Molecular and Phenotypic Characterization of a New Mouse Insertional Mutation That Causes a Defect in the Distal Vertebrae of the Spine

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ABSTRACT

We have identified and characterized the phenotype of a new insertional mutation in one line of transgenic mice. Mice carrying this mutation, which we have designated TgN(Imusdi)37ORpw, display undulations of the vertebrae giving rise to a novel kinky-tail phenotype. Molecular characterization of the insertion site indicates that the transgene integration has occurred without any substantial alterations in the structure of the host sequences. Using probes that flank the insertion site, we have mapped the mutation to chromosome 5 near the semidominant mutation, thick tail (Thl). Thick tail does not complement the TgN(Imusdi)37ORpw mutation; compound mutants containing one copy of each mutation display a more severe phenotype than either mutation individually.

Their phenotype is less severe than that observed for mutations in Th or Wnt-3a. Tail abnormalities observed in int2 mutants may result from the loss of activity of this factor in the posterior primitive streak, a region where int2 is expressed during early development (Wilkinson et al. 1988; Mansour et al. 1993).

Tail abnormalities can also result from defects in patterning mesodermal derivatives during later stages of development. For instance, undulated (un) mutants have a kinked-tail phenotype that appears to result from a defect in somite patterning such that the vertebral bodies do not form properly (Deutsch et al. 1988; Koseki et al. 1993; Wallin et al. 1994). In all un alleles there is a disruption in expression or function of the Pax-1 gene, a member of the paired-box gene family (Balling et al. 1988; Walther et al. 1991; Wallin et al. 1994). Consistent with un phenotypes, Pax-1 is normally expressed in the ventral portion of the somite, in the sclerotome cells that migrate ventromedially and condense around the notochord to form the vertebral bodies (Wallin et al. 1994).

Of the 126 skeletal mutants in the Genetic Variants and Strains of the Laboratory Mouse (Green 1989), 76 include defects of the tail and appendages. Numerous mutations exhibit strictly a tail-length phenotype, while others affect structures of the entire vertebral column (Gruneberg 1963; Green 1989). The phenotypic display of tail mutations can range from the absence or severe shortening of the tail to a slight decrease in length and size. In many cases, fusions, undulations and tail kinks can be observed (Green 1989). As with the mutations described above, the characterization of other mouse mutants with tail defects could provide the means to identify additional genes that function in axial development. For this reason, we have initiated
an effort to characterize a new insertional mutation that causes a defect in the vertebrae. Here we describe the nature of the skeletal phenotype in these animals and the molecular cloning and mapping of the mutant locus. We also show that a dominant mutation called \textit{thick tail} (BEECHER and SEARLE 1980) maps to the same general region on chromosome 5 and does not complement this new mutation.

**MATERIALS AND METHODS**

**Animals:** The transgenic line \textit{TgN(Imusd)37ORpw} was generated as part of a large-scale insertional mutagenesis program in the Biology Division, Oak Ridge National Laboratory. Transgenic mice were generated by the microinjection of a linearized 10-kb DNA fragment containing \( \beta \)-actin chloramphenicol acetyltransferase (\( \beta \)-actin CAT) (GUNNENGE et al. 1987) into fertilized mouse embryos (HOGAN et al. 1986) from the FVB/N mouse line (TARETO et al. 1991). The \textit{TgN(Imusd)37ORpw} line was maintained by intercrossing mutants and backcrossing to wild-type FVB/N mice. The \textit{thick tail} mutation was obtained as a kind gift from A. SEARLE (Harwell) and was maintained in the Mammalian Genetics Section by intercrossing the \textit{Tht} allele to the FVB/N strain.

**Identification of transgenic mice:** Genomic DNA from tail biopsies was isolated, digested with restriction endonucleases, electrophoresed on 0.8% agarose gels (GIBCO-BRL) and Southern blotted to GeneScreen nylon membranes (AUSUBEL et al. 1988). Mutant mice were initially identified by hybridization to sequences from within the transgene. Once the mutant locus was cloned, unique copy sequences from within the region flanking the transgene insertion, probes A–C Figure 2A, were used to distinguish heterozygotes from homozygotes (WOYCHIK et al. 1985; AUSUBEL et al. 1988; SAMBROOK et al. 1989).

**Skeletonal analysis:** Skeletons of \textit{TgN(Imusd)37ORpw}, \textit{thick tail}, and control mice were visualized by staining with Alizarin Red (SELBY 1987). Skeletons were visualized and photographed under a dissecting microscope.

**Genomic cloning and sequencing:** Genomic liver DNA from a \textit{TgN(Imusd)37ORpw} homozygote was partially digested with ScaI and size-fractionated on a 10–40% sucrose gradient. Fragments of 18–20 kb were ligated into AEMBL3 arms to generate a genomic library (SAMBROOK et al. 1989). The library was screened using a radiolabeled probe corresponding to the transgene sequences (SAMBROOK et al. 1989). Transgene-positive \( \lambda \)-clones that also contained the host-flanking sequences were initially identified by hybridization with a probe composed of total mouse genomic DNA (WOYCHIK et al. 1985). To clone the wild-type region corresponding to the mutant locus, FVB/N genomic liver DNA was used to prepare a genomic library that was screened with a probe (probe A, Figure 2) from the genomic region flanking the transgene integration site. All wild-type and mutant fragments were subcloned into the pGEM plasmid vectors (Promega) and sequenced with T7 DNA polymerase (Sequenase, USB) utilizing either the T7 or SP6 universal primers or oligonucleotide primers prepared from a specific region within the insert (SAMBROOK et al. 1989).

**BXD recombinant inbred mapping:** Genomic DNA was isolated from frozen kidneys of C57BL/6J, DBA/2J mice and 25 mice representing the BXD recombinant inbred lines (JENKINS et al. 1982). (RI and parental mice were purchased from the Jackson Laboratory.) To differentiate alleles from the different genetic backgrounds, genomic DNA was digested with \textit{Hinfl} to distinguish the C57BL/6J and DBA/2J alleles fragment sizes of 1.0 and 0.43 kb or 0.3, 0.55 and 0.43 kb, respectively. The genomic DNAs were then Southern blotted and hybridized with probe C, which corresponds to the region flanking the transgene insertion site (Figure 2). Strain distribution patterns from these mice were analyzed with computer software supporting statistical estimates based on SILVER (1985). Strain distribution patterns from these mice were analyzed with RI Map Manager computer software that utilizes statistical estimates based on SILVER (1985).

**Crosses between \textit{thick tail} and \textit{TgN(Imusd)37ORpw}** Reciprocal crosses involving \textit{Tht/+} and \textit{TgN(Imusd)37ORpw} homozygotes were used to generate F1 compound heterozygotes (\textit{Tht/+;} \textit{TgN(Imusd)37ORpw/+}). The \textit{TgN(Imusd)37ORpw} allele was followed by genotyping the animals utilizing genomic DNA from the tail and a probe corresponding to the transgene sequences. The \textit{Tht} dominant allele was followed by scoring the animals for the \textit{thick tail} phenotype (BEECHER et al. 1989).
Wild in the and segregates in tail phenotype. This mutation was named for transgenic mice (GORDON 1992) and will be abbreviation that causes an undulation of the spine and 370Rp-u for the transgene integration site (data not shown). A heterozygous transgenic parents develop zygous for the transgene in this line are phenotypically normal. Approximately 25% of the offspring from heterozygous (1980), which we determined was fully penetrant in the F1 animals.

RESULTS

Generation and phenotypic characterization of the \textit{TgN(Imusd)370Rpw} mutant line: As part of a large-scale insertional mutagenesis program at the Oak Ridge National Laboratory, we identified a new insertional mutation that causes an undulation of the spine and a kinky-tail phenotype. This mutation was named \textit{TgN(Imusd)-370Rpw} (1m, insertional mutation; usd, undulated spine distal) in accordance with the new nomenclature rules for transgenic mice (GORDON 1992) and will be abbreviated \textit{TgN370Rpw}. The transgene in the \textit{TgN370Rpw} line segregates in a simple Mendelian fashion. Mice heterozygous for the transgene in this line are phenotypically normal. Approximately 25% of the offspring from heterozygous transgenic parents develop a specific kinky-tail phenotype, and all of these animals are homozygous for the transgene integration site (data not shown). A total of 563 homozygous offspring analyzed thus far all express the mutant trait, which indicates that the phenotype is essentially 100% penetrant. Based on the recessive genetics of the mutant trait coupled with the fact that none of the numerous other lines that have been prepared with the same transgene DNA construct showed the same kinky-tail defect, we conclude that the phenotype in the \textit{TgN370Rpw} line arose from a mutation caused by the integration of the transgene sequences into the host genomic DNA.

The mutant animals show only the kinky-tail defect and do not contain any obvious soft tissue abnormalities in the mice examined thus far (J. J. SCHRICK and R. P. WOJCHIK, unpublished results). However, since we have not yet conducted an in-depth analysis for any other defects, we cannot exclude the possibility that subtle soft tissue defects may become apparent upon further investigation. To characterize the kinky-tail defect in more detail, we prepared Alizarin-Red-stained skeletons from adult mice (day 65+) using the protocol from SELBY (1987) and systematically analyzed them. All of the skeletal structures in the \textit{TgN370Rpw} mutant mice appear normal except for the distal region of the tail (Figure 1A). In all cases, tails from mutant animals have a disruption in the alignment of the last seven caudal vertebrae (Ca 24–30 in the FVB/N background) caused primarily by misshapen development of the posterior portion of

\begin{figure}[h]
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\caption{Molecular structure of the \textit{TgN370Rpw} mutant and corresponding wild-type locus. (A) The \textit{TgN370Rpw} mutant locus was isolated from a homozygous mutant genomic DNA library using \textit{\beta}-actin CAT, the transgene, as a probe. One genomic clone (X20–12) from the mutant library contained a 1.9-kb \textit{XbaI/EcoRI} fragment immediately downstream from the transgene integration site (probe A) that was used as a probe to isolate the genomic clone, X19–1, from a wild-type FVB/N genomic library. X19–1 spans the transgene insertion site and was used to identify a 0.7-kb \textit{HindIII} fragment (probe B) that was determined to map immediately upstream of the transgene integration site. Probe B was used to isolate a genomic clone (X20B-2) from the mutant library that contains the junction between the transgene and the host sequences upstream of the insertion site. Probe C was used for mapping experiments. \textit{\beta}-transgene; B, \textit{BamHI}; E, \textit{EcoRI}; H, \textit{HindIII}; X, \textit{XbaI}; S, \textit{Sall}. (B) Southern blots with 10 µg of genomic DNA from a wild-type (+/+), heterozygous (\textit{TgN370Rpw}/+) and homozygous (\textit{TgN370Rpw}/\textit{TgN370Rpw}) animal digested with \textit{BamHI}, \textit{HindIII} or \textit{EcoRI} and hybridized with probe A. Numbers refer to the size of the detected fragments in kb. (C) Sequence of the transgene insertion site. A 3.0-kb \textit{HindIII} fragment from \textit{X20B-2}, a 2.6-kb \textit{XbaI/Sall} fragment from X20–12, containing the left and right junction sites, respectively, and a 1.2-kb \textit{XbaI} fragment from X19–1, which spans the insertion site, were sequenced and compared. Host genomic sequence is in uppercase and the transgene sequence is in lowercase. One end of the transgene corresponds to the promoter region of \textit{\beta}-actin (left) and the other end contains the SV40 sequence on the transgene construct (right). The 1A and 1T nucleotides in open boxes represent duplicated nucleotides at the integration site.}
\end{figure}
individual vertebrae (Figure 1A). Consequently, the vertebrae are positioned at right angles to each other rather than in a normal linear arrangement, and it is this defect that may be causing the visible kink in the tail. In mutant neonates, the kinky-tail phenotype first becomes apparent at ~9 days after birth. At this time only a few vertebrae in the tail are affected (Figure 1B).

Cloning the mutant locus: To characterize the mutation in the TgN370Rpw line at the molecular level, we have cloned and characterized both the mutant locus and the corresponding wild-type region (Figure 2A). Flanking sequences were isolated from a genomic library prepared from a TgN370Rpw homozygote using the transgene as a probe (see MATERIALS AND METHODS). Utilizing the cloned sequences, we were able to deduce the structure of the mutant and corresponding wild-type regions (Figure 2A). We confirmed that the cloned region corresponds to the mutant locus by testing genomic DNA from wild-type, heterozygous and homozygous animals within the TgN370Rpw mutant line (Figure 2B). With probe A from one side of the insert, wild-type 6.0- and 12-kb mutant allele-specific EcoRI fragments were detected. Similarly, with probe B (Figure 2A) cloned from the other side of the insertion, 6.0-kb wild-type and 12-kb mutant allele-specific EcoRI fragments were observed (data not shown). With both probes, only the wild-type fragment was detected in wild-type animals, only the mutant allele-specific fragment is observed in the mutant animals, and, as expected, both fragments were detected in animals heterozygous for the mutation. The fact that both probe A and probe B from opposite sides of the transgene insertion detected the same size wild-type fragment indicates that there are no major structural alterations in the host DNA at the transgene integration site. Sequence analysis of the mutant and corresponding wild-type regions indicates that a single T nucleotide is duplicated on one side of the transgene, and a duplication of two nucleotides, TA, has occurred on the other end of the transgene (Figure 2C). Moreover, standard Southern analysis coupled with pulsed-field gel electrophoresis failed to reveal any structural alterations in the mutant region apart from the integration of the transgene (data not shown).

Mapping the TgN370Rpw wild-type locus: To determine if the TgN370Rpw locus is linked to any known mutant locus, we mapped the transgene integration site utilizing recombinant inbred strains (TAYLOR 1978). A single-copy 1.6-kb BamHI/SalI genomic fragment from a region adjacent to the transgene integration site (probe C in Figure 2A) was used to identify a restriction fragment length polymorphism (RFLP) in C57BL/6J and DBA/2J mice. Probe C was hybridized to HinfI-digested genomic DNA from BXD recombinant lines to generate the strain distribution pattern (SDP) shown in Figure 3A. The lowest recombination fraction (ratio of recombinants to informative strains) was found between TgN370Rpw and those for known loci on mouse chromosome 5. TgN370Rpw is most closely linked to Pmv-5 and D5H4S43, in which only one discordant sample was observed with each of these markers. Therefore, we have positioned TgN370Rpw between these two loci with a recombination distance \( \pm SE \) of 1.02 ± 1.06 cM between each marker (Figure 3B). Interestingly, close to the TgN370Rpw transgene integration site is another tail phenotype mutant called thick tail (Tht).

Complementation testing TgN370Rpw and Tht: On the basis of the close proximity of Tht and TgN370Rpw, we performed an allelism test by generating individual animals that were heterozygous for both mutations (see MATERIALS AND METHODS). From heterozygous Tht and homozygous TgN370Rpw parent animals, a total of 52 progeny were generated, 25 of which were compound heterozygotes (Tht/++; TgN370Rpw/+) and 27 were TgN370Rpw heterozygotes (+/++; TgN370Rpw/+). Tht heterozygotes alone have shortened thickened tails that are \( \sim 2/3 \) normal length and develop undulations as the tail grows (BEECHY and SEARLE 1980; J. J. SCHRICK, P. B. SELBY, and R. P. WOVCHEK unpublished data). Compound heterozygotes 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 (Figure 4D). This combined phenotype is never observed in either one of the mutant lines by itself.
FIGURE 4.—Complementation test of the Tht and TgN370Rpw mutations. Alizarin Red preparations of the distal tails from a wild-type FVB/N (+/+) (A), a homozygous TgN370Rpw (B), a heterozygous Tht (Tht+/+) (C), and a compound heterozygous (Tht+/+;TgN370Rpw/+) animal (D). Tails in A and B also shown in Figure 1. In the compound mutant shown in D, the distal tail vertebrae are severely disrupted and appear as small bone fragments near the end, which is unlike that which occurs with either of the mutants individually. Differences in the size of vertebrae between samples is primarily due to variability in magnification of the skeletons.

(Figure 4, B and C). While TgN370Rpw homozygotes possess kinks in the last seven caudal vertebrae as seen in Figure 4B (see also Figure 1A), Tht/+;TgN370Rpw/+ compound mutants (Figure 4D) show a more severe phenotype in which bone fragments, rather than distinct vertebrae, form in the most distal part of the tail. By itself the Tht mutant phenotype does not express any overt skeletal phenotype in the distal vertebrae (see Figure 4C).

DISCUSSION

A novel mouse insertional mutation with a tail defect: We have undertaken the phenotypic and molecular characterization of a new recessive mutation, TgN370Rpw, that causes a kink at the end of the tail. The skeletons of these animals contain an abnormality that is confined to the distal spine and involves a structural abnormality in the individual vertebrae that ultimately gives rise to an undulation of the distal tail. As a first step in beginning to characterize TgN370Rpw at the molecular level, we were able to clone the mutant locus utilizing the transgene as a molecular tag.

Noncomplementation suggests close linkage with Tht: Of the existing mutations that have a defect in the tail, only the pintail mutation has a phenotype that is similar to the TgN370Rpw mutant (BERRY 1960). Since pintail maps to chromosome 4 and TgN370Rpw maps to chromosome 5, the two cannot be allelic. On the other hand, our mapping experiments did reveal that TgN370Rpw maps to a region of mouse chromosome 5 near another mutation, Tht, that causes a defect in the tail. Thick tail (Tht) causes the tail to be short and thickened at birth and to become undulated later in development. This anomaly may be caused by insufficient cartilage formation in the vertebral bodies (GREEN 1989; J. J. SCHRICK, P. B. SELBY, and R. P. WOYCHIK, unpublished results). Compound heterozygotes containing both the Tht and the TgN370Rpw mutant alleles express a unique phenotype that overall is more severe than either mutation by itself. This result could indicate that Tht represents a dominant mutation of the same gene associated with TgN370Rpw. To explore this possibility, we analyzed the Tht allele utilizing DNA probes derived from the region flanking the transgene integration site of TgN370Rpw. Even though Tht may have been induced by radiation, we have been unable to detect any obvious DNA structural alterations with the available probes (J. J. SCHRICK and R. P. WOYCHIK, unpublished results). This result does not rule out the possibility that the two mutations are allelic since Tht may be a point mutation or some other change in the structure of the DNA that cannot be readily detected with our molecular probes. Once we have identified the gene directly associated with the TgN370Rpw mutation, further experiments will be performed to help to resolve this issue.

Similar to the situation described in this report, POHL et al. (1990) characterized a recessive insertional muta-
tion called anterior digit deformity (add), which was initially mapped to a region near the dominant mutation extra-toes (Xi). The recessive add insertional mutation causes subtle defects in the anterior digits of only the forelimbs in the mutant animals, and the existing Xi dominant mutation is characterized by polydactyly on all four limbs (Green 1989). The production of compound heterozygotes between add and Xi generated an enhanced phenotype on both the fore- and hind limbs that was more severe than either allele alone (Pohl et al. 1990). Ultimately, it was determined that the Gli3 gene, which was mapped relative to Xi, was affected by intragenic deletions in the Xi and Xi' alleles (Schimmang et al. 1992; Vortkamp et al. 1992; Hui and Joyner 1993). The transgene insertion in the add mutation integrated within the 5' region of the Gli3 gene, and as a result, negatively influences Gli3 expression and causes a more subtle phenotype than the dominant Xi (Van der Hoeven et al. 1993). Based on the similarity of these data to those described in this report, it may be that the transgene insertion in the TgN370Rpw line has also occurred within a regulatory region of a gene associated with the mutant locus, and that the Tht mutation may be a deletion of the coding sequences of the same gene.

A simple insertional mutation: Previous reports have indicated that some insertional mutations in transgenic mice generated by pronuclear microinjection contain rearrangements and deletions of the host genomic sequences adjacent to the transgene insertion site, thereby complicating the molecular analysis of these mutations (Singh et al. 1991). Our data clearly indicate that it is possible for a transgene to integrate essentially without any rearrangements of the host DNA. Therefore, based on previous reports where it has been shown that a large deletion can occur in the host sequences at the transgene integration site, we are led to suggest that a whole range of different kinds of mutations can probably occur in the host sequences at the transgene integration site, including essentially simple insertions of the exogenous DNA. Although it is presently unknown how often insertional mutations more similar to simple insertions occur as compared to those like large deletions or complex structural alterations, it is becoming increasingly clear that many insertional mutations generated with pronuclear microinjection are like the TgN370Rpw mutation in that they can be readily accessed at the molecular level (reviewed in Meisler 1992; Hodgkinson et al. 1993; Moeyr et al. 1994).

Nature of the developmental defect in the mutant animals: Based on our limited characterization of the phenotype in the TgN370Rpw line, we are speculating that the defect occurs during late somite and tail bud formation. Since the defect is apparent only in the caudal vertebrae in the TgN370Rpw and the Th/t/TgN370Rpw compound mutant animals, we suggest that the defect occurs within the late stage somite or in the sclerotome that gives rise to these vertebrae. Specifically how the defect occurs is presently unclear, although we expect that in situ hybridization experiments with the gene associated with the TgN370Rpw, once it is identified, will be useful in this regard.

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LITERATURE CITED


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