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Genetics

Prenatal lethality in a transgenic mouse line is the result of a chromosomal translocation

(insertional mutagenesis/chromosome rearrangement/developmental arrest/semisterility)

KATHLEEN A. MAHON, PAUL A. OVERBEEK*, AND HEINER WESTPHAL

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **We have produced a line of transgenic mice that is characterized by prenatal lethality. These mice bear a chimeric plasmid containing the long terminal repeat of the Rous sarcoma virus linked to the coding region of the chloramphenicol acetyltransferase gene (pRSV-CAT). Mice heterozygous for the pRSV-CAT integration site are semisterile, producing litters ≈40% of the average size when crossed to normal mice. Approximately 50% of the progeny from such a cross bear the pRSV-CAT sequences and also produce litters of smaller size. An analysis of embryogenesis revealed that normal numbers of embryos implanted, but 60% failed to develop past day 7. Eight other independent transgenic lines containing RSV-CAT show no evidence of embryonic lethality; thus, it is unlikely that the defect observed is due to the direct effects of RSV-CAT expression. We have found that carrier mice bear a reciprocal translocation between chromosomes 6 and 17, T(6A2–6A3;17D–17E1), that can explain the apparent dominant embryonic lethality seen in this line. The site of integration has been localized by *in situ* hybridization at or near the translocation breakpoint in one of the translocated chromosomes (6^{17}). Because the foreign DNA is present in one of the translocated chromosomes, we propose that this rearrangement was elicited by the introduction of foreign DNA.**

It has long been recognized that eukaryotic genes can become inactivated by the insertion of exogenous DNA sequences. Genetic and molecular analyses have revealed that several spontaneous mutations in maize (1, 2), yeast (3), and *Drosophila* (4, 5) are due to the insertion of transposable elements. Similarly, the dilute (*d*) coat color mutation in the mouse results from the integration of an ecotropic murine leukemia virus (6, 7). Genes disrupted in this way can be cloned and analyzed at the molecular level by virtue of their association with the newly integrated DNA.

With the development of methods to experimentally introduce specific DNA sequences into the mouse germ line (for review, see ref. 8), the potential to create new, molecularly accessible mutations by insertional inactivation has been realized. In the Mov-13 mouse strain, established from an embryo infected with Moloney murine leukemia virus, the provirus has integrated into and inactivated the α1(I) collagen gene (9, 10). Microinjection of DNA into the pronuclei of mouse zygotes has generated several mutations, such as recessive prenatal lethality (8, 11, 12), transmission distortion (13), and an abnormality in limb development (14). The latter mutation is an allele of a previously described locus, limb deformity (*ld*) (14), and serves to illustrate the potential role insertional mutagenesis can play in the molecular analysis of genetically well-characterized loci.

In the course of our studies, we generated two transgenic mice that acquired mutations affecting development. These mice were among nine transgenic mice bearing independent insertions of a plasmid containing the long terminal repeat of the Rous sarcoma virus linked to the coding region of the chloramphenicol acetyltransferase gene (pRSV-CAT) (15). Seven of the transgenic lines were phenotypically unremarkable, an indication that the developmental abnormalities observed in two lines were specific for particular integration sites and were not specific for pRSV-CAT sequence or its expression. In one transgenic line, line 2, mice homozygous for the pRSV-CAT sequences display recessive syndactyly of the middle digits on both fore and hind paws (15). Our data clearly suggest that the defect in line 2 mice, like those of the previous examples, results from the insertion of the exogenous DNA into a cellular gene.

The topic of this paper is the other line of pRSV-CAT transgenic mice that showed evidence of mutation, line 5. This line is characterized by embryonic lethality and differs from the previously described examples of insertional inactivation in its genetic characteristics and pattern of transmission. We show here that this lethality is not due to perturbation of genes at the site of integration; instead, this lethality results from gross chromosomal rearrangement.

MATERIALS AND METHODS

Animals, Crosses, and Embryo Isolation. The founder transgenic mouse of line 5 was obtained as described (15) by pronuclear microinjection and was derived from a (FVB/N female × C3H/HeN male) cross. All subsequent crosses were made with the FVB/N strain. The age of embryos was determined by counting the date of the vaginal plug as day 0. One-cell mouse embryos from (FVB/N × FVB/N) and (FVB/N × line 5) crosses were isolated from the oviducts of mated females (day 0.5) and cultured in M16 media (16) under paraffin oil for 5 days. Procedures for embryo isolation and culture are as described elsewhere (17). Postimplantation embryos were isolated by dissection from the uteri of pregnant females at days 6.5–12.5 of gestation.

DNA Hybridization Analysis. Genomic DNA was isolated from tail snips of mice from line 5 and dot blotted onto nitrocellulose filters as described (18). The filters were hybridized to ^{32}P-labeled pRSV-CAT. Copy number was determined by comparison of hybridization signal to that of standards containing known amounts of pRSV-CAT plasmid. A duplicate filter was hybridized to a mouse α2(I) collagen probe to verify that equivalent amounts of DNA were loaded in each well. Southern blotting was done according to standard procedures (19).

Chromosome Preparation and Karyotype Analysis. Spermatocyte chromosomes were prepared from the testes of male mice according to the air-drying technique of Evans *et*

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Abbreviations: RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase.

*Present address: Department of Cell Biology, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030.

al. (20) and stained with Giemsa stain. Mitotic chromosomes were prepared from bone marrow and were G-banded according to a modification of Davisson *et al.* (21). The karyotype was determined by comparison to the standard idiogram of banding patterns for mouse chromosomes (22).

***In Situ* Hybridization.** *In situ* hybridization of meiotic (spermatocyte) and mitotic (bone marrow) chromosome spreads was performed essentially as described (23) except that chromosomes were denatured in 70% formamide/0.6× SSC (1× SSC is 0.15 M sodium chloride/15 mM sodium citrate, pH 7.2) for 2 min at 70°C. Preparations were hybridized with ^{3}H-labeled SP6 RNA transcripts synthesized *in vitro* (24) from the plasmid pAZ1042 (provided by A. Schmidt, Meloy Laboratories, Springfield, VA) containing the 500-base-pair (bp) *Bal* I–*Hin*dIII fragment of pRSV-CAT (25) ligated into the *Sma* I–*Hin*dIII sites of pSP65 (Promega Biotec, Madison, WI). The probe (specific activity, 9×10^9 dpm/μg) was hybridized at a concentration of 1×10^5 dpm/μl. Slides were exposed to Kodak NTB-2 emulsion for 1–4 weeks.

RESULTS

Embryonic Lethality in Line 5. The founder transgenic mouse of line 5 was noted because he fathered unusually small litters when crossed to normal females. Approximately 50% of the progeny carried pRSV-CAT sequence and also produced smaller than normal litters when mated to normal mice. A partial pedigree of line 5 is shown in Fig. 1. There was no evidence for sex bias in the heritability or expression of this trait because both male and female transgenic animals produced small litters in test crosses.

Southern and dot blot analysis revealed that the founder male had two independently segregating integration sites containing 50 and 100 copies of pRSV-CAT sequence, respectively (data not shown), and that the pRSV-CAT sequences at both integration sites were apparently arranged in the multimeric, head-to-tail tandem arrays typical of many previously described multicopy integration sites in transgenic mice (8).

Mice from the line 5 pedigree were extensively mated to normal mice, and litter sizes were recorded. The pRSV-CAT integration site containing 100 copies was genetically separable from the phenotype of reduced litter size, whereas the integration site containing 50 copies cosegregated with small litter size. The average litter size of carrier animals was 3.4 (Table 1); a reduction of 63% from the average litter size of 9.3 recorded for unaffected siblings.

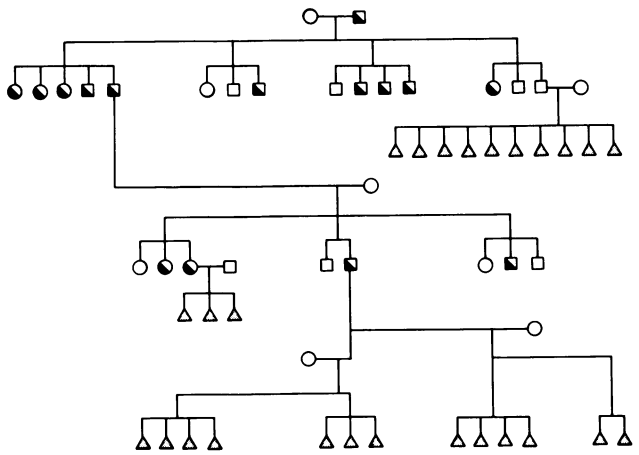

FIG. 1. Representative portion of the pedigree from line 5. The founder male carried pRSV-CAT sequences at two integration sites. For simplicity, segregation of only one site is depicted here; animals with this integration site produced small litters. Males are indicated by squares and females by circles. pRSV-CAT heterozygotes are indicated by the half-filled symbols, and nontransgenic animals are represented by the open symbols. Animals not analyzed for genotype or sex are shown by the hatched triangles.

The observed reduced litter size in line 5 suggested that a proportion of the embryos derived from these crosses were arresting in development before birth and prompted us to monitor development. FVB/N females were mated to transgenic carrier and normal males, killed at various times after mating, and the developing embryos were examined. Preimplantation embryos derived from crosses between carrier mice and normal FVB/N females showed no increased mortality in culture compared with similarly cultured FVB/N wild-type embryos.

An examination of postimplantation development revealed that many embryos derived from crosses with carrier transgenic animals were arrested in development shortly after implantation (between day 5.5 and day 7.5 of gestation). Defective egg cylinders or empty decidua were identifiable by gross inspection and histological analysis as early as day 6.5. The number of normal and defective embryos were recorded for each pregnancy. Although approximately equal numbers of embryos were implanting in both types of crosses, after correcting for the 2.9% mortality observed in noncarrier crosses, it was calculated that 60.3% of the embryos from crosses with carrier males cease development just after implantation (Table 2).

Chromosomal Analysis of Line 5. The characteristics and pattern of transmission of line 5 were not adequately explained by insertional activation or inactivation of a cellular gene at the site of integration. Direct disruption of a cellular gene resulting in dominant lethality could possibly explain why ≈50% of the embryos died in a test cross but cannot explain why the affected parent is viable and why ≈50% of the live progeny survive and pass on the trait with the same frequency as their parents. For this reason we sought evidence of gross chromosomal rearrangement, such as a translocation, as a cause for these results.

In mice heterozygous for a reciprocal translocation, at least 50% of the gametes produced through normal (alternate or adjacent-1) segregation at meiosis will bear duplications and deletions of chromosomal material. Translocation carriers usually produce more than 50% unbalanced gametes because of a higher frequency of nondisjunctional (adjacent-2) segregation (26). Embryos that inherit unbalanced karyotypes generally arrest in development shortly after implantation (26, 27). The surviving progeny will either be normal or balanced translocation carriers.

To determine whether a chromosomal translocation had occurred, chromosomal pairing at meiosis was examined. If a translocation is present, meiotic pairing results in the synapsis of the four chromosomes involved, generating a quadrivalent (or other diagnostic multivalent configurations depending on the extent of pairing) (26). Spermatocyte chromosome preparations were obtained from the testes of the founder mouse and his male progeny (five carrier and five noncarrier). At least 25 spermatocyte cells were scored per animal. A quadrivalent (predominantly chain IV) and 18 bivalents (Fig. 2*A*) were present in most (89%) cells from carrier males. Preparations from noncarrier males in the lineage and wild-type FVB/N males had the normal 20 bivalents. Thus, the founder transgenic mouse of line 5 had acquired a chromosomal translocation that was subsequently transmitted to some of his progeny.

The karyotypes of mitotic chromosomes were analyzed to identify chromosomes involved in the translocation. Fig. 2*B* shows that a reciprocal translocation has occurred between chromosomes 6 and 17. The approximate breakpoints of the translocation appear to be in band 6A2 or 6A3 in the

Table 1. Average litter size of line 5 animals

Cross	Mating pairs, no.	Litter, no.	Progeny, no.	$\bar{X}$ litter size (± SEM), no.
Normal ♀ × carrier* ♂	21 (9)†	45	149	3.31 (± 1.2)
Carrier* ♀ × normal ♂	12	24	84	3.5 (± 1.2)
Total carrier × normal pairs	**33**	**69**	**233**	3.38 (± 1.2)
Normal ♀ × noncarrier‡ ♂	20 (9)†	27	246	9.11 (± 2.2)
Noncarrier‡ ♀ × normal ♂	18	29	276	9.51 (± 1.76)
Total noncarrier × normal pairs	**38**	**56**	**522**	9.32 (± 2.0)

*Transgenic mice heterozygous for the 50-copy pRSV-CAT insertion site.
†Number of males (in parentheses) mated with multiple females.
‡Includes both segregating nontransgenic and transgenic mice with only the 100-copy pRSV-CAT insertion site (see text).

proximal region of chromosome 6 and in band 17D or 17E1 in the distal portion of chromosome 17.

Localization of pRSV-CAT Insertion. In over 52 animals from the line 5 lineage analyzed in detail, the 50-copy pRSV-CAT integration site always cosegregated with the translocation, suggesting that the two were closely linked. To ascertain if the pRSV-CAT sequences were present in one of the translocated chromosomes, *in situ* hybridization was done. Chromosome preparations from the testes of carrier males and the bone marrow of carrier females were denatured and hybridized to ^{3}H-labeled RNA specific for the CAT sequence. Silver grains were found on the quadrivalent in meiotic chromosome preparations (Fig. 2*C*) and on the small translocated chromosome (6^{17}; Fig. 2*B*) in mitotic preparations (Fig. 2*D*) indicating unequivocally that the pRSV-CAT sequence had integrated into one of the chromosomes involved in the translocation. An analysis of 33 metaphase preparations revealed that 44% (41/94) of the grains on 6^{17} were localized over the central region of the chromosome (bands 6A2–A3;17D–E1) in the vicinity of the cytological breakpoint. The remaining silver grains were randomly distributed on the chromosome. In addition, the 100-copy RSV-CAT integration site found not to segregate with the phenotype of semisterility was localized to an autosome not involved in the rearrangement (data not shown).

DISCUSSION

Previous work has shown that mutations can be generated in transgenic mice by insertional inactivation of cellular genes at the site of integration (9–14). Here we describe another type of mutation in transgenic mice that results from gross chromosomal rearrangement. Genetic and cytogenetic analyses demonstrate that line 5, a transgenic mouse line bearing pRSV-CAT sequences, carries a chromosomal translocation between chromosomes 6 and 17. We believe that this rearrangement was elicited by the introduction of the foreign DNA sequences into the mouse embryo.

First, at the level of resolution attainable with the *in situ* hybridization technique, the pRSV-CAT sequences are localized to the cytological breakpoint on translocation marker chromosome 6^{17}. Second, both the integration of the foreign DNA and the chromosomal rearrangement occurred very early in the development of the founder animal of line 5 (probably at the one- or two-cell stage of embryogenesis), strongly suggesting that these were temporally closely linked, if not coincident, events. The translocation was detected cytologically in virtually 100% of the scorable chromosome preparations obtained from both the bone marrow and testis of this mouse, and the transmission of the cosegregating pRSV-CAT sequences revealed no evidence for mosaicism. Taken together with the extremely low rate of spontaneous translocation in laboratory mice (<0.01%) (28, 29), random rearrangement appears unlikely.

Despite close temporal and spatial association of the integration of exogenous DNA and the chromosomal rearrangement, the exact mechanism whereby these events took place is obscure. Whether the integration event initiated the rearrangement or was itself a consequence of the rearrangement cannot be determined unequivocally. Historically, translocations have been induced by a number of mutagenic substances that elicit DNA breakage (30, 31). Chromosomal breakage concomitant with the integration of foreign DNA could serve a similar role. The rearrangement may have been mediated by unequal homologous recombination between pRSV-CAT sequences integrated into nonhomologous chromosomes 6 and 17, leaving pRSV-CAT sequences in only one. Replication or recombination events leading to the generation of tandem repeats of the integrated sequence could potentially facilitate rearrangement. Finally, the possibility that chromosomal breakage originated from mechanical damage sustained during the microinjection procedure cannot be excluded. Cloning and molecular analysis of the mouse sequences surrounding the integrated pRSV-CAT DNA may more precisely denote the nature of the insertion site and the translocation breakpoints and ultimately indicate the mechanism involved.

The role of pRSV-CAT DNA in the generation of the translocation was independent of its sequence. Several transgenic lines were obtained containing the pRSV-CAT construct that showed no signs of gross chromosomal anomalies. Furthermore, at least two other transgenic mice bearing chromosomal translocations have been identified that contain different DNA constructs (our unpublished results; J. W. Gordon, unpublished results cited in ref. 32), although the presence of the foreign DNA at the site of chromosomal breakage in these cases has not yet been demonstrated.

Molecular analysis of a number of integration sites isolated from transgenic mice generated by DNA microinjection has shown that integration is often accompanied by

Table 2. *In utero* mortality in line 5 crosses

Cross	Pregnant ♀, no.	Implantations, no.	Implantations per ♀, no.	Arrested embryos, no.	Arrested, %
Normal ♀ × noncarrier* ♂ (8)†	13	134	10.3 (± 1.5)	4	2.9%
Normal ♀ × carrier‡ ♂ (10)†	15	163	10.8 (± 2.2)	103	63.2%

The litters of pregnant females were examined *in utero* between 7.5 to 12.5 days of gestation.
*Includes both segregating nontransgenic and transgenic males with only the 100-copy pRSV-CAT insertion site.
†Number of males used in crosses.
‡Transgenic mice heterozygous for the 50-copy pRSV-CAT insertion site.

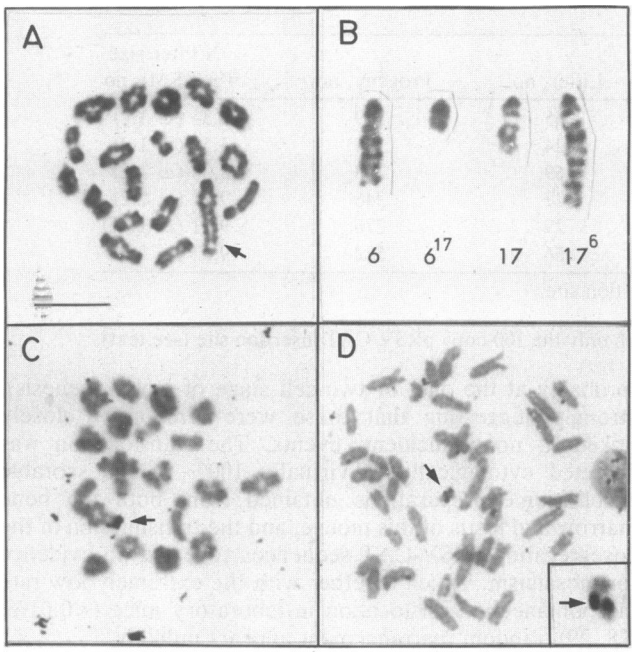

FIG. 2. Chromosome analysis of line 5 mice. Mice were progeny of the original founder mouse and normal FVB/N females and contained only the 50-copy RSV-CAT integration site. (*A*) Meiotic (diakinesis) chromosome preparation from the testis of a carrier male. A chain quadrivalent is indicated by the arrow. Bar = 10 μm. (*B*) Karyotype of Giemsa-banded metaphase chromosomes involved in the translocation—chromosomes 6 and 17 and translocation markers chromosomes 6^{17} and 17^{6}. (*C*) *In situ* hybridization of a ^{3}H-labeled SP6-CAT probe to the DNA in spermatocyte chromosomes from a carrier male. Silver grains over the chain quadrivalent are indicated by the arrow. (*D*) *In situ* localization of RSV-CAT sequence on mitotic chromosomes from a carrier female. An arrow marks the silver grains on the small translocation marker chromosome 6^{17}. (*Inset*) A higher magnification of a representative chromosome 6^{17}. The position of silver grains near the cytological breakpoint is indicated by the arrow.

rearrangement of cellular DNA, ranging from relatively simple deletions and duplications (13, 14) to much more extensive rearrangements (33, 34). Chromosomal translocations may be considered an extreme manifestation of such rearrangement.

The exact cause of embryonic arrest in chromosomally unbalanced embryos is difficult to determine, particularly if the aim is to attribute embryonic arrest to a defect in a specific gene (or genes), because relatively large regions of the chromosomes are duplicated or deficient. The locus or loci responsible for the lethality may be centimorgans away from the actual site of chromosomal breakage. We have identified an animal from line 5 homozygous for the translocation, and chromosomally balanced heterozygotes are phenotypically normal. Thus, the breakpoints of the translocation presented here apparently do not lie within or near an essential gene in such a way as to disturb function.

Our results strongly suggest that the introduction of foreign DNA into the germ line can be mutagenic, not only by the functional disruption of cellular genes at the site of integration, but by eliciting DNA breakage and chromosomal rearrangement. Insertional mutagenesis requires a rather precise insertion into essential gene sequences that comprise a very small percentage of the mammalian genome. Mutations due to chromosomal rearrangement may be recovered more frequently because the exact position of chromosomal breakage and integration is not as important as the chromosomal regions participating in the rearrangement. As more new lines of transgenic mice are generated, the frequency and diversity of insertion-mediated mutations may become apparent.

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