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Genetics

Generation of mice with a 200-kb amyloid precursor protein gene deletion by Cre recombinase-mediated site-specific recombination in embryonic stem cells

(Cre recombination/homologous recombination/embryonic stem cells)

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ABSTRACT **Gene disruptions and deletions of up to 20 kb have been generated by homologous recombination with appropriate targeting vectors in murine embryonic stem (ES) cells. Because we could not obtain a deletion of about 200 kb in the mouse amyloid precursor protein gene by the classical technique, we employed strategies involving the insertion of *loxP* sites upstream and downstream of the region to be deleted by homologous recombination and elicited excision of the *loxP*-flanked region by introduction of a Cre expression vector into the ES cells. In the first approach, the *loxP* sequences were inserted in two successive steps and after each step, ES cell clones were isolated and characterized. Deletion of the *loxP*-flanked sequence was accomplished by introducing the cre gene in a third step. In the second approach, ES cells containing the upstream *loxP* cassette were electroporated simultaneously with the downstream *loxP* targeting vector and the Cre expression plasmid. ES cells were obtained that gave rise to chimeric mice capable of germ-line transmission of the deleted amyloid precursor protein allele.**

The targeted inactivation of a gene is a potentially powerful tool in the study of gene function in the intact organism (1). Gene disruptions, deletions as well as discrete mutations, can be generated in murine embryonic stem (ES) cells by homologous recombination with an engineered gene fragment and the modified ES cells can be used to generate mutant heterozygous and, if the gene is not essential for development, homozygous mice (2). However, the commonly used procedures may still allow the generation of proteins with deletions or truncations with residual function (3, 4) or exhibit transdominant activity (5). We therefore set out to explore methods allowing the deletion of entire, even very long genes.

Deletions of up to about 20 kb have been reported, but we were unable to delete a 200-kb segment of the amyloid precursor protein (APP) gene using the classical gene targeting procedure. We therefore developed a strategy based on the P1 site-specific recombination system (6). P1 Cre recombinase causes efficient recombination between two specific 34-bp sites called *loxP* (7) and has been used in the recombination of genomes of yeast (8), cultured mouse cells (9), mice (10), and plants (11).

We inserted *loxP* sites into the 5′ and 3′ proximal regions of the APP gene by homologous recombination in ES cells and deleted about 200 kb of the gene in between by introducing a Cre recombinase expression vector. The resulting ES cells gave rise to high-grade chimeric mice heterozygous for the deletion and capable of germ-line transmission.

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MATERIALS AND METHODS

Construction of Targeting Vectors. The generic targeting vector (Fig. 1*A*) for the upstream insertion, pNEOUMSLOX(−)TKDT, consists of the neomycin resistance gene (NEO) controlled by the phosphoglycerol kinase promoter (12), the so-called "upstream mouse sequence" (UMS) that purportedly mediates transcription termination (13), the 34-bp *loxP* sequence (7), the herpes simplex virus type I thymidine kinase (TK) gene cassette (14), a TK promoter/diphtheria toxin (DT) cassette (15) in pGEMEX 1 (Promega). A *Pme*I site between the NEO sequence and the vector DNA and an *Sal*I site between the TK and the DT sequence allow insertion of the homology arms. The generic vector for the downstream *loxP* insertion, pHYGROLOX(+)TKDT, consists of the DT cassette, the TK cassette, the hygromycin resistance (HYGRO) cassette (prepared by Michael McBurney), and the *loxP* sequence.

The 1.4-kb *Sac*I–*Sca*I and the 4.4-kb *Sca*I–*Bam*HI fragments of the APP gene around exon 2 were inserted into the *Pme*I and *Sal*I sites of pNEOUMSLOX(−)TKDT to give the upstream targeting vector pAPPDup (Fig. 1*A*). pAPPDdown, the downstream targeting vector, was constructed by inserting the 4.05-kb and the 1.35-kb *Sma*I fragments from around exon 17 into *Sal*I and the *Pme*I sites of pHYGROLOX(+)TKDT (Fig. 1*A*).

Generation of ES Cell Lines with Deletion of the APP Gene. ES cells [line E14.1, derived from 129/Ola mice (16)] were cultured on either mitomycin-C-treated G418-resistant MEFs (17) or irradiated SNLH feeder cells (18) in ES medium (DMEM supplemented with 15% fetal calf serum/0.1 mM β-mercaptoethanol/1 mM sodium pyruvate/1000 units/ml pure leukemia inhibitor factor and penicillin/streptomycin). pAPPDup (20 μg) was linearized with *Sfi*I and electroporated into 1.5×10^7 ES cells in 800 μl of PBS using a Bio-Rad gene pulser (240 V, 500 μF); G418 (400 μg/ml) was added after 24 h. More than 500 G418-resistant colonies appeared, 288 of which were screened in pools of 24 by PCR using primers P1 (5′-AGAAGGAAACAGTCTCTCCTGCATTTGCGT) and P2 (5′-ATTCGCAGCGCATCGCCTTCTATCGCC) (Fig. 1*D*). Clones from positive pools were rescreened singly. The three PCR-positive "NEO-lox" clones (E14.1-N11.2, E14.1-N21.5, and E14.1-N21.6) were confirmed by Southern blot analysis, karyotyped, and tested for biological competence by injecting into blastocysts; all yielded high-grade chimeric mice. No germ-line transmission was observed, as expected for mice expressing the herpes simplex virus-TK gene (19, 20).

When following strategy I, 1.5×10^7 cells of NEO-*loxP* clone E14.1-N21.6 were electroporated as above with pAPPDdown cut with *Sfi*I and *Not*I (Table 1, Exp. 4). After exposure to

Abbreviations: FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil); DT, diphtheria toxin A chain; TK, thymidine kinase; PFGE, pulsed-field gel electrophoresis; ES, embryonic stem; APP, amyloid precursor protein.

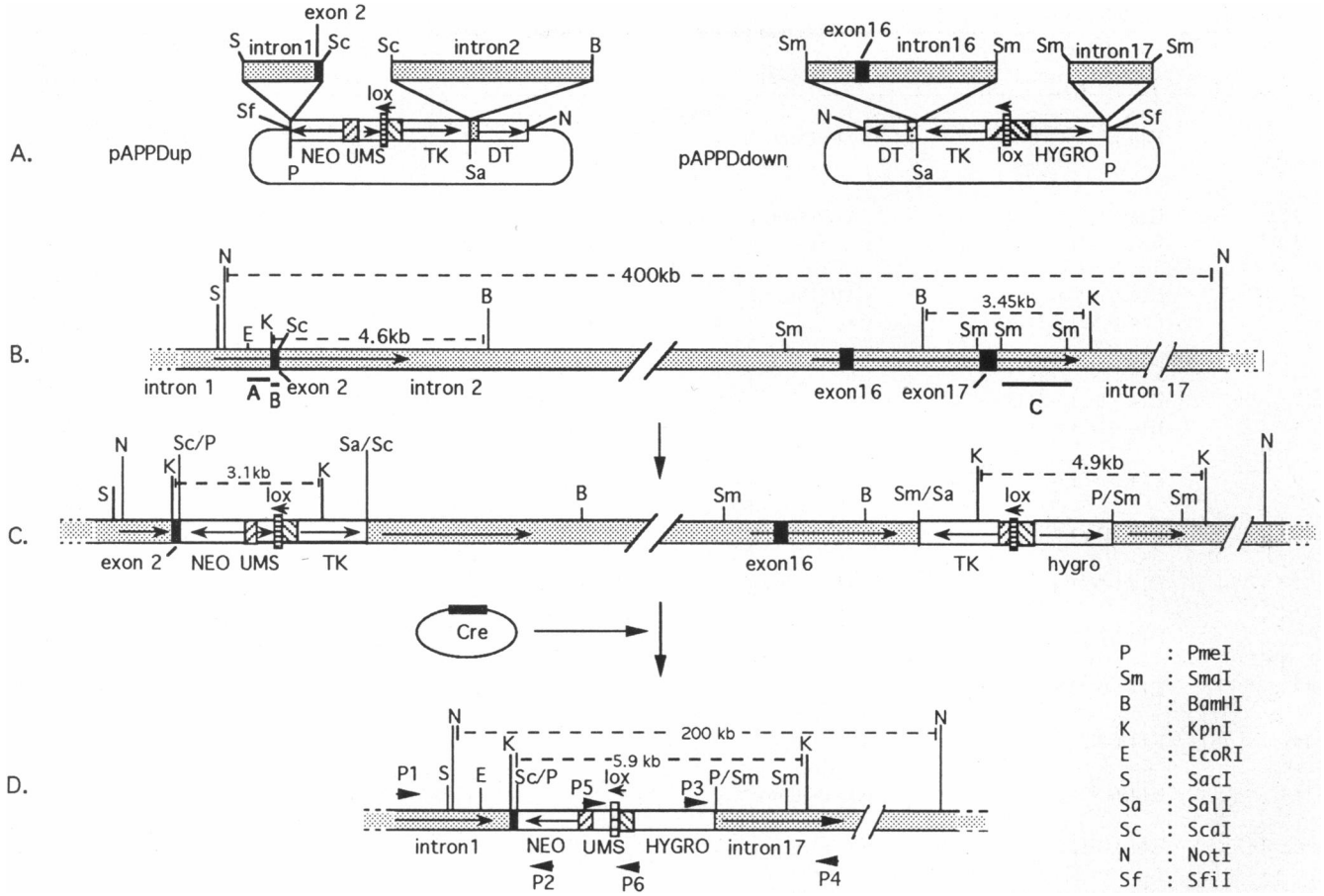

FIG. 1. Maps of targeted APP gene regions and targeting vectors. (*A*) pAPPDup is a targeting vector leading to insertion into exon 2 of a cassette containing the neomycin resistance gene (NEO), the purported transcription termination sequence UMS, the *loxP* sequence, and the TK gene. pAPPDdown leads to replacement of exon 17 and some flanking sequences by a cassette containing the TK gene, the *loxP* sequence, and the hygromycin resistance gene (HYGRO). The DT gene should select against ectopic integration. (*B*) Partial map of the unmodified APP gene around exons 2 and 17. *A*, *B*, and *C* show the derivation of hybridization probes used in Southern blot analyses. (*C*) Partial map after insertion of the upstream and downstream cassettes. (*D*) Partial map after Cre-directed recombination at the *loxP* sites. Arrowheads P1-P6 indicated location of primers used for PCR; long horizontal arrows, direction of transcription; short horizontal arrows above *loxP* sites indicate relative orientation; screened horizontal bars give distances between restriction sites.

G418 (400 μg/ml) and hygromycin (100 μg/ml), 432 colonies were picked and screened by PCR for homologous recombination at the downstream site using primers P3 (5′-CGAGATCAGCAGCCTCTGTTCCACA) and P4 (5′-TCGATTCCTGCAAACCCTTGGG) (Fig. 1*D*). One PCR positive clone (E14.1-N21.6-H5.4) was confirmed by Southern blot analysis. From two additional experiments, three additional clones containing the upstream and downstream cassettes (NEO-*loxP*/HYGRO-*loxP* clones) (Table 1, Exps. 2 and 3) and devoid of ectopic integrations (data not shown) were obtained. Southern blot analyses of *Bam*HI–*Kpn*I-cleaved DNA using probe B (the 160-bp *Kpn*I–*Sca*I fragment of APP exon 2, Fig. 1*B*) or probe C (the 1.35-kb *Sma*I fragment of APP intron 17, Fig. 1*B*) are shown in Fig. 2*A* (lanes 3 and 4).

Each of the 4 NEO-*loxP*/HYGRO-*loxP* clones was electroporated with 20 μg of uncleaved Cre expression plasmid pBS185 (21). Cells were seeded into five 9-cm dishes and grown for 2 days in ES medium with G418 and hygromycin. Cells from one dish were split into three dishes and after 24 h selected in [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil] (FIAU) (0.2 μM). Clone E14.1-N21.5-H15.9 gave about 100 colonies but the other 3 clones none (Table 1, Exps. 5–8). PCR using primers P5 (5′-TGCTCCTGAAGTCCACAATTCACAGTCC) and P6 (5′-CGAGACTAGTGAGACGTGCTACTTCCATTT) (Fig. 1*D*) and Southern blot analysis of *Kpn*I–*Bam*HI-cleaved DNA, using probes B and C (Fig. 1*B*), showed that all 34 FIAU-resistant colonies examined had recombined at the *loxP* sites, as depicted for 3 of them in Fig. 2*A* (lanes 5–7).

In the case of strategy II, 1.5×10^7 NEO-*loxP* ES cells (clone E14.1-N21.6) were coelectroporated with 15 μg of linearized downstream targeting vector pAPPDdown and 15 μg of *Eco*RI-linearized pBS185. After 24 h in G418, hygromycin was added and after 96 h, FIAU was added. The single resulting colony showed the expected deletion (E14.1-N21.6-HC11.12; Table 1, Exp. 9 and Fig. 2*A*, lane 8). Four additional experiments in which several parameters were varied gave four more resistant colonies, three of which were *loxP* recombinants (Table 1, Exps. 10–13).

Pulsed-Field Gel Electrophoresis (PFGE) and Southern Blot Analysis. PFGE was performed on *Not*I-cleaved ES cell DNA using a Bio-Rad DRIII apparatus. Fractionation was for 24 h in 1% agarose (PFGE ultra-pure DNA grade agarose, Bio-Rad) in 0.5× Tris-buffered saline (TBE) buffer at 6 V/cm at 4°C.

Generation of Chimeric Mice. ES cells from lines with a high proportion of undifferentiated cells and >80% euploidy were microinjected into blastocysts of C57BL/6 mice and processed as described (22).

RESULTS

An Attempt to Obtain Large Targeted Deletions by One-Step Homologous Recombination. We first tried to delete 200

Table 1. Gene targeting in ES cells

Exp.	ES cells electroporated*	Plasmid†	Selection‡	Positive clones§	Homologous and/or Cre recombinants
		Strategy I			
1	E14.1	pAPPDup	N	3/288	E14.1-N11.2 E14.1-N21.5 E14.1-N21.6
2	E14.1-N11.2	pAPPDdown	N, H	4★/480	E14.1-N11.2-H23.5
3	E14.1-N21.5	pAPPDdown	N, H	2/228	E14.1-N21.5-H15.2 E14.1-N21.5-H15.9
4	E14.1-N21.6	pAPPDdown	N, H	1/432	E14.1-N21.6-H5.4
5	E14.1-N21.5-H15.9	Cre	N, H, F	34/34	E14.1-N21.5-H15.9 -C1.1 to C3.10
6	E14.1-N21.5-H15.2	Cre	N, H, F	none	—
7	E14.1-N11.2-H23.5	Cre	N, H, F	none	—
8	E14.1-N21.6-H5.4	Cre	N, H, F	none	—
		Strategy II			
9	E14.1-N21.6	pAPPDdown +Cre/*Eco*RI	N, H, F	1	E14.1-N21.6 -HC11.12
10	E14.1-N21.6	pAPPDdown +Cre	N, H, F	1	E14.1-N21.6-HC1.1
11	E14.1-N21.6	pAPPDdown +Cre	N, H, F	1	E14.1-N21.6-HC1.2
12	E14.1-N21.6	pAPPDdown +Cre	N, H, F	1	E14-1-N21.6-HC1.3
13	E14.1-N21.6	pAPPDdown +Cre	N, H, F	none†	—
14	E14.1-N21.6	pAPPDdown +Cre	N, H, F	none	—
15	E14.1-N21.6	pAPPDdown +Cre	N, H, F	none	—
16	E14.1-N11.2	pAPPDdown +Cre	N, H, F	none	—
17	E14.1-N21.5	pAPPDdown +Cre/*Eco*RI	N, H, F	none	—

*About 1.5×10^7 ES cells were electroporated in each experiment.
†pAPPDup and pAPPDdown (Fig. 2*A*) were linearized (see *Materials and Methods*). Cre, Cre expression plasmid pBS185 (21) uncleaved or linearized with *Eco*RI as indicated.
‡N, G418; H, hygromycin; F, FIAU.
§Strategy I: Homologous recombinants/selection-resistant clones screened. (★) Three clones had ectopic TK inserts.
Strategy II: All FIAU-resistant clones except one (+) were Cre-mediated recombinants

kb from the APP gene by introducing a targeting vector containing a neomycin-UMS (NEOUMS) cassette (18) flanked by the 1.4-kb *Sac*I–*Sca*I APP DNA fragment comprising part of intron 1 and exon 2 and the 6.8-kb *Bam*HI fragment containing exon 17 and parts of introns 16 and 17, as well as the herpes simplex virus-TK gene at one end. We performed two electroporations with $\approx 1.5 \times 10^7$ GS1 ES cells as described earlier (23, 24) and screened 816 G418-resistant colonies by PCR, using primers P1 and P2 (Fig. 1*D*); however, no homologous recombinants (<0.12%) were found. In previous experiments using the same cassettes and flanking regions of similar length, but with expected replacements shorter than 2 kb, the yield of homologous recombinants was 1–15%.

Strategies for *loxP*-Mediated Gene Deletion. Two strategies for *loxP*-mediated gene deletion were deployed. In strategy I, cassettes containing the *loxP* sequence (*lox*) were inserted by homologous recombination in two successive steps, upstream and downstream of the DNA segment to be deleted, with the *loxP* sequences in a parallel orientation. After each step, ES cell clones were isolated and characterized. The *lox*-flanked sequence was deleted by introducing the Cre gene in a third step. In strategy II, ES cells containing the upstream *loxP* cassette were electroporated simultaneously with the downstream *loxP* targeting vector and the Cre expression plasmid, without isolating and characterizing ES cells with two *loxP* cassettes.

Gene Deletion by Strategy I. We utilized the agouti-marked ES cell line E14.1 that was known to be very stable (R. Kühn and K. Rajewsky, personal communication). In both strategies described above, each *loxP* cassette contained a TK gene between the *loxP* sequence and the gene segment to be deleted. When ES cells with the upstream and downstream *loxP* cassettes in place are exposed to Cre, selection with FIAU allows survival only if the gene segment flanked by the two TK sequences has been excised. The upstream *loxP* cassette with a neomycin resistance gene (pAPPDup) was targeted to the 3′ end of exon 2. After electroporation, neomycin-resistant ES cell clones were picked. Homologous recombination had taken place in 3 of 288 clones as determined by PCR and Southern blot analysis (data not shown). The three clones had no ectopically inserted cassettes as determined by Southern blot analysis, and generated high-grade chimeras after injection into blastocysts (Table 2). Following strategy I, ES cells containing the upstream *loxP* cassette were electroporated with the vector containing the downstream *loxP* cassette with a hygromycin resistance gene (pAPPDdown), targeted to replace exon 17; ES cell clones with homologous recombination were identified and characterized as above. A Cre expression plasmid was then introduced by electroporation. All clones surviving subsequent exposure to FIAU had undergone deletion of the *loxP*-flanked gene segment, as evidenced by PCR (data not shown).

Clones were further characterized by Southern blot analysis as exemplified in Fig. 2*A* (lanes 5–7). Those that subsequently gave high- grade chimeras were further subjected to PFGE

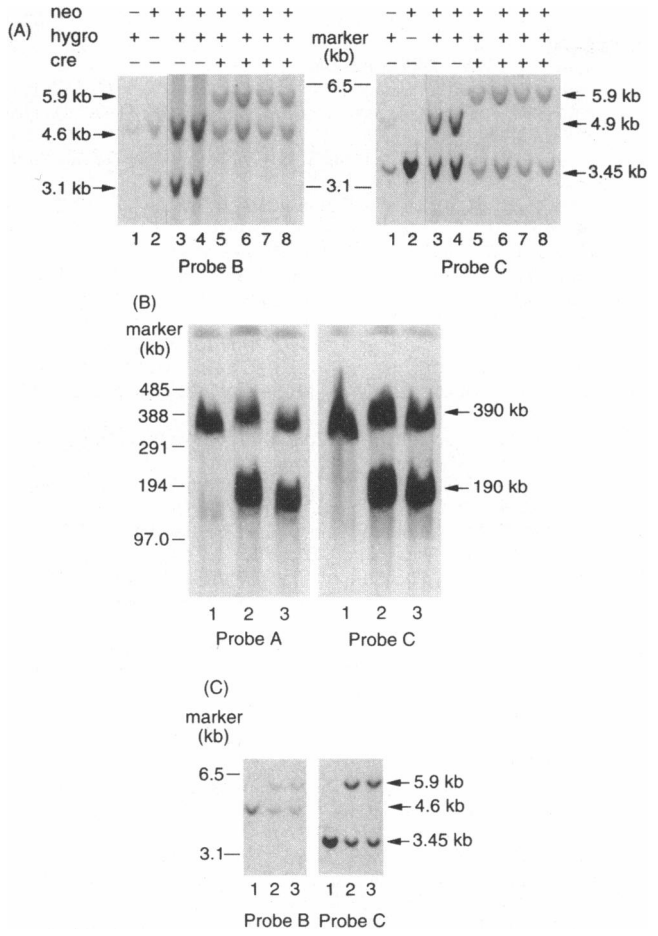

FIG. 2. Analysis of ES cell and mouse tail DNA. Hybridizations were with (upstream) probe A and probe B or (downstream) probe C (see Fig. 1*B*). (*A*) Southern blot analysis of ES cell DNA cleaved with *Bam*HI and *Kpn*I. Lanes: 1, downstream *loxP* cassette inserted (clone GS1-H23.7 from a separate experiment using ES cell line GS1); 2, upstream *loxP* cassette inserted (E14.1-N21.5); 3 and 4, upstream and downstream *loxP* cassettes inserted (E14.1-N21.5-H15.2 and E14.1-N21.5-H15.9); 5–8, upstream and downstream *loxP* cassettes inserted and Cre-recombined (E14.1-N21.5-H15.9-C3.8, E14.1-N21.5-H15.9-C3.9, E14.1-N21.5-H15.9-C3.10, and E14.1-N21.6-HC11.12). For probe B, band at 3.1 kb, allele with NEO-*loxP* insert; 4.6 kb, wild type; 5.9 kb, Cre-recombined. For probe C, band at 3.45 kb, wild type; 4.9 kb, HYGRO-*loxP* insert; 5.9 kb, Cre-recombined. (*B*) PFGE Southern blot analysis of ES cell DNA cleaved with *Not*I. Lanes: 1, wild type; 2 and 3, upstream and downstream *loxP* cassettes inserted and Cre-recombined (E14.1-N21.5-H15.9-C3.9 and E14.1-N21.6-HC11.12). Band at 390 kb, wild-type allele, at 190 kb, Cre-recombined. Marker, λ DNA ladder (Pharmacia). (*C*) Southern blot analysis of mouse tail DNA cleaved with *Bam*HI and *Kpn*I. Lanes: 1, wild-type mouse; 2 and 3, heterozygous mouse derived from clone E14.1-N21.6-HC11.12 (see Table 1, Exp. 9; Table 2, line 6). Bands as in (*A*).

Southern blot analysis. As shown in Fig. 2*B* (lane 1), *Not*I-cleaved wild-type ES cell DNA, after hybridization with upstream probe A or downstream probe C (Fig. 1*B*), gave a single band corresponding to a fragment of about 390 kb, whereas DNA from Cre-recombined clones E14.1-N21.5-H15.9-C3.9 and E14.1-N21.6-HC11–12 (from strategy II; Table 1) resulted in two bands, at about 390 and 190 kb, both with probe A and probe C, showing that a segment of about 200 kb had been excised from the APP segment between the *Not*I sites (Fig. 2*B*, lanes 2 and 3). One of three clones injected into C57BL/6 blastocysts gave rise to four high-grade chimeras that are currently breeding.

Gene Deletion by Strategy II. Nine experiments were carried out following strategy II (Table 1, Exps. 9–17); about 1.5×10^7 ES cells carrying the upstream NEO-*loxP* cassette were co-electroporated with the downstream targeting vector containing the TK-loxP-HYGRO cassette and the Cre expression plasmid. Selection with FIAU yielded a single colony in five of nine electroporations. In four of these electroporations the *loxP*-flanked sequence was deleted, as evidenced by PCR and Southern blot analysis (see Fig. 2*A*, lane 8). The agouti-marked ES cells from one of the four Cre-recombined clones was injected into C57BL/6 blastocysts (Table 2); three of four pups showed high-grade chimerism and were mated to C57BL/6 mice. All resulting pups (24 of 24) were agouti and 11 of 13 examined had the deleted allele, as determined by PCR and Southern blot analysis (Fig. 2*C*), demonstrating efficient germ-line transmission.

Table 2. Outcome of blastocyst injections

ES cell line	Euploid, %*	Blastocysts injection	Surviving pups	Chimeric pups†
E14.1	100	39	1	1
E14.1-N11.2	100	71	4	4
E14.1-N21.5	100	60	3	3
E14.1-N21.6	100	75	3	3
E14.1-N21.5-H15.9	100	24	3	1
E14.1-N21.6-HC11.12	100	83	4	3
E14.1-N21.5-H15.9-C3.8	80	18	0	0
E14.1-N21.5-H15.9-C3.9	90	26	4	4
E14.1-N21.5-H15.9-C3.10	90	25	0	0

*Percentage of cells with 40 chromosomes. Ten chromosomal spreads were evaluated for each clone.
†All pups were about 90% chimeric as estimated from the extent of agouti coat color.

DISCUSSION

We developed a procedure to generate long targeted deletions in ES cell chromosomes based on the insertion of *loxP* sites upstream and downstream of the sequence to be deleted, in parallel orientation, followed by Cre-mediated recombination. Because there is a TK sequence between each *loxP* site and the region to be deleted, cells in which excision has taken place can be selected with FIAU. When the procedure was executed in three steps (strategy I), with isolation and characterization of the ES cell clones after insertion of each *loxP* site, introduction of the Cre expression plasmid resulted in a high yield of FIAU-resistant colonies in one of four ES cell clones, whereas three gave no resistant colonies. This likely reflects the fact that excision only occurs if the two *loxP* sites were integrated syntenically, which should happen in about one-half of the clones. FIAU-resistant clones cannot arise if an ES cell line contains an ectopically integrated TK gene.

Gene deletion could be carried out in only two steps, by first inserting the upstream *loxP* sequence, characterizing the resulting homologous recombinant ES cells and then electroporating simultaneously with the downstream *loxP* targeting vector and the Cre expression plasmid (strategy II). The yield of FIAU-resistant recombinants was low, one per 1.5×10^7 cells electroporated in five of nine experiments, maybe because the probability of inserting the *loxP* cassettes syntenically and introducing the Cre expression plasmid into the same cells is low. Despite the poor yield, the savings in culture time and effort are very considerable as compared to the three-stage procedure.

Many ES cell lines with the desired deletion were obtained by strategies I and II; four clones were injected into blastocysts and in two cases several high-grade chimeric mice resulted. A chimeric mouse derived from the two-stage procedure gave germ-line transmission.

The APP gene comprises 19 exons. The human gene is estimated to be about 400 kb in length (25). In the experiments described above, the *loxP* sites were inserted at the 3′ end of

exon 2 and replacing exon 17 because of the availability of the cognate genomic APP clones. From the PFGE Southern blot analysis we deduced that a 200 kb DNA fragment had been deleted. The remaining APP sequences comprise codons 1–71 and 738–770; the transmembrane region, which is encoded by exon 17 and lies between codons 700 and 723, is thus deleted. Mice homozygous for a deletion of part of the 5′ flanking upstream region and exon 1 of APP showed reactive gliosis and decreased locomotor activity (26); mice expressing a low level of APP lacking amino acids 20–75 had behavioral deficits and increased incidence of agenesis of the corpus callosum (4). It will be of interest to compare the phenotype of mice homozygous for the APP deletion described in this paper with that of the animals described previously.

Cre recombinase technology may be used to delete a gene entirely; however, a resulting phenotype need not necessarily result from the deletion of the cognate coding sequence, because distinct genes may overlap on opposite DNA strands (27), deleted introns may contain independent genes (28) or even genes for transcription factors controlling the expression of other genes (29). To ensure that an observed phenotype is truly due to deletion of the coding sequence of the gene in question, it is necessary to show that the defect can be complemented by introducing a cDNA expressing the cognate protein. This may be an arduous task if several different proteins are derived from the gene due to alternative splicing.

While this manuscript was in preparation, Ramirez-Solis *et al.* (30) reported the generation of deletions of as much as 3–4 centimorgans as well as chromosome rearrangements using Cre-mediated recombination.

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