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Efficient Cre–lox linearisation of BACs: applications to physical mapping and generation of transgenic animals

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ABSTRACT

Due to the size of BAC, PAC and P1 clones, it is often difficult to construct detailed restriction maps, with large number of restriction fragments leading to ambiguity of mapping data. We report the use of Cre recombinase to linearise and asymmetrically introduce label at the unique loxP site of large loxP-containing clones. Subsequent partial digestion allows the direct ordering of restriction fragments. Additionally, BAC DNA linearised using the Cre–lox system has been used successfully to generate transgenic animals.

With the increased need to analyse large clones such as P1 artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) comes the inherent difficulties in mapping. Linearisation at a unique site such as cosN, which is cleaved by λ-terminase, has been used to map lambda and cosmid clones (1). Although BAC clones contain the cosN site, the method is not applicable to P1 or PAC clones. Additionally, the method requires indirect labelling of the partially restricted products using labelled oligonucleotides complementary to the right and left arms of the cleavage product, with inherent reduction in signal intensity. We have utilised a direct method of labelling BAC clones by linearising with Cre recombinase at the unique loxP site (2). The resultant linear molecule, labelled at either strand, can be partially digested with restriction enzymes, and the products analysed by pulse field, or field inversion gel electrophoresis. The partial mapping strategy is outlined in Figure 1a.

Two 40-base oligonucleotides representing the upper strand, lox 1, 5′-TCGACATAACTTCGTATAATGTATGCTATACGAAGTTATG-3′ and lower strand, lox 2, 5′-TCGACATAACTTCGTATAGCATACATTATACGAAGTTATG-3′ of loxP (3) (designed to generate *Sal*I-ends on annealing) were synthesized, using an ABI 381A DNA synthesizer. The deprotected oligonucleotides were purified by two extractions with *n*-butanol (4). Aliquots (20 pmol) of either lox-1 or lox-2 were end-labelled with T4 polynucleotide kinase (Boehringer Mannheim). The labelled oligo was annealed to 20 pmol of its unlabelled complementary oligo in 0.9 M NaCl, 9 mM EDTA, 90 mM Tris–HCl, pH 7.5, using a touch-down lift-off strategy (dropping from 98 to 60°C in 1°C steps—1 min melting steps alternating with 2 min annealing steps at 60°C) in the Hybaid Omnigene. Unincorporated radionucleotides were removed by spinning through a sephadex G-50-packed Spin-X column (Corning Costar Corporation, Cambridge, MA). Since the annealed products included both the required lox-1/lox-2 heterodimer, and also lox-1/lox-1 and lox-2/lox-2 homodimers, one can deduce that 10 pmol of the heterodimer was recovered, equivalent to 20 pmol of Cre recombinase binding sites (assuming 100% recovery).

Cre recombinase reactions were carried out with 1 µl Cre (1 mg/ml) (5) in 50 mM Tris pH 7.5, 33 mM NaCl and 5 mM spermidine with 1 µl (0.2 pmol of binding sites) of labelled loxP and 1.5 µg BAC DNA (prepared by standard alkaline lysis followed by two CsCl gradients), in a 30 µl volume. (1.5 µg of a 150 kb BAC clone contains 0.07 pmol of Cre recombinase binding sites.) After 1 h at 37°C, the reaction was terminated by heating to 70°C for 5 min, and buffer concentration was adjusted to accommodate the restriction enzyme of choice. Products of partial digestion (0.5 µg aliquots) were separated either by pulse field gel electrophoresis [PFGE separating between 20 and 200 kb; 1% Seakem agarose (FMC) in 0.5× TAE (20 mM Tris, 10 mM NaAc, 0.5 mM EDTA pH 8.2); using a CHEF system, 20 s pulse time, 180 V, 36 h], or by field inversion [separating between 5 and 100 kb; 1% Seakem GTG agarose (FMC) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA); using Hoeffer Switchback, 0.6–6.0 s pulse time, 3:1 ratio, 150 V, 28 h] prior to Southern analysis. A typical autoradiograph for a 140 kb BAC clone [BACN10, isolated from a 129 mouse BAC library; Research Genetics Inc.] and the resultant restriction map are presented (Fig. 1b and c).

To test the efficacy of Cre–lox linearisation for large scale production of linearised plasmid, the procedure was scaled-up, using 25 µg BACN10, 10 µl unlabelled loxP and 20 µg Cre. Following gentle extraction of the linearised DNA with phenol/chloroform/isoamylalcohol (P/C/I; 25:24:1), the aqueous phase was carefully mixed with an equal volume of molten 1% low melting point agarose (Ultrapure, Gibco BRL) in 0.5× TAE, and loaded on a preparative PFG (1% Seakem agarose, 22 s pulse time, 180 V, 42 h). Linearisation of the plasmid was almost complete, as judged by ethidium bromide visualisation. The 140 kb fragment was purified as described for YAC DNA (6). Following electrophoresis, the DNA was concentrated in 4% Nusieve (FMC) agarose, and recovered by β-agarase (New England Biolabs) treatment using agarase buffer supplemented with 100 mM NaCl. The resultant DNA solution was centrifuged briefly (Eppendorf centrifuge, 14 000 r.p.m.) and the supernatant carefully transferred to a millipore filter (0.05 µ) and dialysed extensively against injection buffer (10 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 100 mM NaCl). DNA integrity was checked by PFGE, and concentration adjusted to 1 ng/µl.

The fragment was microinjected, using standard protocols (7), into fertilized mouse eggs (CBA/Ca × C57BL/6J F1 mice), and 296 injected embryos were implanted into recipient females. Transgenic animals were identified by Southern blot hybridisation analysis,

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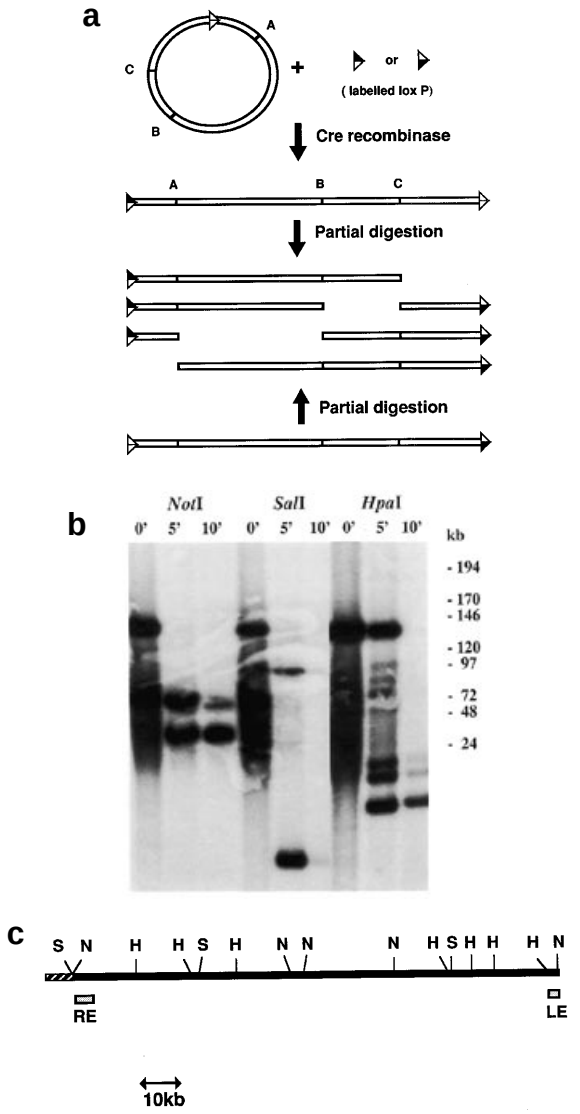

Figure 1. (**a**) Partial mapping strategy for analysis of loxP-containing clones, using Cre recombinase and loxP for linearisation, followed by partial digestion. Triangle denotes loxP sequence, shading represents ^{32}P incorporation into upper or lower strand. (**b**) Autoradiograph of partial mapping products of BACN10 clone. Partial digestions were in 30 µl final volume, using 5 U *Not*I (N), *Sal*I (S) or *Hpa*I (H), (Boehringer Mannheim). Samples (10 µl) were removed at the time points indicated, reactions were terminated (12.5 mM EDTA at 70°C for 10 min), P/C/I extracted and ethanol precipitated. After PFGE, DNA was transferred onto Boehringer positively charged membrane and exposed to X-ray film (or dried down and put to film directly). Migration of the mid-range PFG marker I (New England Biolabs) is noted. (**c**) Map of BACN10 generated by analyzing partial digestion products from each end of the clone, compared with fragments observed on complete digestion. The order of restriction fragments was directly ascertained, or predicted and confirmed by double digestion.

using a vector sequence-specific probe (pBeloBAC; Fig. 2a). Probes generated from the 5′ (RE) and 3’ (LE) ends of the insert (8), were also used to confirm copy number and analyse the integrity of the transgene (Fig. 2b and c). Gross observations from Southern blot hybridization analysis suggested that the transgene remained intact. From 43 pups weaned, five founders were identified, carrying the transgene at copy numbers ranging from 2 to 6.

In conclusion, we have utilised an efficient strategy for linearising BACs at the unique loxP site, which allows partial

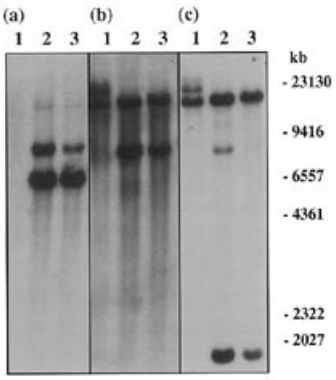

Figure 2. Southern blot hybridization analysis of a non-transgenic littermate (lane 1) and two transgenic founders (lanes 2 and 3) generated using BACN10. The blot was serially probed with: (**a**) pBeloBAC, which hybridised to a 6.5 kb vector-specific fragment, in addition to an 8 kb transgene-specific fragment; (**b**) RE probe; (**c**) LE probe. (See Fig. 1c for location of probes.) RE and LE recognise transgene-specific 8 and 1.9 kb fragments, respectively, in addition to high molecular weight endogenous bands. Migration of *Hin*dIII-digested lambda marker is noted.

mapping of the clones. We found almost complete linearisation of the plasmid, as judged by visualisation on PFGE, allowing large scale purification of the fragment. The Cre–loxP reaction was found to be sensitive to the quality of DNA, working most efficiently on supercoiled plasmid DNA. Although we have only used the method on clones up to 150 kb in size, it should be equally applicable to any plasmids with unique loxP sites, including larger BACs, PACs and P1 clones. [The sequence of the loxP site in the vector should be ascertained to check compatability with the synthetic loxP.] It should additionally be useful for analysing deletion or fusion products of large plasmids (9). Cre–lox linearisation is extremely efficient for large scale preparation of BAC DNA (requiring no detailed knowledge of the insert since loxP occurs within vector sequences) and has been successfully used to generate transgenic mice by microinjection.

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