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Mini-λ: a tractable system for chromosome and BAC engineering

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Abstract

The bacteriophage lambda (λ) recombination system Red has been used for engineering large DNA fragments cloned into P1 and bacterial artificial chromosomes (BAC or PAC) vectors. So far, this recombination system has been utilized by transferring the BAC or PAC clones into bacterial cells that harbor a defective λ prophage. Here we describe the generation of a mini-λ DNA that can provide the Red recombination functions and can be easily introduced by electroporation into any *E. coli* strain, including the DH10B-carrying BACs or PACs. The mini-λ DNA integrates into the bacterial chromosome as a defective prophage. In addition, since it retains attachment sites, it can be excised out to cure the cells of the phage DNA. We describe here the use of the mini-λ recombination system for BAC modification by introducing a selectable marker into the vector sequence of a BAC clone. In addition, using the mini-λ, we create a single missense mutation in the human *BRCA2* gene cloned in a BAC without the use of any selectable marker. The ability to generate recombinants very efficiently demonstrates the usefulness of the mini-λ as a very simple mobile system for *in vivo* genome engineering by homologous recombination, a process named recombineering.

Keywords: Recombineering; Red recombination; Oligo-recombination; BAC engineering; MAMA-PCR

1. Introduction

The Red recombination system of lambda (λ) phage has been recently utilized for manipulation of genomic DNA (Muyrers et al., 1999, 2001; Yu et al., 2000; Copeland et al., 2001). Strains of *E. coli*, like DY331 and DY380, harboring a defective λ prophage have been generated where the expression of phage genes *exo*, *bet*, and *gam*, under control of a temperature-sensitive CI-repressor (Yu et al., 2000; Lee et al., 2001), has been used to induce homologous recombination. The *exo* gene product has 5′–3′ exonuclease activity and the *bet* gene product is a single-strand DNA-binding protein that promotes annealing between complementary DNA strands. The *gam* gene product inhibits the RecBCD nuclease, thus preventing the degradation of linear DNA fragments. Regions of homology as short as 35 bases in the linear targeting vector DNA are sufficient to achieve efficient recombination (Yu et al., 2000; Court et al., 2002).

The defective λ prophage system is extremely valuable as a tool for recombineering. Under repressed conditions, expression of the Red recombination functions is absolutely eliminated, and thus, when combined in a strain with a *recA* mutation, all extraneous homologous recombination is prevented. λ Red expression and homologous recombination are rapidly activated following induction of the prophage, and by 15 min following the initial induction, recombination levels peak (Yu et al., 2000). This peak level generates recombinants at frequencies that are high enough to be detected by screening nonselected colonies arising from the electroporation. This high yield of recombinants has only been reported for the prophage recombineering system (Lee et al., 2001; Swaminathan et al., 2001; Ellis et al., 2001).

Abbreviations: BAC, bacterial artificial chromosome; PAC, P1 artificial chromosome; PCR, polymerase chain reaction; MAMA, mismatch amplification mutation assay.

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Although the strains of *E. coli* are like DY331 or DY380, carrying the defective prophage have been successfully used to modify exogenous DNA cloned in vectors, including plasmids or P1 and bacterial artificial chromosomes (PAC and BAC) (Sternberg, 1992; Shizuya et al., 1992; Ioannou et al., 1994; Copeland et al., 2001; Lee et al., 2001; Swaminathan et al., 2001), they are not ideal for receiving PAC and BAC clones by transformation. Due to reasons not understood, several BAC clones cannot be transformed into DY331 or DY380 cells. Therefore, a more versatile phage system was sought that would generate high yields of recombinants that could be moved directly to strains already containing the PAC and BAC clones. We describe here the generation of a nonreplicating mini-λ circular DNA, which integrates into the bacterial chromosome. This provides a recombination inducing system that can be introduced by electroporation into nearly any *E. coli* strain, including the *recA* mutant DH10B and its derivatives carrying BACs or PACs. In addition to being easily introduced into any host cell, the mini-λ readily excises to cure the cells of the phage DNA. The excision of the mini-λ DNA from the chromosomal DNA also allows purification of the DNA circles from bacterial cells using standard plasmid purification protocol. The mini-λ therefore provides a tractable system to express transiently the phage recombination genes at high levels. We describe the application of this mobile recombineering system by introducing a kanamycin resistance gene (*Neo*) into the vector sequence of a BAC clone. In addition, we use the mini-λ to generate a single-base alteration in the human breast cancer susceptibility gene, *BRCA2*, cloned in a BAC by using single stranded oligonucleotide as targeting vector. Due to the high recombination efficiency, targeted clones were identified directly by PCR-based screening method, without the use of any positive selection marker.

2. Materials and method

2.1. Construction of mini-λ

A W3110 strain, lysogenic for a single copy of λ*cI857*, was used to prepare the two mini-λ constructs carrying in one case the *cat* cassette for chloramphenicol resistance and in the other the *tet* cassette encoding tetracycline resistance. We have described these cassettes and the primers used to amplify them previously (Yu et al., 2000). The orientation of the *tetA* and *tetR* genes making up the *tet* cassette are shown in Fig. 1. The gene in the *cat* cassette (not shown) is oriented in the same direction as *tetR*. A 50-base segment homologous to *cI* was joined to the 5′ end of one primer for each cassette and a 50-base segment homologous to the *tI* terminator region was joined to the other primer at the other end of each cassette. The junction between homology and cassette primer is defined by the slash mark. The full sequences of the four primers used are as follows: *cI*<>*cat*[TGGCGGTGATAATGGTTGCATGTACTAAGGGAGGTTGTATG/TGTGACGGAAGATCACTTCG]; *cI*<>*tet*[TGGCGGTGATAATGGTTGCATGTACTAAGGGAGGTTGTATG/CTCTTGGGTTATCAAGAGGG]; *tI*<>*cat*[ATTAGCGCAAGGTGATTTTTGTCTTCTTGCGCTAATTTTTTA/CCAGCAATAGACATAAGCG]; *tI*<>*tet*[ATTAGCGCAAGGTGATTTTTGTCTTCTTGCGCTAATTTTTTA/CTCGACATCTTGGTTACCGT].

The *cat* and *tet* cassettes were amplified by polymerase chain reaction (PCR) as described previously from pPCR-Script Cam (Stratagene) and *Tn10* templates, respectively. For each cassette, the linear PCR product was electroporated into the W3110 λ lysogen preinduced at 42 °C for 5 min. Preparation of cells and the electroporation mix for selection of the chloramphenicol and tetracycline resistant recombinants has been described (Yu et al., 2000). The respective drug-resistant colonies were tested for the presence of λ immunity and for lysis genes. The parental W3110 λ lysogen is immune to λ*cI* but not to λ*imm21* and carries the lysis gene *R* in the prophage. Like the parental strain, the drug-resistant recombinant cells are immune to λ*cI* but not to λ*imm21*. However, unlike the parental strain, they lack the lysis genes as evidenced by their failure to complement λ*imm21Ram5Ram54*. These drug-resistant cells produce a small circular DNA following temperature induction as described in the text, which is the mini-λ DNA excised (see Fig. 1).

2.2. Purification of mini-λ DNA

Twenty-five microliters of electro-competent DH10B cells were electroporated with 1 μl of mini-λ DNA (25–35 ng) containing a drug selection marker (e.g. *tet*). The electroporated cells were grown at 32 °C for 1 h in LB medium. The cells were then plated on an LB agar plate containing appropriate antibiotics (tetracycline, 12.5 μg/ml, or chloramphenicol, 20 μg/ml) and incubated overnight at 32 °C. Isolated colonies were picked and used either for recombineering or for purification of mini-λ DNA. To purify the mini-λ DNA, 125 ml of LB containing tetracycline in a 1 L flask was inoculated with an isolated tetracycline resistant colony and cultured overnight at 32 °C. The next day, the overnight culture was incubated at 42 °C for 15 min to induce excision of the mini-λ DNA circles. The culture was then chilled on ice for 15 min. The mini-λ DNA was purified from the cells using standard plasmid purification kits (e.g. Qiagen).

Approximately, 10 μg of the mini-λ DNA were obtained that was suspended in 300 μl of 1XTE. Aliquots (50 μl) were frozen at − 20 °C.

2.3. Induction and preparation of competent cells

Bacterial cells containing the mini-λ were induced and made electro-competent as described previously (Yu et al.,

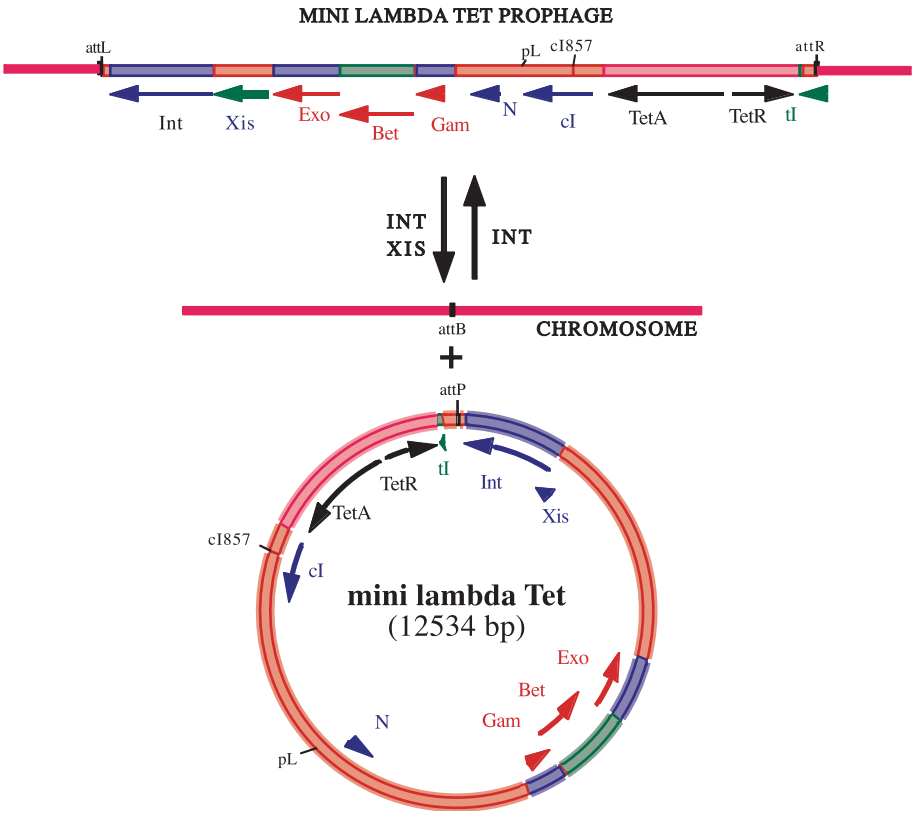

Fig. 1. The mini-λ-Tet construct. The mini-λ circular DNA is a derivative of phage λ circular DNA in which the *tetA* and *tetR* genes replace the lytic operons of λ between *cI* and *tI*, which encode the phage repressor and the *int* transcription terminator, respectively. The repressor is the mutant, *cI857*, causing repressor inactivation at temperatures above 37 °C. The *pL* operon is intact. When induced at 42 °C, transcription from *pL* is allowed and it extends through the *int* genes by action of the positive regulator N. In this way, expression of the Red functions encoded by *gam*, *bet*, and *exo* is ensured. Also, the *int* and *xis* genes are expressed. Int function allows site-specific recombination between *attP* and the chromosome at *attB* following electroporation of mini-λs. Int and Xis together allow the mini-circle DNA to come out of the chromosome by reversal of the integration event during induction process at 42 °C. The integrated mini-λ prophage (not drawn to scale) is shown as part of the chromosome of *E. coli*. The *attL* and *attR* sites are the result of site-specific recombination between *attP* and *attB*.

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 in a 50-ml flask. After being chilled on ice for 10 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.

2.4. BAC engineering using mini-λ

The targeting cassette used to insert a selectable marker into the vector sequence of BAC clone 777 that contains a 165-kb insert, including the human *BRCA2* gene, was generated by PCR. Two 70-mer primers were used to include 50 bases of homology to the BAC vector and the *Neo* gene (under the control of the Tn5 promoter) using a linearized *pGKloxP* (received from Dr. Francis Stewart, EMBL, Heidelberg) plasmid as template. The primers used for PCR were forward primer 5′-CGCGGATCCTTCTATAGTGTCACCTAAATGTCGACGGCCAGGCGGCCGCCATTCTACCGGGTAGGGGAGG-3′ that includes 50 bases of homology to BACe3.6 sequence from nucleotides 2806–2855 (U80929) and 20 base 3′ homology to the 5′ end of the *Neo* gene insert and reverse primer 5′-CCGATGCAAGTGTGTCGCTGTCGACGGTGACCCTATAGTCGAGGGACCTATGTCCTACTCAGGAGAGCGT-3′ with 50 bases of homology to the BAC vector sequence from nucleotides 4865–4914 (U80929) and 20 bases of homology to the 3′ end of the *Neo* gene. The PCR product was purified using PCR purification kit (Qiagen) and used as targeting vector. Targeting vector (300 ng) was electroporated into uninduced or induced electro-competent DH10B cells containing the mini-λ DNA and the BAC 777. Electroporated cells were grown in 1 ml SOC media at 32 °C for an hour and a half and then plated on LB plates containing kanamycin (25 μg/ml).

To confirm the insertion of the *Neo* gene into the BAC vector, 10 kanamycin resistant clones were analyzed. The BAC DNAs were isolated by using a miniprep method described previously (Sinnett et al., 1998). The DNA was digested with *Eco*RI and resolved on an 0.8% agarose gel to differentiate between the recombinant and nonrecombinant clones. Correct insertion of the *Neo* gene is expected to change the 8.7-kb *Eco*RI fragment to a 6.8-kb fragment. This change was also confirmed by Southern analysis using a ^{32}P-labeled probe specific to the BACe3.6 vector (10608–11530).

2.5. Introduction of subtle alteration in BACs

Based on our previous work, a single stranded oligonucleotide as short as 70 bases can be used to generate subtle alterations in BACs (Swaminathan et al., 2001). However, to increase the targeting efficiency and to obtain products of uniform length, a 140-mer targeting vector was generated as described previously (Swaminathan et al., 2001) by using a 100-mer oligonucleotide as template and two 40-mer oligonucleotides as primers for amplification. Standard desalted oligonucleotides were used without any additional purification. The 100-mer oligonucleotide containing the A to G change (G marked in bold) was 5′ACCAATAAGTCTTAATTGGTTTGAAGAACTTTCTTCAGAAGCTCCACCCT-**G**TAATTCTGAACCTGCAGAAGAATCTGAACATAAAAACAACAATTACGAA3′. The 40-mer oligonucleotide used as forward primer was 5′TTTTTTTAAATAGATTTAGGACCAATAAGTCTTAATTGGT3′ and a 40-mer oligo used as reverse primer was 5′TGGAGTTTTAAATAGGTTTGGTTCGTAATTGTTGTTTTTAT3′. The 140-bp PCR product was purified using PCR purification kit (Qiagen) and denatured at 94 °C and chilled on ice. The denatured, single-stranded oligonucleotides were then electroporated into DH10B cells that contain the BAC 777 and the mini-λ, and which had been induced for Red recombination (see Materials and method). BAC 777 contains the full-length human breast cancer susceptibility gene *BRCA2*. The electroporated cells were grown at 32 °C in LB medium for 1.5 h and then 100 μl of various serial dilutions were plated on LB agar plate containing chloramphenicol (20 μg/ml). The plates were incubated at 32 °C overnight. Individual colonies were picked and mixed as pools of four colonies in 10 μl of water. Cell suspension (2 μl) was used in a two-step MAMA-PCR reaction (Cha et al., 1992). The primers used for detection of the A to G change in the BAC by MAMA PCR were forward primer: 5′GTAAAGATGGGGTTTCAACG3′; mismatch detection primer: 5′CTTCTGCAGGTTCAGAATTTC3′. The two-step 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. The primers amplify a 531-bp product.

To confirm the presence of the A to G change in the BAC, an 858-bp region flanking the missense mutation was amplified using PCR primers (forward primer: 5′-GTAAAGATGGGGTTTCAACG-3′ and reverse primer: 5′-CGAGTGAGGAAAAAGAGGCC-3′). The product was purified and sequenced using the reverse primer.

3. Results and discussion

3.1. Recombineering with a wild-type λcI857 prophage

Phage λ infects and establishes itself as a prophage lysogen in most *E. coli* strains including *recA* mutant derivatives. The phage DNA is injected into the cell, circularizes, and integrates by site-specific recombination into the bacterial chromosome as a prophage. We asked whether a λ lysogen of this type carrying a single complete prophage (Powell et al., 1994) could be induced to provide Red functions for recombineering in either *recA*$^+$ or *recA* mutant strains. The prophage we tested carried the *cI857* mutation, which causes the λ repressor to be temperature sensitive (at 42 °C) but able to be renatured and active at 32 °C. Because the prophage carries all of the replication and lytic genes of λ, induction times at 42 °C for longer than 6 min leads to cell death (Weisberg and Gallant, 1967). Therefore, we induced the cultures for only 5 min at 42 °C before electroporating with linear PCR product DNA carrying the *cat* gene cassette encoding chloramphenicol resistance (CmR) following protocols described previously (Yu et al., 2000). The *cat* gene was targeted to replace the *galK* gene of *E. coli*. The *cat* cassette was flanked by 50 bp of DNA homologous to *galK*.

These gene replacement experiments showed that the induced λ prophage generates recombinants that are CmR. This recombination depends upon temperature induction and expression of phage functions but not host RecA function (Table 1). The frequencies of the CmR recombinants observed for *galK* were very similar to those found for the same targets using the defective prophage in strain DY331 when it was induced for 5 min (Yu et al., 2000). However, longer induction times are possible for DY331, and because of that, a 15-min induction can generate many more recombinants.

Following the targeting of *galK*, recombinants were examined for deletion of *galK*. Among 50 CmR recombinants tested, all were phenotypically Gal$^-$ as tested on MacConkey galactose indicator medium. A large percentage (32−40%) of these *gal*< >*cat* recombinants had lost the λ prophage (Table 1). The heat pulse technique used here to induce recombination activity is also commonly used to induce the prophage functions Int and Xis that are required for prophage DNA excision from the chromosome. Here, a 5-min treatment cures the strain of the λ DNA nearly 50% of the time.

A similar experiment was carried out with another λ*cI857* derivative defective in the *cro* gene. The Cro protein is made following induction and acts as a second repressor

Table 1
Gene replacement at *galK* by the *cat* cassette in λ*cI857* lysogens[a]

Host *recA*	Min. at 42 °C	CmR[b]	λ Curing rate[c]
+	0	<1	
+	5	5.1×10^3	0.32
−	5	4.5×10^2	0.35

[a] Cells lysogenic for λ*cI857* prophage were electroporated with 100 ng of the *cat* cassette targeted to replace *galK*.
[b] Total CmR recombinants per 10^8 cells surviving electroporation.
[c] λ Prophage curing rate among recombinants was determined by the number of tetracycline sensitive (loss of λ) among 50 CmR colonies.

to reduce the expression of genes from the *pL* and *pR* promoters including Red. No effect on recombination by Cro was observed (data not shown). In retrospect, this might be expected since Cro normally starts repressing *pL* activity about 8 min after induction (Adhya et al., 1977); here, only 5 min of induction were allowed.

Thus, phage λ provides a mobile system for recombineering. However, because it contains lethal lytic functions, the prophage cannot be induced long enough for its recombination activity to be fully expressed without killing the cell. To overcome this, mini-λ derivatives were created from the λ prophage by recombineering. Like λ, the mini-λ DNA maintains the ability to integrate and excise from the bacterial chromosome but has been deleted for genes encoding the lethal replication and lytic functions of the complete phage.

3.2. Constructing mini-λ prophage

We have generated the mini-λ DNA by using homologous recombination to replace the λ prophage genes from *cro* through *tI* with various drug resistance markers in λ*cI857* lysogens. Two targeting cassettes containing different drug resistance genes (*cat* and *tet*) with appropriate flanking homologies (see Materials and method) were used to generate λ*cI857* drug-resistant derivatives. The drug cassettes replaced a contiguous 36,208 bp segment of prophage DNA from *cro* through *tI* (Fig. 1). This deletion eliminates all replication and lysis genes, creating a replication defective, smaller version of the λ prophage, the mini-λ. The absence of *cro* enables full induction of the *pL* operon at 42 °C.

The *pL* operon of these mini-λs includes the *int* and the *xis* genes as well as the *exo*, *bet*, and *gam* recombination genes essential for recombineering (Fig. 1). Integration of the mini-λ DNA occurs by Int-mediated site-specific recombination between *attP* and *attB* on the chromosome, generating the prophage attachment sites *attL* and *attR* (Fig. 1). Since integration generates attachment sites *attL* and *attR* flanking the mini-λ prophage, inactivation of the repressor at restrictive temperature activates *int* and *xis* gene expression causing site-specific excision of the prophage DNA circle carrying its associated drug marker (Fig. 1). As described in the experiments for full λ prophages above, about 50% of the mini-λ containing cells undergoing induction at 42 °C are cured of the mini-λ prophage. This ability to excise the mini-λ after temperature induction can be used to generate the mini-λ DNA circle free of the chromosome and available for standard plasmid purification methods.

3.3. Mini-λ recombination system: applications in BAC engineering

Because RecA is not required for site-specific Int-mediated recombination, DH10B and other *recA* mutant strains including the DH10B derivatives containing BACs can be used for transformation and for integration of the circular mini-λ DNA by selecting for the appropriate drug marker. We tested if the mini-λ can be efficiently transformed into DH10B cells carrying a BAC. After electroporating about 25–35 ng of the mini-λ DNA carrying the *tet* drug selection marker, 50–100 tetracycline-chloramphenicol-resistant colonies (BAC DNA confers CmR) were obtained.

We then tested the mini-λ recombination system to determine whether it can be used for manipulating BACs by homologous recombination. Using PCR, a linear targeting DNA was generated that contained the *Neo* gene flanked on each side by 50 bases of homology to the plasmid backbone of BAC clone 777. DH10B cells containing the BAC and the mini-λ were induced under conditions found to be optimal for DY380 cells to express recombination functions, and the targeting vector was electroporated. Kanamycin resistant (KmR) recombinant clones were obtained at a frequency of ~ 1 per 7.5×10^5 electroporated cells. Uninduced DH10B cells did not yield any KmR colony. Ten KmR clones from the induced cells were examined to determine whether the *Neo* gene was correctly targeted into the BAC vector. Insertion of the *Neo* into the BAC vector is expected to change an 8.7-kb *Eco*RI fragment to 6.8 kb (Fig. 2A). All 10 clones showed the presence of the 6.8-kb fragment confirming that they were all correctly targeted (Fig. 2B,C). The targeting efficiency obtained here is comparable to those obtained when DY380 cells are used (Lee et al., 2001). The ability to efficiently introduce the *Neo* gene into the BAC by homologous recombination suggests that mini-λ can be used for BAC recombineering.

Next we used an oligonucleotide-based BAC engineering method described previously (Swaminathan et al., 2001) to generate a missense mutation in the BAC 777 that contains the human breast cancer susceptibility gene, *BRCA2*. BACs carrying missense mutations in the *BRCA2* gene can be used to generate transgenic mice. The phenotypes associated with these mutations can be examined in BAC transgenic mice lacking the endogenous *Brca2* gene and provide insight into the functional significance of these mutations. Since no selectable marker is used in the oligonucleotide-based mutagenesis method, the success of this approach relies on high recombination efficiency so that 100–1000 clones can be individually screened by PCR to identify the targeted clones.

We tested if the mini-λ is suitable for this method by generating a missense mutation in codon 42 of the human *BRCA2* gene. The alteration involved changing the codon from TAT to TGT, which changes a tyrosine residue to cysteine in the *BRCA2* protein. This alteration has been associated with cancer predisposition in humans. The targeting vector used to generate this mutation was a 140-bp PCR product containing the single-base alteration in the middle and about 70 bases of homology to the regions flanking the altered base. To generate single-stranded targeting vector,

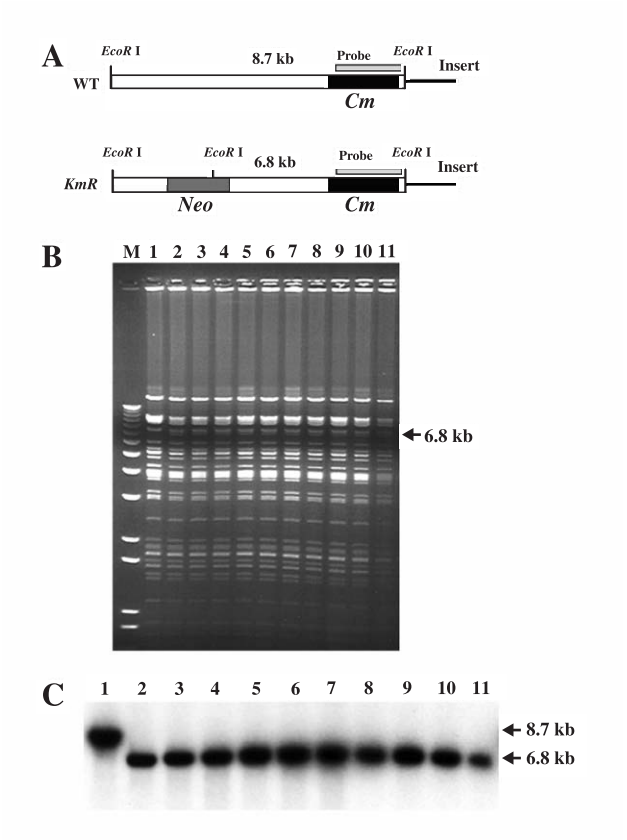

Fig. 2. Confirmation of correctly targeted kanamycin clones of BAC777. (A) Schematic representation of the wild-type (WT) BAC 777 and a recombinant clone (KmR) to show the change in the 8.7-kb *Eco*RI fragment to a 6.8-kb fragment after insertion of the *Neo* gene into BACe3.6 vector. The location of the probe used for Southern analysis is also shown. (B) An agarose gel picture of *Eco*RI-digested BAC DNA shows the presence of 6.8 kb in KmR clones (arrow in lanes 2–11) after the insertion of the *Neo* gene. Wild-type BAC 777 (lane 1) contains an 8.7-kb fragment that is not distinctly visible due to presence of other fragments of similar size. A 1-kb Plus DNA ladder (Invitrogen) is used as molecular weight marker. The smallest fragment represents an 850-bp fragment. (C) To confirm that the 6.8-kb *Eco*RI fragment observed in the KmR clones represented the vector sequence, the *Eco*RI digested BAC DNA was examined by Southern analysis. A radio-labeled probe specific to the vector sequence was hybridized to the Southern blot and the expected 8.7-kb band was observed in wild-type BAC 777, while the KmR clones showed the 6.8-kb band.

the 140-bp PCR product was denatured in water prior to electroporation. As previously described (Swaminathan et al., 2001), denatured PCR products are used to enhance the recombination efficiency by targeting both strands of DNA with long homology arms. DH10B cells containing the BAC and the mini-λ were induced to activate the Red recombination functions. After electroporating the single strands, the cells were grown in LB medium for an hour and a half at 32 °C before dilutions were plated on LB plates containing chloramphenicol. These plates were incubated at 32 °C overnight.

Individual colonies were mixed in pools of four and analyzed by an allele-specific PCR amplification called the mismatch amplification mutation assay-PCR (MAMA-PCR) as described previously (Swaminathan et al., 2001). Briefly, in the MAMA-PCR method, one of the two PCR primers, the "mismatch detection" primer, has two mismatched bases at the 3′ ends with respect to the wild-type sequence (ultimate and penultimate 3′ base), but a single mismatch with the mutated allele (the penultimate 3′ base). The two mismatched bases at the 3′ end of the primer when annealed to the wild-type template fail to amplify a PCR product. However, in the case of the mutant DNA, the primer anneals to the template and allows amplification. Using a two-step PCR cycle consisting of a denaturation step and a common annealing/extension step enhances the specificity of the assay. Three out of the 46 pools that were tested were positive for the single-base change, indicating a targeting frequency of one recombinant per 61 electroporated cells. Once the positive pools were identified, individual colonies were screened to identify the clone containing the mutation (Fig. 3A). The positive clones were sequenced to confirm the A to G base change in codon 42 (Fig. 3B).

The fact that the BACs can be modified with high-targeting efficiency to generate subtle alterations without the use of any selectable marker suggests that the mini-λ recombination system can be useful for rapid modification of BACs. The mini-λ is also a very useful system to manipulate the chromosomal DNA of any *E. coli* strain,

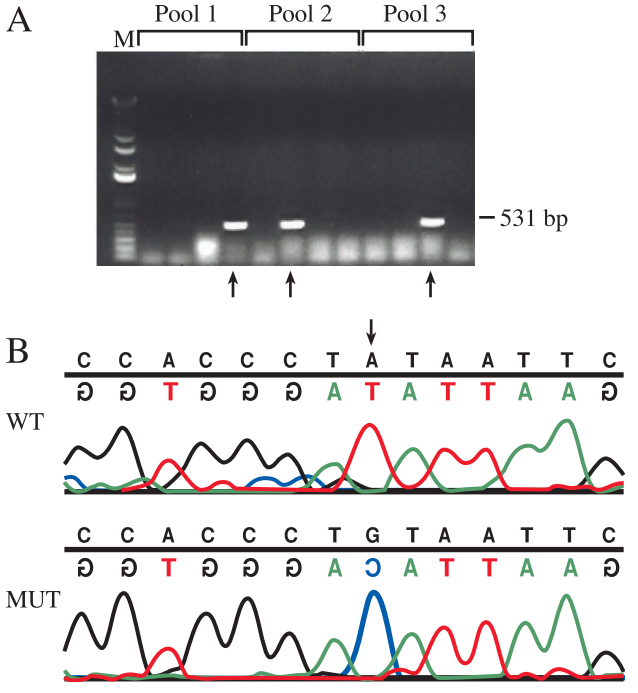

Fig. 3. Detection and confirmation of a single-base change in BAC 777. (A) An agarose gel picture showing the results of MAMA-PCR amplification of individual colonies from three positive pools for an "A to G" change in codon 42. The expected 531-bp product is present in three clones, one from each pool (shown by an arrow). (B) Sequence analysis using a reverse primer to confirm alteration of codon 42 from TAT (Tyr) in wild-type BAC 777 to a TGT (Cys) in one of the targeted clones.

including those with *recA* mutations. Since the mini-λ excises in approximately 50% of the induced cell, recombinants lacking the prophage can be easily obtained. Such cells are not temperature sensitive and can be grown at 37 °C. In the present era of functional genomics, our ability to generate subtle alterations in the large genomic DNA clones carried by PACs or BACs provides a valuable tool for functional dissection of genes as well as for characterization of their regulatory elements.

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