PREPARATIVE PURIFICATION OF λ-DNA FRAGMENTS OBTAINED AFTER Eco RI DIGESTION

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1. Introduction

The study of the expression, and structure of bacterial or eukaryotic genes, as well as the study of the interaction of a particular gene with specific proteins, require the isolation of individual genes, or groups of genes. In bacteria, this can be done with specialized transducing phages, whose DNA is hydrolysed by restriction enzymes, and the interesting DNA fragment is then purified. In a previous publication [1], the purification of the *E. coli lac* gene, via the DNA of a *lac* transducing λ bacteriophage has been reported. In the present work, we describe the purification of all the Eco RI digestion products of the DNA of two mutants of λ bacteriophage.

Two methods have been used: polyacrylamide gel electrophoresis, and isopycnic centrifugation in cesium sulfate gradients in the presence of silver ions. As will be seen, the proper combination of both methods allows the purification of all fragments of λ genomes with either two, or six sites sensitive to Eco RI digestion.

2. Materials and methods

The bacteriophage strains are: λ CI857 S7 (λ), λ plac5 imm 434 CI^{ts} S7 (λ plac imm 434) [1], and λ plac5 CI857 sRIλ3° sRIλ2° sRIλ1° (λ plac II) [2].

The Eco RI, and HpaII restriction enzymes were generous gifts from M. Yaniv and H. Kopeka, respectively.

Extraction of DNA and its hydrolysis by Eco RI endonuclease were as described previously [1].

Analytical and preparative electrophoresis of Eco RI DNA fragments have also been reported [1].

For analytical and preparative isopycnic centrifugation, DNA solutions were extensively dialysed against $Na_2B_4O_7$ 5 mM, pH 9.2 [3]. Before use, DNA solutions were heated to 55°C during 5 min, in order to disrupt the cohesive sites aggregates. Varying amounts of $AgNO_3$ 1 mM were added to the solutions under gentle stirring. Amounts of silver are expressed in number of Ag^+ ions per DNA base. It was assumed that the average mol. wt of DNA per phosphorus atom is equal to 330, and that the extinction of DNA at260 nm is equal to $2 \cdot 10^{-2}\,cm^2\,\mu g^{-1}$. Finally, the density of the DNA-silver solutions was adjusted to $1.500\,g \cdot cm^{-3}$ with the appropriate amount of Cs_2SO_4 crystals (use was made of the Cs_2SO_4 concentration versus density data of Ludlum and Warner [4]) all centrifugations were performed at room temperature. For analytical centrifugation, use was made of a Beckman Model E centrifuge, equipped with monochromator, photoelectric scanner and multiplex attachments. 12 mm and 4° single sector Kel-F centerpieces were used in either a two-cell, or four-cell titanium rotor. Solutions of 1 ml containing 1 to 3 μg of DNA were made up, and 0.7 ml were introduced in the centrifuge cells.
All the scans were performed at 264 nm, the rotor speed was 40 000 rev/min, and equilibrium was usually reached after 48 hr. Preparative centrifugations were performed in Beckman rotors 65 (500 μg of DNA, or less per tube), or 60Ti (500 μg to 1.3 mg of DNA per tube). Polyallomer tubes were used and the gradient volumes were equal to 9 ml, and 25 ml respectively. The tubes were filled up with mineral oil. The 65

rotor was first spun 24 hr at 35 000 rev/min and then 3 days at 29 000 rev/min, and the 60Ti rotor 24 hr at 33 000 rev/min and 4 days at 27 000 rev/min. The tubes were punctured at the bottom and fractionated into 40 to 50 equal vol fractions.

After preparative centrifugation, the silver was removed by extensive dialysis against Tris–HCl 10 mM, pH 7.5, EDTA 1 mM, NaCl 2 M buffer [3], and then against the same buffer devoid of NaCl.

For the measurement of the residual binding of silver to DNA, radioactive [^{110}Ag]No_3 (purchased from C.E.N. Saclay-France) was diluted with non-radioactive $AgNO_3$ to a final specific activity of 0.63Ci/g. A series of 1 ml samples, containing 12 μg of λ plac imm 434 DNA and 0.3 Ag^+ ion per DNA base, were dialysed against the preceding buffer. At various time intervals one sample was removed and its ^{110}Ag content was measured by liquid scintillation in Bray's mixture. At the same time the concentration of ^{110}Ag was measured in the dialysis buffer, which was also renewed.

3. Results and discussion

Fig.1a shows the position of the Eco RI endonuclease cleavage sites on the genetic map of λ plac imm 434 and fig.2c, the electrophoresis pattern of the

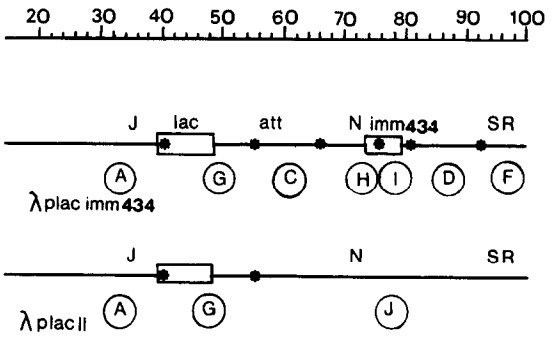

Fig.1. Position of the Eco RI endonuclease clivage sites (*) on the genetic map of the bacteriophages λ plac imm 434 and λ plac II. Letters A to J refer to the bands obtained by gel electrophoresis as shown in fig.2.

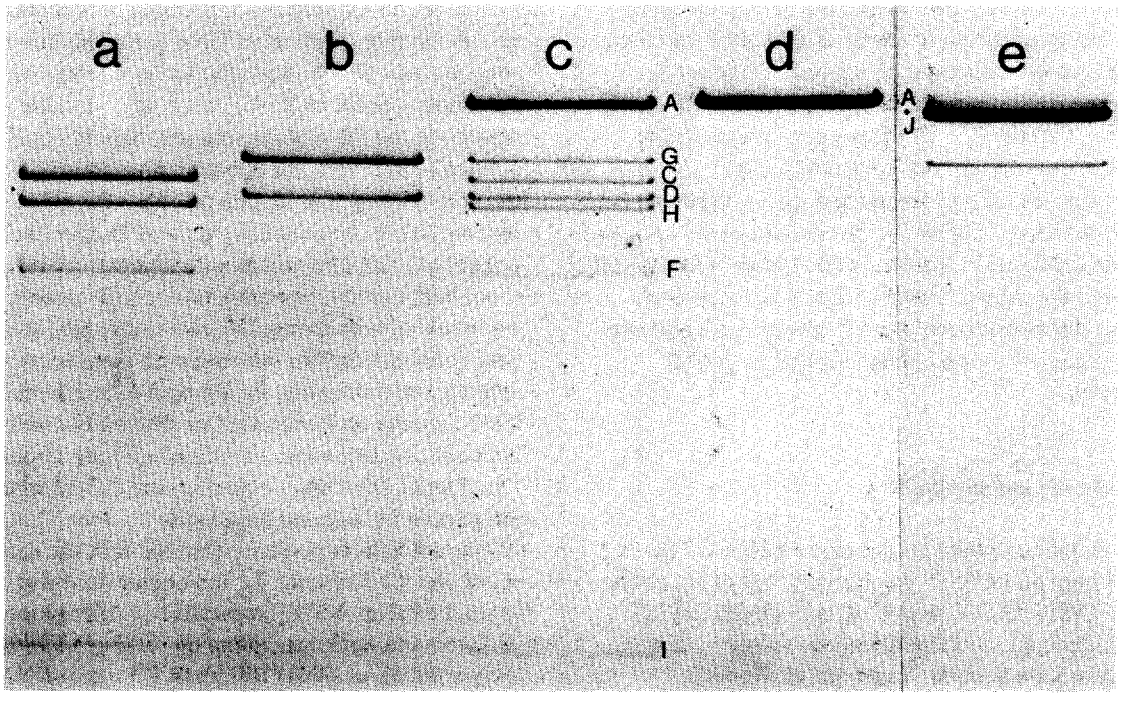

Fig.2. Separation by polyacrylamide gel electrophoresis of Eco RI endonuclease fragments obtained from λ plac imm 434 (c) and λ plac II (e). Patterns d, b, and a correspond to bands α, β, and γ from fig.4a.

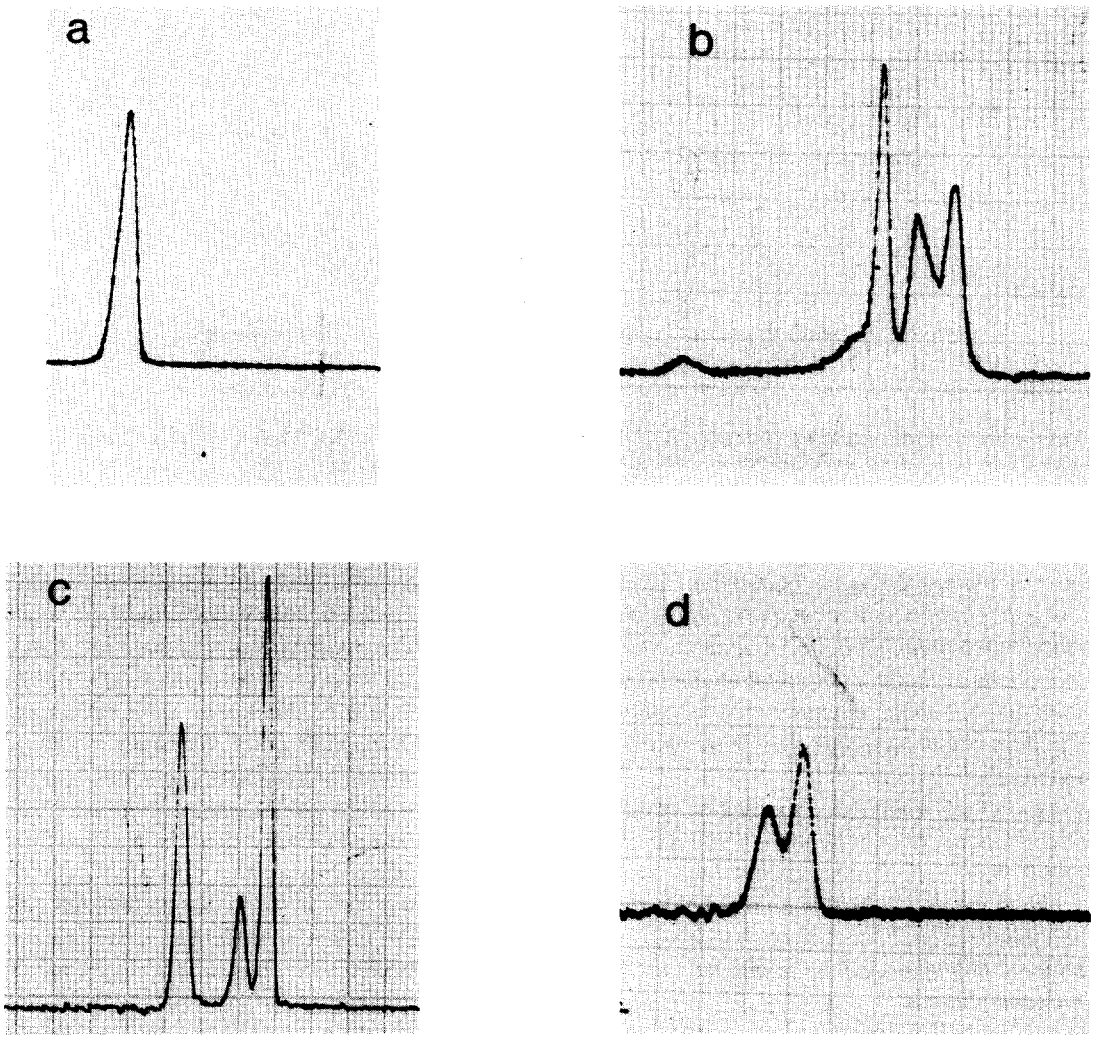

Fig.3. Analytical isopycnic centrifugation in Cs_2SO_4 of the Eco RI hydrolysates of λ plac imm 434 DNA (a, absence of silver; b, 0.30 silver ion per DNA base), λ plac II DNA (c, 0.35 silver ion per base), and DNA from band β of fig.4a (d, 0.30 silver ion per base). Other experimental conditions are given under Materials and methods. The density increases from left to right.

corresponding DNA fragments [1,5]. It appears that the gel sections which contain the A,G,F and I fragments can be cut into separate slices and thus, the respective DNA's can be purified by elution from the gel [1]. But this purification method cannot be used for the C,D and H fragments, which have very close molecular weights, and for this reason remain very close to each other in the gel. Similarly, the G fragment from λ plac II DNA can be purified by electrophoresis [2], but not the A and J fragments (figs.1b, and 2e). The resolving power of agarose gels appears likewise to be insufficient.

In order to purify all the DNA fragments, isopycnic centrifugation in the presence of Ag^+ ions was tried. This method has been used successfully for the purification of animal satellite DNA's [6,7], and for the fractionation of the bovine genome [3]. Fig.3 shows the results of analytical centrifuge runs. In a similar experiment, where some G fragment was added to the DNA samples, it was shown that it banded with the

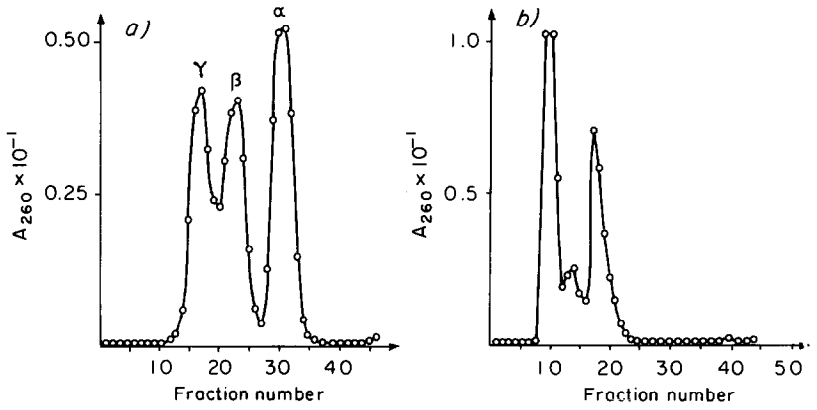

Fig.4. Preparative isopycnic centrifugation in Cs_2SO_4 of 500 μg of λ plac imm 434 DNA (a), and 300 μg of λ plac II DNA (b). See Materials and methods for experimental conditions. The density increases from right to left.

central bands of fig.3b, and 3c. This result, and the relative areas corresponding to the different DNA bands, suggest that for both phage DNA's, the less dense band corresponds to pure A fragment, whereas the other bands of fig.3c correspond to pure G and J fragments. In fig.3b, the asymmetry of the two dense bands, indicates that they contain at least two DNA fragments each.

Fig.4 shows the result of a preparative centrifuge run of both DNA's, from which it appears that the A fragment from both phages, and the J fragment from λ plac II can be purified from one single run. The final purification of the G fragment from λ plac II, is achieved either through an additional centrifuge run, or electrophoresis.

The two dense DNA bands (β and γ) from fig.4a were submitted to an additional, preparative run for further purification (result not shown). The silver present in the β and γ bands, as well as in the previous α band was then removed, and the corresponding DNA's were submitted to analytical electrophoresis. It appears that band β (fig.2b) contains indeed the G fragment, and also the D fragment; band γ (fig.2a), instead, contains the C,H,F and I fragments. It is also confirmed that band α corresponds to pure A fragment (fig.2d). Accordingly. the seven DNA fragments of phage λ plac imm 434 could be purified in two steps: first isopycnic centrifugation in the presence of silver (fig.4a), second, preparative electrophoresis on two high capacity gels [1] with results identical to those of figs.2a and 2b.

Another method for the purification of the two DNA fragments of band β is potentially available. If DNA from this band is recentrifuged without any intermediate treatment, one single band is observed. If, instead, the silver is first removed by dialysis, and then silver is added back, two DNA bands appear in a Cs_2SO_4 gradient (fig.3d). According to the relative aereas defined by both bands, it seems likely that they correspond to the DNA fragments G and D. No attempt was made to further characterize these two DNA bands.

The native structure of the purified DNA fragments rests on the following evidence: a) they have a normal optical spectrum, b) after heat denaturation, their hyperchromicity at 260 nm is of the order of 25% to 30%; c) digestion of the G fragment by Hpa II endonuclease leads to the expected size distribution [1]; d) a mixture of purified A and J fragments of λ plac II, can be used for the production of phage by transfection of *E. coli* (in collaboration with Ph. Kourilsky; unpublished results).

Isopycnic centrifugation of DNA in the presence of Ag^+ ions deserves some additional comments. It is usually assumed, that on behalf of the recovery of the initial buoyant density in CsCl, and of the optical and physiological properties, all the silver can be removed from the DNA with Cl^-, or CN^- ions [8]. We have checked this hypothesis with radioactive silver. It turns out that with an initial concentration of 0.3 silver ion per DNA base, and after extensive dialysis against NaCl 2 M, about 0.03% of the silver

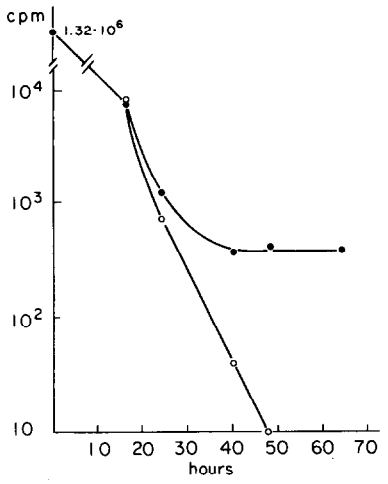

Fig.5. Residual $^{110}Ag^{+}$ on λ plac imm 434 as a function of time of dialysis against NaCl 2 M (●—●), and concentration of $^{110}Ag^{+}$ in the dialysis buffer (○—○). The buffer was renewed at the indicated time intervals.

remains on the DNA (fig.5); with an initial concentration of 0.03 silver ion (10 times higher specific activity) per base, about 0.2% of the silver cannot be removed (result not shown). This indicates that the remaining silver is very low indeed, but still significant. It also confirms that one should distinguish at least two types of binding silver to DNA [8,9], since at low silver concentration, relatively more silver remains bound to DNA. Daune and coworkers [9,10] and Filipski et al. [3] suggest that the amount of silver bound to DNA, not only depends on the overall base composition of a given molecule, but also on the actual base sequence within that molecule. A similar hypothesis is necessary for the interpretation of the buoyant density inversion between the two most dense bands of the λ-wild type Eco RI digested DNA, when the Ag^{+} concentration is raised from 0.25 to 0.35 silver ion per DNA base (fig.6).

Acknowledgements

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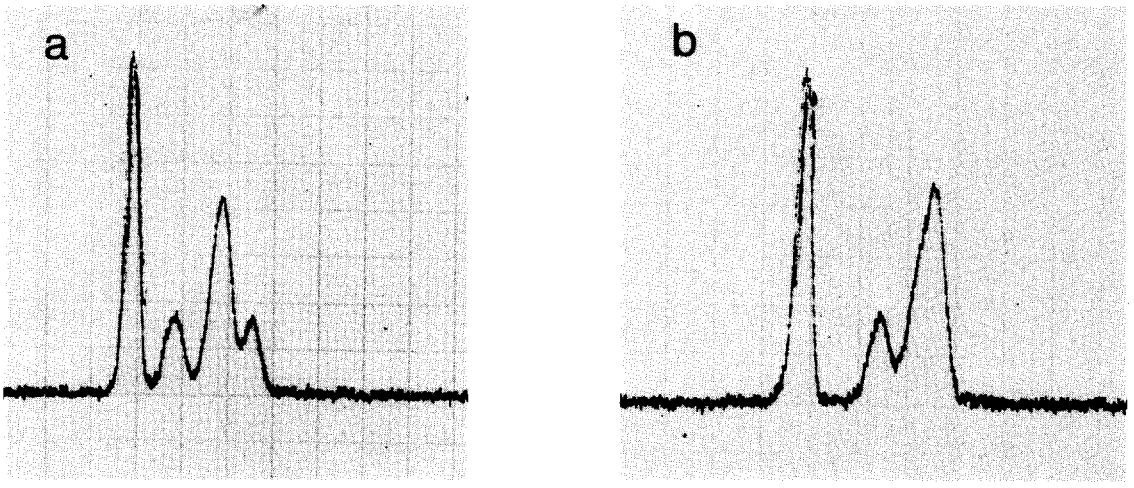

Fig.6. Analytical isopycnic centrifugation of the Eco RI digest of λ DNA at silver concentrations of 0.25 (a), and 0.35 (b) Ag^{+} ion per DNA base. The density increases from left to right.

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