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Evidence for allelism of the recessive insertional mutation *add* and the dominant mouse mutation *extra-toes* (*Xt*)

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Summary

A recessive mutant caused by insertional mutagenesis in transgenic mice has been detected in which the anterior part of the forelimb is disorganized. The morphology of the thumb is always altered and sometimes the adjacent finger has an extra phalanx. This phenotype suggests that a body plan gene is affected. We have named the mutation *add* (anterior digit-pattern deformity). Using the cloned DNA from the flanking region of the integrated transgene, *add* has been mapped close to the centromere of chromosome 13. This position links *add* to a genetically mapped locus called *extra-toes* (*Xt*). The phenotype of the double-mutant *add*/*Xt* as well as the molecular analysis suggest that *add* and *Xt* are allelic.

Key words: morphogenetic mutations, mouse genetics, pattern formation, insertional mutagenesis.

Introduction

Although several dysmorphic mouse mutations are described (Lyon and Searle, 1989), the molecular mechanisms responsible for organizing the mammalian body are unknown. The major reason for this is the inability to define molecularly the affected gene in such a mutant. Several attempts have been started in the last few years to bridge classical genetics and molecular biology. One approach is based on the fact that certain DNA sequence motifs are conserved between species. *Drosophila* homologues have been screened for in the mouse genome, and, following their isolation and chromosome mapping, a comparison is made to known mouse mutations. This approach strongly suggested the molecular identification of the mouse mutations *undulated* using the *pax* gene (Balling *et al.* 1988). Another approach, using a combination of genetic and molecular techniques like walking and jumping, resulted in the isolation of the *T* gene (Herrmann *et al.* 1990). However, the latter approach is only feasible when several mutants of the gene are available. A third approach employed by several laboratories involves screening for developmental mutants by insertional mutagenesis in transgenic mice (Jaenisch *et al.* 1983; Wagner *et al.* 1983; Woychik *et al.* 1985). In such an approach, the gene becomes marked by the integrated DNA and can be cloned out of the genome. We have also tested our transgenic mouse lines (Rüther *et al.* 1987*a*,*b*; Dente *et al.* 1988) for dominant and recessive mutations. Here we describe a recessive mutation (called *add*) that results in a disorganization of the anterior part of the digit pattern of forelimbs. The genetic and molecular analysis suggests that *add* is allelic to the morphogenetic mutation *Xt*, first described by Johnson (1967).

Materials and methods

Mice

Mice of line 358–3 (Dente *et al.* 1988), which carry the *add* mutation, are bred in the mouse colony at the EMBL. This mutation is kept on a mixed C57BL/6×SJL background. Mice carrying the *Xt* mutation were obtained from MRC Radiobiology Unit, Chilton and from the Gesellschaft für Strahlenforschung, München.

Skeleton analysis

To visualize the limb skeleton, the following procedure was used: 24 h in 95% ethanol, clearance of soft tissue in 2% KOH solution and staining of bones in 1% KOH solution containing Alizarin red. Finally the stained limbs were dehydrated by incubation in 30%, 60% and 87% glycerol for several hours.

In situ *hybridisation to mouse chromosomes*

Concavalin A-stimulated lymphocytes of a WMP male mouse in which all the autosomes except 19 are in a form of metacentric Robertsonian translocation were cultured at 37°C for 72 h with 5-bromodeoxyuridine added for the final six hours of culture (60 $\mu g\,ml^{-1}$) to ensure a chromosomal R-banding of good quality. The *SstI* fragment used as probe in Fig. 2 was labelled as cloned insert in pUC19 by nick-translation. Hybridization, autoradiography, staining and banding was performed as described (Mattei *et al.* 1985).

λ and cosmid libraries and DNA analysis

For the cloning of the integration site of the *add* mutation, DNA was isolated from homozygous mice of line 358–3 (Dente *et al.* 1988). This genomic DNA was completely digested with *Hin*dIII and cloned into the λ phage 1151 (Murray, 1983). The λ library was screened using as a probe the f1 origin of replication which is part of the transgene. To obtain the preintegration site, a cosmid library of 129/Sv-SlCP mouse DNA was screened with the *Sst*I DNA fragment isolated from the λ clone as shown in Fig. 2. Positive clones were characterised further as described (Rackwitz *et al.* 1985). Sequence analysis of preintegration site and the flankings of the transgene was performed following subcloning of suitable fragments into plasmid vectors.

Results

Phenotypic characterization of the recessive mouse mutation add

By testing our transgenic mouse lines systematically for recessive mutations, we detected in the course of homozygote breeding in one of our lines (358–3, Dente *et al.* 1988) a deformation of digit 2 on the forepaw (Fig. 1A). The digit appeared bent and connected to the thumb, which is, in mice, only a rudiment. Molecular analysis of the transgene copy number revealed that all mice displaying this phenotype were homozygous for the transgene integration site. When we intercrossed homozygous animals, surprisingly we only observed a transmission of the phenotype to about 60 % of the offspring although all animals were clearly homozygous with respect to the copy number of the transgene (data not shown). To characterize the mutation in more detail, we isolated forelimbs from homozygous animals with and without visible deformations. To visualize the bony part, the soft tissue was cleared and the bones stained. Forelimbs of several dozen mice were treated in this way and have subsequently been analyzed microscopically (Fig1B–H). In all forelimbs isolated from homozygous animals of line 358–3, the thumb shows irregular growth; from slight changes in architecture (Fig. 1C) to very drastic alterations such as an elongation of the thumb by almost 100 % (Fig. 1F,G). Only when the thumb is elongated to a certain extent does digit 2 become bent and connected to the thumb *via* soft tissue (Fig. 1E–H). First appearance of this phenotype during embryonic development was obvious at day 14. Thus, digit formation and phenotypic changes are happening at the same time.

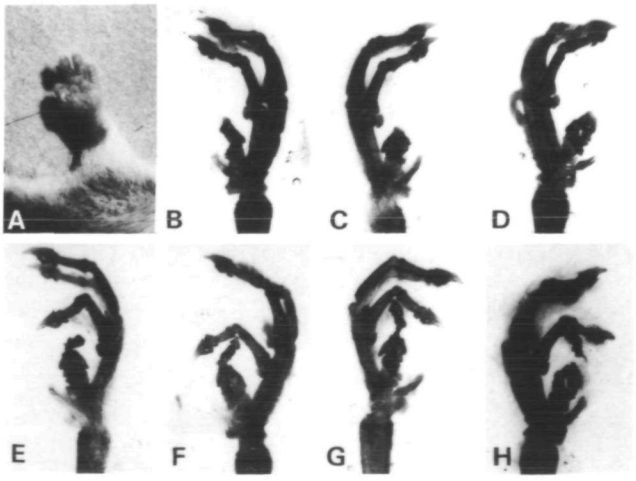

Fig. 1. Phenotype of the forelimb deformation. (A) Left forepaw of a homozygous mouse of line 358–3 with the most obvious phenotype. (B–H) Skeleton of forelimbs of wild-type mouse (B) and homozygous mice of line 358–3 (C–H).

We have never found bone fusion of the thumb and digit 2, but we could detect in about 10 % of the analyzed forelimbs an additional phalanx on digit 2 (Fig. 1H). Thus, the phenotype is polarized such that the structure primarily affected is the thumb, which is the most anterior part of the forelimb. The only other structure sometimes changed on the forelimb is the adjacent finger (digit 2). We have never detected any alteration on digits 3–5 of these mice. The mutated gene interferes with the organization of the anterior part of the digit pattern of forelimb, therefore we suggest *add* (anterior digit pattern deformity) as a name for this mutation.

Molecular characterization of add

To define the *add* mutation molecularly, we cloned the flanking sequences of the transgene from a λ library of homozygous *add* mice. As shown for one flanking side (here defined as 5′, Fig. 2), we could identify unique DNA sequences that can be used as a probe to demonstrate the restriction fragment length polymorphism of the affected *add* allele. Using this probe we have screened a cosmid library to obtain DNA sequences of the preintegration site. Sequencing of both transgenic flanking sites and that of preintegration reveals that the transgene has replaced only one base pair in the course of integration (Fig. 3). In addition, we have found that some unknown mouse DNA has cointegrated with several copies of the transgene.

To map the chromosomal localization of *add*, about one hundred chromosomal metaphases were hybridized with the same probe as used to detect the polymorphism (see legend Fig. 2). In the metaphase cells examined, 106 silver grains were associated with chromosomes and 48 of these (45.3 %) were located on chromosome 13 (Fig. 4). The distribution of grains on this chromosome was not random: 85 % of them mapped to the [A1–A3] region with a maximum in the 13A2 band. Thus, the chromosomal localization of the *add* mutation is most likely the A2 band of chromosome 13. This position suggests a linkage between *add* and a genetically mapped locus called *extra-toes* (*Xt*), a morphogenetic mutation on the proximal part of chromosome 13 (Johnson, 1967; Lyon *et al.* 1967).

Phenotypic characterization of add/Xt *double mutant*

Although the *Xt* mutation shows differences compared to *add* (extra-toes on the hindlimb, dominant mutation), we wondered whether *add* and *Xt* were allelic.

First, the phenotype on the forelimb is almost identical and, second, in the course of breeding several hundred *add* mice we found mice with an extra-toe on one hindlimb with a frequency of less than 1%. Therefore, we mated homozygous *add* mice (males and females) with heterozygous *Xt* mice (*Xt* is homozygous lethal).

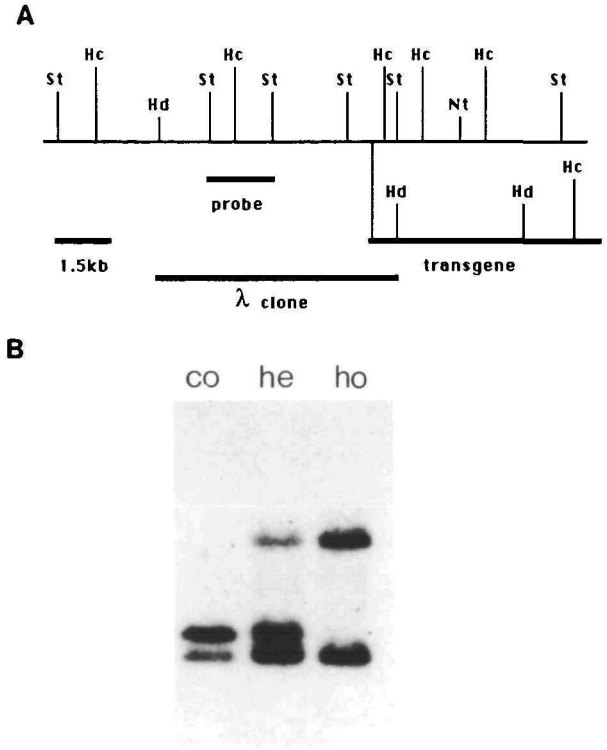

Fig. 2. Mouse genome organization around the transgene integration site. (A) The structure of the normal allele is shown at the top of the figure. The 5′ integration site of the transgene copies is indicated below (only one copy is drawn). The *Sst*I fragment used as the probe in part B of the figure is indicated. The *Hin*dIII fragment cloned from mouse line 358–3 into the λ vector is also illustrated. (B) The polymorphism of the wild-type alleles (co) and of the affected alleles in heterozygous (he) and homozygous (ho) mice of line 358–3 is shown. For this purpose, genomic DNAs have been digested with the restriction enzyme *Hin*cII and the Southern blots were hybridized with the nick-translated *Sst*I fragment indicated in part A of the figure. Abbreviations: St, *Sst*I; Hc, *Hin*cII; Hd, *Hin*dIII; Nt, *Not*I.

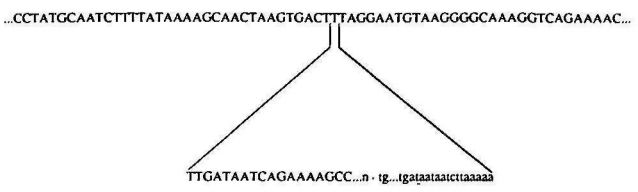

Fig. 3. Sequence of the integration site. At the top of the figure part of the DNA sequence of the wild-type chromosome 13 is presented. Several copies of the transgene DNA are integrated by replacing one base pair as shown in this diagram. The small letters are indicating unknown mouse sequences that cointegrated with the transgene.

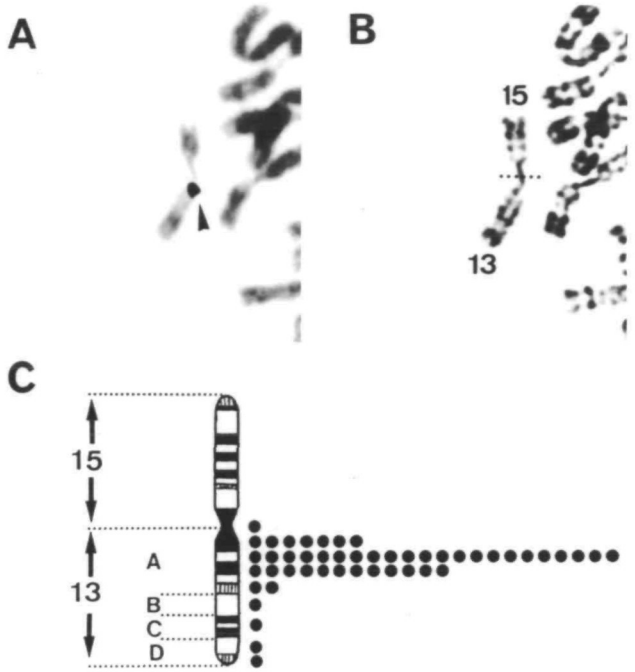

Fig. 4. Localization of the *add* gene to mouse chromosome 13 by *in situ* hybridization. (A) The partial WMP mouse metaphase shows the specific site of hybridization to the proximal part of chromosome 13 (the arrow indicates the silver grains). (B) The chromosomes of the metaphase shown in A were subsequently identified by their characteristic R-banding. (C) Diagram of WMP mouse Rb (13;15) chromosome, indicating the distribution of labelled sites.

50% of the offspring of such a mating should be normal (*add*/+) or with extra-toes (*add*/*Xt*). Indeed, 50% of offspring were normal, but the other 50% showed, besides the extra-toes, an enhanced alteration of fore- and hindlimbs. Skeletal analysis demonstrated the presence of seven digits on both fore- and hindlimbs (Fig. 5). Sometimes two of the additional digits were fused (Fig. 5B) but could still be counted as individual digits. To exclude any specific genetic background for the appearance of seven digits, we have backcrossed the double mutants. Mating of *add*/*Xt* with wild-type mice (C57BL/6×SJL F_1) resulted in 50% normal mice (all positive for the transgene, when tested by Southern blot) and 50% mice with only one extra-toe per hindlimb (all negative for the transgene). We have tested several of the *add*/*Xt* double mutants for other skeletal changes but none could be identified. All the *add*/*Xt* or *Xt*/*add* mice were fertile and seem to have a normal lifespan (the oldest is now more than twelve months).

Molecular analysis of the Xt *locus*

Assuming that *Xt* and *add* are allelic, we were expecting to find restriction fragment length polymorphisms using probes either 5′ or 3′ of the transgene integration site. However, probing of DNA isolated from *Xt* heterozygous animals showed only the wild-type DNA pattern

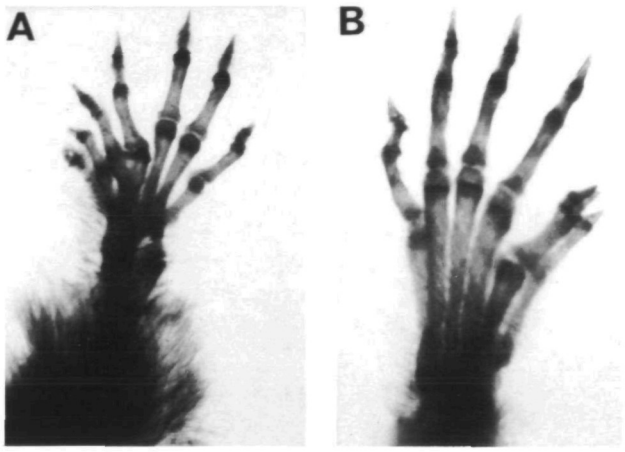

Fig. 5. Phenotype of fore- and hindlimb alterations. Skeletons of (A) fore- and (B) hindlimbs isolated from a *Xt/add* double mutant are shown.

(Fig. 6). By testing DNA isolated from the *add/Xt* double mutant, we obtained the first indication of a deletion on the *Xt* chromosome (Fig. 6) as only the *add* restriction fragment length polymorphism was detectable. For further analysis we have chosen DNA isolated from *Xt* homozygous embryos. Although we have tested unique DNA probes isolated from cosmid clones that cover about 80 kilobases of DNA (≈50 kb 5′ and ≈30 kb 3′ of the transgene integration site), none hybridized to DNA from homozygous *Xt* mice (data not shown). Thus, the length of the deletion on the *Xt* chromosome is at least 80 kilobases.

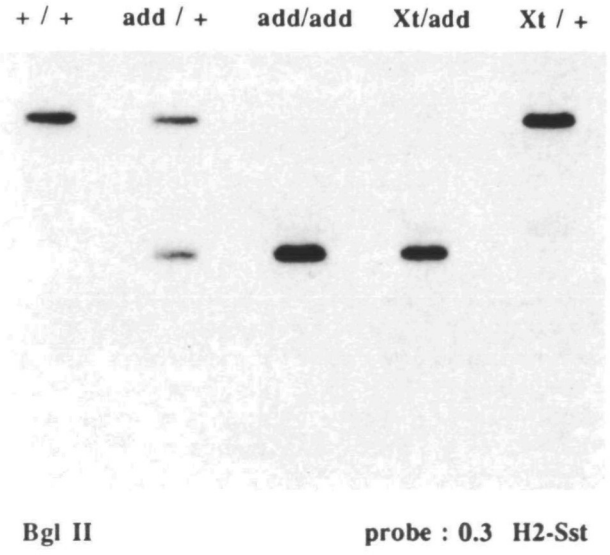

Fig. 6. Restriction fragment length polymorphism of genomic DNA isolated from different mouse genotypes. Genomic DNAs have been digested with *Bgl*II and the Southern blots were hybridized with the labelled H2–*Sst*I probe (see Materials and methods).

Discussion

In this report, we have described a recessive morphogenetic mouse mutation (called *add*) which always affects the organization of the most anterior part of the digit pattern of forelimbs. Occasionally, with a frequency of less than 1% in addition to the forelimb alteration, we have found an extra-toe on the hindlimb. These findings, plus the fact that *add* could be mapped to the proximal part of chromosome 13 (13A2) by *in situ* hybridization, suggested that *add* might be allelic to a genetically mapped mutation called *extra-toes* (*Xt*). The forelimb deformation in *Xt* mice (Johnson, 1967) is very similar to that of the *add* mutant, however, the *Xt* mice have one or more extra digits on the anterior part of the hindlimbs. Since *Xt* is a dominant mutation, every mouse has limb alterations on fore- and hindlimbs. Homozygous *Xt* mice die at birth or *in utero* (Johnson, 1967).

Our mating studies of double mutant *add/Xt* or *Xt/add* mice have already provided some evidence that *add* and *Xt* might be allelic. Each of these double mutant mice displayed a limb phenotype (seven digits on fore- and hindlimbs) which is reminiscent of *Xt* homozygous mice. As described by Johnson (1967), homozygous *Xt* embryos at about day 15 of embryonal development show seven digits or sometimes even more on fore- and hindlimbs. In addition, the homozygote *Xt* mice show multiple abnormalities in the skeleton e.g. vertebrae and thorax. A severe malformation of the brain, central nervous system and sense organs is also reported (Johnson, 1967). We could never detect any of these changes in the *add/Xt* mice.

Additional evidence is provided by molecular analysis of *add* and *Xt*. We could characterize *add* as a mutation caused by the integration of several transgene copies by replacing one basepair of genomic sequence on chromosome 13. Using DNA probes specific for sequences 5′ and 3′ of the integration site, *Xt* was characterized as a deletion of at least 80 kilobases. Although we cannot demonstrate that both mutations are affecting the same transcript, a highly likely interpretation is that these mutations are allelic.

Assuming that both mutations are allelic, how can we explain that the deletion in *Xt* is causing a dominant, and the transgene insertion a recessive, mutation? In principle there are at least two possible explanations. First, one can assume that the presumptive protein encoded by the *Xt/add* gene has at least two functional domains. In the deletion only one domain is affected and therefore a normal functional protein complex is no longer possible, the phenotype being already present in the heterozygote. The insertion would knock out the gene and a phenotype is only visible in homozygous mice. A second possibility is that the *Xt* deletion is removing most of the gene, resulting in a 50% reduction of a protein product in the heterozygous mice, of which the precise concentration for its normal function is crucial (haploinsufficiency). Likewise, the insertion mutation *add* only reduces the expression of one allele but does not abolish it and therefore no

phenotype can be detected in *add/+* mice. However, in *add/add* mice, the reduction of protein is similar to *Xt/+* mice and therefore the phenotype becomes obvious.

Characterization of certain phenotypes in *Drosophila melanogaster* provides a molecular explanation for dominant mutations. One of the most extensive studies has been performed with the *act 88 F* gene, which encodes actin III of the indirect flight muscles. A dominant mutation called KM88 is described, which abolishes mRNA and protein (Okomoto *et al.* 1986). This reduction of about 50% leads to a dominant phenotype due to a filament imbalance in the myofibrils (Beall *et al.* 1989). For the same gene, negative dominant mutations have also been described in which a truncated actin protein (due to either a deletion or a nonsense mutation) affects the assembly of myofibrils (Karlik *et al.* 1984; Okomoto *et al.* 1986).

One observation supports the speculation regarding a negative influence on a gene product concentration in the *add* mice. The phenotypical changes show a strong correlation with a certain genotype, which can actually be quantified, when we simplify the analysis and count only the number of digits: *add/add*, only one thumb is changed; *Xt/+*, in addition one extra-toe; *add/Xt*, two extra-toes and *Xt/Xt*, up to four extra-toes. It is tempting to speculate about the nature of this gene product and it is also unclear why *Xt* is homozygous-lethal. Homozygous *Xt* mice might either die because the gene product is essential in other processes or there might be other gene(s) within the deletion that are recessive mutated, not involved in limb formation, but essential for normal development.

At present we are attempting to detect transcript alterations in the *Xt* and/or *add* mice using DNA probes derived from the genomic region deleted in the *Xt* mice. In parallel, we have begun inducing further mutations on chromosome 13 by chemical mutagenesis of wild-type males and test mating with homozygous *add* females. By using chlorambucil as a reagent there is approximately a 1% possibility of finding more mutations at this locus (Russell *et al.* 1989). With the help of different deletion end points, it should be possible to narrow down the region encoding this gene involved in the pattern formation of the digits. The ultimate proof of allelism of *add* and *Xt* will then be possible by performing a phenotype rescue experiment.

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