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Genetics

Transgene-induced mutation of the murine steel locus

(insertional mutation/developmental genetics/hematopoiesis)

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ABSTRACT **The product of the steel locus is essential for normal development of three distinct populations of stem cells—the neural crest-derived melanoblasts, germ cells, and blood cell precursors. Many mutant alleles at steel are lethal in homozygotes and produce coat color dilution in heterozygotes. We have identified a transgenic mouse with diluted pigmentation that closely resembles that of steel heterozygotes. We have demonstrated that the site of transgene insertion is genetically linked to the phenylalanine hydroxylase locus on mouse chromosome 10. In addition, the chromosome carrying the transgene fails to complement the recessive lethality of the *Sl* allele of steel and the pigmentation defect of the Sl^{pan} allele. The data indicate that the inserted transgene has disrupted the steel locus. The resulting allele, designated Sl^{tg}, provides a molecular tag for isolation of the steel gene, as well as a new allele for characterization of this developmentally important locus.**

Since the initial description of the spontaneous mutant allele *Sl* in 1956 (1), the steel locus has been the subject of intensive investigation because of its pleiotropic effects on development (reviewed in refs. 2–4). Most of the known mutant alleles of steel result in pigmentation dilution in heterozygotes and prenatal lethality in homozygotes. The homozygous defect becomes apparent during midgestation, when the liver fails to initiate hematopoiesis. Primordial germ cells also fail to proliferate in the genital ridges of homozygous embryos, and migration of melanoblasts is abnormal (2). Additional information regarding affected cell types has been obtained from homozygous animals carrying less severe, viable mutant alleles. These homozygotes are white animals with black eyes and are characterized by macrocytic anemia and sterility. Thus, development of three distinct populations of stem-cells appears to be affected by steel: neural-crest-derived melanocytes, hematopoietic cells, and germ cells. Transplantation of homozygous mutant cells to normal hosts has demonstrated that the effects on melanocytes and hematopoietic stem cells are extrinsic to these cell populations, affecting the environment in which they migrate, proliferate, or differentiate (5–7).

To account for these phenotypes, it has been suggested that the steel locus might encode a growth factor required by the affected cells or some essential component of the extracellular matrix in which these cells differentiate. Abnormalities of the dermal extracellular matrix of mice homozygous for the mutant allele Sl^{d} have been described recently (8, 9). Molecular analysis of the steel locus could provide valuable information about the poorly understood process of stem cell development.

Insertion of foreign DNA into mouse chromosomes, by either microinjection or retroviral infection, can result in the disruption of endogenous mouse genes. The inserted DNA then provides a molecular tag for subsequent cloning of the disrupted gene. DNA insertion appears to occur at random sites along the chromosome. Among the thousands of transgenic mice that have been produced by these techniques during the past decade, there are several reported instances of disruption of previously known genes. Retroviral insertion into the collagen structural gene resulted in embryonic lethality at day 12 of gestation (10). Interruption of several other known loci with interesting developmental phenotypes has provided molecular access to the affected genes. These loci include pygmy (11), limb deformity (12), downless (13), hotfoot (14), and Purkinje cell degeneration (15). The transgenic mutants provide a powerful tool for isolation of genes known to play a role in mammalian development. In this report we describe another example, insertional mutation of the steel locus on mouse chromosome 10.

MATERIALS AND METHODS

Animals. Transgenic line Tg6208 was generated as part of a study of the insulin-dependent expression of hybrid amylase/elastase/chloramphenicol acetyltransferase constructs (16). The 17 other transgenic lines in the study were normally pigmented. Transgenic mice were produced by microinjection of a linear 2-kilobase (kb) DNA fragment into fertilized mouse eggs obtained from the mating between (C57BL/6NCr × C3H/HeNCr)F_1 individuals. Tg6208 contains a single copy of the chloramphenicol acetyltransferase gene construct. The line has been maintained by continued backcrossing to strain C57BL/6J.

Inbred C57BL/6J mice and C57BL/6J-*Sl*/+ mutant mice were obtained from The Jackson Laboratory. Mice homozygous for the panda allele of steel, Sl^{pan} (17), were obtained from Verne Chapman and Dennis Stephenson (Roswell Park Memorial Institute, Buffalo, NY).

Identification of Transgenic Mice. Genomic DNA was isolated from tails, and the presence of the transgene was determined by polymerase chain reaction (PCR) with primers complementary to the chloramphenicol acetyltransferase structural gene as described (16).

Southern Blotting. Genomic DNA was digested with restriction endonucleases, electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters. The 1.9-kb *Eco*RI fragment of moPAH8, a full-length mouse phenylalanine hydroxylase cDNA clone (18), was labeled with ^{32}P by the random oligonucleotide-primed method (19) and hybridized to the filters.

RESULTS

Diluted Pigmentation in Transgenic Mice of Line Tg6208. The generation of transgenic line Tg6208 was previously described (16). These transgenic mice have a mutant coat color phenotype, which is illustrated in Fig. 1. The overall dilution of the normal black coat color is especially evident on the belly of transgenic mice. White patches occur frequently on the belly and occasionally on the forehead. The

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Abbreviation: PCR, polymerase chain reaction.

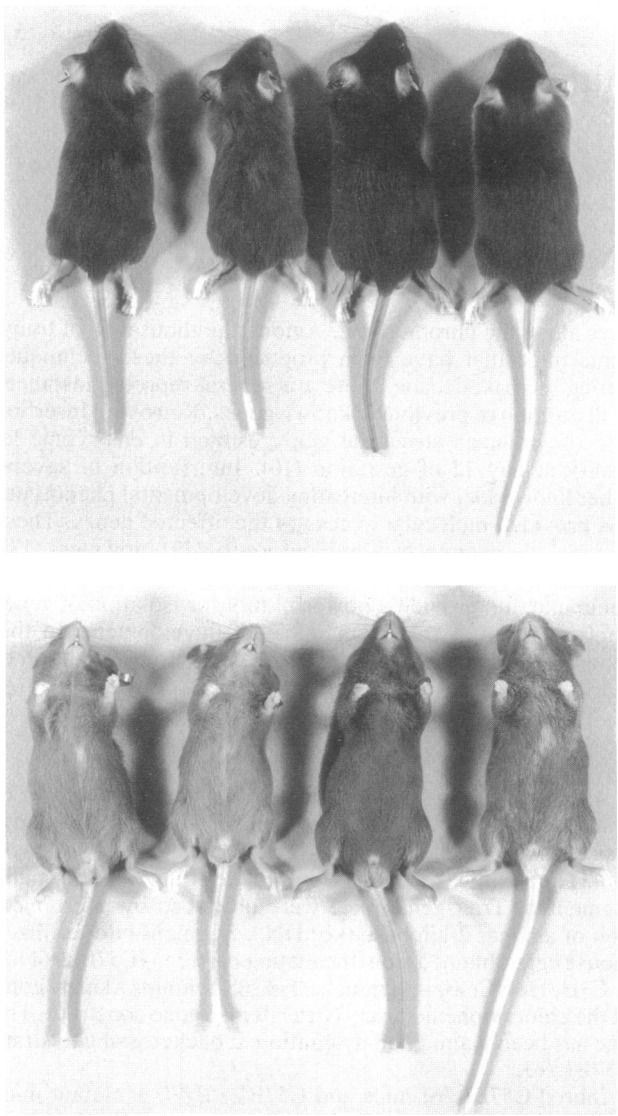

FIG. 1. Coat color phenotype of mice from line Tg6208. Left to right, two transgenic mice, one nontransgenic littermate, and one steel heterozygote (*Sl*/+). Tail tips were removed from three of these mice for preparation of genomic DNA. (*Upper*) Dorsal view. (*Lower*) ventral view.

appearance of the transgenic mice is strikingly similar to heterozygotes for the *Sl* allele at the steel locus (Fig. 1 *Right*). This phenotype did not occur in any other transgenic lines expressing the same reporter gene, indicating that the phenotype is not the result of transgene expression.

During several generations of backcrossing to inbred strain C57BL/6J, the mutant coat color cosegregated with the transgene. Among 156 animals typed for the presence of the transgene by PCR, all 65 transgenic animals had diluted coat color, and all 91 nontransgenic animals had wild-type coat color. The correlation between transgene and coat-color phenotype indicates that the pigmentation defect is the result of insertional mutation of an endogenous gene by the transgene.

Linkage Analysis. To determine whether the diluted pigmentation in Tg6208 is the result of mutation of a known pigmentation locus, we analyzed the segregation of markers linked to the candidate genes varitint-waddler (chromosome 3), dominant spotting, *W* (chromosome 5), and microphthalmia (chromosome 6). The transgene did not cosegregate with any of these markers in a backcross (data not shown).

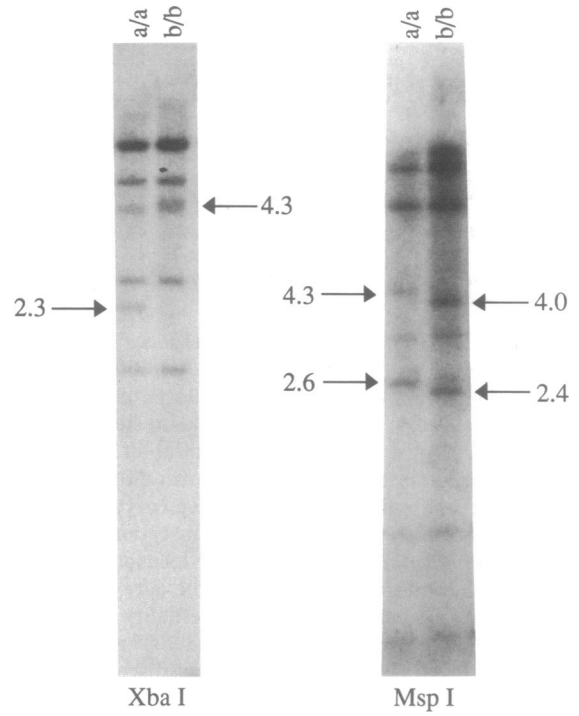

FIG. 2. Restriction fragment length variation at the *Pah* locus. Genomic DNA was digested with *Xba* I or *Msp* I. Southern blots were probed with a labeled *Pah* cDNA probe. The 2.3-kb *Xba* I fragment and the 2.6-kb and 4.3-kb *Msp* I fragments were used as markers to follow the segregation of the Pah^a allele in crosses between Tg6208 and strain C57BL/6J.

Linkage was observed with the phenylalanine hydroxylase locus *Pah* on chromosome 10. We identified a restriction fragment length variant by digestion of genomic DNA with the enzymes *Xba* I and *Msp* I, followed by hybridization with the phenylalanine hydroxylase cDNA (Fig. 2). C3H mice carry the Pah^a allele, and C57BL/6 mice carry the Pah^b allele. These alleles segregate during the propagation of transgenic line Tg6208 by backcrossing with strain C57BL/6J. Transgenic individuals inherited the Pah^a allele from the transgenic founder, a (C57BL/6 × C3H)F_2 individual. In offspring of matings between transgenic mice and C57BL/6J, we observed no recombination between the Pah^a allele and the transgene (Table 1). All transgenic offspring were Pah^a/Pah^b, and all nontransgenic offspring were Pah^b/Pah^b. The observed rate of recombination (0 of 60) indicates that the transgene is located within 6.1 centimorgans of *Pah* ($P < 0.05$). This recombination rate does not differ significantly from the 4% rate of recombination reported between steel and *Pah* (20). The genetic data is therefore consistent with insertion of the transgene at the steel locus in Tg6208.

Complementation Test with Known Alleles of Steel. Hemizygous transgenic mice (Tg/+) were crossed with heterozygotes for the recessive lethal *Sl* allele, and 43 offspring were

Table 1. Linkage of the transgene with *Pah*

Class	Transgene	*Pah*	Progeny, no.
Parental	+	*a/b*	22
	−	*b/b*	38
Recombinant	+	*b/b*	0
	−	*a/b*	0

Transgenic mice of line Tg6208 (Pah^a/Pah^b) were crossed with inbred strain C57BL/6J (Pah^b/Pah^b). Offspring were scored for presence of the transgene by PCR and for *Pah* alleles by Southern blotting with a phenylalanine hydroxylase cDNA probe as described in the text.

Table 2. Failure of transgenic mice to complement the recessive lethality of *Sl*

Genotype	Transgene	Coat color	Progeny, no.
Tg/?	+	Diluted	13
Sl/+	−	Diluted	14
+/+	−	Wild type	16

Transgenic mice (Tg/+) were crossed to C57BL/6J-*Sl*/+. Progeny were scored for the diluted coat color phenotype visually and for presence of the transgene by using PCR. The observed data are consistent with lack of complementation ($P > 0.8$) and differ significantly from the prediction of viability of Tg/*Sl* ($P < 0.05$).

examined. The results of this cross are presented in Table 2. If the steel locus is disrupted by the transgene, offspring inheriting both the transgene and *Sl* would not survive, and three classes of viable offspring would be expected in equal numbers. The results are in good agreement with this prediction. On the other hand, if the transgenic chromosome carried a wild-type allele of steel, then 50% of the offspring of this cross would be transgenic and the other 50% would be equally divided between *Sl*/+ and +/+ mice. This prediction is not consistent with the data. This experiment demonstrates lack of complementation of the recessive lethal phenotype of *Sl* by the transgenic chromosome.

The second cross was with mice homozygous for the viable panda allele Sl^{pan} (17). These homozygotes are white with black ears and black eyes, and males are fertile. Heterozygous Sl^{pan}/+ mice have a normally pigmented coat. Tg6208 females (Tg/+) were mated with Sl^{pan}/Sl^{pan} males. Complementation of the Sl^{pan} allele would result in only pigmented offspring, while noncomplementation would produce equal numbers of pigmented and white mice. Four offspring from this cross were white and transgenic, while the other three were pigmented and nontransgenic. The chromosome with the transgenic insert thus failed to complement the Sl^{pan} allele.

The genetic linkage to *Pah* and the failure to complement two mutant steel alleles indicate that the dominant coat color dilution in Tg6208 is the result of insertional mutation of the steel locus. This new allele of steel is designated Sl^{tg}.

Characterization of the Phenotype of Mice Carrying the Sl^{tg} Allele. Heterozygous Sl^{tg}/+ animals appear to be fully viable. We analyzed a total of 216 offspring of transgenic mice crossed with C57BL/6J. PCR was carried out on 156 animals, and the remaining 58 were typed by pigmentation only. One hundred and twenty-two animals were +/+ and 94 were Sl^{tg}/+.

Because of the difficulty in breeding this transgenic line, we measured testes weight in sexually mature males (Table 3). The relative testes weight of the Sl^{tg}/+ mice was significantly lower ($P < 0.01$) than wild-type controls. Histology revealed the presence of sperm at all stages of development (A. K. Christiansen and M.H.M., unpublished observations).

Hematocrit values and erythrocyte counts for Sl^{tg}/+ heterozygotes were not significantly different from wild type (data not shown). Preliminary observations suggest that Sl^{tg} may be a recessive lethal allele. Overall, these phenotypes of Sl^{tg}/+ heterozygotes are typical of mutant alleles of steel.

Table 3. Reduced testes weight in Sl^{tg}/+ mice

Genotype	Age, weeks	Testes weight, mg	Relative testes weight, mg/g of body weight
Sl^{tg}/+	18 ± 4	138 ± 12	4.4 ± 0.6
+/+	18 ± 3	238 ± 23	5.7 ± 0.6

Data were obtained from sexually mature males between the ages of 12 and 22 weeks. Values are means ± SD for five individuals of each genotype.

DISCUSSION

We have identified a line of transgenic mice with an insertional mutation of the steel locus. Spontaneous and radiation-induced mutations of steel have been studied extensively (2–4). In typical homozygotes, the mutant phenotype becomes apparent at approximately 14 days of gestation when the liver fails to initiate hematopoiesis, and few affected individuals survive to birth. Heterozygotes, however, are only mildly affected, with a characteristic diluted coat color, mild anemia, and small gonads. Compound heterozygotes carrying one severe and one mild allele may be viable and are usually white in coat color and sterile. The phenotype of the Sl^{tg} allele resembles that of the original *Sl* allele, with similar coat-color dilution (Fig. 1) and reduced testes weight in heterozygotes (21). Sl^{tg} also fails to complement the recessive lethality of *Sl*.

Another murine locus with a related phenotype is the *W* locus on chromosome 5. The same cell types are affected by *W* and steel and mutants are nearly identical in phenotype, except that the *W* defect is intrinsic to the affected cells (22). It has recently been established that the *W* locus is identical to *c-kit* and encodes a membrane receptor with tyrosine kinase activity (23, 24). In view of this function of *W*, it is an attractive hypothesis that steel may encode the ligand bound by the c-kit receptor. The transgene causing the Sl^{tg} allele can be used as a probe to clone the disrupted locus. This should lead to characterization of its product and detailed analysis of its role in development. Molecular characterization of this and other interrupted genes which regulate mammalian development can be expected to contribute to fundamental understanding of this important and complex process.

The steel locus is located within a conserved linkage group on mouse chromosome 10 that includes peptidase-2, γ interferon, and phenylalanine hydroxylase (25). Since the relative positions of these loci is conserved on human chromosome region 12q21–q24 (26), the human homolog of steel may be located within this region. This linkage information together with isolation of the steel gene will facilitate analysis of the medical significance of the human gene.

Note Added in Proof. While this paper was in press, several reports appeared in which a mast cell growth factor was identified as a product of the steel locus (reviewed in ref. 27).

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