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Isolation of an allele of reeler by insertional mutagenesis

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ABSTRACT **Reeler (*rl*) is an autosomal recessive mutation that affects migration of postmitotic neurons in the mouse central nervous system. The reeler (*rl/rl*) mouse displays a disruption of laminar structures in both the cerebellum and the forebrain and it exhibits tremors, dystonia, and ataxia. The molecular basis of the reeler phenotype is unknown because the gene involved has not yet been identified. We report here the isolation and characterization of an allele of *rl*, reeler^{transgene} (*rl*^{tg}). This allele was generated by the fortuitous insertion of a transgene, supfos (*sf*), into the mouse *rl* locus. Crosses between *rl*/+ and *rl*^{tg}/+ mice yielded offspring that exhibited the reeler phenotype, indicating that *rl* and *rl*^{tg} are allelic. We cloned the genomic sequences flanking the transgene insertion site from the *rl*^{tg}/*rl*^{tg} mouse genome. Chromosomal mapping studies revealed that the 5′ flanking cellular sequence maps to a locus, *D5Gmrl*, that lies in a region of mouse chromosome 5 that also contains the *rl* locus. Southern blot analysis using a probe derived from the *D5Gmrl* locus revealed no gross structural rearrangement in the *rl* locus. Thus, unlike the two *rl* alleles described previously, *rl*^{tg} provides a molecular probe that can now be used to identify and isolate the *rl* gene.**

Reeler (*rl*) is an autosomal recessive mutation in mice that is manifest by abnormal locomotor behaviors such as tremors, dystonia, and ataxia (1). The cellular basis of the reeler behavioral deficit has been attributed to a disruption of the normal pattern of neuronal lamination in the forebrain and cerebellar cortex (2). This malpositioning of neurons appears to be due to a defect occurring during later stages of neuronal migration (3, 4). In normal mice, the first neurons to be generated usually remain in the deepest cortical positions, while successively younger neurons migrate past the older neurons to occupy sequentially the more superficial layers. This process is referred to as an inside-out mode of development (5–7). In reeler mice, certain populations of neurons, although born at the correct developmental period, fail to migrate past the neurons generated at earlier times (8). This migratory failure results in an inverted stacking of neurons that leads to an outside-in distribution (3). While it has been suggested that the reeler phenotype is due to abnormal cell–cell interactions (9–11), no defective gene or gene product has yet been identified. There is a great deal of interest in elucidating the molecular basis of the reeler phenotype because it would provide insights into the nature of the proteins required for early neuronal migration in mammals.

Here, we describe the isolation of an allele of *rl*, reeler^{transgene} (*rl*^{tg}), by the fortuitous insertion of a transgene, supfos (*sf*), into the mouse *rl* locus. Using *sf* as a probe, we cloned genomic regions flanking the transgene insertion site. Chromosomal mapping studies indicate that one of these flanking sequences is located in a region of mouse chromosome 5 that also contains the *rl* locus. Thus, a DNA marker has been identified that can now be used to identify and isolate the *rl* gene.

MATERIALS AND METHODS

Transgene and Transgenic Mice. The *sf* transgene was generated by inserting a 12-nt sequence (CCGCTCGAGCGG) containing a *Xho* I site between nt 1922 and nt 1923 in the third exon of the mouse c-*fos* (12). A 4.7-kb *sf* fragment was excised from pGEM3Z(H/B)supfos and microinjected into either the male or female pronucleus of fertilized mouse eggs (B6C3F1 × B6D2) to generate transgenic mice (13). Transgenic mice were identified using a PCR assay coupled with *Xho* I digestion. Primers flanking the *Xho* I mutation site of the *sf* transgene (5′ primer, nt 1576–1599; 3′ primer, nt 2510–2533) (12) were used to amplify a 958-bp fragment from nontransgenic DNA and a 970-bp fragment from transgenic DNA. Subsequent digestion of the PCR products with *Xho* I gave rise to two additional fragments of 620 bp and 350 bp unique to the transgenic DNA. The *rl*/+ and *rl*/*rl* mice (inbred strain B6C3Fe) were obtained from The Jackson Laboratory.

Southern Blot Analysis. Genomic DNA isolated from mouse tail or liver tissue was examined by Southern blot analysis (14). The *fos* cDNA probe and 5′-*fos*-specific and 3′-*fos*-specific probes were derived from pSP65c-fos and from a *Hin*dIII–*Xho* I fragment (1.2 kb) and a *Xba* I–*Bam*HI fragment (2.6 kb) of pGEM3Z(H/B)c-fos (15), respectively.

Reverse Transcriptase–PCR (RT–PCR). Brain RNA was isolated from mice that were either untreated or treated with kainic acid for 1 h (15). Kainic acid (5 mg/ml) was injected intraperitoneally to induce seizures. RT–PCR was performed using primers located in two exons of c-*fos*: 5′ primer, nt 1188–1205; 3′ primer, nt 2510–2533 (12).

Construction and Screening of Subgenomic Libraries. The 5′ junction sequence (3.2 kbp) was purified from *Eco*RI/*Ssp* I-digested *sf*/*sf* genomic DNA and cloned into λZAP II (Stratagene) to generate the λZAP-5′fil library. The 3′ junction sequence (4 kbp) was obtained by digestion of *sf*/*sf* DNA with *Eco*RI to generate the λZAP-3′fil library. The libraries were screened with the 5′- and 3′-*fos*-specific probes, respectively. Positive phage clones were plaque-purified and the inserts were sequenced.

Interspecific Mouse Backcross Mapping. The chromosomal positions of the 5′ and 3′ cellular sequences flanking the *sf* insertion site were determined by interspecific mouse backcross mapping (16).

RESULTS AND DISCUSSION

Generation of *sf* Transgenic Mice. To investigate the function of the c-*fos* gene *in vivo*, we generated transgenic mouse

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Abbreviations: cM, centimorgan(s); RT–PCR, reverse transcriptase–PCR.

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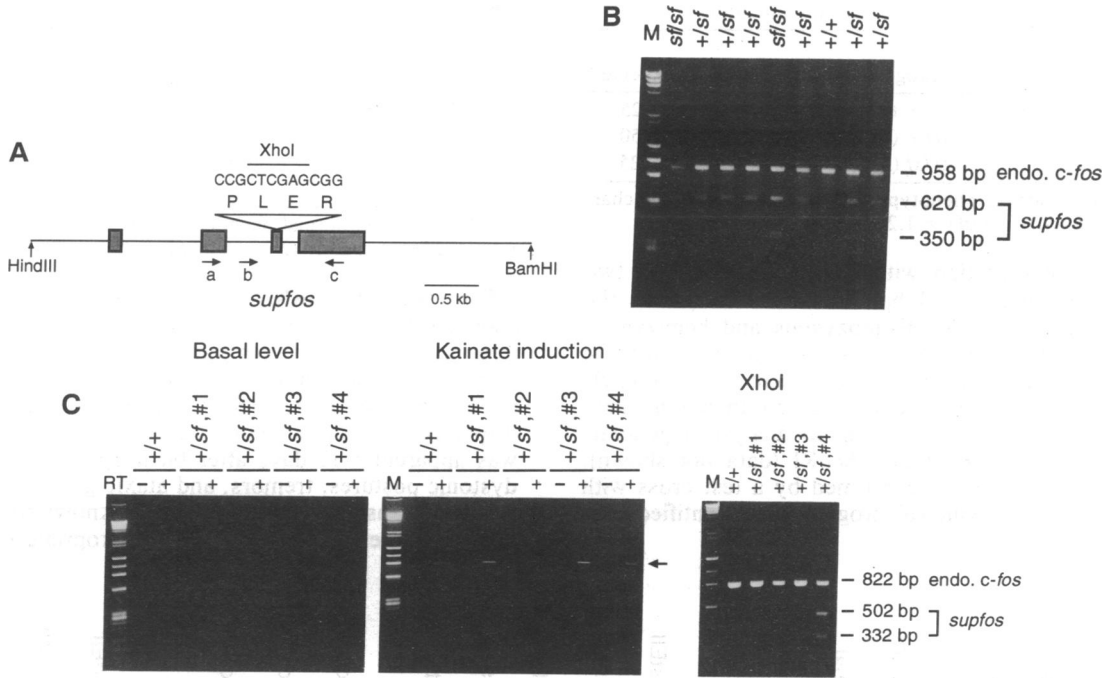

FIG. 1. Molecular identification of *sf* transgenic mice and analysis of transgene expression. (*A*) Structure of the *sf* transgene. The shaded boxes represent four exons of the mouse c-*fos* gene. An in-frame insertion of 12-nt sequence containing a *Xho* I restriction site indicated above the gene was introduced into the third exon of c-*fos*. The corresponding amino acids encoded by the insertion are indicated below the insertion sequence. The arrows below the gene represent the PCR primers used for identification of transgene and its expression. (*B*) PCR analysis of a mouse litter derived from a hemizygous intercross. Genomic DNA was amplified with primers b and c. The products were digested with *Xho* I and resolved on an agarose gel. (*C*) RT–PCR. Brain RNA was isolated from wild-type (+/+) and hemizygous (+/*sf*) adult mice that were unstimulated (Basal level) or stimulated with kainic acid for 1 h (Kainate induction). The four derived transgenic founder lines are indicated by number. RT–PCR was conducted in the presence or absence of reverse transcriptase (RT). PCR products were resolved on an agarose gel. The expression of *sf* transgene was monitored by *Xho* I digestion of reamplified PCR products of kainate-treated samples (XhoI). The sizes of DNA fragments are indicated at the right. Lanes M contain DNA molecular size markers.

lines expressing a mutated c-*fos* gene that functions as a dominant suppressor (17). A 4.7-kb DNA fragment containing the *sf* gene was purified and introduced into the mouse germ line by microinjection (Fig. 1*A*). Four founder transgenic mouse lines were obtained. These were crossed with nontransgenic mice (B6D2 males or B6C3F1 females) to generate hemizygous F_1 progeny. Subsequently, intercrosses were carried out within each founder strain to obtain homozygous transgenic mice. Since the *sf* gene contains a unique *Xho* I restriction enzyme site (Fig. 1*A*), a PCR assay coupled with *Xho* I digestion was designed to identify transgenic mice. Genomic DNA was amplified (Fig. 1*A*, primers b and c) to generate a 958-bp PCR DNA fragment from nontransgenic mice and an additional 970-bp *sf*-specific frag-

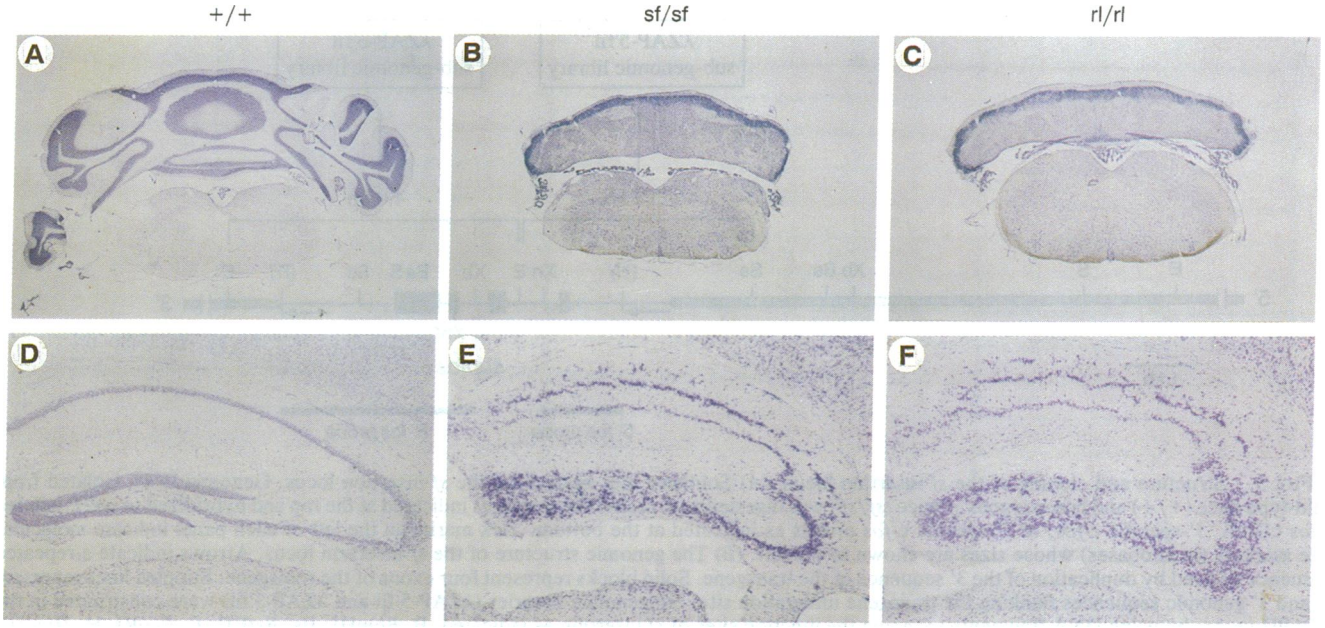

FIG. 2. Neuroanatomic analysis of *sf*/*sf* mouse brain. Coronal sections of cerebellum and hippocampus from wild-type and (+/+) (*A* and *D*), homozygous *sf* (*sf*/*sf*) (*B* and *E*), and homozygous *rl* (*rl*/*rl*) (*C* and *F*) mice are shown.

Table 1. Genetic analysis of the *sf* insertion mutation

Cross	Progeny	No.	%	% expected
sf/+ (WT) × *sf*/+ (WT)	+/+ (WT)	77	28	25
	sf/+ (WT)	132	48	50
	sf/*sf* (MT)	66	24	25

WT, wild type, normal phenotype; MT, mutant phenotype characterized by ataxia. χ^2 (2 df) = 1.240; $P > 0.5$.

ment that, upon digestion with *Xho* I, gave rise to two fragments (620 bp and 350 bp) that were unique to the transgenic mice (Fig. 1*B*). Homozygous and hemizygous mice were distinguished by the PCR assay, as the *sf*-specific fragments in homozygous mice were present at higher levels compared to the endogenous c-*fos* fragment than in hemizygous mice (Fig. 1*B*), or by Southern blot analysis of genomic DNA digested with *Xho* I and *Eco*RI (data not shown). Homozygosity was further confirmed by a test cross with nontransgenic mice in which all progeny were identified to be hemizygous.

Table 2. Allelism of the *sf* insertion and the *rl* loci

Cross	Progeny	No.	%	% expected
sf/+ (WT) × *rl*/+ (WT)	*sf*/+ (WT)	23	27	25
	+/+ (WT)	41	49	50
	rl/+ (WT)			
	sf/*rl* (MT)	20	24	25

WT, wild type, normal phenotype; MT, mutant phenotype characterized by ataxia. χ^2 (2 df) = 0.262; $P > 0.5$.

Expression of sf mRNA in Transgenic Mice. No phenotypic anomalies were observed among hemizygous transgenic mice from all four founder lines. Furthermore, homozygous mice derived from founder lines 1, 2, and 4 also exhibited a normal phenotype. However, homozygous mice of transgenic line 3 displayed a severe locomotor defect. This mutant phenotype was apparent ≈12 days after birth by the presentation of dystonic postures, tremors, and ataxic gait.

The *sf* transgene contains all of the known transcriptional control elements required for the appropriate regulation of

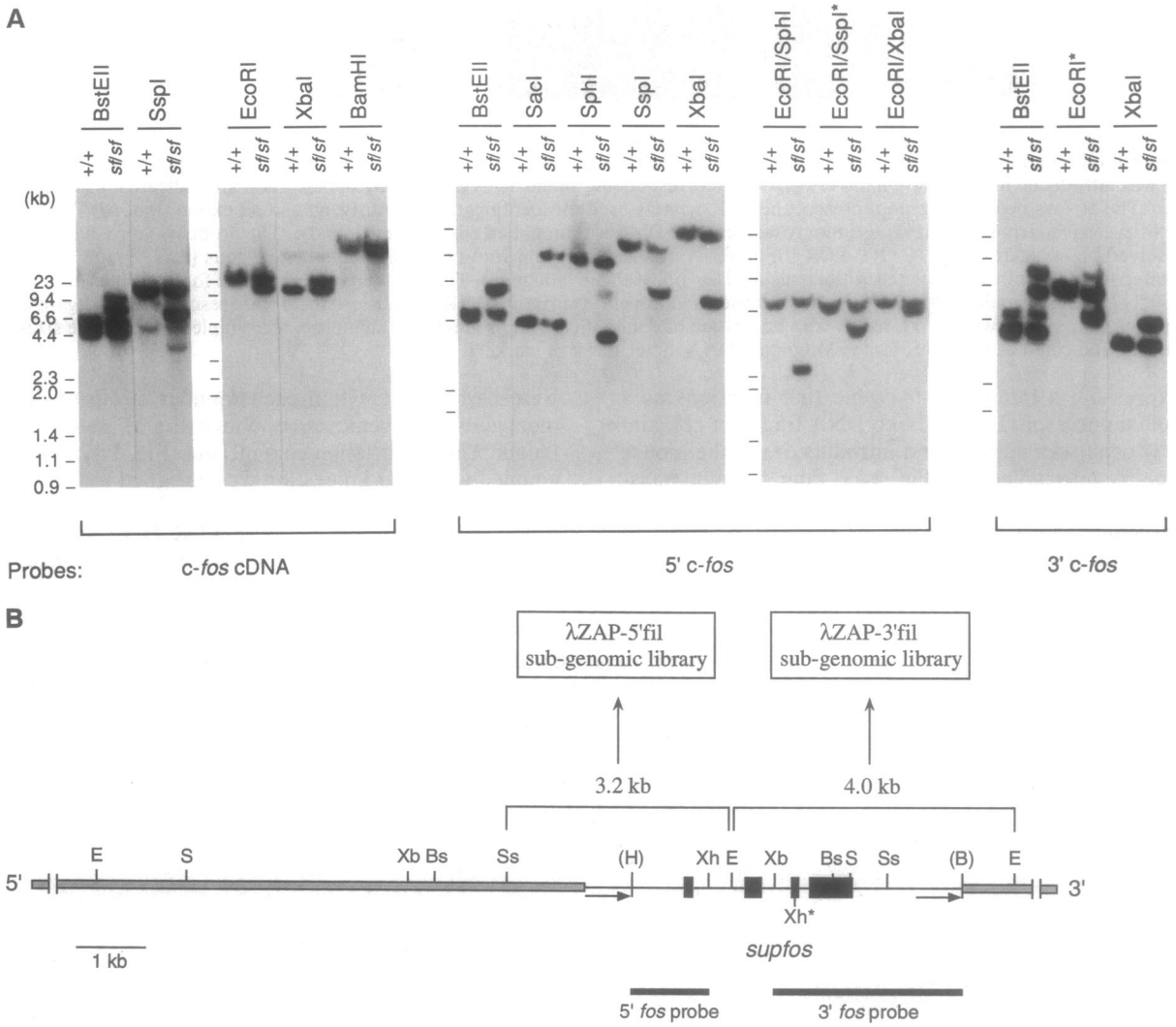

FIG. 3. Structure and cloning of the *sf* insertion locus. (*A*) Southern blot analysis of the *sf* insertion locus. Genomic DNA isolated from wild-type mice (+/+) and homozygous *sf* mice (*sf*/*sf*) was digested with restriction enzymes indicated at the top and hybridized to α-^{32}P-labeled c-*fos* cDNA, 5'-specific c-*fos*, or 3'-specific c-*fos* probes as indicated at the bottom. Tick marks on the left of each panel indicate molecular size markers (in kilobases) whose sizes are shown to the left. (*B*) The genomic structure of the *sf* insertion locus. Arrows indicate a repeated sequence derived by duplication of the 3' sequence of the transgene. Solid blocks represent four exons of the transgene. Stippled lines represent 5' and 3' genomic sequences flanking the transgene integration site. Subgenomic libraries (λZAP-5'fil and λZAP-3'fil) were constructed in the λZAPII vector by using DNA fragments spanning the junction sites of *sf* insertion as indicated. B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; S, *Sac* I; Sp, *Sph* I; Ss, *Ssp* I; Xb, *Xba* I; Xh, *Xho* I. Parentheses indicate that the restriction site was disrupted after integration of the transgene. Xh* is the unique *Xho* I site that distinguishes *sf* from the endogenous c-*fos*.

c-*fos* expression in neurons (18–20). Therefore, we determined whether expression of *sf* was related to this apparently neurological phenotype. To examine expression of *sf* in neurons, brain RNA was isolated from unstimulated mice and from mice after the induction of seizures by treatment with kainic acid for 1 h. Kainic acid has been shown to cause a dramatic but transient increase in c-*fos* expression in many neuronal populations *in vivo* (19). Expression of c-fos and sf RNA was monitored by using a RT–PCR assay (Fig. 1*A*, primers a and c). Neither sf nor c-fos mRNA expression was detected in untreated mice. However, kainate treatment rapidly induced expression of the endogenous c-fos mRNA in all mice examined (Fig. 1*C*). In addition, *sf* was induced in hemizygous mice from three of the four transgenic strains (strains 2–4) (Fig. 1*C*). These findings indicate that the *sf* transgene is capable of responding to neuronal excitation in a manner similar to that of the endogenous c-*fos* gene. However, since the locomotor defect was observed only in strain 3, it cannot be a simple consequence of sf RNA expression.

A Defect in Neurodevelopment Generated by Insertional Mutagenesis. Since there was no correlation between *sf* expression and the locomotor defect, we carried out a genetic analysis within strain 3 to examine the inheritance of the phenotype and its linkage to the *sf* transgene. The mutant phenotype was found to cosegregate with the transgene in an autosomal recessive manner (Table 1). Furthermore, the transmission of both the phenotype and the genotype conformed to a classical mode of Mendelian inheritance (upper 95% confidence limit). These findings suggest that the abnormal phenotype in transgenic mouse strain 3 resulted from a germ-line mutation caused by the insertion of the transgene.

A comparison of the gross brain structure of normal and mutant adult mice revealed that the cerebellum of the mutant mouse was significantly reduced in size and contained little of the typical cortical foliation (data not shown). This anomaly and the ataxic behavior resemble the phenotype caused by the *rl* mutation. This prompted us to compare the cortical structure abnormalities of *sf/sf* and *rl/rl* mice. Fig. 2 shows coronal sections of cerebellum and hippocampus isolated from wild-type (+/+), *sf/sf*, and *rl/rl* adult brains. The wild-type cerebellum exhibits extensive foliation and has the well-defined trilaminar organization (i.e., an outer molecular layer, an intermediate Purkinje cell monolayer, and an inner granule cell layer) (Fig. 2*A*). In contrast, cortical foliation in the *sf* cerebellum is virtually absent. In addition, the cortex is dramatically shrunken and reveals an inverted architecture where an outer granule cell layer lies superficial to the molecular and Purkinje cell layers. The appearance of the *sf* cerebellum is essentially indistinguishable from that of the reeler mouse (compare Fig. 2 *B* and *C*). We also examined the laminar organization of the hippocampal complex. In the wild-type mouse, this structure is characterized by an outer "C-shaped" band of pyramidal neurons called Ammon's Horn. The inferior blade of these pyramidal cells intersects with the dentate gyrus, which consists of densely packed granule cells positioned into a medially pointing "V" (Fig. 2*D*). An examination of the hippocampus revealed that the pyramidal neurons in *sf* mice failed to coalesce into a distinct lamina and are arranged into two loosely organized bands. The granule cells of the dentate gyrus are also loosely packed and appear to merge with a region inferior to the dentate gyrus (Fig. 2*E*). These anomalies are virtually identical to those seen in the hippocampus of the *rl/rl* mouse (Fig. 2*F*). This suggests that the *sf* mutation may result from a disruption of the endogenous *rl* gene by integration of the *sf* transgene.

***sf* Insertion Mutation Is an Allele of the *rl* Gene.** To demonstrate that the mutation was generated by insertion of the *sf* transgene into the *rl* gene, we carried out a genetic complementation analysis. Hemizygous *sf* mice were crossed with heterozygous *rl* mice. Among 84 progeny examined, ≈25% displayed the mutant phenotype (Table 2). Furthermore, all of the mutant mice were hemizygous for *sf*. This demonstrates that the *sf* mutation is allelic to *rl*. In addition, neurohistochemical analysis of *sf/rl* mice revealed similar structural malformations in the cerebellum and hippocampus to those present in both *sf/sf* and *rl/rl* mice (data not shown). Therefore, the *sf*-induced mutation appears to be an allele of the *rl* gene, referred to as reeler^{transgene} (*rl*^{tg}).

Molecular Cloning of Genomic Sequences Flanking the *sf* Insertion Site. The insertion of the *sf* transgene in the *rl* locus offers a unique opportunity to identify and isolate the gene responsible for the reeler phenotype by providing a molecular tag for the locus. As a first step toward this goal, we examined the *sf* insertion locus by Southern blot analysis (Fig. 3*A*) to generate a restriction map (Fig. 3*B*). Quantitative analysis of the *fos* hybridization signal indicated that *sf* was present as a single copy in the locus.

To isolate cellular DNA sequences flanking the transgene, a 3.2-kbp fragment was identified by digestion of *sf/sf* DNA with *Eco*RI and *Ssp* I and hybridization with a 5′-*fos*-specific probe, and a 4.0-kbp *Eco*RI fragment was identified using a 3′-*fos*-specific probe (Fig. 3). These DNA fragments were cloned and characterized by restriction endonuclease and nucleotide sequence analyses. This revealed the presence of a direct repeat of a 669-bp sequence located immediately 5′ of the transgene that was derived by duplication of the most

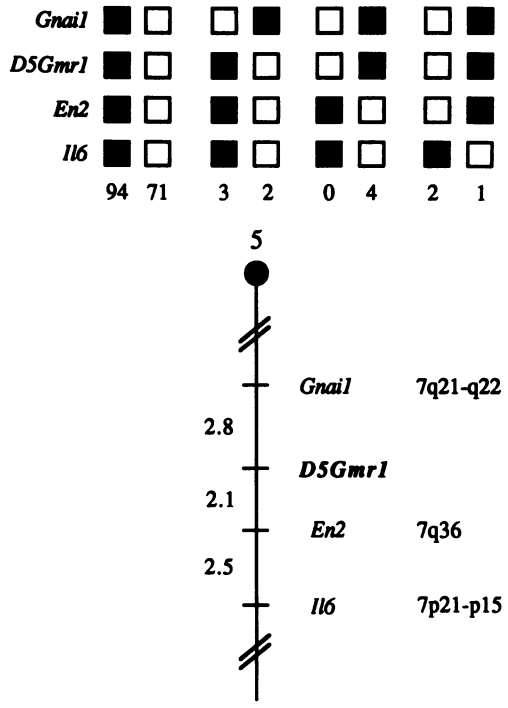

FIG. 4. The 5′ flanking sequence of the *sf* insertion site maps to the same region as the *rl* mutation on mouse chromosome 5. The 5′ flanking sequence (locus symbol, *D5Gmr1*) was placed on mouse chromosome 5 by interspecific backcross analysis. The segregation patterns of *D5Gmr1* and flanking genes in 177 backcross animals that were typed for all loci are shown at the top of the figure. Each column represents the chromosome identified in the backcross progeny. The solid boxes represent the presence of a C57BL/6J allele and open boxes represent the presence of *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at bottom of each column. A partial chromosome 5 linkage map showing the location of *D5Gmr1* in relation to the linked genes is shown. Recombination distances between loci in cM are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right.

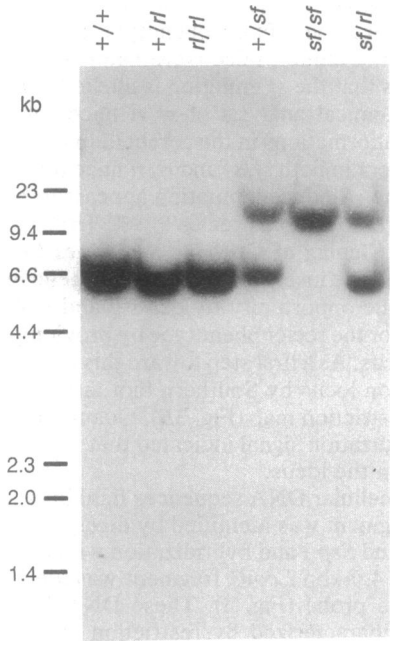

FIG. 5. Southern blot analysis of the reeler mouse DNA. Genomic DNA, isolated from mice of various genotypes as indicated, was digested with *Hin*dIII and hybridized to a α-^{32}P-labeled DNA probe derived from the 5′ flanking region of the *sf* insertion site. Positions of molecular size markers are indicated on the left.

3′ sequence of *sf*. Cellular DNA sequences flanking both the 5′ and 3′ insertion sites (1.1 kbp and 0.7 kbp, respectively) were also identified. Database analysis revealed no significant sequence similarity between either of these two sequences and any known gene.

The 5′ Flanking Sequence of *sf* Maps to the *rl* Locus. The *rl* mutation has been mapped genetically to the proximal region of mouse chromosome 5 (21). To determine whether the cloned 5′ and 3′ sequences flanking the *sf* transgene were located in this region of chromosome 5, restriction fragment length polymorphism analysis was carried out using progeny derived from matings between [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] mice (16). Mapping results indicated that the 5′ probe detected a locus, designated *D5Gmrl*, present in the proximal region of mouse chromosome 5 that was linked to *Gnail*, *En2*, and *Il6* (Fig. 4). Analysis of recombination frequencies among these loci revealed the most likely gene order as centromere–*Gnail*–(2.8 ± 1.2 cM)–*D5Gmrl*–(2.1 ± 1.0 cM)–*En2*–(2.5 ± 1.1 cM)–*Il6*, with the numbers in parentheses indicating the recombination frequencies in centimorgans (cM) (mean ± SEM). The probe from the 3′ region flanking the transgene was mapped to the central region of mouse chromosome 3. The localization of genomic probes from regions flanking the transgene insertion site to different mouse chromosomes suggests that *sf* integration was accompanied by a gross chromosomal rearrangement.

The interspecific map of chromosome 5 was compared to a composite mouse linkage map. The *D5Gmrl* locus on chromosome 5 mapped in a region of the composite map that contains the *rl* mutation. Therefore, the result of the chromosomal mapping study is consistent with the data obtained from the genetic analysis indicating that the *sf* insertion mutation is allelic to the *rl* mutation.

Southern Blot Analysis of the reeler Mouse DNA. To investigate whether the *rl* mutation involves gross structural changes in the *D5Gmrl* locus, we analyzed genomic DNA isolated from homozygous *rl/rl* mice by using a probe derived from the *D5Gmrl* locus. A representative blot is shown in Fig. 5. The probe detected a single DNA fragment in the wild-type +/+, +/*rl*, and *rl/rl* genomes. However, this fragment was disrupted in the *sf/sf* DNA and a new fragment was generated that was unique to the *sf/sf* DNA. As a consequence, the hemizygous +/*sf* and *sf/rl* DNA contained both the wild-type and the *sf*-specific fragments. These results demonstrate that the cloned 5′ flanking region is directly associated with the *sf* insertion site and also indicate that no gross structural rearrangement in the *rl* locus is detected by the probe used in this study.

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