Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice

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A general strategy for selecting insertion mutations in mice has been devised. Constructs lacking a promoter and including a β-galactosidase gene, or a reporter gene encoding a protein with both β-galactosidase and neomycin phosphotransferase activity, were designed so that activation of the reporter gene depends on its insertion within an active transcription unit. Such insertion events create a mutation in the tagged gene and allow its expression to be followed by β-galactosidase activity. Introduction of promoter trap constructs into embryonic stem (ES) cells by electroporation or retroviral infection has led to the derivation of transgenic lines that show a variety of β-galactosidase expression patterns. Intercrossing of heterozygotes from 24 strains that express β-galactosidase identified 9 strains in which homozygosity leads to an embryonic lethality. Because no overt phenotype was detected in the remaining strains, these results suggest that a substantial proportion of mammalian genes identified by this approach are not essential for development.

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Mutations in mice have been generated and collected for many years. To date, >2000 genetic loci have been described (Green 1989), many of which have been identified by linkage to mutations affecting pigmentation, behavior, or morphogenesis. Most of these mutations have arisen either spontaneously or were induced by radiation or chemical mutagenesis. With improved genetic mapping techniques, an increasing number of previously identified mutations have been assigned to cloned genes, allowing an understanding of their normal physiological role (for review, see Wagner 1990). However, this approach is limited to existing mutations, and the identification of the mutated gene can be difficult.

Mutated genes can be identified more readily by insertion mutagenesis in transgenic mice, because the transgene serves both as a mutagen and as a tag to clone the mutated gene (Gridley et al. 1987; Jaenisch 1988). Mutated genes have been identified in transgenic lines generated by retroviral infection (Schnieke et al. 1983; Soriano et al. 1987; Spence et al. 1989; Weiher et al. 1990) or by microinjection of DNA into zygotes (Woychik et al. 1990). However, there are several difficulties associated with this screen: (1) Subtle phenotypes may be missed; (2) DNA rearrangements can occur at the mutated locus using DNA microinjection, complicating the identification of the mutated gene (Gridley et al. 1987); and (3) only 5% of transgenic strains generated by retroviral infection of embryos exhibit an overt phenotype (Spence et al. 1989; Weiher et al. 1990; P. Lawinger and P. Soriano, unpubl.). Because these strains must be bred to homozygosity before mutant phenotypes are identified, this approach is laborious and time consuming. The work presented here was designed to alleviate some of these problems.

To avoid extensive breeding of transgenic lines, a method that would allow screening and selection for mutations in vitro would be useful. Through the use of embryonic stem (ES) cells, transgenic mice that carry preselected mutations can be made. So far, this approach has been used mainly for targeting of specific genes by homologous recombination in ES cells (for review, see Wagner 1990). To identify and mutate new genes, however, other approaches must be used. One approach makes use of the ability of genomic sequences flanking the transgene to influence the pattern of expression of a weak promoter in a temporal- and tissue-specific pattern (Jaenisch et al. 1981; Allen et al. 1988; Kothary et al. 1988; Gossler et al. 1989). These "enhancer traps" have been used extensively in *Drosophila* to identify new genes (O'Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989). In the mouse, however, coincidence of the pattern of expression of the transgene with an endogenous gene has yet to be demonstrated. In addition, this approach does not necessarily mutate the tagged gene. An alternative approach is to use a "promoter trap", in which expression of a reporter gene can initiate only from an endogenous promoter because the reporter gene

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lacks its own promoter (Gossler et al. 1989; Peckham et al. 1989). This approach would combine the advantage of enhancer detection, because the activity of the tagged gene can be assessed in a heterozygous animal by the expression of the reporter gene, and the advantage of functional analysis in homozygous animals, as the insertion should be mutagenic. However, the phenotypic analysis of mice mutated by this approach has been lacking.

We describe the generation of an efficient promoter trap in ES cells using a novel reporter gene. This reporter gene encodes a protein, β-geo, with both β-galactosidase (β-gal) and neomycin phosphotransferase (neo) activities. DNA constructs including this gene were introduced into ES cells by electroporation or retroviral infection, and promoter trap events were selected by acquired resistance to the antibiotic G418. When these cells are used to create chimeric animals and their subsequent transgenic offspring, spatial and temporal expression patterns of the "trapped" genomic promoter can be visualized by staining with the chromogenic substrate X-gal, which produces a blue precipitate upon cleavage by β-gal. Many promoter trap transgenic lines have been derived that display various expression patterns in embryos at different developmental stages. Seven strains did not show β-gal expression. Among 24 strains that exhibit β-gal expression, 9 display recessive lethal phenotypes, whereas no overt defect is observed in the remaining 15 strains.

Results

Construction of promoter traps with the β-galactosidase gene

Promoter traps are based on the integration of a reporter gene lacking a promoter into the genome and its expression from a tagged endogenous promoter. When the reporter gene integrates within an exon in the proper reading frame and orientation, or within a 5′-noncoding exon, a protein that retains reporter gene activity may be formed. To allow for the expression of the reporter gene when it was inserted into an intron, a splice acceptor sequence was included in the constructs. Such promoter traps were designed as either cassettes that could be electroporated into cells or as retroviral vectors.

To determine the frequency of promoter trap events, ES cells were electroporated with pSAβ-galPGKneobpA (SAβ-gal), a construct including a splice acceptor (SA) sequence, a β-gal gene, and a neo gene-expression cassette (PGKneobpA; Soriano et al. 1991a). No enhancer activity has been documented in the phosphoglycerate kinase-1 promoter used to drive neo expression. The β-gal gene includes an ATG codon that can serve either as an initiator (Kozak 1989) or as an internal methionine, as the splice acceptor does not contain stop codons in the same frame as β-gal. ES cells were selected with G418 for 10 days and stained with X-gal to detect activity of β-gal. As a control, ES cells were electroporated with a construct lacking the splice acceptor. Of the total $G418^r$ colonies, 4.5% stained blue when the splice acceptor was included, in contrast to 0.1% when the splice acceptor was omitted (Fig. 1A,B). This result indicates that the majority of insertions leading to expression of the reporter gene have occurred in introns. To determine the number of DNA copies per cell, DNA from colonies that reacted with X-gal was analyzed by Southern blot analysis (Fig. 2). A single insert per cell was observed in five of nine clones. For the remaining lines, concatemeric or complex integration patterns were observed. Breeding analysis suggests that these complex insertions occur at a single chromosomal site (data not shown).

Because complex insertions may be difficult to clone and analyze, the SAβ-gal cassette was inserted in the self-inactivating retroviral vector $pGen^-$, which lacks the viral enhancer sequences and contains a bacterial *supF* gene in the long terminal repeats (LTRs) to facilitate cloning of the provirus and flanking sequences (Soriano et al. 1991b). SAβ-gal was inserted in reverse orientation relative to viral transcription. This was necessary to avoid removing the viral Ψ packaging sequence by splicing from a viral upstream splice acceptor sequence. The resulting retroviral vector is termed ROSAβ-gal (reverse orientation splice acceptor β-gal). To verify that cryptic promoter elements are not present in the viral sequences, ES cells were electroporated with ROSAβ-gal plasmid DNA. A similar percentage of clones with β-gal activity was found with this construct and with SAβ-gal (Fig. 1C).

Virus-producing cells were made with the ROSAβ-gal plasmid, and virus titers were assayed on 3T3 cells. The titer from a population of producer clones was 2×10^5 $G418^r$ cfu/ml. Southern blot analysis of infected cells showed that the provirus had the expected structure, and infections at low multiplicity resulted in single integrants (Fig. 2). Following infection of ES cells, 11% of the G418 clones stain with X-gal (Fig. 1E). This increase in the percentage of blue clones relative to the percentage obtained by electroporation may reflect specificity of proviral integration sites.

Construction of a reporter gene including both β-gal and neo activities

To allow for direct selection of promoter trap events, a novel reporter gene was constructed by placing a neo gene in-frame at the 3′ end of a β-gal gene. Fusion proteins have been made previously with β-gal (Rüther and Müller-Hill 1983) or neo (Reiss et al. 1984; Schwartzberg et al. 1989). The resulting reporter gene, β-geo, gave rise to kanamycin-resistant *Escherichia coli* transformants that demonstrated β-gal activity as well. To test for function in mammalian cells, a phosphoglycerate-kinase-1 promoter β-geo expression cassette, pPGKβ-geobpA, was electroporated into ES cells in parallel with pPGK-neobpA, and both sets of ES cells were selected with G418. Similar numbers of G418-resistant colonies were observed (data not shown). All G418-resistant colonies obtained by transformation with pPGKβ-geobpA stained

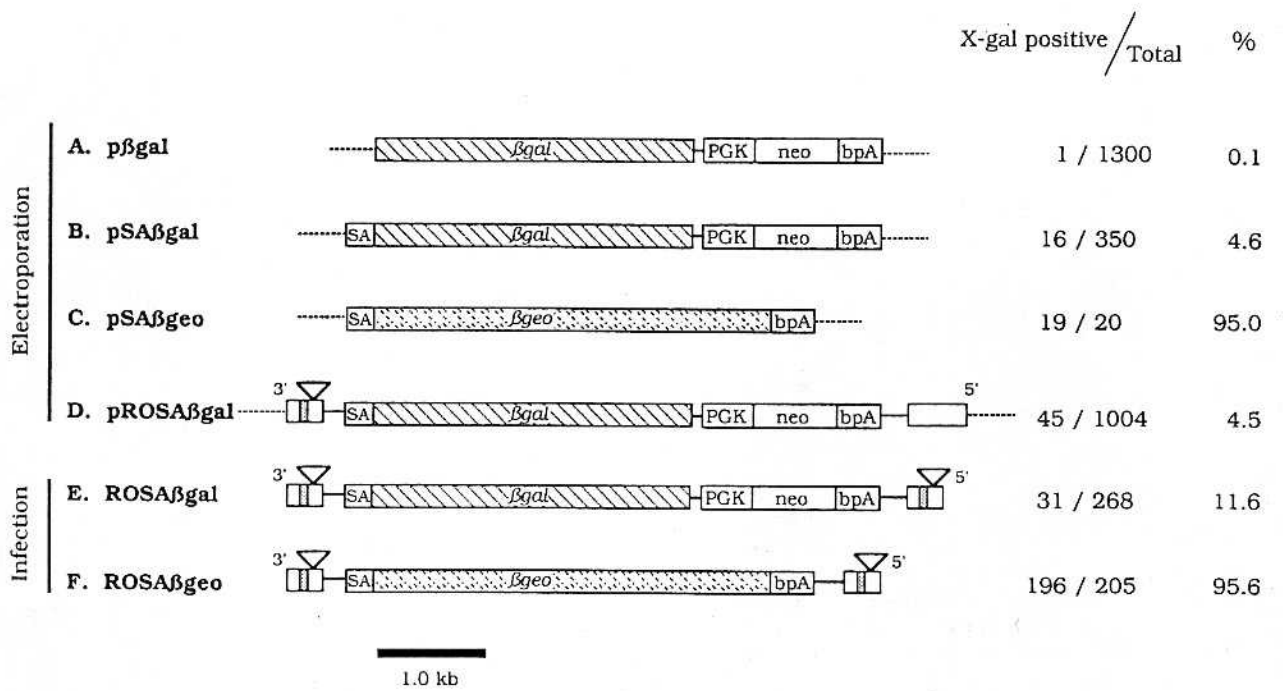

Figure 1. Promoter trap efficiencies. Plasmid constructs (*A–D*) were linearized by digestion with *DraI*, which cuts within the plasmid backbone. Linear DNA was introduced into AB1 ES cells by electroporation and grown for 10 days in media containing G418. The retroviruses shown in proviral form (*E,F*) were used to infect AB1 ES cells at a multiplicity of 0.1. Infected cells were grown in media containing G418 for 10 days. G418^r colonies resulting from the electroporations and infection were stained with X-gal after fixation. The ratio of blue-staining colonies to total G418-resistent colonies is given and is expressed as a percentage. Details of plasmid construction and virus culture are given in Materials and methods. Retroviral vectors and proviruses (*D–F*) are depicted with the 5′ LTR to the right and the 3′ LTR to the left so that the transcriptional direction of the β-gal, neo, and β-geo genes are shown in the conventional 5′ → 3′ left-to-right orientation. (▽) The deletion of viral enhancers. (SA) Splice acceptor; (PGK) phosphoglycerate kinase-1 gene promoter; (bpA) bovine growth hormone gene polyadenylation signal; (broken lines) plasmid sequences.

with X-gal. However, some clones gave lighter patterns of staining than others, and not all cells in a clone stained blue. This observation is in agreement with previous work showing heterogeneity of staining in β-gal colonies (MacGregor et al. 1987). Protein extracts were made from 3T3 cells transformed with pPGKβ-geobpA and analyzed by Western blot with antibodies specific for neo or β-gal. A protein with the expected size of 146 kD was detected by both antisera (data not shown).

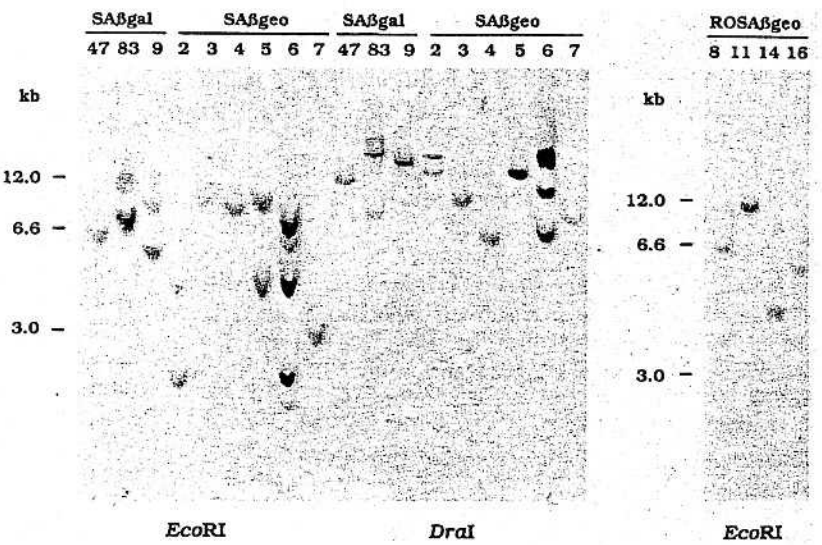

Figure 2. Integration of promoter trap constructs and viruses into the mouse genome. ES cells were electroporated with pSAβ-gal and pSAβ-geo or infected with ROSAβ-geo, and promoter trap events were selected by β-gal expression or G418 resistance. DNA was prepared from individual clones and subjected to Southern blot analysis following digestion with *EcoRI* or *DraI* with a neo probe. *EcoRI* cuts at the end of the β-gal gene and upstream of the neo hybridizing sequences within the β-geo gene; *DraI* does not cut the constructs. Therefore, a simple insertion event should result in a single hybridizing band.

β-Geo promoter trap constructs were made by placing the reporter gene downstream of the splice acceptor sequence (Fig. 1C). The resulting construct, pSAβ-geobpA (SAβ-geo), was used directly as a promoter trap. Alternatively, the construct was inserted in reverse orientation in the retroviral vector pGen$^-$, to yield pGen$^-$ ROSAβ-geobpA. This plasmid was used to make virus-producing cells, and virus titers were assayed on 3T3 cells with G418 selection or β-gal expression. The titer of the resulting virus (ROSAβ-geo; Fig. 1F) from a population of producer clones was 5×10^3 G418^r or β-gal cfu/ml. The lower titer observed with this virus compared to ROSAβ-gal is expected, as β-geo expression depends on proviral insertion downstream of a flanking genomic promoter.

ES cells were electroporated with SAβ-geo or infected with ROSAβ-geo. Almost all of the resulting G418^r colonies stained with X-gal. Generally, these colonies stained more weakly than colonies obtained by electroporation with pPGKβ-geobpA, and ~5% of the G418^r colonies did not stain with X-gal at all.

Expression of the reporter gene in promoter traps originates from upstream promoters

To verify that expression of the reporter gene originates from an upstream promoter, Northern blot analysis was performed on RNA isolated from G418^r ES colonies electroporated with SAβ-geo or infected with ROSAβ-geo. In each of six clonal cell lines electroporated with SAβ-geo, transcripts larger than β-geo (3.9 kb) were observed by using a neo probe, indicating that transcription was initiated from upstream sequences. However, multiple transcripts were detected in five of six cases, as shown in Figure 3 for two cell lines (lanes A,B). This may indicate readthrough transcription patterns due to concatemeric insertions, as Southern blot analysis of these lines revealed complex insertion events in these five cases and a simple insertion event for the remaining sixth line. In contrast, 9 of 12 ROSAβ-geo lines showed transcripts of ~4.4 kb, only slightly larger than the size of the 3.9-kb β-geo structural gene, and only two lines (Fig. 3, lanes 10,12) showed much higher molecular weight transcripts. In two lines, SAβ-geo4 and ROSAβ-geoB, transcripts were only detected using polyadenylated RNA (data not shown). Both of these lines were resistant to G418 but did not stain with X-gal.

To verify that the splice acceptor was used properly in promoter traps, a riboprotection assay was performed with antisense RNA from the 3′ LTR up to the beginning of the β-gal gene. In all cases examined, transcripts were protected up to the splice acceptor, indicating proper use of this element (data not shown). Specific protection within the LTR was not observed, providing further support for the absence of a cryptic promoter.

Germ-line transmission of promoter trap events and staining patterns

Chimeras were generated from ES cells electroporated with SAβ-gal (SAβ-gal9, 47, and 83), SAβ-geo (SAβ-geo2–4), or infected with ROSAβ-geo (ROSAβ-geo1–29). To generate ROSAβ-geo strains, ES cells were infected at a multiplicity of <1, selected with G418, and pooled prior to injection into blastocysts. Because ~15 cells carrying different proviral insertions are injected per blastocyst, more than one transgenic line can be derived from a single chimera. The chimeras were allowed to develop to term and tested for ES cell contribution to the germ line. The ES cell lines used in this work were either AB1 or AB2.1, which have been shown to contribute extensively to the germ line of chimeric animals (McMahon and Bradley 1990; Soriano et al. 1991a). Consistent with previous results, 38 of 45 chimeras tested showed contribution of the ES cells to the germ line, and ROSAβ-geo chimeras transmitted one to three independent proviral genomes to their offspring.

Transgenic offspring were identified by Southern blots of tail DNAs. β-Gal expression patterns were examined by mating transgenic males with wild-type females and staining embryos at different stages of development for

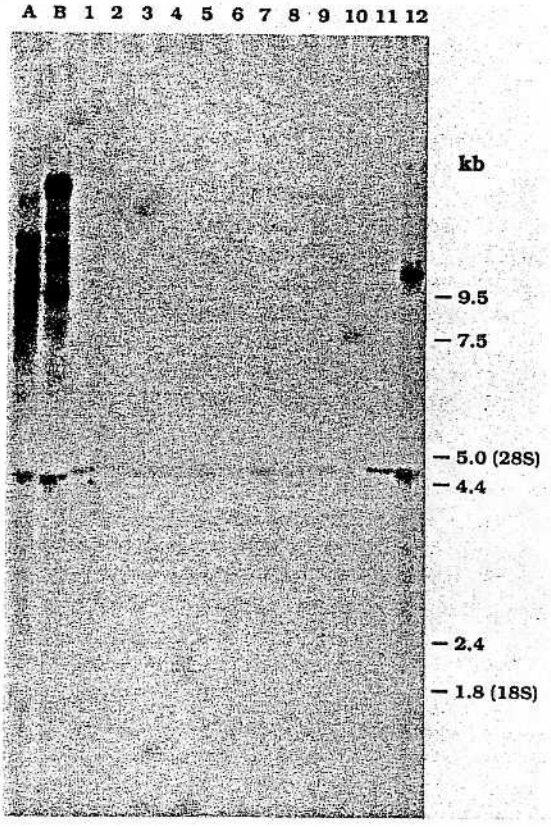

Figure 3. Northern blot analysis of β-geo gene expression in individual clones. Twenty micrograms of total RNA derived from 2 SAβ-geo clones (lanes *A,B*) and 12 ROSAβ-geo clones (lanes *1–12*) was denatured with glyoxal and analyzed by Northern blot with a neo probe. The integrity and amount of RNA in each lane were assessed by running an aliquot of each sample in parallel and staining with ethidium bromide. RNA molecular mass markers were purchased from Bethesda Research Laboratories.

β-gal activity (Fig. 4). Staining patterns differed both spatially and temporally between strains (Table 1). Embryos from all of the SAβ-gal and SAβ-geo strains showed staining at the developmental stages examined. However, 7 of 28 ROSAβ-geo strains did not show staining at any stages of development examined. It is possible that these strains are derived from ROSAβ-geo-infected satellite colonies that were not G418 resistant and were propagated following expansion of ES cell populations in the absence of the antibiotic. Alternatively, it is possible that β-gal expression levels in these strains were too low to allow detection. No phenotypes were observed upon further breeding of six of these strains, and these were not considered further.

Staining patterns differed among all of the strains. Nine strains showed restricted patterns of β-gal expression, in the neural tube alone (SAβ-gal83, ROSAβ-geo2); liver (SAβ-geo3); telencephalon (SAβ-geo4, "Spock"); CNS (ROSAβ-geo9, ROSAβ-geo15); heart (ROSAβ-geo24); in the limb buds, somites, and mandibular arches (ROSAβ-geo21); or in the yolk sac (ROSAβ-geo29). Six strains (SAβ-gal9 and ROSAβ-geo1, 3, 8, 18, and 20) showed widespread staining, with areas of more intense staining. Four strains (SAβ-gal47 and ROSAβ-geo10, 12, and 22) showed light, widespread staining with no area showing higher β-gal activity. Finally, eight strains ([ROSAβ-geo5 ("midnight"), ROSAβ-geo11 ("blue blood"), and ROSAβ-geo16, 23, 25, 26, 27, and 28) exhibited intense staining apparently in all tissues at all stages of development examined and in newborn pups, including red blood cells in ROSAβ-geo11. However, staining for short periods indicated that β-gal expression levels were not uniform throughout the embryo.

Phenotypes associated with promoter trap events

No observable phenotype was observed in heterozygous mice, with two exceptions. In the SAβ-geo2 strain, ES cells contributed to the germ line of the chimeras, as Agouti offspring were derived from matings to C57BL/6J females. However, Southern blot analysis with a neo probe indicated that of 60 Agouti offspring none carried the transgene. It is possible that the transgene inactivates a gene that is imprinted or essential in haploid sperm cells; alternatively, the transgene may be unstable in the germ line (Wilkie et al. 1991). In the ROSAβ-geo25 strain, heterozygous animals were consistently 25–40% smaller than their wild-type littermates. This phenotype was most noticeable in animals younger than 6 weeks.

To determine whether recessive phenotypes were associated with promoter trap mutations, heterozygous mice from 24 strains were intercrossed and the genotype of the offspring was determined by Southern blots of tail DNAs (Soriano et al. 1987). The results of these crosses are presented in Table 2. Homozygous offspring could not be isolated from nine promoter trap lines, indicating that the insertions led to a recessive lethal phenotype. Relatively few offspring derived from heterozygous ROSAβ-geo25 parents were analyzed, as these animals proved to be particularly poor breeders compared to other animals in the same genetic background (hybrid C57BL/6J × 129Sv). This behavior may be attributed to the small size of heterozygotes in this strain.

To determine the nature of the embryonic defect in the homozygotes, embryos derived from heterozygous parents were isolated at different stages of gestation and genotyped by Southern blot analysis (Table 3). In the SAβ-gal47 strain, normal-appearing homozygous embryos were found on embryonic day 9 (E9), but these embryos were generally smaller and in the process of being resorbed by E10–E11. At this stage, heterozygotes showed widespread β-gal staining. In the SAβ-geo4 strain, homozygous embryos could be detected on E9 and were characterized by a small yolk sac surrounding a mass of necrotic cells. No staining was observed in this strain at this stage of development. However, expression of the reporter gene might have been present but at a level below the threshold of X-gal sensitivity. This strain was derived from an ES clone that did not stain with X-gal. Homozygous ROSAβ-geo5 and ROSAβ-geo16 embryos could not be found at E9 among 30 and 26 embryos tested, respectively, indicating that proviral insertion causes lethality at an earlier stage. These strains exhibit ubiquitous staining at all stages of development examined. Phenotypic analysis of homozygous embryos in the other strains with a recessive lethal phenotype is in progress.

In the remaining 15 strains, homozygous animals appeared morphologically normal and did not display behavioral anomalies. Homozygotes in 10 strains tested so far have been able to reproduce. Because promoter traps lead to mutations in the tagged gene, the absence of overt phenotypes in these strains indicates that a substantial proportion of the trapped genes may not be essential for development.

Discussion

We have developed an efficient genetic screen to identify and mutate genes in the mouse. This screen, based on promoter traps, involves the introduction of a reporter gene preceded by a splice acceptor into ES cells. Insertion of the reporter gene into an intron in the correct orientation can lead to expression from the tagged cellular promoter and should also generate a mutation.

An ideal reporter gene for use in promoter traps in ES cells should be innocuous, allow selection for mutagenic insertions, and include a means to easily monitor the activity of the tagged promoter once the ES cell clone has been used to create chimeras and transgenic lines. Therefore, a new reporter gene, β-geo, was constructed. This gene encodes a protein product with both β-gal and neo activities. Electroporation of β-geo expression constructs with a strong promoter produces G418^r colonies, which all show β-gal activity. However, by using SAβ-geo or ROSAβ-geo, 5% of all G418^r colonies do not stain with X-gal. In two instances, chimeric or transgenic embryos resulting from G418^r ES colonies that did not stain with X-gal (ROSAβ-geoB and SAβ-gal4) showed X-gal staining after differentiation. In both instances, Northern blot

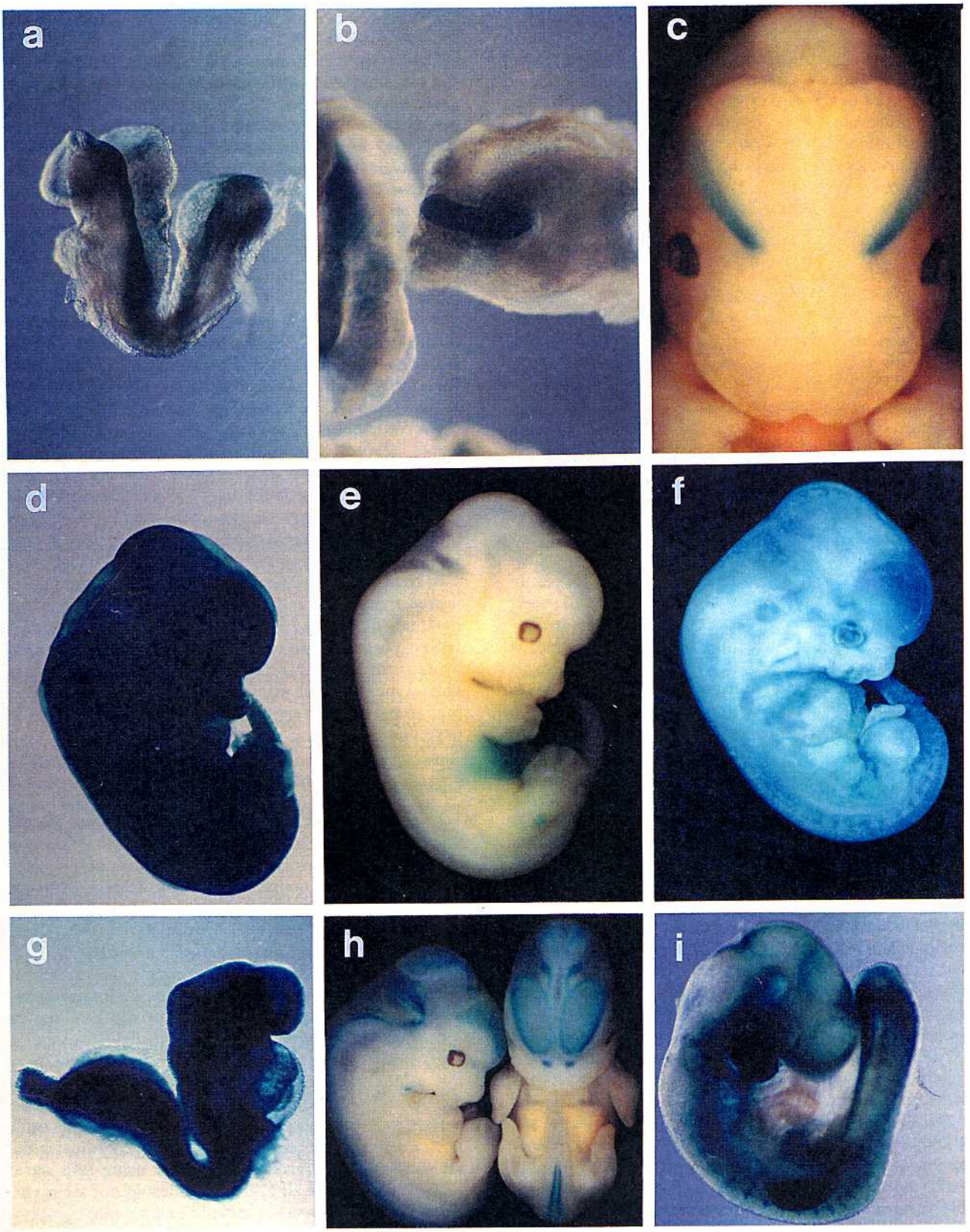

Figure 4. Spatial patterns of expression resulting from promoter trap events. Embryos from different strains and stages were stained with X-gal to reveal β-gal activity. (*A*,*B*) ROSAβ-geoB, E8 (staining was done on chimeric embryos; Gossler et al. 1989); (*C*) SAβ-geo4, E13; (*D*) ROSAβ-geo5, E12; (*E*) SAβ-geo3, E12; (*F*) SAβ-gal9, E12; (*G*) ROSAβ-geo5, E8; (*H*) ROSAβ-geo9, E12; (*I*) ROSAβ-geo21, E9.

Table 1. *Staining patterns of promoter trap lines*

Line	E7	E9	E12
SAβ-gal9	Ws (embryonic)	Ws (E8)	Ws, Tel, Nt
SAβ-gal47	Ws (E8)	Ws (E11)	Ws (E14)
SAβ-gal83[a]	Neg	Neg	Nt
SAβ-geo2[b]	ND	ND	ND
Saβ-geo3	Ws	ND	liver
SAβ-geo4	Neg	Neg	Tel
ROSAβ-geo1	ND	Ws	Ws, Mes, Nt, AER
ROSAβ-geo2	Neg	Neg	Nt
ROSAβ-geo3	Neg	Neg	Ws, Umb, Ht(E11–E12)
ROSAβ-geo5	Ubiq	Ubiq	Ubiq
ROSAβ-geo8	ND	ND	Ws, snout, ears (E14)
ROSAβ-geo9	Neg	Nt	CNS
ROSAβ-geo10	Neg	Ws	Ws
ROSAβ-geo11	Ubiq	Ubiq	Ubiq
ROSAβ-geo12	Neg	Neg	Ws
ROSAβ-geo15	Neg (E8)	Neg	Nt, brain (E10–E12)
ROSAβ-geo16	Ectoderm, EPC	Ubiq	Ubiq
ROSAβ-geo18	Neg	Neg	Ws
ROSAβ-geo20	Ectoderm	Ws	Ws
ROSAβ-geo21	Ectoderm	LB, MA, Som	Ws
ROSAβ-geo22	Neg	Neg	Ws (E13)
ROSAβ-geo23	Ubiq	Ubiq	Ubiq
ROSAβ-geo24	Ectoderm	Neg	Ht
ROSAβ-geo25	Ubiq	Ubiq	Ubiq
ROSAβ-geo26	Ubiq	Ubiq	Ubiq
ROSAβ-geo27	Ubiq	Ubiq	Ubiq
ROSAβ-geo28	ND	Ubiq	Ubiq
ROSAβ-geo29	ExEmEct	YS	ND[c]

Abbreviations: (AER) Apico-ectodermal ridge of limb buds; (EPC) ectoplacental cone; (ExEmEct) extra-embryonic ectoderm; (Ht) heart; (LB) limb buds; (MA) mandibular arches; (Mes) mesencephalon; (ND) not determined; (Neg) no staining; (Nt) neural tube; (Som) somites; (Tel) telencephalon; (Ubiq) ubiquitous; (Umb) umbilical cord; (Ws) widespread; (YS) yolk sac.

Heterozygous transgenic males were mated to wild-type females, and embryos were isolated at different stages of development for staining with X-gal.

[a]ES cells used to generate the SAβ-gal83 line were selected on the basis of X-gal staining following differentiation into embryoid bodies.

[b]SAβ-geo2 chimeras exhibited ES cell contribution to the germ line as evidenced by Agouti offspring derived from crosses to C57BL/6J females; however, none of the offspring carried the transgene.

[c]Staining in the yolk sac could not be determined as a result of background β-gal activity.

analysis indicated that the tagged gene was weakly transcribed in ES cells. G418 selection therefore appears to be more sensitive than X-gal staining, and β-geo may allow for the selection of genes that are expressed strongly in ES cells, as well as genes that are expressed more weakly. β-Geo also may be useful for the isolation of β-gal-expressing cells in instances in which the use of a fluorescence-activated cell sorter (Nolan et al. 1988) is not desirable, or to select for mutations in genes expressed in ES cells by gene targeting and to follow their activity as well (P. Soriano, unpubl.). This would eliminate the need for an additional neo expression cassette to select for the mutations (Le Mouellic et al. 1990; Mansour et al. 1990). In addition, the promoter trap system described in this work may have applications in cell types other than ES cells and be used to trap genes encoding proteins within a biochemical pathway.

It is anticipated that, in general, patterns of expression of β-gal in promoter trap lines should follow that of the tagged gene, except when the insertions have occurred in the proximity of intragenic regulatory regions. This will need to be verified by in situ hybridization with cDNAs once the tagged genes are cloned. Because the juxtaposition of promoters has been shown to create novel tissue specificities in transgenic mice produced either by microinjection or by retroviral infection (Townes et al. 1985; Soriano et al. 1986), it is possible that promoter traps, particularly those based on β-geo, will lead to more faithful expression of the reporter gene than enhancer traps that are based on promoter interactions. Although the retroviral promoter, but not the enhancer, is still present in the retroviral promoter trap lines, it may not be of concern, as it is not active in ES cells and the early embryo; and this block in activity is maintained throughout later development (Jähner et al. 1982). However, as a result of the promoter trap insertion and the inactivation of the cellular gene, de novo methylation may occur and extend into flanking sequences, as has been shown for several retroviral transgenic strains (Jähner and Jaenisch 1985). This may lead to the silencing of the tagged promoter in the animal.

Different patterns of X-gal staining were observed

Table 2. *Genotype of offspring derived from heterozygous parents*

Strain	Wild type	Heterozygous	Homozygous	Total	Fertile[a]
SAβ-gal9	7	19	12	38	+
SAβ-gal47	29	68	0	97	NA
SAβ-gal83	8	14	11	33	+
SAβ-geo3	26	70	21	117	+
SAβ-geo4	38	61	0	99	NA
ROSAβ-geo1	9	20	4	33	ND
ROSAβ-geo2	8	13	1	22	ND
ROSAβ-geo3	15	16	4	35	+
ROSAβ-geo5	39	56	0	95	NA
ROSAβ-geo8	15	36	9	60	+
ROSAβ-geo9	6	10	6	22	+
ROSAβ-geo10	14	39	7	60	+
ROSAβ-geo11	12	21	5	38	+
ROSAβ-geo12	19	27	0	46	NA
ROSAβ-geo15	14	32	9	55	+
ROSAβ-geo16	36	67	0	103	NA
ROSAβ-geo18	13	26	10	49	ND
ROSAβ-geo20	13	38	17	68	+
ROSAβ-geo21	6	11	6	23	ND
ROSAβ-geo22	15	19	10	44	ND
ROSAβ-geo23	11	23	0	34	NA
ROSAβ-geo24	18	35	0	53	NA
ROSAβ-geo25	13	18	0	31	NA
ROSAβ-geo28	17	36	0	53	NA

Offspring derived from double heterozygous matings were analyzed by Southern blot analysis of tail DNA as described in Materials and methods.

[a](+) Homozygous animals that are capable of reproduction; (ND) not determined; (NA) not applicable.

Table 3. *Genotypes of embryos derived from heterozygous parents*

Strain	Gestation	Wild type	Heterozygous	Homozygous		Resorbed embryos
				normal	abnormal	
SAβ-gal47	E9	3	9	2	0	0
	E10–E11	9	13	4	7	0
	E11–E14	0	8	0	0	3
SAβ-geo4	E9	8	20	0	6	0
	E10–E15	5	8	0	0	6

Embryos were isolated at different stages, and genotypes were determined by Southern blot analysis using a neo probe. No DNA could be recovered from the resorbed embryos.

among the transgenic lines produced by promoter trap events. We have observed staining at many stages of development, in tissue-specific or widespread patterns. Restricted staining patterns may prove to be very useful to trace cell lineages in the embryo, as has been done in *Drosophila* (Ghysen and O'Kane 1989). In eight strains, proviral insertion at a cellular locus leads to ubiquitous staining, although staining was not necessarily uniform. Such strains, in particular ROSAβ-geo11, which does not lead to a lethal phenotype, may be very useful for the construction of chimeras and the analysis of prospective potency (Beddington et al. 1989) or serve as marked donor cells for tissue transplant experiments. Previous attempts to generate a transgenic mouse strain exhibiting ubiquitous X-gal staining by expressing β-gal under the control of a constitutive promoter have resulted in variable expression of the transgene (Beddington et al. 1989). This result may be explained if constitutive promoters are very susceptible to position effects when taken out of their normal location in the genome.

The reporter gene used in these experiments has an ATG codon that can serve to initiate translation or allow translation of a fusion protein. Promoter trap constructs in which the ATG was omitted were significantly less efficient (Brenner et al. 1989; Gossler et al. 1989; J. Cannon, G. Friedrich, and P. Soriano, unpubl.). Inclusion of an initiator ATG may be particularly critical in the experiments with retroviral promoter traps because retroviruses preferentially integrate close to hypersensitive sites, which are often found at the 5′ ends of genes (Vijaya et al. 1986; Rohdewold et al. 1987). Possibly, as a result of this specificity, a higher percentage of stained clones was observed after infection with ROSAβ-gal than after electroporation with the plasmid construct, and most of the messages were close to the minimal expected size. This observation is consistent with insertions at the 5′ ends of genes, as 5′ exons tend to be shorter than internal exons and are often <100 bases (Hawkins 1988). Splicing from such small exons would not noticeably increase the minimum size of transcripts when analyzed by Northern blot, as has been observed in previous work with retroviral promoter traps in EC cells (Peckham et al. 1989).

It is probable that in many cases, promoter trap events will create null alleles, because expression of the normal message from the tagged gene could only arise if there is alternate splicing bypassing the promoter trap. To date, alternate splicing has been observed to involve nonconsensus splice acceptor sequences that either lack a polypyrimidine stretch or do not have a normal branchpoint region. Alternate splicing in transgenic mice has only been shown with splice sites normally involved in alternate splicing, as in the case of calcitonin (Crenshaw et al. 1987). The reporter gene in promoter trap lines should not be spliced out of the final mRNA because the adenovirus splice acceptor used in our experiments is a consensus splice acceptor and is not normally involved in alternate splicing. In addition, the majority of retroviral promoter trap events appear to occur at the 5′ ends of genes, making it unlikely for a fusion protein to retain much of its original activity. However, a detailed analysis of several promoter trap transgenic lines will be required to verify that these lines generate null alleles. Retroviral promoter traps used previously either have not used a splice acceptor sequence (Von Melchner et al. 1990) or have been based on the use of a nonconsensus, alternative viral splice acceptor (Brenner et al. 1989; Peckham et al. 1989). Both types of vectors have led to a low frequency of promoter trap events and, in the latter case, may not lead to null mutations, as such viral splices can be bypassed (Nusse 1986).

Twenty-four promoter trap strains have been bred to homozygosity. In nine strains, the insertions lead to embryonic lethality. In two of these strains, SAβ-gal47 and SAβ-geo4, homozygous embryos die at mid-gestation, between E9 and E10. In the ROSAβ-geo5 and ROSAβ-geo16 strains, homozygous embryos die prior to E9. The time of death of these embryos is consistent with the expression of β-gal or β-geo in heterozygotes: The SAβ-gal47 strain exhibits widespread β-gal activity at E10, the ROSAβ-geo5 and ROSAβ-geo16 strains exhibit ubiquitous staining at all stages of development examined, and the SAβ-geo4 strain, while not showing β-gal staining at E9, is derived from a $G418^r$ ES β-geo clone that did not stain with X-gal, indicating that β-geo expression can occur in the absence of β-gal staining. Further molecular analysis of these strains will be required to understand the cause of the defect. The identification of genes tagged by promoter trap approaches should be facilitated by the use of anchored PCR procedures (Frohman et al. 1988) or by selective cloning of the provirus and flanking sequences with *supF* selection (Soriano et al. 1987, 1991b).

The identification of 9 of 24 strains that exhibit an overt phenotype associated with the mutation demonstrates the efficiency of promoter traps as a genetic screen. Previously, only 5 of 102 retroviral transgenic strains resulting from infection of embryos harbor an overt recessive mutation owing to proviral insertion (Spence et al. 1989; Weiher et al. 1990; P. Lawinger and P. Soriano, unpubl.). In addition, among those strains that do not display a phenotype, it is not possible to distinguish those that have not mutated a gene from those that have inactivated a nonessential gene. However, the promoter trap screen described in this work has at least two limitations: (1) The screen is based on genes expressed in ES cells, although it could be used without this selection by examining β-gal expression after differentiation of ES cells into embryoid bodies, as was done in the case of SAβ-gal83; and (2) not all loci may be equally accessible to retroviral infection, although the specificity of proviral integration observed with avian retroviruses (Shih et al. 1988) has not been reported with murine retroviruses.

The observation that 15 of 24 strains do not exhibit an overt phenotype when bred to homozygosity indicates that a substantial proportion of genes accessible by the promoter trap screen are not essential for development. Recently, several genes mutated by retroviral insertion or homologous recombination have not exhibited an overt phenotype (Kuehn et al. 1987; Ziljstra et al. 1990; Fung-Leung et al. 1991; Joyner et al. 1991) or have exhibited a phenotype less severe than anticipated (Soriano et al. 1991a). In *Drosophila*, studies based on chromosomal walks, saturation mutagenesis, and enhancer detector screens suggest that as much as two-thirds of all genes may not be essential for development (for a discussion, see Wilson et al. 1989). Targeted disruptions of genes in yeast have revealed that 70% of the disruptions do not lead to overt phenotypes (Goebl and Petes 1986). An increasing number of genes have been shown to be members of multigene families. Although many of these genes may play important roles in development, mutations in any single member may not result in an overt phenotype or may result in a relatively subtle phenotype, if it can be substituted by another member of the gene family. Therefore, many of the genes that are inaccessible by classic genetics may be identified with promoter traps. Promoter traps combine the ability to select for insertions within genes with the ability to follow the activity of the tagged gene by β-gal expression. Because the insertion should be mutagenic, breeding to homozygosity can be used for functional analysis of the gene. These unique features of the promoter trap screen promise to aid in the understanding of gene activity during early mouse development.

Materials and methods

Construction of plasmids and retroviral vectors

DNA fragments used in the promoter trap vectors were as follows: (1) a 120-bp fragment from pCEM MINX (Robberson et al. 1990) containing the adenovirus major late transcript splice acceptor sequence from the intron 1/exon 2 boundary (kindly provided by Sue Berget); (2) a 3100-bp fragment encoding β-gal in which the bacterial initiation ATG has been replaced by the Moloney murine leukemia virus (MoMuLV) envelope gene ATG (Soriano et al. 1991b); and (3) the PGKneobpA cassette (Soriano et al. 1991a). Individual fragments were cloned in XhopKS$^-$, a derivative of pKS$^-$ (Stratagene) in which the *Xba*I site was converted to an *Xho*I site (kindly provided by Paul Hasty), by using standard procedures. Fragments were then ligated to each other using sites in the polylinker to produce pSAβ-galPGKneobpA.

β-Geo was constructed by inserting a *Bam*HI neo fragment, in which the *Bam*HI sites were provided from a polylinker, into the *Bam*HI site of the pUR289 (Rüther and Müller-Hill 1983). The resulting vector, pUR289neo, has neo-coding sequences in the same translational frame as β-gal. The sequence of nucleotides at the fusion is TGT cag ggg atc ccc cgg gct gca gcc aat atg gga tcg gcc ATT. Codons in lowercase letters indicate nucleotides that are not derived from β-gal or neo-coding sequence (the *Bam*HI site used for cloning is underlined); those in uppercase letters are the β-gal codon encoding amino acid 1021 (Kalnins et al. 1983) and neo codon encoding amino acid 2 (Beck et al. 1982). The β-geo gene was engineered for expression in mammalian cells by exchanging β-gal sequences in pPGKβ-galbpA for β-geo sequences to yield pPGKβ-geobpA. A promoter trap cassette, pSAβ-geobpA, was constructed by ligating the splice acceptor fragment to the β-geobpA fragment. The promoter trap cassettes SAβ-galPGKneobpA or SAβ-geobpA were excised from the plasmid vector with *Xho*I and inserted into the corresponding site of pGen$^-$ (Soriano et al. 1991b) to produce the retroviral vector pGen$^-$ ROSAβ-galPGKneobpA (ROSAβ-gal) or pGen$^-$ ROSAβ-geobpA (ROSAβ-geo). The transcriptional orientation of β-gal and neo are opposite to that of the virus (i.e., RO, for reverse orientation).

Viruses and cell culture

Culture and electroporation of the 129Sv AB1 and AB2.1 ES cell lines were performed as described previously (McMahon and Bradley 1990; Soriano et al. 1991a). ES and feeder cell lines were generously provided by Allan Bradley. Derivation of retroviral producer cell lines in the GP+E86 packaging line, virus titer determination, and ES cell infection were as described previously (Soriano et al. 1991b).

Mouse strains and blastocyst injections

Mouse chimeras were generated as described by Bradley (1987). Briefly, ~15 ES cells were injected into the blastocoel cavity of 3.5-day C57BL/6J embryos. The embryos were transferred to the uteri of 2.5-day pseudopregnant CBA/J × C57BL/6J foster mothers. Chimeric pups were identified by the presence of Agouti hair. The chimeras were bred further with C57BL/6J females to check for contribution of the ES cells to the germ line. Once germ-line transmission had been observed, chimeras were bred to 129Sv mice to maintain the promoter trap lines on an inbred background.

Embryo staining

Embryos were collected at different stages of development, fixed, and stained overnight at room temperature as described previously (Sanes et al. 1986). Embryos were stored in 70% ethanol after staining.

Other molecular biology techniques

Southern blot, Northern blot, and riboprotection were performed as described previously (Soriano et al. 1986).

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