

TECHNOLOGY REPORT

A Reliable lacZ Expression Reporter Cassette for Multipurpose, Knockout-First Alleles

Giuseppe Testa,¹ Julia Schaft,¹ Frank van der Hoeven,² Stefan Glaser,¹ Konstantinos Anastassiadis,¹ Youming Zhang,³ Thomas Hermann,⁴ Wolfgang Stremmel,⁴ and A. Francis Stewart^{1*}

¹Biotec, Technische Universität Dresden, c/o Max Planck Institute of Molecular cell Biology and Genetics, Dresden, Germany

²Deutsches Krebsforschungszentrum, Heidelberg, Germany

³GeneBridges GmbH, c/o Max Planck Institute of Molecular cell Biology and Genetics, Dresden, Germany

⁴Department of Internal Medicine IV, University of Heidelberg, Heidelberg, Germany

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Summary: Alteration of the mouse genome through homologous recombination in embryonic stem (ES) cells is the most accurate and versatile way to dissect gene function in a vertebrate model. Most often, a selectable marker is used to create a knockout allele by replacing an essential part of the gene. However, knockout strategies are limited because the mutation is present constitutively. Conditional approaches based on the Cre-loxP site-specific recombination (SSR) system address this limitation; however, it requires that all parts of the targeted gene remain in ES cells. Here we report success with a “knockout-first” strategy that ablates gene function by insertion of RNA processing signals without deletion of any of the target gene. Incorporation of site-specific recombination target sites creates a multipurpose allele for both knockout and conditional applications. *genesis* 38:151–158, 2004.

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Gene function analysis via homologous recombination in mouse ES cells plays a central role in the biological sciences. In its most straightforward application, homologous recombination is used to delete essential parts of a gene to establish a knockout allele. Knockouts are powerful because the consequences of complete loss of function are established. However, knockouts only report the first role of a gene in development. Because many knockouts are developmentally lethal, analysis of gene function later in development or in the adult is precluded. To circumvent this limitation, strategies for conditional mutagenesis using Cre/loxP site-specific recombination have been developed (Gu *et al.*, 1994). Conditional strategies require the strategic placement of loxP sites into an intact gene to create a conditional allele. Optimally, the loxP sites are placed into introns so that 1) subsequent Cre recombination will delete an

exon, or exons, which encode an essential part of the protein, or produce a shortened mRNA that encodes a frameshift of the encoded protein; and 2) the loxP sites are as close together as possible to ensure efficient Cre recombination. Hence, the design of knockout and conditional alleles differ. Knockout alleles usually require the removal of a substantial part of a gene, ideally all of it, from the genome. Conditional alleles are based on leaving the gene intact.

Strategies to combine the advantages of knockout and conditional alleles offer significant benefits in terms of economy, speed, and number of mouse lines required for the several purposes. These strategies obviously require leaving the gene intact and have been implemented to create allelic series, based on the hypomorphic impact of an FRT or loxP flanked selectable marker (Meyers *et al.*, 1998; Nagy *et al.*, 1998). One strategy, here termed “conditional first,” relies on the creation of a mouse line with a conditional allele, which is then crossed to a Cre deleter for removal of the loxP flanked section for germline transmission of the knockout allele. Here we report exploration of an alternative, “knockout-first” strategy. It is based on inserting a cassette into an intron of an intact gene that produces a knockout at the RNA processing level. A splice acceptor in the cassette captures the RNA transcript and an efficient polyadenylation signal truncates the transcript so that the gene is not transcribed into mRNA downstream of the cassette site. A knockout-first strategy also permits the insertion of an expression reporter like the β -galactosidase gene

* Correspondence to: A. Francis Stewart, Biotec, Technische Universität Dresden, c/o Max Planck Institute of Molecular cell Biology and Genetics, Pfotenhauerstr. 108, Dresden, Germany 01307.
E-mail: stewart@mpi-cbg.de

so that the knockout configuration can serve a second purpose to report the activity of the promoter.

Our starting point was the β geo cassette (Fig. 1), originally developed by Friedrich and Soriano (1991) for inactivation and expression reporting of genes in ES cells (Mountford *et al.*, 1994). This cassette comprises the lacZ-neomycin phosphotransferase fusion downstream of intronic sequences and a splice acceptor site from *engrailed-2* (*en-2*; (Gossler *et al.*, 1989) and the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES; (Kim *et al.*, 1992; Pelletier and Sonenberg, 1988), followed by the SV40 polyadenylation signal, which comprises 200 bp, is bidirectional, and is among the strongest polyadenylation elements described. Our goal was to create “knockout first” targeting vectors based on the β geo cassette flanked by site-specific recombination target sites (SSRTs).

One of the rate-limiting steps in the production of mutant mice until recently has been the assembly of targeting constructs due to the size and site limitations inherent to conventional *E. coli* DNA engineering methods. We developed a method, termed Red/ET, based on homologous recombination in *E. coli* mediated by phage proteins to circumvent these limitations (Copeland *et al.*, 2001; Muyrers *et al.*, 1999, 2001; Yu *et al.*, 2000; Zhang *et al.*, 1998, 2000). For the rapid assembly of targeting constructs, Red/ET permits a key simplification when a dual eukaryotic/*E. coli* promoter is used to drive expression of a dual selectable marker, such as *neo* (kanamycin in *E. coli*, G418 in eukaryotic cells) or *hygro* (hygromycin in both *E. coli* and eukaryotic cells). Thereby, selection can be used in *E. coli* for integration by homologous recombination to create the targeting vector and then reused for homologous recombination in ES cells (Angrand *et al.*, 1999). To adapt the same advantage to the β geo cassette, we altered the linker region between the end of *lacZ* and the beginning of *neo* to include, while keeping the reading frame, the Tn903 promoter to make the β geok cassette (Fig. 1b). β ghygro is a similar variation of β geo (Fig. 1d). Consequently, selection for kanamycin or hygromycin resistance in *E. coli* can be used to identify integrations of the β geo cassette into mouse genomic DNA clones to create targeting vectors.

In the three examples shown here (Figs. 1a,d,e; 2a), the β geo cassette and its variations were flanked by SSRTs, either loxPs or FRTs (FLP recombination targets), so that Cre or FLPe recombination can be used to convert the alleles. Regardless of the differences between the three genes targeted, or the different dispositions of the cassette in these three genes, the splice acceptor of the β geo cassette efficiently trapped the nascent transcript, which was also efficiently polyadenylated at the SV40 site, hence producing a knockout at the RNA processing level.

In order to prevent, respectively, out-of-frame initiation of *neo* and *hygro* translation in *E. coli*, and premature termination and polyadenylation of the β geo and β ghygro fusion in ES cells, the Tn903 promoter was

mutated to change the ATG (at position 34–37 of the linker) into an ACG, and the AATAAA sequence (at position 41–46 of the linker) into AGTAAA. When translated in the *lacZ-neo* or the *lacZ-hygro* fusion, the mutant Tn903 promoter incorporates, respectively, 31 and 27 amino acids between the last residue of *lacZ* (amino acid 1022, glutamine) and the second residue of *neo* (isoleucine) or first residue of *hygro* (methionine). When *neo* is translated in *E. coli*, three residues (glycine, serine, and alanine) are added between the initiating methionine and the originally second residue, isoleucine.

The β geok and β ghygro cassettes are shown in Figure 1a,d, respectively. The splice acceptor and the polyadenylation elements serve to, respectively, trap and truncate transcripts from the targeted loci; concomitantly, the IRES enables translation of the *lacZ-neo* fusion for selection and expression reporting. To facilitate the assembly of the targeting constructs, we flanked the cassettes with target sites for rare restriction endonucleases (shown in Fig. 1a). We used these sites to insert, by ligation, PCR products carrying 50–250 bps of homology to the intended insertion site in the targeting construct plus loxP or minimal FRT sites, which were added to the relevant PCR primer by oligonucleotide synthesis.

A limitation of the β geok and β ghygro cassettes is that, being promoterless cassettes, they can be applied only to genes that are expressed in ES cells. Therefore, we also developed β geok-hygro in which the hygromycin B phosphotransferase gene (*hygro*), under the control of the SV40 promoter, is cloned downstream of the β geok cassette in opposite orientation (Fig. 1e). The SV40 polyA element, through its bidirectionality, serves simultaneously the β geok and *hygro* genes. For genes that are not expressed in ES cells, or whose expression status is unknown, hygromycin selection can be used to identify targeted clones.

We targeted the β geok, β ghygro, and the β geok-hygro cassettes to three unrelated loci, *af4*, *trx2*, and *fatp-4* (Baskaran *et al.*, 1997; Herrmann *et al.*, 2001; Huntsman *et al.*, 1999). Targeted alleles are shown in Figure 2a. As summarized in Table 1, in all cases the splice acceptor-polyA module achieved efficient truncation of the target mRNA, as demonstrated by failure to detect, by RT-PCR or Northern analysis, the full-length mRNAs (Table 1, columns 1 and 2; Fig. 2b–d). ES cell targeting experiments showed that the addition of the prokaryotic expression linker between the *lacZ* and *neo* genes did not affect β geok function; G418 resistant colonies were obtained at the expected frequencies (~500 colonies for 1×10^7 cells transfected with 40 μ g of targeting construct DNA) and for all three loci β -galactosidase staining could be used to report gene expression both in ES cells and in mice (Table 1, column 3, and data not shown).

Minimal FRTs (Ringrose *et al.*, 1999) or loxPs were included in the three alleles and efficient recombination was obtained by crossing to appropriate Cre (Lallemand *et al.*, 1998) and FLPe (Rodriguez *et al.*, 2001) expressing mouse lines (Table 1, columns 4 and 5).

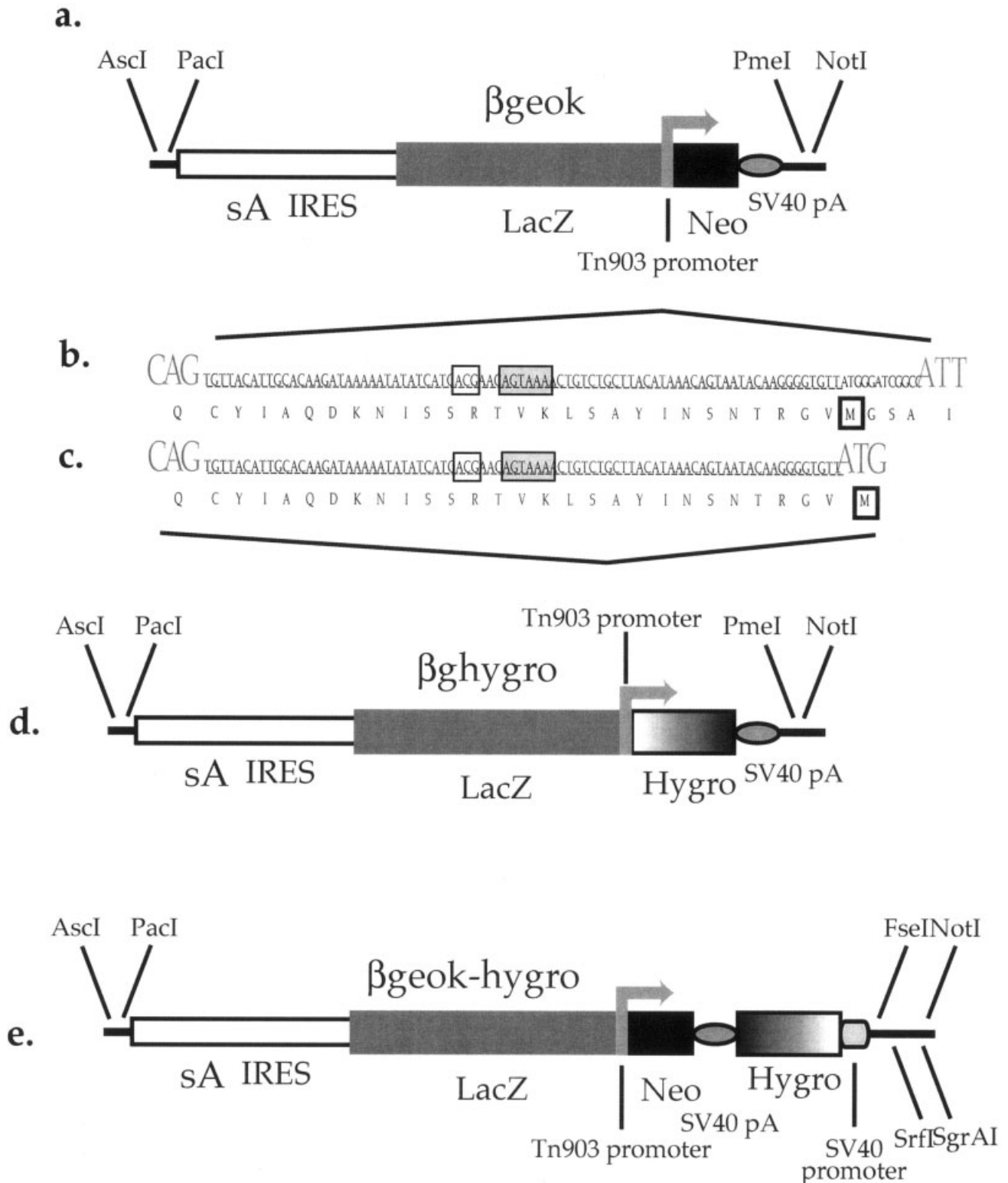


FIG. 1. Architecture of the β geo, β ghygro, and β geo-ghygro cassettes. **a:** Schematic representation of the β geo cassette. Throughout the figure the elements of the construct are drawn to scale as rectangular boxes of different colors. Names are indicated underneath. The gray arrow indicates the prokaryotic promoter from Tn903. **b,c:** Close-up of the prokaryotic expression linker inserted between amino acid 1022 of lacZ (glutamine codon, in bigger letters) and, respectively, the second residue of neo (isoleucine codon, in bigger letters) and the first residue of hygro (methionine codon, in bigger letters). The boxed ACG and AGTAAA sequence are intended mutations of, respectively, ATG and AATAAA present in the original Tn903 promoter. The boxed "M" indicates the initiating methionine of neo and hygro, respectively. **d:** Schematic representation of the β ghygro cassette. **e:** Schematic representation of the β geo-ghygro cassette. hygro, hygromycin B phosphotransferase gene; IRES, internal ribosomal entry site from the encephalomyocarditis virus; lacZ, gene encoding β -galactosidase; neo, neomycin phosphotransferase; pA, SV40 polyadenylation signal; sA, splice acceptor element from engrailed-2.

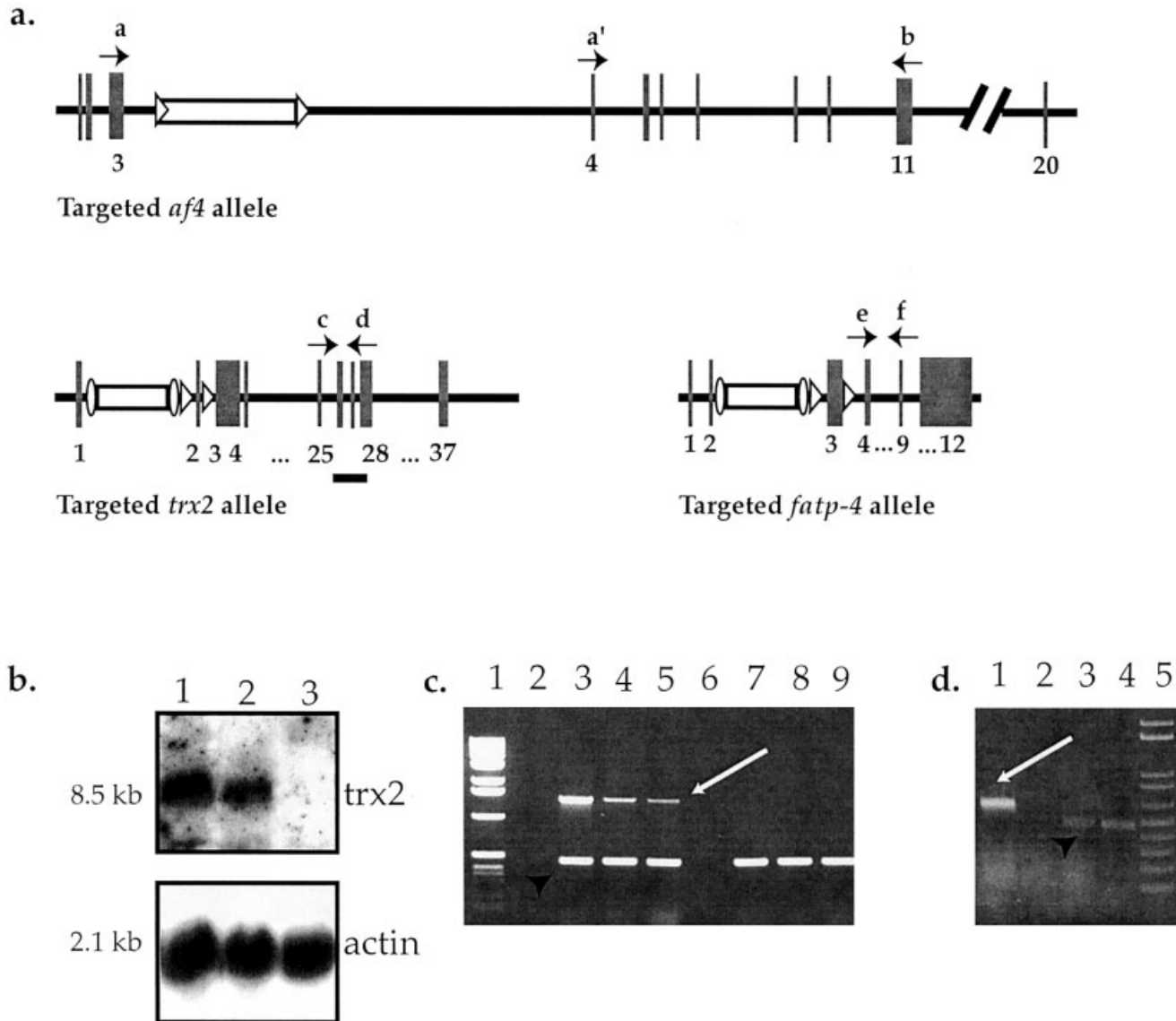


FIG. 2. Functional inactivation of the *af4*, *trx2*, and *fatp-4* genes. **a:** Schematic representations of the targeted alleles of the *af4*, *trx2*, and *fatp-4* genes. The *af4* gene was targeted in intron 3 with the β geok-hygro cassette described in Fig. 1e; the *trx2* gene was targeted in intron 1 with either the β geok or the β ghygro cassettes described in Fig. 1a,d, respectively; the *fatp-4* gene was targeted in intron 2 with the β geok cassette described in Fig. 1a. Relevant exons are represented by gray boxes and numbered underneath. The white rectangular boxes represent, for each gene, the relevant targeting cassette. White triangles indicate loxP sites, while white ovals indicate FRT sites. Arrows above the exons indicate the primers used for the RT-PCR shown in **c** and **d** (and data not shown). The black bar below exons 26–28 of the *trx2* allele represent the probe used for the Northern analysis shown in **b**. **b:** Northern analysis on homozygous mutant mouse ES cells, in which the two alleles of the *trx2* gene have been targeted with, respectively, the β geok and the β ghygro cassettes in two consecutive rounds of electroporation. The upper panel shows hybridization with a gene-specific cDNA probe (CF14) which spans exons 26–28. The lower panel shows a positive control hybridization with a β -actin probe. Lane 1: wildtype ES cells; lane 2: heterozygote ES cells targeted with the β geok cassette; lane 3: homozygote ES cells targeted with both the β geok and the β ghygro cassettes. **c:** RT-PCR analysis on different tissues of homozygote mutant mice for the *af4* allele through simultaneous amplification of an *af4* cDNA fragment of 1430 bp (white arrow, amplified with primers a and b depicted in **a**) and a β -actin cDNA fragment as positive control of 439 bp (black arrowhead). Lane 1: DNA marker; lanes 3–5: thymus, kidney, and liver, respectively, of wildtype littermates; lanes 7–9: thymus, kidney, and liver, respectively, of *af4*^{-/-} littermates. **d:** RT-PCR analysis of homozygote mutant mice for the *fatp-4* allele through amplification of an *fatp-4* cDNA fragment of 612 bp (white arrow, amplified with primers e and f depicted in **a**) and a *fatp-1* cDNA fragment as positive control of 481 bp (black arrowhead). Lanes 1 and 3: cDNA from wildtype mice amplified, respectively, for the *fatp-4* and *fatp-1* gene; lanes 2 and 4: cDNA from *fatp4*^{-/-} littermates amplified, respectively, for the *fatp-4* and *fatp-1* gene. Note the absence of the *fatp-4* amplification product in *fatp4*^{-/-} littermates.

Table 1
Results Obtained with the β geok Cassette on Three Unrelated Loci

Gene	Assay				
	Northern on $-/-$ ES cells	RT-PCR	LacZ expression	FLPe recombination	CRE recombination
<i>Trx2</i>	✓	✓	✓	✓	✓
<i>Af4</i>	n.a.	✓	✓	n.a.	✓
<i>Fatp-4</i>	n.a.	✓	✓	✓	✓

Rows refer to the target genes, while columns indicate the assay used to test the intronic knockout/conditional approach. The *Trx2* and *FATP-4* genes were targeted with the β geok construct, while the *Af4* gene, whose expression status in ES cells was unknown, was targeted with the β geok-hygro construct. Positive results are indicated by a tick in the appropriate table box. The first two columns summarize Northern and RT-PCR analysis to detect full-length transcription from the targeted loci: a tick represents failure to detect the full-length mRNA and hence efficient truncation due to the SV40 polyA element. The third column indicates successful expression of β -galactosidase, which to the extent characterized so far mirrors endogenous expression patterns. Columns 4 and 5 refer to the application of either FLPe or Cre or both FLPe and Cre recombination to the targeted alleles: all three alleles have been recombined successfully through FLPe (except *Af4*, which has no FRTs) and Cre deletors with expected frequencies. In addition, for *trx2*, we have evidence that the early lethal phenotype of the “knockout first” allele is recapitulated by the allele generated after both FLPe and Cre recombination.

Abbreviations: RT-PCR = reverse transcription-polymerase chain reaction; *Trx2* = trithorax 2; *Fatp-4* = fatty acid transporter 4; n.a. = not applicable.

Figure 3 shows a schematic representation of a strategy to make multipurpose knockout/conditional alleles based on a “knockout-first” approach.

To begin with, an exon or group of exons are identified which, in the final allele configuration, will be flanked by loxP sites to set up the conditional knockout. Exons should be chosen whose removal results in either a frameshift of the protein or a loss of a key part of the protein, while ideally keeping the loxP sites as close together as possible for high efficiency of Cre recombination (Ringrose *et al.*, 1999). As shown in Figure 3, step 1, this first goal is achieved via Red/ET recombination in *E. coli* of a loxP flanked PCR-amplified selectable cassette into the desired intron. Following Cre expression in *E. coli* (Buchholz *et al.*, 1996) (Zhang *et al.*, 1998), the selectable marker is removed leaving behind the loxP site (step 2).

Step 3 involves the placement of the β geo cassette in an upstream intron of the gene, which should be selected according to the experimental goal: most upstream introns for a complete knockdown of protein function, and more downstream ones for the creation of potentially informative hypomorphic and/or dominant negative alleles. The β geok or the β geok-hygro cassette (depending on the expression status of the target locus in ES cells) flanked on the 5' and 3' end by, respectively, an FRT site and an loxP site, is cloned into the desired intron by Red/ET recombination in *E. coli*, using kanamycin resistance to select recombinant clones. Upon transfection in ES cells and identification of homologous recombinants, this construct yields, depending on the intron, either a knockout or a hypomorphic allele and reports gene expression through the *lacZ* marker.

Step 4 is the FLPe mediated excision of the β geo (or β geok-hygro) cassette, either in ES cells (Schaft *et al.*, 2001) or in mice (Rodriguez *et al.*, 2000), to restore gene function, leaving only one FRT and one loxP site in the target intron. The resulting conditional allele enables tissue- and time-controlled ablation of gene function by selecting appropriate Cre expressing mouse lines.

Finally, step 5 constitutes a potentially useful configuration of the multipurpose allele; here, only Cre is used to remove the exon(s), while leaving in place the β geok (or β geok-hygro) cassette. The phenotype of this allele should be the same as the knockout-first allele (i.e., before Cre or FLPe recombination). If it is not, this indicates that either the knockout-first allele is not a complete knockout, or that a cis element lies between the loxP sites.

Since such a “knockout-first” approach leaves the gene intact, problems might arise if the “knockout” function of the RNA processing module is bypassed, through alternative splicing or through the presence of additional, downstream promoters. In the three examples described, we did not observe any alternative splicing around the β geo cassette, indicating that the splice acceptor element is robust and relatively locus-independent.

Our evidence so far indicates that none of the three targeted introns is involved in alternative splicing. This does not exclude the possibility that alternative splicing problems may occur if alternatively spliced introns are targeted with the β geo cassette. However, in the case of *af4*^{-/-} littermates, failure to detect by RT-PCR a product with primers, respectively, upstream and downstream of the β geok-hygro cassette (Fig. 2c; primers a and b in Fig. 2a) and the concomitant amplification of a product with primers both located downstream of the β geok-hygro cassette (data not shown; primers a' and b in Fig. 2a), suggest the presence of an alternative *af4* promoter in the third intron downstream of the β geok-hygro cassette. Obviously, the unknown presence of a downstream promoter will confound aspects of the desired allele.

In conclusion, results obtained with three distinct loci highlight the robustness of the β geok system of dual prokaryotic/eukaryotic selectable cassettes. When combined with the use of Cre and FLPe, these cassettes permit the generation of multipurpose alleles based on a reliable “knockout-first” approach.

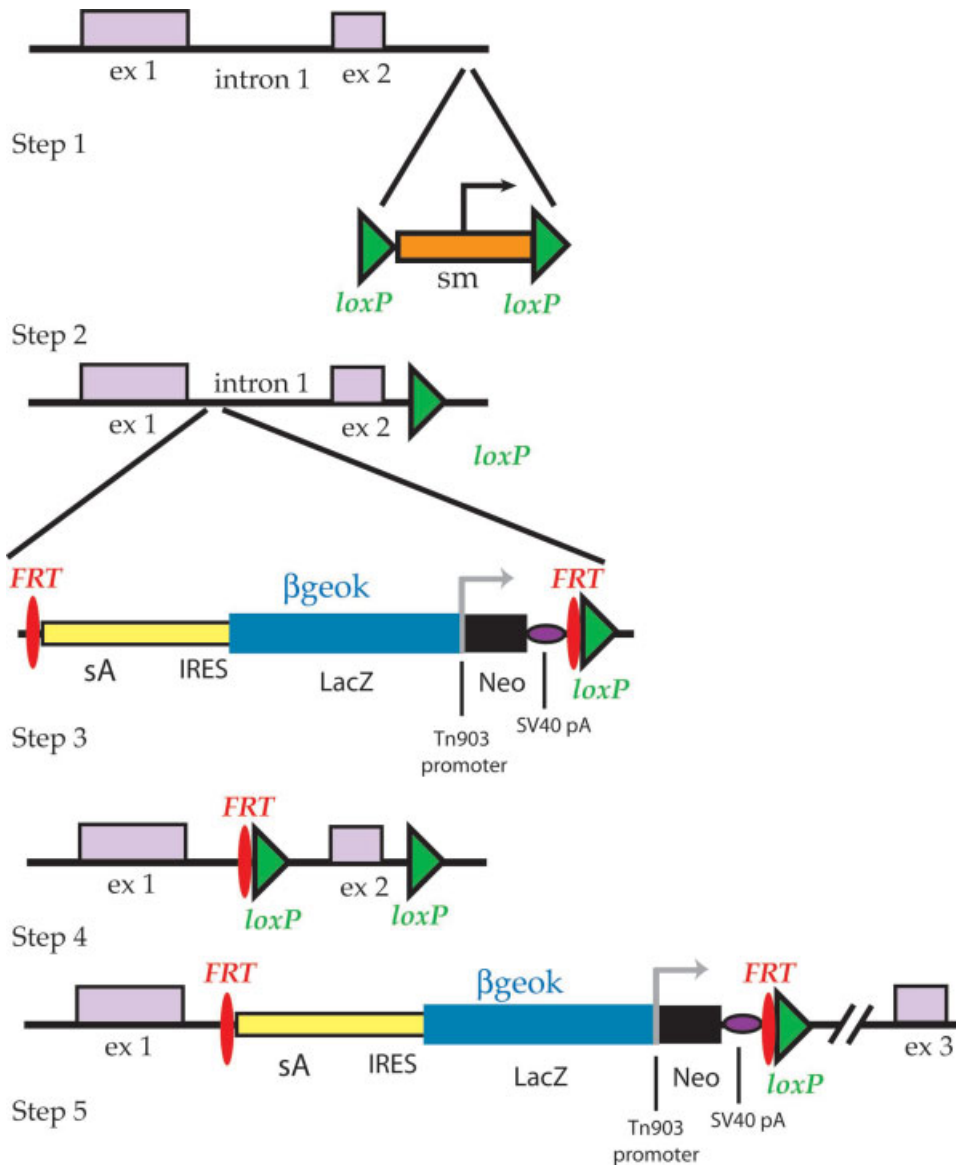


FIG. 3. A strategy for the generation of multipurpose knockout/conditional alleles. Schematic representation of the targeting strategy for multipurpose knockout/conditional alleles. Steps 1–3 are performed in *E. coli*, while steps 4–5 are performed either in ES cells or in mice. Step 1 involves cloning, using Red/ET recombination, of a loxP flanked selectable marker cassette into an intron downstream of the exon(s) to be deleted conditionally. Step 2 is the Cre-mediated excision of the selectable marker inserted in step 1, to leave behind only the loxP site. In step 3 the β geo cassette is placed by homologous recombination into an upstream intron of the target gene: recombinants are identified through kanamycin selection. After homologous recombination in ES cells, the allele depicted in step 3 constitutes the constitutive knockout based on RNA processing. In step 4, FLPe is used to excise the β geo cassette and establish the conditional allele. Step 5 represents a safety variation, in which Cre is used to delete the essential exon (in this case, as an example, exon 2) while leaving in place the intronic knockout cassette. ex, exon; hyg, hygromycin B phosphotransferase gene; FRT, target site for FLP recombinase; loxP, target site for Cre recombinase; IRES, internal ribosomal entry site from the encephalomyocarditis virus; lacZ, gene encoding β -galactosidase; neo, neomycin phosphotransferase; pA, SV40 polyadenylation signal; sA, splice acceptor element from engrailed-2; sm, selectable marker; SV40, SV40 promoter.

MATERIALS AND METHODS

Plasmid Construction

β geo (Mountford *et al.*, 1994) was changed to β geoK and then β geoK-hygro using restriction nucleases and DNA ligase.

The β ghygro plasmid was assembled replacing, via Red/ET recombination, the *neo* gene of β geoK with a promoterless *hygro* gene, according to the protocol described in Zhang *et al.* (2000). Primers EthygroUp and EthygroDw (sequence below) were used to amplify the hygromycin phosphotransferase gene from the pcDNA3 plasmid (Invitrogen, La Jolla, CA); each oligo comprises 50 nucleotides (underlined) of homology to the β geoK plasmid, followed on the 3' by the PCR primer sequence.

EthygroUp: 5'ATATCATCACGAACAGTAAAACTGTC-TGCTTACATAAACAGTAATACAAGGGGTGTTA-TGAAAAAGCCTGAACTCACCGCGACG 3'

EthygroDw: 5'GGGGGAGGTGTGGGAGGTTTTTTAA-AGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCACTATTCCITTTGCCCTCGGACGAGT 3'.

Red/ET Recombination Reactions

All Red/ET recombination reactions to insert the β geoK, β ghygro or β geoK-hygro cassettes in, respectively, the *fatp-4*, *trx2*, and *af4* genomic clones were carried out with the Red/ET expression plasmid pR6K- $\alpha\beta\gamma$ (Zhang *et al.*, 2000). Cells harboring the relevant genomic clone were transformed with the pR6K- $\alpha\beta\gamma$ plasmid according to standard procedures. Single colonies were picked and grown in 5 ml LB medium overnight; 0.7 ml of culture

were then transferred into 70 ml of LB medium (without glucose) and grown at 37°C. 10% glycerol with dH₂O was cooled on ice for at least 3 h. When the cells reached OD₆₀₀ = 0.1–0.15, 0.7 ml of a 10% L-arabinose solution was added to induce protein expression. Cells were harvested at an OD₆₀₀ of 0.25–0.4 followed by centrifugation for 10 min at 7,000 rpm at –5°C and resuspension in ice-cold 10% glycerol (repeated three times). After the final centrifugation, cells were resuspended in 50 µl ice-cold glycerol and electroporated with 0.3–1 µg of linear DNA fragment (PCR product or a fragment excised from a plasmid). Colonies were identified on selection plates containing appropriate combinations of the following antibiotics: chloramphenicol 12.5 µg/ml, ampicillin 50 µg/ml, tetracycline 25 µg/ml, kanamycin 20 µg/ml, hygromycin 100 µg/ml.

Cre Recombination in *E. coli*

Cre-mediated removal of the selectable marker in *E. coli* (i.e., as in step 2, Fig. 3) was carried out with the 705-Cre plasmid, which carries a temperature-sensitive replication origin (pSC101) to enable replication at 30°C; Cre recombinase is under the control of a thermosensitive promoter (cl578), which drives expression within the 37–42°C temperature range (Buchholz *et al.*, 1996). The 705-Cre plasmid was transformed into *E. coli* cells containing the target plasmid with the loxP flanked cassette. After transformation, cells were incubated in 1 ml LB at 30°C for 1.5 h and plated on double selection plates with the relevant antibiotics (15 µg/ml chloramphenicol for the 705-Cre plasmid). After about 24 h at 30°C, single colonies were picked, grown in 0.5 ml LB at 30°C for 2–3 h, selecting only for the antibiotic marker carried by the target plasmid, and then incubated overnight at 37°C before streaking onto plates to get single colonies. Cre recombination, which is often quantitative, was assessed by restriction fingerprinting of target plasmid DNA.

ES Cells

Mouse ES cells of the line E14 were cultured on mouse embryonic fibroblasts (MEFs) in medium supplemented with recombinant leukemia inhibitory factor (LIF) as described previously (Nichols *et al.*, 1990). After electroporation (500 µF, 240 mV) of ~10⁷ cells in 800 µl with the targeting constructs (40 µg per electroporation), cells were seeded at a density of 2,000,000 cells/10 cm plate. Then, 24 h after electroporation, drug selection was started at the following concentrations: G418 (200 µg/ml); hygromycin (160 µg/ml) (Hogan *et al.*, 1994).

Northern Hybridization and RT-PCR

Total RNA from mouse organs and ES cells was extracted using TriReagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. Northern analysis was performed using denaturing formaldehyde gels, with subsequent transfer to Biodyne B membranes (Pall, East Hills, NY). Labeling of probes was done using Highprime (Roche, Nutley, NJ) incorporating [32P]dCTP (Amer-

sham, Arlington Heights, IL). Blots were hybridized at 64°C in 0.5M NaPO₄, 7% SDS, and washed at 64°C in 40 mM, NaPO₄ 1% SDS. Blots were exposed 1–4 days on Biomax MR-1 films (Kodak).

cDNA was synthesized with the Reverse Transcriptase SuperScriptII (from Gibco BRL, Gaithersburg, MD). In a total volume of 12 µl, 4 µl of total RNA (1–5 µg) were combined with 1 µl Oligo(dT) (500 µg/ml), and 1 µl of a dNTP mix at a concentration of 10 mM each. The mixture was heated at 65°C for 5 min and quickly chilled on ice before adding the following components: 4 µl of 5X First-Strand Buffer (provided by the supplier), 2 µl of DTT (0.1M), and 1 µl of RNase inhibitor RNaseOUT (40 units/µl). The components were mixed and incubated at 42°C before adding 1 µl of Reverse Transcriptase (200 units). The reaction was allowed to proceed for 50 min at 42°C and then inactivated by heating at 70°C for 15 min. To remove the RNA from the DNA-RNA hybrid, 1 µl (2 units) of *E. coli* RNaseH was added and the reaction incubated at 37°C for 20 min.

PCR Primers indicated as black arrows in Fig. 2a were:

Primer a: 5'CTTTATCGATTGGGTGACTATGAG-GAGATGAA 3'
 Primer a': 5'GAAGTCCATTGCGTTGAAGAGATT 3'
 Primer b: 5'CTGCCCTCAGCGACACCCCTACTT 3'
 Primer c: 5'TAGAAGCAGCAGAGGAGAACC 3'
 Primer d: 5'GGCTCACCCCTCCCCTGCCATC 3'
 Primer e: 5'AGT GCC CGT CAG CAC AGA GC 3'
 Primer f: 5'TAC ACA AAG GAC AGG ATG CG 3'
 Primer *β-actin* sense: 5'GGCCCAGAGCAAGAGAGG-TATCC 3'
 Primer *β-actin* antisense: 5'ACGCACGATTCCTCT-CAGC 3'
 Primer *fatp-1* sense: 5'TGC GTA TCG TCT GCA AGA CG 3'
 Primer *fatp-1* antisense: 5'CAG AAC TTG AGG AGG CTC TT 3'.

LITERATURE CITED

- Angrand PO, Daigle N, van der Hoeven F, Scholer HR, Stewart AF. 1999. Simplified generation of targeting constructs using ET recombination. *Nucleic Acids Res* 27:e16.
- Baskaran K, Erfurth F, Taborn G, Copeland NG, Gilbert DJ, Jenkins NA, Iannaccone PM, Domer PH. 1997. Cloning and developmental expression of the murine homolog of the acute leukemia proto-oncogene AF4. *Oncogene* 15:1967–1978.
- Buchholz F, Angrand PO, Stewart AF. 1996. A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. *Nucleic Acids Res* 24:3118–3119.
- Copeland NG, Jenkins NA, Court DL. 2001. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* 2:769–779.
- Friedrich G, Soriano P. 1991. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev* 5:1513–1523.
- Gossler A, Joyner AL, Rossant J, Skarnes WC. 1989. Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* 244:463–465.
- Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. 1994. Deletion

- of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* 265:103-106.
- Herrmann T, Buchkremer F, Gosch I, Hall AM, Bernlohr DA, Stremmel W. 2001. Mouse fatty acid transport protein 4 (FATP4): characterization of the gene and functional assessment as a very long chain acyl-CoA synthetase. *Gene* 270:31-40.
- Hogan B, Beddington R, Costantini F, Lacy E. 1994. Manipulating the mouse embryo. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Huntsman DG, Chin SF, Muleris M, Batley SJ, Collins VP, Wiedemann LM, Aparicio S, Caldas C. 1999. MLL2, the second human homolog of the *Drosophila* trithorax gene, maps to 19q13.1 and is amplified in solid tumor cell lines. *Oncogene* 18:7975-7984.
- Kim DG, Kang HM, Jang SK, Shin HS. 1992. Construction of a bifunctional mRNA in the mouse by using the internal ribosomal entry site of the encephalomyocarditis virus. *Mol Cell Biol* 12:3636-3643.
- Lallemant Y, Luria V, Haffner-Krausz R, Lonai P. 1998. Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. *Transgenic Res* 7:105-112.
- Meyers EN, Lewandoski M, Martin GR. 1998. An Fgf8 mutant allelic series generated by Cre- and Ffp-mediated recombination. *Nat Genet* 18:136-141.
- Mountford P, Zevnik B, Duwel A, Nichols J, Li M, Dani C, Robertson M, Chambers I, Smith A. 1994. Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc Natl Acad Sci USA* 91:4303-4307.
- Muyrers JP, Zhang Y, Testa G, Stewart AF. 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* 27:1555-1557.
- Muyrers JP, Zhang Y, Stewart AF. 2001. Techniques: recombinogenic engineering—new options for cloning and manipulating DNA. *Trends Biochem Sci* 26:325-331.
- Nagy A, Moens C, Ivanyi E, Pawling J, Gertsenstein M, Hadjantonakis AK, Pirity M, Rossant J. 1998. Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol* 8:661-664.
- Nichols J, Evans EP, Smith AG. 1990. Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* 110:1341-1348.
- Pelletier J, Sonenberg N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* 334:320-325.
- Ringrose L, Chabanis S, Angrand PO, Woodrooffe C, Stewart AF. 1999. Quantitative comparison of DNA looping in vitro and in vivo: chromatin increases effective DNA flexibility at short distances. *EMBO J* 18:6630-6641.
- Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM. 2000. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet* 25:139-140.
- Schaft J, Ashery-Padan R, van der Hoeven F, Gruss P, Stewart AF. 2001. Efficient FLP recombination in mouse ES cells and oocytes. *genesis* 31:6-10.
- Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 97:5978-5983.
- Zhang Y, Buchholz F, Muyrers JP, Stewart AF. 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat Genet* 20:123-128.
- Zhang Y, Muyrers JP, Testa G, Stewart AF. 2000. DNA cloning by homologous recombination in *Escherichia coli*. *Nat Biotechnol* 18:1314-1317.