A Novel Method for Production of Transgenic Mice from Embryonic Stem Cells

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Contents

Summary

I developed a novel method for production of transgenic mice for the phenotypic rescue experiment of gene knockout mice with ES cells. For this experiment, I showed that puromycin, a protein synthesis inhibitor, can be used for the selection of recombinant ES cells from heterozygously gene-disrupted G418-resistant ES cells. ES cells were killed by culturing with puromycin at a concentration 0.1 μg/ml for 2-days. Puromycin can function independently from G418, because G418-resistant ES cells were also killed under the same conditions. ES cells can acquire puromycin resistance by introducing the *pac* gene, and the recombinant ES cells can survive against the puromycin selection. G418-puromycin double drug resistant ES cells could generate chimeric mice at high rate, and maintained high germline differentiating potency. The *pac* gene was transmitted to offsprings via chimeric mice, and the gene function was maintained among them.

For the rescue experiment by the gene-trap method, I used Fyn knockout mice as a model. GT-2 gene-trap vector was introduced into heterozygously *fyn*-disrupted ES cells. The vector contained the *lacZ* gene and human *fyn* cDNA as reporter genes and the *pac* gene as a selection marker. Among puromycin-resistant ES cells, *lacZ* positive clones were obtained. In these clones, Fyn was also expressed, and the insertions of the GT-2 vector were confirmed by southern blotting analysis. These results indicated that the endogenous promoter could direct the expression of introduced gene. Chimeric mice were produced with the *lacZ* positive clones by the microinjection, and germline chimeras were obtained from three clones. The mice with the purposed genotype [*fyn* (-/-), *gt*/+] were obtained from F1 offsprings between these chimeras and Fyn-deficient mice.

General Introduction

An improvement of the gene targeting method using mouse embryonic stem (ES) cells made it possible for us to analyze functions of the gene *in vivo* by producing the mutant. However, the mutant mice of a certain gene which is expressed in multiple regions might present several types of phenotypic abnormalities (Fig. 1A). In these cases, it is not clear whether these abnormalities are due to abnormality of certain region of the brain or abnormality in certaion stage during development, because the gene expression was lost completely in the mutants. Rescue experiments would be the best way to clarify these correlations. For these experiments, forced gene expression in a restricted region in a knockout mutant would be most appropriate. (Fig. 1B). We usually make transgenic mice by the microinjection of the vector, containing cDNA of the disrupted gene connected to the promoter which is derived from a known gene and confer regional expression, into zygotes of the gene knockout mice in these experiments (Fig. 2A). The correlation could be clarified by the analysis of these rescued mice. Nevertheless, for these experiments, the promoter to directing the expression of the cDNA has to be cloned and well characterized before producing the transgenic mice. Moreover, promoters of the gene directing expression in the specific region of the central nervous system (CNS) have not been cloned yet. If the specific promoters are not available for the rescue experiment, the gene-trap or the enhancer-trap method should be applied to ES cells (Fig. 2B), since the endogenous promoters which are not identified could direct the expression of transgene to the desired region. The gene-trap method has a great advantage in addition to the transgene expression in particular: The promoter of the trapped gene could be cloned by using the sequence of the gene-trap vector. From this advantage, the gene-trap method should be used for the rescue experiments. If we perform the rescue experiment by the gene-trap, the heterozygously gene-disrupted ES cells are preferable to be used because it shortens a period to obtain the transgenic mice with purposed genotypes (Fig. 3A and B). By using these ES cells, the transgenic mice could be obtained in F1 generation, and all the mice in F1 generation could be analyzed as negative or positive controls.

However, the transgenic mice could not be obtained until F2 generation if we start from the wild type ES cells. It takes much longer time and breeding spaces, and much more efforts for us to examine the genotypes of all the mice. However, there are one problem and three questions to overcome in the gene-trap strategy using heterozygously disrupted ES cells for the rescue experiment. The problem is that there are no suitable drugs to be used for the selection of recombinant ES cells, which do not reduce germline differentiating potency of the ES cells after the electroporation of the secondary transgene into G418-resistant ES clone and selection. The *neo* gene could not be used again, because the ES cells have already been selected by G418 resistance for gene targeting. Another selection drug is needed. The questions were as follows: 1) Could the expression of the transgene be directed by the endogenous promoter? The introduced transgene must trap the endogenous promoter to be expressed because it does not contain the promoter. On the other hand, the vector must function in ES cells, since the gene-trap vector for the rescue experiment has much more complex structure than that of the conventional gene-trap. 2) How can I produce chimeric mice from recombinant ES cells efficiently? In the gene-trap method, chimeric mice should be produced from the each of the selected clone independently, because the gene expression depended on a random integration of the gene-trap vector. Nevertheless, we could not treat many embryos with the microinjection method, so I needed another method for production of chimeric mice. 3) Could the transgene be transmitted to offsprings, and could the mice with the purposed genotype be obtained among them?

My purpose in this study is to develop novel methods for overcoming this problem and the questions using Fyn knockout mice as a model. I developed a novel selection method for recombinant G418-resistant ES cells with a protein synthesis inhibitor, puromycin, after introducing the secondary transgene them. Puromycin was shown to function independently from G418, and killed wild type and G418-resistant ES cells efficiently. The ES cells could acquire the puromycin resistance by the electroporation of the *pac* gene, and could be selected by puromycin without affecting the germline differentiating potency. A novel gene-trap vector, GT-2, containing the *lacZ* gene and

human *fyn* cDNA as transgenes and the *pac* gene, was electroporated into the heterozygously *fyn*-disrupted, G418-resistant ES cells and selected by puromycin. The recombinant ES cells which expressed these transgenes were obtained among surviving clones. In chimeric mice production, the aggregation method was examined. By this method, chimeric mice could be produced without the microinjection apparatus. Though germline chimera could be produced by the aggregation method, the result was inferior to that of the microinjection. Consequently, chimeric mice were produced from gene-trapped ES clones by the microinjection. The produced chimeras were crossed with Fyn-deficient mice to obtain F1 offsprings. The transgenic mice with the purposed genotype were obtained among them.

The gene knockout mice could be analyzed in details by the rescue experiment with the gene-trap method.

Figure 1. Rescue experiment of the gene knockout mice to clarify the correlation between a region and a phenotype by the regional gene expression. (A) The knockout of a certain gene which was expressed in wide area might present several types of phenotypical abnormalities. (B) It is possible to clarify the correlation between the region which lost the gene expression and the phenotypic abnormality which is caused by the regional gene expression.

Figure 1

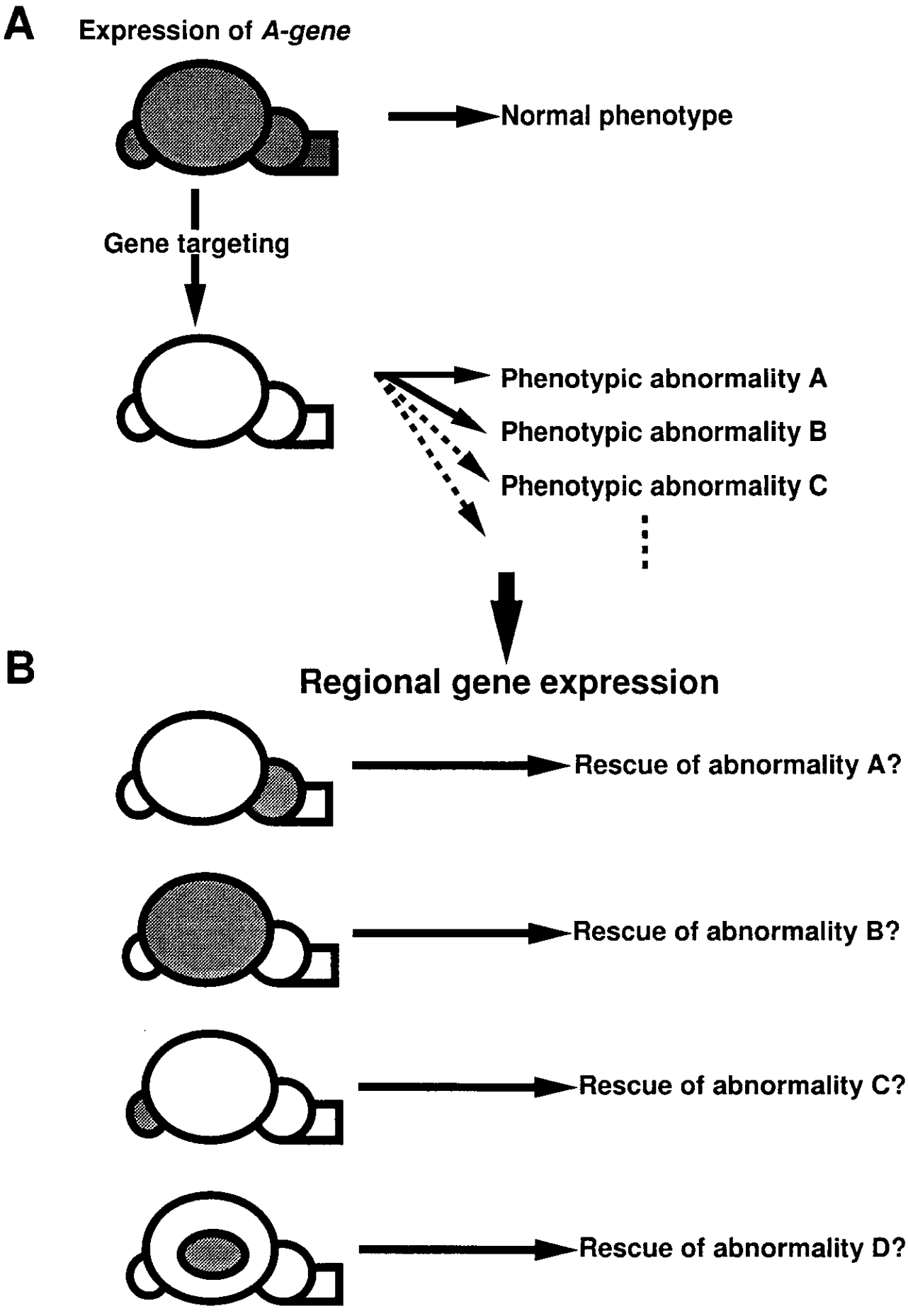
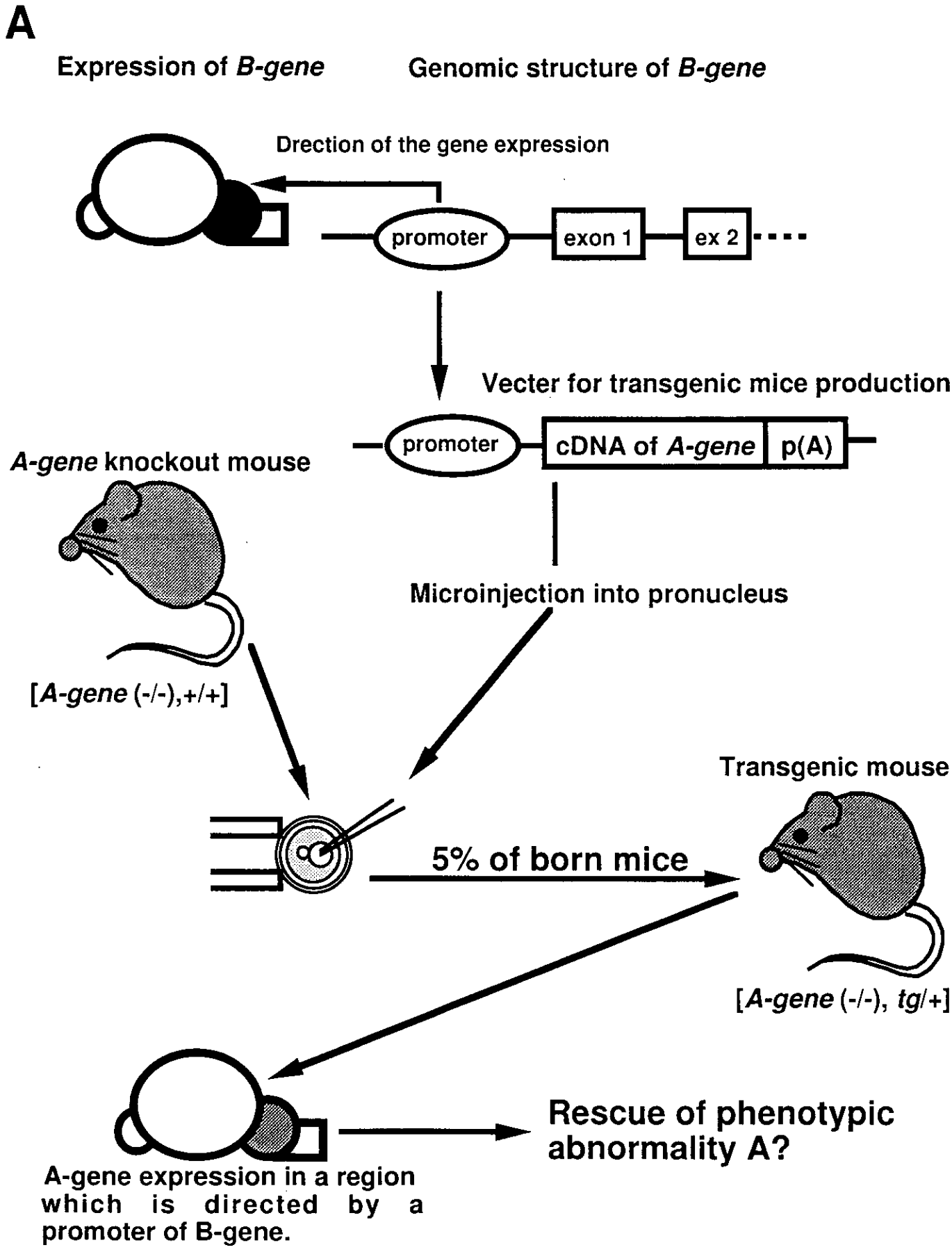

A
Expression of A-gene
Normal phenotype
Gene targeting
Phenotypic abnormality A
Phenotypic abnormality B
Phenotypic abnormality C
B
Regional gene expression
Rescue of abnormality A?
Rescue of abnormality B?
Rescue of abnormality C?
Rescue of abnormality D?

Figure 2. Rescue experiment of the gene knockout mice by introducing the cDNA of the disrupted gene. (A) Production of transgenic mice by introducing a vector which contains the cDNA which is expressed in a region which is directed by the promoter ligated in front of it. (B) If it is not available to use the promoter which directs the gene expression in the purposed region, the gene-trap method should be used with embryonic stem (ES) cells.

Figure 2


A
Expression of B-gene
Genomic structure of B-gene
Drection of the gene expression
promoter
exon 1
ex 2
Vecter for transgenic mice production
promoter
cDNA of A-gene
p(A)
A-gene knockout mouse
Microinjection into pronucleus
[A-gene (-/-),+/+]
Transgenic mouse
5% of born mice
[A-gene (-/-), tg/+]
Rescue of phenotypic abnormality A?
A-gene expression in a region which is directed by a promoter of B-gene.

B

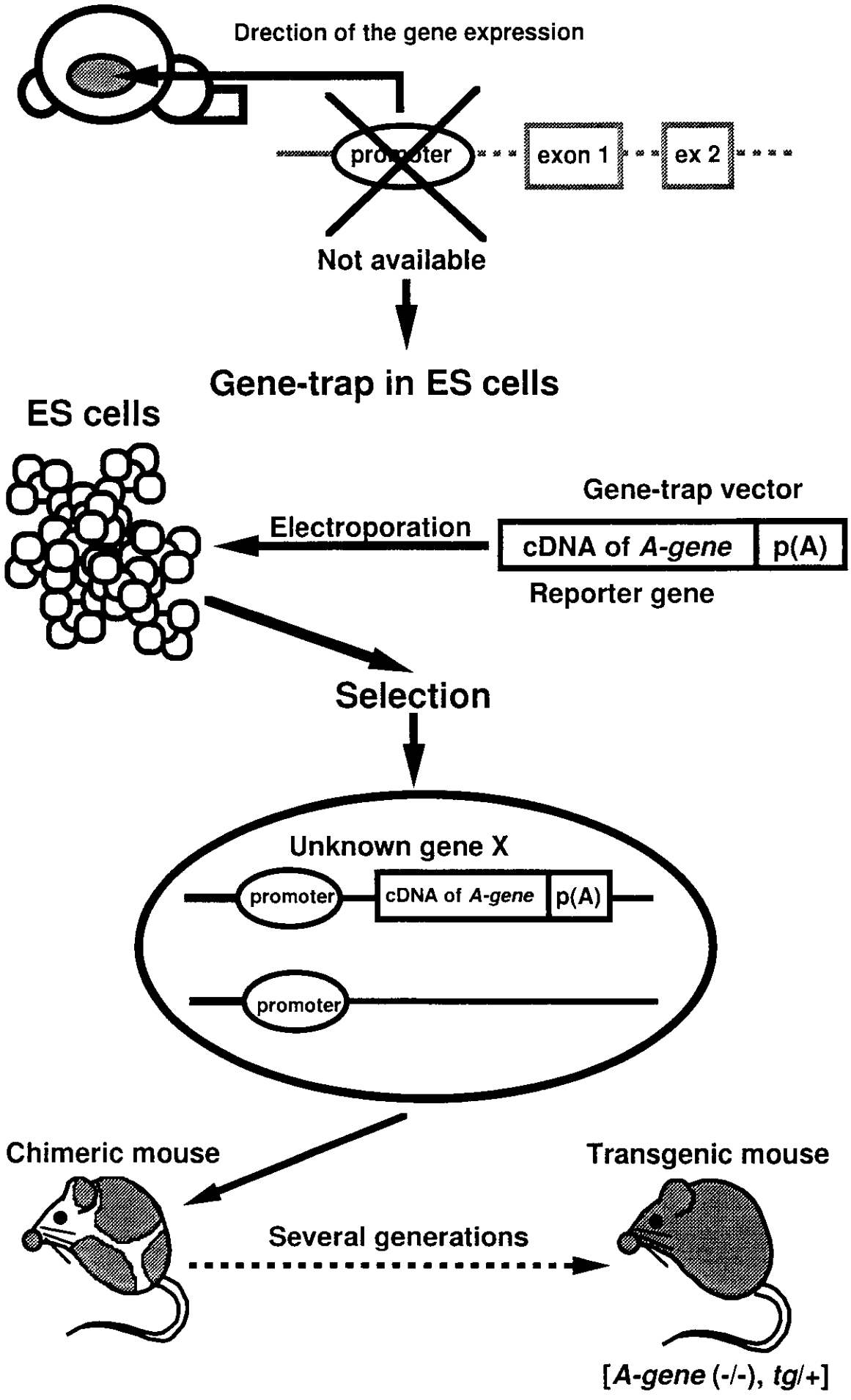
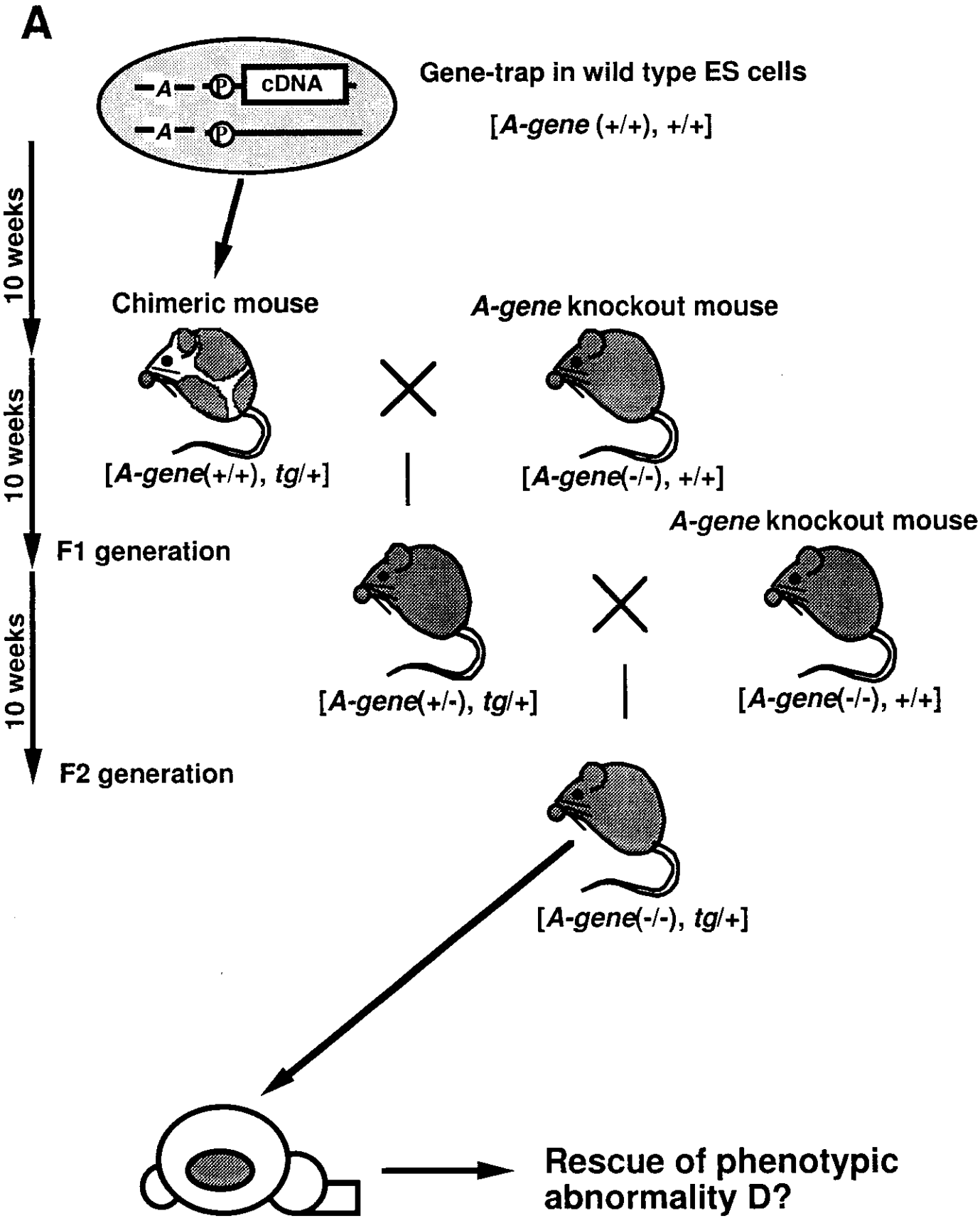

Drection of the gene expression
promoter
exon 1
ex 2
Not available
Gene-trap in ES cells
ES cells
Gene-trap vector
Electroporation
cDNA of A-gene
p(A)
Reporter gene
Selection
Unknown gene X
promoter
cDNA of A-gene
p(A)
promoter
Chimeric mouse
Transgenic mouse
Several generations
[A-gene (-/-), tg/+]

Figure 3. Comparison of the gene-trap in wild type (wt) ES cells and heterozygously gene-disrupted ES cells. (A) The gene-trap with wt ES cells takes about 30 weeks (more than half a year) to obtain the mice which have the purposed genotypes after screening of the ES cells. (B) The gene-trap with heterozygously gene-disrupted ES cells takes about 20 weeks.

Figure 3


A
A
P
cDNA
A
P
Gene-trap in wild type ES cells
[A-gene (+/+), +/+]
10 weeks
Chimeric mouse
A-gene knockout mouse
[A-gene(+/+), tg/+]
[A-gene(-/-), +/+]
10 weeks
F1 generation
A-gene knockout mouse
[A-gene(+/-), tg/+]
[A-gene(-/-), +/+]
10 weeks
F2 generation
[A-gene(-/-), tg/+]
Rescue of phenotypic abnormality D?

B

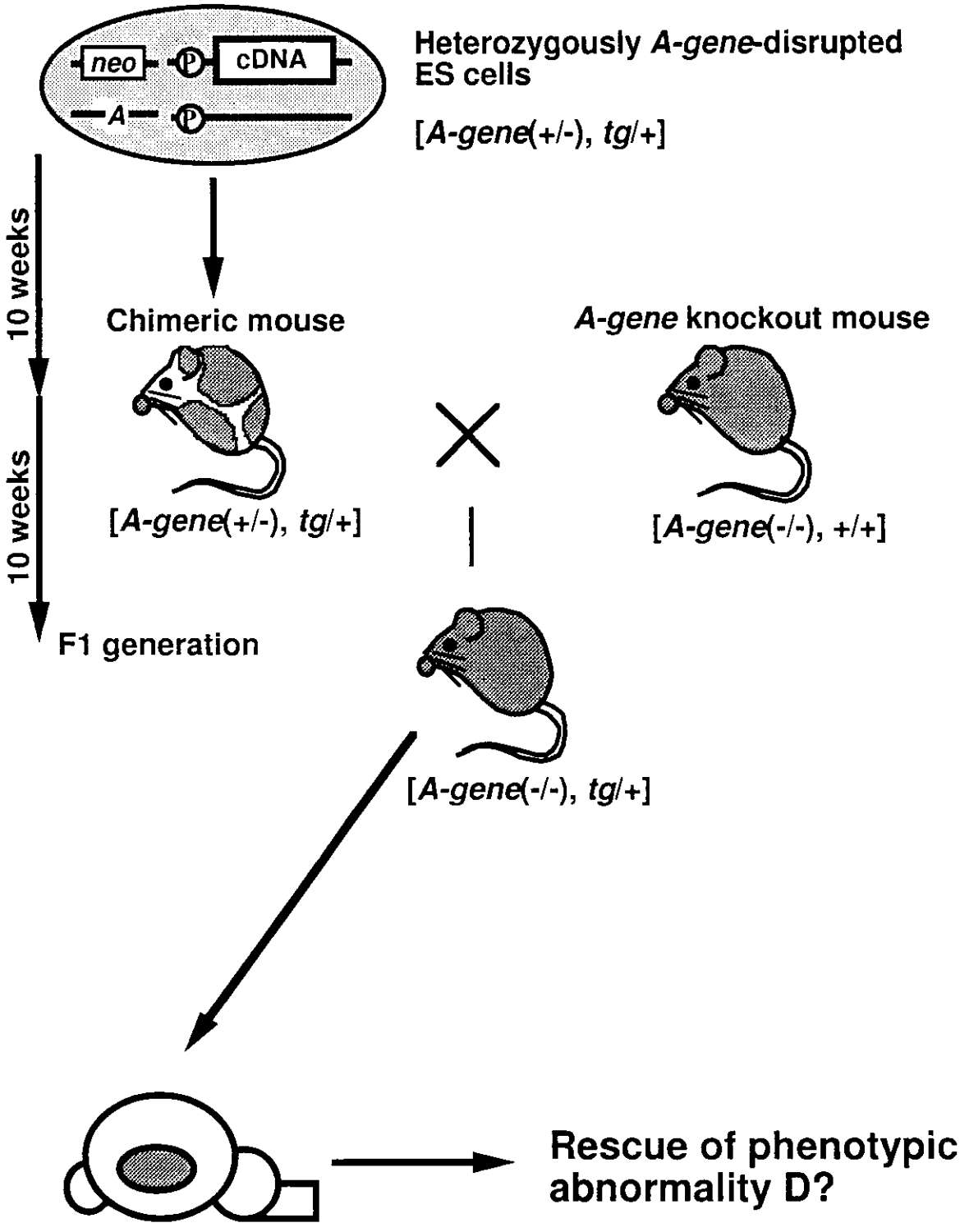
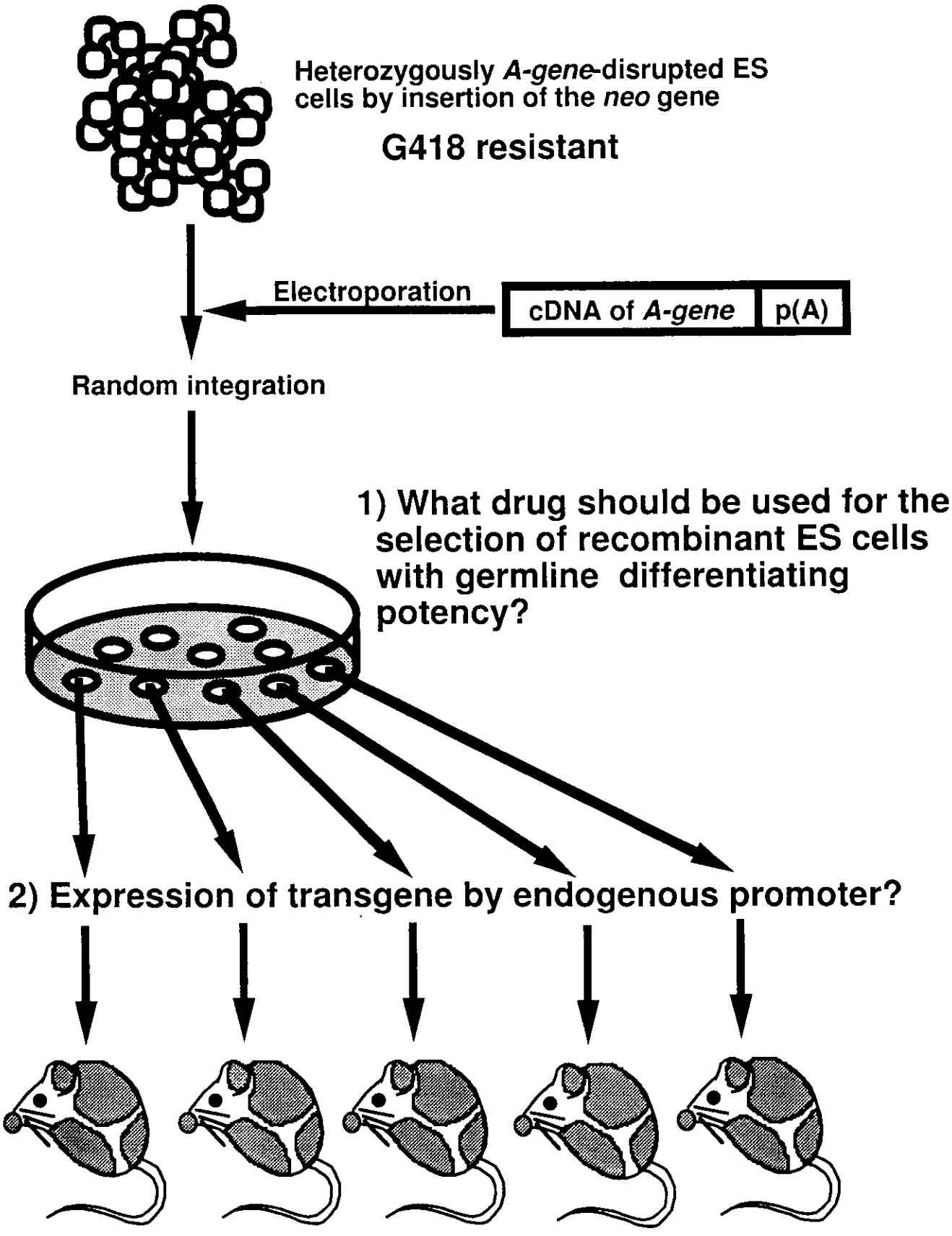

neo
cDNA
A
Heterozygously A-gene-disrupted ES cells
[A-gene(+/-), tg/+]
10 weeks
10 weeks
Chimeric mouse
[A-gene(+/-), tg/+]
A-gene knockout mouse
[A-gene(-/-), +/+]
F1 generation
[A-gene(-/-), tg/+]
Rescue of phenotypic abnormality D?

Figure 4. Four problems are lying in the gene-trap with heterozygously gene-disrupted ES cells for the rescue experiment. 1) What drug should be used for the selection of recombinant ES cells with germline differentiating potency after the introduction of the secondary transgene. 2) Can the transgene be expressed by the direction of the endogenous promoter? 3) The chimeric mice must be produced independently from the each recombinant ES clone as the transgene would be integrated randomly. 4) Can the mice with the purposed genotype?

Figure 4

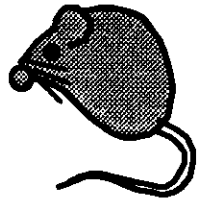

Heterozygously *A-gene*-disrupted ES cells by insertion of the *neo* gene
G418 resistant
Electroporation
cDNA of *A-gene*
p(A)
Random integration
1) What drug should be used for the selection of recombinant ES cells with germline differentiating potency?
2) Expression of transgene by endogenous promoter?
3) Efficient production of chimeric mice?

Chimeric mouse

[*A-gene*(+/-), *tg*/+]

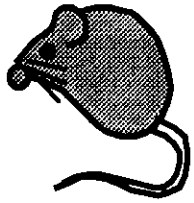
×

A-gene knockout mouse



[*A-gene*(-/-), +/+]

F1 generation



[*A-gene*(-/-), *tg*/+]

4) Can the mice with objected genotype be obtained in F1 generation?

Part 1

Stable Production of Mutant Mice from Double Gene Converted ES Cells. -use of Puromycin and Neomycin resistance as selection markers-

Introduction

Mutant mice production by gene targeting or gene trapping in ES cells has greatly developed mammalian genetics. Efficient colonization into the germline of gene-converted ES cells has primarily been obtained by the *neo* gene followed by G418 selection (1-3). Efforts are being made to utilize other dominant selection drugs, because double gene-converting methods are useful for complete disruption of two alleles of a certain gene in ES cells, simultaneous disruption of two closely linked genes, or to reverse abnormalities of gene-disrupted mice using ectopic expression of a gene. Only double dominant selection of G418 and hygromycin (hyg) has been reported in homozygous gene disruption in cultured ES cells. All the phenotypes in these experiments have been analyzed using ES cells (4) or chimeric mice (5). Gene-disrupted bone marrow cells of the chimeric mice are particularly advantageous for immunological studies, when the cells are transplanted into immune disrupted mice by X-ray irradiation (6). Since the double gene-converted experiments have been limited to the stage of chimeric mice, no evidence has been reported of germline differentiating potency in ES cells doubly selected by G418 and hyg. Other double-drug selections with the *neo* gene and thymidine kinase (7) or hypoxianthine phosphoribosyltransferase genes (8) can be employed to enrich homologous recombinants by positive-negative selection. In this type of selection, however, the negative selection marker genes were removed at the step of homologous integration and only one dominant selection marker remained (7) .

Fyn-deficient mice appeared to have several types of neurological dysfunction: abnormalities in suckling (9), spatial learning (10), and emotional behavior (11) and susceptibility to audiogenic seizure (12). Since Fyn is widely expressed in the central nervous system through the developmental stage (13), it is not clear whether these abnormalities are derived from any brain region or any developmental stage. To determine this, in addition to G418, establishing of a secondary dominant drug which does not inhibit the germline differentiating potency of the ES cells would be advantageous. Gene rescue experiments were individually performed on gene-disrupted

mice. Knockout mice were crossed with transgenic mice which had been produced independently. In this case, F2 offspring from chimeric or transgenic mice were the first generation in which the reverse phenotypes could be analyzed in the absence of a gene. If, however, the transgene could be integrated in the heterozygously gene-disrupted ES cells which are resistant to G418, I can analyze them in the F1 generation by crossing the chimeric male and the gene knockout female. This F1 analysis would make systematic rescue analyses of, for instance, promoter trapping in many chimeric lines possible. Improvement of a secondary dominant drug will allow us to obtain the transgenic mice for the rescue experiment in F1 generation.

An antibiotic of puromycin inhibits growth of animal cells by blocking protein synthesis, because it is a structural analogue of a tRNA molecule linked to an amino acid; the ribosome mistakes it for an authentic amino acid and covalently incorporates it at the carboxyl terminus of the growing polypeptide chain, thereby causing the premature termination and release of this polypeptide. The puromycin-resistant gene was isolated from *Streptomyces aboniger*, and it is encoded by the *pac* gene (14). If expressed in animal cells, this *pac* gene rescues the growth and the protein synthesis in puromycin (15). Here, I provide the first evidence that, in addition to combination of G418 and the *neo* gene, the *pac* gene functions as a dominant selection marker for the ES cells, and these selected cells possess germline-differentiating potency, and that the puromycin resistance stably transmitted into F1 generation of germ-line chimeras, and that puromycin could be used in the stable production of double gene-converted mice.

Materials and Methods

Construction of vectors

The *pac* (puromycin-resistant) gene was inserted between the phosphoglycerate kinase-1 promoter and the polyadenylation signal of the vector, pGKPuro, a gift from Peter W. Laid (Fig. 1) (21).

Cell culture, Electroporation and Screening of recombinant ES cells:

Embryonic stem (ES) cell line

TT2 line was established from an F1 embryo between a C57BL/6 female and a CBA male as F1/1 cells, and had Agouti coat color genetic background and XY karyotype (2) Fz45 line, derived from TT2 line, in which fyn locus was heterozygously targeted by a insertion of one copy of *neo* gene (2). E14 line was derived from an 129/Ola embryo and had a Chinchilla coat color genetic background (16). Table 1 shows all the cell lines and the mouse strains used in this report.

ES cell culture

ES cell culture procedures were similar to those described previously (2). All the frozen ES cell lines were quickly thawed and transferred to a 15 ml tube. ES cell medium [ESM; Dulbecco's modified Eagle's medium (high glucose, GIBCO), 20% fetal calf serum (Biological Industries), 10^4 units of leukemia inhibitory factor (AMRAD, ESGRO), 10^{-4} M β-mercaptoethanol, 0.1 mM nonessential amino acids (Flow Lab.), 1 mM sodium pyruvate (Flow Lab.)] was added and the tube was centrifuged. The ES cells were re-suspended in 5 ml of ES medium, and seeded at 1 X 10^6 cells on a feeder layer in 60 mm dish. The cells were incubated in 95% air/5% CO_2 at 37°C for three days until they became confluent with changing the medium every day. The cells were passaged once in three days at the same density.

Condition for puromycin selection of recombinant ES cells.

Fz45, TT2 and E14 cell-lines were expanded at 1 X 10^3 cells/60 mm tissue culture dishes coated with gelatin (sigma) in ES medium. 24 hr later, the medium was changed to a puromycin-containing medium, and the ES cells were cultured in concentrations of puromycin from 0.05 to 0.5 μg/ml for several days (upto 10 days).

Preparation of embryonic fibroblast (EMFI) cells

Preparation of embryonic fibroblast cells was described in (17). EMFI cells were used as feeder cells for culturing ES cells and for an experiment analyzing the functions of *pac* gene in F1 mice. EMFI cells were established from 14-day embryos of mice. A pregnant mouse was killed by cervical dislocation, and the uterus was dissected out. The uterus was washed with PBS, and the embryos were dissected away from the uterus. After removing livers, the embryos were minced in PBS into cubes. 5 ml of trypsin/EDTA [0.1% trypsin (GIBCO) and 0.02% EDTA in PBS, pH 7.2] per an embryo was flushed into dishes. Cubes were transferred to a 50 ml Falcon tube, and incubated at room temperature for 20 min with gentle shaking. After incubation, the tubes were kept still for 1-2 min to precipitate the debris, and the supernatant was transferred to fresh tubes. An equal volume of FM [10% FCS + DMEM] was added to inactivate trypsin. The tubes were centrifuged at 1000 rpm for 5 min at 4°C to collect trypsinized cells. The pellets were re-suspended in FM and plated at a density of 1-5 X 10^7 cells/10 cm dish. The cells were cultured until they became confluent for 2-3 days. After cultivation, cells were trypsinized, centrifuged at 1000 rpm 5 min at 4°C, re-suspended in FM containing 10% DMSO at 3 X 10^5 cells/ml and frozen as stocks.

Feeder cells:

The TT2 ES cell line was established using primary embryonic fibroblast (EMFI) cells as a feeder layer, and EMFI cells were routinely used for cultivations of ES cells. Preparations of EMFI cells are described below.

Preparation of feeder cells

EMFI cells were prepared by thawing frozen stock. The thawed cells were washed with FM, and splited on to 3-5 100 mm dishes, containing FM. The cells were incubated at 37°C in 95% air/5% CO_2 until they formed confluent monolayer (for 2-3 days). The medium was removed from the confluent dishes and 5 ml of fresh FM containing 10 μg/ml mitomycin C was added. The cells were incubated for 2-3 h and washed three times with 10 ml of PBS per dish. The cells were trypsinized and re-expanded on 60 mm dishes at 1.2 X 10^6 cells/dish in FM. The cells were allowed to attach to the dish overnight.

Electroporation and selection of recombinant ES cells

Electroporation conditions were similar to those that have been described (2).The ES cell line, Fz45 in which one copy of *neo* gene was inserted into the*fyn* gene locus, was cultured on embryonic fibroblast feeder layers that had been treated with mitomycin C as described above. For electroporation, cells were trypsinized and singly re-suspended at a concentration of 4 X 10^7 per milliliter in HBS (25 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM dextrose); cell suspension was electroporated at room temperature in a cuvette with path length of 0.4 cm with one microgram of pGKPuro vector DNA linearized by *XbaI*-digestion by using Bio - Rad Gene Pulser (250 V, 960 μF). The treated cells were plated at 3 X 10^6 cells per 9-cm dish. ES cells were then cultured for 24 h, and selected with 0.1 μg/ml of puromycin (Sigma) for 8 days. After selection, drug resistant colonies were picked, re-expanded on freshly prepared EMFI feeder layers, and screened by PCR (see below). 5 clones of FzP1-5 was confirmed to be the pac gene positive (Fig. 1).

Productions of chimeric mice

Procedures for production of chimeric mice were similar to those described (2).

The oviducts of superovulated CD-1 females were flushed with M2 medium [NaCl 94.6 mM, KCl 4.8 mM, $CaCl_2$ 1.71 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM,

$NaHCO_3$ 4.15 mM, HEPES 20.9 mM, sodium lactate (Sigma) 23.3 mM, sodium pyruvate (Flow Lab.), 0.33 mM, glucose 5.56 mM, BSA (Sigma A-4378) 0.4% (w/v) and Kanamycin A 0.03% (Sigma, w/v) pH 7.2] (17) 50-54 h after treatment with human chorionic gonadotropin to collect 8-cell stage embryos. The collected embryos were cultured in microdrops of M16 [NaCl 94.6 mM, KCl 4.8 mM, $CaCl_2$ 1.71 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, sodium lactate 23.3 mM, sodium pyruvate 0.33 mM, glucose 5.56 mM, BSA 0.4% (w/v) and Kanamycin A 0.03% (w/v) pH 7.2] (17) under paraffin oil at 37°C in 95% air/5% CO_2 for 1-2 h.

ES cells were treated with 0.1% trypsin solution, re-suspended in ES medium and re-expanded to remove feeder cells on tissue culture dish. After 1-h incubation, ES cells were centrifuged and re-suspended in M2 medium + 10% FCS. In small drops of the M2 medium + 10% FCS, microinjection was performed with a micromanipulator (Nikon/Narishige). Ten to twelve ES cells were injected into the perivitelline space of an eight-cell stage CD-1 embryo. Then the embryos were allowed to develop into morulae or blastocyst stage in drops of M16 medium + 10% FCS under 95% air/5% CO_2 at 37°C (O/N) and transferred to the uteri of 2.5 day pseudopregnant CD-1 females. Chimeric pups were identified by the presence of Agouti hair, and chimerism was judged by coat color pigmentation. The chimeras were bred with CD-1 females to check for contribution of the ES cells to the germ line. Genomic DNA of F1 generation was also purified and analyzed by PCR.

Genotype analysis

Preparation of genomic DNA

genomic DNAs from ES cells and tails were prepared according to Laird ey al. (18). The lysis buffer [100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml proteinase K] was added to the tissue or cells. They were incubated at 55°C overnight, washed with an equal volume of phenol, phenol-chloroform and chloroform, and precipitated by isopropanol.

PCR analysis

0.1 μg/ml genomic DNA was dissolved in 25-μl (final volume) of 1X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 μM $MgCl_2$, 1% TritonX-100), containing 0.2 mM dNTPs, 0.2 mM primers, and 50 U/ml Taq polymerase. For the *pac* gene, 4% glycerol was additionally mixed with this reaction buffer, and the PCR was performed at 30 cycles of reaction at 96°C for 45 sec, at 58 °C for 25 sec, and at 72 °C for 3 min. Primers used were 5'Pac (5'-ATG ACC GAG TAC AAG CCA C-3') and 3'Pac (5'-TTA CGG GTC ATG CAC CAG GA-3'). For the *neo* gene-inserted *fyn* locus, PCRs were performed with each primer, FAP0 (5'-TGC ACA CTT AAG TAG GCT-5') and AGN0 (3). The products were analyzed by 2% agarose gel electrophoresis.

Confirmation of transmission of puromycin resistance to F1 generation.

EMFI cells, which were prepared from F1 embryo between chimera mouse and CD-1 female, were expanded at 1 X 10^5 cells/60 mm tissue culture dishes in FM. Medium was changed to the puromycin containing medium 24 h later, and cells were cultured in the presence of puromycin at concentrations ranging from 0.1 to 5 μg/ml for 7 days.

Results

Puromycin sensitivity of ES cells.

To determine the most appropriate concentration of puromycin for the selection of ES cells, drug sensitivity was examined by culturing the ES cells at various concentrations of the drug. ES cell lines, wild type TT2, G418-resistant Fz45 derived from TT2 and E14 cells (Table 1) were cultured in the presence of puromycin of 0.05 to 0.5 μg/ml on gelatin coated dishes for ten days. In these cell lines, no colonies could be obtained at puromycin concentrations of higher than 0.1 μg/ml (Fig. 2A). At these concentrations, cells died after culturing for more than two days (Fig. 2B). This suggested that the pac gene-containing recombinants could be obtained by selection in 0.1 μg/ml for two days, and that puromycin could be used to select cells independently from G418 and *neo* gene combination. I also examined the optimal dose of puromycin for feeder cells which were produced from primary fibroblasts of 14-day wild type embryos; most feeder cells survived at 0.1 μg/ml but died at 0.5 μg/ml. I therefore used this concentration for selection of recombinant ES cells on the feeder cells which did not contain *pac* gene.

Isolation of puromycin and G418-resistant ES cells.

An undifferentiated, highly pluripotent G418-resistant ES cell line Fz45 (10) was electroporated with pGKPac (Fig. 1). After electroporations of 5 X 10^7 Fz45 cells with pGKPac, cells were submitted to selection with 0.1 μg/ml puromycin for 8 days on wild-type feeder cells, and 5 colonies which were puromycin-resistant were obtained with the plasmids of pGKPac (Table 2). Surviving colonies were isolated and expanded, and their DNA was isolated from individual clones to identify recombinant directly by PCR for the presence of the *pac* gene (Fig. 3). The *pac* gene was found in all cells. However, only few clonies (5; Table 2) were obtained by puromycin selection on wild type feeder cells.

Efficient colonization of somatic tissues and germline.

I tested the totipotency of the 4 ES cell lines, Fz45 and FzP1 by producing chimeric

mice. The multipotency of the Fz45 cell lines (agouti coat color and black eyes) was first judged by the coat color of pups after cells were injected into eight-cell stage embryos (Table 3). In control experiments, Fz45 of parent cells yielded 18 chimeras from 180 embryos. In puromycin and G418-resistant cells, FzP1 yielded 20 chimeras from 80 embryos. The 100% chimeras in which ES cells were nearly dominant, were obtained from double drug-resistant ES cells (data not shown). Germline-differentiating potency of the double drug-resistant ES cell lines was tested on these male chimeras by mating with albino CD-1 females. 12 chimeric males (60% of chimeras) from FzP1 produced ES-derived F1 offspring exclusively. Thus double drug-selected FzP1 derived from TT2 cells had similar potency to TT2 cells (2), therefore puromycin and G418 barely influenced the stability of totipotency and germline differentiating potency of TT2 cells in culture. I also confirmed that F1 offspring derived from double drug-resistant ES cells had both the *pac* gene and the *neo* gene inserted into the *fyn* locus in the context of Mendelian ratio by PCR. Fig. 4A and B show the PCR products of genomic DNAs from 14 F1 mice between a chimeric male and a CD-1 female. Six mice contained the *pac* gene, and 8 mice contained the *neo* gene. The bands of the each gene appeared independently.

Transmission of puromycin resistance from ES cells to the F1 generation of germ-line chimeras.

To determine if there is still puromycin resistance after transmission into transgenic mice, primary cultured embryonic fibroblast cells of E14.5 embryos between a chimera and CD-1 were tested for puromycin resistance. Their genotypes were determined by PCR analysis (data not shown). Fibroblast cells of each genotype (*pac* positive, *neo* positive and wild type) were cultured in the presence of puromycin ranging from 0.1 to 5 mg/ml for 7 days. Only in explants from *pac*-positive embryo were the cells resistant to puromycin (1 μg/ml). Examples of mbryonic explant cultures from wild type and *neo*-positive were shown in Fig. 5B and C. All cells from embryos whose DNA was negative for the *pac* gene died during 7 days of the selection. These results conclusively show that

ES cells can stably transmit puromycin resistance through the germ-line and into the following generations, and that *pac* gene and *neo* gene function independently.

Discussion

These experiments have demonstrated that the antibiotic puromycin is effective in the selection of ES cells with high germline differentiating potency. It works at lower concentration of 0.1 μg/ml for the ES cells than 1.0 μg/ml required for feeder cells. This suggests that puromycin blocks the growth of strains with higher growth rate such as the ES cells (cell cycle of ES cells is 20-22 hr) more efficiently. In comparison with other conventional selection drugs, G418 and hyg, the period of selection with puromycin is two days, much shorter than any other selection drug (six to ten days). Since the selection period of ES cells influences their germline differentiating potency, it seems that puromycin is superior as a dominant selection marker to G418 or hyg.

These results also indicated that puromycin could be used together with G418. As described in the Introduction, a drug which could be used secondary to G418 with stable germline differentiating potency is useful for our systemic phenotypic rescue experiments. Puromycin is one of the best candidates for our future analyses on the function of Fyn which regulates suckling, learning or emotional behavior, or ordered cell layers formation of the hippocampus. The drug might also be useful for secondary gene targeting for another genetically linked locus such as the gene of a Hox cluster. Double drug selections have usually been used for the complete disruption of two alleles of a certain gene in ES cells. However, more precise analysis of chimerism, such as GPI (glucose phosphate isomerase) assay, should be performed using several organs of the chimeric mice. I obtained 100% chimeric mice from several ES cell lines selected by puromycin as judged by hair and eye color, male distortion and body size; CD-1 and chimeric mice (body weight 25-28 g) with less than 100% chimerism were larger than the F1 between C57BL/6 and CBA mice (body weight 20-23 g) (data not shown). Using puromycin and *pac* gene, mutant mice production could greatly improve mammalian genetic studies.

Table 1. Mouse strains and cell lines used in this report.

Mouse	Genotype	Genetic background and coat color
CD-1(ICR)	wt	outbred strain, albino
CBA	wt	inbred strain, agouti
C57BL/6	wt	inbred strain, black
129SV/Ola	wt	inbred strain, chinchira
Cell line		
TT2	wt	CBA X C57BL/6 F1, agouti
Fz45	fyn(+/-), G418-r	derived from TT-2, agouti
E14	wt	derived from 129SV/Ola, chinchira

Table 2. Frequencies of surviving colonies by puromycin selection.

Electroporated vector	No. of cells electroporate	No. of surviving colonies
PGK-pac	2×10^7	5
PGK-neo	2×10^7	0

Table 3. Comparison of germ-line differentiating potency between G418-resistant and G418-puromycin double resistant ES cell.

ES cell line	Transgene	No. of embryo transplanted	No. of pups born	(%)	ES contribution in coat		Germ-line chimeric	Germline chimeric /Chimeric (%)
					Chimeric	Non-Chimeric		
neo								
Fz45	PGK-neo	180	36	(20)	18	18	8	44
neo and *pac*								
FzP1	PGK-pac	80	26	(35)	20	6	12	60

Table 4. Resistance to puromycin of embryonic fibroblast cells derived from offsprings of chimera

Cell line	Genotype	Growth in puromycin-containing medium (μg/ml)				
		0	0.1	0.5	1	2
3	[+/+, +/+]	+++	++	-	-	-
5	[neo/+, +/+]	+++	+++	-	-	-
8	[+/+, *pac*/+]	+++	+++	+++	++	-

Figure 1. Schematic showing the transgenes used to drive the expression of the puromycin-resistant gene. pGKPac vector for expression of puromycin-resistant gene, P; PGK1 promoter, p(A); poly A signal of PGK1 gene. Insertions of vectors could be detected by PCR with 5' and 3' primers.

Vector for puromycin-resistant gene

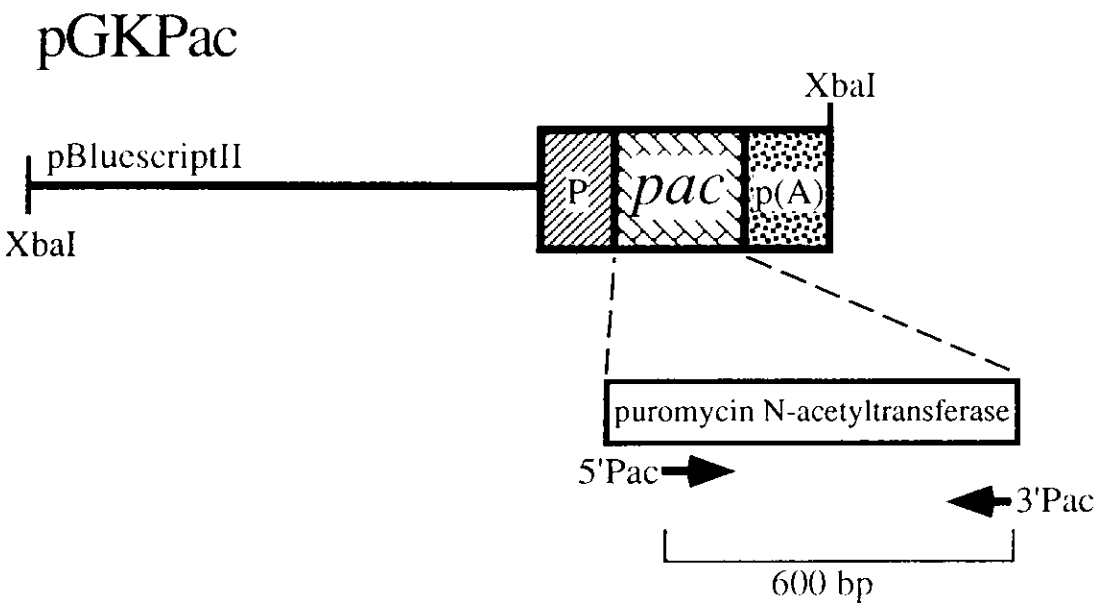


Figure 2. Sensitivity to puromycin. (A) Puromycin dose response of each ES cell line. (B) Selection period of each ES cell line with puromycin at a concentration of 0.1 μg/ml. ; Fz45 (G418-resistant), ; TT2 (wild type), ; E14 (another ES line).

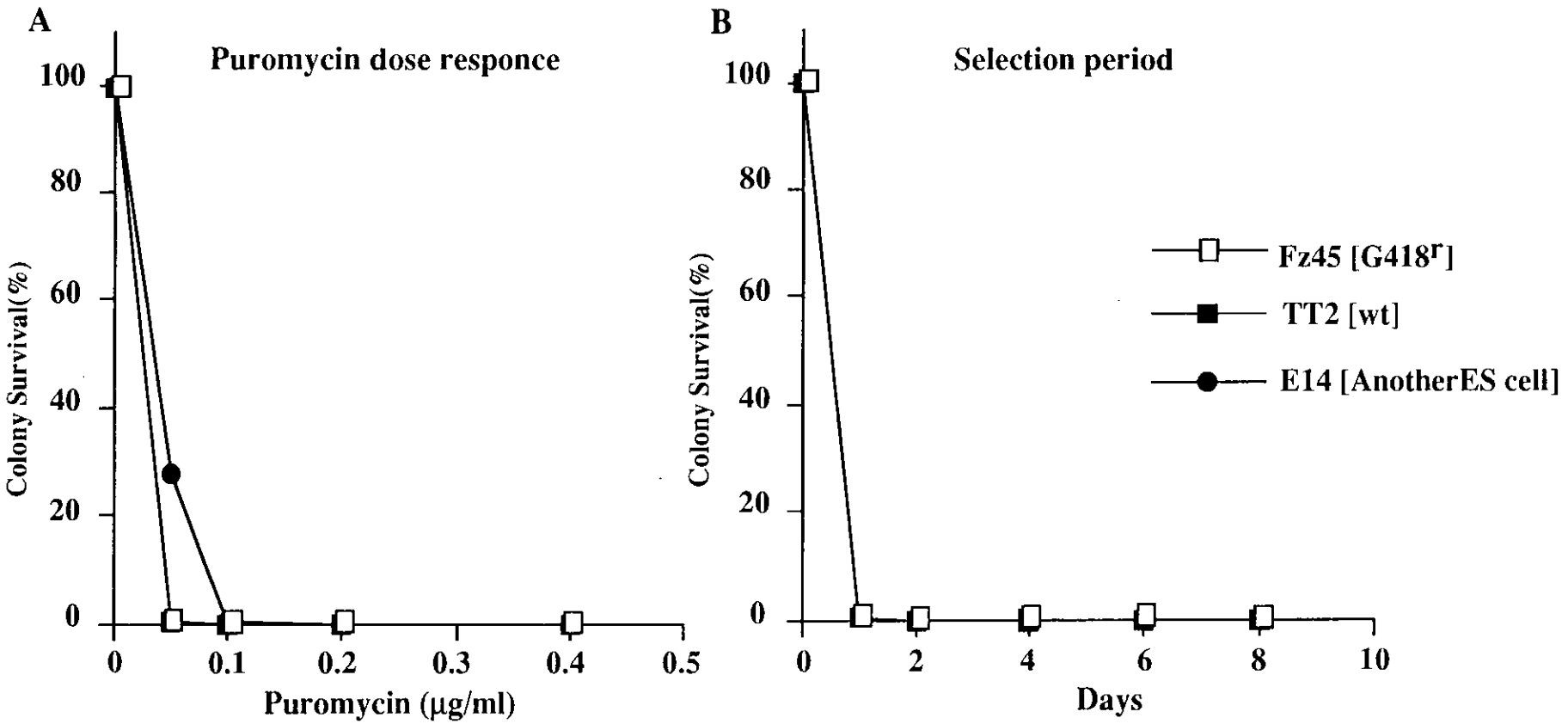

A
Puromycin dose responce
100
80
60
40
20
0
Colony Survival(%)
0
0.1
0.2
0.3
0.4
0.5
Puromycin (μg/ml)
B
Selection period
100
80
60
40
20
0
Colony Survival(%)
0
2
4
6
8
10
Days
Fz45 [G418r]
TT2 [wt]
E14 [AnotherES cell]

Figure 3. PCR analysis of genomic DNA from five ES clones which were selected by puromycin for the presence of *pac* gene. A 600 bp band indicats PCR product of *pac* gene. The arrow indicats a 600 bp band of *pac* gene.

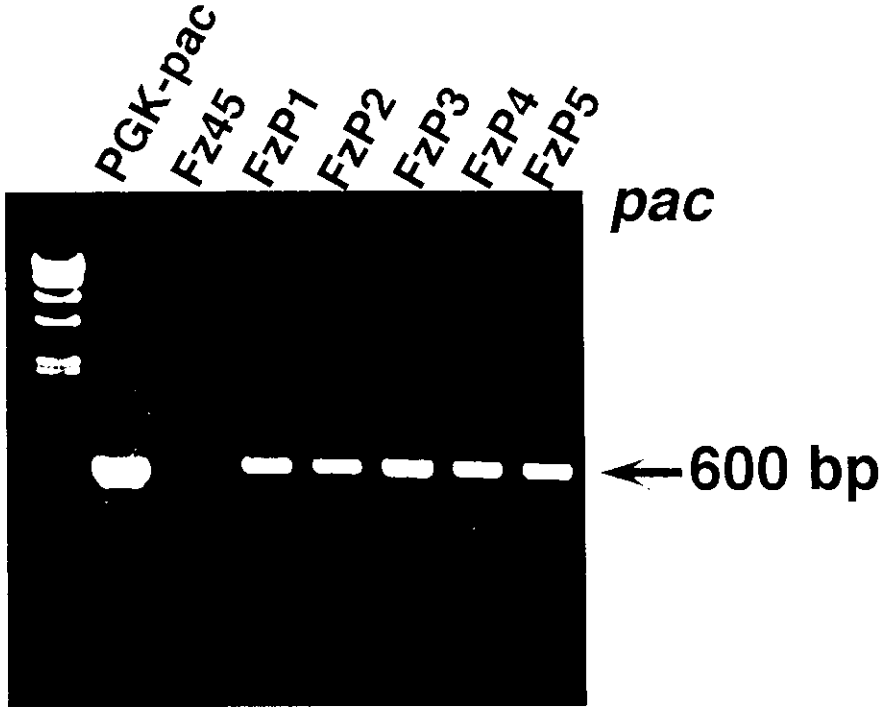
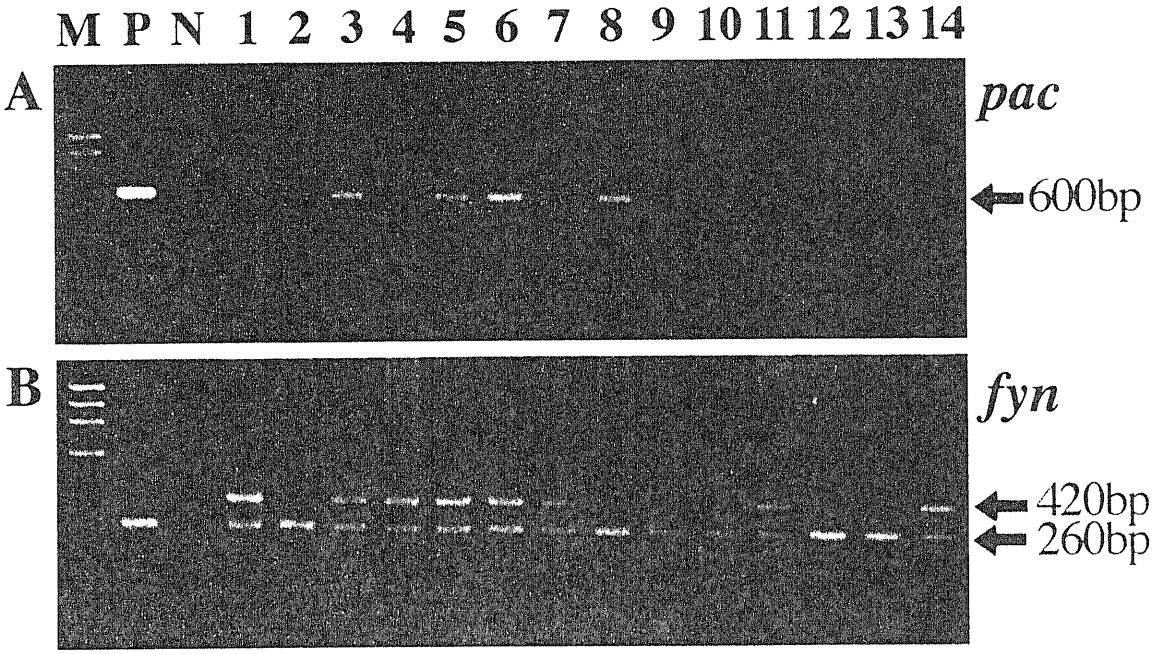

PGK-pac
Fz45
FzP1
FzP2
FzP3
FzP4
FzP5
pac
600 bp

Figure 4. PCR analysis of tail genomic DNA from fourteen F1 mice between FzP1 chimera-1 and CD-1 female (lane 1-14). (A) *pac* gene. (B) targetted *fyn* locus. Lane P; positive control (100 ng of genomic DNA from C57BL/6) and N; negative control (10 pg each of pGKPuro). The *fyn* locus that was amplified by PCR was illustrated (bottom).


M P N 1 2 3 4 5 6 7 8 9 10 11 12 13 14
A
pac
600bp
B
fyn
420bp
260bp

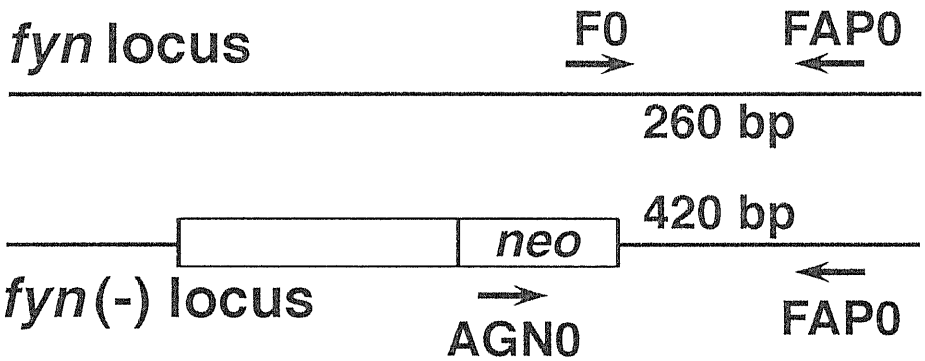
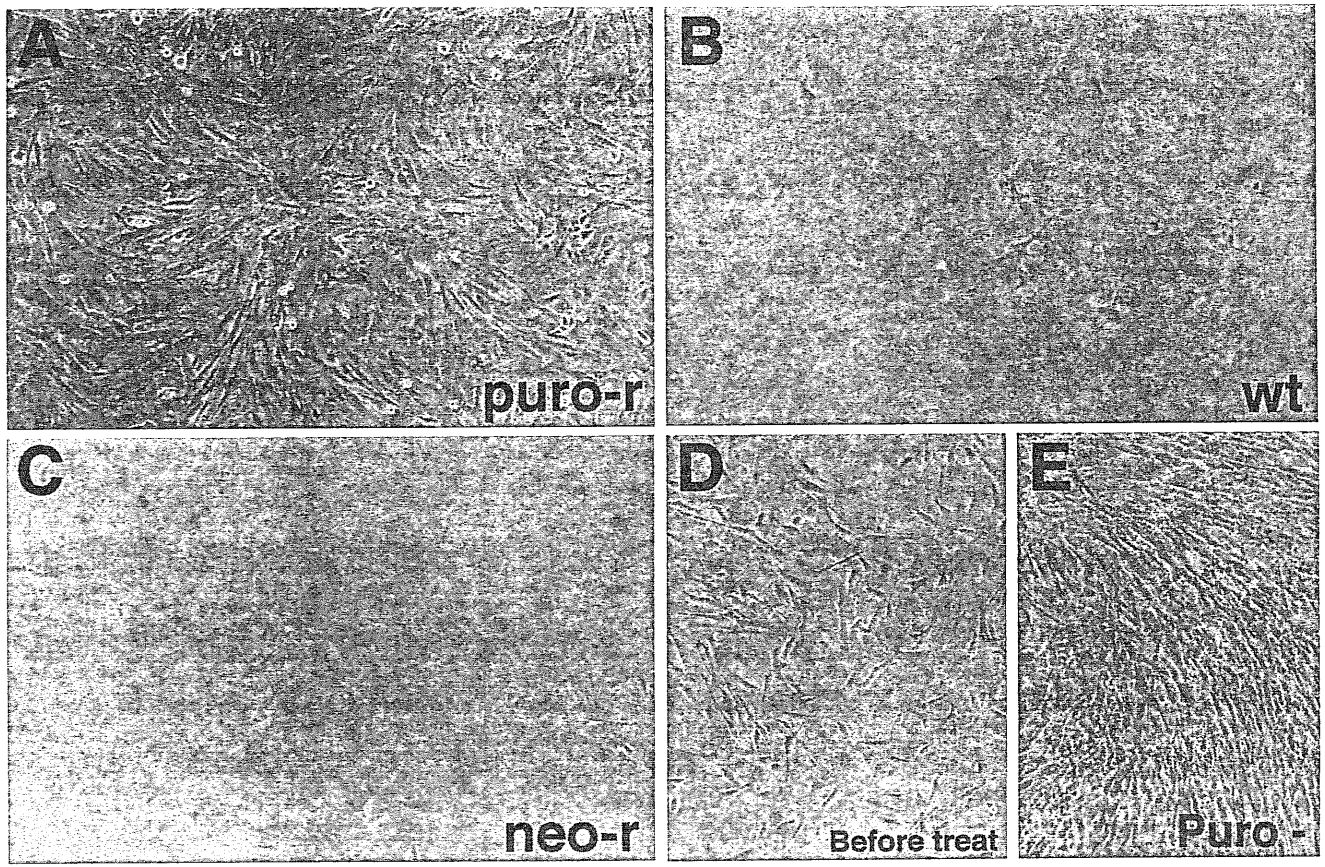

fyn locus
F0
FAP0
260 bp
420 bp
neo
fyn(-) locus
AGN0
FAP0

Figure 5. Culturing of embryonic fibroblast cells from F1 embryos under the presence of puromycin. Embryonic fibroblast cells from F1 embryo were maintained for 7 days in medium containing puromycin (1 μg/ml). (A) *pac* gene contained (puro-r). (B) wild type (wt). (C) *neo* gene contained (neo-r). (D) before treatment. (E) cultured without puromycin (Puro-).


A
puro-r
B
wt
C
neo-r
D
Before treat
E
Puro -

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Part 2

Development of the Gene-trap Method for Rescue Experiment of Knockout Mice

-with Heterozygously Gene-Disrupted Embryonic Stem Cells-

Introduction

Fyn, a non-receptor type protein tyrosine kinase, is expressed in the central nervous system (CNS), especially in brain (1). Fyn-deficient mice produced by gene targeting have behavioral abnormalities in suckling (2), spatial learning (3), and emotional behavior (4), and are susceptible to audiogenic seizure (5). Fyn is widely expressed in the CNS throughout the developmental stage (6), therefore it is not clear whether these abnormality are due to abnormality of certain region of the brain or abnormality in certaion stage during development. Fyn-deficient mice, however, should be useful to examine responsible for regulation of certain behavior.

For the purpose, I produced transgenic mice in which extra transgenic Fyn is expressed in a restricted brain region of Fyn-deficient mice. There are several ways to produce transgenic mice, the most common being the microinjection of transgene DNAs into mouse eggs. There are two problems, however, associated with this microinjection method. First, many promoters confering regional restrict expression in mammalian brain are not known. Second, only 5% of the mice born from microinjected eggs express the transgene (7). It is therefore necessary to produce more than one hundred mice per transgenic construction. Gene-trap or enhancer-trap methods can be performed only with embryonic stem (ES) cells in mouse. These expressions are based on the random integration into the genome of a reporter gene lacking promoter or enhancer. As the reporter gene, the *lacZ* gene can be expressed under the control of a tagged endogenous promoter and/or enhancer (8). Advantage of this strategy is that an unidentified endogenous promoter or enhancer can be used to express the reporter gene and/or a gene interest (I wanted to express the *lacZ* gene and the human *fyn* gene). Since the enhancer-trap method uses a low-activity promoter, the reporter gene would be expressed weakly as a background. I therefore wanted to use the gene-trap strategy. To efficiently perform the gene rescue experiment, I used a novel gene-trap vector for the heterozygously Fyn-disrupted ES cell line (Fig. 1). A novel gene-trap vector, GT-2, containing a *lacZ* and a human *fyn* gene, was used as a co-expressing reporter gene by the use of NTR (5'-

nontranslated region) sequence, and the *pac* gene was used as a selection marker gene for the neomycin-resistant *fyn*-disrupted ES cell line. NTR containing the IRES (internal ribosomal entry site) was derived from encephalomyocarditis virus (EMCV), and enabled efficient translation of the downstream cistron. Furthermore, cap-mediated translation of the upstream cistron could be blocked without affecting translation of the downstream.

In the gene-trap, since many recombinant ES cells should be obtained by random gene integration of transgenes, chimeric mice must be produced efficiently from them. I examined the aggregation method instead of the microinjection method. The aggregation method is a chimeric mice production technique without using microinjection apparatus (9). Eight-cell stage embryos whose zonae pellucidae had been removed are aggregated with clumps of 10-15 ES cells. The R1 ES clone (9) improved this technique, although other ES clones have not yet produced chimeric mice by this method. I also tried the TT2 cell line; the parental line of the Fyn-deficient ES clone (10).

I conclude in this report that :1) the gene-trap vector was efficiently integrated into the G418-resistant ES cell line with the *pac* gene and puromycin selection; 2) ES cell lines expressing the *lacZ* gene could co-express the human *fyn* gene, therefore the NTR functioned in ES cells; 3) ES clones containing the gene-trap reporter genes contributed highly in chimeric mice and were efficiently transmitted to the offsprings via the chimeric male; 4) in the TT2 cell, the microinjection method for production of chimeric and/or transgenic mice more efficiently than the aggregation method; and that 5) 3 lines of mice ontaining *fyn*-cDNA in *fyn*-deficient genotype were obtained.

Materials and methods

Construction of gene trap vector

The gene-trap vector, GT-2 had the following fragments from 5' to 3' (in order): the *XbaI-BamHI* 800 bp fragment of the *fyn* genomic DNA from pGFYN3.0Bm (12), *NcoI-AluI* 36 bp fragment of translational initiation codon of chicken *β-actin* gene, the *BamHI-NcoI* 3.1 kb fragment of n-*lacZ* gene from pactβgal (13), *XbaI-BamHI* 0.6-kb NTR fragment from pNTR-lacZ (14), *MluI* 1.6 kb fragment of human *fyn* cDNA from pSN-MluI (15), *MluI-XbaI* 1.1 kb SV40 polyadenylation signal fragment from pMT2TZ, *EcoRV-SpeI* pBluescriptSK (-) fragment, pGFYN7.7 (12), 1.3kb fragment from pGKPac, and *SalI-XbaI* 800 bp fragment of *fyn* genomic DNA from pGFYN7.7.

Establishment of puromycin-resistant feeder cells

NHL-7 line, a gift from Dr. H. Kondoh, was rendered puromycin-resistant by electroporation with pGKPac vector. For electroporation, cells were trypsinized and singly resuspended at a concentration of 4 X 10^7 per milliliter in HBS (25 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM dextrose); cell suspension was electroporated at room temperature in a cuvette with path length of 0.4 cm with one microgram of pGKPac vector DNA linearized by *XbaI* using a Bio - Rad Gene Pulser (250 V, 960 μF). The treated cells were plated at 3 X 10^6 cells per 9-cm dish. ES cells were then cultured for 24 h, and selected with 5 μg/ml of puromycin (Sigma) for 8 days.

ES cell culture, electroporation and screening of recombinant ES cells

The ES cell line, Fz45 in which one copy of *neo* gene was inserted into *fyn* gene locus, was cultured on EMFI (embryonic fibroblast) layers that had been treated with Mytomycin C as described (10). Electroporation conditions were similar to those in (10). The Fz45 cells were freshly thawed from frozen stock and subjected to electroporation

after one passage. For electroporation, 10 μg of linearized GT-2 vector by *XbaI* was used. The electroporated cells were selected at 1.0 μg/ml of puromycin (Sigma) on NHL-7P feeder layers for 8 days. Surviving colonies were picked up and expanded on freshly prepared feeder layers. These clones were stained by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (see below) to examine gene-trap events. Genomic DNA was extracted from the X-gal stained positive clones and analyzed by PCR (see below).

X-gal staining

To determine the *lacZ* expression at the undifferentiated state of GT-2-containing ES cells, 17 surviving ES clones after puromycin selection were fixed in PBS solution containing 2% formaldehyde for 10 min at room temperature. Fixed samples were washed several times with PBS and stained in X-gal solution (1 μg/ml X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM $K_2Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in 1 X PBS) at 37°C for 12 hr (16).

Western blotting

ES cells and organs of 8-12-week-old mice were homogenized in sample buffer [10% sucrose (w/v), 3% SDS (w/v), 60 mM Tris-HCl (pH 6.8)]. After extracts were spun, the amount of total protein of supernatants (Sl) was quantitated by BCA assay (Pierce). The Sls were treated with β-mercaptoethanol at a final concentration of 2% and boiled for 5 min, then run in reducing conditions on 10% polyacrylamide-SDS gel and transferred to nitrocellulose membrane (21). Equal loading of proteins was verified by Ponceau S staining of the membrane after transfer. All subsequent incubations and washes were done at room temperature. Blots were blocked with 10% nonfat dry milk in Tris-buffered saline [TBS; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl] for 60 min and then incubated for 1 hr in anti-human Fyn antibody (γc3), a gift from Mr. M. Yasuda. After being washed three times for 10 min in TBS, blots were incubated for 1 hr in biotinylated anti-rat IgG antibodies (Amersham), which had been diluted (1:3000) in TBS containing 5% nonfat dry milk. Blots were washed again three times in TBS, and incubated for 30 min

in streptoavidine-conjugated alkaline phosphatase (Amersham), which had been diluted (1:400) in TBS containing 5% nonfat dry milk. Blots were washed with TBS again, and proteins were detected by staining of alkaline phosphatase in staining buffer [3.3 mg/ml nitro-blue tetrazolium (Sigma) and 1.65 mg/ml 5-bromo-4-chloro-indolyl phosphate (Sigma) 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.5)].

Southern blotting analysis

Genomic DNAs of ES cells were prepared according to (19). Ten μg of genomic DNA was digested with *NcoI* in a volume of 200 μl and resulting fragments were separated on 1% agarose gels. After electrophoresis DNA was transferred onto nitrocellulose membrane filters by capillary-blotting (20). A 0.8 kb *SalI-XbaI* fragment of GT-2 vector was used as a probe and labeled with [α-^{32}P] dNTP by random oligo priming. The hybridization was performed in hybridization buffer [6 X SSC (diluted from 20 X stock; 3M NaCl and 0.34M sodium citrate pH 7.0), 5 X Denhart's solution and 10 μg/ml salmon sperm DNA] at 58°C for 12 h. The filters were washed two times at room temparature for 10 min in 2 X SSC and once for 10 min in 2 XSSC 0.1% SDS at 55°C, and exposed to an imaging plate (Fuiji film) for 12 hr.

Generation of chimeric mice

Embryo collections, cultivations and microinjection procedures were performed as described (10).

A brief description of the production of chimeras is as the following. Embryos in 8-, 4- and 8-16-cell (after compaction) stages were used for aggregation as described by Nagy et al. (9). Zonae pellucidae of the embryos were removed by treatment with acidic Tyrode's solution [NaCl 0.8% (W/V), KCl 0.02%, $CaCl_2 \cdot 2H_2O$ 0.024%, $MgCl_2 \cdot 6H_2O$ 0.01%, glucose 0.1%, and polyvinylpyrolidone 0.4% pH 2.5] (17). ES cells which had been plated at low density 2 days prior to aggregation were briefly trypsinized to form clumps of 10-15 loosely connected cells. These clumps were then attached to embryos in aggregation wells made by pressing a darning needle (Clover Co.

#3) into the plastic bottoms of the bacterial culture dishes (18). The aggregates were cultured overnight in microdrops of M16 before transfer into the uterus of 2.5 day pseudopregnant recipients.

Microinjection procedures were also similar to those described in (10). The chimeras with agouti hair were bred with CD-1 females or checked for contribution of ES cells to the germline. After confirming germline transmission, the chimeras were crossed with Fyn-deficient mice to obtain the desired genotype appropriate for the rescue experiment. Genomic DNA of F1 generation was also purified from tails of mice and analyzed by PCR.

Genotype analysis by PCR

Tail genomic DNAs were prepared according to Laird et al. (19). One-tenth μg/ml genomic DNA was dissolved in a final 25 μl vol. of 1X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 1% TritonX-100), containing 0.2 mM dNTPs, 0.2 mM primers, and 50 U/ml Taq polymerase. For the pac gene, 4% glycerol was additionally mixed with this reaction buffer, and the PCR was performed at 30 cycles of reaction at 96°C for 45 sec, at 58 °C for 25 sec, and at 72 °C for 3 min. Primers used were 5'Pac (5'-ATG ACC GAG TAC AAG CCA C-3') and 3'Pac (5'-TTA CGG GTC ATG CAC CAG GA-3'). For *neo* gene inserted *fyn* locus or the human *fyn* gene, the PCRs were performed with each primer, FAP0 (5'-TGC ACA CTT AAG TAG GCT-3') and AGN0 (5'-TGA TAT TGC TGA AGA GCT TGG-3') or FKS01 (5'-TGC AGA ATC CCT GCA GTT GA-3') and FKS02 (5'-GAG CTC TTC CTT CTC CAT CT-3'), respectively. The products were analyzed by 2% agarose gel electrophoresis.

Results

Strategy of the GT-2 gene-trapping

Figure 2 shows the gene-trap vector, GT-2, used in the present study to examine the expression of transgenes by endogenous promoter. This contained a reporter gene cassette which contained the n-*lacZ*, the nuclear location signal added to *lacZ*, and the human *fyn* genes. This was connected to the NTR and followed by SV40 polyadelylation signal [p(A)]; GT-2 also had the PGK-Pac-p(A) cassette as a marker of vector integration. Intron 1 and the splicing acceptor of the *fyn* genomic gene were placed in front of the reporter gene cassette so as to express n-*lacZ*, NTR and human *fyn* under control of promoter/enhancer of the tagged gene; the transcription would then represent the expression of the tagged gene directed by its promoter and enhancer. The transcription of the human *fyn* is able to reinitiate translation at the ribosome re-entry site of the NTR sequence.

In case in which the fusion occurred out-of-frame of the n-*lacZ* sequence, the initiation codon would be placed at the top of the *lacZ* gene in-frame, and the stop codon was placed out-of-frame (22). These steps were as reported by Skarnes et al. (23). The *pac* gene was used for puromycin selection, because in gene rescue of Fyn, the GT-2 vector integrates into the Fz45, G418-resistant ES cell line targeted *fyn* gene locus. Figure 3 shows the product when the GT-2 vector trapped the active gene locus.

Electroporation of GT-2 vector into Fz45 cells and selection of recombinant ES cells.

The feasibility of the strategy was tested in the ES cells. The Fz45 cells were electroporated, and selected by 1.0 μg/ml puromycin for 8 days on the feeder of our newly established puromycin-resistant cell, NHL-7P. More than 200 colonies survived from 2 X 10^7 electroporated Fz45 cells (Table 1), while only 20 were obtained on the feeder layer of the wild-type primary fibroblast cells (data not shown). Seventeen colonies of the 200 were stained blue in the nucleus by the X-gal staining, and the 10

most strongly stained cell lines, KGT34, 41, 43, 47, 59, 65, 77, YGTa8, b2 and b6, were chosen. Figure 4 shows X-gal-positive staining of the KGT59, 65 and YGTa8 clones. No positive staining was observed in the Fz45 parental cell colony.

Using NTR of the GT-2, X-gal positive clones should also express the human Fyn in ES cells ectopically. To confirm this, Western blotting analysis was performed with anti-human Fyn monoclonal antibody (Fig. 5). However, the antibody could also detect the endogenous mouse Fyn protein: in all the clones approximately 60 kdalton band was detected. If the human Fyn protein is expressed, the 60 kdalton band should be more intensively stained than that of the parental Fz45. Specially in KGT65 and YGTa8 lines which were strongly stained by X-gal, a more intensive staining of the 60 kdalton band was observed (Fig. 4). Since strongly X-gal positive clones contained a larger amount of the Fyn protein, at least in KGT65 and YGTa8, the NTR of the GT-2 is functional in ES cells.

To examine the integration of the GT-2 vector, genomic Southern blotting in 9 clones was performed except for KGT77. With a probe of *SalI-XbaI* 0.8 kb *fyn* genomic DNA fragment of the GT-2 vector, a 2 kb band represented endogenous *fyn* genomic DNA, and a 1kb band represented fyn (-) locus. Approximately 12 kb bands were the transgenic bands (Fig. 6). The 12 kb bands differed in intensity corresponding to 1 to 4 copies in comparison with 2 kb endogenous band in each clone, indicating tandem transgene integration. The 1 kb *fyn* (-) bands also appeared in KGT34, 41, 43, 47, 59, 65 and YGTa8. The 13 kb bands appeared in KGT41, YGTb2 and YGTb6. Endogenous 2 kb bands were less stained in YGTa8, b2 and b6 because of an experimental problem. These results showed that transgenic GT-2 vector was integrated but the copy number was independently different among the clones. These results suggested that the strategy of the GT-2 vector was feasible in the cultured ES cells.

Production of chimeric mice

In this gene-trap method, chimeric mice should be made independently from each recombinant ES clone because the GT-2 vector was randomly integrated into the genomic

DNA of ES cells. However, it is time consuming and laborious for us to treat many embryos by the microinjection method, and therefore we searched for more efficient method

Aggregation method with TT2 cells.

Using the aggregation method reported by Nagy et al.(9), 100% chimeric mice could be produced when R1 ES cells were aggregated with the CD-1 embryos from which zonae pellucidae had been removed in M16 medium (Fig. 7). TT2 cells also produced 100% chimeras by the 8-cell microinjection method (16). However when the other ES cell lines, such as E14, CCE or D3, were injected into 8-cell or blastocyst stage embryos, no 100% chimeras have been born. Because of the similarity in the production of 100% chimeric mice in R1 and TT2 cells, I attempted to produce chimeras with TT-2 cells, which was a parental ES line of gene-trap lines, using this aggregation method. A comparison of the results between aggregation and microinjection using the TT2 line is shown in Table 2: the aggregation method was much inferior to the 8-cell microinjection method. In each case, chimeric mice were obtained. By the aggregation method, no 100% chimeras were obtained (data not shown) and only 3 germline chimeras were obtained from 1830 transplanted embryos. On the other hand, by the microinjection method, 15 100% chimeras were obtained and 13 germline chimeras were obtained from 390 transplanted embryos. The result showed that microinjection method is much preferable to produce chimeras to the aggregation method. I, therefore, used conventional 8-cell microinjection to produce chimeric mice.

Production of chimeric mice with the gene-trapped ES cell lines by the microinjection.

The parental and the puromycin selected Fz45 cells highly contributed in chimeric mice and had high germline-differentiating potential (10). Seven of the 10 X-gal positive clones, except for KGT34, 47 and 65, were microinjected into the 8-cell stage CD-1 embryo. When 102 microinjected embryos were transplanted into pseudopregnant CD-1 females, 41 chimeras were obtained with the agouti coat color, 14 of which were mated

and produced ES cell derived agouti progeny. Thirteen of these germline chimeras produced agouti offsprings with 100% frequency (Table 3). All 7 of the X-gal positive clones were able to contribute in chimeric mice, but chimeric pups generated from KGT41 were lethal by deformities. This indicated that the Fz45 containing the GT-2 vector could contribute to chimeras and still retained the germline differentiating potency.

Germline transmission of the GT-2 vector into offsprings

In KGT59, YGTa8 and YGTb6, the genetic background of the parental ES cells was transmitted to the offsprings via chimeric mice and resulted in agouti coat color . To determine the transmission of the transgene into the offsprings, the genotype of the agouti offsprings between chimeras and CD-1 females was analyzed by PCR analysis for both the *pac* gene and the human *fyn* cDNA. Figure 8 shows an example of YGTa8 offsprings. In approximately half of these offsprings, the *pac* gene and human cDNA were detected; this both ratio was estimated as the Mendelian ratio. In KGT59 and YGTb6, similar results were also obtained (data not shown). Among them, [fyn (+/+), gt/+] brains were stained with X-gal to detect the region in which transgene was expressed, but no X-gal staining could be detected (data not shown).

To obtain the mice with the purposed genotype [*fyn* (-/-), *gt*/+], the confirmed germline chimeras were mated with Fyn-deficient mice. Genomic DNAs of the offsprings were analyzed by PCR and the rescue genotype [*fyn* (-/-), *gt*/+] mice were obtaind among F1 offsprings (data not shown). In all the lines; KGT59, YGTa8, b2 and b6, both the pac gene and the human *fyn* cDNA were detected in each individual, and the transgene positive genotype was independently segregated against *fyn* genotype as Mendelism. Pedigree charts of KGT59, YGTa8 and YGTb6 lines are shown in Fig. 10A, B and C.

Their hippocampal formation was analyzed anatomicaly to determine the phenotypic rescue on which cell layers of the Fyn-deficient hippocampus were undulated and disordered (1). The abnormality was, however, not corrected in all the [*fyn*(-/-), *gt*/+] genotypes (data not shown). Proteins of several organs of these offsprings were

analyzed by Western blotting to detect the expressions of Fyn protein. In no case, however, the human Fyn was detected (data not shown).

Discussion

My final goal in the gene rescue experiment in gene knockout mice is to identify a series of mice in which the ectopic *fyn* gene is expressed in restricted brain regions, for example, only in the hippocampus, the CA1 pyramidal cells, or the nuclear of amygdara. Here, I attempted to develop a method for an efficient production of these transgenic mice lines using the gene-trap technique. Because many promoters, active only in the restricted brain regions have not yet been well-characterized, and I had to accomplish an efficient Fyn gene rescue experiment, I used the heterozygously *fyn*-disrupted clone, Fz45, as parental ES cells. Since the Fz45 cell is resistant to G418, an additional drug was necessary to enrich the gene-trapping clones. As described in part 1, the *pac* gene and puromycin have been practicaly used for selection of the Fz45, that is, puromycin-resistant Fz45 clones extensively contributed to the chimeras and differentiated into germ cells. Using the GT-2 vector with a *pac* gene, efficient production of the germline chimeras was also shown. The *pac* gene thus might be applicable to an additional gene conversion strategy for a neomycin-resistant ES clone. The second gene conversion in an ES clone will not require time to produce objective transgenic mice. In this study, the object was [*fyn* (-/-), *gt*/+] genotype. In all 3 lines, this genotype was obtained within 4 months after electroporating the GT-2 vector in the F1 generation of chimeric mice. If transgenic mice were usually produced by DNA-microinjection into zygote, the object would require over 6 month to obtain, as the F2 generation of parental transgenic mice. Furthermore, the F1 offsprings obtained by GT-2 gene-trapping contained the control genotypes: [*fyn* (+/-), *gt*/+], [*fyn* (-/-), +/+], and [fyn (+/-), +/+], as brother or sister (Fig. 3). Therefore phenotypic rescue experiments could begin in the F1 generation offsprings. This strategy might be one of the best for gene rescue experiments in gene-knocked out mice.

A novel GT-2 vector contained two reporter genes: a n-*lacZ* gene to easily monitor the activity of the tagged promoter and human *fyn* cDNA for gene rescue in Fyn-deficient mice. To co-express the two reporter genes, I used an NTR sequence for re-entry of the

ribosome to re-initiate translational initiation. At the ES cell stage, since the strongly X-gal positive clones also overexpressed Fyn proteins, the NTR sequence is functional in ES cells. No over-expression of Fyn was apparent, however, in six X-gal positive clones. These results might indicate: 1) re-initiation translation by the NTR sequence human *fyn* expression became fainter than that of the front gene; n-*lacZ* gene: 2) X-gal staining was more sensitive than immunoblotting for Fyn: 3) the different stages for detection might be the cause of these differences. X-gal staining was done at the time the colonies were picked up, while immunoblotting was done after the passage of clones.

All the *lacZ* positive clones contained GT-2 vectors, but the copy number of the vectors was different. These copy numbers did not reflect the amount of human Fyn or the strength of X-gal staining. Since in the strategy of the gene-trap, one copy integration is the best, further examination will be necessary to determine the concentration of electroporated DNA and the dose of puromycin. This was the first trial of gene-trap strategy, so β-gal positive clones were used to produce chimeric mice; in β-gal negative clones, there would be reporter genes integrated under the promoter which were active in the late stage of development but inactive in ES cells. When chimeric mice were produced from the *lacZ* negative clones, gene-traped lines could be obtained. My final goal is to obtain mice in which the fyn gene is expressed in a restricted brain region, and in future I shall seek to develop an efficient method for producing such desired ES clones.

A new aggregation method, which requires no microinjection apparatus, was examined with the TT2 line, because the characters of this line and the R1 line used for aggregation were similar in F1 genetic background (TT2; CBA X C57Bl/6, R1; 129SV X 129SV/J), host embryo (both, CD-1) and chimerism (both, 100%). However, the result of applying aggregation method to TT2 was not satisfactory as expected. Germline chimeras were obtained by aggregation, but these ratios were very low. Yagi et al. described that TT2 microinjectioned into blastocysts resulted in lower chimerism than 8-cell microinjection (10). However, Papaioannu et al. reported that 8-cell injection with D3 line or E14 line was inferior to blastocyst microinjection (24). These results

suggested that the method to produce chimeric mice differed in each ES cell line, and that the 8-cell microinjection was the best way for the TT2 line to produce chimeras.

These chimeras were mated with Fyn-deficient females to produce Fyn-deficient F1 mice containing reporter genes (Fig. 10). The chimeric mice of KGT 59, YGTa8 and YGTb6 generated F1 mice, and the Fyn-deficient mice containing reporter gene were obtained in these three lines in Mendelian ratio. Human Fyn, however, could not be detected by Western blottings in these F1 mice (Fig. 11). There are three possibilities for the negative result: 1) in all *lacZ* positive clones, GT-2 vector integrated into inactive gene loci at the late developmental stage, and 2) ES cell passage before microinjection lacked active integration transgene loci; Boulter et al. described that expression of v-src in ES cells induced differentiation and aberrant development in chimeric mice (25). ES cells which expressed human Fyn, a src family tyrosine kinase, might have differentiated during cultivation, and ES cells which did not express Fyn might have greatly increased. If so, deformities of chimeric mice generated from the KGT41 line (data not shown) might be induced by the expression of Fyn during development. 3) Fyn expression was stopped during cultivation. Jähner and Jaenisch reported that integrations of foreign DNA, induced *de novo* methylation of flanking host sequences correlated with gene inactivities (26). The integration of foreign DNA led to altered chromatin structures that allowed *de novo* methylations, and these induced gene inactivities. These methylations might have occurred in β-gal positive clones. Further analysis is needed to clarify these phenomena.

This report provides a novel method for a rescue experiment to analyze the phenotype of gene-disrupted animals. Gene-trap combined with puromycin selection is very forceful. A rescue experiment for phenotype of Fyn-deficient mice was attempted, but it could not be applied to a gene-disrupted animals. In lethal phenotypes particular, the effect of the introduced gene can be clearly seen. This method should be helpful in analyzing gene-disrupted animals.

Table 1. Frequencies of survived colonies and *lacZ*-positive colonies.

Electroporated vector	Feeder	No. of cells electroporated	No. of surviving colonies	No. of *lacZ*-positive colonies
GT-2	NHL-7P	2 X10^7	>200	17/96
PGK-pac	NHL-7P	2 X10^7	>200	0/>200

Table2. Comparison between aggregation and microinjection methods for production of chimeric mice.

Developmental stage	No. of ES cells used	No. of embryo transplanted	No. of pups born		ES contribution in coat		Germ-line chimeric
				(%)	Chimeric	Non-chimeric	
Aggregation							
8 cell st.	10-15	1274	202	16	13	189	3
4 cell st.	10-15	342	10	3	3	7	0
8-16 cell st.	15-20	214	28	13	3	25	0
Total		1830	240	13	19	221	3
Microinjection							
8 cell st.	10-12	390	72	18	35	37	13

Table 3. Germ-line differentiating potency of *lacZ*-positive clones.

ES cell line	No. of embryo transplanted	No. of pups born	(%)	ES contribution in coat		Germ-line chimeric	Germline Chimeric / Chimeric (%)
				Chimeric	Non-Chimeric		
KGT41	40	6	(15)	4	2	0*	(0)
KGT43	40	7	(18)	1	6	0	(0)
KGT59	90	16	(18)	6	10	3	(50)
KGT77	40	6	(15)	1	5	0	(0)
YGTa8	200	41	(21)	9	32	2	(22)
YGTb2	40	12	(30)	5	8	1	(20)
YGTb6	40	16	(40)	15	1	8	(53)
Total	510	113	(20)	45	69	14	

*; chimeric pups were died of deformities.

Figure 1. Strategy of rescue experiment by gene-trap. A reporter gene, human *fyn* cDNA was electroporated into heterozygously *fyn*-deficient ES cell Fz 45 line. The electroporated cells were selected by puromycin and expression of β-gal (β-galactsidase). Chimeric male which was produced from selected ES cells was mated with Fyn-deficient female. Four genotypes would be obtained in F1 generation, and all of them could be used for analysis. In the mice with [*fyn* (-/-), gt/+] genotype, the rescue of phenotypic abnormalities could be expected. In the analysis of these mice, the mice with [*fyn* (-/-), +/+] and [*fyn* (+/-), +/+] genotypes could be used as negative and positive control. The mice with [*fyn* (+/-), gt/+] genotype could be used for the analysis of the effect of the transgene under the endogenous *fyn* existence.

Gene-trap for rescue experiment

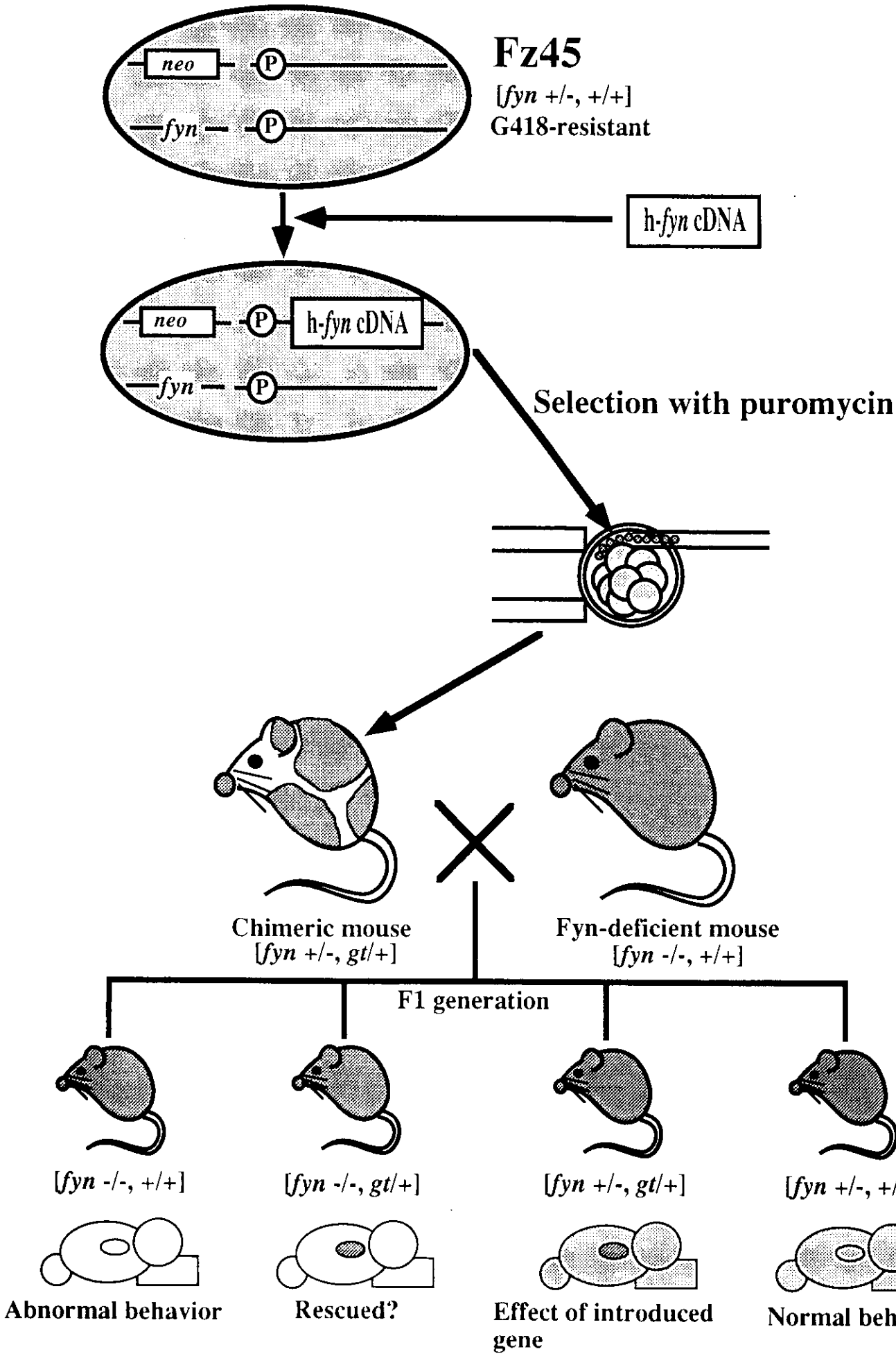


Figure 2. Schematic representation of gene-trap vector, GT-2. The box designated as SA: splicing acceptor sequences fyn gene: the box designated as n-*lacZ*: gene ligated with nuclear transport signal; the box designated as h-fyn: human *fyn* cDNA; The box designated as p(A): polyadenylation sequences from the SV40 large T gene; the box designated as P: promoter sequences from phospho glycerate kinase-1 (PGK) gene; the box designated as *pac*: puromycin N-acetyl transferase gene; the box designated as p(A) at left side of *pac* gene: polyadenylation sequences from PGK gene. The regions which were amplified by PCR for *pac* and human *fyn* were also illustrated.

GT-2 (Gene trap vector)

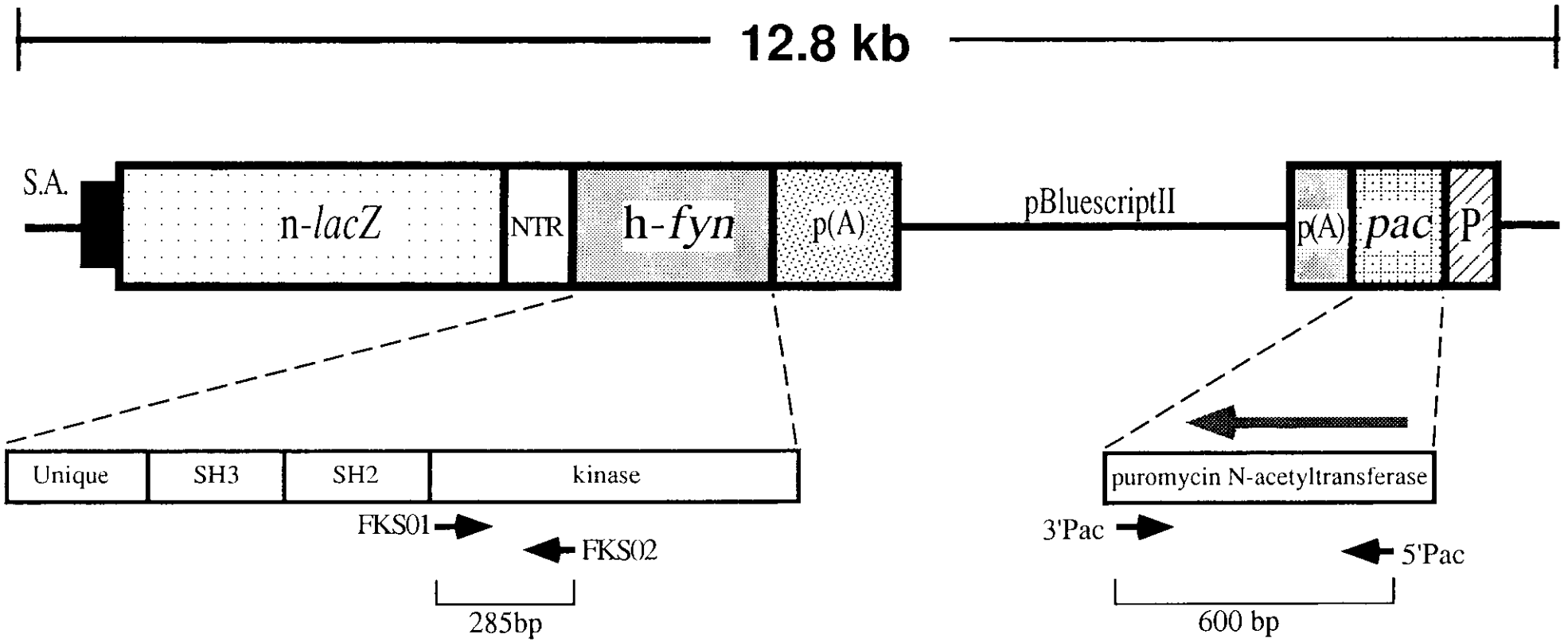


Figure 3. Principle structure and mode of activation of reporter genes in GT-2 vector. Among the random recombinants of gene-trap vector, after introduction of the GT-2 by electroporation into ES cells, the *pac* gene in ES cells directed by pgk-1gene promoter that is active in ES cells. In the survived ES clones, the vector inserted into genes (gene X) will express the reporter genes as a fusion transcript. As a splice acceptor is placed in front of the *lacZ* gene, integrations within or outside the endogenous gene in both orientations should result in *lacZ* gene expression. In these *lacZ* positive clones, human Fyn was also expressed by the effort of NTR sequence.

Gene-Trap with the GT-2 vector

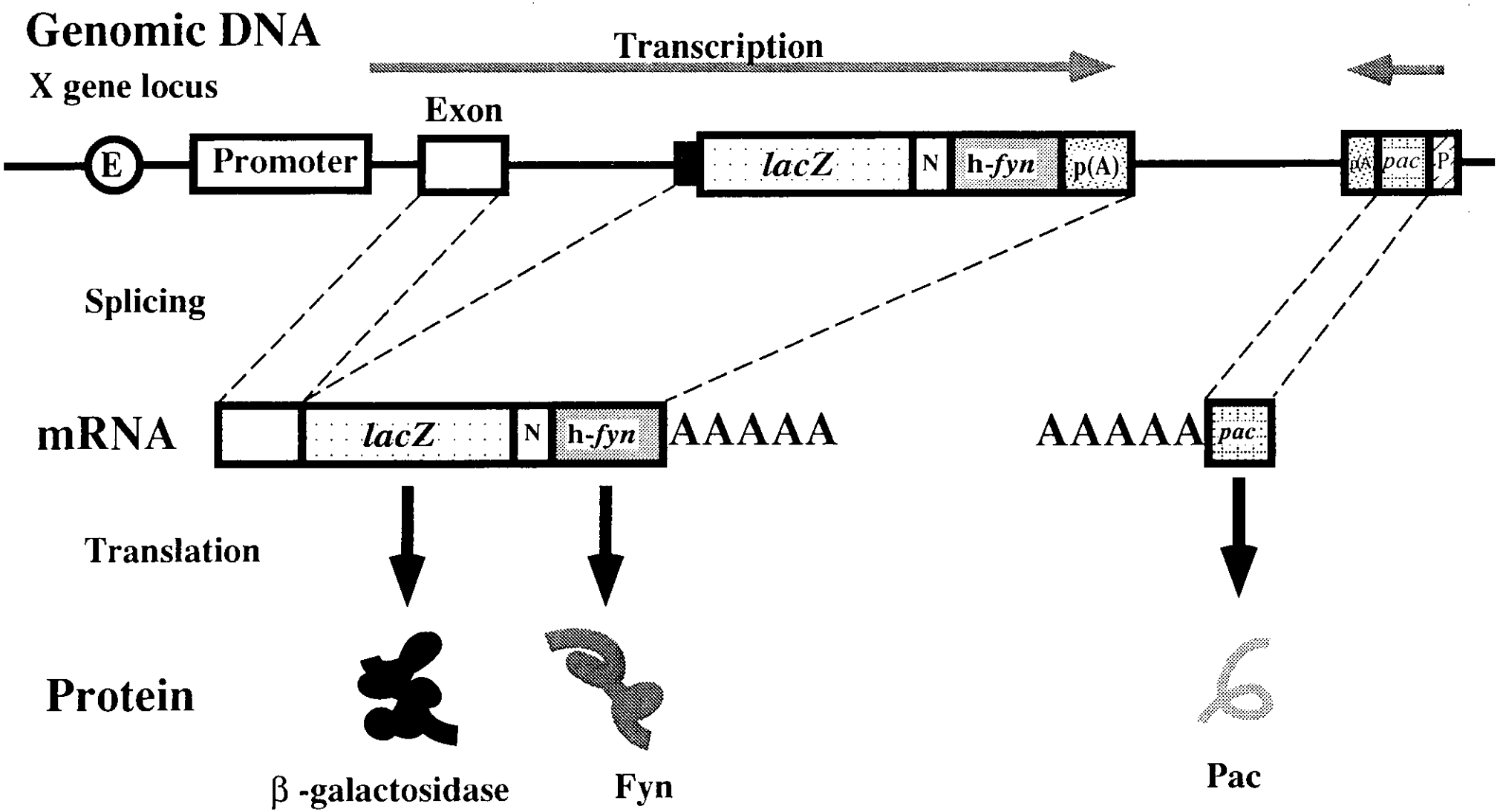


Figure 4. Survived ES cells of puromycin selection were stained by X-gal. Fz 45, parent ES line of gene-trap lines, was not stained by X-gal (top). One of the *lacZ* positive clones, KGT59 was strongly stained by X-gal (bottom).

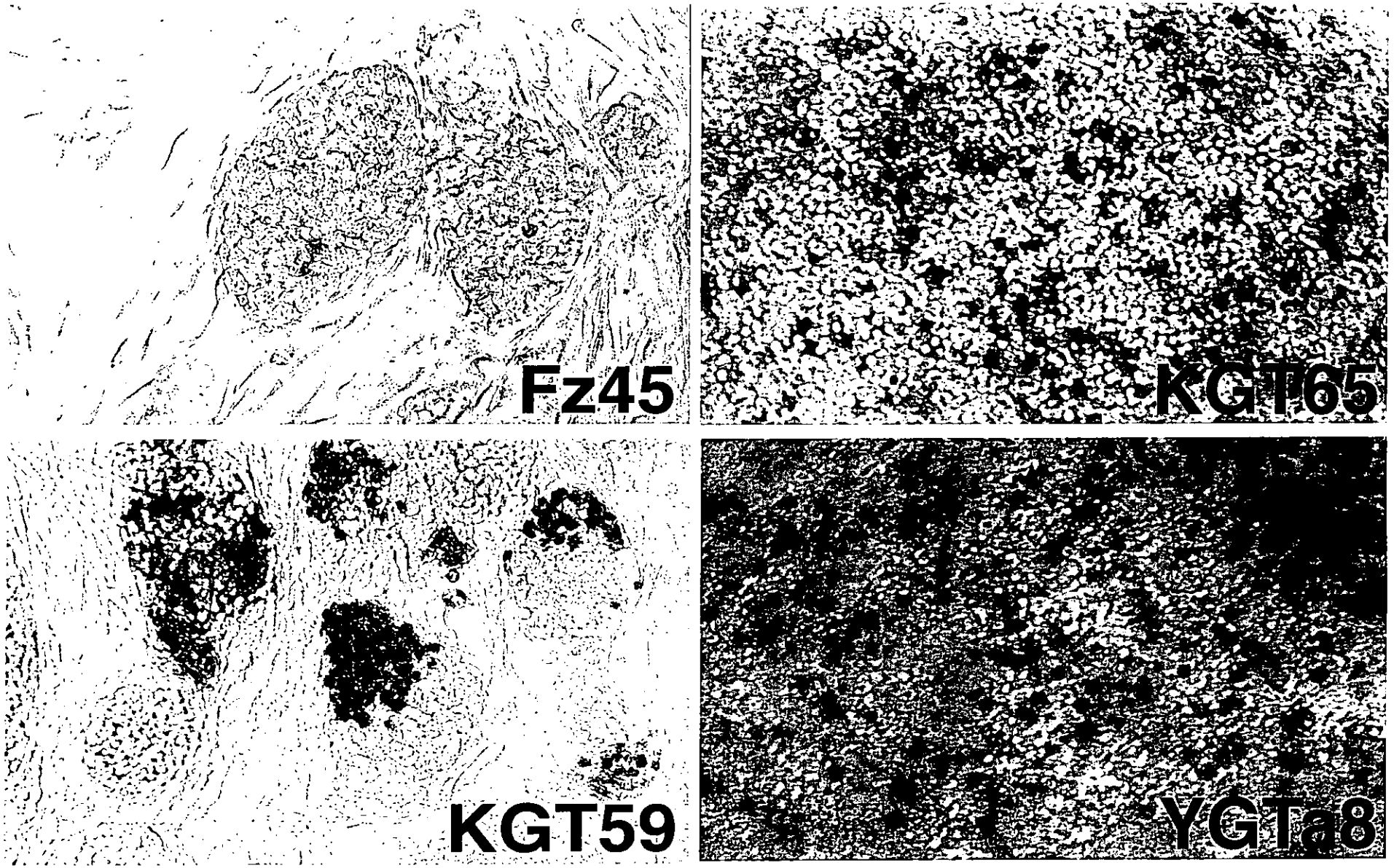
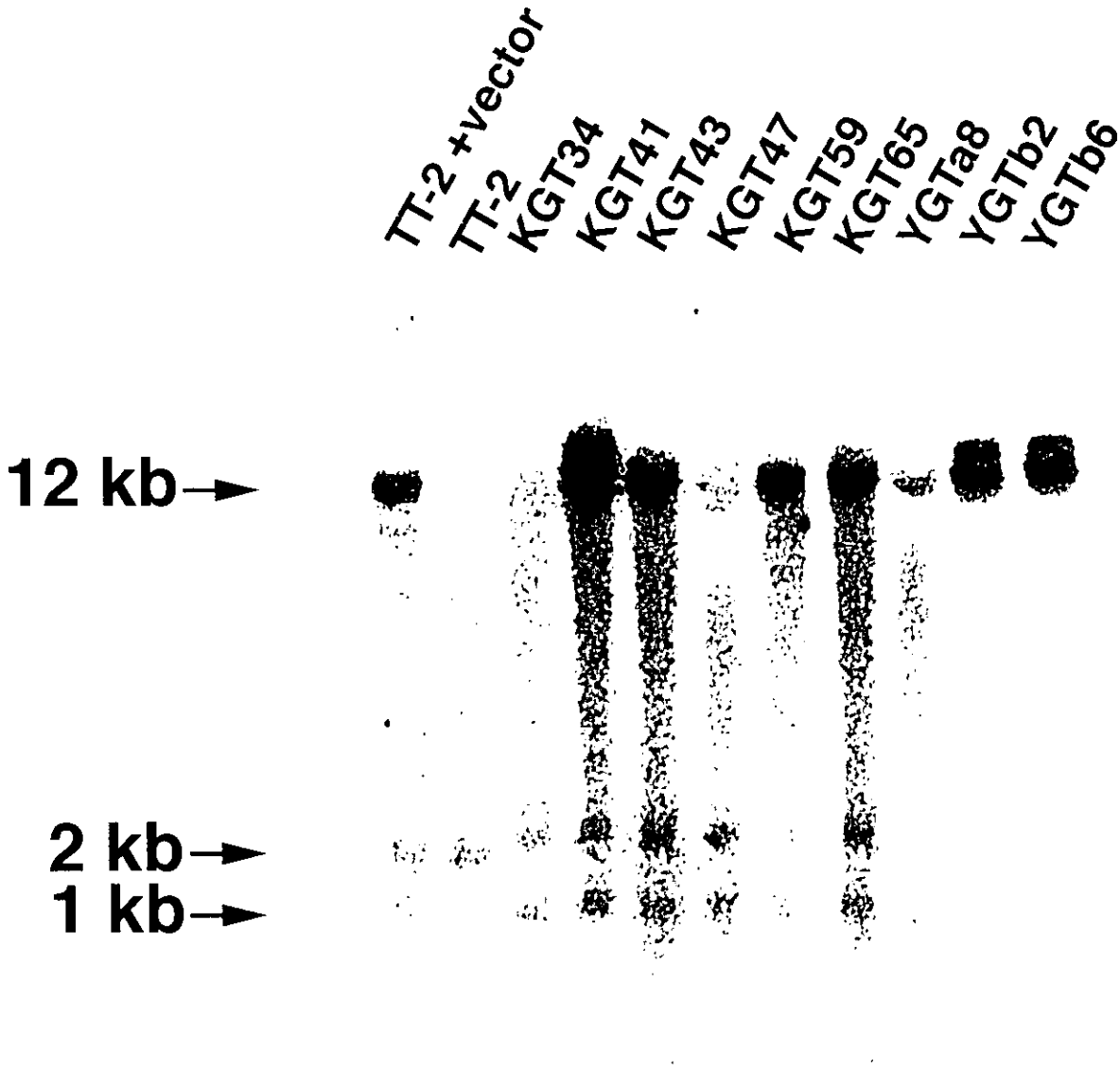

Fz45
KGT65
KGT59
YGTa8

Figure 5. Western blot analysis of *lacZ* positive clones for the expression of human Fyn. A 59 kD band of Fyn was detected by anti-human Fyn monoclonal antibody. The cell lines are indicated above each lane.

wt brain
fyn(-/-)brain
TT-2
E14
FZ45
KGT41
KGT43
KGT59
KGT65
YGTa8
YGTb2
YGTb6
Fyn

Figure 6. Southern blot analysis of *lacZ* positive clones of survived clones. (A) NcoI digest. The cell lines are indicated above each lane. A 12 kb band indicated integrated GT-2 vector. (B) A 0.8 kb SalI/XbaI fragment of GT-2 vector which was used as a probe was illustrated.

A


TT-2 +vector
TT-2
KGT34
KGT41
KGT43
KGT47
KGT59
KGT65
YGTa8
YGTb2
YGTb6
12 kb
2 kb
1 kb

B

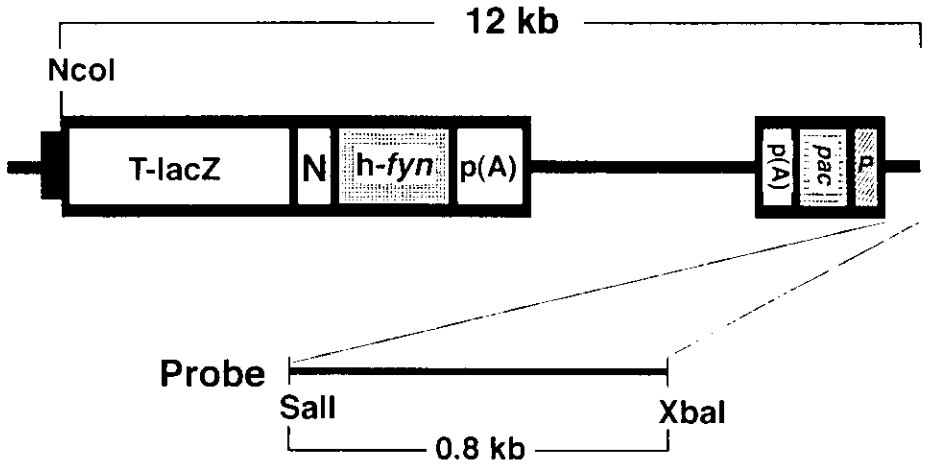

12 kb
NcoI
T-lacZ
N
h-fyn
p(A)
p(A)
pac
p
Probe
SalI
XbaI
0.8 kb

Figure 7. The comparison of procedures between aggregation and microinjection. Microinjection apparatus was not needed in aggregation.

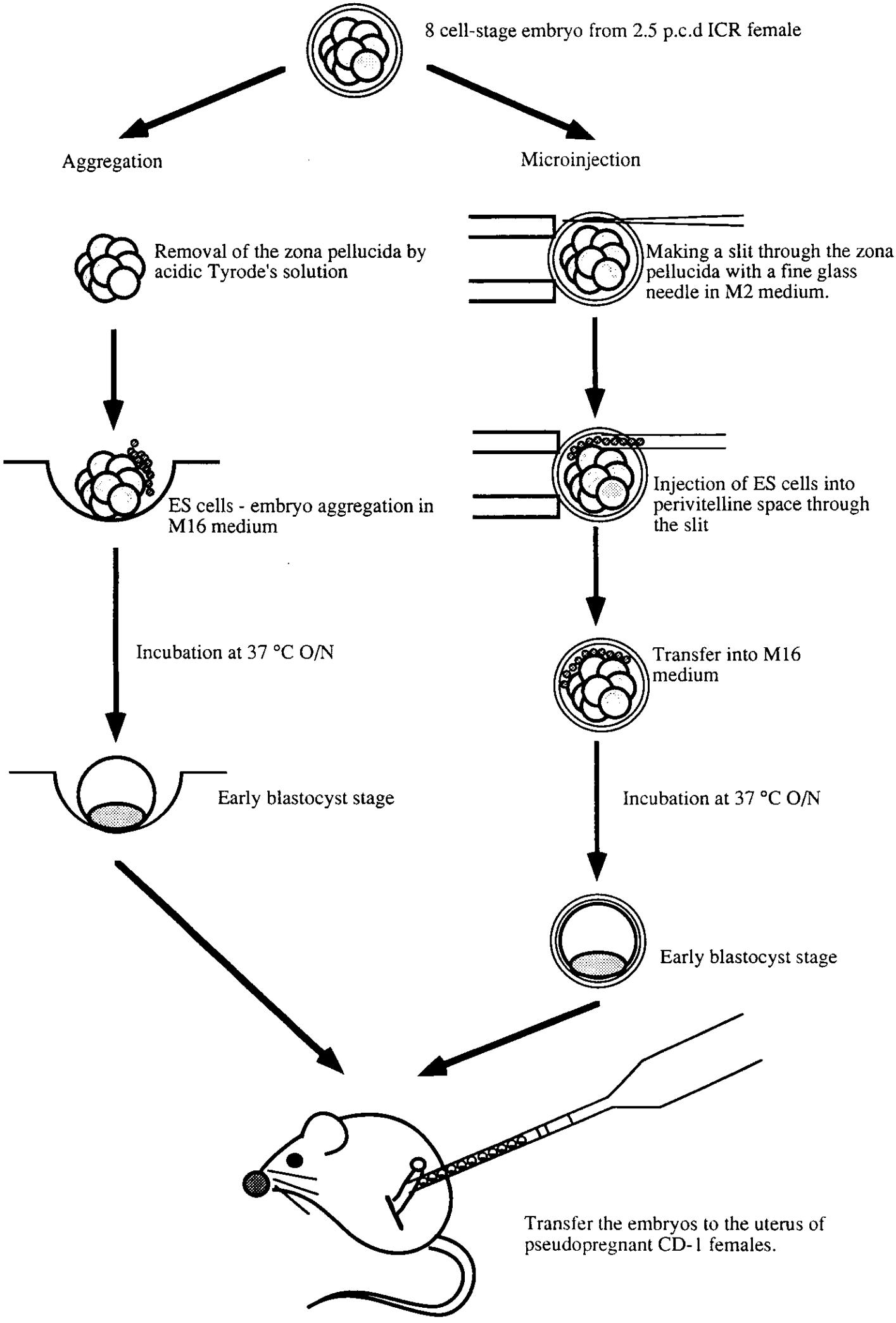

8 cell-stage embryo from 2.5 p.c.d ICR female
Aggregation
Microinjection
Removal of the zona pellucida by acidic Tyrode's solution
Making a slit through the zona pellucida with a fine glass needle in M2 medium.
ES cells - embryo aggregation in M16 medium
Injection of ES cells into perivitelline space through the slit
Incubation at 37 °C O/N
Transfer into M16 medium
Early blastocyst stage
Incubation at 37 °C O/N
Early blastocyst stage
Transfer the embryos to the uterus of pseudopregnant CD-1 females.

Figure 8. PCR analysis of tail DNA from thirteen F1 mice between YGTa8 chimera and CD-1 female (lane 1-13). The *pac* gene (top). The human *fyn* cDNA (bottom).

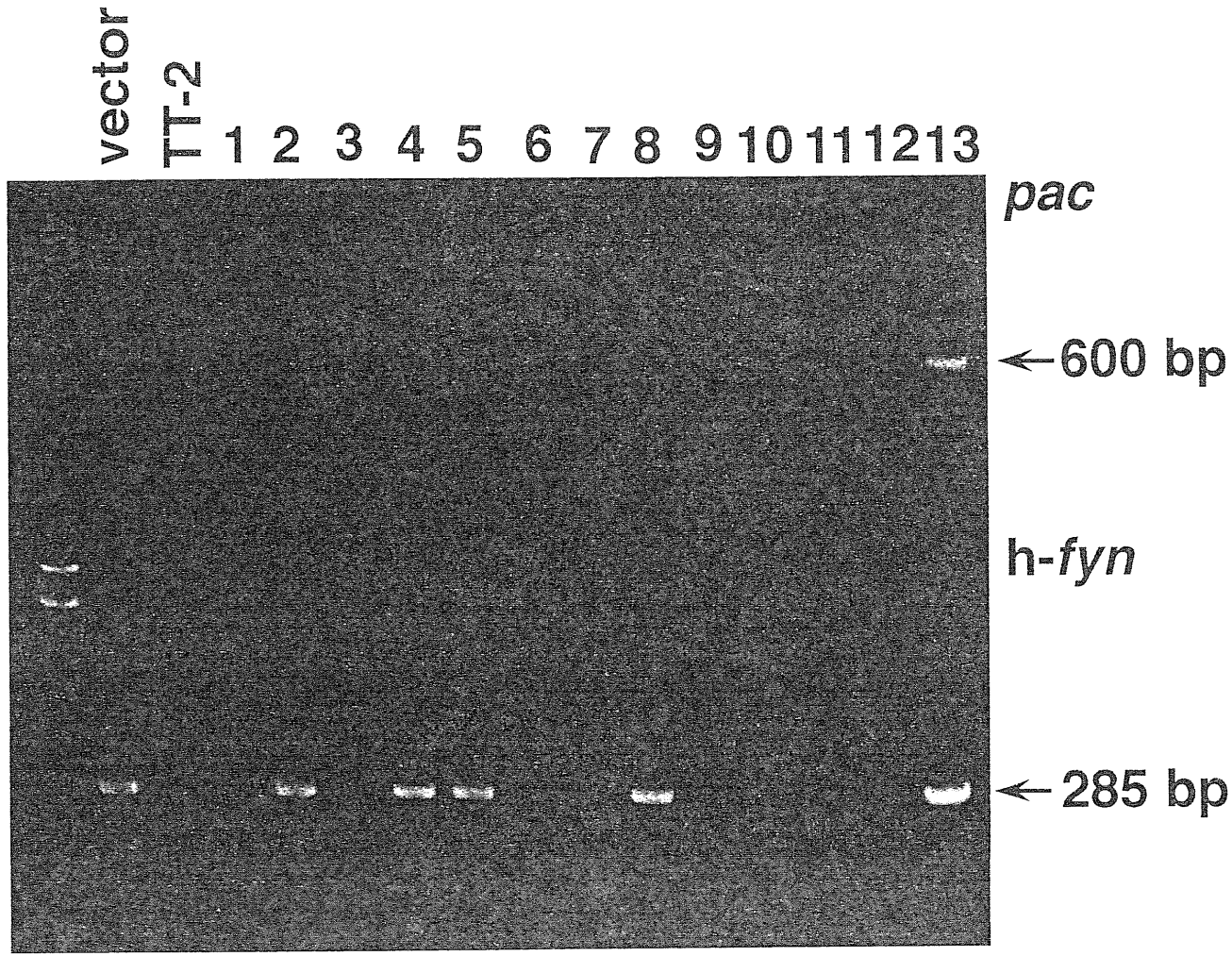
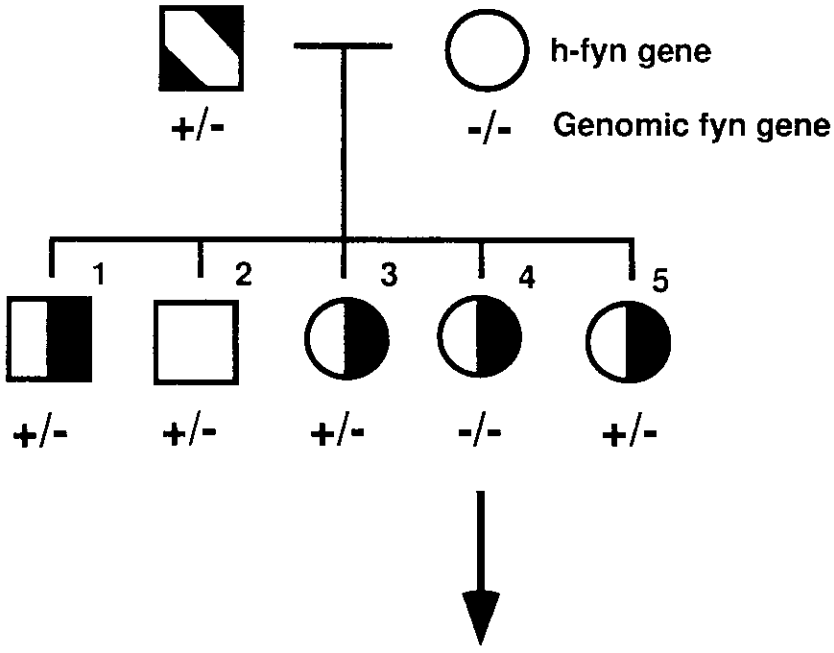

vector
TT-2
1 2 3 4 5 6 7 8 9 10 11 12 13
pac
600 bp
h-fyn
285 bp

Figure 9. Pedigree charts of F1 mice between chimeras of gene-trap lines and Fyn-deficient females. (A) KGT59 line. (B) YGTa8 line. (C) YGTb6 line. The square designates male, and the circle designates female. The figure that is hatched right half designates an animal containing GT-2 vector.

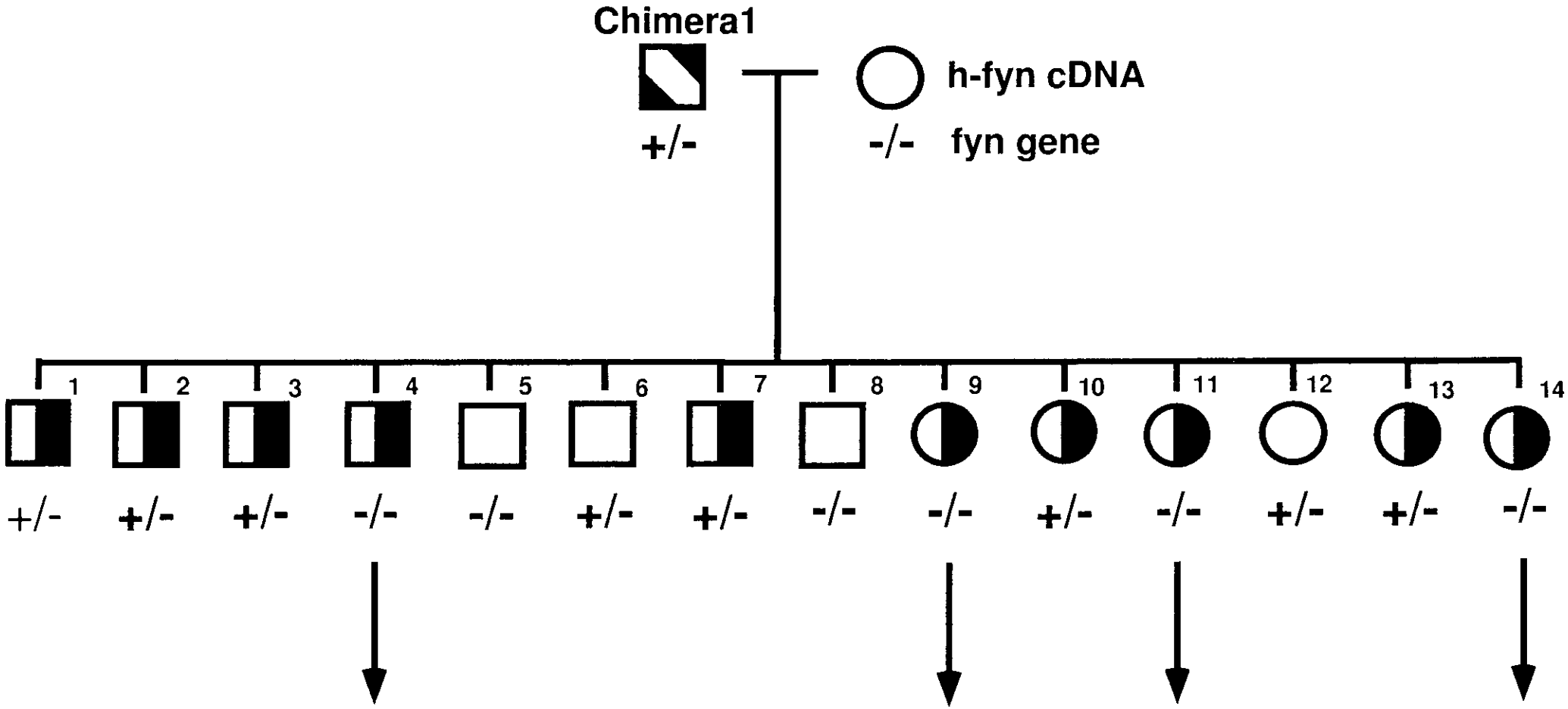
A

Pedigree chart of KGT59



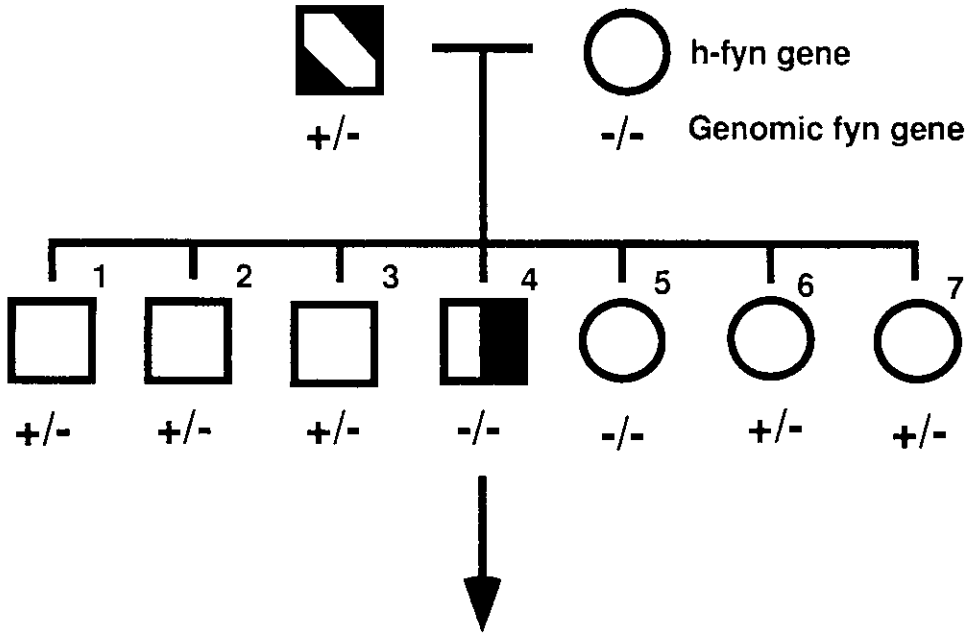
B

Pedigree chart of YGTa8



C

Pedigree chart of YGTb6



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