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It is believed that the primary function of the protein encoded by the NF-1 gene is as a negative regulator of the ras protooncogene. Mutation of the NF-1 gene therefore releases ras function from negative regulation leading to hyperplasia and defects in multiple neural crest derived tissues. We have embarked on the generation of conditional mutant mice at the NF-1 locus and at the ras-Gap locus since these two genes are thought to be the major negative modulators of ras activity. In the first year (Objectives 1 and 2), we have confirmed germline transmission and activity of the NF-1 conditional allele. In addition we have embarked on various cellular and cre-transgenic mediated strategies to further study NF-1 function as proposed. In addition, as outlined in Objective 2, we have constructed a conditional ras-gap allele, introduced it into ES cells, and generated chimeric mice for the propagation of conditional mutant mice. Thus we have achieved all of our proposed goals for the first year and plan to continue along the outlined strategies in year 2.

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Introduction

Von Recklinghausen's neurofibromatosis type 1 (NF1) is a dominant autosomal disorder that strikes approximately 1 in 3500 individuals. The disease in humans results from the mutational inactivation of a single gene. It is characterized by anomalies of diverse cell types, many of which are of the neural crest lineage, including melanocytes and Schwann cells. The severity or penetrance of the different phenotypes can vary greatly between afflicted individuals. However, the most serious life-threatening health situation occurs when neurofibromas/Schwannomas progress into malignant neurofibrosarcomas. The *NF1* gene product, neurofibromin, is a large cytoplasmic protein of over 2,800 amino acids that exhibits structural and functional homology to the GTPase-activating protein (GAP), both of which biochemically function as negative regulators of GTP-bound Ras proteins. The prevailing theory on the mechanism of Neurofibromatosis type 1 is that inactivation of the *NF1* gene results in elevated Ras-GTP levels which causes excessive signaling to downstream effector molecules in the mutant cells. Thus, over-stimulation of signal transduction pathways caused by uncontrolled Ras-GTP levels is thought to play a major role in the development of this disease. Furthermore, oncogenic mutations in *ras* genes that encode dominant constitutively active GTP-bound forms of Ras are frequently observed in a diverse range of human malignancies. Therefore, while it is clear that study of the *NF1* gene is essential to the understanding of this particular disease, the analysis of other proteins that regulate Ras activity in the same way as neurofibromin also merit careful study.

This proposal concerns the development and analysis of mouse models for the study of Neurofibromatosis. In past years, we have isolated the mouse equivalent *NF1* gene and inactivated it by mutation, allowing us to study the function of neurofibromin in great detail [1]. For example, our studies have revealed that the *NF1* gene may have important functions in regulating how neurons survive and in the interplay between neurons and non-neuronal cells [3]. Others have used this mutation to study the effects of *NF1* loss on lymphomas [4]. Our studies of *GAP* mutant mice have also revealed defects in blood vessel formation and in the survival of neuronal cells [2]. Moreover, combination of the *NF1* and *GAP* mutations in mice has revealed genetic interactions during both embryonic development and in tumorigenesis [2] (Henkemeyer et al., manuscript in preparation). The availability of these mouse models has provided important data, but suffers from the drawback that both *NF1* and *GAP* homozygous mutant animals perish during fetal development, thus limiting our analysis to the early phases of embryonic development. In this application, we proposed to generate improved mouse models that will permit analysis of more mature tissues and adult animals in attempts to more closely mimic the human condition. This strategy will permit us to inactivate the *NF1* and *GAP* gene products either separately or in combination in selected tissues, cell types, and at specified times of the animal's life. These animal models will provide powerful tools for understanding the function of the *NF1* gene and the consequences of its inactivation in the regulation of Ras signaling in the cell. Moreover, continued analysis of the genetic interactions of *NF1* and *GAP* should lead to a greater understanding of the biological functions of these Ras GAPs and may help define their role in the development of diseases including Neurofibromatosis type 1. These studies may help in the development of novel strategies to treat Neurofibromatosis related diseases.

This application brings together expertise, background and reagents to study Neurofibromin, GAP and *ras* regulation. Our proposal concerns the establishment of recent advances in gene knockout technology that will permit us to study the consequence of mutations in the *NF1* and *GAP* genes beyond the presently available embryonic stages. We proposed to generate conditional mutant mice through the implementation of a site specific recombinase (Cre) to catalyze recombination when expressed in cells that contain the unique target (*loxP*) sequences. We proposed five Objectives or Specific Aims. Objective 1 extends ongoing studies on *NF1* mutations. Objective 2 proposed to employ identical strategies to that of Objective 1 to generate and obtain germline transmission of a conditional mutation in the *GAP* gene.

Body.

Objective 1 (Parada). Through genetic and biochemical methodologies, we proposed to generate conditional NF1 mutant mice and will examine the neurotrophin requirements of neurons, the biological properties of glia, and the cell signaling pathways regulated by neurofibromin

Objective 1a: We will use two specific strategies employing the Cre/loxP system to generate conditional null mutations at the NF1 locus in mice. This is accomplished by the molecular construction of appropriate recombination cassettes that harbor specifically located loxP sequences, followed by selection of homologous recombinants in ES cells. The ultimate goal of this Objective is to obtain three distinct alleles (null, hypomorph, conditional null) of the NF1 gene present in mice in addition to the wild-type allele.

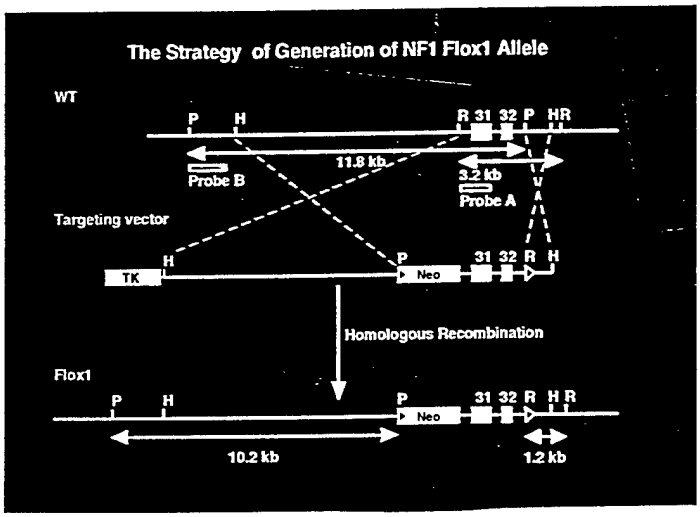


Figure 1

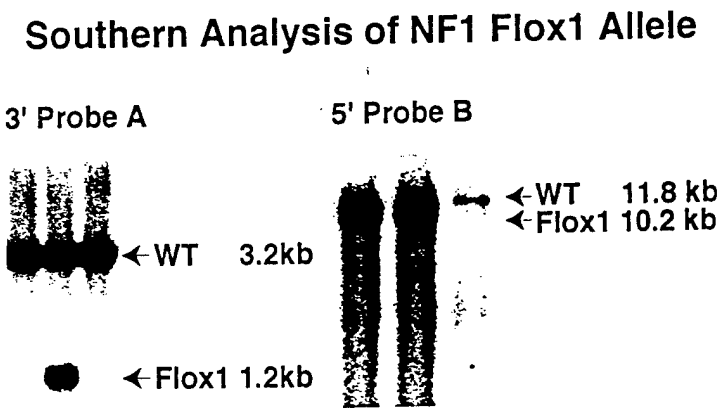


Figure 2

Figure 1 outlines the strategy which we ultimately employed to obtain a *floxed* allele of NF-1 in ES cells. **Figure 2** shows Southern blots which indicate the presence of the floxed allele using both 3' (1.2 kb) and 5' (10.2 kb) external probes to insure that both *lox* sites had been incorporated by the homologous recombination event. The ES cells were injected into C57Bl blastocysts and agouti germ line chimeras were obtained. Figure 3 indicates Southern blots showing the presence of the *floxed* allele with both 5' and 3' probes in the mice. In addition, the *floxed* allele was crossed into the preexisting null allele to make a mouse that has the conditional alllele on one chromosome and the mutant null allele on the other chromosome. As can be seen on the lower panel of **Figure 3**, both the *floxed* allele homozygotes and the compound *flox*/null heterozygote mice are viable, fertile and exhibit no apparent phenotype. We have therefore ceased experiments with alternative flox constructs since this allele appears to give us the requisite conditional allele.

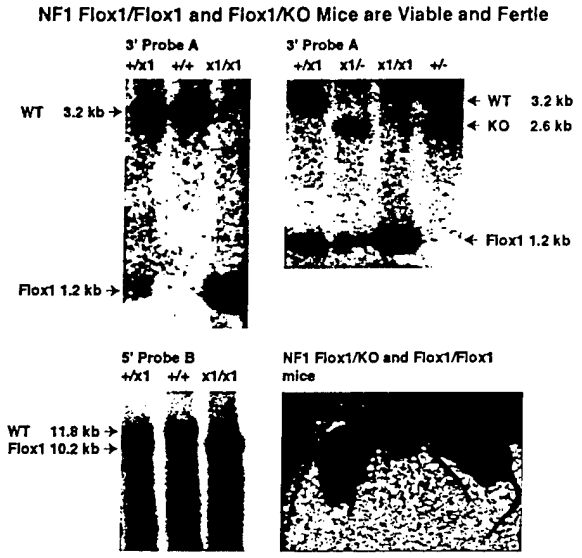


Figure 3

Objective 1b was to assess the properties of sensory, sympathetic, and cortical neurons in response to neurotrophins. To induce NF1 ablation in the conditional mutants, a CMV promoter Cre encoding adenovirus (Adcre) was proposed.

Embryonic DRG Neurons are infected with LacZ Adenovirus

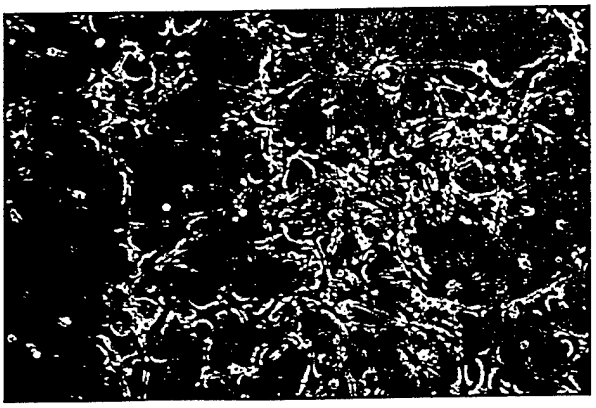


Figure 4

Generation of NF1 Mutant Neurons by Cre Adenovirus

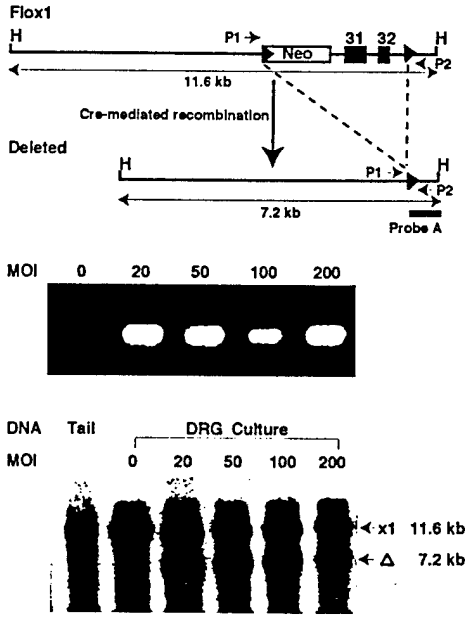


Figure 5

Figure 4 indicates the infectivity of primary sensory neurons with a lacZ expressing adenovirus. These data confirm that we can use the proposed cre-adenovirus t6o induce NF-1 inactivation in

sensory neurons as proposed. **Figure 5** shows a schematic strategy for PCR analysis using primers P1 and P2 (middle panel) and Southern analysis (lower panel) to detect cre-mediated recombination. Primary sensory neurons were infected with CMV-cre adenovirus beginning with multiplicity of infection of 20 (moi = 20) to moi of 200. Both the PCR and Southern data concord that Moi of 20 is sufficient to obtain cre mediated recombination.

E13.5 DRG neurons from NF1*flox* homozygous embryos were cultured in the presence of NGF. Cultures were either infected with the cmv-cre adenovirus, no virus, or an adenovirus that expresses no recombinant protein (**Figure 6**). After 72 hours, NGF was removed from all cultures. The data show that only the neurons exposed to cre adenovirus can survive in the absence of neurotrophins. These crucial data indicate that the previously observed independence from neurotrophins by NF1 mutant neurons is a direct consequence of NF1 absence in the neurons and not to a developmental consequence of the mutations. These studies will now be greatly expanded.

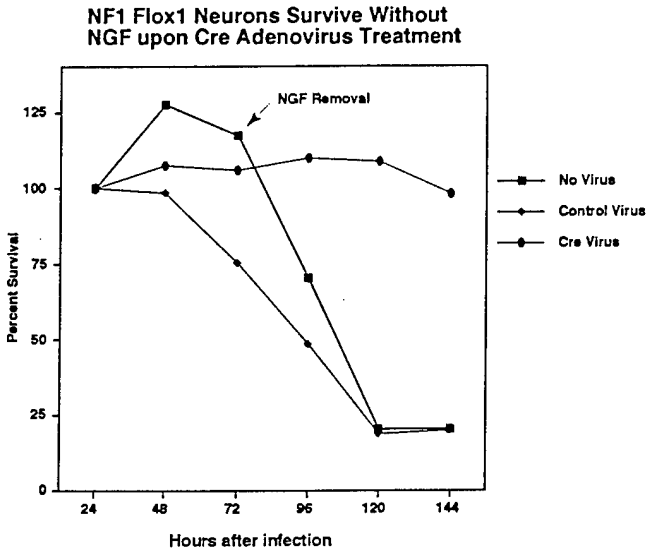


Figure 6

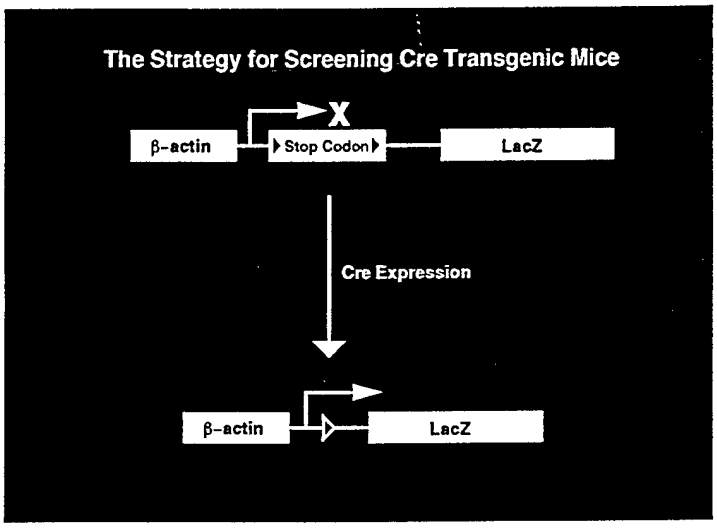


Figure 7

Objective 1c proposed to study the state of the Ras signaling pathway. These experiments are underway and two manuscripts are now in press (Klesse et al, 1998 Oncogene; and Klesse & Parada, 1998, Journal of Neuroscience) that outline the adenoviruses and their power in studying signaling in neurons.

Objective 1d was to generate transgenic stock animals that will afford us specific CRE recombinase expression. These transgenic founders will be crossed into the conditional mutant mice with the aim of knocking out NF1 function in vivo in specific tissues. **Figure 7** outlines the strategy employed to asses the expression pattern of each cre transgenic mouse through a cross to a lacZ reporter mouse generated by Dr. David Anderson. Expression of Cre results in activation of the lacZ gene. **Figure**

8 shows such an experiment in which we have crossed a synapsinI-cre mouse to the reporter mouse. As anticipated, neither the synapsinI or lacZ transgenics exhibit LacZ activity. Only the dual transgenic mice have expression and it is confined to neural tissues. **Figure 9** shows section of synapsinI-cre/lacZ transgencis that better show expression early (E12.%) in the brain spinal cord and DRD and at E15.5 in the DRG.

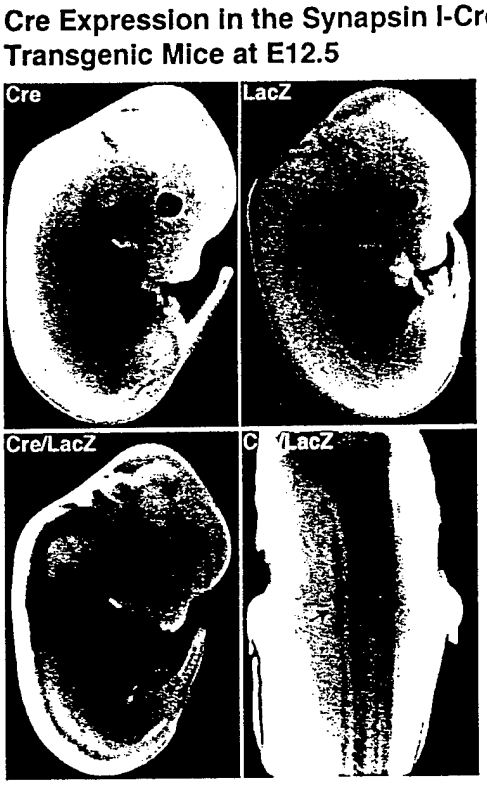


Figure 8

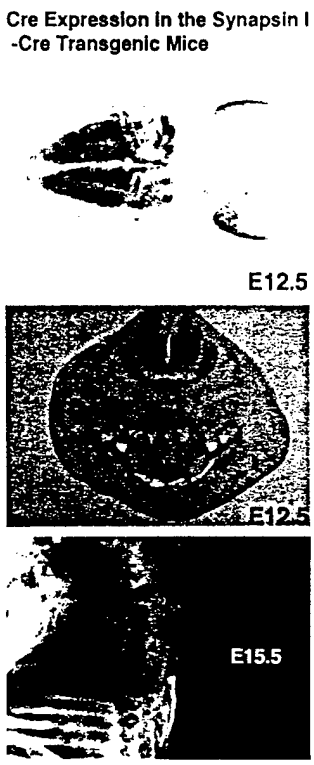


Figure 9

The synapsinI-cre mice have been bred into the floxNF1 background. The resulting mice exhibit several preliminary phenotypes. **Figure 10** shows three syn/NF1flox animals next to an NF1flox littermates. All mice that have loss of NF1 in the nervous system as caused by syn-cre, are runted. **Figure 11** quantitates this effect which is apparent at weaning (3 weeks) and displays about a 50% reduction in weight. In addition, as outlined in **Figure 12**, the synI/NF1flox mice die between 3 and 4 months of age. These data confirm that NF1 function in neurons is essential for survival. The mice are under scrutiny to determine the scope of defects and the ultimate cause of death.

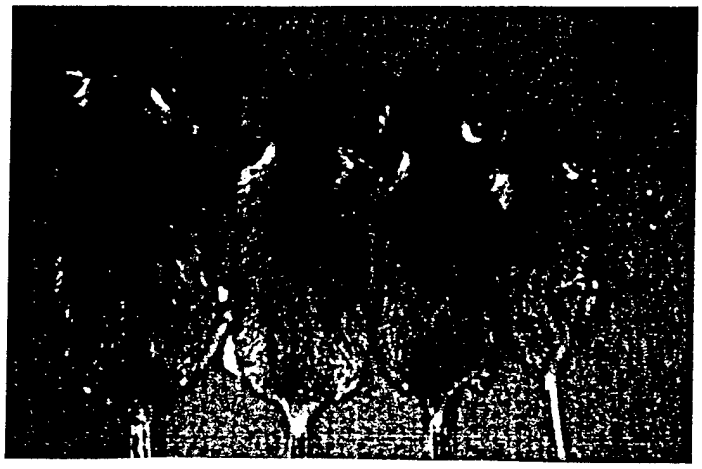


Figure 10

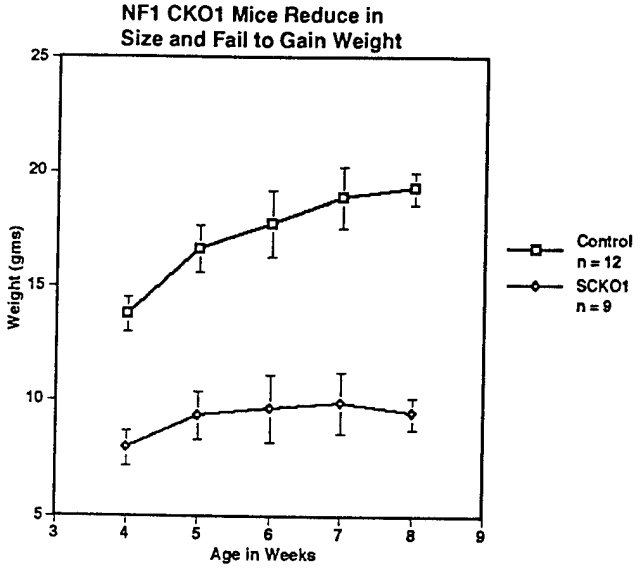


Figure 11

Additional cre-transgenic mice have been made and the work will be expanded, as other cre-mice are available.

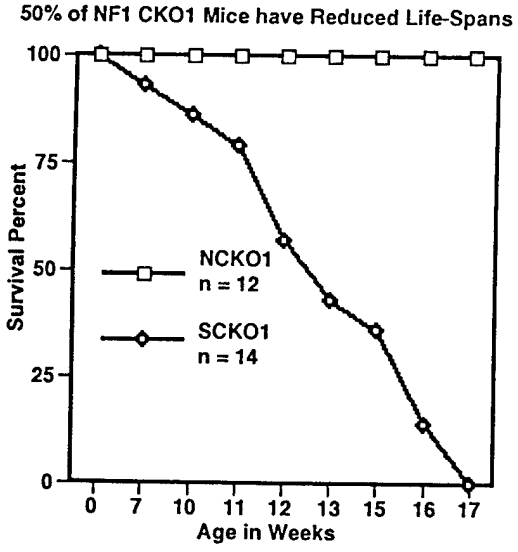


Figure 12

Objective 2a (Henkemeyer laboratory) is listed as Task 7 in the Statement of Work in our original application and was to be accomplished in months 1-10. As documented below and in **Figures 13 and 14**, we have successfully accomplished the main goals of this Task. In brief, we have 1) constructed the necessary plasmid vector to conditionally inactivate *GAP*, 2) have electroporated this vector into murine ES cells, 3) have identified homologous recombinant ES cell lines containing the appropriate *loxP* sequences, and 4) have used these cell lines to generate chimeric male mice that have already (or soon will) transmit the conditional mutation through the germline.

Our previous work has shown that deletion of a 2.4 Kb EcoRI to Asp718 genomic segment of *GAP* including two exons coding for GAP amino acids 172 to 267 disrupts the function of this gene and results in a homozygous lethal mutant phenotype in the mouse [Henkemeyer, 1995 #4]. DNA sequence analysis indicates that inadvertent mRNA splicing around these two deleted exons would lead to a defective fusion transcript containing a frameshift mutation in the open reading frame. Moreover, the protein null nature of this 2.4 Kb deletion was confirmed by immunoblot analysis with both N-terminal and C-terminal GAP-specific antiserum. As this mutation results in an early embryo lethality, it has been difficult to study the function of GAP at later stages of development or in adult life. To aid such analysis, we have designed, constructed and successfully used a new targeting vector to generate a conditional *GAP* mutation that will allow us to selectively delete these two exons by expressing the Cre recombinase from bacteriophage P1. Our strategy for generating the conditional mutation was to insert, by homologous recombination in ES cells, the 34 bp *loxP* sequences which are recognized by Cre recombinase into the introns that flank these exons of *GAP*. **Figure 13** shows the polypeptide domain structure of GAP (A), a cloned, mapped and partially sequenced portion of the wild-type *GAP* genomic DNA (B), the p*GAP-loxP-neo* targeting vector that was used to shuttle in the *loxP* sequences into the *GAP* locus (C), and the desired $GAP^{loxP\text{-}neo}$ homologous recombinant allele following electroporation of this vector into murine ES cells (D). The p*GAP-loxP-neo* targeting vector is a derivative of the original vector (pMGAP.G7.7) used to generate the null allele [Henkemeyer, 1995 #4], however, instead of deleting the 2.4 Kb EcoRI to Asp718 genomic fragment of *GAP*, this new vector retains the 2.4 Kb fragment while flanking it with *loxP* sequences. In this construct we flanked the *neomycin* (*neo*) resistance cassette with *Frt* sites which will allow its removal by expressing the Flp recombinase.

We have electroporated the p*GAP-loxP-neo* targeting vector into ES cells and have screened an extremely large number of potential positive cell lines by Southern blotting with external *GAP* probes located outside of the targeting vector. Out of 2,883 cell lines examined to date, only 2 were recovered that exhibited homologous recombination at the *GAP* gene, VIII-22 and V-40 (see **Figure 14** below). This unexpected result is in stark contrast to what we previously observed using the pMGAP.G7.7 targeting vector where 8 of 108 cell lines isolated exhibited homologous recombination [Henkemeyer, 1995 #4]. This greatly reduced frequency could be due to the presence of the *loxP* and *Frt* sequences present in the new p*GAP-loxP-neo* vector, which may inhibit homologous recombination events. Needless to say, with such a low frequency of events, the bulk of our effort over the past year has been concentrated on screening as many cell lines as possible to recover the desired homologous event.

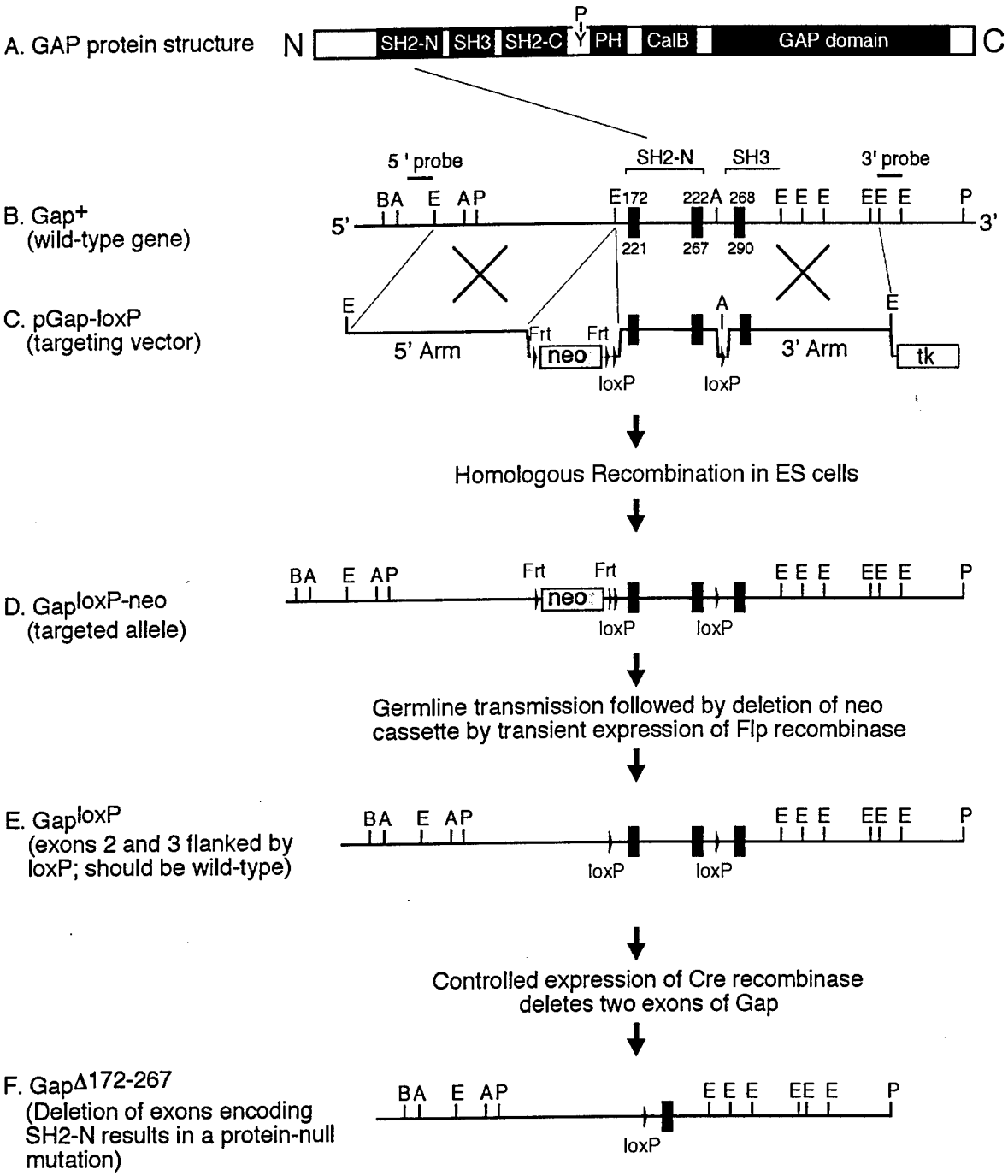


FIGURE 13
L. F. PARADA

We performed Southern blot analysis of purified genomic DNA from the 2 identified cell lines to characterize the homologous recombination into the *GAP* gene (**Figure 14**). Unfortunately, we determined that only one of these 2 cell lines (V-40) had incorporated both desired *loxP* sequences into the *GAP* gene (**Figure 14 C**). Although the sub-optimal VIII-22 cell line only has one *loxP* sequence, it might still provide useful information as the insertion of the *neo* cassette in the *GAP* intron could create a hypomorphic allele. Furthermore, since the VIII-22 cell line was isolated prior to the V-40 line, we went ahead and generated VIII-22 chimeric mice which have already transmitted the mutation through the germline. Genetic studies of this potential hypomorphic allele are presently under way.

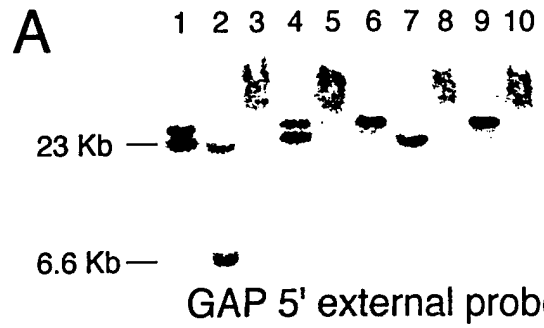
Presently, we have generated a number of chimeric mice with the V-40 cell line that contains both *loxP* sites and are waiting for the pups to age to the point where they will be ready to test for germline transmission. We anticipate no problems in obtaining germline transmission of the conditional mutation using the V-40 chimeric mice.

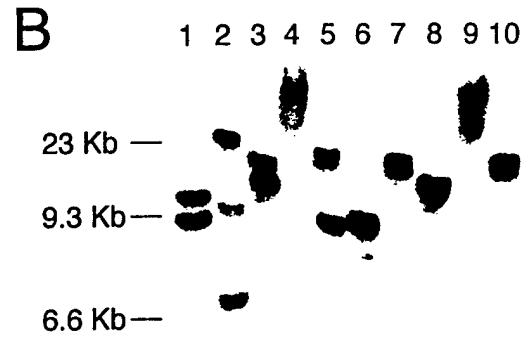
Figure 13. Strategy to generate a conditional *GAP* mutation in ES cells.
A. The polypeptide domain structure of p120 Ras GTPase-Activating Protein, GAP. The protein contains two Src Homology 2 (SH2) domains, an SH3 domain, a pleckstrin homology (PH) domain, a calcium binding (CalB) domain and a C-terminal domain, which contains Ras-GAP function.

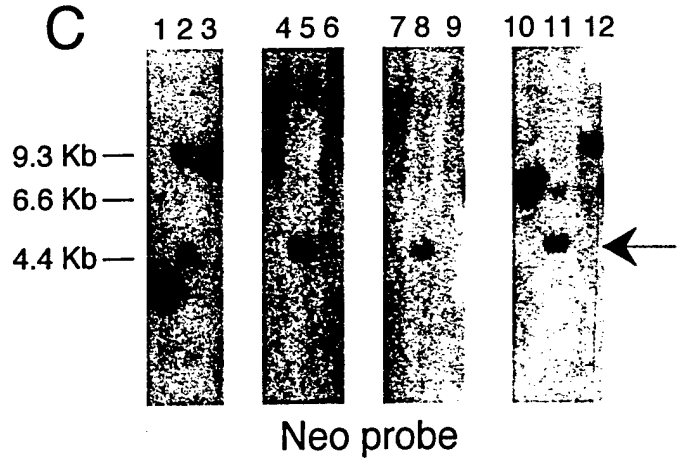
B. Map of cloned genomic DNA corresponding to a portion of the wild-type murine *GAP* gene. Protein-coding exon sequences are denoted by boxes and the location of the two exons encoding the N-terminal SH2 domain are indicated.

C. The p*GAP-loxP-neo* targeting vector used to shuttle *loxP* sequences into the *GAP* locus via homologous recombination in ES cells. The p*GAP-loxP-neo* targeting vector is a derivative of the original vector (pMGAP.G7.7) used to generate the null allele [Henkemeyer, 1995 #4], however, instead of deleting the 2.4 Kb EcoRI (E) to Asp718 (A) genomic fragment of *GAP*, this new vector retains the 2.4 Kb fragment while flanking it with *loxP* sequences. The *neomycin* (*neo*) resistance cassette is flanked by *Frt* sites. The *Frt* sites will allow removal of the *neo* cassette by transient expression of the Flp recombinase. The *thymidine kinase* (*tk*) cassette is utilized for negative selection to help enrich for homologous recombinants. Following electroporation of this targeting vector into ES cells and selection in G418 and gancyclovir, individual colonies were expanded and genomic DNA was screened for the presence of a homologous event by Southern blot analysis using the indicated 5' and 3' external probes.

D. The desired homologous recombination event following electroporation of p*GAP-loxP-neo* into murine ES cells. Out of 2,883 such cell lines examined to date, only 2 were recovered that exhibited homologous recombination at the *GAP* gene, VIII-22 and V-40 (see Figure 2 below). This unexpected result is in stark contrast to what we previously observed using the pMGAP.G7.7 targeting vector where 8 of 108 cell lines isolated exhibited homologous recombination [Henkemeyer, 1995 #4]. This greatly reduced frequency could be due to the presence of the *loxP* and *Frt* sequences present in the new p*GAP-loxP-neo* vector, which may inhibit homologous recombination events. Both of these ES cell lines have been used to obtain chimeric males and germline transmission of the VI-69 cell line has already been obtained.


A
1 2 3 4 5 6 7 8 9 10
23 Kb
6.6 Kb
GAP 5' external probe


B
1 2 3 4 5 6 7 8 9 10
23 Kb
9.3 Kb
6.6 Kb
GAP 3' external probe


C
1 2 3
4 5 6
7 8 9
10 11 12
9.3 Kb
6.6 Kb
4.4 Kb
Neo probe

E. Removal of the *neo* cassette by expression of Flp recombinase. The homologous event generated by the p*GAP-loxP-neo* targeting vector places a *neo* cassette into the intron upstream of the exon encoding GAP residues 172 to 221, which may interfere with the expression of normal levels of GAP. As the conditional allele is to be functionally wild-type in its native state, the *neo* gene will be removed from the *GAP* locus through the *Frt* sequences which flank this cassette. This step involves transient expression of Flp recombinase to excise the *neo* gene, which will leave behind a single *Frt* site and the two *loxP* sequences, one in the intron upstream of the *GAP* exon encoding residues 172 to 221 and the other in the intron downstream of the exon encoding residues 222 to 267.

F. Deletion of *loxP*-flanked *GAP* exons by expression of Cre recombinase. As described in detail in the original grant application and in this progress report, various strategies will be utilized to express Cre recombinase to delete these *GAP* exons to generate a protein-null mutation. This will allow us to inactivate GAP function in specific cell types or at specific times of embryonic development or adult life.

Figure 14. Southern blot of conditional *GAP* targeting in ES cells. Out of 2,883 cell lines screened, only 2 were identified that showed homologous recombination at the GAP locus.

A. Southern blot analysis of genomic DNA using an external probe corresponding to a 5' region of the *GAP* locus. Lanes 1-5 are digests of ES cell DNA from the single *GAP*$^{\text{lox P-neo}}$/+ heterozygous cell line recovered to date (V-40), while lanes 6-10 are digests of wild-type +/+ ES cell DNA as the control. DNA was digested with *Sac* I plus *Sal* I (lanes 1 and 6), *BamH* I (lanes 2 and 7), *Xho* I (lanes 3 and 8), *Xho* I plus *Sac* I (lanes 4 and 9), and *Xho* I plus *Sal* I (lanes 5 and 10). DNA was resolved on an agarose gel and hybridized with the 5' external *GAP* probe. Note that in lanes 6, 7 and 9, only a wild-type band is present, while in lanes 1, 2 and 4, two bands are present, and one of which corresponds to the wild-type allele and the other the mutant allele. As both *Xho* I and *Sal* I are rare site cutters, the single digest with *Xho* I and the double digest with *Xho* I+*Sal* I results in only high molecular weight DNA that cannot be resolved in the agarose gels. For molecular weight standards, the migration of Lambda DNA digested with *Hind* III is indicated.

B. Southern blot analysis of genomic DNA using an external probe corresponding to a 3' region of the *GAP* locus. Lanes 1-5 are digests of ES cell DNA from the single *GAP*$^{\text{lox P-neo}}$/+ heterozygous cell line recovered to date (V-40), while lanes 6-10 are digests of wild-type +/+ ES cell DNA as the control. DNA was digested with *Pst* I (lanes 1 and 6), *Sac* I plus *Sal* I (lanes 2 and 7), *Bam* HI (lanes 3 and 8), *Xho* I (lanes 4 and 9), and *Xho* I plus *Sac*I (lanes 5 and 10). DNA was resolved on an agarose gel and hybridized with the 3' external *GAP* probe. Note that in lanes 6, 7, 8 and 9, only a wild-type band is present, while in lanes 1, 2, 3 and 4, wild-type and mutant-specific bands are present. For molecular weight standards, the migration of Lambda DNA digested with *Hind* III is indicated.

C. Southern blot analysis of genomic DNA using a probe corresponding to the *neo* cassette. Lanes 1, 4, 7 and 10 are digests of wild-type ES cell DNA, lanes 2, 5, 8 and 11 are digests of the *GAP* cell line V-40, and lanes 3, 6, 9 and 12 are digests of the *GAP* cell line (VIII-22). DNA was digested with *Sac* I plus *Sal* I (lanes 1-3), *Sal* I (lanes 4-6), *Xho* I plus *Sal* I (lanes 7-9) and *Xho* I plus *Apa* I (lanes 10-12). DNA was resolved on an agarose gel and hybridized with a *neo* probe. Note that in lanes 5, 8

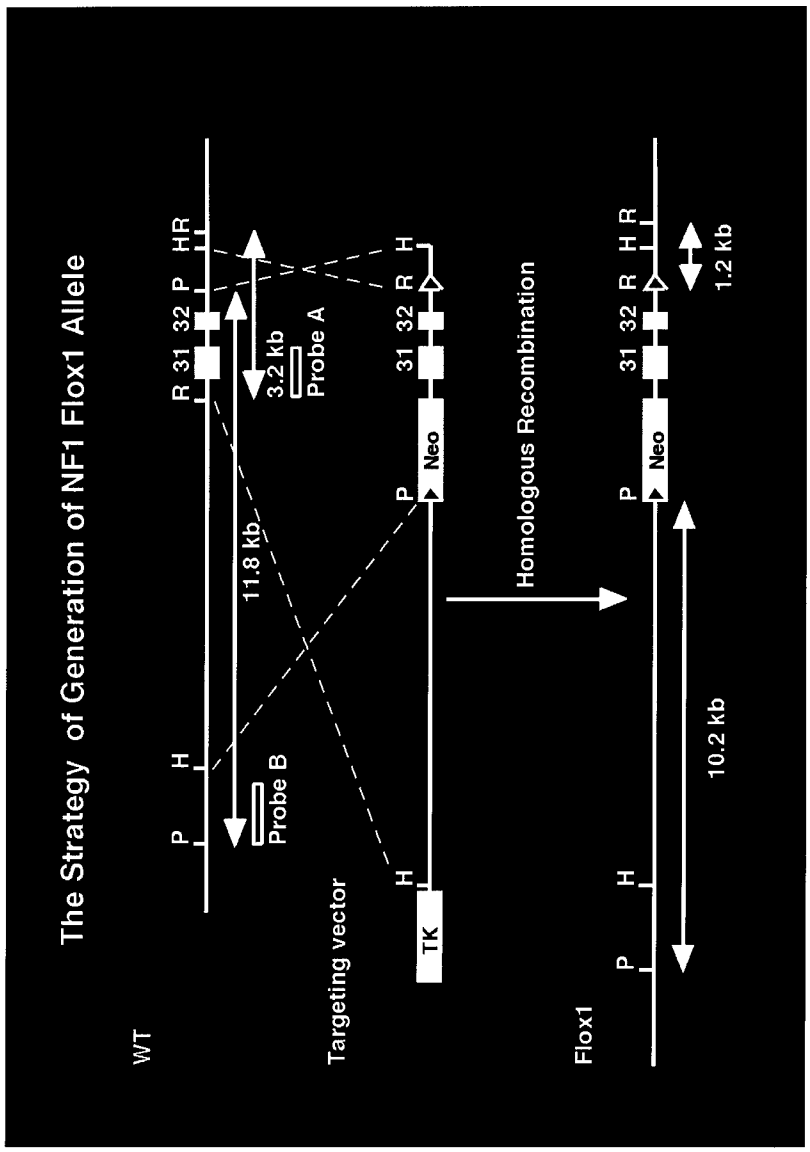
and 11, a 6 Kb band is present indicating the presence of both *loxP* sequences and proper targeting of the *GAP* locus in the V-40 cell line. This fragment is not observed in digests of the wild-type control DNA (lanes 4, 7 and 10) or in DNA from the VIII-22 cell line (lanes 6, 9 and 12). These results indicate that the 3' homologous recombination event that generated the VIII-22 cell line occurred between the two-loxP sites in the targeting vector and, thus, results in a cell line that only contains one *loxP* site in the *GAP* gene.

Conclusions.

Our progress in the first year has been far greater than anticipated. The availability of the various cre transgenic and adenoviruses generated in this first period mean that as the Gap conditional mutants come on line, we will be in a position to move those experiments and the double mutant experiments rapidly.

Appendix Table of Contents

FIGURE 1
L. F. PARADA



Southern Analysis of NF1 Flox1 Allele

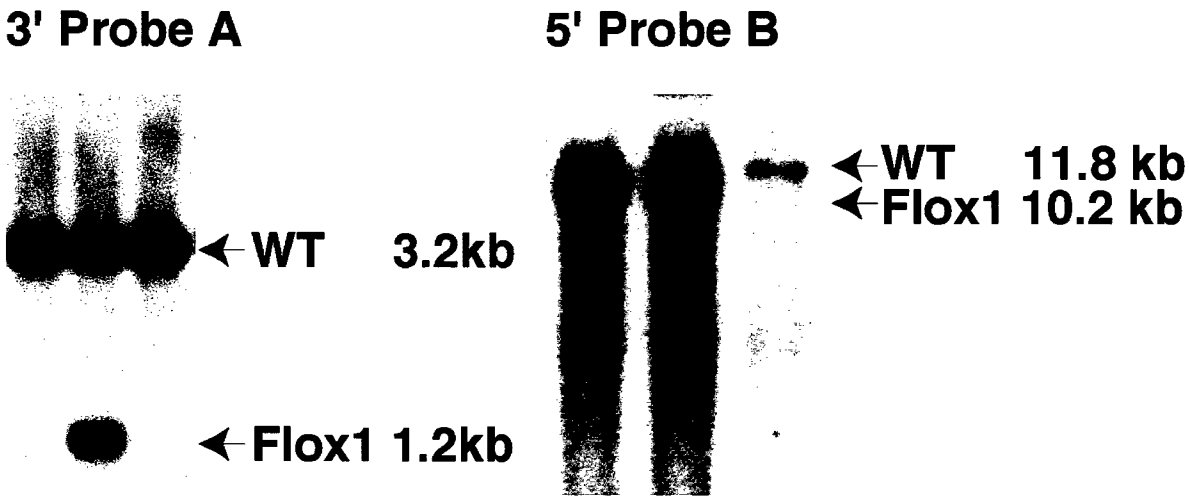


FIGURE 2
L. F. PARADA

NF1 Flox1/Flox1 and Flox1/KO Mice are Viable and Fertle

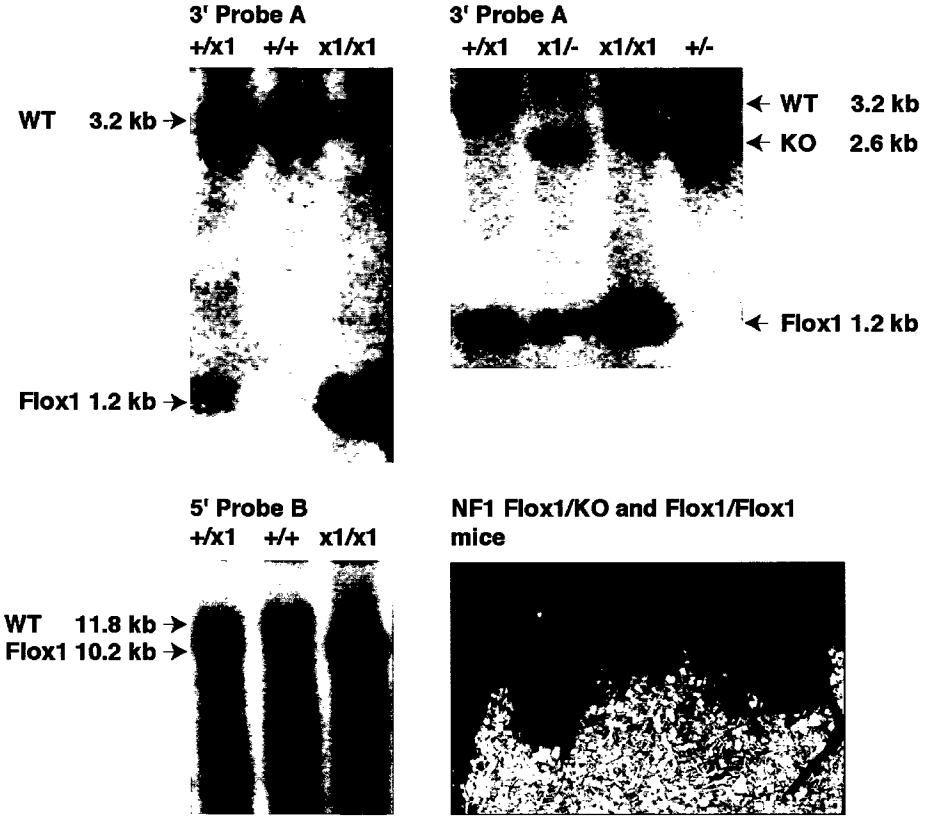


FIGURE 3
L. F. PARADA

Embryonic DRG Neurons are infected with LacZ Adenovirus

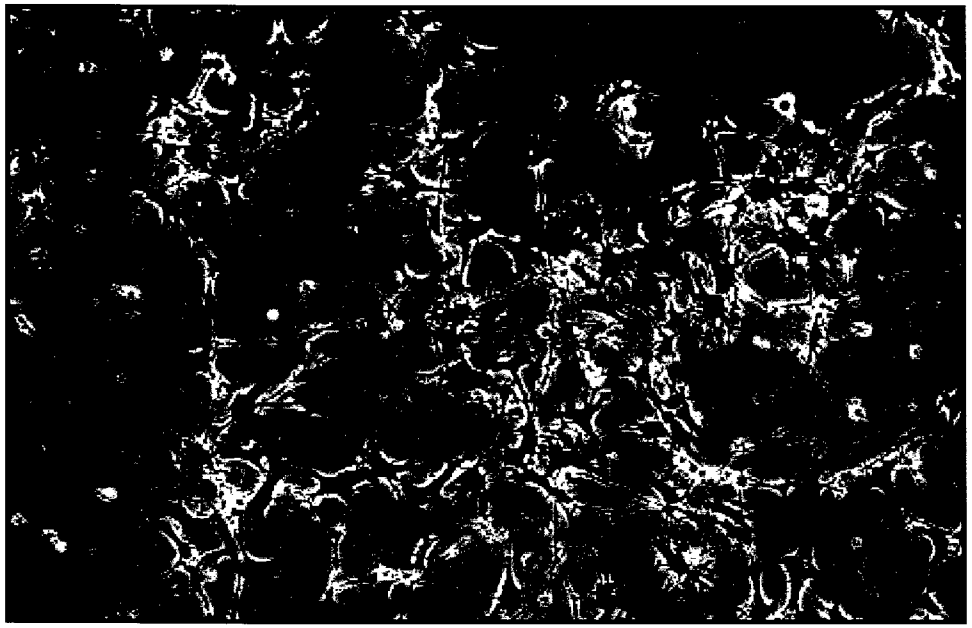
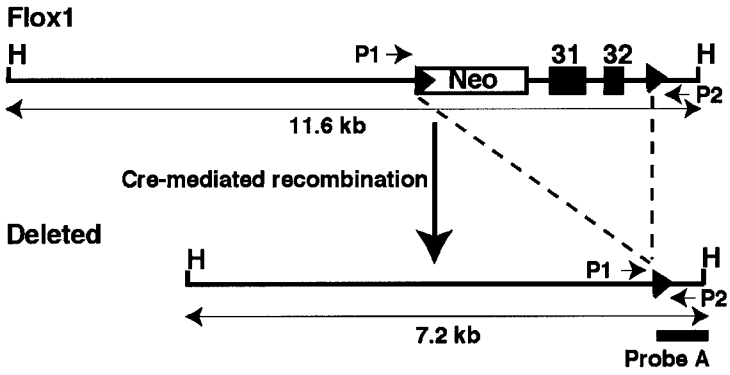
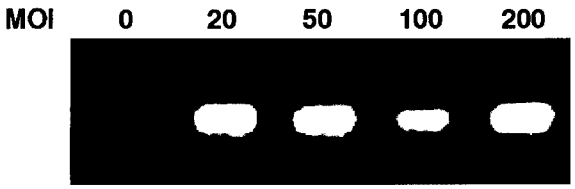


FIGURE 4
L. F. PARADA

Generation of NF1 Mutant Neurons by Cre Adenovirus





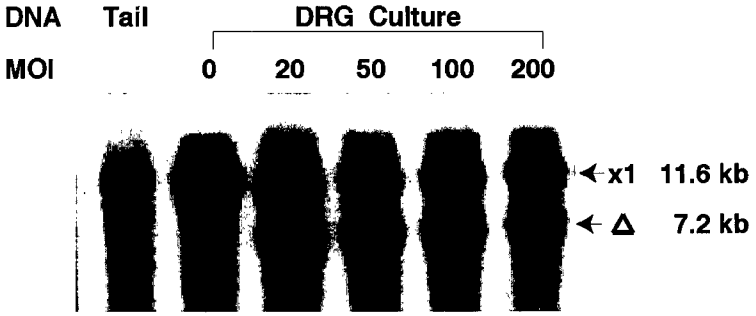


FIGURE 5
L. F. PARADA

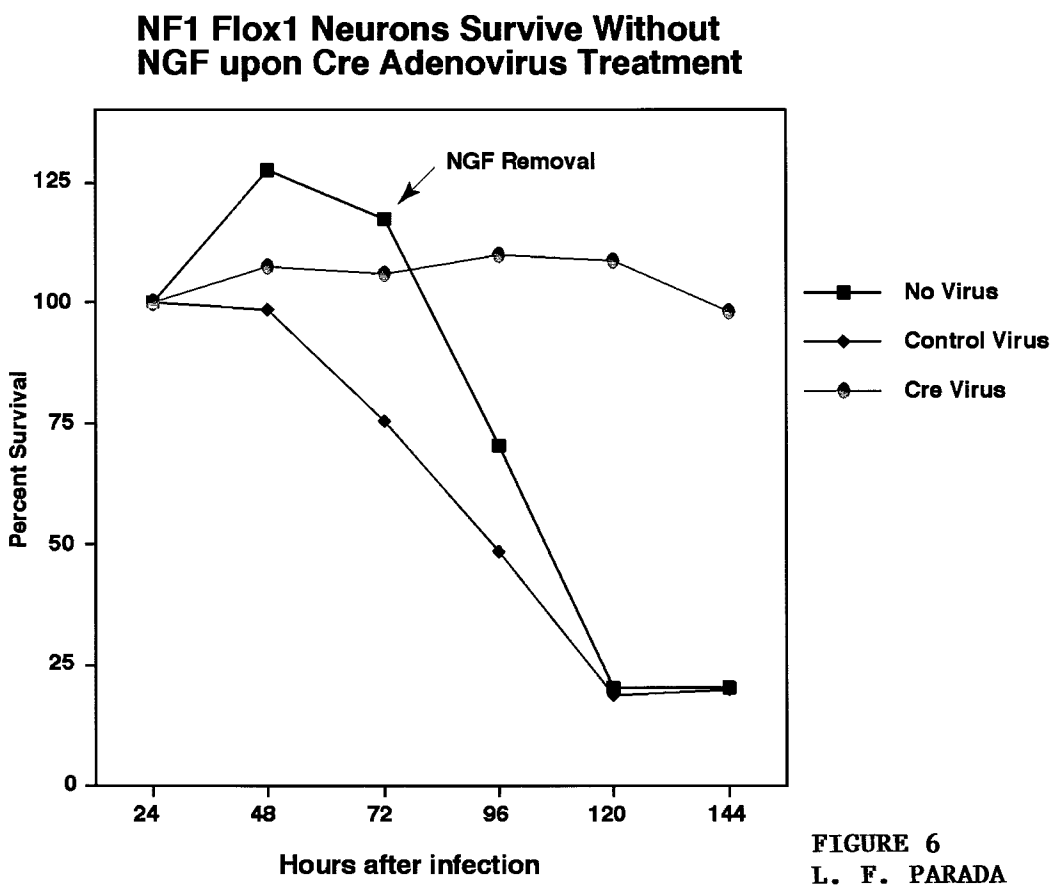
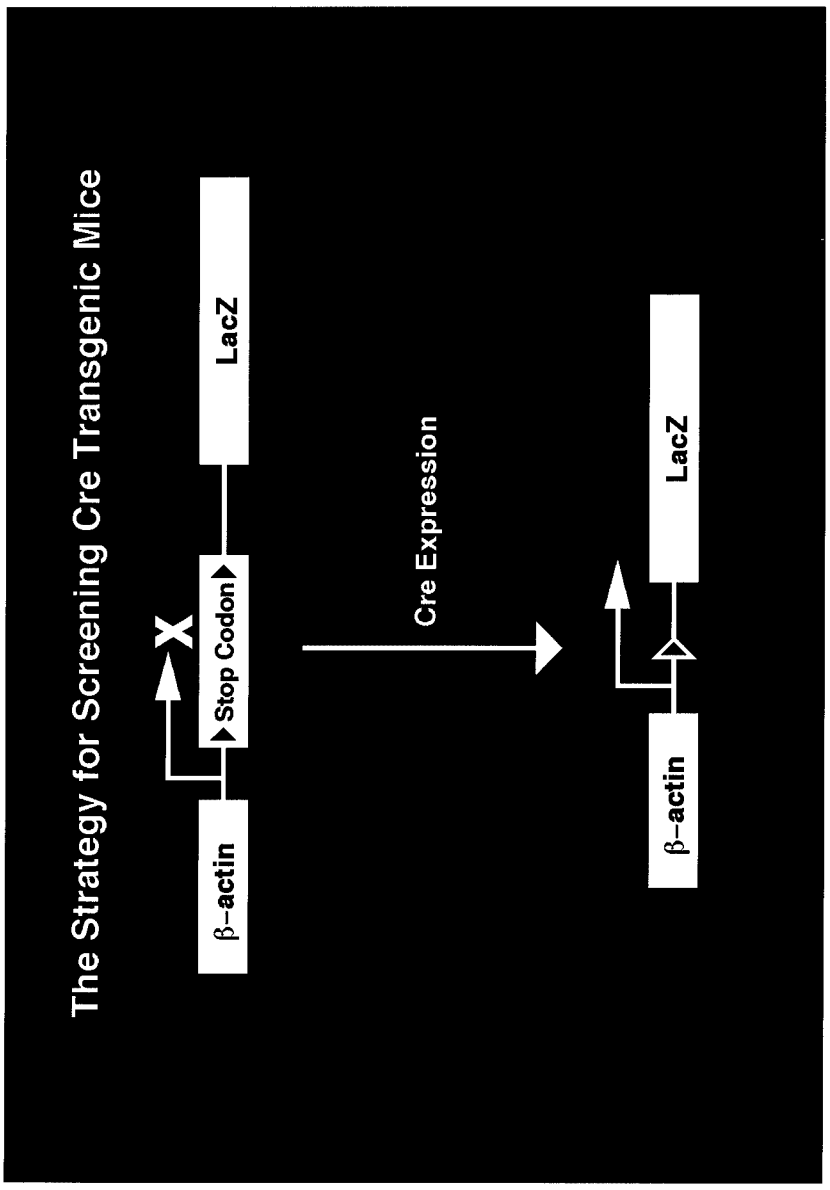


FIGURE 6
L. F. PARADA

FIGURE 7
L. F. PARADA



Cre Expression in the Synapsin I-Cre Transgenic Mice at E12.5

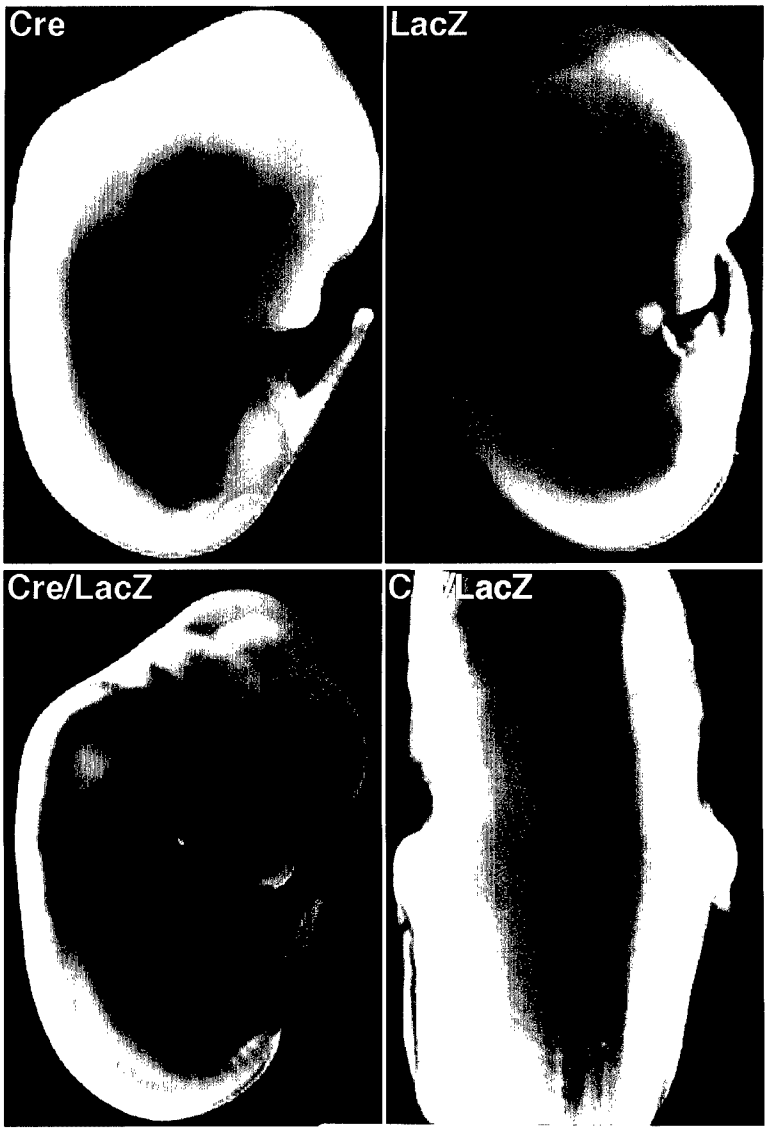


FIGURE 8
L. F. PARADA

Cre Expression in the Synapsin I -Cre Transgenic Mice

E12.5

FIGURE 9
L. F. PARADA

FIGURE 10
L. F. PARADA

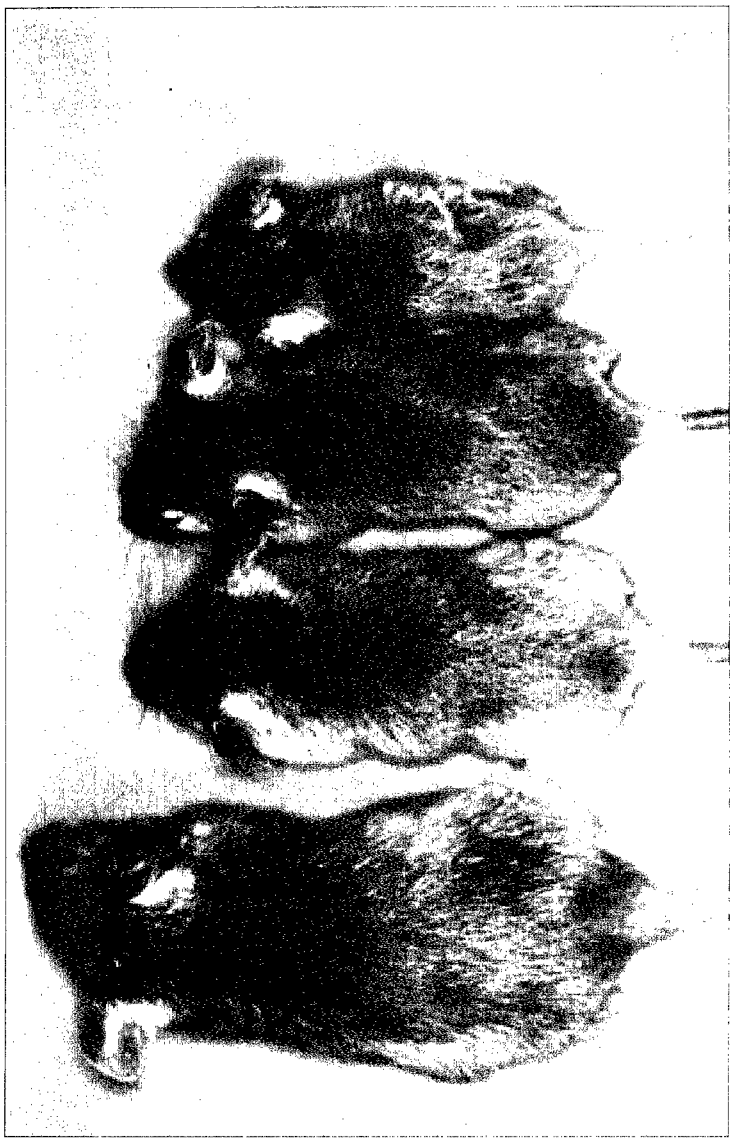
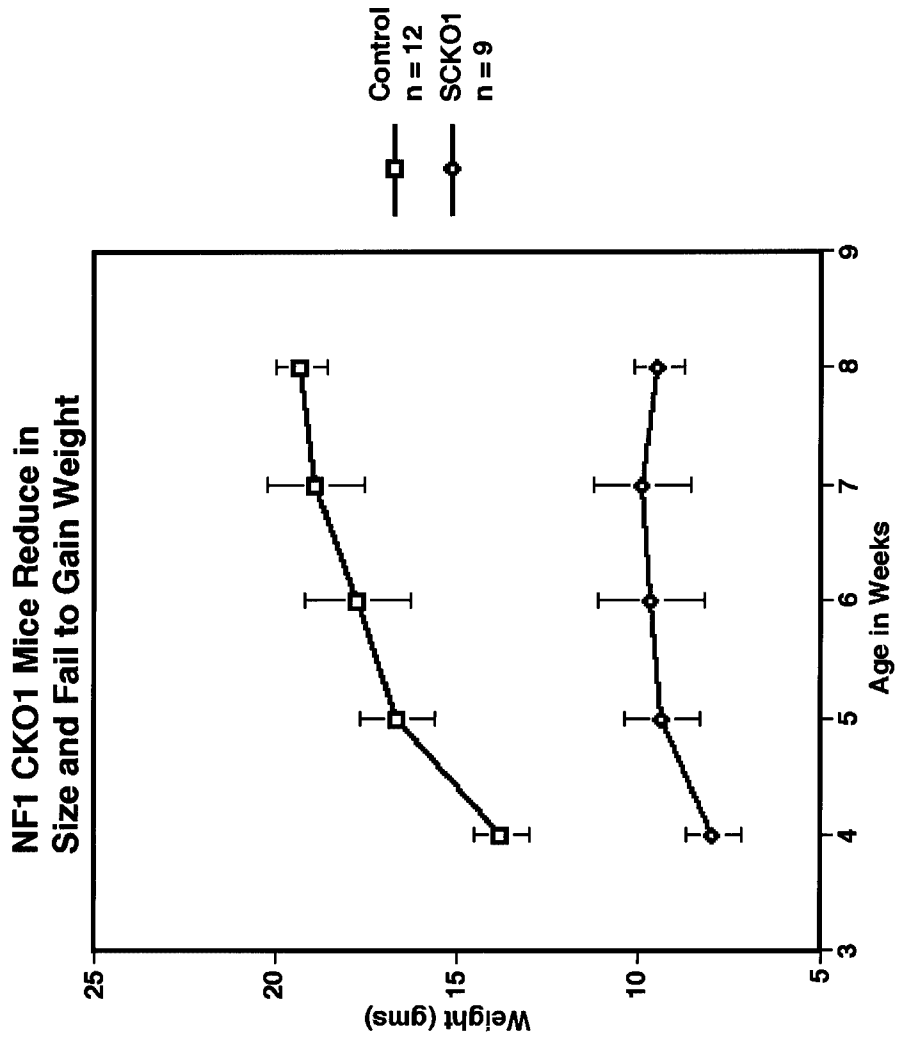


FIGURE 11
L. F. PARADA



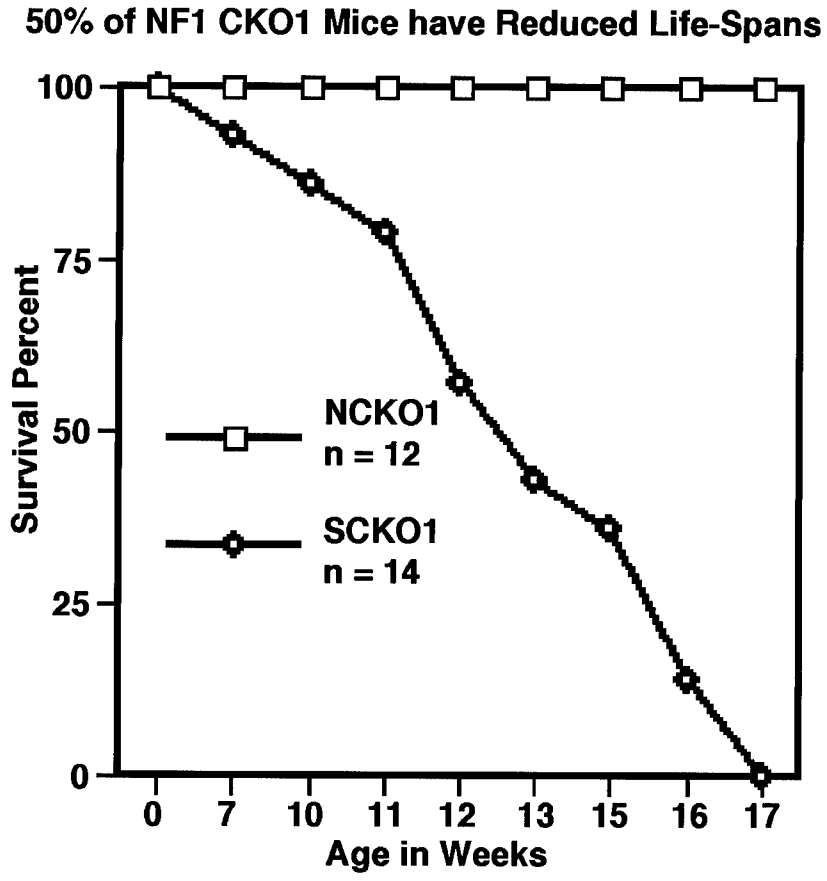


FIGURE 12
L. F. PARADA

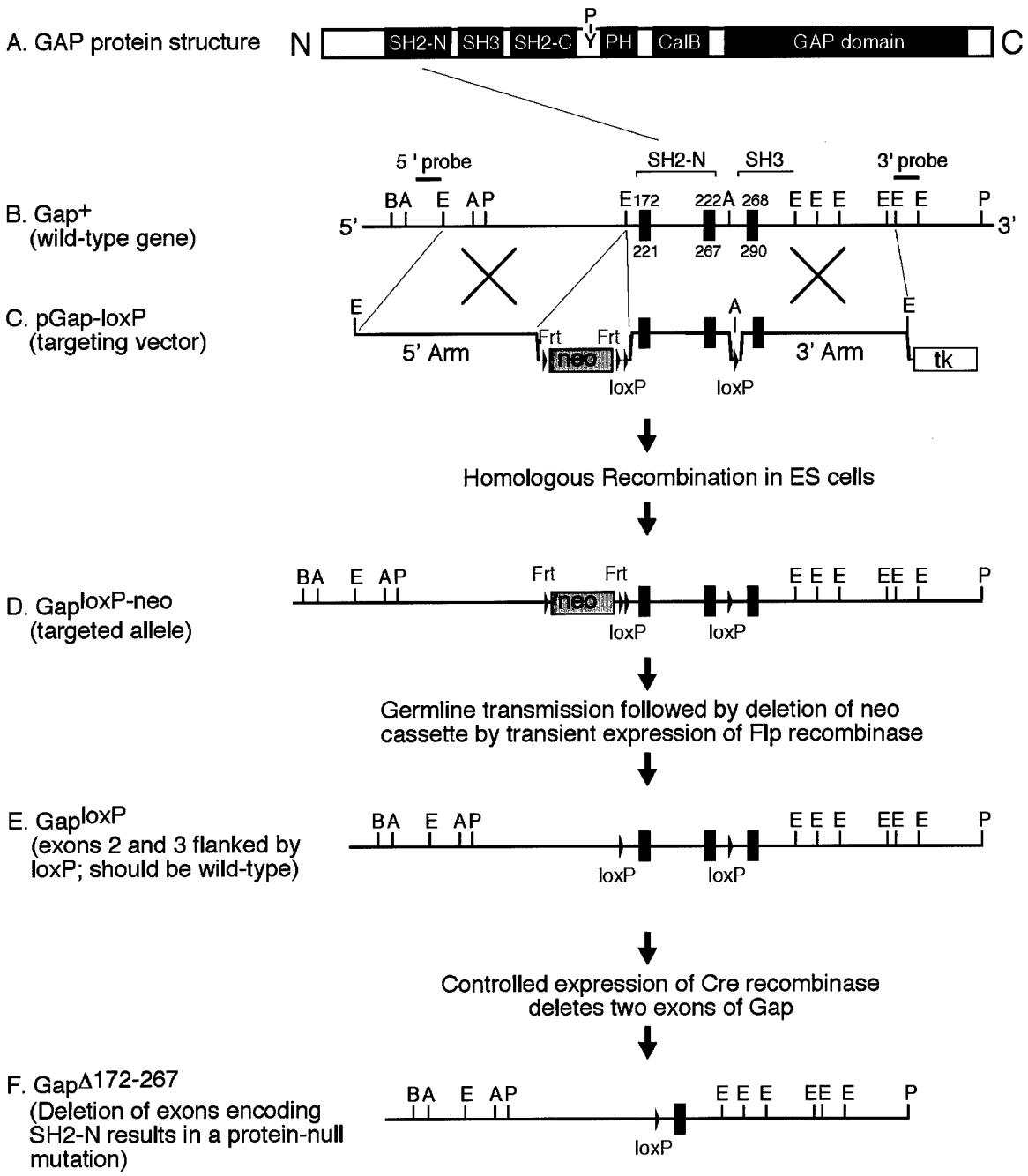


FIGURE 13
L. F. PARADA

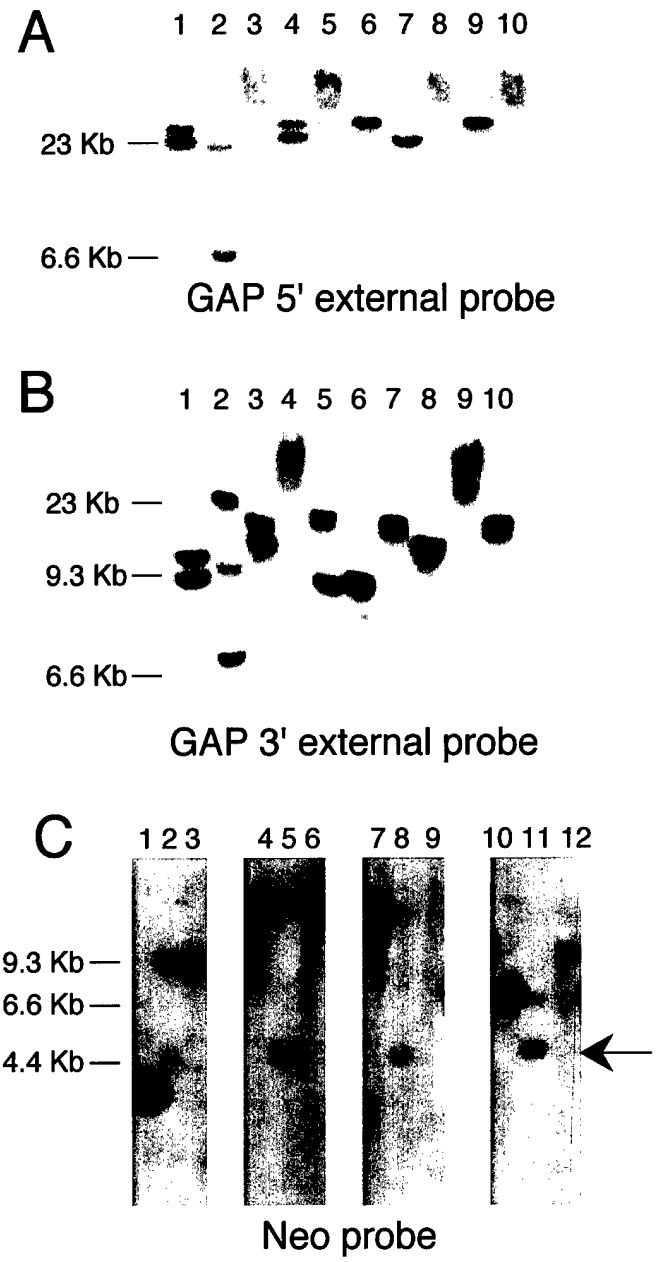


FIGURE 14
L. F. PARADA