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EXPRESSION OF THE REPLICATION REGION OF PHAGE λ DNA CLONED INTO pBR322 IN E. COLI MINICELLS

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Replication region of bacteriophage λ DNA was cloned into pBR322 plasmid by the use of two restriction enzymes — PstI and HindIII. The restriction analysis of four obtained plasmids revealed that λ DNA was cloned in both orientations.

Recombinant plasmids were transferred to the minicell-producing strain of E. coli and synthesis of the plasmid-mediated proteins was analysed by polyacrylamide-gel electrophoresis. All four recombinant plasmids produced λ DNA replication proteins pO and pP as well as some proteins specific for pBR322. The orientation of cloned fragment did not affect the synthesis of λ DNA replication proteins.

Bacteriophage λ proteins, products of O and P genes are essential for the initiation and maintenance of phage DNA replication (Skalka, 1977). They may be identified in the isolated subcellular complexes as proteins of well-defined molecular mass which are absent in preparations obtained from appropriate amber or deletion phage mutants. The superfluous synthesis of bacterial proteins is eliminated by the use of minicells (Lipińska et al., 1980) or is inhibited by UV-irradiation of bacterial cells prior to phage infection (Oppenheim et al., 1979). The first of above-mentioned methods has been used for the localization of phage λ replication proteins in subcellular fraction of minicells (Kuypers et al., 1980; Żylicz & Taylor, 1980). Our aim was to perform analogous studies with bacterial cells, however, since λ replication proteins might form artificial complexes in UV-irradiated bacteria, we sought for another method of their visualization. Contrary to the normal, DNA-containing E. coli cells, the minicells are not able to sustain replication of λ DNA (Witkiewicz & Taylor, 1979). Isolation of complexes composed of phage replication proteins and DNA from normal cells may help to establish a system for the in vitro replication of λ DNA.
The only method for the visualization of plasmid-coded proteins which avoids UV-irradiation is based on the fact that after chloramphenicol amplification and washing out of the antibiotic, the plasmid-coded proteins are synthesized preferentially (Neidhardt et al., 1980). With this type of experiment in mind we cloned the λ DNA fragment containing the replication region (pR-oR-cro-cII-O-P) into pBR322. We describe here the cloning of this region (in both orientations) into two unique restriction sites (PstI and HindIII) and also describe the expression in minicells of λ replication genes present in all four recombinant plasmids. We wanted to explore the possibility that the action of some pBR322 promoters (e.g., β-lactamase promoters p1 and p3; Stüber & Bujard, 1981) superimposed on the action of λ promoter pR may enhance the expression of λ replication genes.

The λ DNA fragment containing the replication region has been cloned by others into BamHI site of pBR322 for construction of a new cloning vector (Rao & Rogers, 1978) and several parts of this region cloned into pBR322 were used in the study on λ DNA replication (Hobom et al., 1978). In neither case, however, the synthesis of λ O and P gene products has been investigated. The synthesis of the O protein in minicells was studied after cloning the O gene into pBR322 containing the lac promoter-operator region (Kuypers et al., 1980).

**MATERIALS AND METHODS**

*Bacteria and plasmids.* The bacterial strain used as recipient for transformation in cloning experiments was E. coli C600 F-(λ<sup>-</sup>thr<sup>-</sup>-leu<sup>-</sup>-tonA<sup>-</sup>-lacY<sup>-</sup>-thi<sup>-</sup>) r<sup>+</sup> m<sup>+</sup> obtained from P. Węgleński. Minicell-producing strain E. coli χ1197 (Frazer & Curtiss, 1975) was obtained from G. Kellenberger-Gujer. Plasmids used for cloning were pBR322 (Bolivar et al., 1977) and λvd1 (Matsubara & Kaiser, 1968).

*Preparation of plasmid DNA.* Plasmid-containing strains were grown in TB medium (10% Trypton Difco, 0.5% NaCl) supplemented with 2 μg/ml of thiamine hydrochloride and appropriate antibiotic at the standard concentration. When A<sub>575</sub> reached 0.8 the bacteria were lysed according to Clewell & Helinski (1969). The cleared lysates were deproteinized by extraction with an equal volume of phenol saturated with 20 mM-Tris, 150 mM-NaCl, 10 mM-EDTA, pH 7.4, followed by extraction with chloroform-ethanol mixture (24:1, v/v). After precipitation with 2 vol. of cold ethanol, DNA was resuspended in TE buffer (10 mM-Tris, 1 mM-EDTA, pH 7.4). Such preparations were used for restriction analysis and transformation. Further purification of closed circular plasmid DNA for cloning experiments was achieved by ethidium bromide/caesium chloride density-gradient centrifugation.

*Enzymes and enzymatic digestions.* Restriction endonucleases EcoRI, PstI and HindIII, and T4 polynucleotide ligase were obtained by standard procedures. Digestions were carried out in 30 μl volumes containing 0.3 - 1.0 μg DNA as specified by Robinson & Landy (1977). For digestion with PstI the same conditions as for HindIII were used. Electrophoresis of digestion products was carried out in 0.8% (Sigma type II) agarose horizontal slab gels in Tris/acetate buffer (40 mM-Tris,
20 mM-sodium acetate, 2 mM-EDTA, 18 mM-NaCl, pH 8.0). The gels were stained for 10 min in 2.5 μg/ml ethidium bromide and photographed under 364 nm irradiation.

*Ligation and transformation.* DNA fragments were mixed in 1:1 molecular ratio. Ligation was carried out at 8°C for 16 h in 100 μl volumes of 66 mM-Tris/HCl, pH 7.6, containing 6.6 mM-MgCl₂, 10 mM-dithiothreitol, 0.1 mM-ATP, 2-3 μg of DNA and 0.5 unit of T4 ligase. Ligation was stopped by phenol/chloroform extraction followed by ethanol precipitation. The precipitate was resuspended in 0.1 mM-CaCl₂ and transformation was performed according to Dagert & Ehrlich (1979). Isolated transformants were assayed for their resistance to tetracycline, ampicillin and λ-infection.

*Other methods.* Isolation of minicells, radioisotope labelling of proteins, polyacrylamide-gel electrophoresis and fluorography were carried out as described previously (Grzesiuk & Taylor, 1977; Grzesiuk et al., 1980).

**RESULTS AND DISCUSSION**

*The cloning procedure.* Hybrid plasmids were constructed by insertion of λ DNA into HindIII or PstI cleavage sites of pBR322. The source of λ DNA was the plasmid λdv1 (6.9 kb) containing the following structural genes of phage λ: *rex, cl, cro, cII, O and P* (Fig. 1). The DNA of λdv1 and pBR322 was digested with the same enzyme and ligated. The cells of *E. coli rᵣ⁻mᵣ⁻* strain were transformed with ligation mixtures and spread on plates containing ampicillin or tetracycline. The strains sensitive to one of these antibiotics and resistant to λ-infection were selected, plasmid DNA isolated and analysed by the use of the appropriate restriction enzymes.

![Restriction map of plasmid λdv1](image)

Fig. 1. The restriction map of plasmid λdv1 representing a fragment of phage λ DNA with the structural genes: *rex, cl, cro, cII, O, P*. The gene products are indicated by boxes; the length of boxes corresponds to the molecular mass of the proteins. The positions of restriction cleavage sites are depicted according to the map of bacteriophage λ given by Daniels *et al.* (1980); *i.e.* EcoRI cut is defined as coordinate 40,000 bp and the left end of λ genome is not zero but 369 bp. The thick line portions represent regions of known DNA sequence. This map and that published for pBR322 by Sutcliffe (1978) served for calculation of the sizes of restriction fragments given in Figs. 3 and 4.

Finally, four different classes of hybrid plasmids (referred to as pIG3, pIG4, pIG7 and pIG8) were selected. The recombinants pIG3 and pIG4 were constructed using PstI. They contained complete λdv1 DNA inserted into *Amp* region of pBR322
Fig. 3. Digestion pattern of hybrid plasmids cleaved with the enzymes used in their construction.  
A. The molecular mass standards — EcoRI restriction fragments of bacteriophage λ DNA (a), and recombinant plasmids: pIG3 (b) and pIG4 (c) cleaved with PstI. The upper bands represent λdv1 fragment (6.90 kb), and the lower bands — pBR322 fragment (4.36 kb). B. The molecular mass standards — HindIII restriction fragments of bacteriophage λ DNA (a) and recombinant plasmids: pIG7 (b) and pIG8 (c). The upper bands represent the larger fragment of λdv1 (6.32 kb) and the lower bands are the pBR322 fragment (4.36 kb).
(Fig. 2A and 3A) in two opposite orientations. The plasmids pIG7 and pIG8 contained the larger of the two fragments obtained after cleavage of λdv1 with endonuclease HindIII. This fragment was inserted into HindIII site of pBR322 (Figs. 2B and 3B). The orientation of λdv1 fragment relative to pBR322 DNA was ascertained by cleavage with EcoRI (Fig. 4).

Fig. 2. Schematic map of recombinant plasmids containing the replication region of bacteriophage λ: cro-cII-O-P. A. Plasmids pIG3 and pIG4: the molecule of λdv1 linearized with PstI was cloned into PstI site of the vector pBR322 in both orientations. B. Plasmids pIG7 and pIG8: the larger of the two fragments of λdv1 produced by the cleavage with endonuclease HindIII was cloned into HindIII site of pBR322 in both orientations.
Expression of plasmid proteins in minicells. The plasmids pBR322, λdvl and four recombinant plasmids were transferred to a minicell-producing strain of E. coli in order to study the synthesis of plasmid proteins in minicells. Purified minicells containing each of the respective plasmids were labelled with $^{14}$C-amino acids and lysed. The proteins were separated by electrophoresis in 15% SDS-polyacrylamide gel. Radioactive proteins were visualized by fluorography. To identify the $O$ and $P$ proteins coded for by λdvl and recombinant plasmids, minicells infected with phage λ and its $O$- and $P$-amber mutants were used as described by Lipińska et al. (1980). The synthesis of λ replication proteins (molecular mass p$O$—34 000 and p$P$—23 000) occurs in minicells harboring λdvl (Fig. 5a). Five other proteins of molecular mass ranging from 35 000 to 26 000 were also found as well as two proteins of much lower molecular mass. Strains containing pBR322 produced four major polypeptides (Fig. 5b). According to Alton et al. (1978), the protein of molecular mass 33 500 located in the upper band, represents the product of Tet$^r$ region, while three other bands of 29 000, 27 000 and 24 000 represent the proteins coded for by the Amp$^r$ region.

All recombinant plasmids produced λ proteins p$O$ and p$P$. They also coded for some proteins specific for pBR322. In pIG7 and pIG8 (Fig. 5c,d), strong bands of 29 000 and 27 000 represent products of Amp$^r$ region while the band of 24 000 is overlapped by a distinct band of $P$ protein. The proteins coded for by the Amp$^r$ region of pBR322 are absent in pIG3 and pIG4 due to inactivation by insertion in the PstI site. It is difficult to distinguish the 34 000 protein specific for Tet$^r$ from λ $O$ protein possessing a similar molecular mass.

The electrophoretic profiles of pIG7 and pIG8 are identical while there is a substantial difference in protein pattern of pIG3 and pIG4 in the region below 22 000. These proteins were not coded for by either λdvl or pBR322, hence, they may represent the products of overlapping transcription/translation of pBR-λdvl junctions.

We could not find any substantial influence of cloning site and/or orientation of the cloned fragment on the synthesis of λ$O$- and $P$-gene products in minicells. It also became evident, that—due to the lack of proteins of similar size—some recombinants are more suitable for the identification of a particular λ protein than others. The plasmids containing λ insertion in PstI site (pIG3 and pIG4) will be used for further studies with λ$P$ protein, and the plasmids with insertion inactivated Tet$^r$ region (pIG7 and pIG8)—for studies with λ $O$-gene products.

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Fig. 4. Estimation of orientation of cloned \( \lambda \) fragments by cleavage of hybrid plasmids with EcoRI. The molecular mass standards — EcoRI restriction fragments of phage \( \lambda \) DNA (a), fragments of plasmid pIG3—8.81 and 2.46 kb (b). The restriction fragments of plasmid pIG4 (5.72 and 5.55 kb) migrate as one heavy band (c). Plasmid pIG7 after EcoRI digestion yields fragments of 8.94 and 1.74 kb (arrow) (d); and pIG8 — fragments of 6.04 and 4.64 kb (e).

Fig. 5. SDS-polyacrylamide gel electrophoresis of plasmid proteins synthesized in minicells. The proteins of plasmid-containing minicells were labelled with \(^{14}\)C-amino acids for 30 min. After lysis of minicells, proteins were separated in 15% SDS-polyacrylamide gel according to Grzesiuk et al. (1980). The fluorogram shows protein pattern of minicells harbouring the plasmids: \( \lambda dv l \) (a), pBR322 (b), pIG7 (c), pIG8 (d), pIG4 (e) and pIG3 (f).
EKSPRESJA REJONU REPLIKACYJNEGO BAKTERIOFAGA λ WKLONOWANEGO DO PLAZMIDU pBR322 W MINIKOMÓRKACH E. COLI

Streszczenie

Rejon replikacyjny bakteriofaga λ został sklonowany do wektora pBR322 przy użyciu dwóch enzymów restrykcyjnych PstI i HindIII. Analiza restrykcyjna otrzymanych czterech plazmidów wykazała, że DNA fagowy został sklonowany w obu możliwych orientacjach w stosunku do DNA wektora.

Hybrydowe plazmidy przeniesiono do produkującego minikomórki szczepu E. coli i badano syntezę białek plazmidowych, stosując metodę elektroforezy poliakrylamidowej.

Wszystkie cztery rekombinanty produkowały fagowe białka replikacyjne pO i pP oraz niektóre białka specyficzne dla pBR322. Orientacja wklonowanego fragmentu nie miała wpływu na syntezę replikacyjnych białek fagowych.

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