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Immunoenzymatic detection of expressed gene fragments cloned in the *lac Z* gene of *E. coli*

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We describe a method which permits the detection of exon fragments. Such DNA was cloned and expressed in the promoter proximal part of the *lac Z* gene of *Escherichia coli*. The resulting antigen-β-galactosidase chimeras are bound to their respective antibodies fixed to polyvinyl sheets. The β-galactosidase part of the chimera permits detection of such clones by histochemical staining. As model DNA, we used the *lac I* gene cleaved with *Hae*III, *Hha*I, or *Hpa*II. Fragments were tailed with poly(dC) and inserted into the poly(dG)-tailed promoter proximal part of the *lac Z* gene. Recombinant clones, isolated on lactose-agar plates, were replica-plated and lysed with chloroform. Polyvinyl sheets coated with antibody against *lac* repressor were placed onto the top of the lysed colonies for immunoadsorption. The immune complexes were made visible after washing by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside in buffered agar. The β-galactosidase activity of the chimera cleaves the colourless histochemical compound to a blue dye at those positions where clones produce the antigen. In the case of the *lac I* gene two types of clones were isolated, carrying the NH_2-terminal part of the *lac* repressor up to codons 27 and 75.

Key words: expression/immunoenzymatic assay/*lac* operon/molecular cloning

Introduction

Identification of clones is the crucial step after cloning of DNA sequences of pro- or eucaryotic sources into plasmid vectors. Immunoassays have been developed which permit the detection of specific clones (Ehrlich *et al.*, 1978; Broome and Gilbert, 1978; Clarke *et al.*, 1979; Kemp and Cowman, 1981). Most of these methods utilize the capacity of antibody to bind to an insoluble polymer (Catt *et al.*, 1967). Antigen released by lysed cells can be recognized by antibodies and the immune complexes can be detected either by a second iodinated [^{125}I]antibody against the antigen, or by binding of iodinated *Staphylococcus aureus* protein A (Ehrlich *et al.*, 1978; Broome and Gilbert, 1978; Clarke *et al.*, 1979). In other cases, enzyme-labelled antibodies have been used to identify antigen-antibody complexes by histochemical staining (Guesdon and Avrameas, 1974; Sanzey *et al.*, 1976). Recently, Kemp and Cowman (1981) reported a method that couples antigen, expressed by recombinant clones, covalently to chemically activated paper.

In most immunoassays described so far, only DNA that codes for intact gene products has been identified. It would be useful to clone small gene fragments from any source that would be suitable for direct identification of the expressed protein parts. We propose to clone such gene fragments coding for antigenic determinants in frame in the promoter proximal part of the *lac Z* gene. This allows immediate detection of antigen-producing clones. The β-galactosidase part of the chimera between antigen and enzyme permits identification of these clones, since the activity of β-galactosidase can be detected histochemically. We have used this method for cloning and direct identification of antigenically active fragments of the *lac I* gene of *Escherichia coli*.

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Results

Construction of vector pUK230

We constructed a plasmid that carried the *lac Z* and *lac Y* genes with suitable restriction sites and a frameshift mutation in the promoter proximal part of the *lac Z* gene. We introduced a small deletion into the polylinker region in the *lac Z* gene (Figure 1) of plasmid pUK217, described earlier by Rüther *et al.* (1981). pUK217 DNA was cut with *Sal*I to completion. The mixture was treated with DNA polymerase I (large

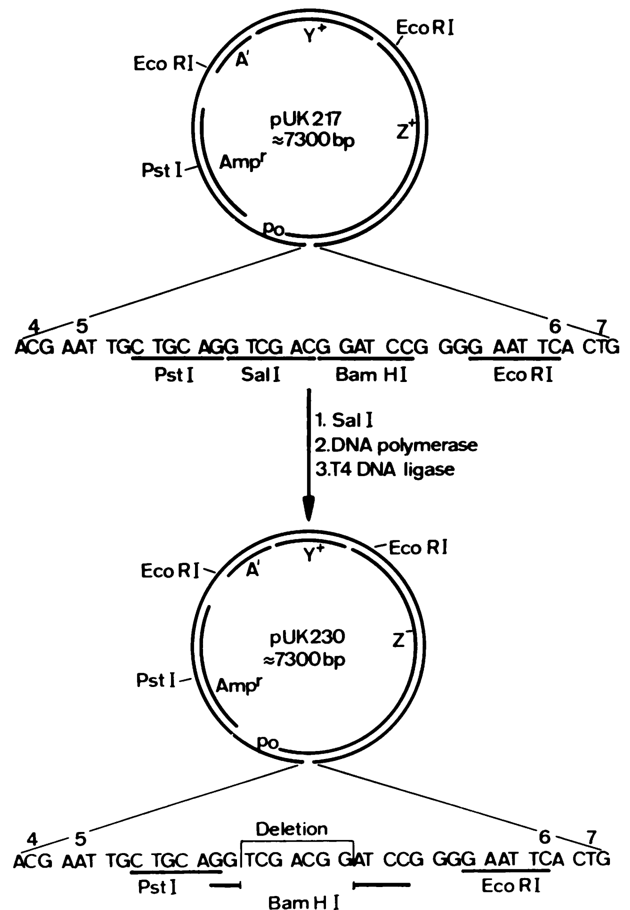

Fig. 1. Construction of pUK230, the vector used for the production of antigen-β-galactosidase chimeras. pUK230 contains a poly-linker region with restriction sites inserted in the nonessential promoter proximal part of the *lac Z* gene. A deletion of seven base pairs has been introduced. The resulting frameshift mutation reduces substantially the background of *lac*$^{+}$ recombinants after tailing and annealing with the C-tailed fragments.

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fragment) and all four nucleotides for 15 min at room temperature. The reaction was stopped by heating for 10 min to 70°C. After blunt end ligation with T4 DNA ligase overnight, the DNA was used to transform *E. coli* strain F′11*recA* ((*lac pro*)$_\Delta$*thi rif*A *str*A *rec*A/F′*lacI*^{Q1} Z^- *pro*$^+$). Lactose-negative colonies were isolated as white colonies, on plates containing rich media, ampicillin, isopropyl-1-thio-β-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). DNA, prepared according to Birnboim and Doly (1979), was used for DNA sequence analysis according to Maxam and Gilbert (1980). Sequence analysis showed a small deletion which had removed seven base pairs of the polylinker. The resulting frame shift allows easy screening for *lac*$^+$ recombinant clones, as only colonies containing molecules which restore the correct reading frame of the *lac Z* gene can grow on lactose-agar, or are blue on indicator plates containing X-gal. The plasmid is called pUK230.

Cloning of the lac I gene fragments

Digestion of pMC7, a pMB9 derivative containing the *lac I* gene and the *lac* I^Q promoter (Calos, 1978) with the restriction enzymes *Hae*III (six *I* gene fragments), *Hha*I (13 *I* gene fragments) and *Hpa*II (five *I* gene fragments) yielded the fragments we used for cloning. In every case the digestion mixture was tailed according to Roychoudhury and Wu (1980). The poly(dC)-tailed fragments were annealed with the poly(dG)-tailed *Bam*HI site of pUK230. We transformed, in the original experiments, BMH 71-18 ((*lac pro*)$_\Delta$*thi sup*E/F′ *lac* I^Q $Z^-\Delta$M15 *pro*$^+$) (Messing *et al.*, 1977) and later CSH50 ((*lac pro*)$_\Delta$*thi ara str*A) (Miller, 1972). CSH50 was used to identify clones possibly competed out by the *lac* repressor made by the *lac* I^Q gene of BMH 71-18. Lactose-positive clones were isolated at 37°C on lactose-agar plates, or as blue colonies on plates containing rich media, X-gal, IPTG, and ampicillin to determine the number of *lac*$^+$ colonies among the transformants (Table I). The frequency of *lac*$^+$ colonies was in the range predicted, as at least every ninth of the insert-carrying clones should be lactose positive.

Immunoadsorption of antigen producing cells

Replica plated, plasmid-containing colonies were grown in glass petri dishes containing rich media, IPTG, and ampicillin at 37°C overnight. The plates were cooled for ~3 h in a refrigerator. The cells were lysed by incubation with 5 ml chloroform for 2 min at room temperature. Chloroform was poured off and evaporated by incubation at 33°C for 5 min. The plates were stored in the cold until use. 450 μg rabbit antibody against *lac* repressor were used to coat five plastic sheets (ϕ 8.2 cm) in 5 ml 0.2 M $NaHCO_3$, pH 9.2 at room temperature (Broome and Gilbert, 1978). The plastic sheets were placed on the surface of the antibody solution for 2 min, avoiding air bubbles. The coated sheets were washed three times in 20 ml wash solution (10 mM potassium phosphate at pH 7.4; 150 mM NaCl, and 0.5% bovine serum albumin (BSA) w/vol). The plastic sheets were then placed onto the lysed colonies. We tried to prevent the formation of air bubbles between the agar and the plastic sheets. After incubation for 1 h at 4°C the plastic sheets were removed. All non-specifically adhering material was removed by washing the plastic sheets, with the help of a syringe, three times with 30 ml wash solution. At the end of the procedure not a trace of the debris of a colony should be visible (Figure 2).

Table I. Number of transformants and immunoenzymatically detected clones

Restriction enzyme used	Transformants total number	*lac*$^+$ among transformants	Immunoenzymatic positive
*Hae*III	234	73	5
*Hha*I	180	14	2
*Hpa*II	312	51	–

Enzymatic identification of antigen-producing cells

The cleaned sheets were placed in separate empty petri dishes with the immune complexes on the upper side. X-gal was added to TMSII-agar at 37°C (see Materials and methods) and then 20 ml of the mixture were poured quickly over one sheet. After incubation for 1 – 12 h at 37°C, the positions of clones producing antigen-β-galactosidase chimeras were visible as blue coloured marks of the size of a lysed colony. The blue dye is the result of the enzymatic activity of the β-galactosidase part of the antigen-β-galactosidase chimera coupled to its antibody. The β-galactosidase part of the chimera hydrolyses the colourless substrate to insoluble blue dye. The positions of the blue dye correspond to colonies producing antigen on the master plate. The sensitivity of the procedure was estimated with the help of a particular *lac* I^+ – *lac* Z^+ fusion (Müller-Hill and Kania, 1974), carrying active *lac* repressor and various *I* gene promoters. The *lac* I^+ promoter which leads to the synthesis of 15 tetrameric molecules/cell could not be detected. The response was visible when we used the 10-fold stronger I^Q promoter (Müller-Hill *et al.*, 1968) or the 100-fold stronger I^{Q1} promoter (Müller-Hill, 1975) (Figure 3). The strength of the response of the clones isolated here was comparable to 1500 I^+-Z^+ chimeras/cell.

Analysis of clones

Screening of the colonies yielded seven positive clones (Table I). Five were identified as containing *lac I* gene fragments produced by *Hae*III, two others by *Hha*I. Small amounts of DNA were prepared according to Birnboim and Doly (1979). The cloned fragments were cut using the flanking *Pst*I and *Eco*RI sites of the polylinker in the promoter

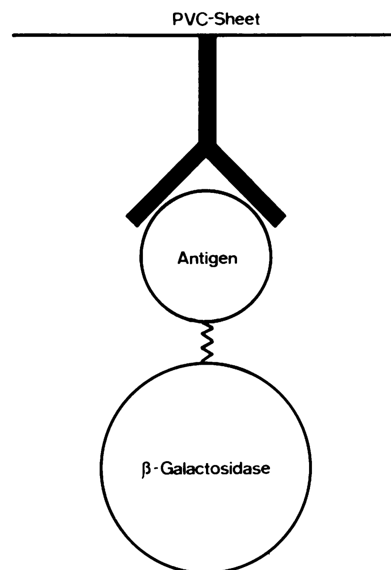

Fig. 2. Binding of an antigen-β-galactosidase chimera to antibody fixed to a polyvinyl sheet. The activity of the β-galactosidase part of the chimera allows detection of the antigen by histochemical staining.

proximal part of the *lac Z* gene of pUK230. After labelling the *Eco*RI site by fill-in reaction with DNA polymerase I (large fragment) and [α^{32}P]dATP (Rüther *et al.*, 1981), the fragments were separated on polyacrylamide gels, eluted, and used for DNA sequence analysis according to Maxam and Gilbert (1980). The sequence data were compared with the known *lac I* gene sequence (Farabaugh, 1978). The fragments obtained could be localized on two restriction fragments of the *lac I* gene. The five inserts containing *Hae*III fragments showed nearly identical sizes of 250 bp. Similarly, the two clones containing *Hha*I fragments showed an insert size of ~300 bp. DNA sequence analysis demonstrated that all five *Hae*III clones contained the same DNA fragment, coding for the NH_2-terminal 27 amino acids of the *lac* repressor. Similarly, both *Hha*I clones coded for the NH_2-terminal fragment of 75 amino acids of *lac* repressor (Figure 4).

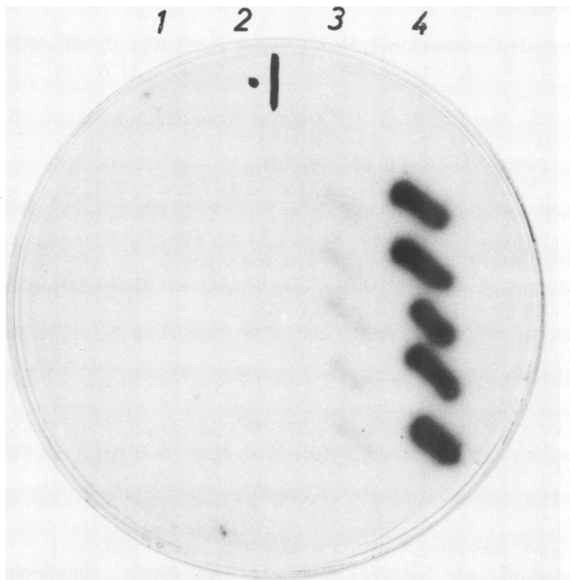

Fig. 3. Histochemical detection of *lac* repressor-β-galactosidase chimeras (Müller-Hill and Kania, 1974). **First lane:** *lac* constitutive F′ $I^- Z^+$ colonies; **second lane:** F′ $I^+ - Z^+$ colonies; **third lane:** F′ $I^Q - Z^+$ colonies; **fourth lane:** F′ $I^{Q1} - Z^+$ colonies. The positions of the F′ $I^- - Z^+$ colonies are completely blank. The same is true for the positions F′ $I^+ - Z^+$. The positions of the F′ $I^Q - Z^+$ colonies are grey (light blue) and the positions of the F′ $I^{Q1} - Z^+$ colonies are black (dark blue).

Discussion

The data presented establish the successful cloning of DNA fragments coding for antigenic determinants in the promoter proximal part of the *lac Z* gene. One particular *Hae*III fragment of the *lac I* gene was isolated five times and a *Hha*I fragment twice. Both fragments code for the NH_2-terminal part of the *lac I* gene. The smaller one codes for 27 residues, the larger one for 75 residues. Both the *Hae*III and *Hha*I clones contain the Shine-Dalgarno sequence in front of the *I* gene. The *Hae*III clones also contain the I^Q promoter and an UGA nonsense codon in phase upstream from the *I* gene. Thus, in the *Hae*III clones, translation presumably always begins with the GUG start codon of the *I* gene whereas in the *Hha*I clones the start codon of the *I* gene may be used when the (dG) tails upstream are out of phase. This situation increases the frequency of immunoenzymatic-positive clones 3-fold over the normal situation where the start codon of the *Z* gene is used. It is, however, not obvious why we could isolate only these fragments. Analysis of the *lac* repressor according to Hopp and Woods (1981) indicates that other restriction fragments of the *lac I* gene should also show some antigenicity. It is possible that the antiserum we used was rather special and that other antisera would behave differently. It is also possible that some regions with predicted antigenicity are not on the surface of intact *lac* repressor, or that the strong antigenic determinants consist of different parts far apart in the sequence of *lac* repressor. We suspected first that the *lac*

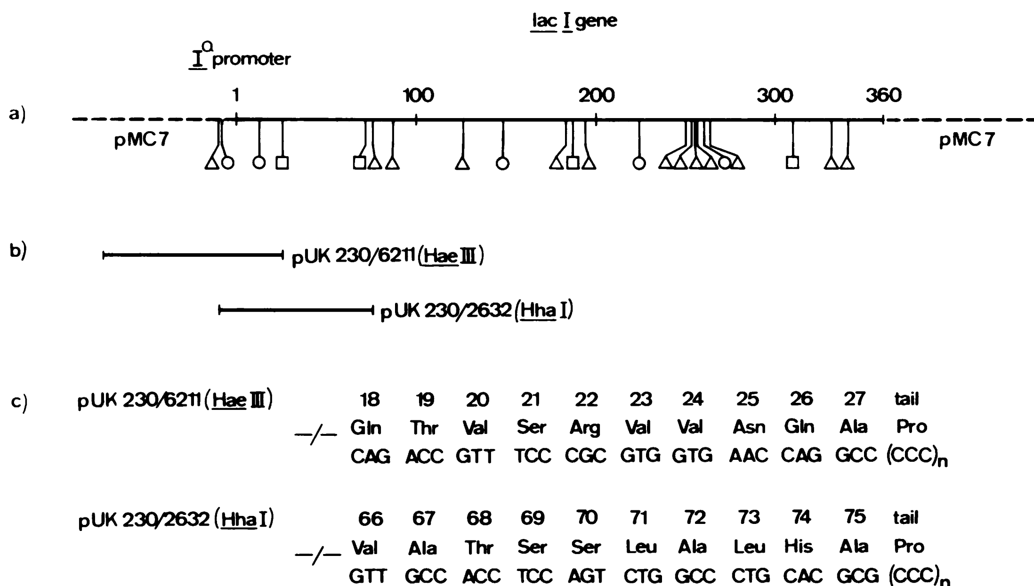

Fig. 4. (a) The *lac I* gene drawn to scale. The line is numbered from codon 1 to codon 360. The sites of cleavage of the restriction enzymes used in this work (*Hae*III□, *Hha*I△, *Hpa*II○) are indicated. **(b)** The five clones, containing identical *Hae*III fragments and the two clones containing identical *Hha*I fragments, are indicated. **(c)** To show which fragment of the *lac I* gene was cloned, the *Pst*I-*Eco*RI fragment was labelled in the *Eco*RI site and the DNA sequence was determined according to Maxam and Gilbert (1980). The *Hae*III-produced fragments code for the NH_2 terminus up to codon 27, as the *Hha*I clones code for the NH_2-terminal fragment up to codon 75. In pUK230/2632 the length of the poly(dC) tail was determined to be n = 3, in pUK230/6211 the number of n was >3.

repressor present in the BMH 71-18 background had effectively competed out the weak antigenic clones. Thus, we used CSH50, which does not contain *lac* repressor and which renders the clones somewhat unstable. We again found one *Hha*I clone and five *Hae*III clones, which were similar to the ones found in the other background. The use of restriction enzymes may also limit the isolation of gene fragments. They may cleave inside a DNA region coding for an antigenic determinant, or they may yield protein fragments that do not fold into their usual conformation. To circumvent this limitation, one might use random DNA fragments produced by partial DNaseI digestion, rather than complete digestion with restriction enzymes. Preliminary experiments indicate that such an approach can indeed be used successfully (data not shown).

The method may be used for various purposes. A library of *E. coli* DNA may be screened for fragments coding for antigenically related material. It has been shown for example that the NH_2 termini of *gal* repressor and *lac* repressor are homologous in sequence (Wilcken-Bergmann and Müller-Hill, 1982). This suggests the existence of a family of such homologous repressors. Preliminary experiments (data not shown) have indicated that a fragment coding for the NH_2 terminus of *gal* repressor can be isolated with our method from DNA carrying the *gal R* gene, using antibody directed against *lac* repressor.

In a similar way libraries from various fragmented cDNAs may be screened for fragments coding for distinct antigenic sites. Colony hybridization (Grunstein and Hogness, 1975) would then allow isolation of clones containing the complete corresponding cDNA or genomic DNA. Finally, the method may be used to analyse antigenic sites. Random cleavage of relevant DNA with DNaseI and cloning of small fragments would allow the contiguous regions which are recognized by antibody to be defined. The same procedure may be used at higher resolution when monoclonal antibodies are available that inhibit defined functions of the protein analysed. Also, DNA of viruses or parasites could be screened for the strongest antigenic determinants. Antigenically active polypeptides identified in this manner may be synthesized chemically and used directly as vaccine after suitable coupling.

Materials and methods

E. coli K12 strains have been described: BMH 71-18 (*lac pro*)$_\Delta$*thi sup*E/F′ *lac*I^QZ^-ΔM15*pro*$^+$) (Messing *et al.*, 1977); CSH50 ((*lac pro*)$_\Delta$*thi ara str*A) (Miller, 1972) and F′11recA ((*lac pro*)$_\Delta$*thi rif*A *str*A *rec*A/F′ *lac*$I^{Q1}Z^-$*pro*$^+$) (Rüther *et al.*, 1981). Plasmid pMC7 has been described by Calos (1978), pUK217 by Rüther *et al.* (1981). Restriction endonucleases *Pst*I, *Sal*I, *Bam*HI, *Hae*III, *Hha*I, *Hpa*II, and DNA polymerase I (large fragment) were purchased from BRL (Neu Isenburg); *Eco*RI from Boehringer (Mannheim) and terminal transferase from P-L Biochemicals (USA). Ligase was prepared according to Tait *et al.* (1980). [α^{32}P]dNTPs (400 and 3000 Ci/mmol) were obtained from Amersham (Braunschweig), dNTPs from Sigma (München). IPTG and X-gal were purchased from Bachem (USA). Polyvinyl (Benecor) was obtained from Benecke (Vinnhorst). Transformation, gel electrophoresis, and ligase reaction were performed as described (Mandel and Higa, 1970; Bolivar *et al.*, 1977). Isolation of gene fragments and DNA sequencing follows the method of Maxam and Gilbert (1980). Large amounts of DNA were prepared according to Clewell (1972), small amounts according to Birnboim and Doly (1979). Digestions with restriction enzymes were performed as described by Rüther *et al.* (1981). Antibody directed against *lac* repressor (18 mg/ml) prepared by ammonium sulfate precipitation followed by DEAE cellulose chromatography was a gift from H.-W. Griesser.

To stain the plastic sheets the following procedure was used: 2 x TMSII buffer (20 mM Tris HCl, pH 7.5; 20 mM MgAc; 400 mM KCl; 0.2 mM EDTA; 100 mM β-mercaptoethanol) (Gilbert and Müller-Hill, 1966) was mixed with an equal volume of melted agar (3 g agar/100 ml H_2O). The mixture was cooled to 37°C and X-gal (0.16 mg/ml final concentration) was added in a small volume of dimethylformamide. The mixture (20 ml/sheet) was then poured over the sheets in empty plates.

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