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Rapid and efficient cosmid cloning

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ABSTRACT

We present a procedure for cosmid cloning that allows rapid and efficient cloning of individual DNA fragments of between 32kb and 45kb. By appropriate treatment of the cloning vector, pJB8, we make left-hand and right-hand vector ends that are incapable of self-ligation but which accept dephosphorylated insert DNA fragments. The inserted fragments are generated by partial digestion with *Mbo*I or *Sau*3A and are dephosphorylated to prevent ligation and insertion of non-contiguous fragments. The method eliminates the need to size the insert DNA fragments and prevents formation of clones containing short or multiple inserts. 1µg of target *Drosophila* DNA gives about $5x10^5$ clones, with an average insert size of 38kb. We also describe a rapid and efficient method for preparing plasmid and cosmid DNA.

INTRODUCTION

A major advance in the study of eukaryotic gene organisation has been the development of methods for the purification and propagation in bacteria of individual DNA fragments. Two major approaches have been adopted: (a) cloning of double-stranded cDNA copies of mRNA sequences into plasmids; (b) insertion of genomic DNA fragments into bacterial vectors. The former technique provides an immediate enrichment for expressed sequences but the latter permits the study of surrounding (and internal) control regions that are not necessarily represented in the mature mRNA. Initially, bacterial vectors (1) were used for genomic cloning but bacteriophage vectors have become more popular because they accept larger DNA inserts (approx. 15-20kb) and can be efficiently packaged *in vitro* into infectious phage particles (2,3).

Recently, plasmid vectors that can be packaged into λ phage heads ("cosmids") have been constructed (4). Cosmids accept larger inserts than λ phage (30-45kb) and can be efficiently introduced into host bacteria through *in vitro* packaging. Despite these apparent advantages, cosmids have not been much used, possibly because various side reactions that

reduce efficiency have been associated with their preparation. Even with sized chromosomal DNA fragments, cosmids can be recovered that contain non-contiguous DNA fragments ligated to form a single insert (5), leading to a distorted view of the original genomic organisation. Although the problem can be overcome by dephosphorylating the inserted DNA ("target"), this method is very sensitive to the exact ratio of target and vector DNAs (6) and tends to give smaller inserts because of vector-to-vector ligation.

In this paper, we present a method for cosmid cloning that overcomes both the above problems. Our procedure inserts unique DNA fragments of about 30-45kb into cosmid vectors with high efficiency, with none of the above side reactions. It also avoids the need to size the target DNA fragments. In addition, we describe quick and convenient protocols for both analytical and preparative DNA extractions of plasmids and cosmids.

MATERIALS AND METHODS

*Homer*I (amp^r) was constructed and donated by W. Chia (7). It is derived from the disabled plasmid pAT153 (8). *Sal*I and T4 DNA ligase was a gift of Dr. A. Udvardy. Unless otherwise indicated, enzymes were supplied by New England Biolabs Inc. and used according to their recommendations. Nitro cellulose filters were purchased from Sartorius GmbH.

Preparation of pJB8. *Homer*I DNA (1µg) was cut with *Eco*RI and the staggered ends filled in with T4 DNA-polymerase (2OU) in the presence of all 4 deoxy-triphosphates. Synthetic *Bam*HI linker (2.5µg; Collaborative Research) was phosphorylated with polynucleotide kinase (2U) and ligated to the linearised *Homer*I DNA with T4 DNA ligase (0.5U) and RNA ligase (7U) for 20 h at 16°C. After phenol extraction, the mixture was digested with *Bam*HI (2OU) for 1 h at 37°C, and the digested linker removed by chromatography on Sephadex G-15O. The DNA was circularised with T4 DNA ligase and used to transform Ca-treated HB1O1. pJB8 was one of the transformants and proved to have a *Bam*HI site in the position of the *Eco*RI site of *Homer*I (Fig. 1).

All the buffer changes and removal of phenol were achieved by spinning the reactions through Sephadex G-5O (0.8ml) in a 1ml syringe barrel packed with polymer wool (9).

Preparation of vector for cosmid cloning. pJB8 (12µg) was digested with either *Hin*dIII or *Sal*I (Fig. 2). Each reaction was terminated by incubating for 1O min at 7O° with 0.1% diethylpyrocarbonate (Sigma) and spun through a Sephadex G-5O column equilibrated with H_2O. The linear plasmids were each dephosphorylated with calf intestinal phosphatase (Boehringer; O.1U)

for 30 min at 37°C and the enzyme inactivated for 1 h at 70°C (10). The efficiency of linearisation and dephosphorylation was checked by ligation and transformation into HB101 and found to be better than 99.5%. (This is necessary to eliminate the formation of tandem vectors during ligation). The vector DNA was cut with *Bam*HI and its ability to religate was checked before use. An equimolar mixture of the *Bam*-cleaved dephosphorylated *Hin*dIII-treated pJB8 and *Bam*-cleaved dephosphorylated *Sal*I-treated pJB8 is used for cloning (Fig. 2).

Target DNA preparation. *Drosophila* DNA was made from adult flies (11). About 20µg DNA (from 100 flies) was digested with *Mbo*I in 200µl 10mM Tris pH 7.5, 10mM $MgCl_2$, 20mM NaCl, 10mM 2-mercaptoethanol for 10 min at 37°C with occasional gentle mixing. The reaction was stopped by heating to 70°C for 12 min. The extent of the above reaction was assayed by electrophoresis on a 0.2% agarose gel, supported by a bed of 0.8% agarose. The DNA was dephosphorylated for 40 min at 37°C with calf intestinal phosphatase (0.25U), and the phosphatase was inactivated at 70°C for 1 h. The nucleic acid was precipitated with ethanol and redissolved in 50µl TE (10mM Tris pH 8.0, 1mM EDTA).

Ligation and *in vitro* packaging. Target DNA was ligated with an excess of the vector mixture (Fig. 2). We used 3.5µl of vector DNA (240µg/ml) plus 1µl of target DNA in 8µl for 4 h at 22°C. *In vitro* packaging was by a modification due to V. Pirrotta (personal communication) of the method of Sternberg *et al.* (12) using strains BHB2688 and BHB2690 (13), and gives $\sim 5 \times 10^8$ pfu/µg λ DNA. In summary, a freeze-thaw lysate is made from BHB2688 and a sonic extract from BHB2690 and the DNA packaged in a putrescine-spermidine buffer. The packaged cosmids are diluted in phage buffer and stored at 4°C over $CHCl_3$.

Infection of host bacteria. HB101 (*hsdR*$^-$ *rec*A$^-$) is grown in L Broth containing 0.4% maltose until just saturating. After harvesting, the culture is stored 2-fold concentrated in 10mM $MgSO_4$. It is important that its *rec*A phenotype be tested by assaying UV sensitivity. The cosmids (in up to 20µl) are added to 10µl HB101, and incubated at 37°C for 10 min. 100µl L Broth is added and incubated for a further 30 min at 37°C. The mixture is spread on a 9cm dish of L Agar containing 100µg/ml ampicillin. Single colonies were picked for estimating insert size. For large scale screening of cosmid clones, the volumes were doubled and a 14cm dish used.

DNA extraction from plasmid or cosmid cultures. We have devised a rapid and efficient method of making DNA from analytical or preparative plasmid and

cosmid cultures by combining the methods of Davis *et al.* (14) with the alkaline lysis method of Birnboim and Doly (15).

Analytical DNA extractions ("miniprep"). 1ml of a saturated culture is harvested in a 1.5ml microcentrifuge tube (20 sec in a Beckman Microfuge), resuspended in 100µl 50mM Glucose, 25mM Tris pH 8.0, 10mM EDTA (Solution I) and incubated for 5 min at 22°C. 200µl 0.2N NaOH, 1% SDS (Solution II; made weekly from a 10N NaOH stock) is added, mixed gently and put on ice for 5 min. 150µl precooled 5M KOAc pH 4.8 (Solution III; final concentration = 3M KOAc+2M HOAc) is added, mixed gently, and after 5 min on ice, the precipitated protein, dodecyl sulphate and chromosomal DNA removed by centrifugation for 1 min. 2 vol. EtOH is added to the supernatant, incubated for 2 min at room temperature, and the nucleic acid is precipitated with a 1 min centrifugation. The pellet is washed with 70% EtOH, dried, and taken up in 50µl TE. 2µl portions (containing 100-500ng of DNA) are digested with restriction enzymes in buffers containing 2µg/ml boiled RNAase and analysed on horizontal agarose gels in a Tris-Borate-EDTA buffer. Cosmid DNA is purified from 5ml cultures using double the above volumes and precipitating the nucleic acid with 0.6 vol. iso-propanol.

Preparative extractions. For 1 litre cultures (or less) : The protocol for cosmid cultures is scaled up 40-fold and the DNA purified by isopycnic centrifugation in a CsCl-ethidium bromide gradient. (Residual acid should be neutralised with tris-base before centrifugation).

We find that yields of plasmid and cosmid DNA are superior to Triton-lysis methods and can be up to 10mg DNA/litre. Chloramphenicol amplified cultures can be similarly extracted.

High-density plating of cosmid clones. We have modified the screening method of Hanahan and Meselson (16). The bacteria are spread on a thick well-dried L Agar plate (+100µg/ml Ampicillin) and the colonies grown to diameters of 0.2-0.4mm. A dry nitrocellulose filter (not sterilised) is carefully layered onto the plate and allowed to wet. After marking the plate and filter with a needle, the filter is removed - most of the bacteria should adhere to the filter. The colonies on the master-plate are grown up again and the plate stored at 4°C in a sealed bag. The replica filter is placed (colonies up) on a chloramphenicol plate (200µg/ml in L Agar) and a second dry filter carefully placed on top to form a sandwich. The second filter is marked and the sandwich incubated overnight at 37°C. The sandwich is removed and worked up as follows: 30 sec in 0.5N NaOH; 2M NaCl; >1 min in 0.5M Tris pH 7.0; 3M NaCl. The 2 filters are peeled apart to give 2

duplicate filters and washed separately in 2xSSC, 0.1% SDS (removing bacterial debris with a tissue) and 2xSSC (5). (SSC is 15mM tri-sodium citrate, 0.15M NaCl). They are blotted dry and baked at 80°C. Master plates can be stored up to 6 weeks. If they are not allowed to dry, the filters can be screened with a succession of probes, removing previous hybridisation with 0.1M NaOH (10 min at room temperature). Hybridisation to nick-translated probes is as described by Jeffreys *et al.* (17).

RESULTS

Cosmid vector pJB8

We have modified the 5.3kb cosmid vector *Homer*I (gift of W. Chia) by introducing a synthetic *Bam*HI linker into its *Eco*RI site (Fig. 1, see Materials and Methods). This allows insertion of DNA fragments generated by partial cleavage with *Mbo*I or *Sau*3A. The *Bam*HI linker has recreated flanking *Eco*RI sites so that *Eco*RI excises the inserted DNA from hybrid cosmids (Fig. 1).

Fig. 2 illustrates our cloning procedure that allows the dephosphorylation of both target and vector in such a way as to overcome the side-reactions of: (a) cosmids containing non-contiguous inserts, (b) cosmids containing multiple vectors and multiple inserts. If we cut pJB8 with *Hin*dIII, dephosphorylate, and then cut with *Bam*HI, we make a left-hand vector *cos* fragment that cannot ligate in tandem but which accepts dephosphorylated target DNA. Similarly, cutting pJB8 with *Sal*I, dephos-

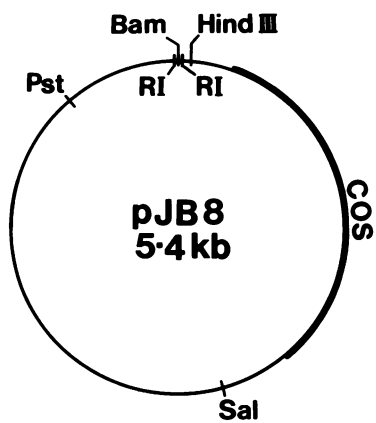

Figure 1. Restriction map of pJB8. The thickened region represents the BglII fragment from λ that contains the cos site (7).

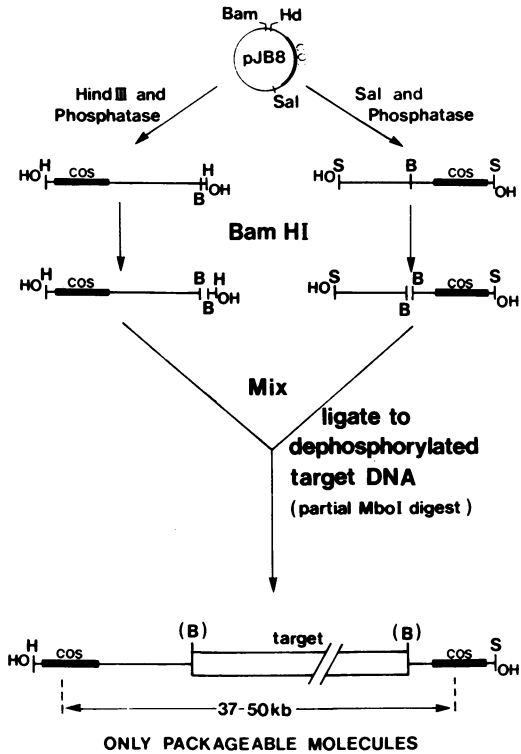

Figure 2. Cosmid cloning method

phorylation, and cutting with *Bam*HI yields a right-hand vector *cos* fragment. Neither digest alone can be used as a cloning vector as only molecules with 2 *cos* sites separated by 37-50kb (18) can be packaged, but an equimolar mixture of the two digests will allow packaging of appropriately sized target fragments (Fig. 2). Dephosphorylation of the linear vector molecules prevents the formation of tandem vectors and eliminates background colonies lacking inserts.

Target DNA is made by partial *Mbo*I digestion of large genomic DNA to a mean size of 35-45kb followed by dephosphorylation with calf intestinal phosphatase. The frequency of *Mbo*I sites (about every 250bp on average) means that such fragments are essentially random. Partial digestion with restriction enzymes recognising a 6bp sequence can lead to selective loss of specific fragments (6). Ligation of an excess of vector-mixture to dephosphorylated target DNA gives only one kind of packageable molecule

(Fig. 2) : those containing the left-hand *Hin*dIII-*Bam*HI *cos* fragment, a target fragment of 32-45kb and the right-hand *Sal*I-*Bam*HI *cos* fragment. Dephosphorylation of both vector and target DNA eliminates all the side reactions described above. Moreover, because of the size-specificity of the packaging reaction, it is not necessary to purify the correct size-fraction of the target DNA from the partial digest.

We have packaged *Drosophila* DNA prepared from adult flies and made cosmid "libraries" by infecting HB101. We recover about $5x10^5$ clones/μg of *Drosophila* DNA. (The estimate of DNA concentration is only approximate). As a guide, we recover about 240,000 clones from 2 fly-equivalents of DNA, an efficiency of about 10^5 clones/adult fly.

Characterisation of the cosmids.

We have assayed the integrity of our cosmids by analysing "minipreps" of randomly picked clones (see Materials and Methods). Fig. 3 shows *Eco*RI digests of DNA from 16 clones. The *Eco*RI sites that flank the site of insertion in pJB8 (Fig. 1) mean that *Eco*RI cuts out a characteristic vector band and that the insert can be sized by summing the remaining bands. Fig. 3 shows that each clone has only a single vector fragment and hence only a single insert. (Where the vector band is overly intense, it separates into 2 bands on a 0.3% gel).

The clones in Fig. 3 have an average insert-size of 33kb ± 3kb (n=16), somewhat smaller than expected. This may be due to our failure to check the *rec*A phenotype of the HB101 host as recombination within the insert would give rise to smaller cosmids. A subsequent experiment using a pure *rec*A HB101 stock had inserts sized 38.8kb + 3.7 kb (n=14). This (32kb-45kb) is the size range expected from the specificity of packaging and suggests that cosmids are stably propagated.

DISCUSSION

Cosmid cloning allows the isolation of large regions of the genome intact. However, previous methods of preparing cosmids have tended to give unsatisfactory clones that were either shorter than expected, or had potentially misleading non-contiguous inserts. The cloning protocol presented in Fig. 2 overcomes both side reactions. It is designed for the pJB8 vector but can be adapted for other cosmid vectors. Any two restriction enzymes that give a left-hand and a right-hand *cos* fragment will suffice, *e.g.* in our scheme, *Cla*I could be substituted for *Hin*dIII and *Ava*I can be substituted for *Sal*I. As sizing of the insert DNA is avoided,

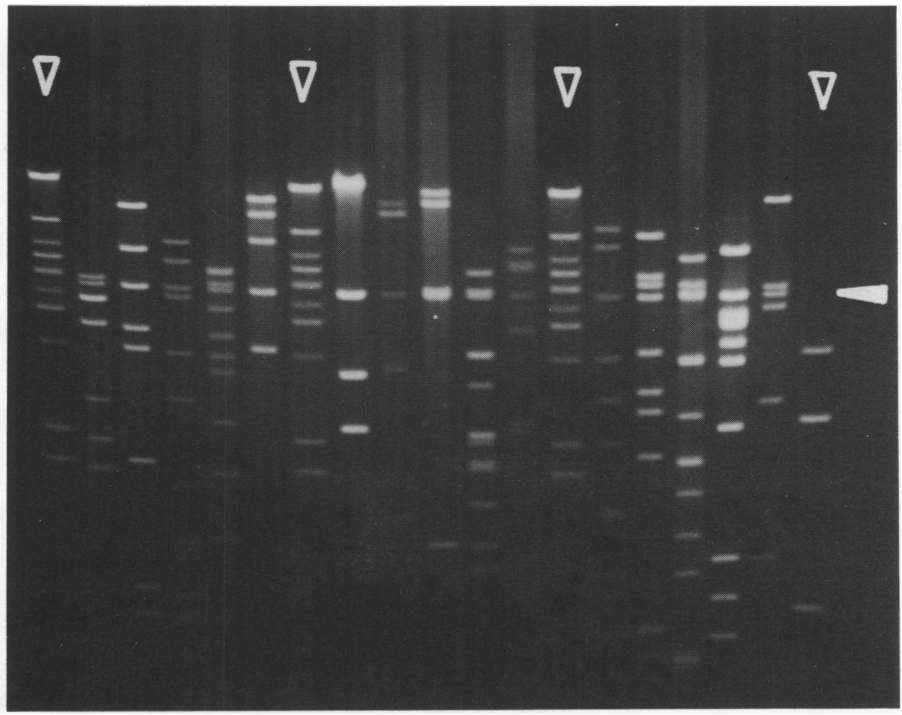

Figure 3. *Eco*RI-digests of cosmid DNA. 2µl of analytical-scale DNA preparations (Materials and Methods) from randomly picked cosmid clones were digested with *Eco*RI and run on a 0.8% agarose gel. Tracks containing marker DNA fragments are indicated by the hollow arrowheads. The pJB8 band is indicated by the solid arrowhead. Larger DNA fragments were sized on a 0.3% agarose gel.

lowered efficiency of cloning due to loss or breakage of the target DNA is minimised. Moreover, the complex manipulations necessary when using sheared DNA fragments as target (19) are avoided by the use of *Mbo*I digestion to give essentially random fragments.

The lack of side-reactions and elimination of background transformants mean that cosmid cloning is an alternative to phage λ for the recovery of chromosomal DNA fragments. The choice between the two will depend on circumstances, but we note some advantage of using cosmids. Fewer colonies need be screened. The high density screening method of plasmids (16) is fully applicable to cosmids so that a complex eukaryotic genome can be screened on a few filters. For *Drosophila*, 4300 colonies are a genome-equivalent and the probability of detecting a specific sequence is 0.95 if

13,000 clones are screened. The packaged cosmids are stable refrigerated for many months and our preliminary experiments suggest that they can be quick frozen and stored at -70°C in the presence of 10% glycerol without loss of infectivity. Using a simplification of the Hanahan-Meselson colony screening method (Materials and Methods), we have recovered several cosmid clones derived from the 87A7 and 87C1 heat-shock loci and from the 63F ecdysterone-induced locus (unpublished results). They have inserts sized between 32kb and 38kb and appear to propagate stably (unpublished observations). The high efficiency of cosmid production allows direct screening of recombinants, avoiding the loss of poorly growing clones that occurs during amplification. The clones can be stored frozen on filters (16), or freshly screened as required. Because of the larger insert, cloning of large chromosome regions ("walking") can be more rapidly achieved than with λ. Single cosmids can give up to 45kb of DNA and it is easy to prepare end fragments as probes for walking because the insert can be excised exactly with *Eco*RI.

Our experience has been that cosmids can propagate without noticeable instability. Thus, we find only appropriate-sized inserts and no evidence of reduction in size during propagation. The use of a *rec*A host is clearly important and we can say that most cloned sequences appear stable. Moreover, clones containing up to four copies of SV40 (7) or two β globin sequences (5) appear to propagate without excision. Instability during propagation in phage λ has been reported for duplicated α-globin or polyoma sequences (20,21,22).

The analytical and preparative DNA isolation procedures described give high yields of both cosmid and plasmid DNA. It takes about 60 min to make DNA from 20 cultures suitable for restriction enzyme digestion. After phenol extraction and gel filtration, the DNA can be used for subcloning or *in situ* hybridisation to polytene chromosomes (unpublished observations). The same method can be used to purify any covalently closed circular DNA and has been used to prepare extrachromosomal circular molecules from *Drosophila* tissue culture cells (A.J. Flavell and D. Ish-Horowicz, manuscript in preparation).

A final advantage of the use of cosmids lies in the desire to achieve *in vivo* expression of cloned DNA. Experiments on the nuclease sensitivity of native chromatin suggest that differences in chromatin configuration of active and inactive genes extend outside the directly transcribed regions (23). If correct chromatin packaging requires the flanking regions, the integrity of large DNA fragments may be necessary for correct expression.

Indeed many eukaryotic genes extend over long regions due to their interruption by non-coding intervening regions. In such cases, correct *in vivo* expression of cloned DNA may demand the use of the long DNA fragments that are only obtainable with cosmid cloning.

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