TECHNOLOGY REPORT

Heterogeneous Populations of ES Cells in the Generation of a Floxed *Presenilin-1* Allele

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Summary: Generation of a floxed *Presenilin-1* (*PS1*) allele involved two recombination events in the embryonic stem (ES) cells. First, a targeting vector containing a loxP site in intron 1 and a floxed *CMV-HYG/TK* double selection cassette in intron 3 was integrated into the *PS1* locus by homologous recombination. The use of a negative selection cassette, *PGK-DTA*, dramatically increased the recombination efficiency within the targeted locus (75-fold). Second, an expression vector encoding Cre recombinase was introduced to excise the floxed *CMV-HYG/TK* cassette via site-specific recombination. However, all five ES cell clones testing positive for the proper removal of the *CMV-HYG/TK* cassette also contained a proportion of ES cells in which recombination had occurred between the distal loxP sites in introns 1 and 3, resulting in excision of the entire floxed region. It is therefore critical to screen for possible recombination events involving all 3 loxP sites, in order to identify ES cells clones bearing high proportions of the desired ES cells. genesis 26:5–8, 2000. © 2000 Wiley-Liss, Inc.

Key words: mouse, conditional knockout

Presenilin-1 (*PS1*) is a major gene responsible for familial Alzheimer's disease. We previously generated mice with a targeted germ-line disruption of the *PS1* gene, and mutant mice homozygous for the resulting null allele exhibited perinatal lethality, severe skeletal malformation, and specific defects in neurogenesis, indicating a critical role for PS1 in skeletal and neural development (Shen *et al.*, 1997). To investigate the role of PS1 in the adult brain, we have employed the Cre/loxP technology to generate a floxed *PS1* mouse, which will be crossed to specific Cre transgenic mice to generate regionally and temporally restricted *PS1* knockout mice.

The targeting vector (Fig. 1A) contains a loxP site flanked by *Spe* I and *Nhe* I restriction sites in *PS1* intron 1 (loxP1) and a floxed positive and negative selection cassette (*CMV-HYG/TK*) in *PS1* intron 3 (loxP2 and loxP3). The cassette encodes a fusion protein with hygromycin-resistance (HYG) and thymidine kinase (TK) activities under the control of the CMV promoter. Another negative selection cassette (*PGK-DTA*), which encodes diphtheria toxin under the control of the *phosphoglycerate kinase* promoter (*PGK*), was included in the targeting construct to allow enrichment for the correctly targeted embryonic stem (ES) cell clones (Soriano, 1997).

The linearized targeting vector was transfected into J1 ES cells by electroporation. In the presence of the positive selection drug, hygromycin B, only those clones in which the *CMV-HYG/TK* selection cassette has been integrated and the *PGK-DTA* cassette has been removed by homologous recombination would survive. After selection in hygromycin-containing medium (120 μg/ml) for 7.5 days, 192 of these ES cell clones were picked and expanded. It is important to distinguish ES cells that have undergone homologous recombination in the 5′ homologous region from those undergoing homologous recombination within the floxed region, which results in exclusion of loxP1. Genomic DNA derived from 96 individual ES cell clones was subjected to *Spe* I digestion followed by hybridization with the 5′ probe. The 7.7 and 9.0 kb *Spe* I fragments represent the homologous recombinants in the 5′ and floxed regions, respectively (Fig. 1B). Southern analysis showed that 7 ES cell clones underwent targeting events in the 5′ homologous region (≈5 kb), and 8 clones exhibited targeting events in the floxed region (≈1 kb). Interestingly, the frequency of homologous recombination within the floxed region appears similar to that within the 5′ region, despite its much smaller size. In addition, the use of the negative selection cassette *PGK-DTA* has markedly increased the targeting frequency (≈75-fold), compared to our previous disruption of the *PS1* locus using the same 5′ and 3′ homologous regions (Shen *et al.*, 1997). These 15 ES cell clones were expanded, and their genomic DNA was digested with *Spe* I and *Bam*HI followed by hybridization with the 5′ and 3′ probes, respectively. Seven of the

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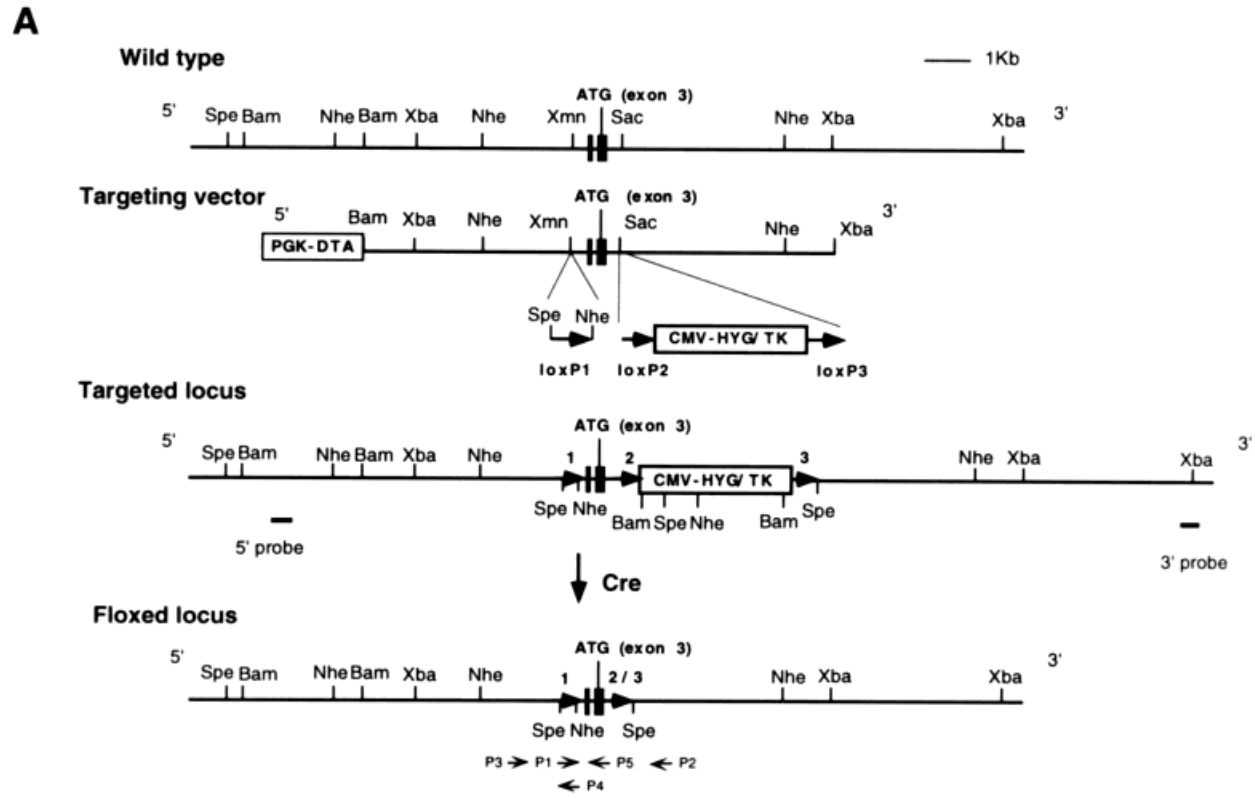
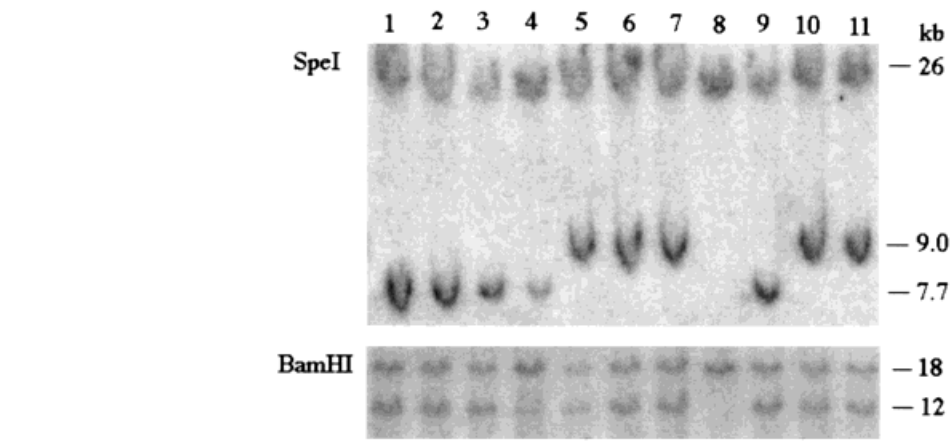

A
Wild type
1Kb
ATG (exon 3)
5'
Spe Bam
Nhe Bam Xba
Nhe
Xmn
Sac
Nhe Xba
Xba
3'
Targeting vector
PGK-DTA
Spe Nhe
CMV-HYG/ TK
loxP1
loxP2
loxP3
Targeted locus
1
2
3
5' probe
3' probe
Cre
Floxed locus
2 / 3
P3
P1
P5
P2
P4


B
1 2 3 4 5 6 7 8 9 10 11
kb
SpeI
26
9.0
7.7
BamHI
18
12

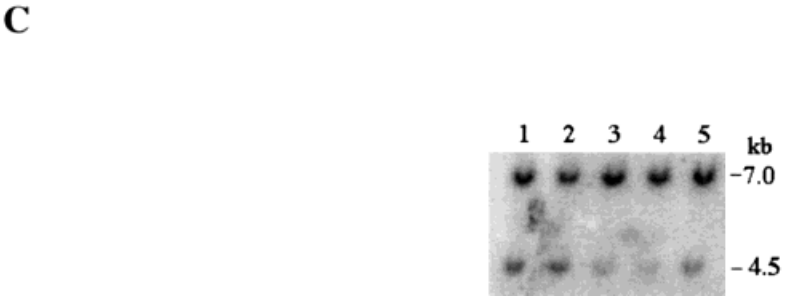

C
1 2 3 4 5
kb
7.0
4.5

FIG 1

Table 1
Screening for the Correct Site-Specific Recombination Event

PCR	Primers	Wild-type	loxP 1,2,3	loxP 1,2/3	loxP 1/3
	P1-P2	—	4.7 kb/N.D.[a]	1420 bp	—
	P3-P4	—	153 bp	153 bp	153 bp
	P1-P5	—	633 bp	633 bp	—
	P3-P2	1292 bp	4.6 kb/N.D.	1527 bp	376 bp
Southern	Probe	Wild-type	loxP 1,2,3	loxP 1,2/3	loxP 1/3
Spe I	Exons 2 + 3	26 kb	1.4 kb	1.2 kb	—
Nhe I	Exons 2 + 3	7 kb	1.8 kb	4.5 kb	—

[a]N.D., not detected.

FIG 1. Generation of a floxed *PS1* allele. **(A)** Targeting strategy and restriction map of the locus. The *PS1* exons 2 and 3, which were removed in *PS1* −/− mice, are flanked with a loxP site and the floxed *CMV-HYG/TK* selection cassette. The loxP1 site and the floxed selection cassette were inserted in *PS1* introns 1 and 3, located 340 bp upstream of and 360 bp downstream of the intron/exon boundaries, respectively. Therefore, it is highly unlikely that the loxP sequence (loxP1 and loxP2/3) will interfere with the splicing of introns 1 and 3. The loxP sites are indicated as arrowheads. Multiple *Xmn* I sites are present in the locus, but only the relevant one is shown. The loxP1 site and the floxed selection cassette are not drawn to scale. P1 and P4 are primers specific for loxP1 used for cloning and PCR. P3, P5 and P2 are PCR primers specific for *PS1* intron 1, exon 3 and intron 3, respectively. 5′ and 3′ probes are 0.5 and 0.7 kb fragments used for screening homologous recombinants, and are located outside of the 5′ and 3′ homologous regions. **(B)** Southern analysis of targeted clones. DNA derived from ES cell clones was digested with *Spe* I and *Bam*H I, followed by hybridization with 5′ and 3′ probes, respectively. The 26 kb *Spe* I fragment and the 18 kb *Bam*H I fragment represents the wild-type *PS1* locus. The 7.7 and 9.0 kb Spe I fragments correspond to homologous recombination occurring in the 5′ and floxed homologous regions due to the introduction of a *Spe* I site along with loxP1 and the Spe I site within the double selection cassette, respectively (Fig. 1A). The 12 kb *Bam*H I fragment represents the homologous recombinant in the 3′ homologous region. Lanes 1–4 and 9 correspond to ES cell clones carrying correct homologous recombination events in 5′ and 3′ homologous regions, whereas Lane 8 shows control ES cells. Lanes 5-7, 10, and 11 represent those ES cell clones carrying homologous recombination events in the floxed and 3′ homologous regions. The intensities of the 18 kb and 12 kb *Bam*H I bands are similar in B37 (Lane 2) and B38 (Lane 3) ES cell clones, indicating homogeneous population of targeted ES cells. **(C)** Southern analysis of properly recombined ES cell clones. DNA derived from 5 ES cell clones testing positive by PCR for the proper recombination between loxP sites 2 and 3 was digested with *Nhe* I, followed by hybridization with a probe specific for *PS1* exons 2 and 3. The 7 and 4.5 kb fragments represent the wild-type and the loxP 1, 2/3 alleles, respectively. The intensity of the 4.5 kb band is lower than the 7 kb band, suggesting heterogeneous populations of ES cells containing the loxP1, 2/3 and the loxP1/3. B37-16 (lane 5) and B38-9 (lane 1) ES cell clones, which contain higher percentages of ES cells carrying the loxP1, 2/3 locus, were injected into blastocysts to obtain chimeras. **Methods:** The 46-mer P1 and P4 containing *Spe* I, loxP, and *Nhe* I sites were annealed and inserted into the *Xmn* I site of *PS1* intron 1. The floxed *CMV-HYG/TK* cassette was inserted into the *Sac* I site of *PS1* intron 3. The targeting vector was thoroughly examined with over 10 single and double restriction digestions. It was further verified by sequencing the floxed region, all three loxP sites, and adjacent regions. The sequences of the PCR primers are as follows: P1, 5′ ACTAGTATAACTTCGTATAATGTATGCTATACGAAGTTATGCTAGC; P2, 5′ GGTTTCCCTCCATCTTGGTTG; P3, 5′ TCAACTCCTCCAGAGTCAGG; P4, 5′ GCTAGCATAACTTCGTATAGCATACATTATACGAAGTTATACTAGT; P5, 5′ TCTGGAAGTAGGACAAAGGTG.

15 ES cell clones were confirmed to carry correct homologous recombination events in the 5′ and 3′ homologous regions (5 shown in Fig. 1B).

Two of the 7 confirmed ES cell clones, B37 and B38, were transiently transfected by electroporation with 25 μg of a vector expressing Cre recombinase under the control of the *CMV* promoter (O'Gorman *et al.*, 1997), in order to remove the *CMV-HYG/TK* double selection cassette. Since three loxP sites are present in the targeted *PS1* locus, Cre recombinase can mediate three distinct site-specific recombination events between any pair of loxP sites: loxP 1 and 2; loxP 1 and 3; and loxP 2 and 3. After 24 h of culturing, negative selection with gancyclovir (2 μM) was used to enrich for the desired recombination event between loxP sites 2 and 3. ES cells were plated at low densities (5–10 $\times$ 10^4 cells/100 cm^2) to ensure effective negative selection. Six days after growth in the selection medium, 160 gancyclovir-resistant ES cell clones were picked and expanded. Genomic DNA derived from 50 of these ES cell clones was screened by polymerase chain reaction (PCR) using a 5′ primer specific for loxP1 (P1) and a 3′ primer located in *PS1* intron 3 (P2; Fig. 1A). Five ES cell clones (B37-4, -16, B38-9, -44, and -46) yielded the expected 1.4 kb product, indicating recombination between loxP sites 2 and 3 and retention of loxP1 (Table 1). The PCR product was sequenced to confirm the presence of loxP1, loxP2/3, and the floxed region. PCR using primers P3-P4 and P1-P5 also yielded the expected amplification products from these five positive clones (Table 1).

Twenty of the remaining negative clones were analyzed by PCR using P3 and P2 for recombination between loxP1 and loxP3. All 20 tested clones yielded a 376 bp product that was not observed in the parent clones B37 and B38, indicating excision of the entire floxed region (Table 1). This result also indicates a high efficiency of negative selection, most likely due to low plating density. Surprisingly, the 376 bp product was also detected in the five positive clones, suggesting mixed populations of ES cells carrying loxP1/3 and loxP2/3 recombination events. These five clones were further tested by Southern analysis using *Nbe* I and *Spe* I digestion followed by hybridization with a probe specific for *PS1* exons 2 and 3. In addition to a 7 kb *Nbe* I fragment representing the wild-type *PS1* locus, a 4.5 kb

Nhe I band representing the correctly recombined loxP 1, 2/3 locus was detected (Fig. 1C). However, the intensity of the 4.5 kb *Nhe* I band among these five ES cell clones varied but was generally lower in comparison to the 7 kb band. Similar results were also obtained using *Spe* I digestion (Table 1). These findings further confirmed the heterogeneity of these ES cell populations carrying both loxP1/3 and 2/3 recombination events. The simplest explanation for this heterogeneity in the pattern of Cre recombination within a "clonal" population would be the occurrence of subsequent recombination events following ES cell division due to persistent Cre expression or activity.

The five ES cell clones carrying the targeted, floxed *PS1* locus were expanded and similarly confirmed by PCR and Southern analysis. Two of the confirmed ES cell clones (B37-16 and B38-9) containing higher percentages of the correctly recombined ES cells (loxP1, 2/3) were injected into C57BL/6J and Balb/c mouse blastocysts. Sixteen and five chimeric mice were obtained from the B37-16 and B38-9 clones, respectively. The chimeras were then mated to C57BL/6 mice to obtain heterozygous floxed F1 mice. Genotyping of 247 F1 mice by PCR and Southern analysis showed that only 26 of them carried the properly recombined floxed *PS1* locus with loxP1 and 2/3, whereas 46 carried the loxP1/3 locus. This result is consistent with the heterogeneity of these two ES cell populations in B37-16 and B38-9 clones. The 26 heterozygous floxed F1 mice were derived from two B37-16 and one B38-9 chimeras.

In summary, our analysis demonstrates the importance of thorough screening of ES cell clones for correct recombination events. Homologous recombinants obtained following transfection of the targeting vector and appropriate selection should always be verified by Southern analysis using external 5′ and 3′ probes. Selection for correct site-specific recombinants presents the additional complexity of multiple possible recombination events produced by Cre expression. An informal survey of multiple investigators undertaking similar approaches in the generation of floxed mice indicates that heterogeneity with respect to Cre-mediated recombination events is relatively common in positive ES cell clones. If ES cells with the desired recombination pattern actually represent a small minority of the injected ES cell population, germ-line transmission of the floxed locus may be difficult to obtain. Therefore, it is critical to design a screening strategy that can distinguish among the wild-type, lox1,2,3, lox1,2/3, and lox1/3 loci. If PCR screening is used, PCR-positive clones should be subjected to Southern analysis, which allows selection of those ES cell clones containing higher percentages of the correctly recombined ES cells for injection.

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