Development 1990 Supplement, 73–79
Printed in Great Britain © The Company of Biologists Limited 1990

A transgene insertional mutation at an imprinted locus in the mouse genome

JULIE A. DeLOIA and DAVOR SOLTER

The Wistar Institute of Anatomy and Biology, 36th and Spruce Streets, Philadelphia, PA 19104, USA

Summary

Genetic imprinting in mice results in functional differences in the oocyte and spermatocyte genomes, as evidenced by both genetic and pronuclear transfer experiments. To gain insights into the molecular mechansims involved in the imprinting process, researchers have studied methylation phenotypes and expression of hemizygous transgenes associated with parental origin. In this report, we describe a transgenic mouse lineage in which expression of both the transgene and an endogenous gene at the insertion site are determined by the parent of origin. The mutation caused by transgene insertion shows variable expressivity and incomplete penetrance in addition to a modified dominant pattern of inheritance.

Key words: transgene, insertional mutation, imprinting, mouse.

Introduction

In mice, the paternal and maternal genomes are not functionally equivalent during the early stages of development (for review see Solter, 1988). This observation suggests that embryonic genomes are differentially modified in each sex during gametogenesis. Thus, specific genes probably bear an imprint which predetermines transcriptional activity, unique to each parental lineage. This functional imprinting is believed to be responsible for the opposite and complementary phenotypes seen in gynogenetic embryos, which contain two maternally derived pronuclei, and androgenetic embryos, which contain two paternally derived pronuclei (McGrath and Solter, 1984; Surani *et al.* 1984). Studies on the mechanism of imprinting and the nature of the imprint require the isolation of suitable molecular probes.

In lieu of having isolated endogenous imprinted genes, researchers have used transgenes as molecular indicators of imprinting. Specifically, the addition or removal of methyl groups on CpG residues within transgenes was studied. Methylation was ascertained since it is an epigenetic DNA modification known to affect gene expression, is transmissable to daughter cells, and is thought to be involved in imprinting. This approach has enabled indentification of methylation phenotypes dependent on parental origins for a number of transgenes. Transgenes are unique within the mouse genome, and thus, when hemizygous, can easily be scored for any methylation changes associated with passage through each parental lineage.

However, the generalization of these transgene data to mechanisms of endogenous imprinting may not be wholly appropriate for the following reasons. There is a relatively high frequency of transgenes that show methylation differences (for a review, see Surani *et al.* 1988), higher than what would be expected from the mouse genetic imprinting map (Beechey *et al.* 1989 and Cattanach, 1986); with one exception (Sapienza *et al.* 1987), paternal transmission is associated with transgene hypomethylation; the possibility of nonrandom integration of the transgene into the host genome exists; and, transgenes may be treated as foreign DNA and not as endogenous DNA at insertion site.

Transgenes do display some features that we could predict for imprinted endogenous genes. An imprint must be eraseable such that at the conclusion of gametogenesis the imprinting effect of only the most recent germ cell lineage remains. For example, a transgene that has been passed through a male and then a female should bear the methylation pattern typical of maternal inheritance. In general, transgene methylation phenotypes are reversible upon passage through the opposite lineage, although there are exceptions, such as the hepatitis B surface antigen gene, which was permanently hypermethylated with maternal transmission (Hadchouel *et al.* 1987).

An important consequence of imprinting should be its effect on gene expression. Swain *et al.* (1987) were the first to identify a transgenic lineage in which parental origin of the transgene c-*myc* fused to the Rous sarcoma virus (RSV)-LTR determined both the extent of methylation and expression of the transgene. Only

mice receiving the paternally contributed RSV-*myc* fusion gene expressed the transgene in myocardial tissue, which is consistent with the promoter specificity of the RSV-LTR to mesodermal derivatives in transgenic mice (Overbeek *et al.* 1986). Transgene hypermethylation and transcriptional inhibition were observed when this construct was maternally inherited. McGowan *et al.* (1989) described another line of mice in which methylation and transgene expression (the *Escherichia coli lacZ* driven by the murine hsp68 promoter) were concordant. Transgene expression, limited to the floor of the developing neural tube from 10–14 days gestation, was found only when the transgene was hypomethylated. Those authors also described an intermediate methylation phenotype that was correlated with an intermediate level of transgene expression. This intermediate methylation phenotype seemed to be controlled by trans-acting factors which vary according to the genetic background of the nontransgenic female. Sapienza *et al.* (1989) have also reported methylation intermediates of a troponin I transgene related to the nontransgenic maternal genotype.

To summarize, transgene methylation phenotypes are often associated with and dependent on parental legacy. Methylation modification is apparently sequence-independent, since a diverse array of transgenes has been modified. Transgene expression can be affected by the pattern of methylation, which appears to be either permissive or restrictive for transgene expression. However, the temporal and spatial regulation of transcription is more strongly influenced by regulatory elements unique to each transgene. An assumption in all of these studies is that methylation phenotypes reflect the chromosomal insertion site, even though the location of most of the transgenes within the genome is unknown.

In this report, we provide evidence to support this assumption, by showing that in one transgenic mouse line parental origin controls both transgene expression and presumably expression of the endogenous gene at the transgene insertion site. In these mice, phenotypic abnormalities occur as a consequence of transgene insertional mutagenesis, but only with paternal inheritance. Similarly, transgene expression, which in this line is evidenced by the appearance of skin papillomas, occurs only in those phenotypically abnormal animals. From this correlation of endogenous gene and transgene expression, we suggest that the transgene has inserted into a region of the genome which is imprinted such that the endogenous allele is more transcriptionally active in male-derived chromosomes. Perturbation of the paternally inherited gene by insertion of the transgene results in a decrease in gene product levels below some critical value necessary for normal development. Conversely, expression of the transgene becomes possible because it inserts in a transcriptionally active domain. The mutation was studied in detail to determine when and where the endogenous gene is expressed.

A transgene insertion that causes paw deformity

Line *Adp* (Acrodysplasia) was derived from zygote microinjection of a linearized plasmid (pL116), a derivative of pdBPV-MMTneo (Law *et al.* 1983), containing the entire genome of the bovine papilloma virus (BPV) and the human growth hormone-releasing factor (hGRF) under the transcriptional control of the mouse MT-1 promoter. Upon breeding, the original founder male for this line was identified as carrying an insertional mutation; approximately half of the offspring presented with deformity of both fore- and hindlimbs (Fig. 1). Both male and female progeny were affected equally, a transmission pattern consistent with an autosomal dominant mutation. However, with further breeding, a significant number of mice that carried the transgene appeared to be phenotypically normal, specifically, those that inherited the transgene maternally. Mice that received the transgene from males were deformed, indicating a modified dominant pattern of inheritance (DeLoia *et al.* unpublished data).

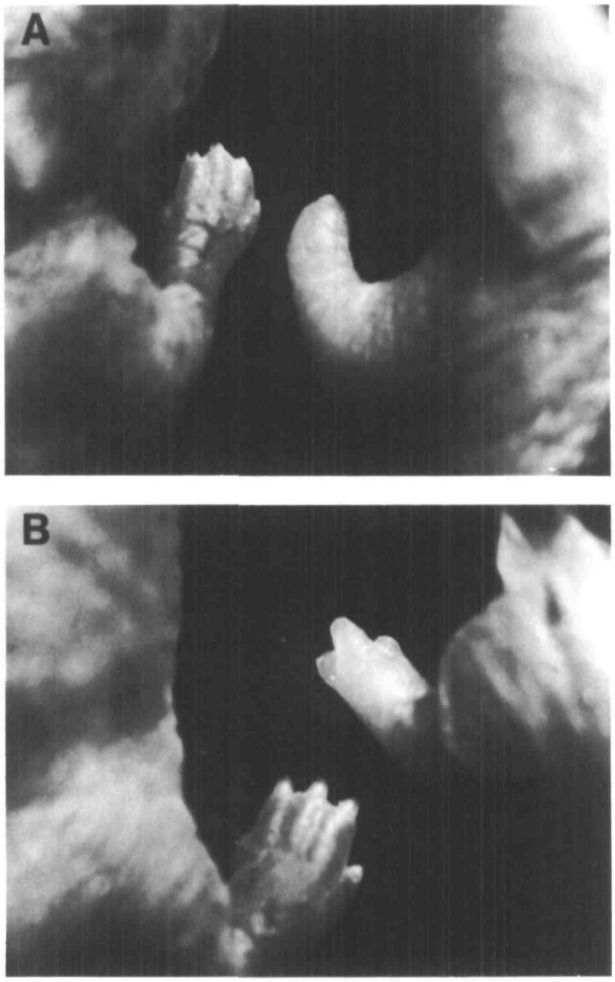

Fig. 1. Paw deformity in transgenic mice. Two littermates, one normal (left side) and one deformed (right side) are shown. In A, the forepaws of the transgenic animal have no distinct digit formation; the hindpaws (B) are not as severely affected, which is typical in this line of mice. Magnification is 8×.

The mutation also shows variable expression, ranging in severity from barely detectable to complete absence of digits (Fig. 2), and incomplete penetrance, not all mice with paternally inherited transgenes are affected.

Other laboratories have reported insertional mutations affecting digit or limb development in transgenic mice. One of these mutations mapped to a previously identified limb mutation, limb deformity (*ld*) (Woychik *et al.* 1985), whereas two others, *legless* (McNeish *et al.* 1988) and a mutation causing fusion of the digits (Overbeek *et al.* 1986), did not. These new mutations were all detected as autosomal recessive disorders when the transgenic lines were bred to obtain transgene homozygosity. In contrast, *Adp* mice express the mutation as heterozygotes, with a modified dominant transmission pattern.

Transgene expression in *Adp* mice

Northern analysis of total RNA collected from limb bud regions revealed no evidence of either BPV or hGRF expression during the critical period of limb development, embryonic days 12–14, suggesting that the observed mutation in *Adp* mice is not due to interference in the developmental pathway by transgene product. Rather, it seems more likely that the observed phenotype is a consequence of integration of the transgene into or near an endogenous gene necessary for normal development. BPV is expressed in the *Adp* line in adult animals, as evidenced by the appearance of papillomas at about 6 months of age. Interestingly, only deformed mice developed papillomas, whereas non-deformed transgene-bearing animals survive up to a year without papilloma formation. Restriction of BPV expression to a single parental lineage does not appear to be sequence-dependent since another BPV transgenic line does not show any such restricted pattern of expression (Lacey *et al.* 1986). From the data, we conclude that the transgene has inserted into an endogenous gene that functions in limb bud development, and postulate that this integration site controls expression of the transgene.

Developmental analysis of the mutation

Microscopic examination of the skeletal structures of normal and mutant animals, using alizarin red S stain to visualize ossified structures and alcian blue for chondrified structures (McLeod, 1980), revealed ossification of the radius, ulna, metacarpals and phalanges, but not of the carpals in a newborn forepaw of a nondeformed mouse (Fig. 2A). Figures B–D show skeletal structures of deformed mice, with diferent degrees of deformity; four digits, with noticeable but not severe involvement of the phalanges, metacarpals and carpals (Fig. 2B); a paw with only the fifth digit, and loss of almost all phalanges and metacarpals, with apparent fusion of the metacarpals from digits 4 and 5 (Fig. 2C); and no phalanges and only two very shortened metacarpals with reduction and fusion of the carpals (Fig. 2D). The loss of normal structure of the digits makes it difficult to identify precisely which of the digits is absent or abnormal. Regardless of the degree of deformity seen in the small bones (carpals, metacarpals, phalanges), the long bones were not affected. Thus the *Adp* mutation appears to affect the number, size and shape of the small bones; synostosis of some of these bones was frequently observed.

Analysis of skeletons of newborn mice permitted detection of the bones affected by the mutation. We then addressed the question of when during development the small bones of *Adp* mice were affected by the mutation. Bone formation involves three distinct processes: (1) formation of the blastema, a clustering of precartilage mesenchymal cells that give rise to specific structures of the limb; (2) secretion of cartilage matrix or chondrification by chondrocytes in the blastema; (3) removal of the cartilage matrix by invading osteoclasts and replacement with osseous tissue deposited by osteoblasts. In mice, chondrification centers are present in the metacarpals, metatarsals and tarsals by embryonic day 13 (day 0 is day of vaginal plug) (Rugh, 1968). To determine if chondrification centers in the small bones of deformed animals were forming at the appropriate time, females with staged pregnancies were collected and the fetuses processed for visualization of cartilage (McCleod, 1980). Fig. 3 shows limb buds from normal and deformed embryos at 13.5 days *post coitum*. In both the fore- and hindlimb buds, only three digit rays are evident in the deformed animal, compared to five rays in the normal littermate. Although reduced in number and size, the mutant digit rays have begun the process of chondrification on schedule, suggesting that the primary defect in this mutation occurs earlier than embryonic day 13.5, and affects blastema formation. In fact, examination of whole fetuses prior to the onset of chondrification revealed that the mutant phenotype was already apparent by 12.5 days *post coitum* (Fig. 4). By this time in normal development, indentations between the digit rays are evident, causing the limb buds to appear more polygonal than round (Fig. 4B, left), whereas the mutant embryo still has rounded limb buds (Fig. 4B, right). This difference is even more pronounced 24 h later (Fig. 4A). At 11.5 days *post coitum*, before the blastemas of the five digits have formed, the mutant and normal embryos appear similar (Fig. 4C).

The apical ectodermal ridge is intact in mutant embryos

Limb development is one of the most extensively studied systems of pattern formation in vertebrates and much is known about the establishment of the anteroposterior and the proximodistal axes of polarity. Establishment of the anteroposterior axis requires release of the morphogen retinoic acid by a group of mesenchymal cells on the distal posterior margin of the limb called the zone of polarizing activity (ZPA) (for a review, see Brockes, 1989). In chick embryos, surgical

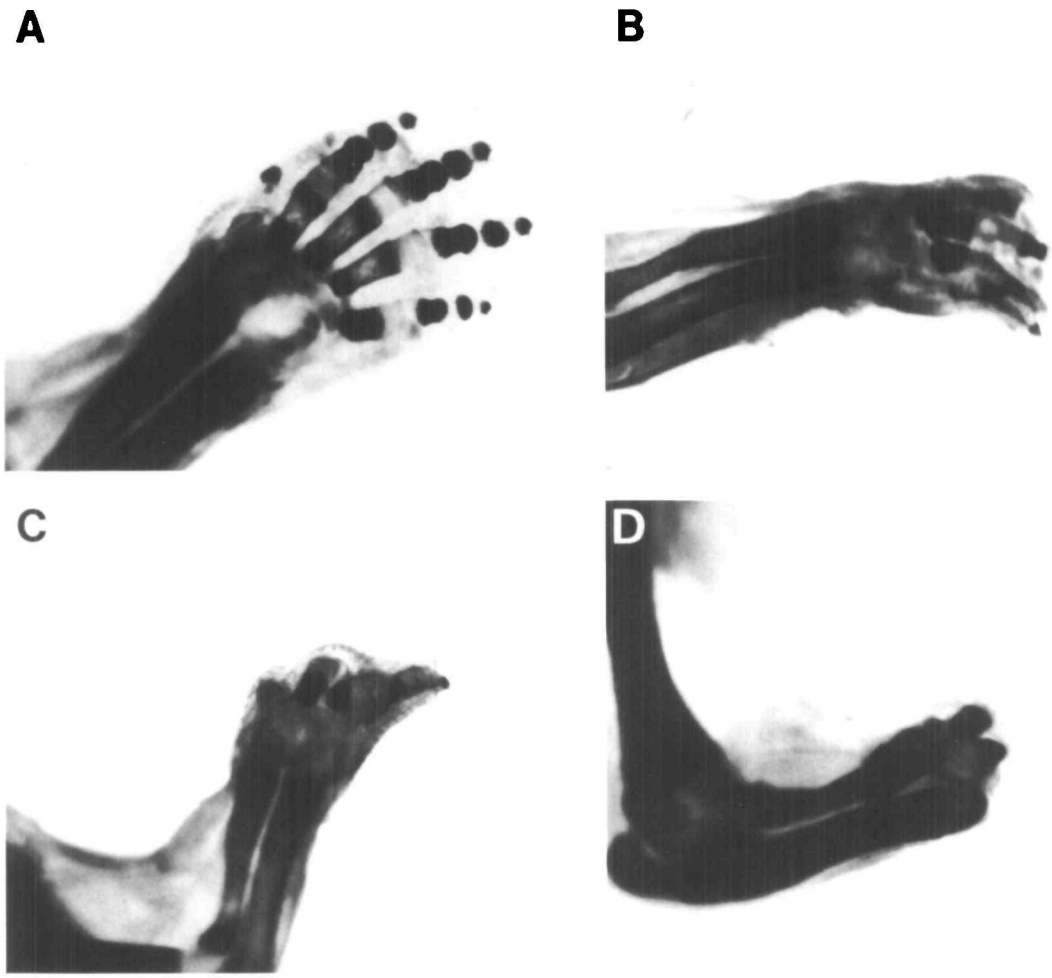

Fig. 2. Skeletal preparations of normal and deformed newborn pups. Animals were processed by the procedure of McLeod (1980). Following death, the skin and viscera were removed and the carcasses were fixed in 95% ethanol for at least 5 days and acetone for 2 days. Staining was done in 0.3% alcian blue 8GS and 0.1% alizarin red S for 3 days at 37°C. Soft tissue was cleared in 1% KOH followed by graded solutions of glycerol in 1% KOH over a 2 week period. All of the limbs shown are right forelimbs, oriented ventral side downward. (A) A nondeformed control; (B–D) 3 different transgenic animals showing the variation of deformity present in this line, with progressively more loss of distal structures. Carpals, metacarpals and phalanges are affected; long bones of the limb appear normal. Magnification is 20×.

removal of the ZPA results in lack of digit formation, whereas the implantation of a second ZPA on the anterior margin causes duplication of the anteroposterior axis with consequent duplication of digits (Tickle *et al.* 1975). A second structure believed to be involved in anteroposterior organization is the apical ectodermal ridge (AER), a band of columnar epithelial cells at the distal border of the developing limb. The AER is also crucial for the proximodistal axis; its presence keeps the underlying mesenchyme developmentally labile and permits continued growth of the limb at the distal margin. Studies with chick embryos showed that complete removal of the AER results in dramatic shortening of the proximodistal axis, with loss of progressively more distal structures related to the timing of removal. Partial AER removal causes digit loss along the anteroposterior axis (Rowe and Fallon, 1987).

To determine whether the integrity of the AER was affected by this mutation, we used a genetic cross between our line of mice and a second transgenic line, 671 (kindly provided by R. Behringer and R. L. Brinster), in which the transgene *Escherichia coli lacZ*, is under the transcriptional control of the murine Hox 1.4 promoter and *lacZ* expression within the limb bud is restricted to the AER (R. Behringer, personal communication). Expression of *lacZ* is easily ascertained by an enzyme assay for the protein product, β-galactosidase. By crossing 671 females with *Adp* males, we obtained deformed fetuses with *lacZ* expression confined to the AER of the limbs. Fig. 5 shows such a mouse at 13.5 days *post coitum*. Both fore- and hindlimb buds show developmental defects (compare with developmentally normal limb buds in Fig. 4A). These mice lack the digit rays and indentations, but the AER is revealed as a single, intact band

along the distal border of the limb buds. Thus, the AER is probably not the primary site of the mutation. The primary defect must occur earlier in development, before day 12.5 and most probably at the time of blastema formation.

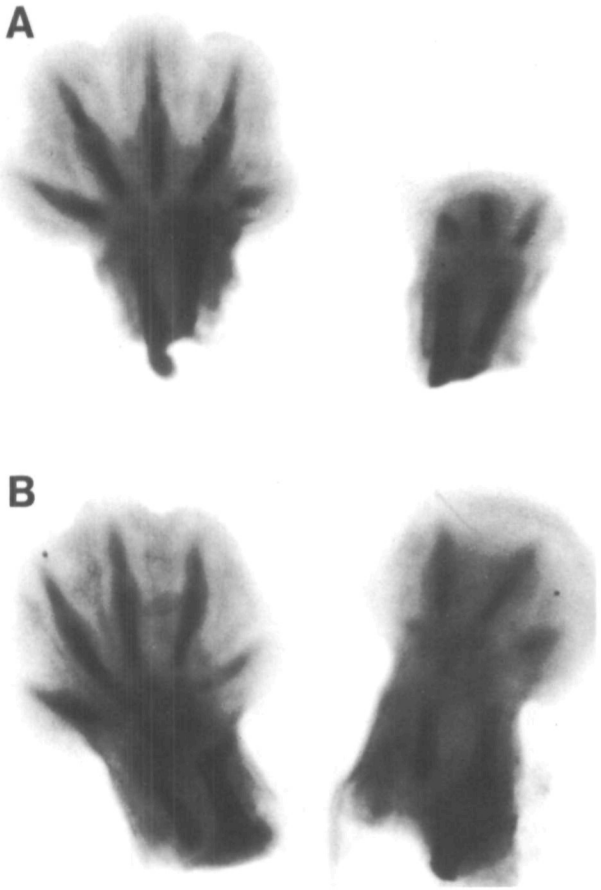

Fig. 3. Pattern formation in mutant limbs. (A) Cartilage formation in the forelimbs and (B) hindlimbs of normal (left side) and deformed (right side) littermates collected at 13.5 days *post coitum* Carcasses were prepared as before with some modifications (McLeod, 1980). Mice were kept in acetone for 7 days in lieu of skinning and put directly into 20% glycerol in 1% KOH following staining to avoid disintegration of fetuses. By this stage the deformed fetuses have fewer and shorter digit rays than normal fetuses. Magnification is 25×.

Discussion

The concept of genomic imprinting is now well established; however, the mechanism underlying the imprinting process has remained elusive. Molecular studies have been hindered by lack of any cloned imprinted sequences. In this report, we have described a transgenic line in which expression of both the

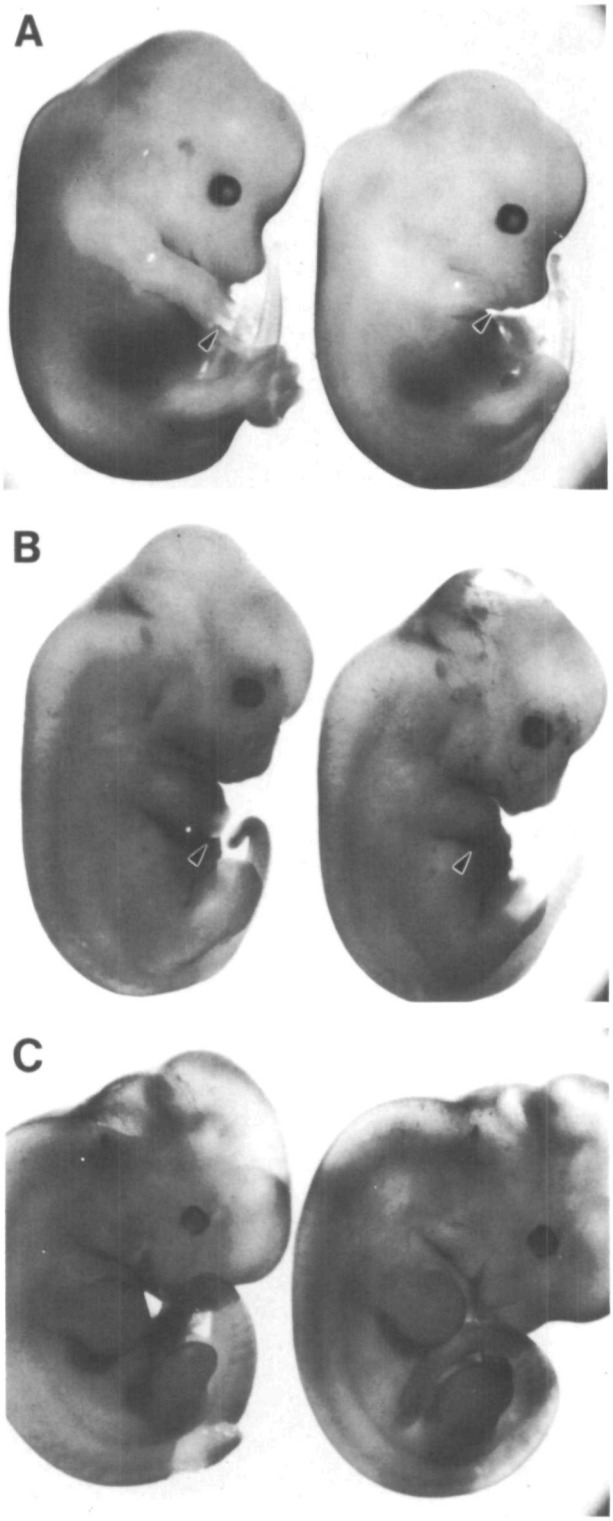

Fig. 4. Macroscopic morphology of developing limbs in transgenic fetuses. Fetuses were fixed in 0.02% glutaraldehyde, their extraembryonic membranes removed and analyzed by the polymerase chain reaction for the presence of the transgene. Nontransgenic mice are on the left side of each pair of littermates. (A) 13.5-day fetuses, magnification 8×; (B) 12.5-day fetuses, magnification 10×; and (C) 11.5-day fetuses, magnification 12.5×. Arrows indicate the indentations between the digits in normal mice and where these indentations should appear in the deformed fetuses. By 12.5 days, the developmental retardation is clearly visible in the mutants.

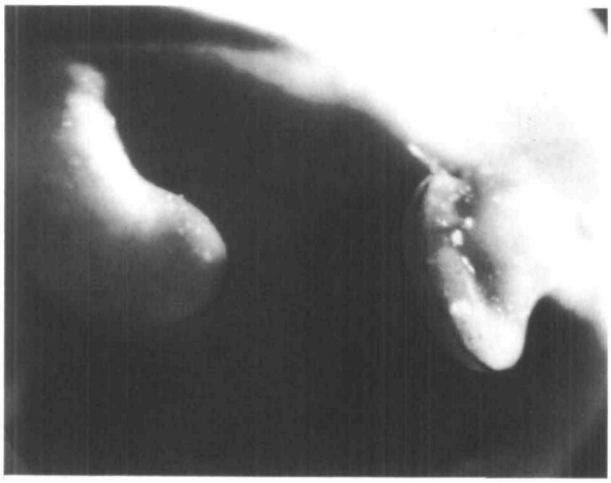

Fig. 5. Detection of *lacZ* expression in the AER of transgenic fetuses. Fetuses that were compound heterozygotes for two transgenes, a *lacZ* gene driven by the Hox 1.4 promoter and pL116 were assayed for the presence of β-galactosidase activity in the AER. Whole fetuses were fixed in 0.2% glutaraldehyde and stained overnight in a buffer containing X-gal (2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP40 in phosphate-buffered saline, pH 7.3). The photograph is of a single fetus at embryonic day 13.5, showing that the AER of deformed fetuses remains intact. Magnification is 32×.

transgene and the neighboring endogenous gene show imprinting effects. The coupling of expression of these endogenous and exogenous genes can result from integration of the transgene into an imprinted region of the host genome, which affects the transgene, or from modification of the transgene on integration into the genome, an effect which might then spread to neighboring sequences. We are attempting to clone the endogenous gene to discriminate between these two possibilities.

The mutation in the *Adp* line of mice is unlike other previously described limb mutations (Green, 1981). The inheritance pattern is a modified dominant transmission; only mice that inherit the transgene paternally are affected. Additionally, penetrance is incomplete; not all mice with the paternally contributed transgene are deformed, and those that are show variation in severity of deformity. Incomplete penetrance and variable expression might result from allelic differences in gene activity determined by parental inheritance, such that maternal genes express 0–50% activity and paternal genes express 50–100% activity. Disruption at the paternal allele would cause deformity, the severity of which depends on the activity of the intact maternal allele. The paternally active gene would compensate disruption of the maternal allele, with no apparent phenotypic anomaly. A second explanation for the incomplete penetrance, which does not necessarily exclude the first possibility, is somatic mosaicism of endogenous gene expression. Erasure of the imprint, which inactivates the maternal allele, may occur gradually over time, such that partial but incomplete erasure occurs in the developing limb, permitting some transcriptional activity of the maternal gene and partial rescue of the phenotype. The intermediate levels of transgene methylation and expression reported by McGowan *et al.* (1989) suggest that mosaicism between cells within individual tissues does occur. Interestingly, in T^{hp}, a deletion mutation on mouse chromosome 17 which is lethal with maternal inheritance, the affected fetuses die over a period of several days rather than at a specific time in development. Survival of affected fetuses varies with genetic background; 33% of maternally inherited T^{hp} animals survive to birth on a Balb/c background, while only 20% survive on a C57BL background. In the case of paternal inheritance, tail malformations that are concordant with the mutation also show a degree of phenotypic heterozygosity. Additionally, there is reduced litter size with paternal inheritance of T^{hp}, suggestive that there is embryonic loss *in utero* (Johnson, 1974).

Close examination of the mutation in *Adp* mice provides insight as to when during development the endogenous gene normally functions. The observed mutation affects only the small bones of the fore- and hind-paws, with variation involving the number of small bones affected. The primary site of gene action appears to be the blastema organization, with chondrification and ossification occurring on schedule. Any gene(s) suspected of mutation should be expressed during this time. Of the transgene insertional mutations involving limb development that have been reported (Woychik *et al.* 1985; Overbeek *et al.* 1986; and McNeish *et al.* 1988), one of these (Woychik *et al.* 1985) involved insertion of the transgene into a known site, the *ld* locus. Using the transgene as a starting site for cloning, those authors have isolated the putative *ld* gene and used it to analyze *ld* mutations (Zeller *et al.* 1989). A similar strategy might be fruitful in identifying and isolating the endogenous imprinted gene in *Adp* mice.

Special thanks to R. Behringer and R. L. Brinster for the use of transgenic line 671 and to Jenny Price, who prepared the *Adp* lineage and brought its pattern of expression to our attention. This work was supported in part by US Public Health grants HD-17720, HD-21355 and HD-23291 from NICHD and by grant CD-286 from the American Cancer Society. J.A.D. was supported by grant CA-09171 from NCI.

References

Beechey, C. V., Cattanach, B. M. and Searle, A. G. (1989). Genetic Imprinting Map. *Mouse News Letter* **84**, 48–49.

Brockes, J. P. (1989). Retinoids, homeobox genes and limb morphogenesis. *Neuron* **2**, 1285–1299.

Cattanach, B. M. (1986). Parental origin effects in mice. *J. Embryol. exp Morph.* **97 Supplement**, 137–150.

Green, M. C. (1981). *Genetic Variants and Strains of the Laboratory Mouse*, Gustav Fischer Verlag, Stuttgart.

Hadchouel, M., Farza, H., Simon, D., Tiollais, P and Pourcel, C. (1987). Maternal inhibition of hepatitis B surface antigen gene expression in transgenic mice correlates with *de novo* methylation. *Nature, Lond* **329**, 454–456.

JOHNSON, D. R. (1974). Hairpin-tail: A case of post-reductional gene action in the mouse egg? *Genetics* **76**, 795–805.

LACEY, M., ALPERT, S. AND HANAHAN, D. (1986). Bovine papillomavirus genome elicits skin tumours in transgenic mice. *Nature, Lond.* **322**, 609–612.

LAW, M. F., BYRNE, J. C. AND HOWLEY, P. M. (1983). A stable bovine papillomavirus hybrid plasmid that expresses a dominant selective trait. *Molec. cell. Biol.* **3**, 2110–2115.

MCGRATH, J. AND SOLTER, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–183.

MCGOWAN, R., CAMPBELL, R., PETERSON, A. AND SAPIENZA, C. (1989). Cellular mosaicism in the methylation and expression of hemizygous loci in the mouse. *Genes and Dev.* **3**, 1669–1676.

MCLEOD, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299–301.

MCNEISH, J. D., SCOTT, W. J., JR AND POTTER, S. S. (1988). Legless, a novel mutation found in PHT1-1 transgenic mice. *Science* **241**, 837–839.

OVERBEEK, P. A., LAI, S.P., VAN QUILL, K. R. AND WESTPHAL, H. (1986). Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. *Science* **231**, 1574–1577.

ROEW, D. A. AND FALLON, J. F. (1981). The effect of removing posterior apical ectodermal ridge of the chick wing and leg on pattern formation. *J. Embryol. exp. Morph.* **65**, 309–325.

RUGH, R. (1968). *The Mouse Its Reproduction and Development.* Burgess Publishing Company, Minneapolis, MN.

SAPIENZA, C., PAQUETTE, J., TRAN, T. H. AND PETERSON, A. (1989). Epigenetic and genetic factors affect transgene methylation imprinting. *Development* **107**, 165–168

SAPIENZA, C. J., PETERSON, A. C., ROSSANT, J. AND BALLING, R. (1987). Degree of methylation of transgenes is dependent on gamete of origin. *Nature, Lond.* **328**, 251–254.

SOLTER, D. (1988). Differential imprinting and expression of maternal paternal genomes. *A. Rev. Genet.* **22**, 137–146.

SURANI, M. A. H., BARTON, S. C. AND NORRIS, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis *Nature, Lond.* **308**, 548–550.

SURANI, M. A. H., REIK, W. AND ALLEN, N. D. (1988). Transgenes as molecular probes for imprinting. *Trends Genet.* **4**, 59–62.

SWAIN, J. L., STEWART, T. A. AND LEDER, P. (1987). Parental legacy determines methylation and expression of an autosomal transgene: A molecular mechanism for parental imprinting. *Cell* **50**, 719–727.

TICKLE, C., SUMMERBELL, D. AND WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature, Lond.* **254**, 199–202.

WOYCHIK, R. P., STEWART, T. A., DAVIS, L. G., D'EUSTACHIO, P. D. AND LEDER, P. (1985). An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. *Nature, Lond.* **318**, 36–40.

ZELLER, R., JACKSON-GRUSBY, L. AND LEDER, P. (1989). The limb deformity gene is required for apical ectodermal ridge differentiation and anteroposterior limb pattern formation. *Genes and Dev.* **3**, 1481–1492.