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Methods

A New Method for Rapidly Generating Gene-Targeting Vectors by Engineering BACs Through Homologous Recombination in Bacteria

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Generating knockout mice is still an expensive and highly time-consuming process. Target construct generation, the first labor-intensive step in this process, requires the manipulation of large fragments of DNA and numerous, and often cumbersome, cloning steps. Here we show the development of a rapid approach for generating targeting constructs that capitalizes on efficient homologous recombination between linear DNA fragments and circular plasmids in *Escherichia coli* ("recombineering"), the availability of bacterial artificial chromosomes (BACs), and the accessibility of the sequence of the mouse genome. Employing recombineering, we demonstrate with only 1–2 template plasmids, short homologies (40–50bp) between donor and target DNA, and one subcloning step that we can efficiently manipulate BACs in situ to generate a complicated targeting vector. This procedure avoids the need to construct or screen genomic libraries and permits the generation of most standard, conditional, or knock-in targeting vectors, often within two weeks.

The generation of knockout mice through gene targeting by homologous recombination in embryonic stem (ES) cells is still an expensive and highly time-consuming process. Knockout construct assembly is the first step in a laborious process that can take up to a year even in the most technically skilled laboratories. Typically, the process requires screening a genomic library to obtain the gene of interest, followed by restriction mapping and numerous cloning steps.

One strategy that can avoid time-consuming subcloning steps and may offer a more efficient approach for generating targeting constructs is the use of homologous recombination in *Escherichia coli* (Eggleston and West 1996). The basis of the technology is to utilize strains of *E. coli* that can efficiently carry out homologous recombination between short terminal homology regions on a linear PCR-derived DNA fragment and sequences on a recipient plasmid. This strategy has been referred to as either recombineering—for recombinogenic engineering (Copeland et al. 2001; Court et al. 2002)—or recombination cloning (Zhang et al. 2002). This approach has been utilized to alter various DNA targets, including the *E. coli* chromosome, high copy number plasmids, and bacterial artificial chromosomes (BACs; Zhang et al. 1998, 2002; Angrand et al. 1999; Murrers et al. 1999; Datsenko and Wanner 2000; Yu et al. 2000; Copeland et al. 2001; Lee et al. 2001; Liu et al. 2003).

While two groups have employed this strategy for the construction of gene targeting vectors, simplifying some of the time-consuming steps for targeting vector construction, these approaches still required gene isolation or the construction of genomic libraries and/or numerous complex gene manipulations

(Angrand et al. 1999; Zhang et al. 2002). We desired a construction strategy that would preclude the need to screen for and subsequently map genomic sequences and, most importantly, would avoid all of the cumbersome cloning steps. To simplify the process, we reasoned that one could identify the genomic sequence of the gene of interest from publicly available databases and then purchase an *E. coli* clone containing a BAC encompassing these sequences rather than employing standard genomic library screening. With the appropriately constructed template vectors (described below) and the essential recombination machinery, we could then use homologous recombination in *E. coli* to manipulate directly the gene of interest in the BAC. Finally, in one step, we could remove the manipulated sequence within the BAC by identifying flanking restriction sites from the published sequence, and under appropriate antibiotic selection, generate a targeting construct. Overall, this approach would obviate the need for the numerous time-consuming cloning steps typically required for generating complicated targeting constructs.

RESULTS AND DISCUSSION

We sought to generate a variety of constructs for the conditional targeting of the *NOD2/CARD15* gene (the murine homolog of a novel Crohn's disease gene; Hugot et al. 2001; Ogura et al. 2001). From available sequence databases, we identified the *NOD2* gene and purchased a BAC clone containing all 11 coding exons. The overall strategy for generating a conditional targeting vector was to employ two successive recombination steps to introduce a *loxP* site 5' to exon 3 and an aminoglycoside phosphotransferase (*aph*) gene cassette (mediating kanamycin resistance or neomycin resistance when expressed in prokaryotes or eukaryotes, respectively) flanked by *loxP* sites ("floxed") 3' to exon 3 in the BAC clone (Fig. 1). To mediate each homologous recombination step, we introduced a plasmid containing the arabinose-inducible Red

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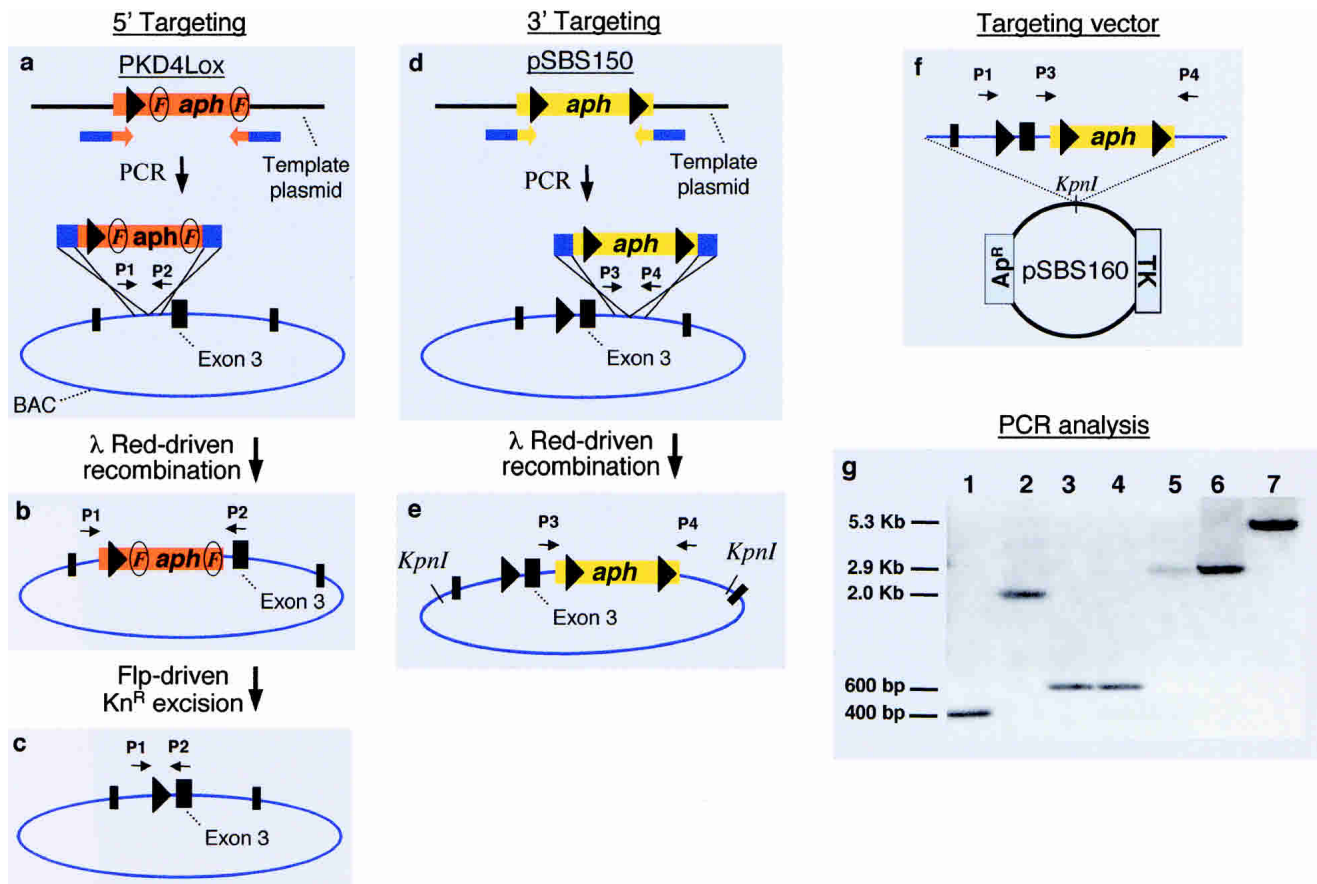


Figure 1 Conditional knockout vector construction. (a–f) Sequential steps leading to a *NOD2* exon 3 recombinant BAC and targeting vector (see text for details). (g) Gel electrophoresis depicting PCR products confirming the recombinants schematized in diagrams: (a) lane 1 (WT-BAC), (b) lane 2 ($BAC^{loxP/rr/aph/rr}$), and (c) lane 3 (BAC^{loxP}), generated with primers 1 (P1) and 2 (P2); (d) lane 4 (BAC^{loxP}) and (e) lane 5 ($BAC^{loxP-loxP/aph/loxP}$), generated with P3 and P4; and (f) lanes 6,7 (targeting construct) using respectively P3 and P4, and P1 and P4. Circled “F”, *frt* site; large arrowhead, *loxP* site.

recombination system from bacteriophage λ (Murphy 1998; Datsenko and Wanner 2000) into the BAC containing strain.

In the first recombination step, intronic BAC sequences 5' to exon 3 were targeted with PCR products containing a *loxP* site and an *frt*-flanked *aph* gene (Fig. 1a). These PCR products were amplified with 60-bp primer pairs consisting of 40 bp of sequence homologous to the intronic region flanking the targeted exon, and 20 bp complementary to the template plasmid containing the DNA to be inserted (Fig. 1a). The template plasmid, pKD4Lox, was derived from pKD4 (Datsenko and Wanner 2000), a conditional replicon (*oriR γ*) that requires the *pir* gene product (absent in most *E. coli* strains that carry BACs) for replication. Transformation with the *loxP/aph*-containing PCR fragment resulted in several kanamycin-resistant (Kn^R) recombinants (Fig. 1b). Following transformation with a temperature-sensitive replicon that expresses the *flp* recombinase (Cherepanov and Wackernagel 1995), which recognizes *frt* sites, the *aph* gene was excised by site specific recombination (Fig. 1c).

The second step was to manipulate BAC intronic sequence 3' to exon 3. Kanamycin-sensitive strains isolated above (Fig. 1c) were targeted with a PCR product containing a floxed *aph* cassette generated from pSBS150. This pKD4-based template contains the *aph* gene under the control of both prokaryotic and eukaryotic promoters to allow selection in bacterial and ES cells (Fig. 1d). From the genomic sequence, we identified a restriction enzyme site (*KpnI*) flanking the manipulated exon that would yield a fragment of sufficient length for homologous recombina-

tion in ES cells (Fig. 1e). To isolate the construct, the recombinant BAC was digested with *KpnI*, and the fragments were subcloned into a multicopy cloning vector (e.g., pBluescript or a *thymidine kinase* [*TK*]-containing plasmid; Fig. 1f), and selected for ampicillin resistant (Ap^R) and Kn^R colonies. The presence of the *aph* gene within the region of the BAC of interest allowed, upon kanamycin selection, enrichment of clones containing only the desired DNA fragments. PCR (Fig. 1g), restriction enzyme, and sequencing analyses confirmed the desired products in all steps.

We also developed a similar method for a “one-step” targeting strategy that would be applicable for either conditional knockout targeting construct generation or for the “knock-in” of a mutated exon. We first generated pSBS156, another conditional replicon. This plasmid contained a unique *EcoRI* site to allow the cloning of a wild-type or “mutated” exon of interest flanked by a *loxP* sequence and a floxed *aph* gene cassette (Fig. 2a). Subsequently, we developed a *NOD2* “knock-in” template by introducing into pSBS156 a mutated exon 10 that corresponded to the mutant genotype in CD (Fig. 2b, pSBS158; Hugot et al. 2001; Ogura et al. 2001). Homologous recombination between linear PCR fragments and the BAC generated Kn^R recombinants containing the mutated exon and a 3' floxed *aph* gene, but not the 5' *loxP*. To circumvent this recombination that occurred within the exon (which would be incompatible with a conditional targeting construct), we increased the homology arm in the sense primer to 50 bp, and selected a target sequence located 100 bp upstream of the mutated exon as the 5' region for homologous

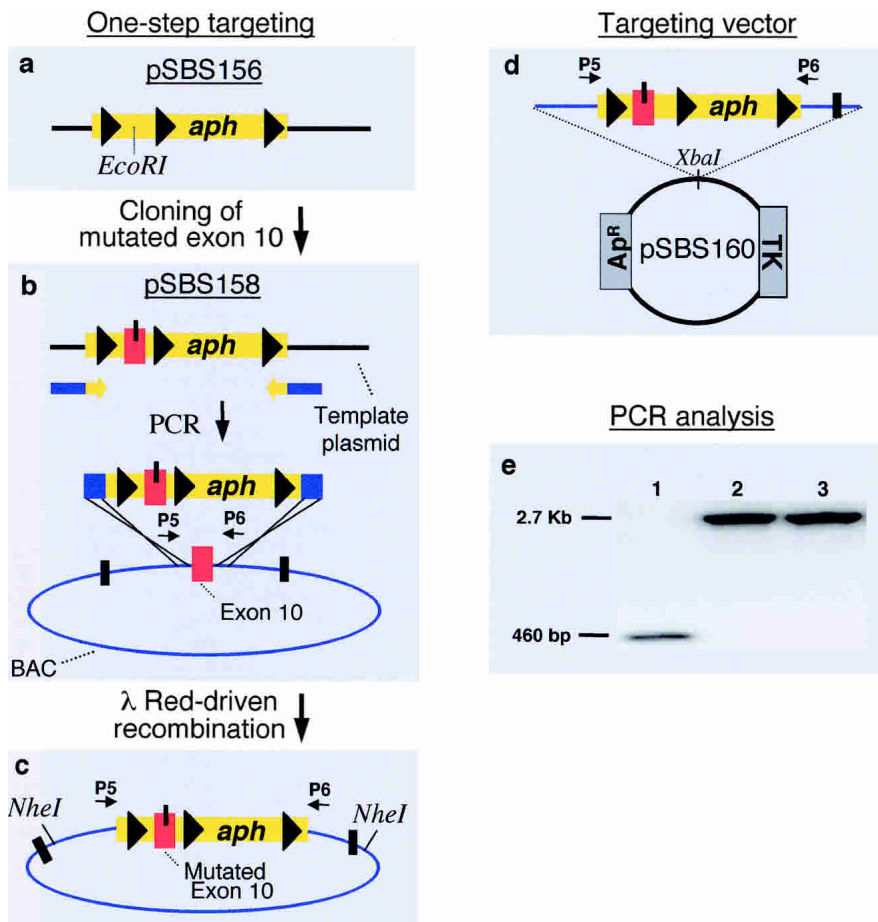


Figure 2 “Knock-in” vector construction. (a–d) Sequential steps leading to a mutated *NOD2* exon 10 “knock-in” BAC and targeting construct (see text for details); (e) Gel electrophoresis depicting PCR products confirming the recombinants schematized in diagrams: (c) lane 1 (WT-BAC), (d) lane 2 (BAC^{loxP/exon 10m/loxP/aph/loxP}), and (e) lane 3 (the targeting vector), generated with P5 and P6.

recombination. Appropriate recombinants were identified and confirmed (Fig. 2c,e). Restriction analyses of the recombinant BACs revealed the absence of unwanted internal rearrangements (data not shown). A targeting construct was generated by digesting the recombinant BAC with *NheI*, followed by subcloning into a *TK*-containing vector and selection on ampicillin/kanamycin plates (Fig. 2d).

Although not described here, construction of simple targeting vectors that aim to replace a portion of a gene with an *aph* gene cassette can be generated using pSBS150 as template. PCR primers homologous to the sequence flanking the deleted regions can be used to generate a linear fragment for homologous recombination with the BAC. It was previously shown that primers designed in this way efficiently loop out intervening sequence with replacement by the selectable marker (Zhang et al. 1998; Angrand et al. 1999; Datsenko and Wanner 2000; Yu et al. 2000).

Two groups previously described methods for the construction of targeting vectors utilizing recombinering (Angrand et al. 1999; Zhang et al. 2002). Both of these approaches required screening genomic libraries and did not utilize the availability of BACs. Here we describe a simple and rapid method of generating complex gene knockout targeting constructs using recombination engineering requiring only one or two plasmid templates (Figs. 1, 2). This approach exploits the commercial availability of mouse genomic BAC clones spanning known sequences in the mouse genome. Since they can accommodate 5–10-fold larger

inserts than bacteriophage, the use of BACs ensures that sufficient sequence is available to generate large regions of homology required for efficient homologous recombination in ES cells. Furthermore, this strategy avoids library construction and most subcloning steps that are characteristic of conditional targeting construct generation, and can be applied to the rapid generation of most standard, conditional, or knock-in targeting vectors.

While our work was being finalized for publication, another recombinering-based approach that manipulated BACs for the generation of targeting vectors was reported (Liu et al. 2003). Those authors employ gap-repair for the subcloning of a BAC fragment into a recipient plasmid, followed by further targeting of this plasmid to construct a conditional targeting vector. Although both approaches utilize recombinering and BAC DNA sequences for vector construction, there are unique and notable distinctions. Because we did not use Cre recombinase in the BAC targeting process, the presence of *loxP* sites in the BAC vectors has not posed any constraints to the insertion of further *loxP* sites for conditional targeting vector construction. On the other hand, the presence of three *loxP* sites leads to three possibilities of recombinants when the Cre recombinase is expressed in ES cells; the utilization of additional sequences (e.g., *frt* sites) which orient site-specific deletion can further simplify this process (Liu et al. 2003). In addition, in our approach, the requirement for unique restriction

sites in the BAC that are located outside the regions of homology is one potential obstacle. Although we have been able to generate three different targeting constructs using this approach without difficulty, one can circumvent this obstacle by subcloning via gap repair (Liu et al. 2003).

Most importantly, in our studies, we manipulated BACs *in situ* in the original host, which avoids numerous subcloning steps and permits additional uses of this technology. Furthermore, we utilized a plasmid, rather a prophage, to encode the lambda Red recombination system and achieved efficient recombination utilizing only two templates for all targetings and very short terminal homologies (40–50 bp). Short homologies did not pose any constraint to our targetings. We achieved a specific targeting efficiency of $65\% \pm 30\%$ for eight unique targetings within four different genes (average total number of colonies tested per targeting: 13 ± 9 ; average number of correct colonies identified per targeting: 7 ± 3). Aberrant recombination in the alternative approach employing gap repair may have resulted from short homologies between the donor sequences and repetitive sequences in the target DNA (Liu et al. 2003). It is likely that this potential problem can be avoided by surveying the BAC sequence prior to designing primers, to avoid using repetitive sequences for the site of recombination. Finally, manipulating BACs directly can permit the use of the entire BAC as a targeting construct in ES cells—avoiding constraints on the size of the homology arms.

METHODS

Construction of Template Plasmids

The template plasmids pKD4Lox, pSBS150, and pSBS158 were derived from pKD4, and each maintained in *pir*⁺ bacteria (Datsenko and Wanner 2000). The use of pKD4 as the backbone was essential to prevent replication of the template plasmid in transformed strains.

pKD4Lox was constructed by cloning a single *loxP* site upstream of the *fit*-flanked TN5-*aph* segment in pKD4. pSBS150 was constructed by cloning a floxed *aph* gene (driven by both TN5 and PGK promoters) in the pKD4 vector double-digested with *Bam*HI/*Nco*I. To allow single targeting of BACs (see below), pSBS156 was generated from pSBS150 by cloning a *loxP* site upstream of the floxed *aph* gene. pSBS158 was constructed by cloning the mutated CARD15 exon (see text) into a unique *Eco*RI site located between the *loxP* sequence and the floxed *aph* gene cassette in pSBS156 (see Fig. 2b).

Generation of BAC Targeting Constructs

PCR products for BAC targeting were generated with 60–74-bp primer pairs using Advantage 2 polymerase (Clontech). Within each primer, the first 40–54 bp (homology arm) was homologous to the BAC sequence, and the next 20 bp was complementary to the template plasmid of interest. The 60-bp sequences to prime pKD4Lox, pSBS150, and pSBS158 (and further BAC targeting) were, respectively: CCTTGTTTAACCATATCAAAGTTCCTCCG GCTGTAT TCCTGCTCTTGAGCGATTGTAGGC (forward) and GGACCCAGGGGCTTCC TAAACCTGGTCTTCCCTCG CAA ACATATGAATATCCTCCTTAG (reverse), ATT CCACCTCTGC CACCCTTCATACAACACCAGCCAGACCCCGATCATA TTCAATA ACCC (forward) and GCTATAGTTATTTGTTCT GAAACTAAGGCCAGAAAGAGGG CTATGCTACTCCCGT CAATAA (reverse), AGCCTGGCAATGAGCTGTGGCTCCT CAGCCCTTCCCTCCCTTCCCAGTGTGGGTACCGGGGG ATCCGGAAACCCTT (forward) and ATACCAAATGTTG GTCA AAAGTATCTTCAGAATTCCACCCTATG CTACTCCGTC AATAA (reverse). Each PCR product (1.6-, 2.2-, and 2.5-Kb in length for pKD4Lox, pSBS150, and pSBS158, respectively) was purified (QIAquick PCR purification kit, QIAGEN), digested with *Dpn*I (New England Biolabs), ethanol-precipitated, and resuspended in Tris-Cl buffer pH 8.5.

Two-Step BAC Gene Targeting

Bacteria carrying the target BAC and pKD46 (λ Red recombination system) were grown in 100-mL LB cultures with chloramphenicol (12.5 μ g/mL), ampicillin (100 μ g/mL), and L-arabinose (150 μ g/mL, Sigma) at 30°C to an OD₆₀₀ of ~0.6. Electrocompetent bacteria were transformed with 100 ng of the pKD4Lox-amplified and *aph* gene-containing PCR construct. Chloramphenicol-resistant (*Cm*^R)/*Kn*^R transformants were first replated to confirm adequate resistance, and then screened by PCR (using P1 and P2 primers, AGCCCTGCCCCCTT CTATTT and TCACAGCGGGACCTACACAG, respectively; see Fig. 1), and the PCR products were sequenced to confirm the desired targeting. Positive colonies were grown overnight at 42°C to cure pKD46 (confirmation was performed by testing ampicillin sensitivity). In order to remove the *aph* gene, a single *Cm*^R/*Kn*^R and ampicillin-sensitive colony was transformed with pCP20 (an Ap^R and temperature-sensitive replicon containing the thermally-induced *flp* recombinase gene; Cherepanov and Wackernagel 1995) and grown overnight at 30°C on chloramphenicol/ampicillin plates. Some colonies were selected again in chloramphenicol plates at 43°C, and then tested for kanamycin sensitivity (i.e., loss of the *aph* gene) and loss of ampicillin resistance (i.e., the curing of pCP20).

A single *Cm*^R and kanamycin-sensitive colony was transformed with pKD46. Chloramphenicol/ampicillin selected transformants were then transformed with 100 ng of the pSBS150-

amplified (containing the floxed *aph* gene) PCR product. As detailed above, to confirm the desired targeting, *Cm*^R/*Kn*^R transformants were first screened by PCR and sequencing (using P3 and P4 primers, TGGGCCTGTGACAAATGCTACTG and GT-TGTGGGGATGGAT GGGGTTATT, respectively; see Fig. 1), and then cured of pKD46.

Mutation of the Target Exon

PCR amplification of *mNOD2/CARD15* exon 10 was performed in the BAC clone RP23–447G4, and the product was subcloned in PCR4-TOPO (TOPO TA cloning kit, Invitrogen). A mutation analogous to the 3020incC frameshift mutation in *NOD2/CARD15* found in Crohn's disease patients (Hugot et al. 2001; Ogura et al. 2001) was introduced into this subclone, using site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene).

One-Step BAC Gene Targeting

pSBS158 was used as the template for the one-step BAC targeting in *E. coli*. This targeting used the same BAC clone and was performed as described above for the two-step targeting protocol. Confirmation of the desired recombinant BAC was performed by PCR screening and sequencing (using primers P5 and P6, TGGGCCTGTGACAAATGCTACTG and GTTGTGGGGATG-GATGGGGTTATT, respectively; see Fig. 2).

Generation of the Targeting Vector

In both targeting strategies, the desired recombinant BAC DNA (~50 μ g) was digested with a suitable restriction enzyme in order to achieve homology arms with appropriate size for further homologous recombination in ES cells. Following digestion, BAC DNA was shotgun-cloned into a linearized pBluescript-derived *TK*-containing vector, and the desired transformants were selected by growth on ampicillin/kanamycin plates.

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