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pUR 250 allows rapid chemical sequencing of both DNA strands of its inserts

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ABSTRACT

A multipurpose plasmid, pUR 250, has been constructed. It contains seven unique cloning sites (Hind III, Xba I, Sal I, Acc I, Hinc II, BamH I and EcoR I) in a small region of its lac Z gene part. Insertion of foreign DNA into the plasmid can be easily detected. Plasmid DNA isolated from a clone by a rapid method can be used to determine the DNA sequence of the insert from both ends directly without isolation of labeled fragment.

INTRODUCTION

DNA sequencing has become one of the most important tools for the analysis of pro-and eukaryotic genes. Efforts to simplify the sequencing routine have been reported for the enzymatic (1,2) and the chemical method(3). DNA cloned into the plasmid pUR 222 (3) can be sequenced rapidly by the Maxam & Gilbert method (4), because use of this plasmid obviates the need to isolate labeled fragments for sequencing. However,this simplification applies only to sequencing of cloned DNA in one orientation.

Here a new plasmid, pUR 250, is described which overcomes this limitation. The plasmid contains seven unique cloning sites, Hind III,Xba I, Sal I, Acc I, Hinc II, BamH I and EcoR I. Cloning in any of these sites can be monitored on appropiate indicator plates by the appearance of white colonies in a background of blue colonies which contain only vector DNA (5). DNA cloned into the central sites Sal I, Acc I and Hinc II, can be sequenced from both ends without isolation of labeled fragments, because the cloned fragment can be labeled selectively in the lower or upper strand. Plasmid DNA from mini preparations is sufficently pure to derive sequence information up to 600 bases.

MATERIALS & METHODS

Plasmids pUR 219, pUR 222 and pUC 9 have been described in ref.(3,6).

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E.coli K12 RRIΔM15 is a derivative of RRI (7) and has the the genotype : leu pro thi strA hsd r^- m^- lac ZΔM15 F' lac I^Q ZΔM15 pro^+.

Synthetic oligonucleotide fragments dAGCTTTCTAGAG and dTCGACTCTAGAA were purchased from Applied Biosystems, USA.

Restriction enzymes Xba I, Hinc II and Acc I were from Biolabs, USA, Hind III, BamH I and EcoR I from Boehringer,Mannheim and Hae II, Hae III and DNA polymerase I (large fragment) from BRL, Neu-Isenburg. T4 DNA ligase was isolated from a lambda lysogenic strain (8,9). All reactions with restriction enzymes were performed as described (3).

Preparation of plasmid DNA: 5 ml over-night culture of plasmid carrying cells are collected by centrifugation and lysed gently at room temperature with 1 ml lysis mix (1 % Brij 58, 0.4% Na-deoxycholate, 50 mM Tris-HCl, 50 mM EDTA pH 7.6) after 10 minutes incubation with 25% sucrose (in 50 mM Tris-HCl pH 7.6) ,0.1 ml lysozyme solution (10 mg/ml) and 0.1 ml 0.5 M EDTA pH 7.6. After centrifugation for 30' at 20 000 rpm (Sorvall,SS34) the supernatant is added to 0.5 ml of a solution containing 5 M $NaClO_4$ (final concentration 1 M). The solution is extracted twice with chloroform, DNA is collected by the addition of 0.5 ml isopropanol and centrifugation for 5' at 15 000 rpm (Sorvall,SS34). The dried pellet (15',RT) is dissolved in 100 ul DNA buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.6). DNA prepared in this way is pure enough for subsequent cloning and sequencing steps.

Labeling of DNA has been described in ref.(3).

RESULTS

Construction of plasmid pUR 250

Since the Pst I site of plasmid pUR 222 and also pUC 9 (6) can not be labeled in a DNA polymerase I catalysed reaction, this site was changed into an Xba I site.

Therefore a duplex of two 12 bp synthetic DNA fragments(see Materials & Methods) containing an Xba I site and Hind III and Sal I ends were cloned into the plasmid pUC 9 (see Fig.1). This was done without phosphorylation of the synthetic DNA to avoid polymeric forms of this DNA. The plasmid pUR 245 contains all the sites desired, but the polylinker region results in a frameshift and leads to a lac α^- phenotype. Therefore the polylinker regions of pUR 222 and pUR 245 were combined (Fig.2) and as a result plasmid pUR 250 was obtained (Fig.3).

Shotgun cloning into plasmid pUR 250

To demonstrate the general use of the plasmid pUR 250, the blunt end

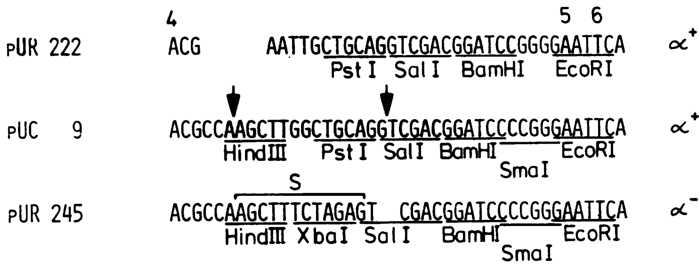


Fig. 1 Construction of plasmid pUR 245. Plasmid pUR 245 was constructed by insertion of a duplex of two synthetic DNA fragments containing an Xba I site between the Hind III and Sal I sites of plasmid pUC 9 (6). The cutting positions of these enzymes are indicated by arrows. Nonphosphorylated synthetic DNA fragments were used to avoid the generation of polymeric forms in the ligation reaction. The insertion of the DNA fragments (S) generates a frameshift as indicated by a gap in the sequence of pUR 245. In order to re-establish a correct reading frame the polylinker regions of pUR 245 and pUR 222 were combined (see Fig.2). Codon numbers of the original lac Z gene are given above the sequence.

Hinc II site was used for cloning. Cloning into the site of restriction enzyme Hinc II is difficult because enzyme batches of all firms tested contain a second nucleolitic activity. Cutting with Hinc II leads to deletions at the end of DNA cut and these deletions may have the same effect as inserts (see below).

0.5 ug DNA of pUR 250 was cleaved with Hinc II and 1 ug of an EcoR I fragment containing the lac Z gene of E.coli was cleaved with Hae III. Both DNA digests were mixed and ligated in a volume of 50 ul. E.coli RRIΔM15 cells were transformed with 1 ul of the ligation mixture. This strain was used because it yields the highest amount of plasmid DNA in the mini preparation described in Materials & Methods. Nearly 800 colonies were obtained on indicator plates (3,5). 40% were white or light blue, the rest dark blue. Plasmid DNA from white and light blue colonies was prepared and analysed by cutting with Hind III and EcoR I. 50% of these plasmids contained inserts. These results show that Hinc II can be used for cloning of blunt ended DNA fragments with a total efficiency of 20 % recombinant clones.

Sequencing of DNA from one clone in both orientations without isolation of the DNA fragment to be sequenced

The general scheme of the preparation of DNA for sequencing is shown in Fig.4. Some of the Hae III lac Z gene clones were sequenced in the following way: 5 ul (0.5 -1 ug of DNA) of the plasmid preparation were digested in 20 ul volume of buffer with BamH I or Xba I. Labeling, recutting with EcoRI

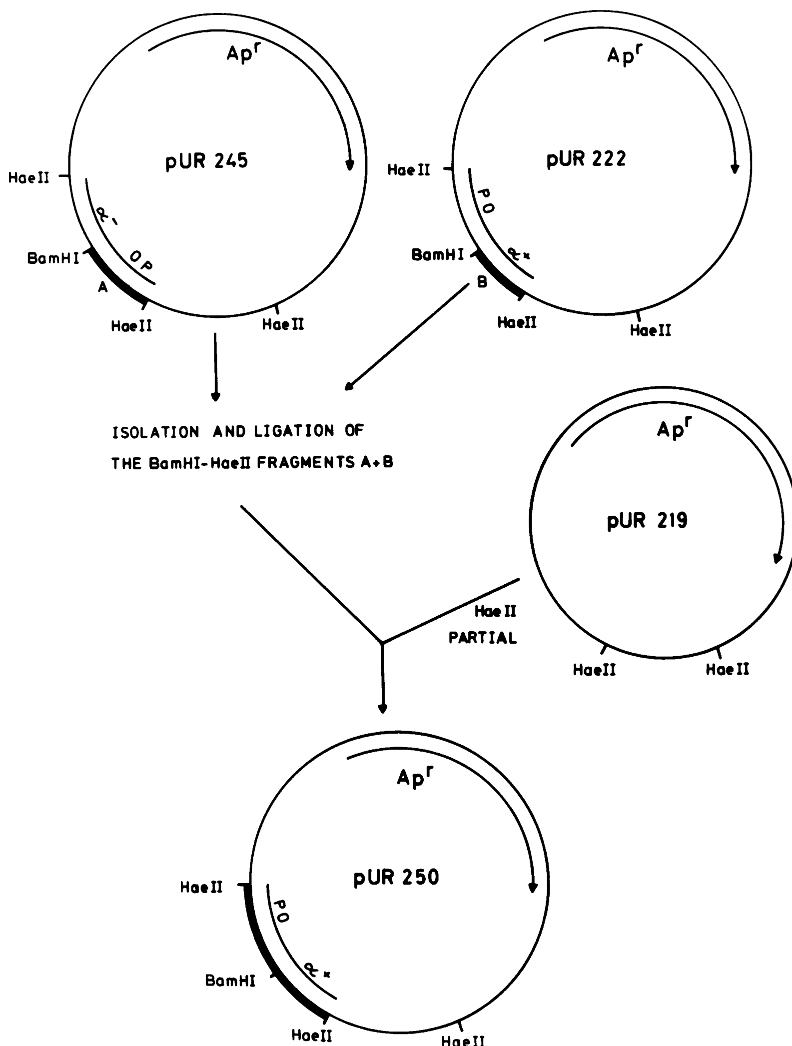


Fig. 2 Construction of plasmid pUR 250. The combined polylinker regions of plasmids pUR 245 and pUR 222 contain all desired restriction sites and allow the expression of an active α - peptide of the lac Z gene. Therefore the BamH I - Hae II fragments A and B of these plasmids were isolated and ligated. After recutting with Hae II these fragments were cloned into the partially Hae II digested plasmid pUR 219. This plasmid is identical to pUR 222 but does not contain the Hae II fragment carrying the lac promotor, operator and the beginning of the lac Z gene which codes for the α - peptide. As a result plasmid pUR 250 was obtained.

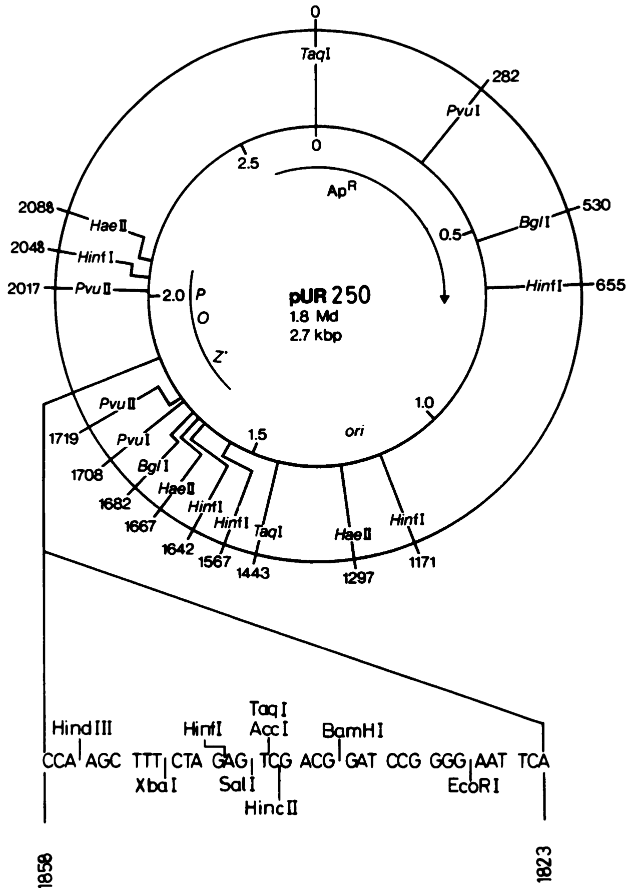
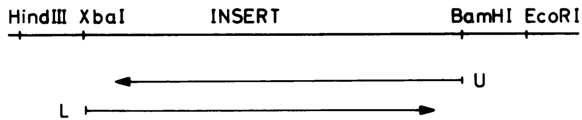


Fig. 3 Detailed restriction map of the plasmid pUR 250

or Hind III and purification of this DNA were done as described in (3). DNA was sequenced according to the procedure of Maxam & Gilbert (4). A typical autoradiogram is shown in Fig.5. There seems to be a compression of the sequence (see arrows in Fig.5) in the lower strand (labeling of the Xba I site) which does not occur in the sequence ladder of the upper strand (labeling of the BamH I site). This is a good example for the immediate correction of a sequencing artefact by simultaneously getting information of both strands in this vector. The sequence of the lac Z Hae III fragment codes for the amino acid residues 881 - 896 of the known β- galactosidase protein sequence (10).



A. UPPER STRAND (U)
BamH I
FILL-IN WITH POLI (COLD G, HOT A)
EcoRI
SEQUENCING

B. LOWER STRAND (L)
Xba I
FILL-IN WITH POLI (HOT C)
Hind III
SEQUENCING

Fig. 4 Preparation of inserts cloned into the plasmid pUR 250 for sequencing. Details of the procedure are described in Results and ref.(3). The labeling of the BamH I site was carried out with dGTP and $[\alpha^{32}P]$ dATP because this protocol yields a higher incorperation of isotope than labeling with $[\alpha^{32}P]$ dGTP alone.

DISCUSSION

In a previous paper the method of direct sequencing with plasmid DNA was described (3) and some problems were discussed which have now been overcome: Previously plasmid DNA isolated by the method of Birnboim & Doly (11) was used. On the gels a background of faint bands in all positions was usually observed. To distinguish between C and T was sometimes impossible. To eliminate this problem I compared different strains of E.coli and different methods of plasmid preparation. As described in Results the strain RRIΔM15 gives high yield of plasmid DNA when the other method of DNA preparation is used (see Materials & Methods). The sequence shown in Fig.5 is a typical result.

In addition, by the development of the plasmid pUR 250, it is now possible to sequence both strands of DNA from one preparation. An obvious advantage is that a given fragment does not have to be cloned in both orientations to obtain the sequence information in order to overcome possible errors such as compression in one strand (see Fig.5).

The plasmid pUR 250 can be used to clone all types of DNA fragments. In the experiment described in Results I have shown that blunt end cloning into the Hinc II site of pUR 250 can be carried out with high efficiency. Fragments created by restriction enzymes Hpa II or Taq I can be cloned into

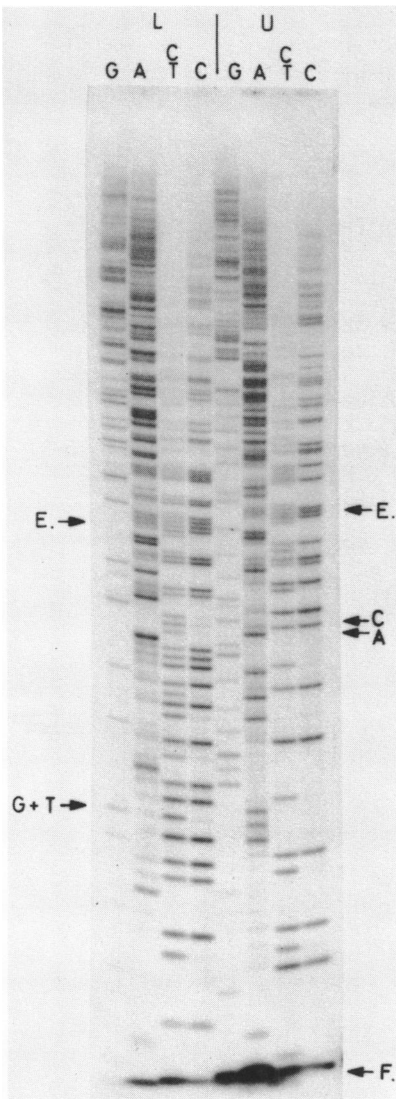


Fig. 5 Direct sequence analysis of both DNA strands of a insert in pUR 250.
A Hae III fragment of the lac Z gene was cloned into the Hinc II site of plasmid pUR 250. Plasmid DNA was prepared by a rapid method as described in Materials&Methods and sequenced without isolation of the labeled fragments. The autoradiogram of the sequence on a 12% polyacrylamid gel is shown. The sequence codes for the amino acid residues 881-896 of β-galactosidase (10). The sequence of the lower strand(L) shows a compression of two bases(G+T) at one position but the sequence of the upper strand(U) clearly shows C and A in two positions at the complementary strand. The small labeled fragments obtained by the recutting step(see Fig.4) are presented on the autoradiogram(F). The ends of the inserted DNA fragment are indicated (E).

Acc I site, fragments with any ends can be cloned after tailing into a tailed Hinc II, Sal I or any of the other sites (12).

This system now routinely yields sequence information up to 300 bases in either direction in a minimum of time and effort. Therefore in combination with the modified procedure plasmid pUR 250 is a real improvement in DNA sequencing.

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REFERENCES

1. Messing,J.,Crea,R. and Seeburg,P.H. (1981) Nucl.Acids Res.9, 309 - 321
2. Sanger,F.,Nicklen,S.and Coulson,A.R. (1977) Proc.Natl.Acad.Sci.USA 74, 5463 - 5467
3. Rüther,U.,Koenen,M.,Otto,K.and Müller-Hill,B. (1981) Nucl.Acids Res. 9, 4087 - 4098
4. Maxam,A.M.and Gilbert,W. (1980) in: Methods in Enzymology Grossman,L. and Moldave,K.,eds,Vol.65, pp.499 - 560, Academic Press,New York
5. Rüther,U. (1980) Molec.gen.Genet. 178, 475 - 477
6. Messing,J. (1981) in Third Cleveland Symposium on Macromolecules: Recombinant DNA, ed.A.G.Walton, pp.143 - 153,Elsevier,Amsterdam
7. Bolivar,F.,Rodriguez,R.L.,Betlach,M.C.and Boyer,H.W. (1977) Gene 2,75-93
8. Murray,N.E.,Bruce,S.A.and Murray,K. (1979) J.Mol.Biol. 132, 493-505
9. Tait,R.C.,Rodriguez,R.L. and West,jr.R.W. (1980) J.Biol.Chem. 255, 813 - 815
10. Fowler,A.V.and Zabin,J. (1978) J.Biol.Chem. 253, 5521 - 5525
11. Birnboim,H.C.and Doly,J. (1979) Nucl.Acids Res. 7, 1513 - 1523
12. Rüther,U.,Koenen,M.,Sippel,A.E.and Müller-Hill,B. (1982) Proc.Natl.Acad. Sci.USA, submitted