LARGE SCALE PURIFICATION OF A BACTERIAL GENE

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1. Introduction

The answer to many questions about the biosynthesis of nucleic acids and proteins requires studies with isolated genes. These questions include problems about (a) the structure of the genes, (b) the mechanisms of genetic transcription and their regulation, and (c) the mechanisms of protein synthesis.

In bacteria, preparations of a chromosomal cistron can be enriched at least 100-fold by preparing specialized transducing phages which carry bacterial genes and it is possible to prepare by genetic manipulations specialized transducing phage which carry any specified bacterial cistron. Using these methods, transducing phages bearing different operons: lactose [1], arabinose [2], galactose [3], tryptophan [4], maltose [5–7] have been isolated. As an example, DNA of *lac* transducing bacteriophages (λ or φ80) has been widely used as a model for the study of genetic expression in vitro.

These genetic manipulations can be used to enrich selected genes up to a certain level but they do not yield preparations pure enough for many experiments dealing with transcription in vitro or with structural properties where it is important to have a gene preparation that is free from other operons such as viral operons. Several authors have studied the question of a further purification after the isolation of DNA from λ transducing bacteriophage. One method for the preparation of bacterial genes has been developed by Shapiro et al. and allows the isolation of the *lac* operon DNA free of viral genes [8]. They used two transducing bacteriophages having in common in thei chromosomes only the DNA of the integrated *lac* operon. Other authors, have isolated among DNA fragments obtained by sonication those which bind specifically to the *lac* repressor, form a complex with it and are retained on filters [9,10].

The first method of Shapiro et al. allows the isolation of pure *lac* operon DNA, free of viral genes, but it requires many delicate steps and yields only very small quantities of DNA. The second method allows the easy preparation of great quantities of DNA, but the fragments which contain the *lac* gene are heterogenous in size.

In this work we have structured a transducing bacteriophage (λ *imm434 plac*$_5$) which facilitates, in few steps, the preparation of homogenous DNA fragments containing the *lac* operon. For this purpose (a) the DNA of the λ transducing bacteriophage is cleaved at specific sites by the *Eco*RI endonuclease (b) the resulting fragments are separated by polyacrylamide gel electrophoresis. (The 434 immunity is necessary for a good resolution) (c) the DNA fragment containing the *lac* operon is then eluted, purified and concentrated by chromatography on hydroxy-apatite.

2. Materials and methods

The bacteriophage strains used were λ *CI857 S7*, λ *plac*$_5$ *CI857 S7* and λ *imm434 CI*^{ts}*S7* kindly provided by Ph. Kourilsky.

The bacterial strains used are listed below:

M72 F$^-$*thi lac*$_{amber}$ *trp*$_{amber}$ *Str* (from J. Beckwith)
C600 F$^-$*thi thr leu lac Sup*E [11]
AR1002 F$^-$*thi thr leu lac Sup* E (spontaneous *lac* derivative of Y10) [12].
M5224 F$^-$*thi lac*$_{amber}$ *trp*$_{amber}$ *str chlA*, derivate of Δ H1 [13] lysogenic for λ *CI857 bio256* (from Ph. Kourilsky)

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Lysates were made in liquid L-broth rich medium. Bacteriophage assays were done either on tryptone broth agar or on MacConkey lactose agar (Difco medium).

*Eco*RI endonuclease was generously made available to us by P. Yot. HpaII endonuclease was a generous gift of B. Allet.

The bacteriophage λ *plac*$_5$ *imm434 CI*^{ts}*S7* was constructed by genetic cross between λ *plac*$_5$ *CI857 S7* and λ *imm434 CI*^{ts}*S7*. Irradiated C600 strain was doubly infected and incubated for 90 min at 37°C. A lysate was obtained after addition of chloroform and plated with the λ immune indicator M5224 on MacConkey lactose plates at 30°C. As the MacConkey lactose allows S7 bacteriophages to grow [14] plaques were obtained. Lysogenic bacteria giving red colonies were picked in the rare red plaques and isolated several times on MacConkey lactose at 30°C. The recombinants were obtained as prophage. We thus first verified that the bacteria lysogenic for the prophage were *lac*$^+$ and thermosensitive. Clones were then heat-induced in liquid medium, the resultant bacteriophages were examined genetically. They were *lac*$^+$, *CI*^{ts}, *S7*, and had the immunity 434. Bacteriophage particles were purified by two successive CsCl gradients. The bacteriophage DNA was extracted twice 30 min by an equal volume of phenol at 4°C. Hydrolysis by *Eco*RI endonuclease was performed on DNA (4–6 A_{260} units/ml) in Tris–HCl 100 mM pH 7.4, $MgCl_2$ 10 mM. The DNA was digested for 2 hr at 37°C. The quantity of enzyme was chosen to give complete hydrolysis. EDTA was then added to a final concentration of 10 mM. DNA was extracted by a 10 min phenol treatment and dialyzed for 24 hr against Tris–HCl 10 mM, pH 8.0, EDTA 1 mM. Hydrolysis by HpaII endonuclease was performed as described by Allet [16].

Analysis of Eco RI fragments was performed by polyacrylamide gel electrophoresis in a slab of 0.4 × 14 cm section and 8 cm height containing a linear gradient of polyacrylamide from 2.5% at the origin to a final concentration of 7.5%. Gel and the reservoirs buffer was 40 mM Tris–acetate pH 8.0, 20 mM sodium acetate. Electrophoresis was carried out in an electric field of 6 V/cm for 24 hr at room temperature [15, 16]. Analysis of HpaII fragments was performed in a slab of a section of 0.4 × 14 cm and of 12 cm height containing a linear gradient of polyacrylamide from 2.5% at the origin to a final concentration of 14%. Electrophoresis was carried out for 12 hr. The DNA was stained by soaking the gel in a 0.02% solution of methylene blue.

For preparative electrophoresis of Eco RI fragments, the same conditions were used in a gel of 14 cm section and 7 cm height. The sample, 2.5–3 ml of DNA solution (6 A_{260} units/ml) was loaded all along the gel section. The gel slice (of thickness of 1–2 mm) corresponding to the *lac* band was cut out with a tighten metallic wire. The gel slice was homogenized and put into a tube of 2 cm of diameter having at the bottom a scintered glass plate and below a dialysis tubing of 1.5 cm of diameter and 6 cm of length was attached. The tubing contained the buffer used for the electrophoresis. An electric potential was applied between the top of the tube and the bottom of the dialysis tubing. To minimize the heating, the current was kept below 30 mA per tube and the electro-elution was done at 4°C. We verified, with [^{32}P]DNA, that under these conditions 95% of DNA is eluted.

Purification and concentration of the DNA on hydroxyapatite column were performed as described by Bernardi [17]. Hydroxy-apatite suspension in 1 mM phosphate, pH 6.8 buffer was a generous gift of A. Prunell. The DNA solution from the dialysis tubing was loaded on a 0.5 × 5 mm column. In the first step the acrylamide which eluted with the DNA from the gel slice, was washed from the column with a 10 mM phosphate buffer overnight. In the second step the DNA was eluted from the column with a 0.5 M phosphate buffer. In both steps the flow rate was 8 ml/h.

3. Results and discussion

When the DNA of λ *plac*$_5$ is hydrolyzed by the *Eco*RI endonuclease and the products are analyzed by polyacrylamide gel electrophoresis, the pattern obtained displays 6 bands (fig. 1). The comparison of the patterns of λ and λ *plac* allows to deduce the physical map of λ *plac* [15] (fig. 2). Band G corresponds to the fragment which contains the lactose operon (refered to as *lac* fragment) in the DNA of λ *plac*$_5$ (the band will be referred to as the *lac* band). This *lac* band forms a doublet with the band B which corresponds to the immunity region and thus, technically, it is impossible to cut out the slice of gel which would contain only the *lac* fragment.

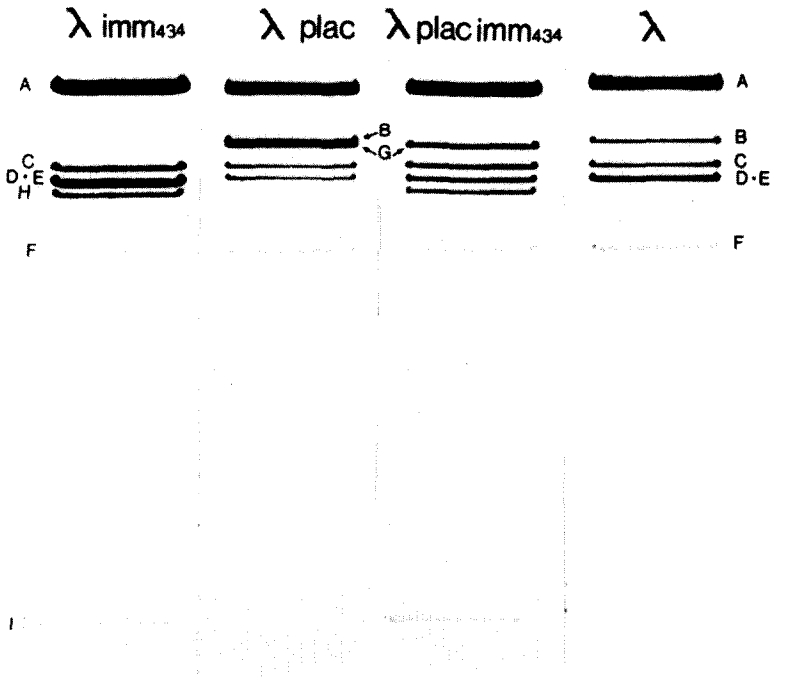
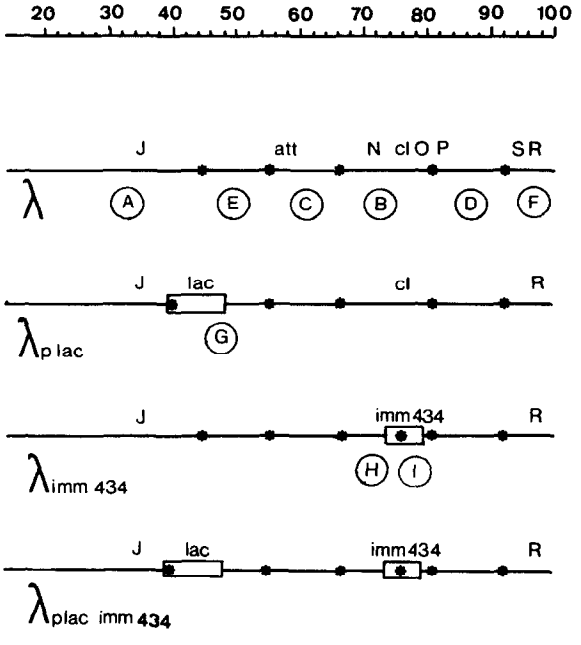


Fig. 1. Separation by polyacrylamide gel electrophoresis of *Eco* RI endonuclease fragments obtained from λ *CI857 S7*, λ *plac*$_5$ *CI857 S7*, λ *imm434 CI*^{TS} *S7*, and λ *plac*$_5$ *imm434 CI*^{TS} *S7* DNA.



The comparison of the patterns of λ and λ *imm 434* shows that the band B of λ (immunity region) disappeared and is replaced by two bands H and I (fig. 1). This proves the existence of a new site of cleavage which must be located in the region of non homology between λ *imm 434* and λ [15]. From the results of the electrophoresis one can estimate the mol. wt. of the fragments H and I: respectively 3.2×10^6 and 1×10^6. The location of the 434 immunity on the λ genome shows that the fragment I is at the right of the fragment H (fig. 2). Consequently the λ *plac imm434* bacteriophage would give a pattern where the *lac* fragment would no longer be contamin-

Fig. 2. Position of the *Eco* RI endonuclease cleavage sites on the genetic map of the bacteriophages. Letters A to I correspond to the bands obtained by gel electrophoresis as showed in fig. 1. Asterisks refer to the *Eco* RI endonuclease sites on the genetic maps.

ated by a fragment of a similar molecular weight as in the case of λ *plac*$_5$. As expected, the pattern shows that the lac is clearly separated from the other bands (fig. 1). Electrophoresis of *Eco*RI cleaved DNA from such a bacteriophage would permit preparative isolation of the *lac* band. Up to 750 μg of hydrolysed DNA can be run in a standard slab of 1 × 14 cm section, with a very good resolution of the *lac* band. After elution from hydroxyapatite, the absence of acrylamide was verified from the ultraviolet spectrum of the sample. Starting with 750 μg of DNA we obtained 70 to 75 μg of *lac* fragment; as this fragment represents 15% of the genome the recovery after the total purification is 60 to 70%. The losses come from the

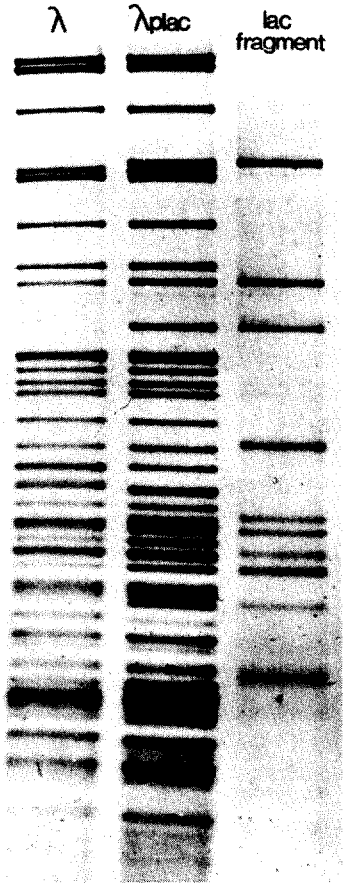


Fig. 3. Polyacrylamide gel electrophoresis of the *Eco*RI endonuclease *lac* fragment compared to the pattern of λ *plac*$_5$ *imm434 CI*^{ts} *S7*.

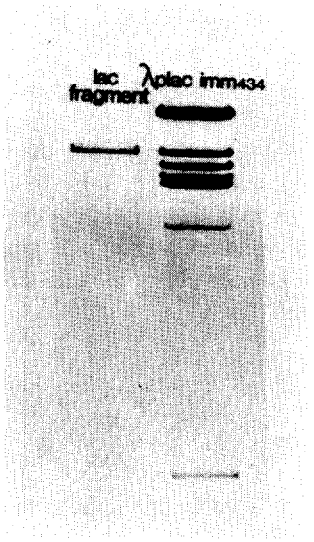


Fig. 4. Separation by polyacrylamide gel electrophoresis of HpaII endonuclease fragments obtained from λ *CI857 S7* and λ *plac*$_5$ *CI857 S7* DNA and *lac* fragment DNA.

accumulation of losses of 5 to 10% during the different steps as verified with [^{32}P]radioactive DNA.

We have attempted to verify that the different steps of the purification do not alter the structure of the DNA fragment. (a) An aliquot of the DNA which had been purified by the described method (1–2 μg) was analyzed on a gel in parallel with a total hydrolysate of λ *plac imm434* (fig. 3). The purified fragment migrates at the expected position. (b) The thermal denaturation showed an hyperchromicity of 25% which corresponds to native DNA. (c) When the purified DNA was run on an alkaline gradient a unique peak was observed showing no nicks in the fragment. (d) The fragment was hydrolyzed by the enzyme HpaII endonuclease and analyzed by gel electrophoresis (fig. 4). The comparison with the similar patterns for λ and λ *plac* showed that the bands of λ *plac* which do not exist in λ (those corresponding to the *lac* fragment) are in the purified fragment.

4. Conclusion

The method developed here can be extended to the purification of other DNA segments. The possibil-

ity of obtaining a homogeneous preparation of DNA containing a functional genetic unit with the control genes (promotor, operator and β-galactosidase structural gene) facilitates in vitro transcription experiments and the study of the regulatory mechanisms. However, the presence of λ sequences (b_2 region) in the fragment remains a disadvantage. In addition to this use, our method yields enough pure homogeneous DNA to permit physico-chemical studies of protein–DNA interaction.

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