

Segmental genomic replacement in embryonic stem cells by double *lox* targeting

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ABSTRACT

We have applied Cre-mediated double *lox* recombination to embryonic stem (ES) cells to facilitate repeated knock-ins at a target locus, thus helping to assure correct temporal and spatial transgene expression in mice. Using homologous recombination, we inserted a double *lox* cassette a few nucleotides before the authentic ATG start of MHL-1, the gene coding for the major subunit of the asialoglycoprotein receptor. The cassette carries a marker gene bounded by heterospecific *lox* sites that cannot recombine with each other, but which can undergo recombination with like sites on an incoming double *lox* targeting vector. Cre-mediated replacement of the *lox*-delimited genomic segment at MHL-1 with targeting DNA occurs at a frequency three times that of random DNA integration and is sufficiently robust that correctly targeted ES colonies can be identified by PCR screening without relying on any drug selection procedure. Moreover, double *lox* recombination at the MHL-1 locus is not dependent on the presence or absence of a transcriptionally active promoter at the genomic target. The strategy and vectors described here are generally applicable to designing double *lox* targeted knock-ins at any locus in ES cells and should prove useful in more precise molecular engineering of the mouse genome.

INTRODUCTION

The directed introduction of mutations into the mouse genome by molecular techniques has become a powerful strategy for understanding gene function and genetic regulatory mechanisms in vertebrates (1,2). Either complete ablation of an endogenous gene or replacement of the wild-type allele by a mutant allele carrying just a single nucleotide change can be readily achieved by homologous recombination, or gene targeting, in embryonic stem (ES) cells. Gene targeting also provides a means to circumvent the position effects on gene expression often encountered in the production of transgenic animals that results in spatially variable gene expression and even unexpected tissue misexpression due to random integration of the transgene (3). For example, homologous recombination has been used to direct integration of a transgene to a defined ectopic locus to help minimize position effects on gene expression (4). Similarly, homologous recombination-based 'knock-in' strategies have been used to directly place a transgene under the control of the promoter of an endogenous gene to give precisely targeted transgene expression (5).

In gene targeting, homologous sequences on the targeting DNA are used to direct precise recombination to the endogenous target locus. Because the frequency of targeting events after transfection is low, it is necessary to select for a drug resistance marker present on the targeting vector. Moreover, the resulting drug-resistant clones must then be rigorously screened by PCR and/or Southern blotting since only a small fraction of them will have correctly modified the target locus. Although a single round of homologous recombination and selection is sufficient to disrupt and/or delete the coding region of a gene of interest, the introduction of a point mutation generally requires additional steps, for example removal of the selectable marker by Cre-mediated recombination (6,7).

Recently we described a double *lox* strategy (8) for Cre-mediated replacement of any predesignated genomic segment in cultured NIH 3T3 cells with DNA from an incoming targeting vector and showed that the frequency of targeted DNA insertion is 20- to 100-fold more efficient than Cre-mediated insertion at a single chromosomal *lox* site (9). The strategy is based on the observation that two *lox* sites that differ in the central spacer region do not recombine whereas *lox* sites having the same spacer region recombine efficiently (10). This allows the placement into the genome of uniquely designed *lox* sites that can then be individually and specifically targeted by 'single *lox*' targeting vectors having the cognate specificity (11,12). By including two heterospecific *lox* sites on the targeting vectors, DNA can be delivered into a genomic double *lox* target by a double crossover mechanism, i.e. Cre-mediated recombination occurs between each genomic *lox* site and its cognate *lox* site on the targeting vector. This results in replacement of the genomic interval by one from the targeting vector. Similar work has demonstrated the feasibility of the double *lox* strategy in cultured MEL cells (13). Moreover, analogous strategies have also been demonstrated for the related FLP site-specific DNA

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recombinase, both in yeast (14) and at randomly selected sites in mammalian cells (15,16), using a wild-type FRT site and a modified FRT site that has an altered spacer region.

In this work we combine targeted homologous recombination with the double *lox* strategy in ES cells to allow precise and repeated knock-ins of any targeting DNA to a defined site just downstream of the endogenous liver-specific promoter of MHL-1, the gene coding for the major subunit of the asialoglycoprotein receptor. Targeted insertion events can be recovered by either positive or negative selection strategies. Moreover, recombination is sufficiently high that correctly targeted cells can be identified without the use of drug selection. We further show that the frequency of Cre-mediated targeting is insensitive to the presence or absence of a transcriptionally active gene at the target locus.

MATERIALS AND METHODS

Vector construction

The heterospecific double *lox* vector pBS524 (Fig. 1B) was derived from pBluescript KSII (Stratagene) and carries the 1.8 kb *Xho*I–*Xba*I fragment from pPNT (17) containing the PGK*neo* gene (here referred to as *neo*). Synthetic oligos were used to flank *neo* with a number of unique restriction sites and also *lox511* and the modified *loxP* site *loxG33* (8). Replacement of the *Spe*I–*Bgl*II *neo* fragment of pBS524 with the corresponding *hyg* fragment from pPWL512 (18) generated pBS528. The *Bam*HI–*Hin*dIII fragment from pPNT containing the PGK*tk* gene was blunt end ligated into the unique *Apa*I site adjacent to *lox511* in pBS528 to generate pBS545. The *Kpn*I–*Bam*HI *lacZ* fragment from p324 (19) was inserted into the corresponding sites of the double *lox* vector pBS397 (8) to generate the double *lox lacZ* fusion vector pBS559. Similarly, the *Xba*I–*Hin*dIII polylinker region from pBluescript KSII replaced the *Xba*I–*Hin*dIII *neo* fragment of pBS397 to give pSS20. To generate pSS66 the *Bgl*II–*Nhe*I double *lox* fragment of pSS20 was inserted into the *Bam*HI and *Xba*I sites of a pBluescript KSII derivative in which the *Kpn*I–*Sma*I region had been deleted. The Cre expression vector pBS513 (20) carries the *cre* gene under the control of the EF1α promoter (21). Substitution of the wild-type green flourescent protein (GFP) coding region in pBS377 (22) by the *Xba*I–*Kpn*I EGFP fragment from pEGFP-1 (Clontech) generated the EF1α–EGFP vector pBS502.

A λ phage FIX II (Stratagene) clone carrying 15 kb of the mouse 129SVJ genomic MHL-1 locus (a gift of W. Berlin, FDA, Bethesda, MD) was used for construction of the MHL-1 targeting vector. To generate the *loxP*^{2}*neo* cassette plasmid pBS479, the PGK*neo* gene from pPNT (17) was inserted into the *Hin*dIII–*Bam*HI sites of pBS246 (23). The MHL-1 targeting vector pBS531 was constructed from pBluescript KSII so that the final vector carries a unique *Kpn*I site, the 4.33 kb *Eco*RI–*Bsp*HI 5' region of MHL-1, the *loxP*^{2}*neo* cassette from pBS479, an *Eco*RI site, a synthetic *lox511* site and a 4.0 kb region of MHL-1 3' homology that extends from a *Sac*I site in exon 3 to just after the poly(A) site (Fig. 2A). All regions of plasmids obtained using synthetic oligos were confirmed by DNA sequencing.

Cell culture and homologous targeting

Standard procedures were used for propagation of J1 ES cells (24) and primary embryo fibroblast feeders (EFF), electroporation, drug selection and DNA preparation for PCR/Southern analysis (25). Culture media and reagents were from Life Technologies, unless otherwise indicated. Fetal bovine serum was from HyClone. Homologous placement of the double *lox* target at MHL-1 was by electroporation (0.35 kV and 0.5 mF using a Bio-Rad Gene Pulser II) of 5×10^6 J1 cells in electroporation buffer (Specialty Media) with 20 μg of the targeting vector pBS531 (linearized with *Kpn*I) and selection for resistance to 350 μg/ml G418 1 day later. PCR and Southern analysis indicated that correct homologous targeting had occurred in six of 100 candidate clones.

Site-specific targeting

Double *lox* ES cell lines were co-electroporated as above with 25 μg pBS513 (EF1α–*cre*) and 25 μg of the appropriate double *lox* targeting vector, e.g. pBS545 (*hyg tk*), pBS524 (*neo*) or pBS559 (*lacZ*). Resistance to G418 (350 μg/ml) or hygromycin B (200 μg/ml; Boehringer Mannheim) was selected for 24 h after electroporation and colonies were picked for analysis, propagation and storage 7–9 days later.

Double *lox* targeting by TK$^-$ negative selection of 9tk ES cells was performed in the following fashion. After co-electroporation with pBS524 and pBS513, cells were plated on 60 mm plates with EFF. Four days later cells were trypsinized and replated (1×10^5 cells/100 mm dish) with EFF and either 350 μg/ml G418 or 2.5 μM gancyclovir (GCV) (Roche). For negative selection, the GCV-containing medium was replaced with drug-free medium after 20 h and cells were incubated for 24 h, after which time a second round of selection for 24 h with 2.5 μM GCV was imposed. Cells doubly selected for resistance to both G418 and GCV were subjected to only a single round of GCV selection.

FACS sorting

To enrich for targeted cells using FACS, double *lox* ES cells were co-electroporated with 25 μg pBS513, 25 μg of the targeting vector pBS524 and 2.5 μg of the GFP vector pBS502. Cells were incubated for 3 days without selection and then sorted as described previously (22).

PCR and Southern analysis

Southern analyses were performed as previously described (8) using 20 μg DNA/gel lane. Detection of homologous targeting of MHL-1 was with oligonucleotides BSB292 (5'-AGCATTTATCACATAAGCTCG-3', located 4410 bp upstream of the coding ATG of MHL-1) and BSB131 (5'-ATCTCCTGTCATCTCACCTTGC-3', *neo*-specific) using 35 cycles of amplification with ExTaq polymerase (Takara): 30 s at 94°C; 30 s at 61°C; 5 min at 68°C. Cre-mediated targeting was detected with BSB340 (5'-CTGGCCTCGAACCTACAGAG-3', located 260 bp upstream of the coding ATG of MHL-1) and BSB131 for *neo* vectors or BSB341 (5'-GAGAGCCTGACCTATTGCATCTCC-3', *hyg*-specific) for *hyg* vectors. DNA was amplified for 35 cycles using AmpliTaq polymerase (Perkin-Elmer): 30 s at 94°C; 30 s at 60°C; 2 min at 72°C.

β-Galactosidase assay

β-Galactosidase in cell extracts was assayed (26) by incubation for 30 min at 37°C with the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside. Fluorescence was measured at 450 nm after excitation at 360 nm using a Packard Fluoro-Count reader.

RESULTS

Modification of the MHL-1 locus for Cre-mediated targeting

The Cre-based strategy for repeatable gene knock-ins at a defined locus is shown in Figure 1A. In the first step, two heterospecific *lox* sites are placed at the target locus in ES cells by homologous recombination, using a selectable marker such as *neo*. A *lox* site having *loxP* specificity is positioned just downstream of the promoter at the target endogenous locus and in the orientation shown so that the first AUG encountered by a transcript from this promoter lies downstream of the *lox* site. The heterospecific *lox511* site is positioned on the other side of the *neo*-selectable marker. The *loxP* and *lox511* sites differ from each other in the sequence of the spacer region of the recombination site and, hence, are unable to recombine with each other. Because both sites are proficient for self × self recombination, they can still recombine with cognate *lox* sites on an incoming targeting vector, as described below. Thus, the DNA interval bounded by these two heterospecific *lox* sites defines the region targeted for segmental replacement by site-specific recombination. If needed, a second (negative) selectable marker can be included in this genomic interval (see below).

In the second step, the modified locus is targeted using a double *lox* vector carrying the desired gene and Cre recombinase. Since recombination occurs only between *lox* sites having the same specificity, the selectable marker(s) at the modified locus is replaced by the corresponding segment on the double *lox* targeting vector by what is formally a site-specific double crossover event, thus transcriptionally fusing the desired gene to a pre-selected endogenous promoter. Figure 1B shows a series of double *lox* vectors constructed to facilitate redesign of the endogenous locus, or, alternatively, to serve as double *lox* vectors for Cre-mediated replacement recombination in the second step of the strategy.

To implement the strategy in ES cells, we modified the endogenous MHL-1 gene, which codes for the major subunit of the mouse asialoglycoprotein receptor and which is expressed post-natally, predominantly in the liver and testes (27,28). Based on the sequence of the MHL-1 gene (manuscript in preparation) we positioned the double *lox* target 2 nt before the translational start of the MHL-1 coding region in exon 2 (Fig. 2A). Thus, the coding portion of exon 2 for asialoglycoprotein was replaced with a *loxP* site and the *neo*-selectable marker, leaving the 5'-untranslated region untouched. In this instance, we also incorporated a second *loxP* site into the homologous targeting vector so that the *neo* gene was flanked by two directly repeated *loxP* sites and could thus be removed, if desired, by simply transfecting with a Cre expression vector. To enable subsequent double *lox* targeting, the heterospecific *lox511* site was included downstream of *neo*.

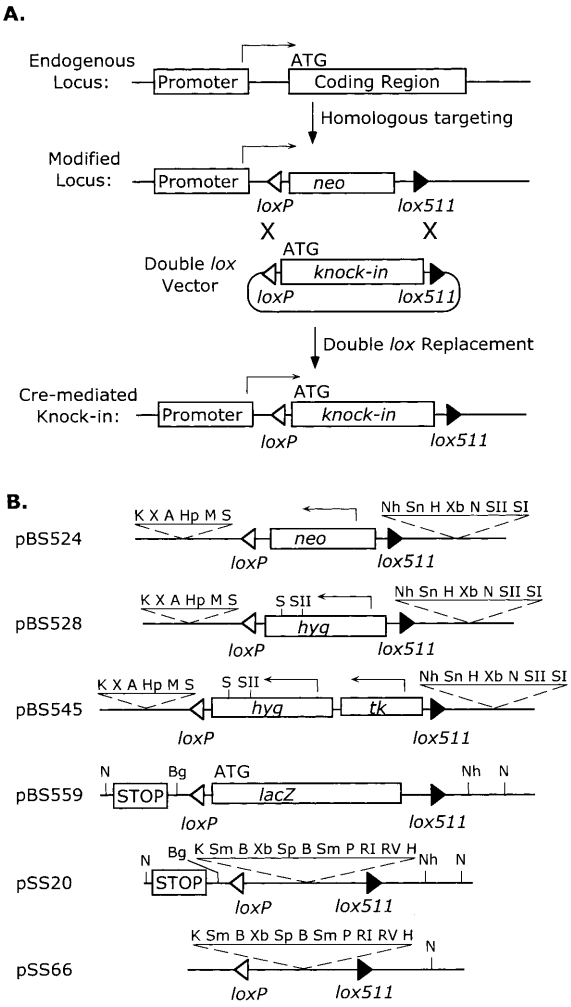

Figure 1. Double *lox* replacement. (**A**) Strategy for Cre-mediated knock-in. Homologous recombination is used to place a double *lox* cassette immediately 5' to the position of the natural ATG start for the coding region of the target endogenous locus. In the diagram, the coding region of the locus has been ablated by insertion of a *loxP*–PGK*neo*–*lox511* cassette. A 'knock-in' gene is then targeted to the modified locus by introduction of a double *lox* targeting vector along with a Cre transient expression vector. Double crossover recombination replaces the PGK*neo* cassette by the knock-in gene such that it is now under the control of the promoter at the target mouse locus. (**B**) Double *lox* vectors. All vectors carry the *amp*^{r} marker for selection in *E.coli*. Restriction sites: A, *Avr*II; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; N, *Not*I; Nh, *Nhe*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I; SI, *Sac*I; SII, *Sac*II; Sm, *Sma*I; Sn, *Sna*BI; Sp, *Spe*I; X, *Xho*I; Xb, *Xba*I.

An initial PCR diagnostic screen (data not shown) following transfection of the J1 ES cell line with the homologous targeting vector pBS531 yielded a number of candidate clones. As shown in Figure 2 for two isolates, Southern blotting confirmed that both the 5'-end (Fig. 2B, junction fragment 4.6 kb) and 3'-end (Fig. 2C, junction fragment 6.5 kb) of the modified locus were correctly targeted and that they also carried one untargeted allele. Of these, clone 1H was chosen for subsequent double *lox* targeting experiments.

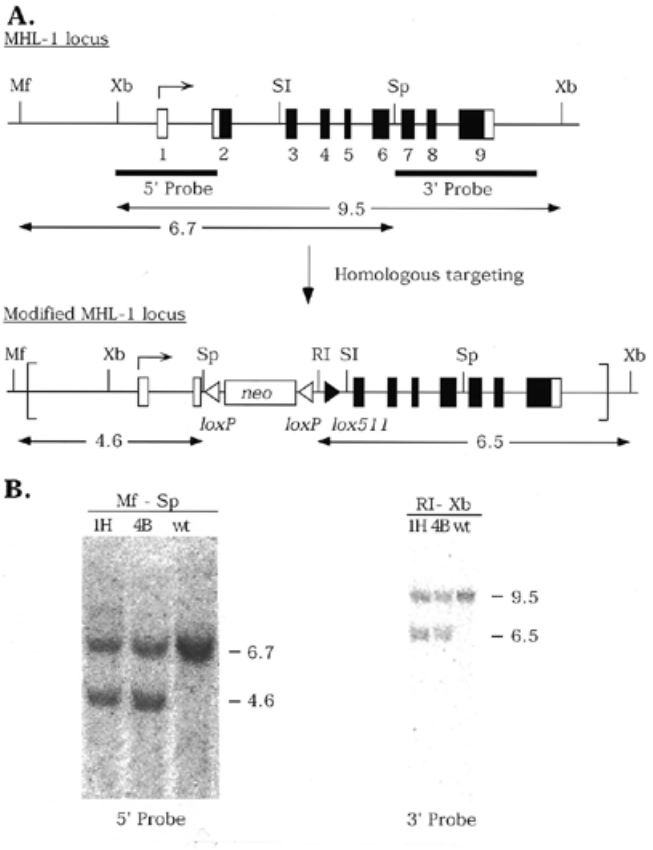

Figure 2. Placement of the double *lox* cassette at MHL-1. (**A**) Both the wild-type MHL-1 locus and the allele modified by homologous gene targeting are shown. The thin arrow indicates the transcriptional start. Non-coding (white) and coding (filled) regions of exons (numbered 1–9) are shown, as are the *loxP* (open triangles) and *lox511* (filled triangle) sites. The structure of the targeting vector pBS531, along with the extent of MHL-1 homology used, is indicated by enclosure in brackets at the modified MHL-1 locus. Diagnostic restriction fragment sizes are shown that distinguish the modified from the wild-type locus. (**B**) Southern analysis of homologously targeted ES lines. DNA from candidate Neo^r clones (1H and 4B) and ES cell wild-type DNA was digested with either *Mfe*I + *Spe*I (5' probe) or *Eco*RI + *Xba*I (3' probe). Restriction sites: Mf, *Mfe*I; others as in Figure 1 legend.

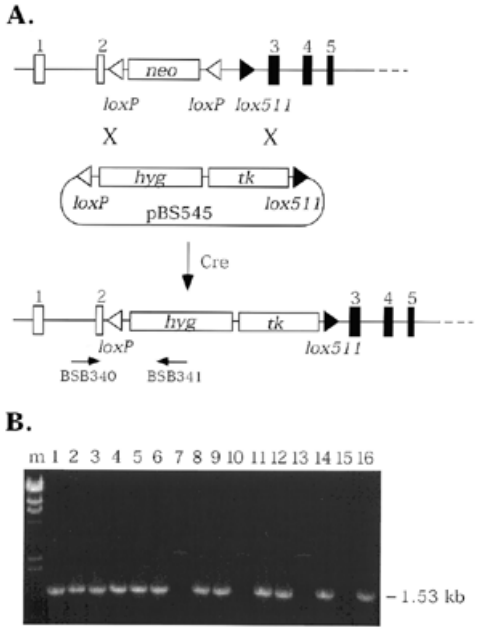

Figure 3. Replacement of *neo* at MHL-1 by double *lox* targeting. (**A**) Cre-mediated recombination between compatible heterospecific *lox* sites on the genomic target in ES cell line 1H and the double *lox* targeting vector pBS545 leads to replacement of the genomic *neo* gene by the plasmid-borne *hyg* and *tk* genes. Exons are numbered as in Figure 2 and the diagnostic PCR oligos BSB340 and BSB341 (predicted product 1.53 kb) are shown. (**B**) Detection of the predicted leftward MHL-1/*loxP*/*hyg* junction by PCR. ES cell line 1H was co-transfected with pBS545 and the Cre expression vector pBS513, followed by selection for resistance to hygromycin B. The PCR left junction analysis of 16 randomly chosen Hyg^r colonies is shown (m, size markers).

Exchange of selectable markers at MHL-1

The marker exchange strategy is diagrammed in Figure 3A. Co-transfection of the 1H ES cell line by the double *lox* vector pBS545 and the Cre expression vector leads to recombination between genomic and plasmid-borne *loxP* sites and, similarly, between pairs of *lox511* sites. The *neo* marker is thereby evicted from the genome and replaced by the *hyg tk* cassette from the plasmid vector. From several hundred Hyg^r colonies obtained after co-electroporation of 1H with the pBS545 double *lox* vector and the Cre expression plasmid, 16 were randomly chosen for PCR analysis. Of these, 12 (75%) exhibited the predicted 1.4 kb junction fragment (Fig. 3B). The remaining four Hyg^r clones were most likely random integrants of pBS545 elsewhere into the genome.

Southern analysis (Fig. 4) confirmed that the 12 candidate replacement clones were precise replacements of the *neo* gene by the *hyg tk* cassette, exhibiting both the predicted 5' 4.6 kb *Nco*I junction (Fig. 4B) and the predicted 3' 6.4 kb *Nco*I–*Avr*II junction (Fig. 4C). One of these, no. 9, was designated 9tk and chosen for subsequent targeting experiments, described below. In contrast, the four presumptive random integrants all retain the *Avr*II site located between the target genomic heterospecific *lox* sites (Fig. 4A), as indicated by the 3.3 kb 3' *Nco*I–*Avr*II junction fragment they share with the parental 1H cell line (Fig. 4C). As expected after transfection with the Cre plasmid, excision of the *neo* gene by *loxP*×*loxP* recombination did occur, as evidenced by the absence of the 5' 3.8 kb *Nco*I fragment (Fig. 4B) and the generation of a larger *Nco*I fragment that co-migrates with the wild-type 7.5 kb band. Note that, theoretically, the *hyg tk* cassette could integrate by recombination with the *lox511* site and the internal *loxP* site at the genomic target, leaving the *neo* gene in place. However, we did not observe any Hyg^r clones that retain the *neo* gene, presumably because *loxP*×*loxP* recombination is so efficient that the *neo* gene is almost always excised in cells expressing Cre (23).

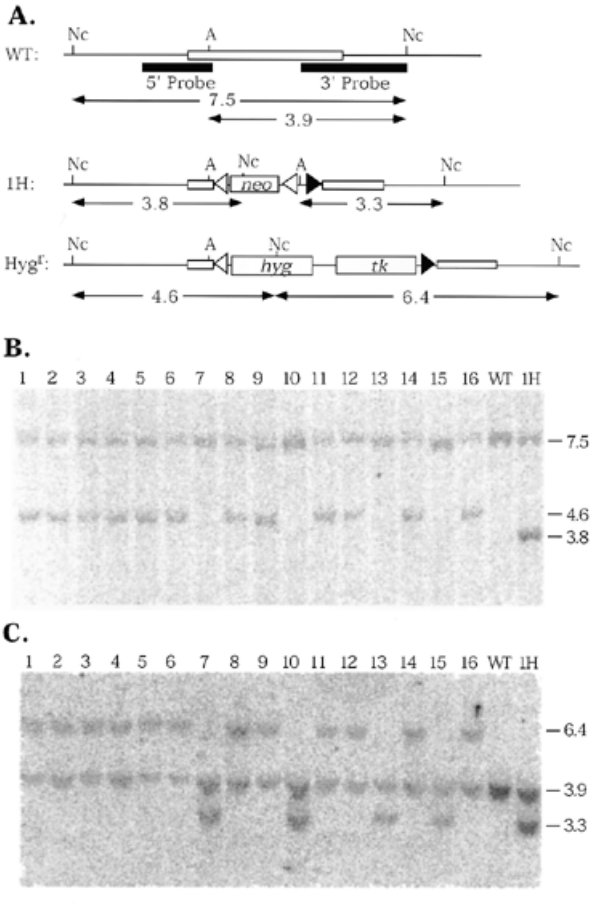

Figure 4. Southern analysis of double *lox* replacements. (**A**) The genomic structures of the wild-type allele (white box) and of both the *neo* (cell line 1H) and the Cre-mediated *hyg* replacement alleles are shown. (**B**) DNA from candidate replacement clones (1–16), wild-type ES cell DNA and the double *lox neo* parental line (1H) were digested with *Nco*I and subjected to Southern analysis using the 5' MHL-1 probe (Fig. 2). (**C**) DNA, as in (B), was digested with both *Nco*I (Nc) and *Avr*II (A) and analyzed with the 3' probe.

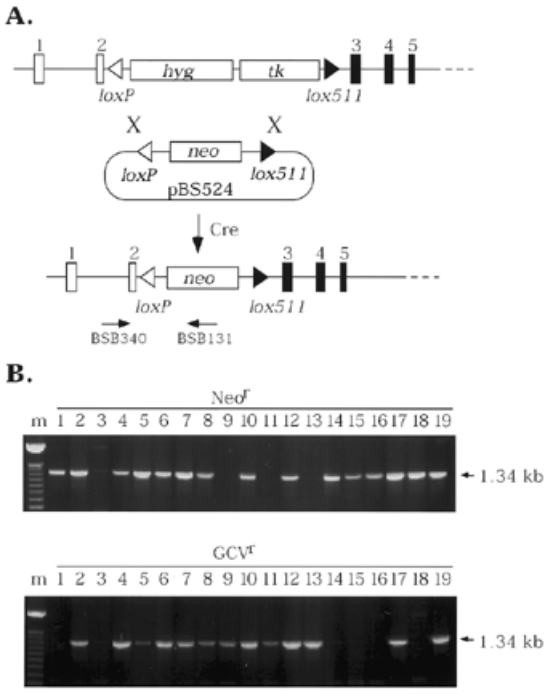

Figure 5. Replacement of *hyg*/*tk* at MHL-1 by double *lox* targeting. (**A**) Cre-mediated recombination between compatible heterospecific *lox* sites on the genomic target in ES cell line 9tk and the double *lox* targeting vector pBS524, respectively, leads to replacement of the genomic *hyg* and *tk* genes by the plasmid-borne *neo* gene. Exons are numbered as in Figure 2 and the diagnostic PCR oligos BSB340 and BSB131 (predicted product 1.34 kb) are shown. (**B**) Detection of the predicted leftward MHL-1/*loxP*/*neo* junction by PCR. ES cell line 9tk was co-transfected with pBS524 and the Cre expression vector pBS513, followed by selection for resistance to G418. The PCR left junction analysis of 19 randomly chosen Neo^r colonies and a second additional set of 19 randomly chosen GCV^r colonies is shown (m, size markers).

Replacement by negative selection

Because cell line 9tk carries the HSV *tk* gene at the MHL-1 locus, we asked whether Cre-mediated double *lox* replacement of *tk* by DNA from an incoming replacement vector could be selected directly using the drug GCV (Fig. 5A). To compare the efficiency of negative selection using GCV with positive selection using G418, 9tk cells were transfected with a Cre expression vector and the double *lox* vector pBS524 which carries the *neo* gene. Selection for replacement recombination was then carried out either by selecting for resistance to G418 or to GCV or to both antibiotics simultaneously. PCR analysis of progeny clones that were obtained by either resistance to G418 or GCV showed that in both cases a substantial majority were specific replacement integrants at the MHL-1 target locus (Fig. 5B). These data are summarized in Table 1. About 70–80% of either positively or negatively selected transformants of 9tk ES cells were precise replacements, a frequency similar to that obtained by positive selection for Hyg^r replacements with the parental 1H ES cells. Dual selection of transfected 9tk cells for resistance to both G418 and GCV markedly increased the frequency of site-specific replacement. Of 34 analyzed isolates, all were precisely targeted recombinants.

Integration of the Cre-expressing plasmid into the genome could occur independently at low frequency by random, illegitimate recombination and thus some of the double *lox* integrants potentially could harbor one or more copies of the *cre* plasmid elsewhere in the genome. Examination of the double *lox* integrants, however, showed that integration of the *cre* gene occured in <1% of the double *lox* integrants (data not shown), as expected.

Replacement recombinants without selection

Previously we had shown that in cultured fibroblasts we had engineered to have a double *lox* target in the genome, double *lox* targeting occurred at an absolute frequency of 2–10%, i.e. Cre specifically targeted exogenous DNA to the desired

Table 1. Cre-mediated replacement targeting

ES cell line	MHL-1 resident gene	Replacement construct	Selection	% Replacement[a]
1H	*neo*	pBS545 (*hyg/tk*)	Hyg^r	a: 71 (24)
				b: 56 (16)
9tk	*hyg*/*tk*	pBS524 (*neo*)	Neo^r	a: 75 (20)
				b: 87 (24)
			GCV^r	a: 65 (19)
				b: 73 (19)
			Neo^r GCV^r	a: 100 (20)
				b: 100 (14)

[a]Duplicate experiments (a and b) for each cell line were performed by co-electroporation with the Cre expression plasmid and the indicated replacement vector, giving ~200–2000 colonies resistant to the indicated antibiotic(s). The number of independent colonies analyzed by PCR from each experiment for determination of replacement percentage are given in parentheses.

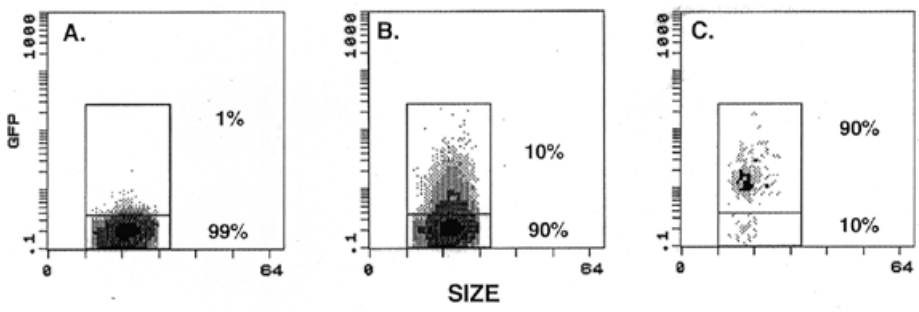

Figure 6. Preparation of transfected ES cells by FACS. The *hyg*/*tk* ES cell line 9tk was co-transfected with the Cre expression plasmid pBS513, the double *lox* targeting vector pBS524 and plasmid pBS502 that expresses GFP. FACS was performed 3 days after electroporation. (**A**) Non-transfected 9tk ES cells. (**B**) Transfected 9tk ES cells. About 10% of cells exhibited fluorescence above background and were recovered by sorting. (**C**) Re-analysis by FACS of sorted fluorescent cells from (B).

genomic locus in about one of every 10 non-selected cells that had taken up DNA (8). We expected that similar results would be obtained in ES cells, thus obviating the need for any selectable marker in double *lox* replacement strategies and allowing site-specific integrants to be identified by PCR screening methods. However, this assumes either that the transfection efficiency is quite high or that non-transfected cells can be eliminated.

We therefore co-electroporated the 9tk ES cell line with three plasmids: a Cre expression vector, the double *lox neo* vector pBS524 and the GFP expression vector pBS502. Fluorescence cytometry indicated that 10% of the cells had taken up DNA, as evidenced by expression of GFP (Fig. 6). These cells were sorted to yield a population from which most non-fluorescent (non-transfected) cells had been removed (Fig. 6C). Individual colonies were obtained in the absence of drug selection from this (transiently) fluorescent population of cells and screened by PCR to detect double *lox* replacements. Of 96 isolates analyzed, two exhibited the correct PCR band diagnostic of double *lox* replacement of the resident *hyg* gene by *neo* (Fig. 7). Experiments using a GFP*cre* fusion expression plasmid (22) gave similar results (data not shown). Thus, double *lox* replacements in ES cells can be isolated without reliance on drug selection using PCR screening methods.

Targeting of a silent locus

The frequency of homologous or site-specific recombination at a locus may well depend on factors that are locus specific. For example, Cre-mediated single *lox* integrative targeting varies by up to 50-fold in human osteosarcoma cells having the *lox* target at different chromosomal sites (29). Because transcription at a genomic locus can itself modulate homologous recombination (30), we asked whether or not transcriptional activity might affect double *lox* targeting at MHL-1. MHL-1 itself is expressed only after birth in the liver and in the testes (27,28). However, the modified locus carrrying the double *lox* target in 1H and 9tk, respectively, express the *neo* and *hyg* genes at the MHL-1 locus in ES cells under the control of the PGK promoter. To determine whether we could attain the same incidence of double *lox* targeting at a silent locus, we replaced the *hyg tk*

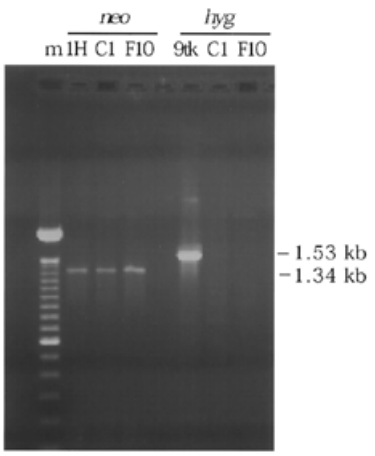

Figure 7. Confirmation of non-selective targeting. Of 96 random colonies obtained from the sorted cells of Figure 6, two clones, C1 and F10, exhibited the diagnostic MHL-1/*neo* junction by PCR analysis (conditions as in Fig. 5). Cell line 1H is a positive control for the MHL-1/*neo* junction. Simultaneously, clones C1 and F10 have lost the MHL-1/*hyg* junction present in the parental 9tk ES cells (PCR conditions as in Fig. 3; m, size markers).

cassette at the MHL-1 locus in 9tk with a *lacZ* reporter gene by double *lox* replacement using pBS559 (Fig. 1) and GCV selection. The resulting cell line, LacZ6, carries the *lacZ* gene under the control of the MHL-1 promoter, but no selectable marker and hence no active promoter element. As expected, only barely detectable background levels of β-galactosidase activity were detected in both the LacZ6 and the parental 9tk cells (data not shown), confirming that the MHL-1 promoter is silent in ES cells.

Both 9tk (having active *hyg* and *tk* genes) and LacZ6 were used for Cre-mediated targeting using the double *lox neo* vector pBS524. Double *lox* targeting yielded similar numbers of G418^r clones for both cell lines (Table 2). Because these G418^r colonies are comprised of both site-specific replacements and also of random integrants, we conducted PCR analysis to obtain the percentage of integrants that were actual double *lox* replacements. A difference in recombination frequency at MHL-1 between the two cell lines would be detected as a change in the percentage of double *lox* replacements, since non-Cre-mediated random integration should be unaffected by the status of the MHL-1 locus. With both cell lines, ~75% of the selected colonies were double *lox* replacements, indicating that a silent MHL-1 locus is no less accessible to targeting than one carrying an active gene.

DISCUSSION

We have shown that Cre mediates efficient replacement of a predefined genomic segment in ES cells with DNA from a double *lox* targeting vector. The strategy relies on the observation that Cre recombines two *lox* sites having the same spacer, but performs recombination inefficiently between *lox* sites having different spacers, i.e. heterospecific *lox* sites. In this work we used homologous recombination to place a double *lox* target, consisting of one *lox* site with *loxP* specificity and a second, heterospecific *lox* site called *lox511*, at the MHL-1 locus. Subsequent Cre-mediated targeting with a similarly configured double *lox* targeting vector gave high efficiency replacement of the *lox*-delimited genomic interval with DNA from the targeting vector. As shown previously in cultured fibroblasts, Cre-mediated replacement is 3- to 4-fold more frequent than random illegitimate integration of the targeting vector and results in simultaneous eviction of a target segment and integration of the targeting DNA segment. Recombinants can be selected either by having a positive selection marker present on the incoming double *lox* targeting vector or by designing the target genomic to carry a negative selectable marker that is then evicted by Cre-mediated replacement. Because replacement occurs at high frequency, recombinants can also be readily isolated without use of drug selection, if desired, by enriching for transfected cells using a GFP expression plasmid. In this latter case it is important to note that the FACS enrichment process should be performed with care as it potentially could be detrimental to ES cells. Nevertheless, efficient germline transmission of sorted ES cells has been previously reported (31).

Table 2. Targeting of a silent locus

ES cell line	Neo^r colonies		% Replacement[a]
	–Cre	+Cre	
LacZ6	180	>700	75 (40)
9tk	200	>700	76 (38)

[a]The number of independent colonies analyzed by PCR (Fig. 5) for determination of replacement percentage are given in parentheses.

LoxP site integration targets that have been randomly placed into the genome of cultured cells differ in their ability to be targeted for Cre-mediated integration (29). For homologous recombination, one of the many factors that can influence the recombinational potential of a locus is whether or not it is transcriptionally active (32,33), perhaps by making the locus more accessible. For double *lox* targeting at the silent MHL-1 locus in ES cells, we show here that there is no difference in Cre-mediated integrative recombination in cells either having or not having a transcriptionally active gene at the double *lox* target. The ability of Cre to efficiently target even a silent MHL-1 locus bodes well for the general utility of the double *lox* integration strategy at any chromosomal locus.

In the work described here, we purposefully configured the genomic double *lox* target such that replacement recombination can result in a knock-in at MHL-1 of a desired gene on the double *lox* targeting vector and we describe a number of vectors useful for this purpose. Importantly, the strategy can be generalized to any genomic locus. There are a number of attractive features to this strategy for use in the construction of genetically engineered mouse lines. First, a series of cDNAs can easily be placed at exactly the same genomic locus and under the control of an endogenous control element, thus minimizing variation in the level and pattern of gene transgene expression and allowing

more accurate assessment of gene function. In a similar fashion, double *lox* replacement strategies should prove useful in the analysis of promoter function during development and differentiation. Second, the molecular manipulation is vastly simplified since there is no requirement for inclusion of genomic homology on the targeting vector. Third, the strategy also permits the use of the same targeting vector to deliver the same DNA to any double *lox* genomic locus. By assembling a collection of ES cell lines having double *lox* knock-in targets placed at selected loci (for example, at a liver-, heart- or brain-specific gene), the same DNA targeting vector can be used to express a desired gene with any of a variety of desired expression profiles, simply by choosing the appropriate ES cell line. Fourth, the simple cloning procedures and the relative insensitivity of Cre-mediated recombination to vector size suggest that even quite large fragments of DNA can be targeted efficiently to a defined genomic locus. For these reasons we anticipate that segmental genomic replacement by Cre-mediated double *lox* recombination will become an important tool for the genomic engineering of mice and other eukaryotes.

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REFERENCES

1. Doetschman,T., Gregg,R.G., Maeda,N., Hooper,M.L., Melton,D.W., Thompson,S. and Smithies,O. (1987) *Nature*, **330**, 576–578.
2. Thomas,K.R. and Capecchi,M.R. (1987) *Cell*, **51**, 503–512.
3. Hatada,S., Kuziel,W., Smithies,O. and Maeda,N. (1999) *J. Biol. Chem.*, **274**, 948–955.
4. Bronson,S.K., Plaehn,E.G., Kluckman,K.D., Hagaman,J.R., Maeda,N. and Smithies,O. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 9067–9072.
5. Hanks,M., Wurst,W., Anson-Cartwright,L., Auerbach,A.B. and Joyner,A.L. (1995) *Science*, **269**, 679–682.
6. Gu,H., Zou,Y.R. and Rajewsky,K. (1993) *Cell*, **73**, 1155–1164.
7. Lakso,M., Pichel,J.G., Gorman,J.R., Sauer,B., Okamoto,Y., Lee,E., Alt,F.W. and Westphal,H. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 5860–5865.
8. Bethke,B. and Sauer,B. (1997) *Nucleic Acids Res.*, **25**, 2828–2834.
9. Sauer,B. and Henderson,N. (1990) *New Biologist*, **2**, 441–449.
10. Hoess,R.H., Wierzbicki,A. and Abremski,K. (1986) *Nucleic Acids Res.*, **14**, 2287–2300.
11. Sauer,B. (1992) *J. Mol. Biol.*, **223**, 911–928.
12. Sauer,B. (1996) *Nucleic Acids Res.*, **24**, 4608–4613.
13. Bouhassira,E.E., Westerman,K. and Leboulch,P. (1997) *Blood*, **90**, 3332–3344.
14. Saveliev,S.V., Fessing,M.Y., Kopylov,A.M. and Kirjanov,G.I. (1993) *Curr. Genet.*, **24**, 26–31.
15. Seibler,J. and Bode,J. (1997) *Biochemistry*, **36**, 1740–1747.
16. Seibler,J., Schubeler,D., Fiering,S., Groudine,M. and Bode,J. (1998) *Biochemistry*, **37**, 6229–6234.
17. Tybulewicz,V.L., Crawford,C.E., Jackson,P.K., Bronson,R.T. and Mulligan,R.C. (1991) *Cell*, **65**, 1153–1163.
18. Johnson,K., Lerner,C., Di Lacio,L., Laird,P., Sharpe,A. and Simpson,E. (1995) *Nucleic Acids Res.*, **23**, 1273–1275.
19. Eustice,D.C., Feldman,P.A., Colberg-Poley,A.M., Buckery,R.M. and Neubauer,R.H. (1991) *BioTechniques*, **11**, 739–743.
20. Le,Y., Miller,J.L. and Sauer,B. (1999) *Anal. Biochem.*, **270**, 334–336.
21. Mizushima,S. and Nagata,S. (1990) *Nucleic Acids Res.*, **18**, 5322.
22. Gagneten,S., Le,Y., Miller,J. and Sauer,B. (1997) *Nucleic Acids Res.*, **25**, 3326–3331.
23. Sauer,B. (1993) *Methods Enzymol.*, **225**, 890–900.
24. Schnieke,A., Harbers,K. and Jaenisch,R. (1983) *Nature*, **304**, 315–320.
25. Ramírez-Solis,R., Davis,A.C. and Bradley,A. (1993) *Methods Enzymol.*, **225**, 855–878.
26. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Huber,B.E. (1992) *Mol. Pharmacol.*, **41**, 639–644.
28. Monroe,R.S. and Huber,B.E. (1994) *Gene*, **148**, 237–244.
29. Baubonis,W. and Sauer,B. (1993) *Nucleic Acids Res.*, **21**, 2025–2029.
30. Gangloff,S., Lieber,M.R. and Rothstein,R. (1994) *Experientia*, **50**, 261–269.
31. Reddy,S., Rayburn,H., von Melchner,H. and Ruley,H.E. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6721–6725.
32. Thomas,B.J. and Rothstein,R. (1989) *Cell*, **56**, 619–630.
33. Nickoloff,J.A. (1992) *Mol. Cell. Biol.*, **12**, 5311–5318.