Nucleic Acids Research, 1997, Vol. 25, No. 11 **2205–2212**

Isolating large nested deletions in bacterial and P1 artificial chromosomes by *in vivo* P1 packaging of products of Cre-catalysed recombination between the endogenous and a transposed *loxP* site

Pradeep K. Chatterjee* and Jonathon S. Coren[1]

Department of Medicine, SUNY Health Science Center, 750 East Adams Street, Syracuse, NY 13210, USA and [1]Program in Genetics, Dupont Merck Pharmaceutical Company, Experimental Station, PO Box 80336, Wilmington, DE 19880, USA

Received December 10, 1996; Revised and Accepted April 14, 1997

ABSTRACT

A general approach for isolating large nested deletions in P1 artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) by retrofitting with a *loxP* site-containing Tn10 mini-transposon is described. Cre-mediated recombination between the *loxP* site existing in these clones and one introduced by transposition leads to deletions and inversions of the DNA between these sites. Large deletions are selectively recovered by transducing the retrofitted PAC or BAC clones with P1 phage. The requirement that both *loxP* sites in the cointegrate be packaged into a P1 head ensures that only large deletions are rescued. PCR analyses identified these deletions as products of legitimate recombination between *loxP* sites mediated by Cre protein. BACs produce deletions much more efficiently than PACs although the former cannot be induced to greater than unit copy in cells. Mammalian cell-responsive antibiotic resistance markers are introduced as part of the transposon into genomic clone deletions for subsequent functional analysis. Most importantly, the *loxP* site retrofitting and P1 transduction can be performed in the same bacterial host containing these clones directly isolated from PAC or BAC libraries. These procedures should facilitate physical and functional mapping of genes and regulatory elements in these large plasmids.

INTRODUCTION

Libraries of DNA fragments from the genomes of numerous organisms have been constructed in cloning systems as diverse as yeast artificial chromosomes (YACs) (1), cosmids (2), bacteriophage P1 (3–8), F-factor derived bacterial artificial chromosomes (BACs) (9,10), P1 artificial chromosomes (PACs) (11), P1 vector-based bacteriophage T4 packaging system (12) and the human artificial episomal chromosomes (HAECs) (13). Despite the availability of several vector systems capable of faithful propagation and easy manipulation of high molecular weight DNA, the study of genes and other regulatory sequences in clones from these systems has invariably been conducted using subcloning strategies (14–16). Physical mapping of genes have also relied upon PCR and hybridization analysis of insert DNA subcloned into smaller vectors. Often the manipulations that go along with the ordering of small pieces of DNA in a large uncharacterized clone become cumbersome in approaches using such reconstruction strategies.

Genomic cloning systems that produce good yields of relatively pure DNA offer the advantage of having their plasmids used directly in biological experiments after modification. A general retrofitting procedure capable of introducing such alterations was recently described in the P1 system (17). In this approach sequences of up to 10 kb are readily inserted into the genomic DNA as part of a Tn10 mini-transposon. The near random distribution of Tn10 insertions allows one to also generate nested deletions from one end of the insert by Cre catalyzed recombination between the P1 vector *loxP* site and another introduced by transposition (17). However, deletions are only one half of the products of this recombination process. Recombination between *loxP* sites that are in opposite orientation leads to a less desirable inversion product in half the population (18–21). For P1 clones, the recovery of both recombination products by transduction with P1 phage is very efficient (17). The deletions in this approach can be distinguished from the inversions only after analyzing the size of the DNA isolated from a retrofitted clone.

PACs and BACs are also amenable to such a retrofitting strategy for generating nested deletions since there is a *loxP* site within the vector sequences in both of these cloning systems (9,11,22). Because plasmids from these systems are generally much larger than P1, the transduction procedure can be used here to select for deletions that are large enough that the DNA fits into a P1 head. By supplying Cre protein *in trans* from P1 vir^s during transduction (17), the need for transferring the PAC or BAC plasmids to a cre^+ background is avoided. Thus clones from existing PAC and BAC libraries can be used directly to generate nested deletion sets for numerous mapping studies. Antibiotic

*To whom correspondence should be addressed. Tel: +1 315 464 4536; Fax: +1 315 464 5797; Email: chatterp@vax.cs.hscsyr.edu

resistance markers selectable in mammalian cells can be introduced into genomic clone deletions as part of the *loxP* transposon. This should allow functional characterization of the deleted sequences in suitable mammalian cells.

Here we demonstrate the use of this strategy to isolate large nested deletion sets in (i) the PAC system with newly designed transposons carrying a *loxP* site, a chloramphenicol resistance gene and the mammalian cell-selectable marker for puromycin resistance (Fig. 1B); and (ii) the BAC system with a different transposon which carries a kanamycin resistance gene and a *loxP* site. The RSVneomycin gene present in this transposon (Fig. 1B) can confer resistance both to kanamycin in bacteria and neomycin in mammalian cells.

MATERIALS AND METHODS

Bacterial strains, vectors and media

PAC clones #2, 5, 9 and 27 and BAC clones #10, 11, 18, 21, 22, 29 and CLC5BAC were gifts from Drs David Smoller and Kirk Findlay (Genome Systems Inc. MO, USA). These were isolated from the respective libraries constructed with human genomic DNA. The host DH10B containing the PAC and BAC plasmids is *recA* and expresses the $lacI^q$ repressor constitutively.

Isogenic cre^- and cre^+ strains, NS3516 and NS3529 respectively, were described earlier (5,23). The virulent form of P1 phage (P1 vir^s) used to transduce P1, PAC and BAC deletions has also been described (5). The P1/*apex* clone in the cre^- host NS3516 was described earlier (17).

P1 vir^s stocks were made by a modification of the procedure outlined in (24,25) and described in detail in (17). Phage titers of 8×10^{10} p.f.u./ml were obtained using this procedure.

All Tn10 mini-transposon plasmids were propagated in $lacI^q$ strains (e.g. NS3516) overexpressing lac repressor. The transposon donating plasmid pTnRSVneo/loxP (17) served as the starting point for construction of all transposon plasmids described here. pTn(Minimal)/loxP was constructed by replacing the *Not*I–*Asc*I fragment containing the RSVneo gene with a *Not*I–*Asc*I synthetic oligonucleotide duplex containing a *Bgl*II site (Fig. 1B). pTnPGKpuro/loxP was constructed similarly by replacing the same *Not*I–*Asc*I fragment with a *Not*I–*Sac*I fragment containing the PGKpuro gene from plasmid pPGKpuroBPA (obtained from Dr Alan Bradly, Baylor College of Medicine, Houston) and a synthetic oligonucleotide adapter with *Sac*I–*Asc*I ends. Transposon plasmid pTnpuro/loxP devoid of *Not*I and *Bgl*II sites was constructed by blunt end ligating the *Not*I–*Asc*I large fragment from pTnRSVneo/loxP to the 2 kb fragment containing the puromycin gene from pPUR3 obtained from Clontech. This puromycin gene lacks the *Bgl*II site.

The transposon donating plasmid pTnBAC/loxP used in BACs was constructed by destroying the chloramphenicol resistance gene in pTnRSVneo/loxP. This was achieved by replacing the smallest *Not*I–*Eco*RI fragment (size 1.6 kb) with a synthetic oligonucleotide adapter.

Inserting *loxP*-Tn10 into PACs and BACs

Retrofitting PAC and BAC clones with *loxP* transposons was performed in a manner similar to that described earlier for P1 clones (17). Transposon plasmids pTnRSVneo/loxP and pTnPGKpuro/loxP or pTnBAC/loxP were first introduced separately into PAC or BAC plasmid-containing DH10B cells by calcium chloride treatment (26). Cells harboring both a transposon plasmid and a PAC or BAC plasmid were selected for resistance to all three antibiotics, kanamycin or chloramphenicol (from PAC or BAC respectively) and chloramphenicol or neomycin/kanamycin (from the PAC or BAC transposon plasmids respectively) and ampicillin (from both transposon plasmids) on L agar plates containing 25 µg/ml of each antibiotic. A single transformed colony was grown to an A_{600} of 0.1 in 10 ml of L broth containing 25 µg/ml each of kanamycin and chloramphenicol. The transposase gene in the transposon plasmid was induced by adding 100 µl of a 0.1 M solution of isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were shaken vigorously for 4–5 h at 37°C, concentrated 10-fold by centrifugation in a Sorval table top centrifuge at 3000 r.p.m. for 10 min at 4°C and the pellet resuspended by gentle vortexing in 1 ml of prewarmed L broth containing 5 mM calcium chloride. Cells were infected with a P1 vir^s lysate at a multiplicity of infection (m.o.i.) of 2 and incubated at 37°C for 10 min without shaking. Then 2 ml of prewarmed L broth containing 5 mM calcium chloride was added and incubation continued at 37°C for another 2 h with vigorous shaking. Lysis was enhanced by adding 100 µl of chloroform to the culture, vortexing and incubating an additional 1 min at 37°C, and vortexing again. Lysed cultures were centrifuged at 7000 r.p.m. for 10 min at 4°C in a SS 34 Sorval rotor. The P1 lysate was stored at 4°C. This phage lysate contains the population of retrofitted, headful-sized PAC or BAC DNA. Cre^- NS3516 cells were infected with this lysate to regenerate the PAC or BAC deletion plasmid (5,17). This transformation from phage to plasmid was performed as described earlier for infection with P1 vir^s except that sufficient NS3516 cells were used to give an m.o.i. of 0.5. Infected cells were shaken at 37°C for 1 h after dilution, concentrated 10-fold and aliquots spread on L agar plates containing kanamycin, chloramphenicol or ampicillin alone or kanamycin and chloramphenicol together to select for retrofitted PACs or BACs.

Isolating plasmid DNA from PAC and BAC deletions

Plasmid DNA from PAC or BAC deletions were isolated by the alkaline-SDS procedure after inducing cells with IPTG (for PACs only) as described earlier for P1 DNA (17).

PCR analyses

PCR reactions were set up with reagents from Perkin Elmer and used a 1 min annealing at 60°C followed by a 4 min polymerization at 72°C. Sequence of primers used for analyzing deletions in P1 and PACs are: CM-1, d(CAACGGTGGTATATCCAGTG) and P1-loxP, d(CTCTGTCAGAAACGGCCTTACG). Relative orientations are indicated in Figure 1A. Primers used for analyzing deletions in BACs are: Bac-1, d(GAGCTCGGACATGAGGTTG), Neo-1, d(GAGCAAGGTGAGATGACAGGAG), Bac-2, d(CCGTATTCAGTGTCGCTG), Neo-2, d(CTGCCTCGTCCTGCAGTTCATTC), Bac-3, d(CAATTATGACGCAGGTATCG) and Neo-3, d(GAATGAACTGCAGGACGAGGCAG). Bac-2 and Neo-2 are in the same direction as Bac-1 and Neo-1 respectively, while Bac-3 and Neo-3 are opposite in orientation to Bac-1 and Neo-1 respectively.

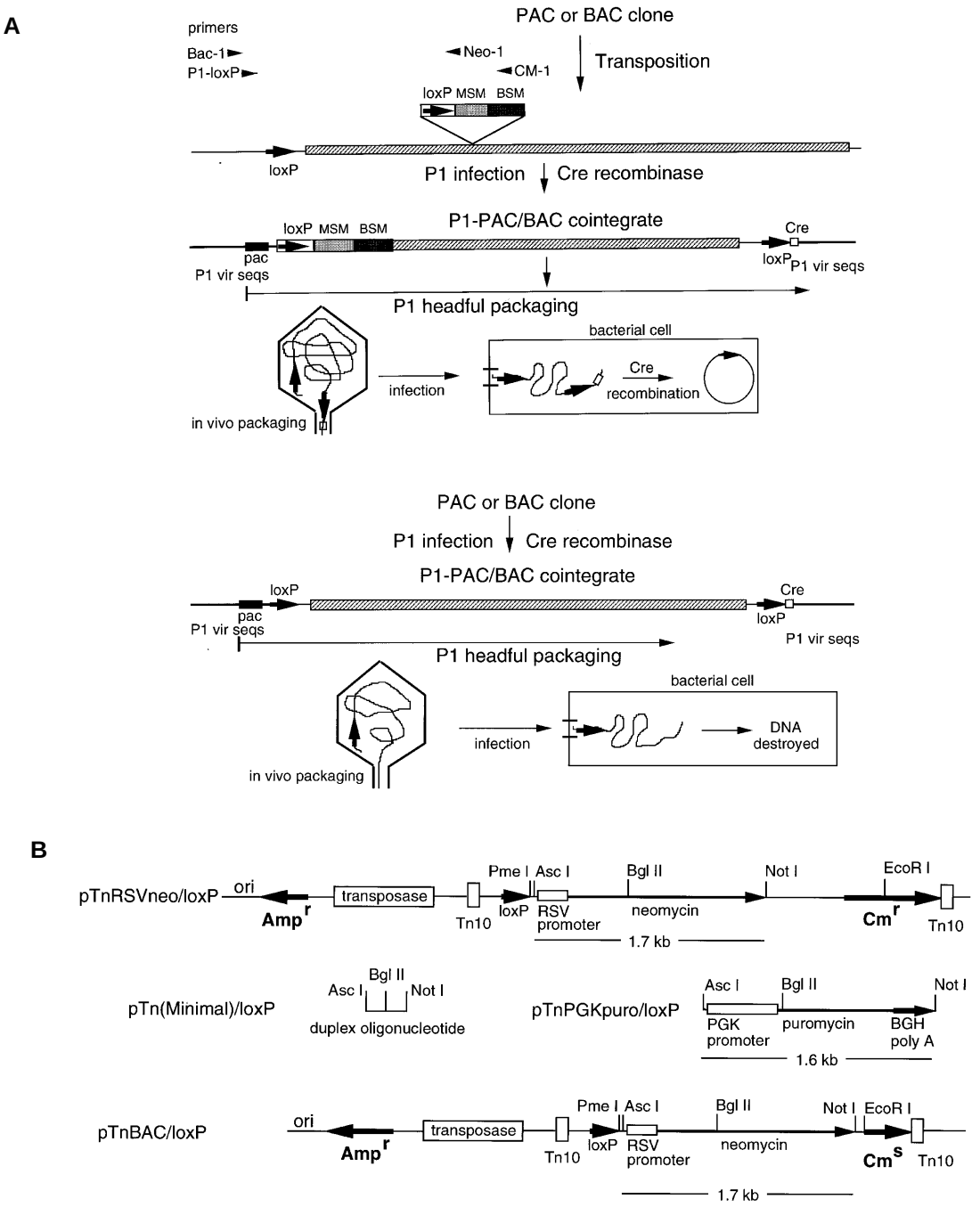

Figure 1. Scheme for selective recovery of PAC and BAC deletions. (**A**) Insertion of a transposon containing a *loxP* site, a bacterial selectable marker (BSM) and a mammalian cell selectable marker (MSM) into the genomic insert of a PAC or BAC clone is shown. Cre protein synthesized early during infection by P1 vir^s mediates recombination of *loxP* sites both to create a deletion in the insert and form the P1-PAC/BAC cointegrate. P1 packaging initiates at the *pac* site (supplied by P1 vir^s) and proceeds till the phage head is full. Packaging of the downstream *loxP* site and *Cre* gene enables the DNA between the *loxP* sites to be recovered as a circular molecule after infection. In the absence of packaging of the downstream *loxP* site, the DNA is destroyed after infection as indicated in the lower panel. The location and direction of PCR primers used to characterize PAC or BAC deletions are indicated. (**B**) Structure of four transposon donating plasmids used in this study. Important general features include a transposase gene located outside the 70 bp inverted repeat ends of the transposon (indicated here as open boxes Tn10) and a *loxP* site and bacterial resistance marker within the transposon boundaries.

Gel electrophoresis of large plasmid DNA

Plasmid DNAs digested with restriction enzymes were electrophoresed in 0.9% agarose gels in 1× TAE buffer (26) at 23 V for 18–20 h to analyse DNA fragments < 15 kb.

*Not*I digested P1, PAC or BAC DNAs were analyzed by FIGE in 0.7% agarose gels in 1× TBE buffer (26). Samples were electrophoresed for 1 h at 80 V without switching and then continued for 20–40 h with a switching regimen of 1 s forward, 0.3 s reverse (ramp factor 0.5). Gels were occasionally rerun after

staining with ethidium bromide and photography to enhance differences in mobility between linear PAC DNA fragments and small supercoiled transposon plasmid molecules.

RESULTS

Retrofitting clones with a *loxP* transposon

The PAC system was developed to clone greater than 100 kb DNA using a modified version of the P1 vector and replacing the *in vitro* P1 headful packaging and infection procedures with electroporation (11). The BAC vector is F-factor derived with different regulatory elements that do not allow increasing plasmid copy number with IPTG. BACs contain a chloramphenicol instead of a kanamycin resistance gene (9,22) and a *loxP* site, and thus plasmids from this system can be used for generating nested deletion sets as described earlier for P1 clones (17). Retrofitting was initiated by introducing the transposon donating plasmid into the same cell containing the PAC or BAC plasmid. The chloramphenicol resistance gene in pTnRSVneo/loxP, pTn(Minimal)/loxP and pTnPGKpuro/loxP and the neomycin–kanamycin resistance gene in pTnBAC/loxP are located within the 70 bp inverted repeat ends of the transposon and is inserted along with the *loxP* sequence into the target DNA during transposition while the ampicillin resistance gene and the gene encoding transposase enzyme which lie outside the boundaries of the transposon are left behind.

Treatment of cells containing a PAC or BAC plasmid and a transposon donating plasmid with IPTG induces expression of the transposase gene and increases PAC plasmid number (17,21,27). Transduction with P1 phage efficiently recovers the low percentage of retrofitted PAC or BAC DNA as a packaged phage. The infecting P1 vir^s provides the PAC or BAC plasmid with a *pac* site when forming the cointegrate via Cre mediated *loxP* recombination (Fig. 1A; ref. 17). Packaging initiated at the *pac* site allows recovery of that DNA which has undergone not only a transposon insertion but also a deletion large enough to enable the second *loxP* site in the cointegrate to fit into the P1 head such that the DNA is able to cyclize and survive after entering a suitable host. *Cre*$^-$ NS3516 cells were used for infection with the phage lysate because earlier studies (5,17) had indicated more efficient plasmid DNA recovery from this host. Again, Cre protein expressed early in infection from the *pac-loxP* P1 vir^s transduced fragment (Fig. 1A; ref. 17), circularizes the infecting DNA between the two *loxP* sites (21).

Isolating nested deletions in P1 and PACs

A P1 clone with a 90 kb DNA insert containing the mouse AP endonuclease (*apex*) gene and four PAC clones with inserts ranging in size from 30 to 170 kb were used in this study. Results summarized in Table 1 show that the P1/*apex* plasmid is efficiently transduced with P1 vir^s in the absence of a transposon donating plasmid and produces a large number of colonies resistant to kanamycin. Colonies resistant to both antibiotics arise only if a transposon plasmid such as pTn(Minimal)/loxP or pTnPGKpuro/loxP was originally present in the same cell as the P1/*apex* plasmid and appear 10% as frequently as those resistant to kanamycin alone.

Table 1. Recovery of P1 and PAC clones after retrofitting

	kan^r	$kan^r + cam^r$
P1 (apex) (90 kb insert)	++++	0
P1 (apex) + pTn(Minimal)/loxP	++++	++
P1 (apex) + pTnPGKpuro/loxP	++++	++
PAC-2 (120 kb insert)	0	0
PAC-2 + pTn(Minimal)/loxP	++	++
PAC-2 + pTnPGKpuro/loxP	++	++
PAC-5 (100 kb insert)	++	0
PAC-5 + pTn(Minimal)/loxP	++	++
PAC-5 + pTnPGKpuro/loxP	++	++
PAC-9 (30 kb insert)	+++	0
PAC-9 + pTn(Minimal)/loxP	+++	++
PAC-9 + pTnPGKpuro/loxP	+++	++

+, 5–10 colonies; ++, 20–50 colonies; +++, 100–200 colonies; ++++, 400–500 colonies.

Analysis of P1 DNA indicates that while colonies resistant to kanamycin alone contain P1/*apex* DNA indistinguishable from the parent, those isolated from clones resistant to both drugs contain either deletions or inversions. Lane 2 in Figure 2A shows a field inversion gel pattern of *Not*I digested DNA from the P1/*apex* clone without transposon insertions. Lanes 3 and 4 show *Not*I digests of DNA from two clones containing inversions while lanes 5 and 6 show the DNA of two clones with deletions. Note that transposon insertion introduces not only 3–5 kb of DNA sequence but also a *Not*I restriction site. The inversions contain two plasmid populations because the colonies selected on kanamycin and chloramphenicol plates did not undergo further single colony purifications (17). Therefore the sum of the molecular weights of DNA bands in a *Not*I digest is expected to be slightly more than twice the starting clone size.

*Bgl*II digests of the same DNA samples are probably more informative as seen in lanes 8–12 of Figure 2A. Because *Bgl*II sites are more frequent, the DNA from deletion clones (lanes 11 and 12) produce a pattern largely similar to the parent with clusters of bands from the deleted region absent. In contrast, DNA from the inversions (lanes 9 and 10) give a pattern with minor differences. Clones containing deletions produce higher yields of plasmid DNA than inversions.

PAC-9 with a 30 kb insert was expected to produce results similar to a P1 clone upon retrofitting. Table 1 indicates that although the results are qualitatively similar to P1/*apex*, the overall efficiency of isolating retrofitted clones is ~4-fold lower. Metabolic differences between the host strains containing these plasmids might be responsible for this.

In the absence of a transposon donating plasmid both PAC-2 (120 kb insert) and PAC-27 (170 kb insert) fail to produce kanamycin resistant colonies upon transduction with P1 vir^s (Table 1, data for PAC-27 not shown). The greater than headful length of DNA between the two *loxP* sites in the P1 vir-PAC cointegrate precludes the downstream *loxP* site from the P1 head when packaging is initiated at the *pac* site (Fig. 1A). Consequently the PAC DNA in the P1 phage derived from this cointegrate is destroyed upon entering a cell.

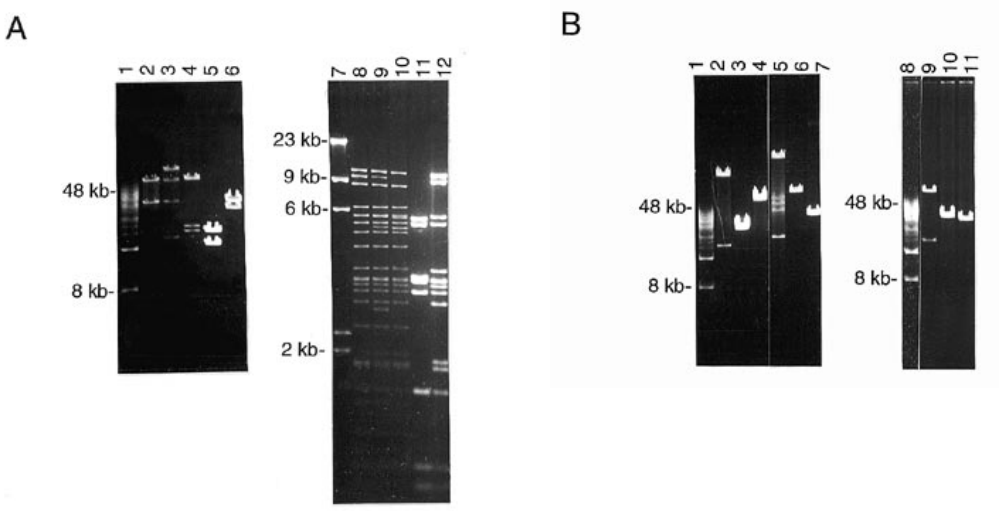

Figure 2. Analysis of plasmid DNA isolated from retrofitted P1/*apex*, PAC-2, PAC-27 and PAC-5 clones. (**A**) Lanes 2–6 display *Not*I digested DNA from P1/*apex* clones analysed by FIGE while lanes 8–12 show a *Bgl*II digest of the same DNA in standard gel electrophoresis. Lanes 1 and 7 show molecular weight standards. Lanes 2 and 8 contain the DNA isolated from clones prior to retrofitting. (**B**) FIGE of *Not*I digested DNA isolated from the starting PAC-2 clone (lane 2) and two of its deletion derivates, lanes 3 and 4. Lane 5 shows the DNA from the starting PAC-27 clone and lanes 6 and 7 show two of its deletion derivatives. DNA from PAC-5 and two of its deletion derivatives are shown in lanes 9 and 10, 11 respectively. Lanes 1 and 8 contain high molecular weight DNA standards.

The downstream *loxP* site in the cointegrate can be packaged into the same P1 head with the upstream one in either of two situations: (i) by creating a deletion large enough to reduce the size of the intervening PAC DNA; and (ii) by forming the cointegrate using the transposon-inserted *loxP* site rather than the PAC *loxP* site provided the inserted *loxP* is in the same orientation and placed sufficiently distant from it. The two pathways lead to identical products. Because Cre-mediated *loxP* recombination is much more efficient when the *loxP* sites are intramolecular rather than intermolecular, the first pathway is expected to operate (20). The analysis suggests that only clones undergoing large deletions would be efficiently recovered. It is not surprising therefore that all colonies arising from PAC-2 or PAC-27 retrofitted with a *loxP* site contain deletions as shown in lanes 2–7 of Figure 2B. Inversions arising from insertion of the transposon *loxP* site in an orientation opposite to that of the PAC *loxP* site do not allow their DNA to be recovered.

Clones such as PAC-5 which contain an insert of 100 kb DNA produce a unique pattern of colonies. This insert size appears to be slightly over the upper limit of 95 kb that is believed to be the insert-DNA packaging capacity of P1 phage (21). In the absence of a transposon plasmid, PAC-5 consistently produced only a few colonies upon transduction with P1 vir^{s} (10% as many produced by PAC-9) on kanamycin plates. This is in sharp contrast to PAC-2 or PAC-27 which produced zero colonies. The result is also distinctly different from PAC-9 or P1/*apex* which produced 10 and 40 times as many colonies respectively. Retrofitting with a *loxP* transposon produced colonies with only deletions at slightly higher frequencies than PAC-2 or PAC-27 as shown in lanes 9–11 in Figure 2B.

Isolating nested deletions in BACs

BAC clones produced nested deletions efficiently upon inserting a *loxP* site. The transposon TnBAC/loxP (Fig. 1B) used to retrofit BAC clones contains the RSVneo gene which confers resistance to kanamycin in *Escherichia coli* and neomycin in mammalian cells (28). Although BAC plasmids cannot be induced to multiple copies, they produced almost 10 times more colonies with

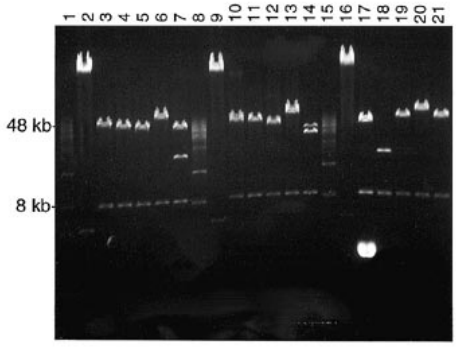

Figure 3. FIGE analysis of *Not*I digested DNA isolated from BAC deletions. Lanes 2, 9 and 16 contain the DNA from the starting clones BAC-10, BAC-11 and BAC-18 respectively. Lanes 3–7, 10–14 and 17–21 show the DNA isolated from five deletion derivates of BAC-10, BAC-11 and BAC-18 respectively. Lanes 1, 8 and 15 contain high molecular weight DNA standards.

deletions than PACs using similar conditions. Six BAC clones with inserts between 120 and 180 kb isolated from a human library were used in this analysis. As in PACs, P1 transduction alone failed to recover BAC plasmids of this size class. However, a less-than P1 headful-sized BAC clone with a 80 kb insert containing the human CLC5 gene was efficiently transduced under identical conditions (data not shown). BAC clones resistant to both chloramphenicol and kanamycin were recovered upon retrofitting. Analysis of plasmid DNA indicates the presence of large deletions of 40–90 kb. FIGE of *Not*I digested DNA from 15 such clones is shown in Figure 3.

Characterizing PAC and BAC deletions

Success in recovering PAC and BAC clones after retrofitting with a *loxP* transposon using the P1 transduction procedure relies upon the generation of large deletions. It is crucial therefore to determine whether the deletions in these large plasmids are due to genuine recombination between *loxP* sites mediated by Cre protein. PCR was used to demonstrate this.

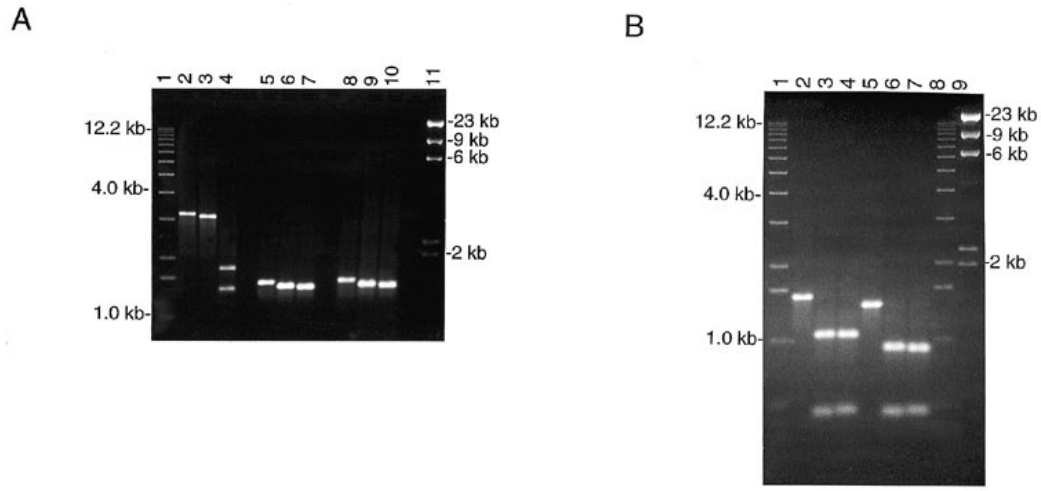

Figure 4. PCR analyses of DNA isolated from PAC and BAC deletions. (**A**) Gel electrophoresis of PCR product obtained with the primer set CM-1 and P1-loxP using the following templates: deletion derivatives of PAC-2 obtained by retrofitting with TnRSVneo/loxP (lane 2), and Tn(Minimal)/loxP (lane 5), a deletion clone of P1/*Apex* obtained by retrofitting with Tn(Minimal)/loxP (lane 8). Lanes 1 and 11 contain molecular weight standards. Lanes 3, 6 and 9 show *Pme*I digests and lanes 4, 7 and 10 show *Not*I digests of the DNA obtained from the PCR samples shown in lanes 2, 5 and 8 respectively. (**B**) Identical PCR analyses of DNA from BAC deletions. Lanes 2 and 5 contain the PCR product obtained by using the primer pairs Neo-1/Bac-1 and Neo-2/Bac-1 respectively, with DNA from the BAC-10 deletion shown in lane 7 of Figure 3 as template. Lanes 3, 4 and 6, 7 display the digestion pattern of the PCR products shown in lanes 2 and 5 with *Pme*I (lanes 3 and 6) and *Asc*I (lanes 4 and 7).

The sequence and location of primers are shown in Materials and Methods and Figure 1A. Primer CM-1 was designed from the N-terminus of the chloramphenicol resistance gene going outward toward the *loxP* site in the transposon and primer P1-loxP was designed from sequences between the P1 plasmid replicon and the *loxP* site in the P1 vector (sequences 13034–13013 in the pAd10SacB II map) such that primer extension by *Taq* polymerase would continue toward the *loxP* site in the P1 vector and into the genomic insert. A deletion between the *loxP* site existing in P1 or PAC and the transposed *loxP* site would generate a PCR product of ~3.2 kb when retrofitted with pTnRSVneo/loxP, a 1.5 kb product with pTn(Minimal)/loxP and a 3 kb product with pTnPGKpuro/loxP. DNA from a number of PAC deletion clones and a few deletion and inversion clones arising from P1/*apex* after retrofitting with different *loxP* site containing transposons was analysed by PCR. All PAC deletion clones arising from insertion of TnRSVneo/loxP transposon produce the expected 3.2 kb DNA band with digestion patterns predicted from the restriction map of the transposon (Fig. 1B) as shown in lanes 2 and 3, 4 of Figure 4A. PAC deletion clones and P1/*apex* deletion and inversion clones isolated after insertion of Tn(Minimal)/loxP transposon produced the predicted 1.5 kb DNA band with its characteristic digestion pattern shown in lanes 5 and 6, 7 and 9, 10 respectively, in Figure 4A. Note that the inversion product is a mixture of two plasmids (17), one of which has the same relative configuration of the two primers as the deletion product. P1 and PAC clones with deletions or inversions (for P1 and smaller PACs) retrofitted with the TnPGKpuro/loxP transposon failed to produce a PCR product under identical conditions although the frequency of clones obtained were indistinguishable from that isolated with the Tn(Minimal)/loxP transposon. The puromycin DNA sequence revealed a high GC content and this property could account for the lack of a PCR product due to formation of stable secondary structures in the template. Nevertheless, PCR reactions with transposon donating plasmids or the starting P1 and PAC plasmids as templates failed to produce the specific bands obtained with the PAC and P1 deletion clones (data not shown).

An identical analysis performed with several BAC deletions confirm that they also arise from genuine *loxP* recombinations mediated by Cre protein. PCR primers from the transposon plasmid pTnBAC/loxP were designed from sequences within the neomycin gene as shown in Figure 1A and Materials and Methods. These were paired in different combinations with primers designed from sequences within the BeloBAC vector in the region 6913–7024 and used in PCR reactions with the original BAC plasmids or deletions derived from them as templates. Four out of the nine combinations of primer pairs (one of each pair chosen from the neomycin gene and the other from the BeloBAC vector sequences) gave PCR products of the correct size with the BAC deletion clone templates as predicted (Fig. 4B). Digestion patterns of the 1.5 kb PCR product with *Pme*I (lanes 3 and 6) or *Asc*I (lanes 4 and 7) indicate that the products arise from the correct location on the template DNA. No products were observed when either the original BAC clone or the transposon donating plasmid pTnBAC/loxP were used as templates (data not shown).

Copurification of transposon donating plasmid with PAC and BAC deletions

Analysis of plasmid DNA reveals that ~10% of colonies with PAC or BAC deletion plasmids also contain the much smaller transposon donating plasmid. Examples of these are shown in lanes 2 and 3 of Figure 5 for PAC-27 retrofitted with either Tn(Minimal)/loxP or TnPGKpuro/loxP transposons respectively and lane 17 of Figure 3 for a BAC clone. The lowest molecular weight band in lanes 2 and 3 arises from linearized transposon plasmid since each contains a single *Not*I site (Fig. 1B). Although the transposon plasmid bands copurifying in these samples are of original size, small deletions are also observed occasionally (for example lane 17 in Fig. 3 and lane 13 in Fig. 5).

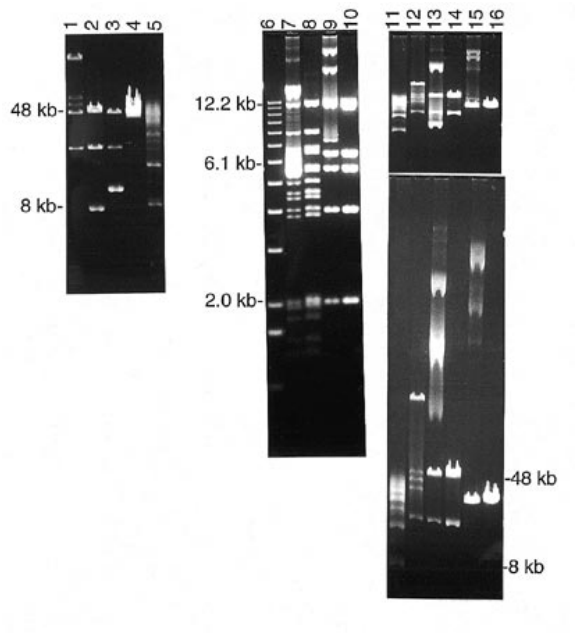

Figure 5. Analyses of transposon plasmid copurifying with PACs. FIGE analysis of *Not*I digested DNA from PAC-27 (lane 1), and deletion derivatives isolated after retrofitting with Tn(Minimal)/loxP transposon (lanes 2 and 4), and TnPGKpuro/loxP transposon (lane 3). Lanes 5 and 11 show high molecular weight DNA standards. Lanes 6–16 analyze loss of transposon plasmid by repeated single-colony purification of two PAC-27 deletions. Lanes 7–10 show the *Bgl*II digests of plasmid DNA after regular electrophoresis. Lanes 7, 9 and 8, 10 contain DNA from clones before and after the curing process respectively. FIGE analysis of the same samples is shown in lanes 13–16. Lane 12 contains DNA from PAC-27 before retrofitting. The upper panel in lanes 11–16 shows the ethidium bromide stained gel after 16 h of FIGE. The lower panel shows the same gel electrophoresed an additional 20 h after ethidium bromide staining.

Copurification of the transposon plasmid with PAC deletions was investigated with the goal of establishing conditions for eliminating the contaminating smaller plasmid. A *loxP* transposon plasmid similar to pTnPGKpuro/loxP but devoid of *Not*I or *Bgl*II sites (named as pTnpuro/loxP) was constructed (see Materials and Methods) and used to retrofit PAC-27. Purified plasmid DNA from two PAC-27 deletions is analysed in lanes 7, 9, 13 and 15 (Fig. 5). Note that the transposon plasmid appears as supercoils in these restriction enzyme digests.

The transposon plasmid copurifying with PAC or BAC deletions can be eliminated by removing the selection for antibiotic resistance expressed by the smaller plasmid (chloramphenicol for PACs and kanamycin for BACs). PAC deletion clones containing transposon plasmid were single-colony purified twice in the absence of selection for chloramphenicol. Plasmid DNA before and after single-colony purification is shown in lanes 7, 9, 13, 15, and 8, 10, 14, 16 respectively in Figure 5. The absence of supercoiled DNA bands in lanes 8, 10, 14 and 16 indicates that the PAC deletion clones are cured of the transposon plasmid. Cured PAC deletion clones continued to be resistant to chloramphenicol in addition to kanamycin, as expected from the presence of part of the transposon inserted in them. Identical results were obtained with BACs where the selection for kanamycin was removed during single-colony purification (data not shown).

DISCUSSION

Retrofitting plasmids from a genomic library should allow one to study the genes and regulatory sequences in a clone without resorting to laborious subcloning experiments. A procedure for retrofitting clones directly derived from a P1 library with a Tn10 mini-transposon containing a variety of sequence signals was described recently (17). Such a scheme is easily adaptable to the PAC system because identical regulatory elements operate in these two systems. Owing to their larger size PAC clones, after retrofitting, need to be recovered by electroporation into suitable hosts. While that is true for retrofitting in general, the present study focuses on one specific kind of retrofitting namely, the insertion of a *loxP* site and a mammalian cell-specific selectable marker into a PAC clone with the goal of generating nested deletion sets. This should prove highly useful not only in physical mapping studies of genes and regulatory sequences but also in the functional characterization of a clone by reintroducing it into suitable mammalian cells or animals. Although different DNA elements regulate BACs, the presence of the unique *loxP* site at one end of the insert DNA enables us to extend this retrofitting technology also to that system. Cre-mediated recombination between the *loxP* site in PACs or BACs and the one introduced via transposition can lead to two alternative events: deletion of the DNA between them if the two *loxP* sites are in the same orientation or inversion of the DNA if they are in the opposite orientation (19–21). In this study P1 transduction (5,17) was used to selectively recover PAC and BAC plasmids containing large deletions after retrofitting with a *loxP* transposon. Because the transduction with P1 vir^{s} provides Cre protein *in trans*, such deletions between *loxP* sites can occur in the same *cre*$^{-}$ host containing the PAC or BAC plasmid. This procedure therefore circumvents the need to transfer large PACs or BACs from their original hosts to a *cre*$^{+}$ background, minimizing potential disruptions in the DNA that might lead to rearrangements upon subsequent electroporation (11).

The transposon plasmid pTnRSVneo/loxP has been used in an earlier study (17). As noted in the previous study and described here in an earlier section, transduction with P1 vir^{s} efficiently transfers both the PAC deletion plasmid and the transposon plasmid by forming a packaging-competent cointegrate via the *loxP* site existing in either plasmid (Fig. 1B). The modest activity of the neomycin gene in *E.coli*, generating resistance to kanamycin, leads to a high background of clones (almost 10 times that of a retrofitted P1) resistant to both kanamycin and chloramphenicol arising not from a retrofitted P1 clone but originating instead from the starting transposon donating plasmid. This problem became more severe in PACs where only one out of 30 colonies resistant to kanamycin and chloramphenicol proved to be a PAC deletion (data not shown). As noted earlier (17), one could check for ampicillin sensitivity of clones resistant to both kanamycin and chloramphenicol and discard the large majority that are ampicillin resistant (Fig. 1B). In an effort to eliminate this problem we describe several new transposons in which the RSVneomycin gene is replaced by a duplex oligonucleotide as in pTn(Minimal)/loxP or a PGKpuromycin gene as in pTnPGKpuro/loxP (Fig. 1B). Both transposon plasmids gave identical results in retrofitting experiments, similar to those obtained with pTnRSVneo/loxP after the background of clones containing only the transposon plasmid was subtracted.

The ability of the RSVneo gene in pTnRSVneo/loxP to confer resistance to kanamycin in bacteria was exploited in the design of the transposon plasmid pTnBAC/loxP used for BACs. Since BACs contain the chloramphenicol resistance gene, the retrofitting plasmid was designed to be devoid of this resistance marker and the ability of the RSVneo gene to confer resistance to kanamycin was used as a positive selection for isolating clones with TnBAC/loxP insertions.

The occasional copurification of transposon plasmid with a PAC or BAC deletion plasmid (Figs 5 and 3) is not surprising since multiple less-than-headful size DNA molecules with *loxP* sites have been known to be packaged in a P1 head (29). After circularizing the P1 vir^s DNA forms cointegrates with both the *loxP* transposon plasmid and the PAC or BAC deletion plasmid. Yield of P1 vir^s-transposon plasmid cointegrates is probably higher in cells because the IPTG induction might not produce large numbers of PACs due to their slower replication compared with the smaller multicopy transposon plasmid. There should also be no selection between the two kinds of cointegrates during packaging of the DNA into a P1 head. Because more than one DNA molecule can occupy the same head, a transposon plasmid is capable of entering the same cell with the PAC plasmid at a low frequency and circularize via its *loxP* sites (29). It is also possible that due to the large intracellular pool of transposon plasmid, the majority of phage produced in the lysate contain only transposon plasmid DNA. Then one would speculate that DNA from more than one particle is stabilized in cells when this lysate is used in a subsequent infection. As long as the selection for both drugs is maintained, both plasmids will be propagated because they contain different replication origins. However the smaller plasmid is readily lost once the selection for its drug resistance is removed (Fig. 5). Deletions in the transposon plasmid are relatively rare and might arise from *loxP* site insertions into themselves.

The 10-fold higher yield of BAC deletions compared with PACs is somewhat difficult to explain. It is conceivable that the added load of replicating large PAC plasmids after IPTG induction is deleterious to cells. It is also possible that the relatively rare retrofitted PAC plasmid is inefficiently transduced by P1 vir^s because of competition by the large majority that do not contain a transposon. Distinctions between these and other possibilities can be made definitively with a transposon vector system in which the transposase gene is inducible independently of the P1 lytic replicon in PACs by something other than IPTG.

The results with PAC-5 suggest a degree of flexibility in the amount of DNA that can be packaged in a P1 head. In the absence of retrofitting, the slightly greater than P1 headful sized DNA in this clone is transduced infrequently by P1 vir^s. Restriction enzyme digests of DNA from several transduced clones show them to be indistinguishable from the parent (data not shown). The PAC-5 DNA appears to be packaged into the P1 head a little more tightly at times so as to include the downstream *loxP* site and *cre* gene. Alternatively, a slight variation in capsid dimensions in a small subset of P1 phage particles capable of packaging a few extra kilobases of DNA cannot be ruled out as a possible explanation.

In conclusion, we believe that the procedures described here for isolating large nested deletions in PACs and BACs should prove useful not only in high resolution mapping of genes and regulatory elements but also in developing strategies for creating internal deletions in these large plasmids for gene disruption or 'targeting' experiments.

ACKNOWLEDGEMENTS

We would like to thank Dr Nancy Shepherd for valuable suggestions and encouragement during the work, Drs David Smoller and Kirk Findlay of Genome Systems for gifts of PAC and BAC clones used in this study and for numerous helpful discussions, Dr Bruce Birren for providing the sequence of the BeloBAC vector, and Drs Allan Jaffe, Richard Hoopes, Jr and Joseph Dinchuk, for helpful discussions.

REFERENCES

1 Burke, D.T., Carle, G.F. and Olson, M.V. (1987) *Science* **236**, 806–812.
2 Murray, N. (1986) In Hendrix, R., Roberts, J., Stahl, F. and Weisberg, R. (eds), *Phage Lambda and Molecular Cloning, Lambda II.* Cold Spring Harbor Laboratory press, NY, pp. 395–432.
3 Pierce, J.C., Sauer, B. and Sternberg, N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2056–2060.
4 Hoheisel, J.D., Maier, E., Mott, R., McCarthy, L., Grigoriev, A.V., Schalkwyk, L.C., Nizetic, D., Francis, F. and Lehrach, H. (1993) *Cell* **73**, 109–120.
5 Sternberg, N., Smoller, D. and Braden, T. (1994) *Genetic Anal. Tech. Appl.* **11**, 171–180.
6 Shepherd, N.S., Pfrogner, B.D., Coulby, J.N., Ackerman, S.L., Vaidyanathan, G., Sauer, R.H., Balkenhol, T.C. and Sternberg, N. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2629–2633.
7 Smoller, D., Petrov, D. and Hartl, D. (1991) *Chromosoma* **100**, 487–494.
8 Pierce, J.C., Sternberg, N. and Sauer, B. (1992) *Mammal. Genome* **3**, 550–558.
9 Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y. and Simon, M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8794–8797.
10 Wang, M., Shouse, S., Lipes, B., Kim, U.-J., Shizuya, H. and Lai, E. (1996) *Genome Res.* **6**, 612–619.
11 Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A. and de Jong, P.J. (1994) *Nature Genet.* **6**, 84–89.
12 Rao, V.B., Thaker, V. and Black, L.W. (1992) *Gene* **113**, 25–33.
13 Sun, T.Q., Fernstermacher, D.A. and Vos, J.M. (1994) *Nature Genet.* **8**, 33–41.
14 Krizman, D.B. and Berget, S.M. (1993) *Nucleic Acids Res.* **21**, 5198–5202.
15 Nisson, P.E., Ally, A. and Watkins, P.C. (1994) *PCR Methods Appl.* **4**, S24–S39.
16 Ramirez-Solis, R., Davis, A.C. and Bradley, A. (1993) *Methods Enzymol.* **225**, 855–878.
17 Chatterjee, P.K. and Sternberg, N.L. (1996) *Genet. Anal. Biomol. Eng.* **13**, 33–42.
18 Abremski, K., Hoess, R. and Sternberg, N. (1983) *Cell* **32**, 1301–1311.
19 Hoess, R.H. and Abremski, K. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1026–1029.
20 Yarmolinsky, M. and Sternberg, N. (1988) *The Bacteriophages.* New York, Plenum, pp. 291–438.
21 Sternberg, N. (1994) *Mammal. Genome* **5**, 397–404.
22 Sheng, Y., Mancino, V. and Birren, B. (1995) *Nucleic Acids Res.* **23**, 1990–1996.
23 Sternberg, N. (1994) *Current Protocols in Human Genetics Supplement 1*, New York, Green and John Wiley and Sons, 5.3.17–5.3.20.
24 Cohen, G. and Sternberg, N. (1989) *J. Mol. Biol.* **207**, 99–109.
25 Silhavy, T.J., Berman, M.L. and Enquist, L.W. (1984) *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 93–94.
26 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27 Elliot, T. and Roth, J.R. (1988) *Mol. Gen. Genet.* **213**, 332–338.
28 McCormick, S.P.A., Linton, M.F. and Young, S.G. (1994) *Genetic Anal. Tech. Appl.* **11**, 158–164.
29 Coren, J.S., Pierce, J.C. and Sternberg, N. (1995) *J. Mol. Biol.* **249**, 176–184.