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TECHNOLOGY REPORT

Rapid Engineering of Bacterial Artificial Chromosomes Using Oligonucleotides

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Summary: A rapid method obviating the use of selectable markers to genetically manipulate large DNA inserts cloned into bacterial artificial chromosomes is described. Mutations such as single-base changes, deletions, and insertions can be recombined into a BAC by using synthetic single-stranded oligonucleotides as targeting vectors. The oligonucleotides include the mutated sequence flanked by short homology arms of 35–70 bases on either side that recombine with the BAC. In the absence of any selectable marker, modified BACs are identified by specific PCR amplification of the mutated BAC from cultures of pooled bacterial cells. Each pool represents about 10 electroporated cells from the original recombination mixture. Subsequently, individual clones containing the desired alteration are identified from the positive pools. Using this BAC modification method, we have observed a frequency of one recombinant clone per 90–260 electroporated cells. The combination of high targeting frequency and the sensitive yet selective PCR-based screening method makes BAC manipulation using oligonucleotides both rapid and simple. genesis 29:14–21, 2001. **Published 2001 Wiley-Liss, Inc.†**

Key words: BAC manipulation; phage recombination system; oligonucleotides

The bacterial artificial chromosome (BAC) is a useful vector for cloning large DNA fragments (Shizuya *et al.,* 1992). This is due to the stable maintenance of the DNA insert and use of standard plasmid purification methods to obtain pure BAC DNA. BACs are maintained in a *recA*$^-$ genetic background to prevent genomic rearrangement. However, this restricts the manipulation of the insert DNA using conventional homologous recombination techniques. A temperature-sensitive shuttle vector expressing recA protein has been used for BAC manipulation (Yang *et al.,* 1997). Recently, recombination systems that are independent of recA protein have been developed and used to modify BACs. These utilize an inducible promoter to transiently express bacterial *recE* and *recT* genes or analogous genes in bacteriophage lambda, *exo,* and *bet* and require 50 bases of homology for recombination (Zhang *et al.,* 1998; Muyrers *et al.,* 1999). These also require the *gam* gene function, which inhibits the recBCD nuclease from degrading linear DNA fragments. The *recE* and *exo* gene products have 5′-3′ exonuclease activity, and the *recT* and *bet* gene products are single-strand DNA binding proteins that promote annealing. These recombination systems have been used to disrupt any functional gene in BACs by insertion of a selectable marker (e.g., neomycin resistance gene). In addition, a two-step method has been used to generate subtle changes in the DNA, such as deletions, insertions, or single-base changes (Blomfield *et al.,* 1991; Zhang *et al.,* 1998; Muyrers *et al.,* 1999; E-C. Lee *et al.,* manuscript submitted). The first step involves targeting a positive selection marker along with a counter-selectable marker (e.g., the *SacB* gene, which is lethal to cells in the presence of sucrose) to the region of interest. In the second step, a DNA fragment containing the mutation is targeted to the same region, and cells are selected for loss of the counter-selectable marker.

Recently, an *Escherichia coli* strain harboring a defective lambda prophage was developed that promotes much higher recombination efficiencies. In this strain, the prophage provides the recombination genes *exo, bet,* and *gam* under the control of a temperature-sensitive λ cI-repressor (Yu *et al.,* 2000). These genes are switched on by inactivation of the repressor by transiently shifting the culture from 32°C to 42°C. This system has been efficiently used (one targeted clone per 500 electroporated cells) to generate mutations in bacterial genes in a single step using a 70-bp DNA fragment as targeting vector (Yu *et al.,* 2000). In similar experi-

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ments, 70-mer single-stranded oligonucleotides have been used as targeting vectors and found to be three- to five-fold more efficient (H. M. Ellis, D. Yu, and D. L. Court, manuscript in preparation). Although no selection marker was introduced in the targeting vector, the bacterial genes that were targeted were themselves selectable.

To make this system suitable for manipulation of BACs, *E. coli* strain DY380 was generated by introduction of the λ prophage into the BAC host strain, DH10B (E-C. Lee *et al.,* manuscript submitted). We have used these cells to develop a rapid single-step method for generation of subtle changes in any non-selectable gene in BAC clones using oligonucleotides as targeting vectors. A PCR-based selective amplification screen has been designed to identify targeted clones. We have used oligonucleotides to generate a single-base change, a deletion, and an insertion in different regions of a BAC containing *Brca2,* the murine homolog of human breast cancer susceptibility gene *BRCA2.* Such modified BACs can be used to generate multiple alleles of *Brca2* and the associated phenotype analyzed in BAC transgenic mice on a *Brca2*-deficient genetic background.

Single-Base Alteration

In order to differentiate between the endogenous wild-type *Brca2* allele and the transgene, we attempted to generate a single-base change (T to G) in intron 11 to disrupt an *Eco*RV site. The targeting vector used to generate this change consisted of a 70-mer single-stranded oligonucleotide containing 34 and 35 bases of 5′ and 3′ homology arms and the mutant base in the middle. DY380 cells containing murine *Brca2* BAC, pBAC421, were electroporated with oligonucleotides and screened to identify the targeted clone.

Our first attempt to identify targeted clones by colony hybridization using radiolabeled oligonucleotides (17, 19, or 21-mer) containing the mismatched base in the middle resulted in too many false-positive clones (data not shown). We then tested a PCR-based screen on pools of cells to selectively amplify the DNA containing the T to G change. The electroporated cells were diluted to about 10 cells per 500 μl LB medium and cultured in 96-deep-well plates for 24 h at 32°C. To identify the pool of cells containing recombinant clones, 10 μl of culture from each well was analyzed by PCR. Initial selective amplification by PCR was based on using one of the two detection primers that would be a perfect match with the mutant template but would have a single 3′ ultimate base mismatched to the wild-type DNA. However, this approach yielded an amplification product even when the wild-type DNA was used as the template (data not shown).

Subsequently, we attempted to identify recombinant clones by an allele-specific PCR amplification called the mismatch amplification mutation assay-PCR (MAMA-PCR; Cha *et al.,* 1992). In the MAMA-PCR method, one of the two PCR primers has two mismatched bases at the 3′ end with respect to the wild-type sequence (ultimate and penultimate 3′ base) but a single mismatch at the penultimate 3′ base with the mutated allele (Fig. 1a). The two mismatched bases at the 3′ end of the primer when annealed to the wild-type template provide a conformation that is very inefficiently extended by DNA polymerase. However, in the case of the mutant DNA, the ultimate 3′ base anneals to the complementary base, allowing sufficient priming and amplification. The specificity of the assay is enhanced by using a two-step PCR cycle consisting of a denaturation step and a common annealing/extension step. This method has been successfully applied for allelic discrimination of multiple genes (Cha *et al.,* 1992; Glaab and Skopek, 1999; Zirnstein *et al.,* 1999).

We performed MAMA-PCR using a reverse primer with two mismatched bases at the 3′ end and a perfectly matched forward primer on pools of electroporated cells. Under conditions of denaturation at 94°C followed by annealing and extension at a common temperature of 60°C instead of 55°C and 72°C, respectively, a specific PCR amplification was obtained only from clones containing the mutation. In addition, increasing the number of cycles of amplification from 35 cycles to 40 and gradual cooling and heating of PCR reactions improved the yield of the PCR product. Consequently, the PCR product was visible on an agarose gel, obviating the use of fluorescent primers or detection by Southern blot analysis (Fig. 1b). An added advantage of this two-step PCR reaction is its short run time, allowing rapid identification of positive pools and subsequently individual clones.

Eleven of the 93 pools tested positive for the single-base change, indicating a targeting frequency of one recombinant per 90 electroporated cells. When we used a 70-mer oligonucleotide complementary to the one used above to target the other strand of DNA, we obtained a targeting frequency of one per 205 electroporated cells. Analysis of 24–50 single colonies from dilutions of five positive pools yielded several recombinants from each pool. Five independent clones were confirmed by Southern analysis (Fig. 2) and sequencing (Fig. 4a; Table 1) for the disruption of the *Eco*RV site. The result of the Southern analysis also shows absence of random insertion of the targeting vector in the BAC DNA.

Deletion

Using oligonucleotides, we attempted to generate an 87-base (nucleotides 3553–3639, U65594) in-frame deletion in exon 11 of *Brca2.* This mutant allele will be used to understand the biological role of the 29 amino acids encoded by these 87 bases. These amino acids are expected to be functionally important since they are identical between the murine and human BRCA2 proteins (Sharan and Bradley, 1997). In the first set of experiments, we used a 100-mer oligonucleotide as the targeting vector with 50 bases of homology to each side of the

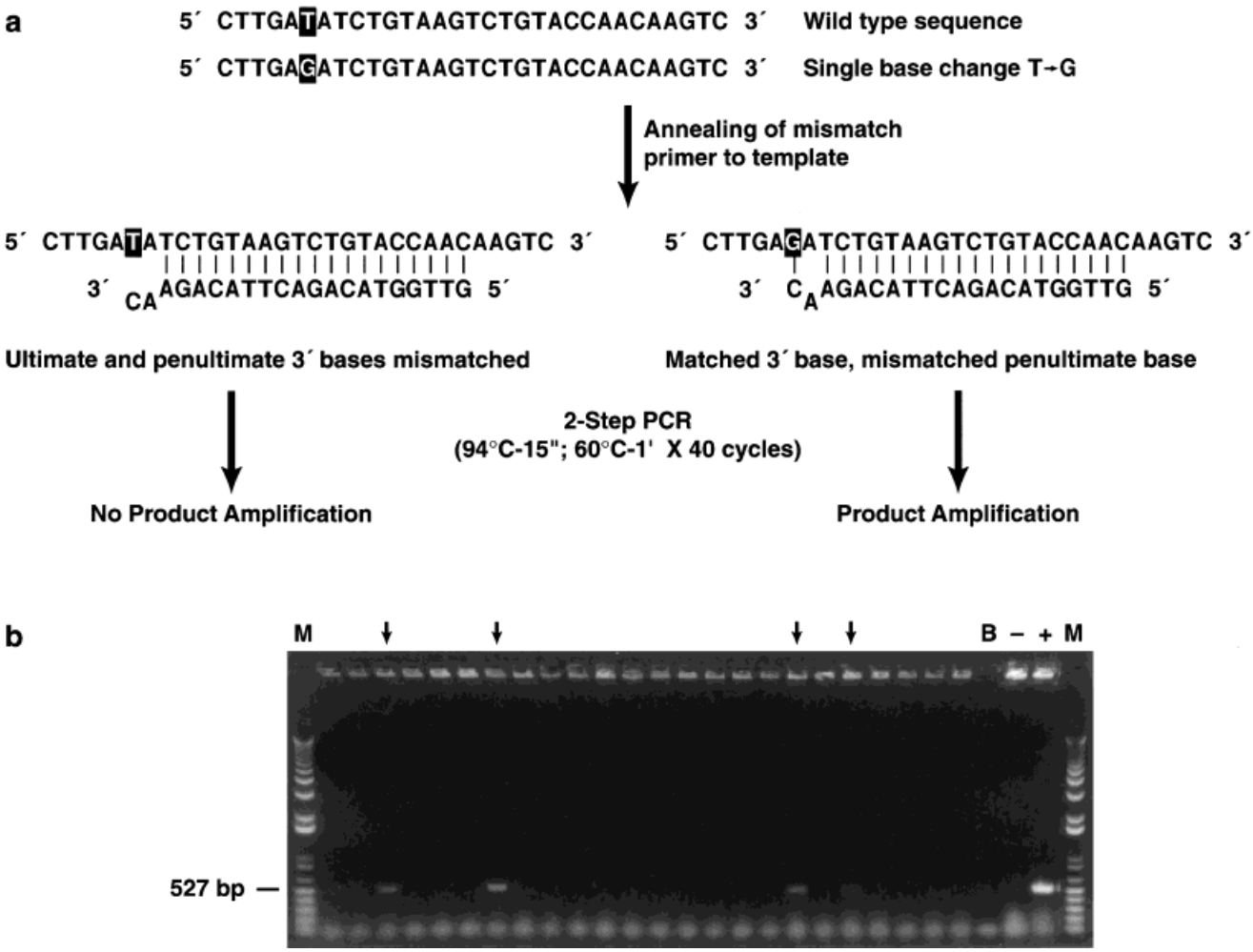

FIG. 1 Schematic representation of mismatch amplification mutation assay-PCR (MAMA-PCR). (**a**) A detection primer (reverse primer) with a two-base mismatch at the 3′ end does not support proper amplification of a PCR product from the wild-type sequence under the two-step PCR conditions. The same primer anneals with the altered sequence through the ultimate 3′ base match and supports amplification under identical PCR conditions. (**b**) An agarose gel picture showing the products of MAMA-PCR to identify positive pools of cells in the 96-well plate. Specific amplification of 527 bp is observed in lanes marked with an arrow that contain the recombinant BAC with the single-base alteration of T → G. "M" represents the DNA size marker lane; blank PCR reaction "B" contains no DNA; the negative control lane marked as "−" contains wild-type pBAC421 BAC DNA amplified with mismatch primers; and "+" marks the PCR-positive control where pBAC421 DNA is amplified with primers without any mismatch.

deletion. No targeted clone was obtained from the 6,000 cells that were analyzed. Based on the assumption that the single-base change described above was obtained at a high targeting frequency since the length of homology was 70 bases (with one mismatch in the middle), we tested whether increasing the length of homology would enhance the recombination frequency. Since the maximum size of synthetic oligonucleotides is 100 bases, we used a PCR-based approach to increase the length of oligonucleotides so that it would have 70 bases of homology to either side of the deletion. The 140-mer targeting oligonucleotide was generated by synthesizing two new 40-mer oligonucleotides. Each of the 40-mer primers had 20 bases of overlapping homology to either end of the 100-mer and an additional 20 bases to increase the length of homology. A PCR was performed using the original 100-mer as the template and the 40-mers as PCR primers (Fig. 3a). The resultant PCR product was 140 bp in size. It was denatured to obtain single-stranded 140-mer oligonucleotide prior to electroporation into DY380 cells.

We performed colony hybridization to detect targeted clones using a 20-mer oligonucleotide specific to the deletion junction fragment with 10 bases of sequence from either side of the deletion. This resulted in many false-positive clones (data not shown). We then used the above described MAMA-PCR method to identify recombinant clones. In this case, the mismatch-containing

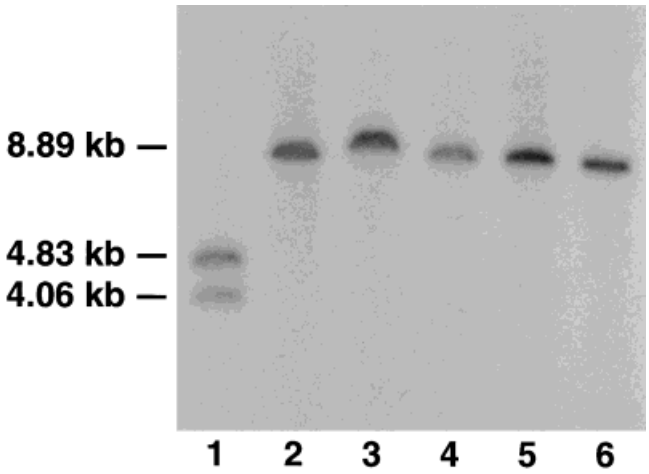

FIG. 2 Southern analysis of recombinant BACs. DNA from a wild-type BAC and five recombinant BACs containing disrupted *Eco*RV site were digested with *Eco*RV restriction enzyme and examined by Southern analysis. The probe used for hybridization is the 70-mer oligonucleotide used as the targeting vector. The probe hybridizes to two *Eco*RV fragments (4.06 kb and 4.83 kb) in the wild-type BAC DNA (lane 1) but a single 8.89 kb fragment in the recombinant BACs (lanes 2–6), demonstrating the disruption of the *Eco*RV site in these clones.

Table 1
Summary of Targeting Efficiency

Mutation	Targeting frequency	No. of clones positive by sequencing/No. of clones sequenced
Single-base change	1:90	5/5
Single-base change (complementary strand)	1:205	ND[a]
87-bp deletion	1:120	10/10
1.93-kb deletion	1:185	6/6
FLAG insertion	1:130	6/6

[a]Not determined.

primer used to detect the deletion was a 20-mer with 18 bases from the region flanking the proximal end of the deleted region and the last two bases flanking the distal end of the deletion. Using the 140-mer targeting vector, we obtained one targeted clone per 120 electroporated cells. Generation of the 87 bp deletion was confirmed by sequencing 10 independent clones (Fig. 4b; Table 1).

We generated the point mutations in the BAC by using single-stranded oligonucleotide with 35 bases of homology on either side of the mutation. Our initial attempts to generate a deletion using 50 bases of homologies to each side of the deletion did not yield frequent recombinants. Based on our experience, two factors could be involved. One is that the length of the homology might be limiting in this segment. The second is that the two strands of complementary DNA that can be used for generating this deletion can have different efficiencies of recombination. Although in the case of the single-base change that we have described we observed about two-fold variation, in other cases up to 40-fold variation in recombination efficiency has been observed (H. M. Ellis, D. Yu, and D. L. Court, manuscript in preparation). By using denatured PCR products, this problem is resolved because both single-strand products are available for recombination. In addition, it overcomes the size limitation of synthetic oligonucleotides. We have used a 140-mer oligonucleotide to delete the 1.93-kb *SacB* gene present in the pBACe3.6 vector at a frequency of one in 185 cells, demonstrating that the method can be used to generate larger deletions.

Insertion

A large number of missense mutations have been identified in the human *BRCA2* gene in cancer patients. To understand the role of these mutations, we are generating mice carrying these mutations in the murine *Brca2* gene. To distinguish between wild-type Brca2 protein and the mutant protein, we are inserting at the N-terminus of the Brca2 protein an eight-amino acid FLAG peptide sequence that can be used as a tag. We attempted to insert the 24-base sequence encoding the FLAG octapeptide into the 5′ end of the murine *Brca2* gene by using oligonucleotides. We generated a 164-mer targeting vector by PCR using two 94-mer oligonucleotides, one forward and one reverse. At the 5′ end of each 94-mer were 70 bases of homology to the region flanking the insertion site followed by the 24-base FLAG sequence at the 3′ end. These two oligonucleotides had complementary FLAG sequence and were used as both template and primer to amplify a 164 bp product (Fig. 3b). The denatured 164 bp PCR product was used as targeting vector. Targeted clones were identified by PCR on culture pools and then on individual colonies. Standard amplification conditions (94°C-1′; 55°C-1′; 72°C-2′ $\times$ 35 cycles) were used with a forward primer specific to the FLAG sequence and a reverse primer containing sequence present in both wild-type and recombinant clones. PCR products specific to the clones containing the FLAG insert were identified at a frequency of one per 130 electroporated cells. Six individual targeted clones were sequenced to confirm the FLAG sequence insertion (Fig. 4c; Table 1).

In the era of functional genomics, the simple protocol described here for rapid manipulation of BACs will prove to be a valuable tool with a wide range of applications. For example, as described here, this approach can be used to generate subtle changes to obtain multiple alleles of any gene. Reporter lines with series of deletions in the promoter region can be used to identify *cis*-regulatory elements. In positional cloning projects, often multiple candidate genes are present on the BAC that is able to rescue the mutant phenotype. The BAC can be modified to generate multiple clones, each with a mutation in one of the candidate genes. Based on the results of rescue experiments using the modified BACs, the gene involved could be identified in a relatively short time.

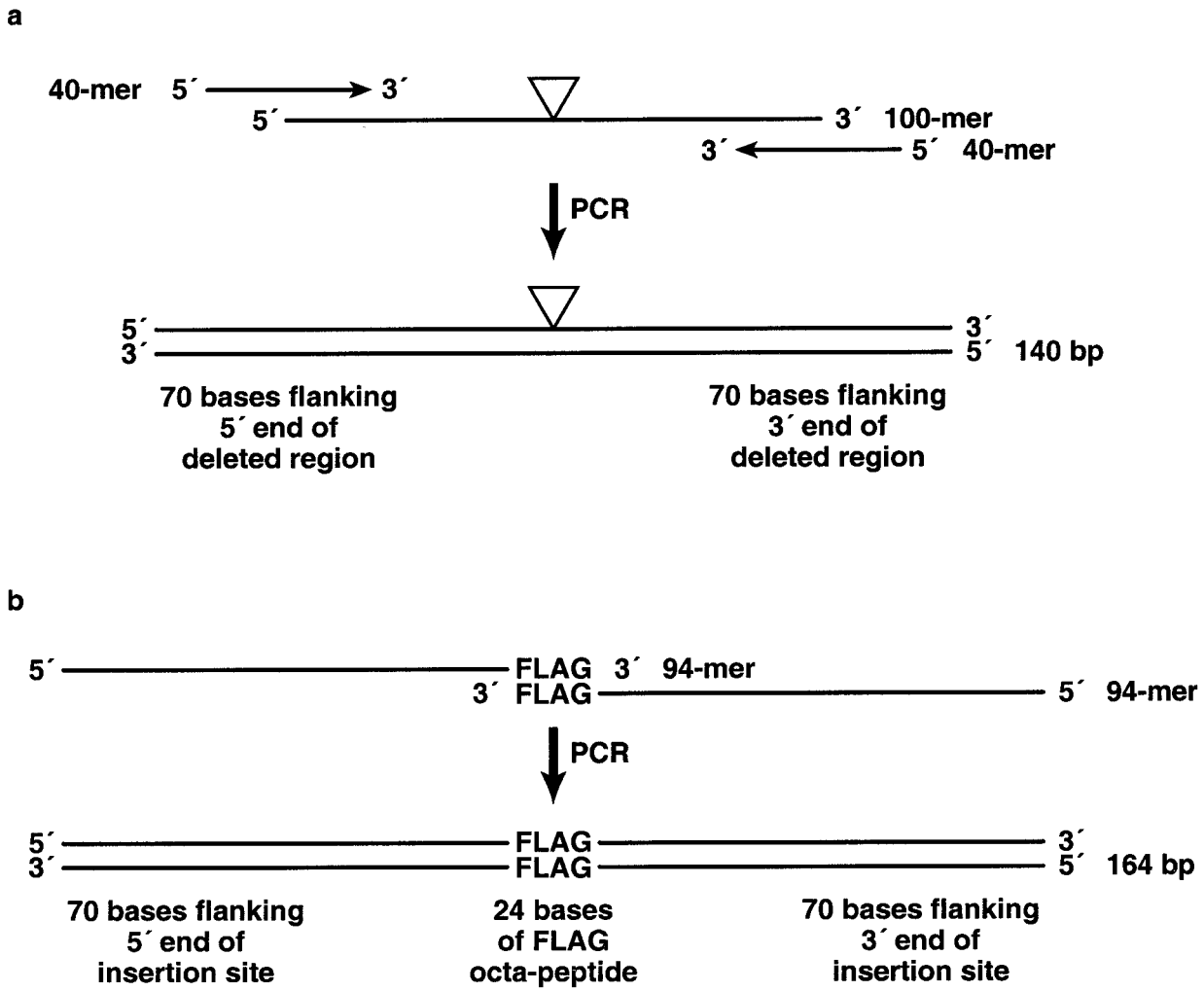

FIG. 3 Schematic representation of the PCR method used to increase the length of homology in targeting vectors. (**a**) A 140 bp targeting vector is obtained from a 100-mer and two 40-mers to generate an 87 bp deletion in exon 11 of the murine *Brca2* gene. (**b**) A 164 bp targeting vector is obtained from two 94-mers to generate an insertion of 24 bp FLAG sequence in the *Brca2* gene.

METHODS

Bacterial Strain

E. coli strain DY380 (E-C. Lee *et al.,* manuscript submitted), carrying a defective λ prophage harboring the recombination genes *exo, bet,* and *gam* under the control of temperature-sensitive cI-repressor, was used for BAC modification. BAC clone pBAC421 was isolated from the Roswell Park Cancer Institute murine genomic library (RPCI-22) in BAC vector pBACe3.6 containing the chloramphenicol resistance gene. The BAC, pBAC421, contains a 220 kb insert that includes the full-length *Brca2* gene, the murine homolog of human breast cancer susceptibility gene *BRCA2.* pBAC421 was introduced into DY380 cells by electroporation and grown in LB medium containing chloramphenicol (25 μg/ml).

Induction and Preparation of Competent Cells

DY380 cells containing pBAC421 were induced and made electro-competent as described by Yu *et al.* (2000). Briefly, 10 ml of cells, grown at 32°C to an OD_{600} of 0.6, were induced at 42°C for 15 min. After being chilled on ice for 15 min, cells were washed with ice-cold water three times and resuspended in 50 μl of ice-cold sterile water and used immediately for electroporation.

Targeting Vectors

Single-strand oligonucleotides were used to create single-base changes. The unpurified oligonucleotides were resuspended in water and directly used for electroporation. A 70-mer synthetic oligonucleotide, 5′-TGTGGAG-TTTAGAAGACAGTGATTTAGAACTTGAGATCTGTAAG-

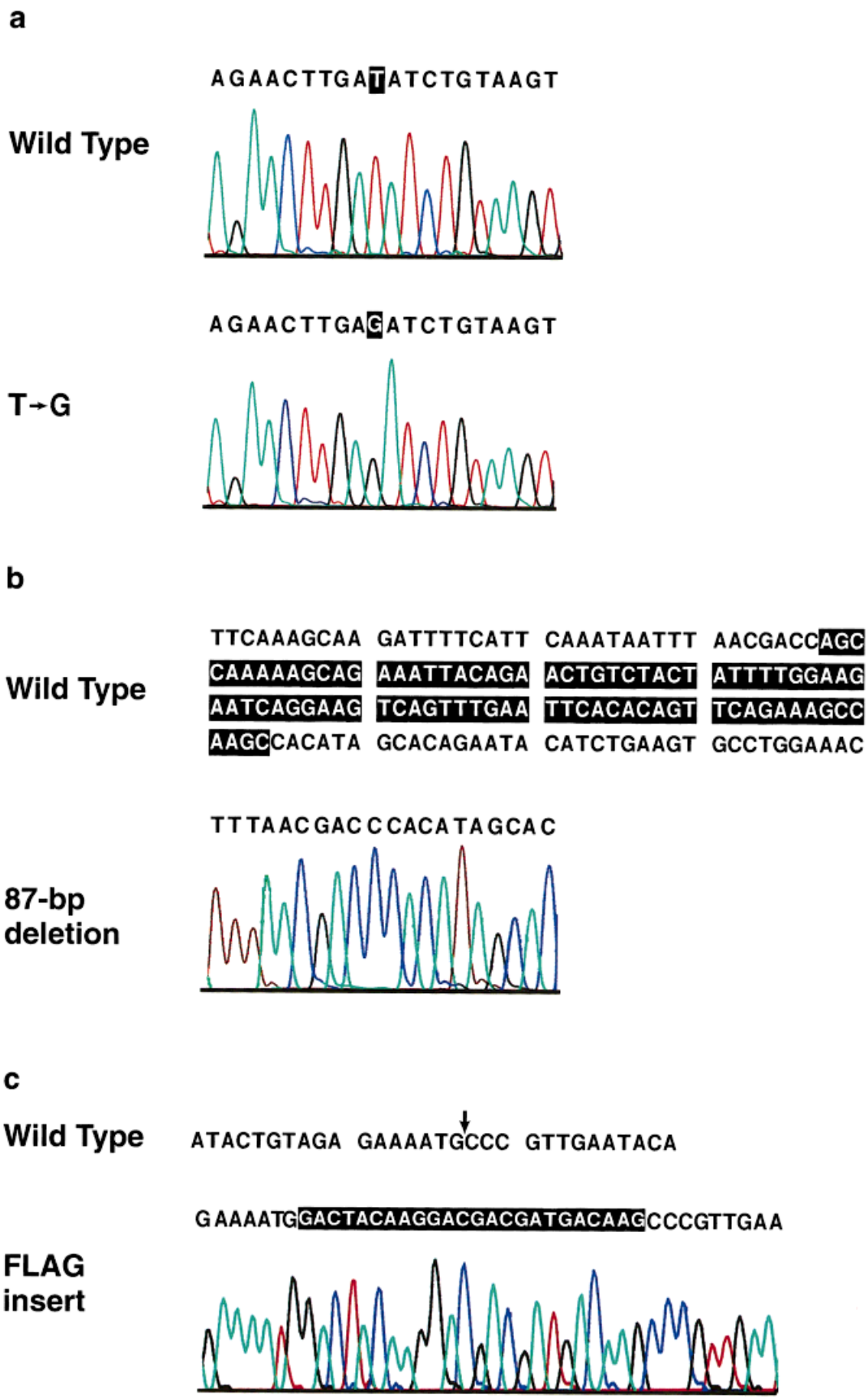

FIG. 4 Sequence confirmation of manipulated BACs showing the single-base change from T to G (**a**), showing the 87 bp deletion in the murine *Brca2* gene (**b**), and showing insertion of FLAG sequence (**c**).

TCTGTACCAACAAGTCATATTTTAAG-3′, with a single-base alteration from T → G in the middle (in bold) was used to target and disrupt an *Eco*RV restriction site in intron 11 of murine *Brca2* gene.

PCR using overlapping primer sets was used to extend homology arms in targeting cassettes to generate deletion and insertion (Fig. 3). The targeting cassette for deletion was generated by using three primers: a 100-mer oligonucleotide with 50-base homology from either side of the region to be deleted, 5′-CCTCAGATGTTATCTTCAAAGCAAGATTTTCATTCAAATAATTTAACGACCCACATAGCACAGAATACATCTGAAGTGCCTGGAAACCAGATGGTTGTTT-3′; and two 40-mer external primers, one forward and one reverse, with 20-base overlaps on either side of the 100-mer, 5′-ATGAAGATACTGACACAGCACCTCAGATGTTATCTTCAAA-3′ and 5′-ACTCCTTAGAAGCGGTACTTAAAACAACCATCTGGTTTCCA-3′. Combination-PCR was carried out by mixing 10 ng of the 100-mer and 300 ng of the 40-mers in a 50-μl PCR reaction using the Expand™ High Fidelity PCR system (Boehringer Mannheim). The resultant PCR product, a 140-mer targeting cassette, was purified by using the QIAquick PCR Purification kit (Qiagen) and reconstituted in sterile water. The PCR product was denatured at 94°C for 10 min and quick-chilled on ice prior to use in electroporations.

The targeting cassette for insertion of the 24 bp sequence encoding the FLAG peptide was prepared using two synthetic 94-mer oligonucleotides, one forward and another reverse, each containing the FLAG sequence insert and 70 bases of homology to one side of the insertion site. Amounts of 300 ng each of the forward primer 5′-TGCAGTGTCTGTTATTTTCTCTTTTGCAGACTTCCTTACCGAGCATCGGAGAAATACTGTAGAGAAAATGGACTACAAGGACGACGATGACAAG-3′ and the reverse primer 5′-CTAAATCTGCTGTGCTGCATCTCGCCTTAAAAATTTCCCAAAAAGTTGGTCTCCTTTTGTATTCAACGGGCTTGTCATCGTCGTCCTTGTAGTC-3′ were used in a PCR reaction as described above for the deletion targeting cassette.

PCR-Based Screen to Identify Targeted Clone

50 μl of freshly prepared electro-competent cells (approximately 10^8) were transformed with 300 ng of purified linear targeting cassette. Cells were resuspended in 1 ml of SOC medium. The cells were diluted and plated in 2.2-ml-deep-well plates (Marsh Biomedical Products) at about 10 cells per well in 500 μl LB media (containing chloramphenicol to maintain the BAC). The cells were grown at 32°C for about 24 h. The number of cells per well was determined by plating serial dilutions of electroporated cells on agar plates and generally found to be in the range of 5–45 cells per well.

After 24 h, 10 μl of culture from each pool was analyzed by PCR. Detection of point mutants utilized mismatch primers designed to amplify only the recombinants. A detection primer containing a two-base mismatch to the wild-type sequence but only a penultimate-base mismatch to the point mutation at its 3′ end, 5′-GTTGGTACAGACTTACAGAAC-3′, was utilized along with a forward primer, 5′-CCAACTCTCTCAGATGGAGA-3′, in a two-step PCR. The PCR condition included denaturation for 4 min at 94°C followed by 40 cycles of 94°C for 15 s and 60°C for 1 min (a common annealing and extension temperature) and a final extension at 72°C for 7 min. These primers amplify a 527-bp product. The amplification products were visible on an agarose gel when PCR was performed on a standard thermocycler where a single heating/cooling block is used for denaturation and annealing/extension steps as compared to a RoboCycler (Staratgene) where there are different blocks for each step. The gradual cooling of the block may help in proper annealing of the primers as compared to when the temperature is directly shifted from 94°C to 60°C, thus enhancing the yield of the amplification product. Under similar PCR conditions, a deletion was detected with the mismatch PCR primers containing 18 bases identical to the sequence flanking the 5′ end of the deleted region and two bases flanking the 3′ end of the deleted region. This forward primer 5′-TCAAATAATTTAACGACCCA-3′ used with reverse primer 5′-AAGAGCAGAACAAATCCTCC-3′ in a two-step PCR amplifies a 270-bp fragment in clones with the deletion. Insertions were detected by selective amplification utilizing a forward primer specific to the FLAG sequence, 5′-GACTACAAGGACGACGATGA-3′, along with a reverse primer, 5′-TCTGAGACAGAGGCTCTCTA-3′, specific to *Brca2* sequence. The expected size of PCR product from recombinants with the FLAG insert is 292 bp.

Southern Analysis of Clones Containing *Eco*RV Disruption

BAC DNA from wild-type pBAC421 and five independent clones with disrupted *Eco*RV site were digested with *Eco*RV and transferred to Hybond$^+$ membrane (Amersham) and analyzed using standard procedures (Sambrook *et al.,* 1989). The Southern blot was hybridized at 50°C with 70-mer targeting vector that was end-labeled with γ-^{32}P-dATP. The blot was washed with 6 × SSC solution at room temperature.

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