the proximal tubules was followed rapidly by marked dilation and cyst formation of the collecting tubules. Similarly, in human ARPKD, the advanced lesions are localized to the collecting tubules (Bernstein and Gardner, 1986; Lippert, 1991), while proximal tubular cysts can be demonstrated at early stages of the disease (Avner, 1988,1993).

We were able to establish that the cloned *Tg737* cDNA can rescue the kidney lesions in the mutant animals (Yoder *et al.*, 1996)**.** Like humans with ARPKD, the insertional mutants with PKD lose their ability to concentrate their urine, which is a functional defect associated with the kidney lesions. Expression of the *Tg737* cDNA as a transgene in the mutant background

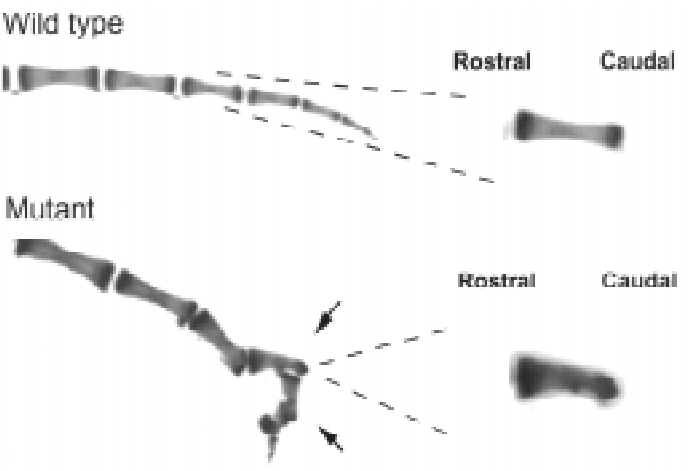

Fig. 2. Alizarin staining preparations of the tail of an adult *TgN370Rpw* insertional mutant compared to a wild-type littermate. *The kinks in the mutant tail bone are indicated by arrows, which show the defective caudal region of the ossifying vertebrae. One specific vertebrae, Ca25, from normal and mutant tails is shown at the side.*

effectively rescued the urine concentrating function in the mutant animals (Yoder *et al.*, 1996). Analysis of the kidneys of the rescued animals also revealed a substantial improvement in the histology of the kidneys. At the developmental time period when most of the mutants die from the disease, the rescued animals have kidneys that are essentially free of cysts (Yoder *et al.*, 1996). These experiments provided a direct link between the *Tg737* gene and the kidney lesions in the mutant animals.

We have also been able to use the *TgN737Rpw* model for ARPKD to study the disease pathway of polycystic kidney disease. A consistent feature of the development of ARPKD in humans and mice is a distension of the renal collecting tubules caused by a localized proliferation and aberrant secretion of epithelial cells (Bernstein and Gardner, 1986; Avner, 1988,1993; Lippert, 1991). The expanding structures develop into cysts that are filled with fluid containing biologically active ligands for the epidermal growth factor receptor (EGFR), such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α) (Gattone *et al.*, 1990; Horikoshi *et al.*, 1991; Avner, 1995). The EGFR is normally localized at the basolateral surfaces of the collecting tubule epithelium, but becomes mislocalized to the apical surface on the cells lining the cystic structures. The mislocalization of the EGFR is known to occur in both humans with ARPKD and in the *TgN737Rpw* and other mutant lines of mice that develop recessive polycystic kidney disease (Wilson *et al.*, 1993; Avner, 1995; Avner and Sweeney, 1995; Du and Wilson, 1995; Orellana *et al.*, 1995). Along with this mislocalization to the apical surface, there is an increase in EGFR mRNA and protein, as well as in the tyrosine kinase activity of the EGFR in the diseased kidneys (Wilson *et al.*, 1993; Avner and Sweeney, 1995; Orellana *et al.*, 1995).

We were interested in determining whether the increased EGFR tyrosine kinase activity in the collecting tubule is part of an autocrine/paracrine cycle that drives the cellular proliferation which is required for cyst formation and enlargement (Avner, 1993). To test this possibility and to establish the role of increased

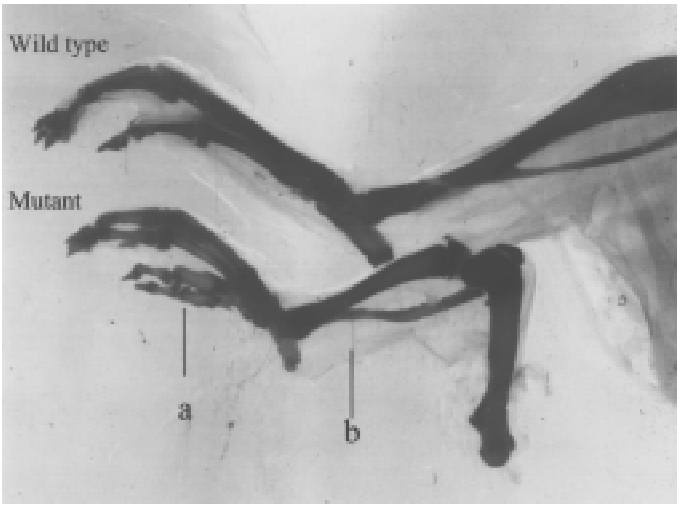

Fig. 3. Hind-limb skeletal defect in the adult *TgN737Rpw* insertional mutants. *Most notable are: a, preaxial polydactyly, and b, detachment of the tibia and fibula.*

EGFR tyrosine kinase activity in the formation of renal cysts *in vivo*, we used a genetic approach to decrease the tyrosine kinase activity of the EGFR in the *TgN737Rpw* model for ARPKD. To accomplish this we crossed the *TgN737Rpw* mutant mice with the *waved-2 (wa2)* mutation. The *wa2* mutation is a point mutation in the coding region of the EGFR gene that leads to a protein that is otherwise normal except that it exhibits only about 10% of its normal tyrosine kinase activity (Luetteke *et al.*, 1994). As expected, introduction of the *wa2* mutation blocked the increase in tyrosine kinase activity of the EGFR to varying degrees in the mutant kidneys, and, most important, this decrease in the tyrosine kinase activity of the EGFR correlated with a decrease in the formation of renal cysts in the collecting duct (Richards *et al.*, 1998). These results suggest that changes in the activity of the EGFR contribute directly to the formation of cysts in the collecting ducts. This is an important finding with respect to the development of clinical therapies for ARPKD, because it suggests that drugs that target the tyrosine kinase activity of the EGFR may potentially be therapeutic in ARPKD.

Another new recessive insertional mutation arose in our laboratory in the *TgN2742Rpw* transgene line. Homozygotes in this transgenic line develop a form of deafness that models the neuroepithelial class of deafness in humans (Schuknecht, 1993). Mutations that affect the sensory neuroepithelia are thought to be a major cause of inner ear defects in humans (Fraser, 1976; Schuknecht, 1993). Mutants in this line were easy to recognize because they exhibit a behavioral phenotype consisting of bi-directional circling, hyperactivity, head tossing in the vertical plane, and an inability to swim (due to loss of orientation). Audiometric evaluations showed that the homozygous animals are deaf (Alagramam, Erway, Woychik *et al.,* manuscript in preparation). Even at postnatal day 10, homozygotes can be easily distinguished from their normal littermates because of their noticeable unstable gait and their propensity to move in circles.

Histological examination of inner ear specimens from adult *TgN2742Rpw* mutant animals revealed inner ear abnormalities affecting both the cochlear and vestibular portions of the membranous labyrinth. At approximately one year of age, the organ of Corti is almost entirely missing throughout all the cochlear turns, and no inner or outer hair cells or supporting cells are present (Fig. 5A,B). The spiral ganglion shows severely reduced numbers of neurons, which probably reflects ganglion cell degeneration secondary to loss of the inner hair cells. Although scattered strial atrophy affecting primarily the apical cochlear turn has been noted, the stria vascularis in these mutants appears mostly normal. In addition, Reissner's membrane and the membranous wall of the saccule are in their normal position, indicating that strial pathology is not a primary defect in these animals. The vestibular apparatus in the *TgN2742Rpw* insertional mutants shows pathology largely restricted to the saccule. In adults, the neuroepithelium of the saccular macula is completely absent, with no recognizable hair cells or supporting cells (Fig. 5C,D). The neuroepithelia of the utricle and cristae of the semicircular ducts appear normal. Sparsely distributed, abnormally large otoconia have been noted overlying the utricular macula in some specimens. The loss of saccular neuroepithelium and otoconial anomalies in the utricle are consistent with the compromised balance and circling behavior consistently observed in the homozygous mutants in this line.

Utilizing the transgene as a molecular tag, we have been able to clone the mutant region associated with the *TgN2742Rpw* insertion site (Kwon, Alagramam, Woychik *et al.*, manuscript in preparation). The mutant locus was mapped to a region on chromosome 10, where three spontaneous, recessive mutation causing deafness have been mapped: *Ames Waltzer (av)*, *Waltzer (v),* and *Jackson circler (jc).* After performing a complementation test on these three mutants, we determined that the insertional mutation in the transgenic line fails to complement *av,* which suggests that the mutation in the *TgN2742Rpw* transgenic line is allelic to *av.* (Alagramam, Woychik *et al.*, manuscript in preparation). Therefore, like the *limb deformity* mutation, it appears that the *TgN2742Rpw* insertional mutation is allelic with an existing spontaneous mutation. Access to the mutant region with the probes from the insertional mutation should allow us to identify and characterize the gene directly associated with the mutant phenotype.

Nature of insertional mutations

Based on the molecular analysis of insertional mutations, it is becoming increasingly apparent that many insertional mutations are not exactly what the name implies, i.e., they are not necessarily simple mutations that arise through the insertion of an exogenous fragment of DNA. For example, large deletions estimated to be in excess of 500 kb have been reported for the *downless* and *legless* insertional alleles (McNeish *et al.*, 1988; Shawlot *et al.*, 1989), and insertional alleles of *limb deformity*, *fused* and other mutations are associated with a more modest sized deletion (Woychik *et al.*, 1985; Vogt *et al.*, 1992; Perry *et al.*, 1995). Also, a translocation that arose in a line of transgenic mice was shown to have integrated the transgene at the translocation breakpoint (Overbeek *et al.*, 1986). In other instances, a small translocated fragment has been shown to have co-integrated with the exogenously added transgene fragment (Pohl *et al.*, 1990; Moyer *et al.*, 1994). Therefore, insertional mutations generated by the pronuclear microinjection procedure may be comprised of simple

transgene insertions (as in the *TgN370Rpw* mutation described above), or of variable sized deletions or other chromosomal structural alterations like translocations in addition to the transgene integration.

The mechanism for transgene integration has not been studied to any great extent, so it is impossible to know exactly why the integration of the transgene causes structural changes in the chromosomes. It is noteworthy that ionizing radiation causes deletions, translocations and inversions, which are exactly like the kinds of structural changes in the DNA that are associated with insertional mutations. But unlike radiation-induced breakpoints, the unique feature of insertional mutations is that the DNA breakpoints are "tagged" with the integrated transgene, which allows the DNA breakpoints to be accessed directly at the molecular level. Due to the similarity in the spectrum of mutations arising from insertional and radiation-induced mutagenesis, we predict that the mechanism of transgene integration is similar to that which causes the DNA structural changes in cells exposed to ionizing radiation. In much the same way that radiation causes double-stranded breaks in the DNA, it is possible that the physical manipulation and microinjection of the zygote causes DNA breaks. Repair of the DNA strand breaks could lead to an integration of the exogenously added DNA at the breakpoints in the host chromosomes. Given this scenario, there is a greater chance that there will be chromosomal changes at the transgene integration site if the host chromosomes become more highly fragmented during the microinjection process.

Large deletions at the transgene insertion site can complicate efforts to identify the gene that is directly associated with the mutant phenotype. Deletions of multiple genes will often express the phenotype of the first acting essential gene within the deleted region, and the challenge then becomes one of identifying that critical gene. Once the mutant region has been cloned and characterized, it may be necessary to use positional cloning techniques to identify candidate genes within the deletion interval. The insertional mutation in this case does not provide immediate molecular access to the gene associated with the phenotype. Candidate genes from the mutant locus must be rigorously tested to prove that the affected geneis associated with the phenotype. This can be accomplished by, for example, targeted mutagenesis using homologous recombination in ES cells or transgenic 'rescue' experiments.

On the other hand, deletions that arise at transgene integration sites can be viewed as having certain advantages and may represent useful tools for the mouse geneticist. Deletions have been used as powerful genetic tools both in the mouse and in other model organisms, most notably *Drosophila*. Deletions have been extensively used at the Oak Ridge National Laboratory for defining the functions of novel genes and as mapping tools for single gene mutations (Rinchik and Russell, 1990). For example, Rinchik and colleagues used deletions at the albino *(c)* and pink-eye dilution *(p)* regions on mouse chromosome 7 to conduct ethylnitrosourea saturation mutagenesis experiments (Rinchik *et al*., 1990; Rinchik, 1991). This strategy for saturation mutagenesis is becoming an increasingly popular means of conducting mutagenesis experiments in mice (Justice *et al*., 1997). The fact that deletions produced by transgenic mice are "tagged" at the molecular level with the transgene potentially makes them easier to use for genetic mapping and saturation mutagenesis experiments, particularly if the transgene contains a gene that confers a dominant coat-color trait (Overbeek *et al*., 1991; Kucera *et al*., 1996).

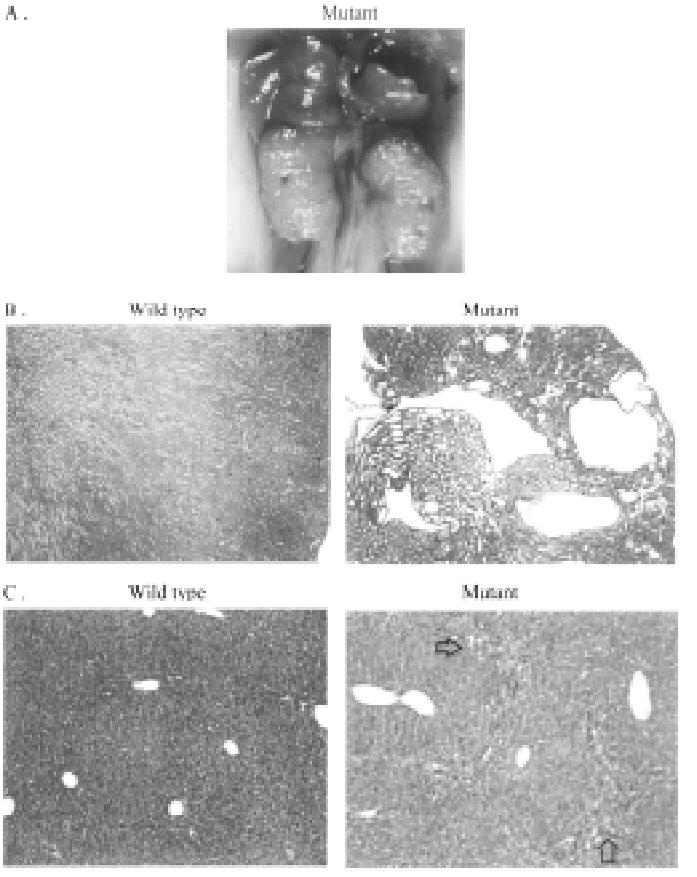

Fig. 4. The appearance of the kidney and liver in the TgN737Rpw insertional mutant. (A) *The kidney appears slightly larger than normal and the presence of multiple cysts is apparent upon gross examination. The liver appears pale with a distinct reticular pattern.* **(B)** *Hematoxylin and eosin stain cross sections showing the renal cortex and medulla from a wild-type (FVB/N) mouse and a mutant (TgN737Rpw) littermate. In the mutant mouse, cystic dilations are apparent in the cortex and medulla.* **(C)** *Hematoxylin and eosin stain cross sections from a normal liver of a wild-type mouse compared to a mutant littermate. Hyperplastic and dysplastic bile ductules are observed in the liver of the mutant animal (arrows).*

Deletions are also unique in that they can potentially give rise to syndromic phenotypes that are caused by the simultaneous elimination of entire blocks of genes. One notable example of this is the varying phenotypic effects of deletions of the *p* locus in the mouse (Silvers, 1979; Rinchik and Russell, 1990). Rinchik, Nicholls and their colleagues cloned the gene directly responsible for the pigmentation defect and also determined that some of the other traits exhibited by these mutants were due to the deletion of additionally linked genes (Rinchik *et al*., 1993). The same is also true for the deletions at the Prader-Willi/Angelman complex in humans (Nicholls *et al*., 1992,1993). Therefore, even though insertional mutations that are associated with large deletions of host DNA may be more difficult to initially characterize at the molecular level, they can also be viewed as useful tools for the mouse geneticist.

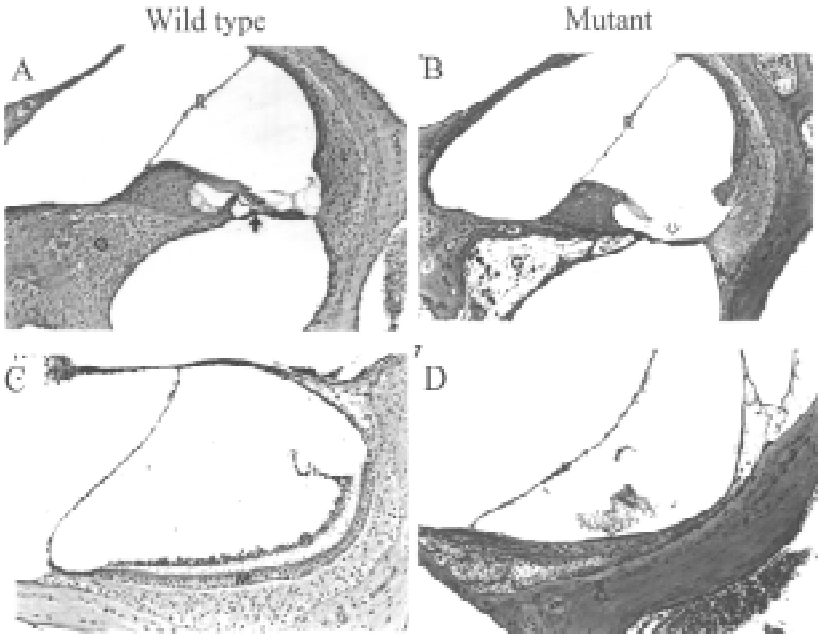

Fig. 5. Histopathology of the inner ear in the transgenic line, *TgN2742Rpw*. (A) *Cross section of middle cochlear turn of normal mouse (100X). The parts of cochlea are indicated as follows: Arrow, Organ of Corti; G, spiral ganglion; R, Reissner's membrane; V, Stria vascularis; L, Spiral ligament.* **(B)** *Cross section of middle cochlear turn of the mutant. The organ of Corti is entirely absent (open arrow) and the spiral ganglion cells are reduced in number.* **(C)** *Cross section of macular neuroepithelium (M) from the saccule of a normal mouse (200X). O, Otoconial membrane.* **(D)** *Cross section of macular neuroepithelium of the mutant. The neuroepithelium is absent (open arrow).*

Finally, although it was originally assumed that insertional mutations occur randomly throughout the mouse genome, analysis of the *limb deformity* locus has raised the possibility that there may be preferred sites for transgene integration. Two randomly generated insertional mutations in completely different transgenic lines were analyzed at the molecular level. The first of these was shown to be a small deletion that did not remove a coding exon (Woychik *et al.*, 1985). The second was shown to be a larger deletion which removed some exons of the gene (Vogt *et al.*, 1992). Also, a third allele of *limb deformity*, called *Is1Gso*, arose in a radiation exposure experiment at the Oak Ridge National Laboratory (Woychik *et al.*, 1990a). We cloned the proximal inversion breakpoint of the *Is1Gso* allele and demonstrated that it occurs within a few kb of the transgene insertion site of the original *limb deformity* insertional allele. The fact that two different independent insertional mutations and a radiation allele show changes in the same general region of the chromosome raises the possibility that the *limb deformity* locus represents a fragile site on the chromosome that is more highly susceptible to DNA breakage. If this proves to be the case, it may be that transgenes integrate at preferred sites on the mouse genome, and that these sites potentially represent regions of the genome that have a configuration that makes them more susceptible to double-stranded breaks.

Cloning insertional mutations

As we alluded to above, the molecular characterization of insertional mutations generated with the pronuclear microinjection procedure is not necessarily as simple as cloning a single copy gene. A number of features of this class of mutations can make the cloning process considerably more challenging than an insertional mutation generated by retroviral insertion (Gridley *et al.*, 1987,1990; Soriano *et al.*, 1987). High copy-number transgenes can add a significant extra effort to the cloning of sequences flanking the transgene integration site, and once the flanking sequences are cloned it is critical to evaluate whether a deletion or other structural alteration has occurred at the transgene integration site.

In most instances, in fact, it would be prudent to start the analysis of an insertional mutation by karyotyping the chromosomes of animals heterozygous for the transgene. Any obvious chromosomal structural alterations that are seen at the level of G-banded chromosomes, particularly if these involve a translocation, inversion, or large deletions, can be very useful in helping to establish the structure of the mutant locus.

There are now a number of options available for cloning sequences which flank the inserted transgene. The most direct approach is to generate a genomic library from the mutant DNA and screen the library using a probe corresponding to the sequences within the transgene. In the event that the integrated transgene complex is large (i.e., contains a high copy-number of transgene fragments), it will be necessary to isolate and characterize several transgene-positive clones to expect to recover clones that also contain the single-copy flanking sequences.

If the integrated transgene contains a selectable marker in *E. coli*, the sequences flanking the transgene insertion site can be quickly identified using a plasmid rescue procedure that was originally used for cloning the *limb deformity* locus (Woychik *et al.*, 1985). In this case the transgene contained the ampicillin resistance (amp^R) gene from pBR322 along with the *MMTR-myc* expression cassette (Stewart *et al.*, 1984). Using a modified version of the cosmid vector c2RB (Bates and Swift, 1983), where its own amp^R gene was inactivated, a cosmid library using partial Sau3A generated fragments with DNA from an animal carrying the insertional mutation was generated. After plating the library on ampicillin containing plates, only a limited number of clones grew on the selection plates. The cosmid clones that grew on ampicillin plates were those with an insert containing the amp^R gene derived from the integrated transgene. Of these amp^R clones, some contained only a portion of the concatemerized transgene, while others contained a portion of the transgene along with the sequences flanking the transgene integration site. This same approach was also used to clone the sequences flanking the transgene insertion site in the *TgN2742Rpw* hearing-loss mutant that we are currently analyzing (see above).

Finally, with the increasing saturation of the mouse genome with microsatellite markers (Silver, 1995), it is now also possible to consider using standard positional cloning techniques to characterize the mutant locus. For this approach, an approximate chromosomal position could be established using fluorescence *in situ* hybridization (FISH) procedures with a transgene-specific

probe. With this completed, it should be possible to clone and fine-structure map the mutant region utilizing the available microsatellite markers. In this case, the integrated transgene could be used as a landmark on the mutant chromosome to position the mutant locus relative to the microsatellite markers.

Future prospects

Insertional mutations that arise in transgenic mice produced with the pronuclear microinjection procedure have proven to be useful tools for studying mammalian development and for generating mouse models of human disease. Although we and many other groups world-wide have identified and characterized several insertional mutations at the molecular level, we predict that this form of germline mutagenesis has been an underutilized source of new mutations in the mouse. With the hundreds and perhaps thousands of transgenic lines that have been produced over the past decade, it is likely that many potentially useful insertional mutations in transgenic lines have gone undetected.

As long as the pronuclear microinjection procedure continues to be the method of choice for producing transgenic mice, insertional mutations will continue to be a potential source of interesting new mutations in the mouse. As with any form of mutagenesis, careful screening of transgenic lines will be necessary to detect useful phenotypes caused by the integration of the transgene. Our experience is that insertional mutations can give rise to a wide spectrum of different kinds of phenotypes. While some phenotypes arising in transgenic mice will have obvious visible or relatively easy to detect abnormalities, others will require careful analysis of the individual animals. This will be particularly the case for those recessive mutations which cause prenatal developmental lethalities. Also, since it is now clear that some insertional mutations may be associated with deletions and other structural alterations of the DNA at the transgene integration site, it is possible that insertional mutations in transgenic mice may prove to be a rich source of the kind of mutations that were previously only available through exposure to radiation, e.g., deletions. Insertional mutations will always have the advantage that the mutant locus is tagged at the molecular level, and with the new tools becoming available from the genome program, it is likely that even those insertional mutations which involve deletions or chromosomal structural alterations will be readily accessible at the molecular level.

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