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A system for rapid generation of coat color-tagged knockouts and defined chromosomal rearrangements in mice

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ABSTRACT

Gene targeting in mouse embryonic stem (ES) cells can be used to generate single gene mutations or defined multi-megabase chromosomal rearrangements when applied with the Cre–*loxP* recombination system. While single knockouts are essential for uncovering functions of cloned genes, chromosomal rearrangements are great genetic tools for mapping, mutagenesis screens and functional genomics. The conventional approach to generate mice with targeted alterations of the genome requires extensive molecular cloning to build targeting vectors and DNA-based genotyping for stock maintenance. Here we describe the design and construction of a two-library system to facilitate high throughput gene targeting and chromosomal engineering. The unique feature of these libraries is that once a clone is isolated, it is essentially ready to be used for insertional targeting in ES cells. The two libraries each bear a complementary set of genetic markers tailored so that the vector can be used for Cre–*loxP* -based chromosome engineering as well as single knockouts. By incorporating mouse coat color markers into the vectors, we illustrate a widely applicable method for stock maintenance of ES cell-derived mice with single gene knockouts or more extensive chromosomal rearrangements.

INTRODUCTION

Gene targeting in mouse embryonic stem (ES) cells currently represents the dominant practice in generating new mutants for functional analyses of mammalian genes (1,2). Although this technology is highly effective, its application in high throughput situations is limited by the molecular cloning required to build large numbers of targeting vectors and by DNA-based genotyping methods for stock maintenance.

An alternative approach to analyze gene function is to perform phenotype-driven mutagenesis screens. This approach does not require any prior knowledge of the genes under study and the responsible genes can be isolated by positional cloning and functional rescue. Stocks of well-mapped chromosomal deletions (deficiencies) have proven to be an invaluable tool in screens for recessive mutations in *Drosophila* over the years (3). These screens have utilized segmental haploidy in combination with point mutagenesis to recover recessive mutations. The use of chromosomal deletions as a genetic tool in mice has mainly been restricted to regions surrounding several visible markers (4). Although limited in overall size, these deletion panels have been instrumental in the genetic and molecular dissection of the chromosomal regions involved (4–6). However, most regions of the mouse genome are not accessible to this approach due to the lack of specifically marked deletions.

Recently, a Cre–*loxP*-based chromosome engineering technology was developed to make defined chromosomal rearrangements (7,8). The strategy involves the introduction of the *loxP* recombination sequence into two pre-defined chromosomal loci by consecutive steps of gene targeting. This is followed by transient expression of Cre, which catalyzes site-specific recombination between the *loxP* sites, leading to the desired chromosomal rearrangement. Reconstitution of a functional drug resistance gene, *Hprt*, from two non-functional halves that are linked to the two *loxP* sites provides positive selection for this event. Depending on the position and relative orientation of the two *loxP* sites with regard to centromere and telomere, different recombination products can be generated, such as deletions, duplications, inversions and translocations (7,8). It was also shown that various chromosomal rearrangements extending over several megabases can then be transmitted through the germline (7,9).

A panel of mice with deletions collectively covering the entire mouse genome would greatly enhance our ability to functionally analyze the mouse genome. With more than 6000 well-mapped microsatellite markers available in mice (10), it is now possible to systematically generate such a panel of deletion mice each spanning a few megabases at a defined location. One of the rate limiting steps in the Cre–*loxP* chromosome engineering technology is the molecular cloning required to construct targeting vectors for multiple deletion end-points. In addition, maintaining and using a large deletion panel in screens for recessive mutations involves a substantial genotyping effort with current DNA-based methods. Therefore, the ability to visually identify mice which carry deletions is highly desirable.

To maximize the efficiency of generating targeted knockouts and chromosomal deletions, we designed and constructed two 129S5 (or 129S5/SvEvBrd, previously named 129/SvEvBrd)

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(11,12) genomic libraries in which the genetic elements required for gene targeting, chromosome engineering and coat color tagging are available in the vector backbone. Any clone isolated from the libraries is essentially ready to be used in targeting experiments for single gene knockouts and/or as an end-point for chromosomal engineering. This virtually eliminates the molecular cloning for individual targeting vector construction, makes it feasible to generate panels of mice with a variety of genetic alterations and enables their visual genotyping.

MATERIALS AND METHODS

Construction of the *5′hprt* and *3′hprt* phage vectors

Our general strategy was to construct customized λ phage vectors containing all of the elements required for plasmid rescue, gene targeting, coat color tagging and chromosome engineering while retaining sufficient space to accommodate 7–14 kb of genomic DNA. The *5′hprt* cassette pL13 (originally named *hprtΔ3′–Neo*) and *3′hprt* cassettes pG7 (originally named *hprtΔ5′*) and pG12 (originally named *Puro–hprtΔ5′*) have been described previously (7).

5′hprt library vector. In order to remove the redundant *hprt* sequence following the *loxP* site that includes part of intron 2 and exons 3–6 in pL13, pL13 was cut with *Bam*HI and *Hin*dIII, filled in with Klenow and the 1.4 (*5′hprt*) and 4.9 kb (*Neo* and pSKII+) fragments were re-ligated with the correct orientation chosen to obtain pBZ7. A variant of pSKII+ with a different polylinker site *Asc*I–*Bam*HI–*Asc*I–*Hin*dIII–*Bgl*II–*Not*I–*Xba*I–*Xho*I (pBZ13) was generated by cutting pSKII+ (pBluescript IISK+; Stratagene) with *Pvu*II and ligating to a pair of complementary oligonucleotides (5′-GGC GCG CCG GAT CCG GCG CGC CAA GCT TCA GAT CTG CGG CCG CTC TAG AAC TCG AG-3′). A 2.5 kb *Xba*I–*Xho*I fragment (*ori*, *Amp*) from pBZ13 was ligated to a 1.9 kb *Xho*I–*Xba*I(partial) fragment (*loxP–Neo*) from pBZ7 to make pBZ15. A 4.4 kb *Hin*dIII–*Bgl*II fragment from pBZ15 was then ligated to a 1.4 kb *Bam*HI–*Eco*RI fragment (*5′hprt–loxP*) from pL13 and a 4.1 kb fragment (*Tyrosinase* minigene) from TyBS (13) to obtain pBZ16 by a three-way ligation. pBZ16 has two tandemly arrayed *loxP* sites flanking a *Not*I site. The 9.9 kb pBZ16 was then linearized at the *Not*I site and used to replace the stuffer region of the λFixII phage vector (Stratagene) to obtain the *5′hprt* phage vector (Fig. 1A). This vector is ~39 kb in size and can accommodate a genomic insert of up to 14 kb. The following 6 bp restriction endonucleases will not cut in the vector sequence of an excised *5′hprt* library plasmid: *Aat*II, *Afl*II, *Bsi*WI, *Bst*BI, *Cla*I, *Hpa*I, *Kpn*I, *Mlu*I, *Nde*I, *Nhe*I, *Nru*I, *Pml*I, *Sac*II, *Sal*I and *Sma*I. A *Bam*HI site may or may not be present at the vector/insert junctions.

3′hprt library vector. The *Eco*RI and *Bam*HI sites of pG12 were removed by Klenow fill-in to obtain pBZ18. To make a variant of pSKII+ with an alternative polylinker site *Asc*I–*Bam*HI–*Asc*I–*Spe*I–*Bgl*II–*Not*I–*Pst*I–*Hin*dIII (pBZ19), pSKII+ was cut with *Pvu*II and ligated to a pair of complementary oligonucleotides (5′-GGC GCG CCG GAT CCG GCG CGC CAC TAG TAT TCA AGA TCT GCG GCC GCC TGC AGT TAA TAA GCT T-3′). A 0.4 kb *Pst*I fragment from pBZ18 containing *loxP* and the first part of *3′hprt* was cloned into the *Pst*I site in pBZ19 to make pBZ23. The *Eco*RI site in pK14A (the *K14Agouti* transgene) (14) was converted to a *Bgl*II site by ligation of the self-complementary oligonucleotide (5′-AAT TGC AGA TCT GC-3′), resulting in pBZ26. A 3.5 kb *Bgl*II–*Hin*dIII fragment from pBZ26 and a 1.8 kb *Bam*HI–*Pst*I(partial) fragment (second part of *3′hprt*) from *PGK–Hprt* (15) were ligated to the 3.0 kb *Hin*dIII–*Pst*I(partial) fragment from pBZ23 to obtain pBZ58 by a three-way ligation. The following sites in the puromycin resistance gene in pG12 were removed: the adjacent *Sac*I and *Cla*I sites by T4 polymerase fill-in; the *Bgl*II site by ligation of the self-complementary oligonucleotide (5′-GAT CCA CTG CAG TG-3′). The resulting 2.2 kb *Bam*HI–*Spe*I(partial) fragment (*Puro–loxP* without *Sac*I, *Cla*I or *Bgl*II sites) was then ligated to an 8.3 kb *Bgl*II–*Spe*I(partial) fragment (*loxP–3′hprt–K14Agouti*) from pBZ58 to obtain pBZ60. pBZ60 has two tandemly arrayed *loxP* sites flanking a *Not*I site. The 10.5 kb pBZ60 was then linearized at the *Not*I site and used to replace the stuffer region of the λFixII phage vector to obtain the *3′hprt* phage vector (Fig. 1C). This vector is ~39.5 kb in size and can accommodate an insert of up to 13.5 kb. The following 6 bp restriction endonucleases will not cut in the vector sequence of an excised *3′hprt* library plasmid: *Afl*II, *Bgl*II, *Bst*BI, *Cla*I, *Eco*RI, *Fsp*I, *Hpa*I, *Kpn*I, *Mfe*I, *Mlu*I, *Nco*I, *Nde*I, *Nru*I, *Pml*I, *Sac*I and *Sal*I. A *Bam*HI site may or may not be present at the vector/insert junctions.

Construction of the *5′hprt* and *3′hprt* libraries

Genomic DNA was prepared from the liver of male 129S5 mice. *Sau*3AI partially digested DNA was fractionated on a 10–40% sucrose gradient and the fractions containing the highest proportion of 7–14 kb fragments were used in the *5′hprt* library construction. For the *3′hprt* library, partially digested DNA was run on a 0.7% agarose gel and DNA fragments of 8–14 kb were purified from the agarose gel by agarase treatment (New England BioLabs). The phage vectors were digested with *Bam*HI (Boehringer Mannheim Biochemicals) and dephosphorylated with shrimp alkaline phosphatase (US Biochemicals). A typical ligation reaction consisted of 1 μg properly prepared phage vector arms and 0.15 μg genomic DNA fragments with 2.5 U of T4 DNA ligase (Boehringer Mannheim Biochemicals) in 5 μl total volume. Packaging reactions were performed with Transpack packaging extract (Stratagene). The primary libraries were then titered, amplified once using host strain XL1-Blue MRA (P2) (Stratagene) and stored at –80°C in 7% dimethylsulfoxide. The complexity of the *5′hprt* library is ~1.1×10^7 p.f.u. (26× genome equivalents). That of the *3′hprt* library is ~2.5×10^7 p.f.u. (75× genome equivalents).

Characterization of the *5′hprt* and *3′hprt* libraries

The percentage of recombinants and the average insert size were estimated by sampling a pool of randomly picked clones from the libraries.

5′hprt library. Eleven phage clones and 17 excised plasmid clones were analyzed by *Asc*I digestion. Plasmid excision was performed using the Cre-expressing bacterial strain BNN132 as described (16). Only one out of the 28 clones (combined total) did not appear to have an insert (~96% recombinants).

3′hprt library. Eight randomly picked phage clones all contained an insert. All the eight clones were individually excised as plasmids by infection of BNN132.

Given the large number of cloning steps, the neomycin resistance gene, the *loxP* site and *5′hprt* in the *5′hprt* library and the puromycin resistance gene, the *loxP* site and *3′hprt* in the

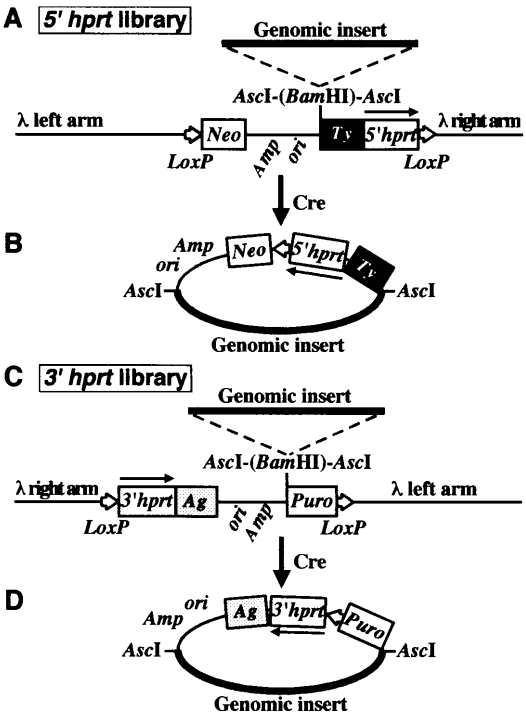

Figure 1. Two libraries of targeting vectors. (**A**) A representative phage clone from the *5′hprt* library which can be automatically subcloned into a plasmid (**B**) by *in vivo* excision with a Cre-expressing bacterial strain. The *5′hprt* library backbone contains the neomycin resistance gene (*Neo*), a *loxP* site, *5′hprt* and a *Tyrosinase* minigene (*Ty*). The *Tyrosinase* gene gives coat color to an otherwise albino mouse. (**C**) A representative phage clone from the *3′hprt* library which can be excised into a plasmid (**D**). The *3′hprt* library backbone contains the puromycin resistance gene (*Puro*), a *loxP* site, *3′hprt* and the *K14Agouti* transgene (*Ag*).

3′hprt library were tested in ES cells and confirmed to be functional (data not shown). To test the *Tyrosinase* and *K14Agouti* minigenes, transgenic mice were generated with a representative clone from the *5′hprt* and *3′hprt* libraries, respectively. In the case of *Tyrosinase*, three out of 12 transgenic founders showed detectable coat color ranging from light gray to gray in an albino background (Results). For *K14Agouti*, three out of three transgenic founders showed yellowing of the fur in a wild-type *Agouti* background (data not shown). These observations recapitulated published results obtained with these constructs (13,14).

Targeting in embryonic stem cells and germline transmission

ES cell growth, electroporation, drug selection, targeting detection and germline transmission were performed as described previously (15,17). Targeting of *D11Mit142*, *D11Mit71* and *D11Mit69* were performed in a hybrid ES cell line ER3.4 (E.Regel and A.Bradley, unpublished results) which was made from an XY embryo from a 129S7/SvEvBrd-*Hprt*$^{b\text{-}m2}$ × C57BL/6-*Tyr*$^{c\text{-}Brd}$ cross (9,11,12). Transient Cre expression and selection of recombination products by HAT were performed as described (7,9).

Fluorescence *in situ* hybridization

Metaphase chromosome spreads from ES cells were prepared as described (18). FISH was performed with three BAC probes following a standard protocol (19).

RESULTS

Although not widely used, insertion vectors are highly mutagenic and are known to undergo very efficient gene targeting in the mammalian genome (20). Moreover, insertion vectors are ideal for constructing a library of vectors because only one contiguous genomic fragment is used as the region of homology (21,22). We designed and constructed two genomic libraries, the *5′hprt* and *3′hprt* libraries, with all the molecular elements required for gene targeting, coat color tagging and chromosome engineering incorporated into the vector backbone (Fig. 1). Two libraries are necessary for chromosome engineering since each rearrangement requires the successive targeting of two end-points with the complementary halves of the *hprt* minigene and different positive selection markers. Specifically, the *5′hprt* library vector (Fig. 1A) carries the neomycin resistance gene for gene targeting, a *loxP* site and the 5′-half *hprt* minigene for chromosome engineering and a *Tyrosinase* minigene (the gene mutated in the mouse albino mutant) for coat color tagging (13). Similarly, the *3′hprt* library vector (Fig. 1C) carries the puromycin resistance gene, a *loxP* site, the 3′-half *hprt* minigene and an *Agouti* transgene under the control of the K14 promoter (*K14Agouti*) (14). Two sets of vectors are also useful because this enables both alleles of a gene to be mutated in ES cells by using complementary vectors which carry different positive selection cassettes.

The libraries were constructed in phage vectors with automatic plasmid excision capabilities (Fig. 1). This was achieved by including in the phage vector a plasmid backbone (replication origin and ampicillin resistance gene) and two tandemly arrayed *loxP* sites that flank all the elements except the phage arms (Fig. 1). Any phage clone can be excised into a plasmid by Cre-induced *loxP* site recombination in a Cre-expressing bacterial strain, BNN132 (16). The *loxP* site that is retained in the excised plasmid will serve in the Cre–*loxP*-based chromosome engineering strategy. The genomic insert is flanked by rare cutter *Asc*I sites which can be used to convert the orientation of the insert relative to the vector sequence in one subcloning step. This makes it possible to target the same locus with two alternative *loxP* orientations in order to construct different chromosomal rearrangements (7). As λ phage has a maximum packaging capacity of ~53 kb DNA, the genetic elements in the customized phage vectors were kept to a minimum size whenever possible so that the size of the genomic inserts would be maximized. To provide as many choices as possible for linearization or gapping (see below) of the targeting vector several unique restriction sites in the vectors were destroyed (Materials and Methods). The average insert size of the *5′hprt* library is ~7.6 kb, while that of the *3′hprt* library is ~9.1 kb, suitable for insertional targeting.

To determine whether the libraries can simplify gene targeting, primer pairs which amplify several *Mit* SSLP markers (10) or genes were used to isolate specific clones for several gene targeting and chromosome engineering projects. A PCR-based screening method was used on arrayed versions of the libraries (as described in 23), which enabled clones to be isolated in a few days. Isolated phage clones were then automatically subcloned

into the plasmid form through *in vivo* excision by BNN132. The insert of each clone was mapped with restriction enzymes known not to cut in the vector backbone (see Materials and Methods).

Although non-overlapping regions of contiguous clones can be used to develop probes for detecting targeting, an alternative streamlined strategy was developed. This takes advantage of the double-strand gap repair in an insertional (O-type) gene targeting event (24–29). An 'external' probe can be derived from the same clone which provides the region of homology for gene targeting. In normal insertional targeting, the targeting vector is linearized within the region of homology to stimulate the single crossover event between the vector and the chromosomal DNA (21). In gap repair insertional targeting, a gap is present in the homologous sequence of the vector. During targeted recombination, the gap is repaired from the chromosomal DNA template. This will lead to a duplication of the entire region of homology including the repaired gap sequence, which will flank the inserted vector sequence. Two strategies can be used to detect recombinants. A PCR approach can be used with a pair of primers specific to the gap sequence and the proximal vector sequence, which requires some sequence information of the gap region. Alternatively, the 'gap' region can be used as the probe on Southern blots with an appropriate restriction digest.

The preparation of a gapped targeting vector from a representative clone, *D11Mit142*, isolated from the *3′hprt* library is illustrated in Figure 2. After isolation of the clone and excision into a plasmid, the insert was mapped with a number of restriction enzymes, including *Bgl*II and *Eco*RI (Fig. 2A). Based on this map, either *Bgl*II or *Eco*RI can be used to create the gap. *Bgl*II was selected because it gives several choices for a gap probe (Fig. 2). The plasmid was digested with *Bgl*II and the purified vector fragment was re-circularized by ligation. This removed the mid portion of the insert, creating a 3.3 kb gap in the region of homology (Fig 2C). Southern hybridization tests showed that a 2.3 kb fragment within the gap region was the best probe. *Eco*RI was selected as the diagnostic enzyme for targeting detection. During the targeting event, the 3.3 kb gap is repaired, resulting in a novel 18 kb fragment detected by the probe in addition to the 7.2 kb wild-type fragment (Fig. 2C and D). This vector has a total homology of 5 kb and a gap of 3.3 kb and gave a 12% targeting frequency. Several loci have been successfully targeted with vectors from these libraries. The sizes of the regions of homology and the gap, along with the corresponding targeting frequencies, are shown in Table 1. Targeting frequencies in the range 4–64% were obtained.

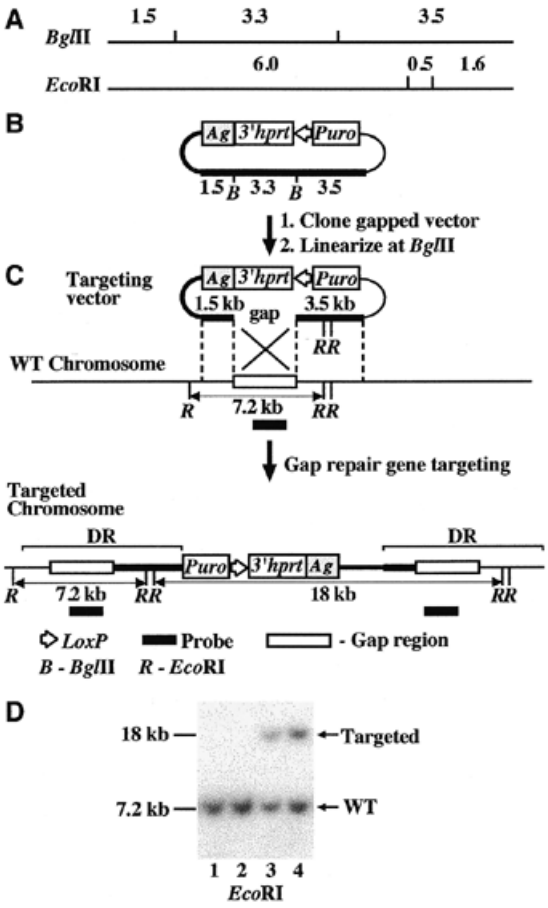

Figure 2. Gap repair targeting at *D11Mit142*. (**A**) Restriction maps of the genomic insert of a *D11Mit142* clone from the *3′hprt* library. The insert can be cut with *Bgl*II into 1.5 and 3.5 kb flanking fragments and three internal fragments of 2.3, 0.8 and 0.2 kb (which add up to 3.3 kb, shown as a single fragment for simplicity). (**B**) The excised *D11Mit142* plasmid clone shown in its entirety. The internal *Bgl*II fragments are removed by one step of subcloning to create a gap in the region of homology to obtain a gapped targeting vector in (C). (**C**) The gapped vector is linearized at the *Bgl*II site and targeted to the *D11Mit142* locus. An 18 kb *Eco*RI targeted fragment is detected in addition to the wild-type 7.2 kb *Eco*RI fragment. DR, duplicated region. (**D**) Mini-Southern analysis for targeting detection. As the 7.2 kb fragment is present in both the targeted and wild-type chromosomes the intensity of the 18 kb fragment relative to the 7.2 kb fragment is 1:2. Lanes 1 and 2, ES clones with random integration events; lanes 3 and 4, targeted ES cell clones.

Table 1. Targeting frequencies of gapped insertion vectors

Locus	Library	Region of homology (kb)			Gap size (kb)	Targeting frequency (%)	
		LA	RA	Total		OriA	OriB
D11Mit142	*3′hprt*	1.5	3.5	5.0	3.3	11	12
D11Mit71	*3′hprt*	2.3	3.9	6.2	4.7	4	5
D4Mit51	*3′hprt*	0.9	5.6	6.5	1.4	22	33
D4Mit70	*3′hprt*	2.0	5.0	7.0	1.8	33	64
$p63^{Brdm1}$	*3′hprt*	2.3	4.0	6.3	1.9	15	ND
$p63^{Brdm2}$	*3′hprt*	2.8	3.8	6.6	3.7	23	ND
D4Mit190	*5′hprt*	1.6	3.0	4.6	1.4	20	ND

The region of homology is divided into two parts (LA, left arm; RA, right arm) by the gap. The gap is excluded in calculating the total size of the region of homology. The targeting frequencies for the two alternative *loxP* orientations are shown separately, as indicated by OriA and OriB. ND, not determined. Two *p63* targeted cell lines were constructed using two independently isolated clones, designated $p63^{Brdm1}$ and $p63^{Brdm2}$.

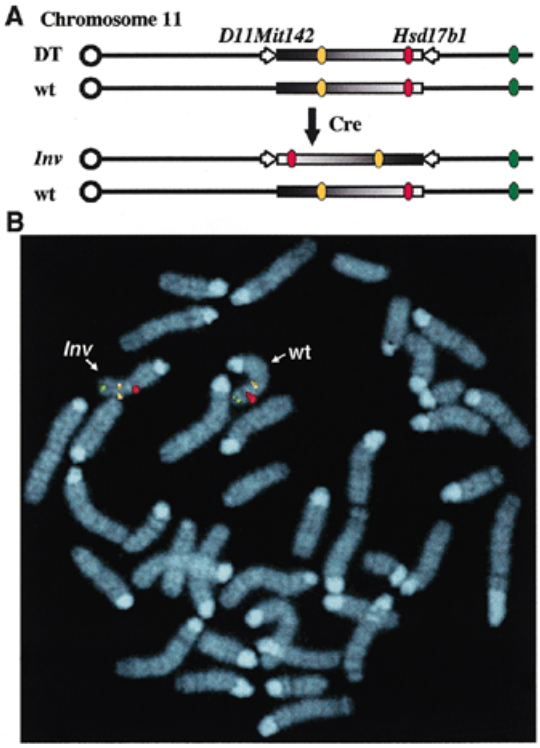

Figure 3. Construction of a 30 cM inversion on chromosome 11 and confirmation by FISH. (**A**) *D11Mit142* was targeted (Fig. 2) in an ES cell line already targeted at the *Hsd17b1* locus on chromosome 11. As the two *loxP* sites are in the opposite orientation, expression of Cre creates an inversion. The *loxP* site is indicated by an open arrow. DT, doubly targeted; wt, wild-type; *Inv*, inversion. The three BAC probes used in FISH analysis are indicated in different colors (yellow, *D11Mit320*; red, *D11Mit263*; green, *D11Mit11*). (**B**) FISH analysis on the inversion product.

To determine whether clones from the libraries can be successfully used to construct chromosomal rearrangements, the gapped *D11Mit142* targeting vector (Fig. 2C) was targeted in an ES cell line which had already been targeted at the *Hsd17b1* locus on chromosome 11 with the *5′hprt* cassette (Fig. 3A). Following Cre expression, HAT-resistant colonies were obtained and the recombination products were analyzed by fluorescence *in situ* hybridization (FISH). This configuration of the *loxP* sites generated a 30 cM inversion after Cre recombination (Fig. 3A and B). To target the *D11Mit142* locus with the opposite *loxP* orientation, the insert of the gapped *D11Mit142* targeting vector described above was flipped by using *Asc*I restriction and re-ligation. *Hsd17b1–D11Mit142* doubly targeted cell lines generated with the flipped vector were used to construct the corresponding deletion/duplication (data not shown).

To determine the extent to which the targeted single copy *K14Agouti* marker affected the coat color, four alleles on three chromosomes were examined, each targeted at a different locus. These were targeted at two genes (*p63* and *Wnt3*) and two SSLP loci (*D4Mit51* and *D4Mit70*), respectively. Heterozygous *p63* mice were consistently yellowish in color (butterscotch) and were clearly distinguishable from the wild-type littermates (Fig. 4A). In line with previous transgenic studies (14), the *K14Agouti* transgene is dominant over wild-type *Agouti*, i.e. the yellowish coat color is manifested in a wild-type *Agouti* background.

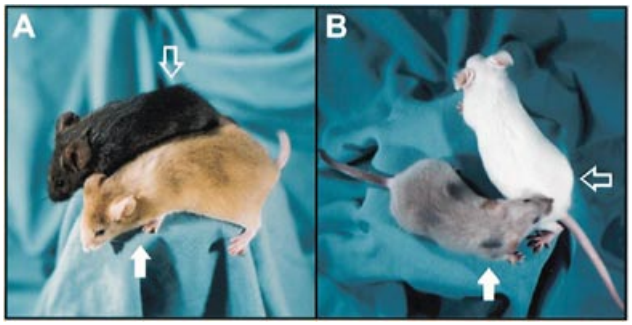

Figure 4. Coat color conferred by *K14Agouti* and *Tyrosinase* genes. (**A**) Germline mice of a *p63* targeted clone. Open arrow, wild-type littermate (with one copy of wild-type *Agouti*); solid arrow, $p63^{Brdm1}$ heterozygote (with one copy of *K14Agouti* targeted at *p63* in addition to one copy of the wild-type *Agouti*). (**B**) A transgenic founder (solid arrow) made with a construct from the *5′hprt* library (containing the *Tyrosinase* gene) with an albino non-transgenic littermate (open arrow).

Heterozygous mice targeted at *Wnt3* do not show the same degree of 'yellowing' of the coat color. However, the mice can be genotyped visually since the tail and the back of the ear lobes of these mice are consistently lighter than their wild-type littermates (data not shown). Mice targeted at one of the two SSLP loci on chromosome 4, *D4Mit51* or *D4Mit70*, showed a lightening of the tail and ear regions similar to that seen in the *Wnt3* targeted mice.

To test the *Tyrosinase* minigene used in the *5′hprt* library, mice with the *Tyrosinase* minigene targeted at the *D11Mit69* locus were generated. Because the wild-type tyrosinase locus on chromosome 7 is dominant, the targeted allele was bred into an albino background by crossing with C57BL/6-$Tyr^{c\text{-}Brd}$ mice (9). Neither heterozygous nor homozygous mice on the albino background were distinguishable from their wild-type albino littermates. Southern blot analysis with a methylation-sensitive enzyme *Sal*I indicated that this locus was methylated in tail DNA (data not shown). As *D11Mit69* is at the distal end of mouse chromosome 11, this transgene silencing may be a position effect. To test whether the *Tyrosinase* minigene in the 5′*hprt* library vector was functional, a clone corresponding to the mouse *Per1* gene was isolated from this library, linearized in the insert and used to generate transgenic mice. Three out of 12 transgenic founders showed a grayish coat color, as exemplified in Figure 4B. This result indicated that the *Tyrosinase* gene in the *5′hprt* library is functional.

DISCUSSION

The two-library system described here facilitates high throughput knockouts of single genes and rapid and systematic generation of targeted chromosomal rearrangements, as well as visual genotyping of the modified alleles. The use of insertion vectors for gene targeting has made it possible to design and construct libraries of targeting vectors by incorporating all of the genetic elements required for gene targeting and chromosome engineering into the vector backbone. Vectors from these libraries have a high targeting frequency and will reliably mutate their target, which is consistent with the known mutagenicity of insertional targeting events at several loci such as *Hprt* (30) and *CFTR* (31,32).

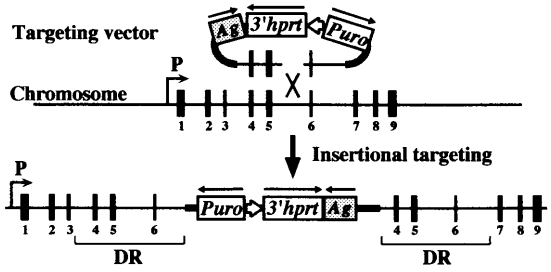

Figure 5. Mutagenic insertional targeting. The chromosome and the region of homology in the vector are indicated by thin lines. The vector sequence is indicated by thick lines. When the vector is linearized in the region of homology and insertionally targeted into the homologous locus, the entire region of homology is duplicated (independent of the linearization site or the presence of a gap within the homology region). The duplicated regions (DR) flank the vector sequence. To generate a truncation mutation, the region of homology in the vector needs to lack both 5′ and 3′ parts of the gene so that neither of the duplicated regions is a complete gene. Both duplication of the middle exon(s) and insertion of the vector sequences contribute to the mutagenic potential of insertional targeting.

Insertional targeting results in duplication of the region of homology contained in the vector. Consequently, mutagenicity is most readily ensured by selecting a clone which contains the middle exon(s) but lacks both the 5′ and the 3′ parts of the gene, as illustrated in Figure 5. For a typical gene that occupies a 20 kb (or more) genomic locus, this can be easily achieved by isolating a clone corresponding to the mid portion of the gene and confirming the absence of the 5′ and 3′ exons. There are several possible mechanisms for insertional targeted mutagenesis. Insertion of the vector sequence into the target locus may lead to a truncated and/or unstable transcript. For instance, two different null mutants of the mouse *p63* gene, a *p53* gene family member, have been rapidly generated using two insertion vectors independently isolated from the *3′hprt* library (Table 1; 33). If splicing over the vector sequence occurs, a transcript containing the duplicated exon(s) will be produced. Exon duplication in the transcript may lead to a change of the open reading frame. If an in-frame transcript is produced, mutagenicity is also possible, since the duplication may disturb protein folding. As long as the region of homology contains one (or more) intact exon(s) that normally does not undergo alternative splicing, the likelihood of producing a wild-type transcript by exon skipping is minimized (32). In addition, spontaneous homologous recombination between the duplicated regions may restore a wild-type gene. However, such reversion events occur at a low frequency (~10^{-6}) (34) and have not been reported to present problems in analyzing mutants generated with insertion vectors. There are fewer constraints when the vectors from these libraries are used solely for chromosome engineering purposes since it is not necessary to mutate the end-points of the rearrangement.

An alternative strategy to generate gene targeting constructs based on an *in vitro* transposon reaction has recently been described (35). This strategy requires fine mapping, two steps of subcloning, a transposition, transformation and PCR screening procedure to obtain the targeting construct. Other approaches to build replacement vectors based on homologous recombination in yeast (36) or in *Escherichia coli* (37) have been developed. These strategies have the ability to construct pre-defined complex targeting vectors but require multiple steps of molecular manipulations and some sequence information.

The libraries described here have the following unique features or advantages. (i) Automatic excision of the plasmid from the phage vector, eliminating subcloning of the insert. (ii) An average insert size of 7–10 kb suitable for insertional targeting. In most cases, even after deleting a gap, high targeting frequencies can be obtained with an average of 10–30%. (iii) The excised plasmid lacks the recognition sequence of more than 10 ‘six cutter’ restriction enzymes in the backbone which can be used for linearization and gap creation. (iv) A Southern-based gap repair-dependent strategy provides a streamlined method to obtain an external probe for detecting targeting. (v) Mouse coat color genes are used to visually tag the targeted alleles, facilitating stock maintenance and genetic screens. This system is suitable for single gene knockouts and is particularly adapted for rapid generation of panels of chromosomal deletions.

One of the most efficient ways to maintain a recessive lethal mutation or to follow the targeted allele in a cross is to mark the locus with a visible genetic marker. Although marking transgenic mice with coat color genes has been described by several groups (14,38–40), marking a targeted allele has not been reported. Here we document that *K14Agouti* is very effective as a coat color tag for several targeted loci. The *Tyrosinase* minigene is more variable in its activity. For instance, it was not functional at one locus (*D11Mit69*) but is functional at another (*p63*; our preliminary data). Thus this marker is position dependent (13). The position dependence of the two coat color markers are being investigated at other loci. In deletion-assisted point mutagenesis screens, coat color-tagged deletions are crucial for the strategy (4,5). Our results mark a significant step towards this goal.

Recently, a method to generate a deletion series based on irradiation of an ES cell line targeted with a negatively selectable marker has been described (41). This approach is efficient for generating a nested deletion series around a single targeted locus. The Cre–*loxP* strategy to make deletions has the advantage of making precise and pre-defined deletions, rendering greater control over the experimental system. Furthermore, deletions created by radiation cannot be directly tagged with a coat color marker. Finally, with the irradiation approach it is not easy to identify inversions, duplications and translocations, which are useful in creating balancer chromosomes, in mapping studies and in modeling certain human disorders. The libraries described here greatly enhance the efficiency and effectiveness of the Cre–*loxP*-based chromosome engineering strategy and allow for rapid generation of coat color-marked deletion series.

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