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Simple and highly efficient BAC recombineering using *galK* selection

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ABSTRACT

Recombineering allows DNA cloned in *Escherichia coli* to be modified via lambda (λ) Red-mediated homologous recombination, obviating the need for restriction enzymes and DNA ligases to modify DNA. Here, we describe the construction of three new recombineering strains (SW102, SW105 and SW106) that allow bacterial artificial chromosomes (BACs) to be modified using *galK* positive/negative selection. This two-step selection procedure allows DNA to be modified without introducing an unwanted selectable marker at the modification site. All three strains contain an otherwise complete galactose operon, except for a precise deletion of the *galK* gene, and a defective temperature-sensitive λ prophage that makes recombineering possible. SW105 and SW106 cells in addition carry L-arabinose-inducible *Cre* or *Flp* genes, respectively. The *galK* function can be selected both for and against. This feature greatly reduces the background seen in other negative-selection schemes, and *galK* selection is considerably more efficient than other related selection methods published. We also show how *galK* selection can be used to rapidly introduce point mutations, deletions and *loxP* sites into BAC DNA and thus facilitate functional studies of SNP and/or disease-causing point mutations, the identification of long-range regulatory elements and the construction of conditional targeting vectors.

INTRODUCTION

Bacterial artificial chromosomes (BACs) have become the DNA of choice for genomic sequencing due to their high stability and large insert size (100–300 kb) (1). BACs are also being used more and more for making transgenic mice, since, in many cases, all of the important regulatory sequences required for normal gene expression can be found on a single BAC (2,3). Many laboratories also use BACs as the starting point for making gene-targeting constructs for manipulating mouse genes using ES cell technology (knock-outs, knock-ins and conditional targeting using Cre/*loxP*) (4,5).

Recombineering (recombination-mediated genetic engineering) makes it possible to modify BAC DNA via homologous recombination [reviewed in (6,7)]. Recombineering is made possible through the use of three λ Red-encoded genes: *exo*, *bet* and *gam*. *exo* encodes a 5′–3′ exonuclease that produces 3′ overhangs from introduced double-stranded DNA targeting cassettes (dsDNA). *bet* encodes a pairing protein that binds to the 3′ overhangs and mediates its annealing and homologous recombination with complementary DNA present on the BAC. At the same time, *gam* encodes an inhibitor of the *Escherichia coli* RecBCD exonuclease and thereby protects the linear DNA-targeting cassette from degradation by RecBCD. λ Red (or the corresponding *RecE* and *RecT* genes of the prophage Rac) can be expressed from a multicopy plasmid using an inducible promoter (8,9). Alternatively, these genes can be expressed from a stably integrated defective λ prophage, where *exo*, *bet* and *gam* are controlled by the strong phage promoter *p*L, under stringent control of the temperature-sensitive repressor, *cI857* (10,11). In the prophage system, *exo*, *bet* and *gam* are not expressed when the bacteria are kept at 32°C. By shifting the bacteria to 42°C for as little as 15 min, the genes are rapidly induced to very high levels and homologous recombination is very efficient.

Using recombineering, one can easily subclone a genomic fragment from a BAC by gap repair, either for use as a transgene directly or for subsequent manipulation to make a gene-targeting construct. The introduction of selectable markers into a BAC is also very easy using recombineering. However, a major limitation to the usefulness of BACs is the ease and efficiency with which one can make subtle and 'seamless'

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mutations like point mutations and clean deletions or introduce in-frame fusions of cDNAs or epitope tags without leaving at the same time a selectable marker or a *loxP*/*Frt* site at the modification site. A handful of methods for making such mutations, all based on homologous recombination in *E.coli*, have been developed. One method is *RecA* dependent and relies on the use of a shuttle vector and two recombination steps: integration followed by the resolution of the co-integrate (2,12). This method requires considerable up-front effort using traditional cloning with restriction enzymes and ligation. A simpler and more widely used method is based on positive/negative selection using e.g. a *sacB–neo* fusion gene (8). *neo* (kanamycin) resistance is used for positive selection while sucrose toxicity resulting from *sacB* expression is used for negative selection. A major drawback of this selection system is that spontaneous point mutations in the *sacB* portion of the *sacB-neo* fusion gene can occur without influencing the bacteria's ability to grow on kanamycin. These *sacB* mutants significantly increase the background after negative selection. A related method is based on counterselection using a recognition site for a rare restriction enzyme like I-SceI (13). For this method to work efficiently, the I-SceI restriction enzyme has to be induced *in trans*, and since there is no selection for maintaining the correct recognition sequence for this enzyme, the background due to point mutation or deletion of the restriction site is high. A method for BAC modification without selection has also been developed (14). Although relatively efficient, this method relies on a PCR-based screening of the resulting colonies to identify the desired clones. Since there is no selection step, the number of manipulations made possible by this selection procedure is more limited.

Here, we report the development of a novel and highly efficient *galK*-based positive/negative selection system for the manipulation of BACs. The *E.coli* galactose operon consists of four genes: *galE*, *galT*, *galK* and *galM*, which are necessary for growth and utilization of galactose as the only carbon source. The *galK* gene product, galactokinase, catalyzes the first step in the galactose degradation pathway, phosphorylating galactose to galactose-1-phosphate. Galactokinase also efficiently catalyzes the phosphorylation of a galactose analog, 2-deoxy-galactose (DOG). The product of this reaction cannot be further metabolized, leading to a toxic build-up of 2-deoxy-galactose-1-phosphate (15). Thus, both positive and negative selection can be conferred by *galK*. Because *galK* is used for both selection steps, background following negative selection is reduced and no colony screening is necessary. The small size of the *galK* cassette (around 1200 bp plus homology arms) also makes it easier to amplify by PCR and to introduce into bacteria using electroporation.

MATERIALS AND METHODS

General recombineering and *galK* selection

Recombineering was performed as follows: 500 µl of an overnight culture was diluted in 25 ml Luria–Bertani (LB) medium with or without chloramphenicol selection (12.5 µg/ml) in a 50 ml baffled conical flask and grown at 32°C in a shaking waterbath to an OD_{600} of 0.6. Then, 10 ml was transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for exactly 15 min in a shaking waterbath. The remaining culture was left at 32°C as the uninduced control. After 15 min the two samples, induced and uninduced, were briefly cooled in an ice/waterbath slurry and then transferred to two 15 ml Falcon tubes (BD Biosciences) and pelleted using 5000 r.p.m. (eppendorf centrifuge 5804R, rotor A-4-44) at 0°C for 5 min. The supernatant was poured off and the pellet was resuspended in 1 ml ice-cold ddH_2O by gently swirling the tubes in an ice/waterbath slurry. Subsequently, 9 ml ice-cold ddH_2O was added and the samples pelleted again. This step was repeated once more. After the second washing and centrifugation step, the supernatant was removed, and the pellet (~50 µl each) was kept on ice until electroporated with PCR product, gel-purified fragment or double-stranded oligo. An aliquot of 25 µl was used for each electroporation in a 0.1 cm cuvette (BioRad) at 25 µF, 1.75 kV and 200 Ω. After electroporation the bacteria were recovered in 1 ml LB (15 ml Falcon tube) for 1 h in a 32°C shaking waterbath. For the counterselection step (see below), the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32°C shaking waterbath. After the recovery period, the bacteria were washed twice in 1× M9 salts as follows: 1 ml culture was pelleted in an eppendorf tube at 13 200 r.p.m. for 15 s and the supernatant was removed with a pipette. The pellet was resuspended in 1 ml of 1× M9 salts, and pelleted again. This washing step was repeated once more. After the second wash, the supernatant was removed and the pellet was resuspended in 1 ml of 1× M9 salts before plating serial dilutions (100 µl, 100 µl each of 1:10, 1:100 and 1:1000 dilutions) on minimal medium plates (see below). Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal medium. The uninduced samples routinely had a higher degree of lysis/bacterial death after electroporation, so the uninduced samples were diluted in 0.25–0.75 ml 1× M9 salts in the final step to make up for the difference. Detailed protocols for recombineering can also be found at our website (http://recombineering.ncifcrf.gov).

Minimal media and indicator plates

Minimal media and the indicator plates were prepared using standard methods (16). We added supplements as indicated.

Washing solution: 1× M9 medium.
Gal positive selection: M63 + agar (15 g/l; Difco, BD Biosciences) + D-galactose (0.2%; Sigma) + D-biotin (1 mg/l; Sigma) + L-leucine (45 mg/l; Sigma) and ± chloramphenicol (12.5 µg/ml; Sigma).
Gal counterselection: M63 + agar + glycerol (0.2%; Fischer) + D-biotin (1 mg/l) + L-leucine (45 mg/l) + DOG (0.2%; Ferro Pfanstiehl) and ± chloramphenicol (12.5 µg/ml).
Gal indicator plates: MacConkey agar (Difco, BD Biosciences) + D-galactose (1%) and ± chloramphenicol (12.5 µg/ml). Plates were prepared using manufacturer's instructions.

Bacterial strains

The strains used in this paper are listed in Table 1. SW101 was derived from DY380 by a homologous recombinational exchange of the mutated *gal* operon leader sequence with a

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Table 1. Recombineering reagents used in this work

	Genotype	Phenotype	Reference
Strains			
W3110		Gal^+	(17)
DH10B	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*lacX74 deoR endA1 araD139* Δ(*ara, leu*) *7697 rpsL recA1 nupG* ϕ80d*lacZ*Δ*M15 galU galK*	Gal^-	(18)
DY380	DH10B [λ*c*1857 (*cro-bioA*)<>*Tet*] *galK*$^+$ *gal490*	Gal^-	(11)
SW101	DY380 *gal*$^+$	Gal^+	This work
SW102	SW101 Δ*galK*	Gal^-	This work
EL250	DY380 (*cro-bioA*)<>araC-P_{BAD} *Flpe*	Gal^-	(11)
EL350	DY380 (*cro-bioA*)<>*araC*-P_{BAD} *Cre*	Gal^-	(11)
SW103	EL250 *gal*$^+$	Gal^+	This work
SW104	EL350 *gal*$^+$	Gal^+	This work
SW105	SW103 Δ*galK*	Gal^-	This work
SW106	SW104 Δ*galK*	Gal^-	This work
Selection cassette			
p*galK*	*galK* wild-type gene driven by the *em 7* promoter		This work

<> indicates the result of a homologous recombination event. Gal^+: the ability to use galactose as the sole carbon source. Gal^-: resistance to DOG and lack of the ability to grow on galactose as the sole carbon source.

441 bp PCR product (Expand High-Fidelity PCR System; Roche Applied Science) containing the wild-type *gal* operon leader sequence, made using W3110 (17) bacteria as the template. The primers used to amplify the wild-type leader were (Integrated DNA Technologies, Inc.): F 5′-CGACGCATG-CAGGCATGAA-3′ and R 5′-AGTGGATCACGGTGTC-GATA-3′ and the PCR conditions were: 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, for 35 cycles. The PCR product was gel purified (GFX kit; Amersham Biosciences) and eluted in 50 µl ddH_2O. An aliquot of 2.5 µl was used in the recombineering experiment as described above and the bacteria were plated on M63 minimal medium + galactose + leucine + biotin. The plates were incubated at 32°C for 2–3 days. A few of the many resulting colonies were streaked for single colonies on indicator plates and a single dark red Gal^+ colony was used in the next step. SW102 was derived from SW101 in the following way: two homology arms flanking the *galK* open reading frame (ORF) were PCR amplified using SW101 bacteria as template and the following primers (Integrated DNA Technologies, Inc.); recognition sites for restriction enzymes are underlined: 5′arm F: 5′-AAATAACTCGAGCAGCTGC-ACGCGCACTTT-3′; 5′arm R: 5′-AAATAAGAATTCTTCT-TACACTCCGGATTCGC-3′; 3′arm F: 5′-AAATAAGAAT-TCTGTAAACCATCACAAGGAGCAG-3′; 3′arm R: 5′-AA-TAAAGCGGCCGCCAGCTGGTTAACGCCCTGA-3′. PCR conditions were 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, for 35 cycles. The 5′ homology arm (171 bp) was digested with XhoI and EcoRI, and the 3′ arm (335 bp) with EcoRI and NotI. The digested PCR products were gel purified and triple-ligated into a vector digested with XhoI and NotI. The targeting cassette was then released from the backbone using XhoI and NotI digestion, followed by gel purification. The cassette was eluted in 50 µl ddH_2O, and 2.5 µl was used for recombineering as described above. After 4.5 h of outgrowth in LB media and two washes in 1× M9 salts, serial dilutions of the bacteria were plated on minimal plates containing glycerol as carbon source, leucine and biotin, plus DOG for selection against *galK*. The plates were incubated for 3 days at 32°C. A few of the many resulting colonies were streaked for single white/colorless (Gal^-) colonies on indicator plates followed by PCR analysis and sequencing to check for correct deletion of the *galK* gene. A single, verified colony was expanded and a glycerol stock was made and used for initiation of all subsequent experiments. SW103 and SW104 were derived from EL250 and EL350, respectively, using the same method as for the derivation of SW101. SW105 and SW106 were derived from SW103 and SW104, respectively, using the same method as for the derivation of SW102 from SW101. SW105 expresses *Flp* under the control of an arabinose-inducible promoter and SW106 expresses *Cre* under the same promoter.

Construction of p*galK*

Two rounds of PCR were performed to construct a *galK* ORF driven by the prokaryotic em7 promoter. The first round was performed using *galK* ORF 1st F and *galK* ORF R primers using W3110 bacteria (17) as template. The resulting PCR product was then used as template for a second round of PCR using *galK* ORF 2nd F and *galK* ORF R primers. The product from the second round of PCR was gel purified and digested with EcoRI and BamHI and cloned into EcoRI and BamHI digested pBluescript SK^-. p*galK* was verified by sequencing [the plasmid sequence is available from our website (http://recombineering.ncifcrf.gov)].The primer sequences were (restriction sites and the *galK* ATG are underlined and the *em7* promoter is in italics): *galK* ORF 1st F: 5′-*CCCAGGAGGCAGATC*ATGAGTCTGAAAGAAAAAA-CTGAAAGAAAAAACACAATCTCTGT-3′; *galK* ORF 2nd F: AATAAAGAATTCCT*GTTGACAATTAATCATCG-GCATAGTATATCGGCATAGTATAATACGACAAGGTGA-GGAACTAAACCCAGGAGGCAGATC*ATG; *galK* ORF R: AATAAAGGATCCTCAGCACTGTCCTGCTCCTT-3′. PCR conditions for both rounds were 94°C for 15 s, 60°C for 30 s and 72°C for 1.5 min, for 30 cycles. Primers were from Invitrogen.

PCR amplification of the *galK* targeting cassette

Sequences of all *galK* primers (Invitrogen) used in the experiments described in this paper are given below. *em7-galK* was PCR amplified using 1 ng p*galK* as template and the following

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conditions: 94°C for 15 s, 60°C for 30 s and 72°C for 1 min, for 30 cycles. After completion of PCR, 2 µl DpnI was added to each 25 µl reaction and incubated for 2 h at 37°C to remove any plasmid template. The DpnI-digested reaction mix was run on a 1% agarose gel over night, and the PCR product was purified and eluted in 50 µl ddH_2O. An aliquot of 2.5 µl was used for each experiment. For making the *Nras* G12D substitution the following primers were used for the first step, introducing *galK*. Homology to *Nras* sequence is in italics and the sequence recognizing *em7-galK* is underlined: *Nras galK* F: 5′-*TTTTTGCTGGTGTGAAATGACTGAGTACAAACTGGTGGTGGTTGGAGCAG*CCTGTTGACAATTAATCATCCGCA-3′; *Nras galK* R: 5′-*CAAAGTGGTTCTGGATTAGCTGGATCGTCAAGGCGCTTTTCCCAACACCA*TCAGCACTGTCCTGCTCCTT-3′. For making 50, 75 and 100 kb deletions in the RP23-341F12 BAC, the following primers were used (homology to BAC sequence is in italics and the sequence recognizing *em7-galK* is underlined): *galK* F 341F12 start: 5′-*ACTCCCACTGGAAGCTTTTTACAAAACATGTGTTGCTGACATGTTGACAG*CCTGTTGACAATTAATCATCGGCA-3′; *galK* R 341F12 50 kb: 5′-*ACCCAAACCAAACAACATCCAAACCAAAAACACAGACAAAACCAAATATG*TCAGCACTGTCCTGCTCCTT-3′; *galK* R 341F12 75 kb: 5′-*ACACTAAGCCAAACTCCTTGCCTGGGCTATTTCTCTTTGTTTTTCCAAAT*TCAGCACTGTCCTGCTCCTT-3′; *galK* R 341F12 100 kb: 5′-*TATGTGTCTGTGTGTGTATGTACAGTTCTTTGTTTTTGTTTTTTTTCTTTT*TCAGCACTGTCCTGCTCCTT-3′. For insertion of a *loxP511* site in the RP23-341F12 BAC, the following primers were used for the first step (introduction of *galK*). Homology to BAC sequence is in italics and the sequence recognizing *em7-galK* is underlined: 95 kb *loxP511 galK* F: 5′-*GGACAGAGGCGTGACAGACGTGTGAGCTCCGTGGACAACTCTCCCCGAAG*CCTGTTGACAATTAATCATCGGCA-3′; 95 kb *loxP511 galK* R: 5′-*GACTCTGAGCAGCAACGGCTGAGCCTCACTTGAGAGGGTCCCTGAGTCAC*TCAGCACTGTCCTGCTCCTT-3′.

Oligos for recombineering

Oligos used to replace the *galK*-targeting cassette were obtained from Invitrogen. dsDNA was used and the oligos (sense and antisense) annealed *in vitro*: 10 µg of each oligo (sense and antisense) was mixed in an eppendorf tube in a total volume of 100 µl of 1× PCR buffer (Expand High Fidelity PCR kit, Roche Applied Science) and boiled for 5 min, allowed to cool to room temperature for 30 min, ethanol precipitated and resuspended in 100 µl ddH_2O to a final concentration of 200 ng/µl double-stranded oligo. An aliquot of 1 µl (200 ng) was used in the recombineering experiments. To introduce the G12D (G<>A) point mutation in the *Nras* BAC CITB 50J2, the following oligos were used for the second step (the introduced adenosine/thymidine base pair is underlined, the flanking sequences are homologous to the *Nras* BAC sequence): G12D S: 5′-TTTTTGCTGGTGTGAAATGACTGAGTACAAACTGGTGGTGGTTGGAGCAGATGGTGTTGGGAAAAGCGCCTTGACGATCCAGCTAATCCAGAACCACTTT-3′; G12D AS: 5′-AAAGTGGTTCTGGATTAGCTGGATCGTCAAGGCGCTTTTCCCAACACCATCTGCTCCAACCACCACCAGTTTGTACTCAGTCATTTCACACCAGCAAAAA-3′. To introduce a *loxP511* site in the RP23-341F12 BAC, the following oligos were used for the second step (*loxP511* is underlined, the flanking sequences are homologous to the BAC sequence around position 95 kb): 95 kb *loxP511* S: 5′-ACGTGTGAGCTCCGTGGACAACTCTCCCCGAAGATAACTTCGTATAGTATACATTATACGAAGTTATGTGACTCAGGGACCCTCTCAAGTGAGGCTCAGC-3′; 95 kb *loxP511* AS: 5′-GCTGAGCCTCACTTGAGAGGGTCCCTGAGTCACATAACTTCGTATAATGTATACTATACGAAGTTATCTTCGGGGAGAGTTGTCCACGGAGCTCACACGT-3′.

Verification of positive recombinants

In the G12D *Nras* experiment, the selected Gal⁻ clones were analyzed by SpeI digestion of BAC miniprep DNA using unmodified CITB 50J2 BAC DNA as a control. Clones without rearrangements were analyzed by PCR using 1 µl BAC miniprep DNA as the template. The PCR products were gel purified and sequenced using the same primers as were used for PCR. Primers flanking the targeted mutation were: *Nras* test F: 5′-CACTCATCTGCAAGGAATGCT-3′; *Nras* test R: 5′-CCTCAGTAAGCACGAACTTGT-3′. PCR conditions were 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, for 30 cycles. Modifications of the RP23-341F12 BAC (50, 75 and 100 kb deletions and the introduction of a *loxP511* site) were tested by SpeI restriction analysis of BAC miniprep DNA and compared with unmodified 341F12 BAC DNA. In the *loxP511* experiment, clones 3, 5 and 6 were further tested for correct insertion of the *loxP511* site by transforming 1 µl of BAC miniprep DNA into electrocompetent and arabinose-induced EL350 cells (11) and plating on LB plates with chloramphenicol. Two colonies from each starting clone were tested by SpeI digestion of BAC miniprep DNA for the 95 kb Cre-mediated deletion. Finally, the Cre-recombined clones were tested by PCR with one primer mapping to the end of the pBACe3.6 BAC backbone and the other mapping to a position 95 kb away on the wild-type BAC. The primers (Invitrogen) used for this analysis were: 95 kb *loxP511* check F: 5′-GCGGATGAATGGCAGAAATTC-3′; 95 kb *LoxP511* check R: 5′-TTTGCCAGACTGGTGCCTAA-3′. PCR conditions were 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, for 30 cycles. The resulting PCR bands were gel purified and confirmed by sequencing using the same primers as were used for the PCR amplification. The follow-up experiment for testing the source of the observed BAC deletions was done as described above.

BACs

Mouse BACs were obtained from Invitrogen. The RP23-341F12 BAC (C57BL/6 DNA) was chosen based on position within the mouse *Ebfaz* gene using the UCSC genome browser (http://genome.ucsc.edu/). The CITB 50J2 BAC (CJ7/129/SV DNA) was identified by screening a CITB mouse BAC library (Invitrogen) with an *Nras* genomic probe using standard hybridization methods. The BAC clones were streaked for single colonies and characterized by PCR and restriction analysis before proceeding to the recombineering experiments. Two methods were used for DNA preparation. For BAC minipreps (1–1.5 µg), we used the following protocol: 5 ml overnight LB culture (15 ml Falcon tube) was pelleted for 5 min at 5000 r.p.m., the supernatant removed and the pellet was dissolved in 250 µl buffer P1 (miniprep kit, Qiagen) and

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transferred to an eppendorf tube. An aliquot of 250 µl P2 buffer was added followed by mixing by inversion and incubation for <5 min at room temperature. An aliquot of 250 µl N3 buffer was added followed by mixing and incubation on ice for 5 min. The supernatant was cleared by two rounds of centrifugation at 13 200 r.p.m. for 5 min in a tabletop centrifuge. Each time the supernatant was transferred to a new tube. DNA was precipitated by adding 750 µl isopropanol, mixing and incubating on ice for 10 min, and centrifugation for 10 min at 13 200 r.p.m. The pellet was washed once in 70% ethanol and the dry pellet was dissolved in 50 µl TE. An aliquot of 40 µl (~1 µg) was used for restriction analysis, and 1 µl was used as template for PCR analysis or transformation of electrocompetent bacteria. Large-scale preparations of BAC DNA (25–100 µg) were done using the Nucleobond BAC maxi kit from Clontech (BD Biosciences) following the manufacturer's protocol.

RESULTS

Generation of SW102 cells

To see whether we could develop a more efficient selective system for BAC recombineering, we looked for a single selectable marker that could be used for both positive and negative selection. We focused on *E.coli galK* because both selection steps could be done using *galK* and its small size makes it easy to amplify by PCR. We previously reported the development of a bacterial strain, DY380 that is readily transformable with BAC DNA due to its DH10B origin (1). DY380 cells also harbor the defective λ prophage required for recombineering. The defective prophage in DY380 was transferred into DH10B with a P1 phage lysate obtained from DY363 cells [for details, see (11)]. DY363 cells carry a 1200 bp IS2 insertion element, *gal490*, in the mRNA leader sequence of the galactose operon, preventing *gal* gene transcription. Since the galactose operon is directly proximal to the site of insertion of the defective prophage, the *gal490* mutation was also transferred to DY380 during P1 transduction. Thus, DY380 is phenotypically Gal^- (galactose minus) and therefore unable to grow on galactose minimal medium.

Recombineering was used to correct this problem. From the wild-type *E.coli* strain, W3110 (17), we PCR amplified a 441 bp fragment from the wild-type *gal* promoter, which spans the region containing the IS2 insertion element in DY380. This PCR product was introduced into DY380 and Gal^+ recombinants were selected for growth on galactose minimal medium (see Materials and Methods). We named this strain SW101 (Table 1). This strain is identical to DY380 except that it lacks the IS2 insertion element and has been made wild type for the *gal* operon. We then made a precise deletion of the *galK* gene, leaving all other genes in the galactose operon intact. This was achieved by PCR amplifying two homology arms of 171 and 335 bp, respectively, flanking the *galK* ORF and cloning them together into a plasmid using three-way ligation. Since the ORF of *galK* overlaps that of *galM*, we left behind the last 33 bp of *galK* (Figure 1) to allow for proper translation of *galM*. The targeting cassette, consisting of a 512 bp linear fragment, was electroporated into heat-induced and electrocompetent SW101 cells, and the recombinant clones selected on minimal medium containing glycerol as the carbon source and DOG for selection against *galK*. The genotype of the resulting strain, SW102 (Table 1), was confirmed by PCR analysis and sequencing of the modified region (Figure 1). This strain now harbors not only the defective λ prophage, but also a functional *gal* operon, except for the deletion of *galK*. The λ prophage is located between the galactose operon and the biotin operon, and the *bio* operon in these cells is nonfunctional, causing a biotin requirement. DH10B, and all derived strains, including SW102, are also deficient in leucine metabolism (18). When using minimal medium, one therefore needs to add both biotin and leucine to the plates to allow SW102 growth (see Materials and Methods).

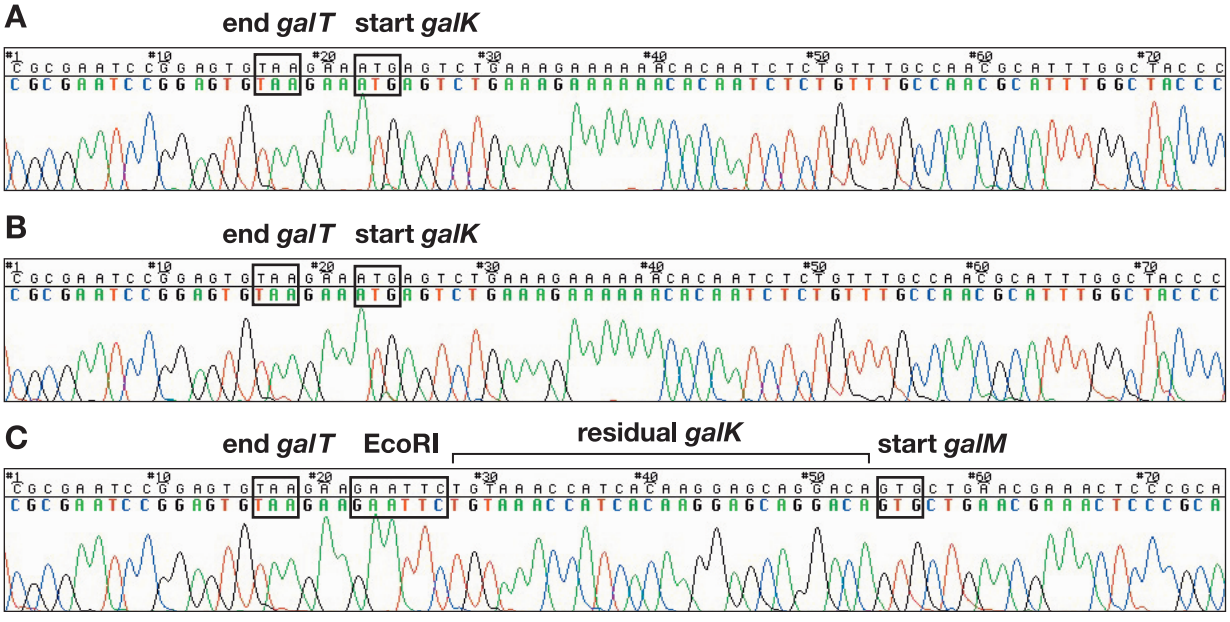

Figure 1. Sequence analysis of the galactose operon in strains DY380 (**A**), SW101 (**B**) and SW102 (**C**). In SW102, the ORF of *galK* was deleted, leaving only 33 bp of *galK* behind to make sure that translation of *galM* is initiated properly. EcoRI: the restriction site used to clone the 5′ and 3′ homology arms flanking *galK*.

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galK expression cassette

Having produced a bacterial strain, SW102, which is *galK* defective, the next step was to make a *galK* expression cassette that could be used to restore the bacteria's ability to grow on galactose by providing *galK in trans*. This was achieved by PCR amplification of the wild-type *galK* ORF from W3110 cells. We then added a minimal bacterial promoter, em7, using a two-step PCR approach (see Materials and Methods), and cloned the expression cassette into pBluescript SK$^-$. We call this plasmid p*galK* (Table 1). The constitutively active *galK* expression cassette can easily be amplified by PCR with homology arms added to the primers (see Materials and Methods).

Making a single base pair substitution

The general scheme for making mutations in BACs using *galK* selection is depicted in Figure 2. To test the *galK* selection system for BAC recombineering, we decided to introduce a point mutation into a BAC containing the murine *Nras* gene (CITB clone 50J2). The sequence of the glycine-coding codon 12 is GGT. By changing this codon to GAT we would obtain the desired mutation, G12D. In order to introduce this mutation into the BAC, we first amplified the *galK* expression cassette by PCR using primers with 50 bp of homology to either side of the second position of the GGT codon. Following homologous recombination, this targeting would introduce a 1 bp deletion into codon 12 in addition to inserting the *galK* selection cassette. Instead of deleting the basepair, the *galK* cassette could have been inserted right next to the basepair instead. SW102 cells containing the 50J2 BAC were heat-induced and made electrocompetent, and then electroporated with the *galK* cassette. Gal$^+$ recombinant colonies were selected for growth on galactose minimal medium with chloramphenicol to maintain the BAC. Bacteria grow more slowly on minimal media than on rich media, and we generally pick colonies after 2–3 days. For this first step, we do not expect any background colonies on the non-induced control plates if the p*galK* plasmid is properly eliminated (see Materials and Methods). To purify the Gal$^+$ colonies, we streaked a few colonies on MacConkey galactose indicator plates to obtain single, bright pink/red Gal$^+$ colonies. One of these single-cloned colonies was picked to initiate a culture for the next step, counterselection. We find that there is no need to analyze the Gal$^+$ colonies further, before proceeding to counterselection.

A 100 bp dsDNA oligo was then prepared by annealing two complementary oligos having 49 and 50 bp homology, respectively, to either side of the desired mutation, a single A/T bp. An aliquot of 200 ng of this oligo was then electroporated into heat-induced and electrocompetent SW102 Gal$^+$ cells containing the *galK* modified 50J2 BAC. After electroporation, the bacteria were allowed 4.5 h outgrowth in a 32°C shaking waterbath. The bacteria were then washed in M9 salts to remove any rich medium, and plated on minimal medium with glycerol, DOG and chloramphenicol. The 4.5 h outgrowth is necessary to obtain complete segregation of the recombinant BACs containing the mutation. After 3 days, we obtained colonies with a ratio of 10–100:1 when comparing plates with heat-induced to non-induced bacteria. We picked 12 colonies from the heat-induced plates for BAC minipreps,

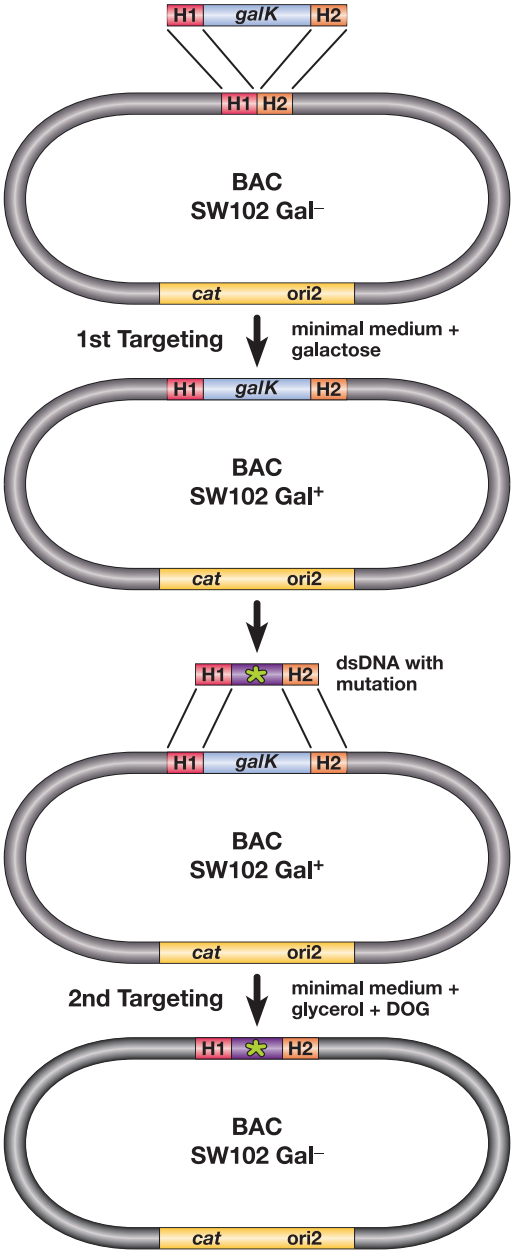

Figure 2. Overview of the *galK* selection scheme. The result of the first targeting event is the insertion of constitutively active *galK* into a defined position on the BAC by selection on minimal medium containing galactose and chloramphenicol to select for the maintenance of the BAC. The bacteria are now phenotypically Gal$^+$. Next, the *galK* cassette is replaced by a dsDNA oligo, a PCR product, or a cloned dsDNA fragment carrying a desired mutation (indicated by a star) and flanked by the same homology arms used in the first selection step. This is achieved by negative selection using minimal medium containing 2-deoxy-galactose (DOG) with glycerol as the sole carbon source. The bacteria become phenotypically Gal$^-$. H1 and H2, homology arms 1 and 2, respectively; *cat*, chloramphenicol acetyl transferase gene; ori2, BAC origin of replication; *galK*, *E.coli* galactokinase gene driven by a minimal promoter.

followed by SpeI restriction analysis (Figure 3A). Ten out of the twelve clones had the same restriction pattern as the unmodified 50J2 BAC DNA, suggesting that the desired replacement of the *galK* gene by the point mutation had occurred. This was confirmed by PCR amplification followed

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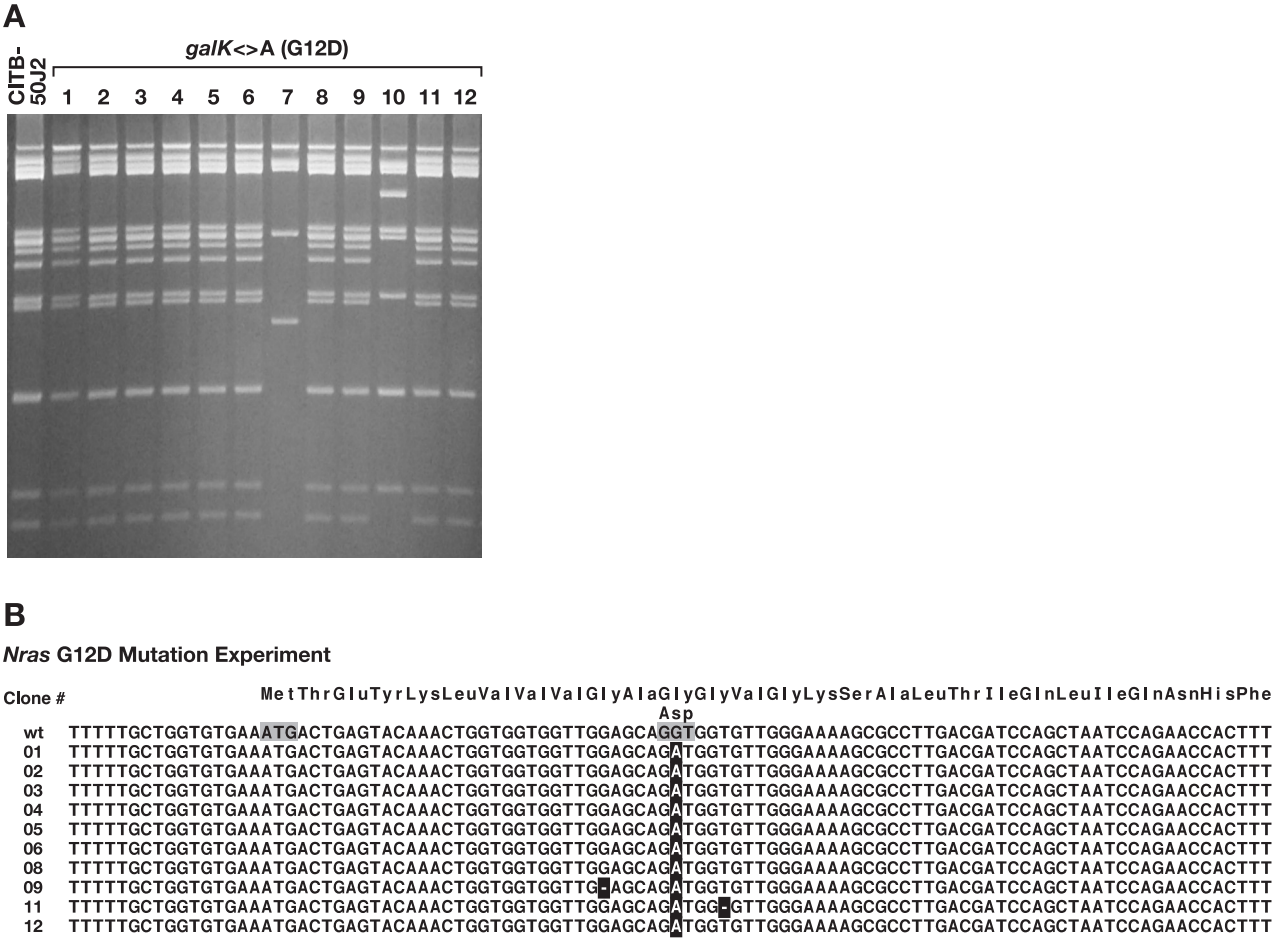

Figure 3. Introduction of a G12D mutation in the *Nras* gene. (**A**) SpeI restriction analysis of BAC miniprep DNA. First lane is the unmodified CITB-50J2 *Nras* BAC. Lanes 1–12 show digestion patterns of 12 clones counterselected for the substitution of *galK* with an oligo containing the G→A substitution for the second position of codon 12 of *Nras*. Clones 7 and 10 had internal deletions, indicating that DOG resistance was achieved by spontaneous deletion and not homologous recombination. These two clones were not analyzed further. (**B**) Sequence analysis of a PCR product spanning the modified region from clones 1–6, 8–9 and 11–12. All clones had the intended substitution (highlighted). However, clones 9 and 11 also had an internal basepair deletion indicated by a minus (highlighted). The *Nras* ATG and codon 12 are indicated (shadow).

by sequencing of the modified region of the 10 BAC clones (Figure 3B). All 10 sequenced clones had the desired point mutation. The effective recombination efficiency in this experiment, however, was 8/12 (67%) since 2 of the 10 clones also had an additional single base pair deletion (clones 9 and 11). These deletions probably occurred during oligo synthesis, since we did not purify the oligos beyond desalting (19). Because of the high efficiency in this system, there was no need to pre-screen the selected colonies prior to picking for minipreps.

Large BAC deletions

A drawback of using BACs for the production of transgenic mice is the frequent presence of other genes on the BAC in addition to the gene of interest. This is especially a problem for BAC complementation used in positional cloning, where a BAC is tested for its ability to rescue a loss-of-function mutant phenotype by making BAC transgenic mice. If complementation is achieved, it is impossible to know which gene on the BAC is responsible for the rescue. Therefore, we decided to see whether *galK* selection could be used to make large, specific and clean deletions in BACs so as to remove unwanted genes (a process called BAC trimming). As a model system, we chose a mouse BAC from the C57BL/6-derived library RP23, RP23-341F12, since we could obtain the BAC sequence directly from the UCSC genomic web browser (see Materials and Methods). We then used PCR to amplify the *galK* selection cassette with primers containing homology to the BAC. The forward primer contained 50 bp of homology to the very 5′ end of the BAC insert, and this primer was combined with three different reverse primers, having 50 bp of homology to positions 50, 75 and 100 kb away from the forward primer's homology (Figure 4A). SW102 bacteria containing the 341F12 BAC were heat-induced and made electrocompetent, and then transformed with the three different selection cassettes, followed by the selection on minimal medium with galactose. From the many resulting Gal^+ colonies, we picked four from each experiment and then did a SpeI restriction analysis of the BAC miniprep DNAs from them (Figure 4B). All four colonies from each experiment had the desired deletion (50, 75 and 100 kb, respectively). The *galK* selection cassette can then be

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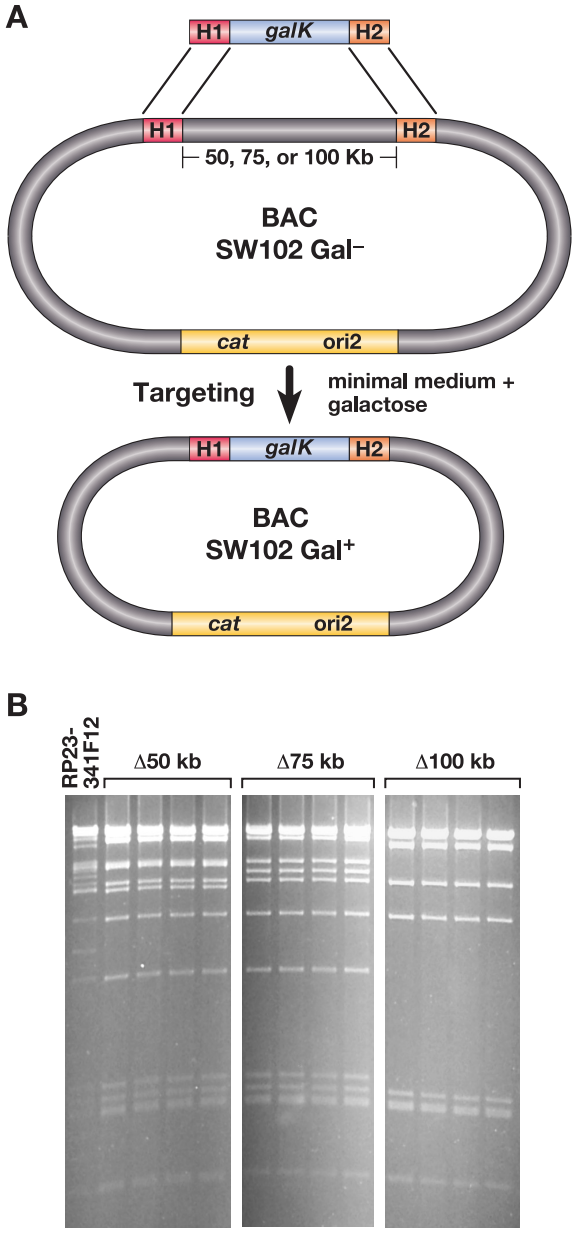

Figure 4. BAC trimming using *galK* selection. (**A**) Illustration of the design of the deletion experiment. Homology arm 1 (H1) was held constant, and H2 was separated from H1 by either 50, 75 or 100 kb. (**B**) SpeI restriction analysis of BAC miniprep DNA from 12 clones showing deletions of 50, 75 and 100 kb, respectively, after the insertion of the *galK* selection cassette. The first lane is unmodified RP23-341F12 BAC DNA, which was included as a control. All tested clones had the intended deletion.

removed with an oligo using *galK* negative selection to cleanly delete the *galK* cassette from the BAC as described in the previous experiment (data not shown).

Insertion of a mutant *loxP511* site into a BAC

Encouraged by the efficiency of this selection system, we decided to see whether we could introduce a single 34 bp *loxP511* site cleanly into BAC DNA using *galK* positive/negative selection. It has been previously shown that *loxP* and *loxP511* sites cannot recombine with each other; therefore, the introduced *loxP511* site can only recombine with the *loxP511* site present in the BAC vector backbone and not the wild-type site (Figure 5A) (20). We used *galK* positive selection to insert a PCR-amplified *galK* cassette flanked by 50 bp homology arms to a position 95 kb away from the mutant *loxP511* site in the BAC RP23-341F12 vector backbone (Figure 5A). We then replaced the *galK* cassette using DOG counterselection with a double-stranded 100 bp oligo containing a 34 bp *loxP511* site flanked by two 33 bp homology arms. In this experiment, we observed less than a 10-fold difference in the number of colonies on the plates from heat-induced and non-induced bacteria, suggesting fewer recombinants. This lower frequency of recombinants, when compared with the *Nras* G12D substitution experiment, is likely explained by the shorter homology arms used in this latter experiment, as it has been shown that the efficiency of recombination increase four orders of magnitude when homology length is increased from 20 to 40 bp (10). We analyzed six BAC minipreps from potential recombinants by digesting with SpeI and comparing the restriction patterns with that of wild-type RP23-341F12 BAC DNA (Figure 5B). Three of six (50%) colonies had exactly the same restriction pattern as the unmodified RP23-341F12 BAC, suggesting that DOG resistance had selected for the desired homologous recombination products in these three cases, whereas the other three clones apparently became DOG resistant due to the selection of BACs that carry large deletions spanning *galK*.

The 100 bp oligo was designed so that the *loxP511* site would be inserted in the same orientation as the *loxP511* site in the BAC vector backbone. To confirm that the BACs with the wild-type restriction pattern (3, 5 and 6) had the *loxP511* site correctly inserted, we electroporated these three BACs into electrocompetent and L-arabinose induced EL350 cells, which carry an L-arabinose-inducible *Cre* gene, and then plated the cells on LB plates containing chloramphenicol to select for the BACs. Two colonies generated from each recombinant *loxP511* clone were then tested by SpeI digestion of BAC miniprep DNA, and all had the expected, Cre-mediated, 95 kb deletion (Figure 5C). Recombination was confirmed by PCR using a forward primer from the BAC vector backbone and a reverse primer mapping to a position distal to the reverse homology arm used to insert the *galK* cassette. These primers are 95 kb apart on wild-type RP23-341F12 DNA and only 378 bp apart on Cre/*loxP511* recombined DNA. PCR analysis of all six clones produced a band of the expected length, whereas no product could be amplified from unmodified RP23-341F12 BAC DNA (Figure 5D).

Source of background deletions following DOG selection

In the *Nras* G12D substitution and *loxP511* insertion experiments, a background of DOG-resistant bacteria, which lacked the desired mutation and instead carried unwanted deletions that spanned the *galK* selection cassette, were observed. The relative ratio of colonies containing BACs with deletions was also higher when short homology arms were used. This was expected since homologous recombination is more efficient with longer homology arms (10) that would increase the relative frequency of correctly targeted BACs. The source of the deletions could be λ Red-mediated, since proteins capable of mediating recombination are expressed when the Red genes

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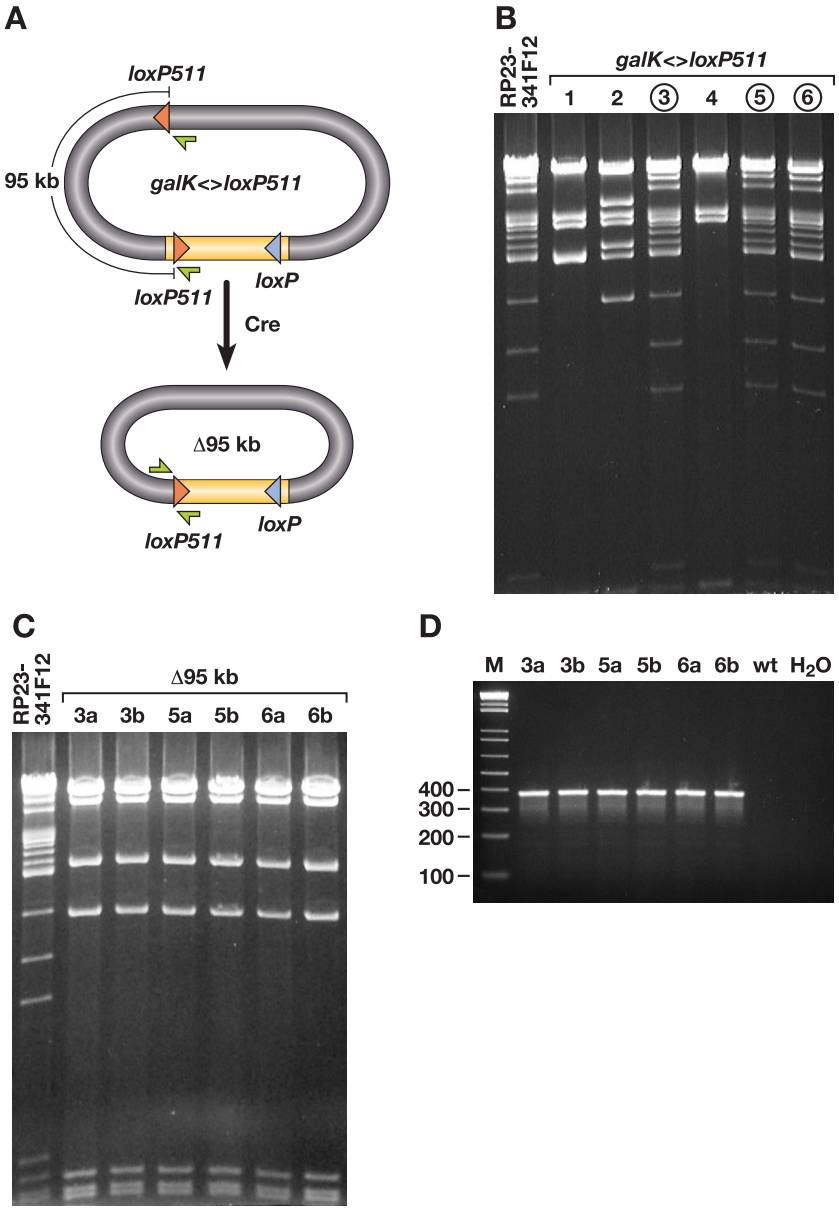

Figure 5. Insertion of a *loxP511* site. (**A**) The location of the wild-type and mutant *loxP* sites in the BAC backbone are indicated along with the extra mutant *loxP511* site that was introduced into the BAC genomic insert via *galK* counterselection. The 95 kb region deleted by Cre-mediated recombination between the two *loxP511* sites is indicated, and PCR primers used to confirm the deletion are shown as small arrows. (**B**) SpeI restriction analysis of six miniprep clones selected for the replacement of *galK* with a dsDNA oligo containing the mutant *loxP511* site. Clones 3, 5 and 6 (circles) had the same restriction pattern as the unmodified BAC, indicating that DOG resistance occurred due to the intended homologous recombination event. Clones 1, 2 and 4 had large deletions and were not analyzed further. (**C**) SpeI restriction analysis of BAC miniprep DNA from clones 3, 5 and 6 after transformation into Cre-induced EL350 cells. Two clones from each parental clone were tested. The restriction pattern shows that the 95 kb region flanked by two *loxP511* sites is deleted from all clones analyzed, confirming the correct insertion of *loxP511* in clones 3, 5 and 6. (**D**) PCR analysis of the six clones from (C) with one primer mapping to the BAC backbone and the other to a position distal to the inserted *loxP511* site.

are activated. Alternatively, the deletions could be induced by the large amount of oligos (200 ng) used in these experiments or they could simply represent spontaneous deletions that occur at low level during normal BAC replication and that are found by DOG selection. To distinguish between these possibilities, we repeated the DOG selection step used to insert the *loxP511* oligo into BAC RP23-341F12 DNA using four differently treated bacteria samples: non-induced bacteria without the 100 bp *loxP511* oligo (Figure 6A), non-induced bacteria with the oligo (Figure 6B), induced bacteria without the oligo (Figure 6C) and induced bacteria with the oligo (Figure 6D). The number of resistant colonies obtained from the four experiments varied by <10-fold (data not shown). Ten colonies from each electroporation were then analyzed by SpeI digestion of BAC miniprep DNA and compared with unmodified RP23-341F12 BAC DNA (Figure 6A–D). Unwanted *galK* region deletions were observed in all 10 colonies for both of the non-induced samples (Figure 6A and B). These deletions are therefore unlikely to be Red-mediated since these samples were not heat-induced. We also observed

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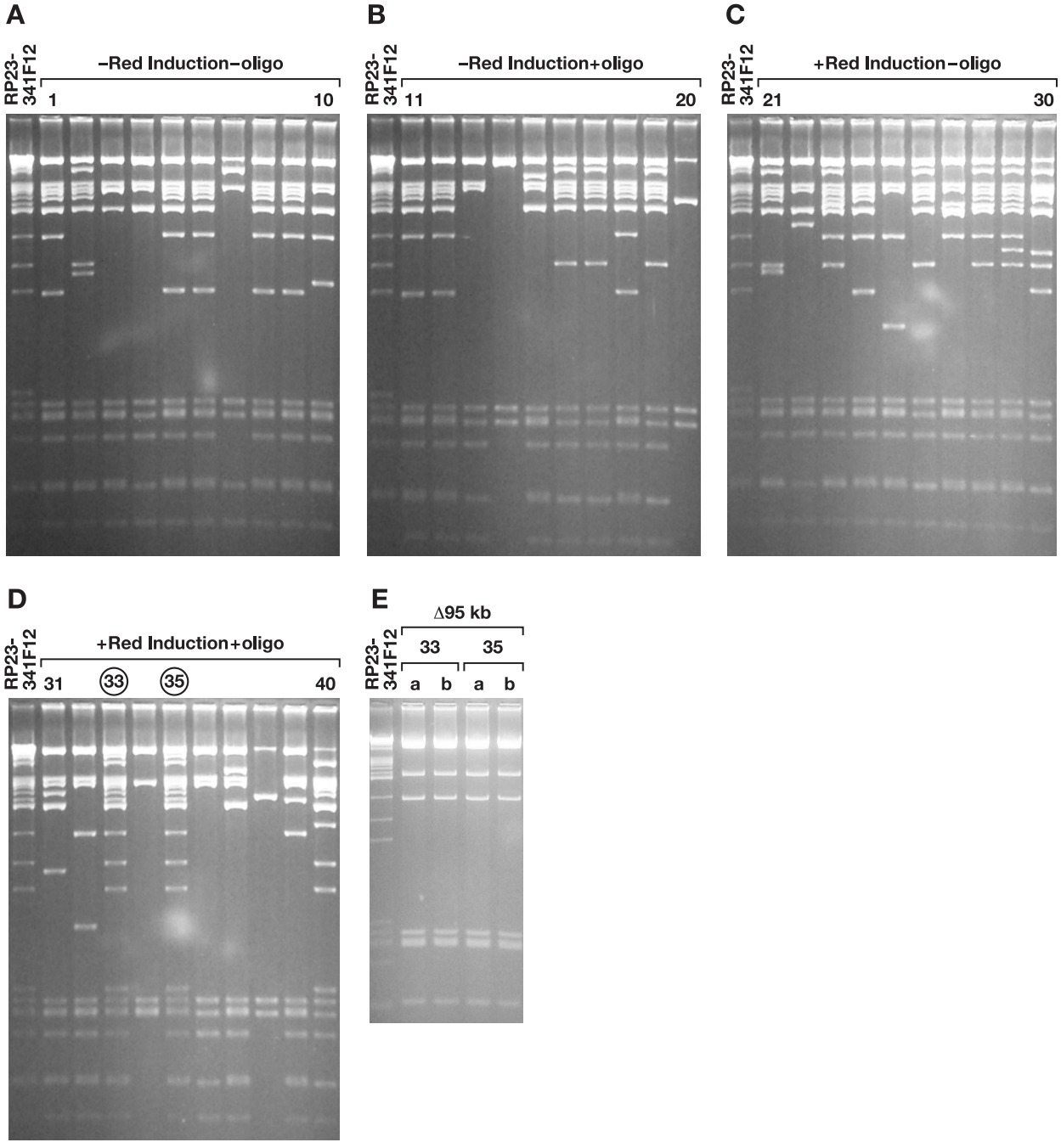

Figure 6. Same experiment as in Figure 5 with modifications as indicated at the top of each panel. (**A**) SpeI digest of 10 minipreps from a control experiment without heat-induction and without the *loxP511* dsDNA oligo. (**B**) SpeI digest of 10 minipreps from a control experiment without heat-induction but with the *loxP511* dsDNA oligo. (**C**) SpeI digest of 10 minipreps from a control experiment with heat-induction but without the *loxP511* dsDNA oligo. (**D**) SpeI digest of 10 minipreps from an experiment with heat-induction and with the *loxP511* dsDNA oligo (comparable with Figure 5B). Clones with the parental digestion pattern indicating DOG resistance due to homologous recombination (clones 33 and 35, circles) are only seen in (D). DOG resistance in all other clones likely occurred due to internal deletions of the BACs. (**E**) SpeI restriction analysis of BAC miniprep DNA from clones 33 and 35 after transformation into Cre-induced EL350 cells. Two clones from each parental clone were tested. The restriction pattern shows that the 95 kb region flanked by two *loxP511* sites is deleted from all the clones analyzed, confirming the correct insertion of *loxP511* in clones 33 and 35 (compare with Figure 5C).

deletions in both the absence and presence of oligo, indicating that the deletions are not oligo induced. We therefore conclude that these are spontaneous deletions that occur at low level during normal BAC replication in the DH10B background.

Non-deleted colonies with the parental SpeI fingerprint were only observed in the Red-induced sample that received oligo (Figure 6D, clones 33 and 35). These clones were subsequently electroporated into EL350 Cre-expressing cells to confirm they contained the introduced *loxP511* site. Two colonies from each original clone were tested by SpeI digestion of the BAC miniprep DNA. As shown in Figure 6E, each colony has undergone Cre-mediated deletion, confirming that these

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colonies were correctly targeted and contain the introduced *loxP511* site (2 out of 10 clones, a 20% efficiency in this experiment).

Generation of SW105 and SW106 cells

The two DY380-derived bacterial strains EL250 and EL350 contain the defective λ prophage needed for recombineering in addition to L-arabinose inducible *Flp* or *Cre* genes, respectively (11). Both strains have proven to be very useful for BAC modification (11), and EL350 is now used routinely for making conditional targeting vectors for ES cell knock-out experiments (4). To further enhance the usefulness of these strains we decided to transfer the *galK* selection system into them. This was done as previously described for SW102. The *galK* IS2 element present in both strains was replaced with the wild-type *gal* promoter and the *galK* gene in the *gal* operon deleted using DOG selection. The resulting strains, SW105 (*Flp*) and SW106 (*Cre*) (Table 1), were then tested to confirm that they still contained inducible *Flp* and *Cre* genes by transforming arabinose-induced and electrocompetent cells with plasmids containing a *neo* gene flanked by *Frt* or *loxP* sites, PL451 and PL452 (4), respectively. Using SW105 or SW106 it is now possible to introduce a point mutation or an informative restriction site into a BAC, retrieve a fragment from this BAC containing the introduced mutation(s) into a plasmid backbone using gap repair, and turn the retrieved fragment into a conditional targeting vector—all using only one bacterial strain, and only a single initial BAC transformation.

DISCUSSION

Here, we describe a new recombineering-based *E.coli* BAC modification system that makes use of *galK* positive selection for growth on galactose minimal medium and *galK* negative selection (counterselection) for growth on DOG. This modification system has several advantages compared with the other related BAC modification systems. First, the *galK* selection cassette is small (1231 bp + homology arms) compared with, for example, the *sacB–neo* cassette (3 kb), making PCR amplification and transformation into bacteria easier. Two homology arms are easily added to the *galK* cassette by including these sequences in the 5′ ends of the primers used for amplification (see Materials and Methods). Second, since *galK* is used for both selection steps, mutations occurring in *galK* during PCR amplification will be selected against during positive selection, significantly reducing the risk of DOG resistance from PCR mutations in *galK* during negative selection. Third, even when very short (33 bp) homology arms were used, the frequency of recombinants among the analyzed DOG-resistant clones was still 20–50%. To our knowledge, successful BAC modification with such short homology arms has never been reported. Finally, since *galK* recombineering is so efficient, little screening is required following selection, reducing the overall hands-on-time to a minimum. *galK* selection requires growth on minimal medium in both selection steps; although perhaps not used routinely in most molecular biology laboratories where BAC modification is needed, these plates are fairly simple to make and should not prevent anyone from using this method.

For all such counterselection schemes there will be background since during negative selection any event leading to the loss of the counterselectable marker will result in survival. In our system, virtually all background appears to result from deletions that span the inserted *galK* gene. These deletions likely occur during BAC replication in the 4.5 h outgrowth phase, since we have shown that these deletions occur independently of Red induction and in the absence or presence of oligo. It is well established that in *recA* defective bacteria, BACs are very stable compared with other large insert vectors, such as yeast artificial chromosomes (YACs). However, using counterselection, rare spontaneous deletions are seen because of the strong selection force. This spontaneous deletion background is not a problem, however, due to the high frequency of homologous recombination obtained with the defective λ prophage system.

Increasing the length of homology arms used for recombineering will reduce the relative number of background deletions observed following negative selection since the percentage of colonies containing BACs that eliminated *galK* by homologous recombination will be increased. Oligos with longer homology arms can be produced by annealing two oligos with overlapping 3′ ends (21). These overlapping oligos (5′ single strand overhangs) are then filled in by DNA polymerase *in vivo* (or *in vitro*) and subsequently serve as the substrate for the λ Red proteins, exo and bet. Longer homology arms can also be added by traditional cloning using restriction enzymes and DNA ligase (4).

A number of uses can be imagined for the strains described here. In principle, our strains can be used to make virtually any kind of BAC modification one can imagine, including point mutations or clean deletions or insertions of everything from cDNA to *loxP* sites or small epitope tags. BAC trimming, the specific deletion of BAC DNA flanking a gene of interest could also be used to remove genes from BACs prior to making BAC transgenic mice so that only the gene of interest on the BAC is analyzed. This should be of particular interest for BAC rescue experiments in positional cloning. Of course, trimming and modification can be combined, since the *galK* selection cassette can be recycled so that the same BAC can be modified several times. BAC modification could also be the starting point for constructing a gene-targeting vector to allow for more sophisticated gene targeting in mouse ES cells. The desired mutation(s) could first be created in the BAC followed by retrieval into a plasmid vector containing a negative-selection marker like thymidine kinase using gap repair (4). Finally, a *neo* marker could be introduced to allow for positive selection in ES cells. Alternatively, the retrieved fragment could be modified so that the end result is a conditional targeting vector. Strain SW106, which in addition to *galK* selection, can be induced to express Cre recombinase, makes it possible to perform all of the steps needed to construct a conditional targeting vector starting with only a single BAC transformation step.

Furthermore, in experiments where BAC transgenic mice are used to analyze the effect of deleting long-range regulators of gene expression, *galK* DOG counterselection can even be used, in the absence of an oligo, to generate a series of deletions around the *galK* insertion site, and the effect on gene regulation studied.

The *Flp* or *Cre* ORFs contained in strains SW105 and SW106, respectively, can also be replaced for any desired

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gene, thus creating tight arabinose-inducible expression for any gene of interest. This is done by first replacing the *Cre/Flpe* ORF with *galK* and select for Gal^+ recombinants. The *galK* cassette is then replaced by the ORF of the gene of interest by DOG selection for Gal^- recombinants.

Finally, the strains described here should be very useful for genetic manipulation not only of BACs, but also the *E.coli* genome itself.

All recombineering reagents discussed in this work are freely available upon request. To obtain these materials, please follow the directions listed on our website (http://recombineering.ncifcrf.gov). Detailed protocols for recombineering, including *galK* selection, can also be downloaded directly from our website.

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