Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3927–3930, October 1974

Bacteriophage λ Having *Eco*RI Endonuclease Sites Only in the Nonessential Region of the Genome

(restriction-resistant mutant/genetic cross/gel electrophoresis)

ALAIN RAMBACH AND PIERRE TIOLLAIS

Unités de Génétique Moléculaire et de Physico-chimie des Macromolécules Biologiques, Institut Pasteur, 25, rue du Docteur Roux, 75015 Paris

Communicated by Jacques Monod, July 8, 1974

ABSTRACT **A derivative of λ *b221* that has lost by mutation all *Eco*RI restriction sites has been isolated by alternative growth on restrictive and nonrestrictive strains. It has an efficiency of plating equal to 1 on the restrictive strain. Genetic cross of this bacteriophage with λ *plac5 imm21* gave rise to recombinants of intermediate restricting ratios. The analysis of the *Eco*RI endonuclease-cleaved DNA by polyacrylamide gel electrophoresis, compared with the genetic results, has permitted identification of *Eco*RI endonuclease cleavage sites in the recombinants. The genotypes are: λ *plac5 CI857 sRIλ3*^{0} *sRIλ2*^{0} *sRIλ1*^{0} and λ *plac5 CI857 sRIλ2*^{0} *sRIλ1*^{0}. The remaining cleavage sites, respectively, *sRIlac sRIλ4* and *sRIlac sRIλ4 sRIλ3*, are all located in a region nonessential for bacteriophage multiplication. The involvement of these mutant bacteriophages as vector for foreign genes are discussed.**

In the study of gene expression an important step is the isolation of specific DNA fragments. The use of transducing bacteriophages (1) has allowed the isolation of defined genes in prokaryotic systems, and the study of their expression *in vivo* and *in vitro*. Such methods do not exist for eukaryotic systems. In order to study isolated eukaryotic genes, one would like to insert specific sequences from eukaryotic chromosomal or extrachromosomal DNA into structures that can be self-replicated inside the bacteria, since this would allow the direct isolation of specific DNA fragments.

One method of gene insertion involves the introduction of the same cohesive ends in the chosen DNA and the self-replicating DNA, followed by the joining of the two structures either *in vitro* with ligase or *in vivo* inside the bacteria. To create the cohesive ends, a restriction endonuclease can be used. These enzymes recognize sites on DNA that have specific nucleotide sequences. To be useful for the preparation of DNA fragments to be fused, such an endonuclease must hydrolyze the DNA at a limited number of sites so that the generated DNA fragments can contain intact genes, and it must generate complementary ends long enough to be cohesive. The *Eco*RI endonuclease can be used for this purpose since it creates cohesive ends of 4 nucleotides at sites that are separated by, on the average, a few thousand base pairs. Using a similar method, Jackson *et al.* (2) have fused simian virus 40 DNA *in vitro* with a fragment of λ DNA, λ dv*gal*. Gene insertion has also been carried out with antibiotic-resistance transferable plasmid as a self-replicating structure (3). The DNA of the RTF plasmid was cleaved with the *Eco*RI endonuclease and joined, *in vitro* or *in vivo*, with fragments of DNA generated with the same endonuclease. The authors succeeded in inserting fragments of DNA of a *Staphylococcus* plasmid into an RTF plasmid replicating in *Escherichia coli* (4). The same method could be used to insert other genes.

Our goal here was to isolate a plaque-forming derivative of bacteriophage λ that could be used as a vector for foreign genes. In our opinion, λ bacteriophage has two advantages as a general transducing particle: (*a*) the hybrid DNA can be prepared in great quantity since each bacterial cell can produce several hundred λ DNA copies and the hybrid DNA can be purified easily as a component of bacteriophage particles, and (*b*) the detailed knowledge of the λ genome might be very useful for further studies on the hybrid DNA, e.g., in studying the expression of the foreign genes *in vivo* or *in vitro*.

For our purpose we had to isolate a λ bacteriophage whose DNA would have sites of *Eco*RI endonuclease cleavage located (*a*) only in a region that is nonessential for bacteriophage multiplication and (*b*) such that the fragments of DNA that contain the essential genes would have a total length inferior to that of the wild-type λ DNA and would be able to be encapsulated after integration of foreign DNA.

We have constructed such a bacteriophage in two steps: isolation of a multiple mutant that is resistant to the *Eco*RI endonuclease followed by genetic cross, giving rise to bacteriophage recombinants having ony two and three sites of *Eco*RI endonuclease cleavage, all located in a single region that is nonessential for bacteriophage multiplication.

MATERIALS AND METHODS

Bacteriophages. The strains used were λ*CI857* (5), λ*b221 CI857* (6), λ*plac5 imm21* (P. Kourilsky, personal gift), and λ*plac5 CI857 S7* (7). Following the convention suggested by Arber and Linn (8), we use the symbol *sRI*λ to designate an *Eco*RI restriction site of λ*plac5*. We number the sites as described in Fig. 1. We use the symbol *sRIλ1*^{0} to indicate the loss of *sRIλ1* by mutation (8).

Bacterial Strains. W3350 is a *gal*$^-$ *E. coli* K12 strain. RY12 is a derivative of W3350, which contains the plasmid RTF1 conferring the *Eco*RI host specificity (this strain was kindly sent to us by H. W. Boyer). AR1002 is a spontaneous *lac*$^-$ mutant of a nonlysogenic derivate of Y10 (9).

Media. 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).

Enzyme. *Eco*RI endonuclease was purified according to Yoshumori (10). The enzyme was generously made available to us by P. Yot.

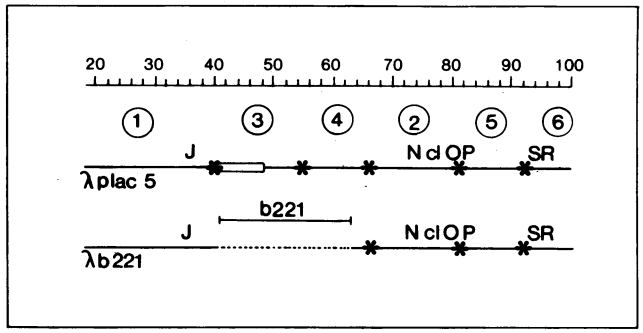

FIG. 1. Position of the *Eco*RI endonuclease cleavage sites on the genetic map of λ according to Allet *et al.* (12). The numbers in circles refer to the position of the fragments in the gel after electrophoresis. Asterisks refer to the *sRI* sites from the right to left, respectively, *sRIλ1*, *sRIλ2*, *sRIλ3*, *sRIλ4*, and *sRIlac*.

Measurement of Efficiency of Plating on EcoRI Restrictive Host. Bacteriophages with no *Eco*RI modification were obtained by growth on W3350 or by induction on lysogenic derivatives of AR1002 and will be referred to as λ · K bacteriophages. The titer of bacteriophages on the RY12 restrictive host divided by the titer on the W3350 host defines the efficiency of plating.

Isolation of Restriction-Resistant Mutant. Lysates were alternately made on RY12 and W3350 by the method of Arber and Kühnlein (11). After each passage on the nonrestrictive host the efficiency of plating of the lysate was determined, and the enrichment procedure was continued until the efficiency of plating reached 1. Restriction-resistant mutants were then purified by simple plaque isolation, and their properties were tested.

Bacteriophage Cross. The restriction-resistant mutant was crossed at 37° with λ*plac5 imm21* in the irradiated AR1002 strain. Resulting lysates were plated at 30° on the lysogenic strain AR1002 (λ*imm21*) on MacConkey lactose plates. Bacteriophage clones carrying the *lac* genes were picked in red plaques.

Propagation of Mutated Bacteriophages and Extraction of DNA. Thermosensitive derivatives of AR1002 *lac*⁻ strain lysogenic for *plac5* recombinant bacteriophages were isolated from the center of red plaques. Lysogenic bacteria were grown in L-broth medium at 32° to a titer of about 5×10^8 bacteria per ml. They were kept at 42° for 20 min and finally at 37° for 40 min. Chloroform was added and the lysate, after being made 0.5 M in NaCl, 10% (w/v) in polyethyleneglycol 6000, and 3% (w/v) in sodium dextran sulfate 500, was allowed to stand overnight at 4°. The sediment was centrifuged at low speed and the precipitate was resuspended in 3% of the original volume of 10 mM Tris·HCl (pH 7.5), 10 mM $MgSO_4$ buffer (Tris–Mg buffer). The suspension was cleared by a low-speed centrifugation, and the bacteriophages were then pelleted by a high-speed centrifugation. The pellet was resuspended in a small volume of the previous buffer and layered on a CsCl step gradient. After centrifugation for 2 hr at 25000 rpm in a SW50 rotor, the band formed by the bacteriophages was collected and centrifuged in a CsCl equilibrium gradient. The purified bacteriophages were then dialyzed for 24 hr against Tris–Mg buffer.

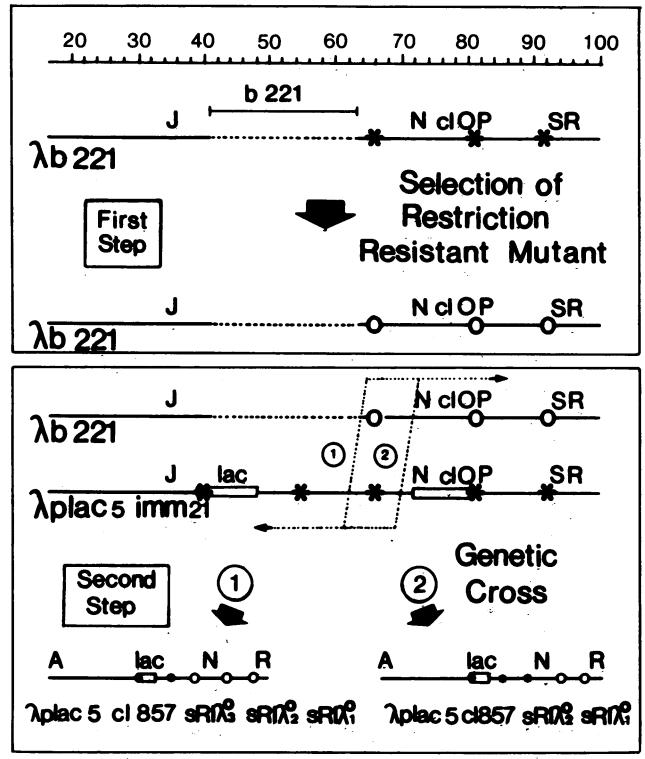

FIG. 2. General protocol for construction of bacteriophage with only 2 or 3 of *Eco*RI endonuclease sites. *, *Eco*RI endonuclease-sensitive site; O, mutated site.

For extraction of DNA, the bacteriophages were resuspended in 10 mM Tris·HCl (pH 8) at 12 A_{260} units/ml. An equal volume of freshly distilled phenol, previously equilibrated with the same buffer, was added and the DNA was extracted for 30 min at 4° with occasional gentle stirring. The aqueous phase was treated with phenol and the re-extracted DNA was dialyzed for 24 hr against 20 mM Tris·HCl (pH 8), 1 mM EDTA, 0.5 M NaCl, 20 mM 2-mercaptoethanol, and for 24 hr against 20 mM Tris·HCl (pH 8). The DNA was stored at 4°.

Hydrolysis of DNA by EcoRI Endonuclease and Analysis of the Products by Polyacrylamide Gel Electrophoresis. DNA (8 A_{260} units/ml) in 0.1 M Tris·HCl (pH 7.4), 10 mM $MgCl_2$ was incubated with *Eco*RI endonuclease. The incubation time 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 10 mM tris·HCl (pH 8), 1 mM EDTA. Aliquots of the DNA solution containing 10 μg of DNA were loaded into the sample wells of the gel. The separating gel was a $0.4 \times 12 \times 10$-cm slab containing a linear concentration of polyacrylamide from 2.5% at the origin to a final concentration of 7.5% (12). The buffer, used for the gel and the reservoirs, was 40 mM Tris-acetate (pH 8), 20 mM sodium acetate. Electrophoresis was carried out in an electric field of 6 V/cm for 24 hr at room temperature. The DNA was stained by soaking the gel in a 0.02% solution of methylene blue for 2 hr, and excess stain was removed by washing the gel with water (12).

RESULTS

General Procedure. It is practically impossible to isolate directly from the λ wild type, which has several *Eco*RI re-

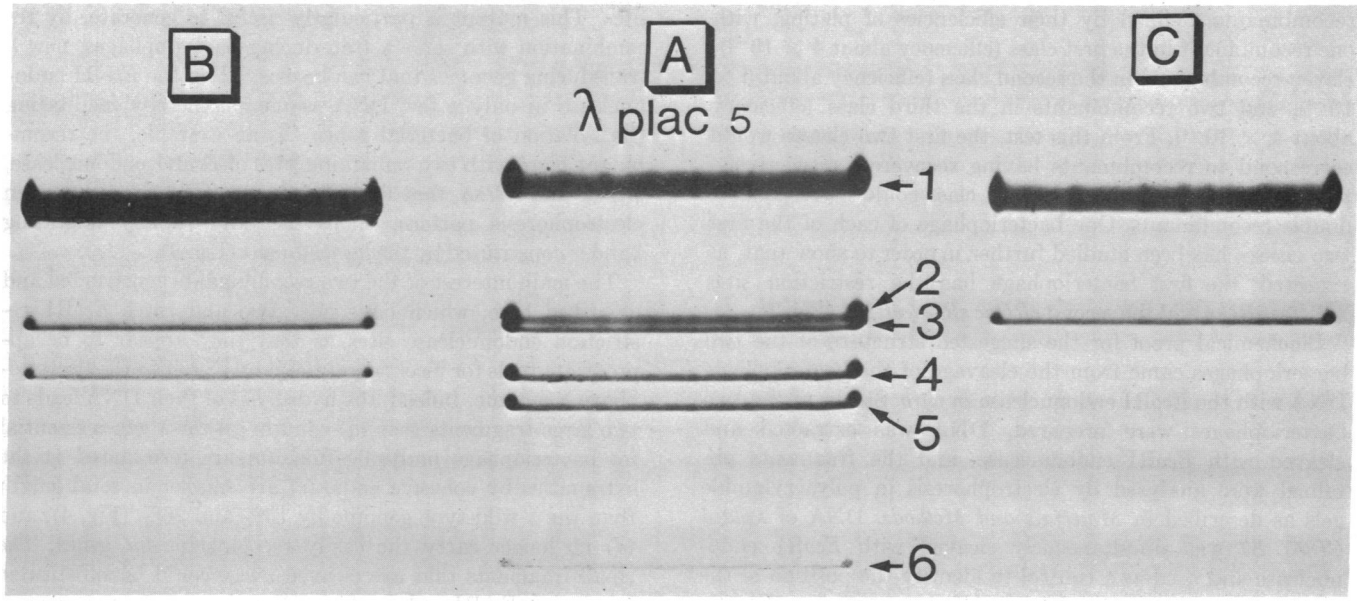

FIG. 3. Separation by polyacrylamide gel electrophoresis of *Eco*RI endonuclease fragments obtained from bacteriophage DNA. Gel electrophoresis was done as described in *Materials and Methods*. Bacteriophages studied are λ*plac5 CI857 S7* (A) and the two *plac5 CI857* recombinants which have, respectively, three and two sites of *Eco*RI restriction (B and C).

striction sites, the desired λ derivative, with only a few *Eco*RI restriction sites all located in the appropriate region of λ. Indeed, the method of isolation of bacteriophages that have lost restriction sites by mutation does not allow any choice as to the λ sites that will disappear. We thus decided to proceed in the two steps presented in Fig. 2: (*a*) isolation of a multiple mutant of λ that has lost all *Eco*RI restriction sites and (*b*) construction by genetic cross of bacteriophages having *Eco*RI restriction sites in the appropriate λ region.

Selection of a Restriction-Resistant Mutant. Our goal here was to isolate a bacteriophage that has lost all its *Eco*RI restriction sites. Since λ possesses five *Eco*RI restriction sites, as demonstrated biochemically (12), a direct selection for a mutant with no sites appeared to us to be very difficult. It was easier to start from a bacteriophage having less than five sites. Comparison of the location of the *Eco*RI endonuclease sites along the λ DNA (12) with the genetic map of λ derivatives (13) led us to choose the deletion mutant λ*b221*. This bacteriophage was expected to carry only three *Eco*RI sites (see Fig. 1).

Since the method of selection of a restriction resistant mutant is based on the correlation of the efficiency on a restrictive host with the number of remaining restriction sites (11), it was necessary to verify that in the *Eco*RI system also the efficiency of plating of a bacteriophage decreases with the number of remaining sites. For that purpose we have measured the efficiency of plating on a restrictive strain of λ*b221 CI857* and λ*CI857*. The measurements, 7×10^{-3} for λ*b221 CI857* and 9×10^{-4} for λ*CI857*, showed that the efficiency of plating of the deleted bacteriophage is in fact higher than for λ*CI857*.

The deletion mutant λ*b221 CI857* was then submitted to the selection procedure (Fig. 2): it was alternately grown on restrictive and nonrestrictive strains as described in *Materials and Methods*. After 14 passages on the two strains, a bacteriophage clone was isolated that had an efficiency of plating on the restrictive strain equal to 1. This result suggests that the isolated bacteriophage has lost its three *Eco*RI restriction sites.

Construction and Characterization of Bacteriophages with Restriction Sites Only in a Region Nonessential for Multiplication. The region of the genome between genes J and N is not essential for bacteriophage multiplication. We tried to introduce *Eco*RI restriction sites inside this region by recombination. For that purpose we crossed our restriction-resistant bacteriophage with the λ strain λ*plac5 imm21*, as indicated in Fig. 2. Recombinants that had inherited the *CI857* marker of the *b221* parent and the *plac5* marker of the other parent were isolated and tested for restriction properties. Fourteen *plac5 CI857* recombinant clones were isolated and further studied.

Their efficiency of plating on the restricting strain has been tested. The results allowed us to distinguish three classes of

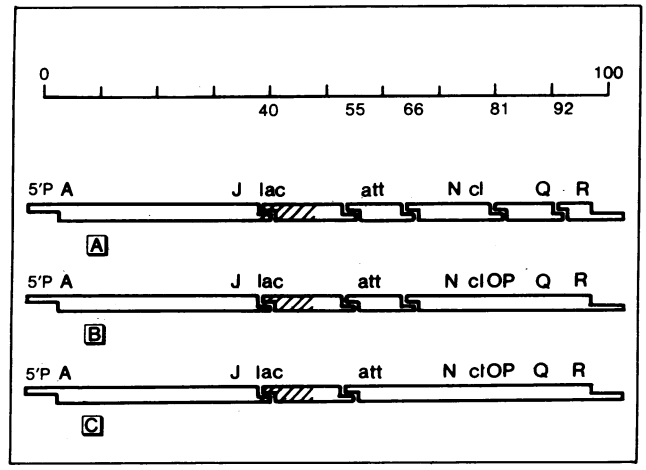

FIG. 4. Cleavage pattern with *Eco*RI endonuclease of the constructed bacteriophages. Bacteriophages studied are: (A) λ*plac5 CI857 S7*; (B) λ*plac5 CI857 sRIλ2⁰ sRIλ1⁰*; and (C) λ*plac5 CI857 sRIλ3⁰ sRIλ2⁰ sRIλ1⁰*.

recombinants, defined by their efficiencies of plating, with one recombinant in the first class (efficiency about 4×10^{-2}), eleven recombinants in the second class (efficiency about 5×10^{-3}), and two recombinants in the third class (efficiency about 4×10^{-4}). From this test, the first two classes would correspond to recombinants having recovered, respectively, two and three sites, and the third class could correspond to double recombinants. One bacteriophage of each of the first two classes has been studied further in order to show that, as expected, the first bacteriophage has the restriction sites λ*sRIlac sRIλ4* and the second *sRIlac sRIλ4 sRIλ3* (Fig. 2).

Biochemical proof for the suggested structure of the two bacteriophages came from the cleavage of the bacteriophage DNA with the *Eco*RI endonuclease *in vitro*. Stocks of the two bacteriophages were prepared, DNA was extracted and cleaved with *Eco*RI endonuclease, and the fragments obtained were analyzed by electrophoresis in polyacrylamide gels as described in *Materials and Methods*. DNA of λ*plac5 CI857 S7* was simultaneously cleaved with *Eco*RI endonuclease and used as a control to identify the position of the bands and to deduce the location of the remaining cleavage sites. In the first bacteriophage pattern, the disappearance of bands *2*, *4*, *5*, and *6* and the existence of band *3* prove that the DNA contains two cleavage sites: *sRIlac* and *sRIλ4* (Figs. 1 and 3). In the second bacteriophage pattern, the disappearance of bands *2*, *5*, and *6* and the existence of bands *3* and *4* prove that the DNA contains three cleavage sites: *sRIlac*, *sRIλ4*, and *sRIλ3* (Figs. 1 and 3).

DISCUSSION

In the course of the construction of the presented bacteriophages, a mutant of bacteriophage λ that is *Eco*RI restriction-resistant has been isolated. The genetic characteristics of this restriction-resistant bacteriophage are an efficiency of plating on the restrictive strain equal to 1 and the ability to give rise, by genetic cross, to recombinants of intermediate restriction ratios. These results, in addition to the biochemical characteristics of the two recombinants obtained from it by genetic cross (existence of, respectively, only two and three sites of *Eco*RI endonuclease), lead us to propose for the restriction-resistant bacteriophage the following genotype: λ*b221 CI857 sRIλ3*^{0} *sRIλ2*^{0} *sRIλ1*^{0}. This shows that in the *Eco*RI restriction system there is a correlation between the efficiency of plating on a restrictive strain and the number of restriction endonuclease sites present on the DNA. Such a correlation is not found in all systems: T7 DNA, for instance, is cleaved at several sites by the restriction endonuclease B *in vitro*, but the T7 bacteriophage itself is not restricted *in vivo* (14).

The *Eco*RI restriction-resistant mutant, which we designate λ^0*Eco*RI, makes possible the construction of various recombinants that have recovered defined *Eco*RI restriction sites. This mutant is particularly useful to generate, by recombination with some λ transducing bacteriophages, new λ transducing genomes that can be cleaved by the *Eco*RI endonuclease in only a few DNA segments, thereby facilitating the isolation of bacterial genes. As an example, the recombinant *plac5* with two remaining sites of *Eco*RI endonuclease, *sRIlac* and *sRIλ4*, that we have constructed here displays an electrophoresis pattern where the only rapidly migrating band is constituted by the *lac* fragment (Fig. 3).

The main interest of the two recombinants constructed and described here, which have only two and three *Eco*RI restriction endonuclease sites, is that they appear to be appropriate tools for insertion of foreign DNA into the bacteriophage λ genome. Indeed, the hydrolysis of their DNA leads to two large fragments that (*a*) contain all the λ genes essential for bacteriophage multiplication, (*b*) are terminated at the extremities by cohesive ends, (*c*) are smaller in total length than the λ wild-type genome by 15% and 26% (Fig. 4), and (*d*) no longer carry the easily recognizable *lac* genes. The small fragments that are cleaved away could be substituted by foreign *Eco*RI endonuclease DNA fragments.

We are grateful to Bernard Allet for helpful advice for the gel separation technique and to Henri Buc for encouragement and helpful discussions. The work was aided by grants from the Centre National de la Recherche Scientifique, the Centre d'Etudes Nucléaires, the National Institute of Health, the Collège de France, the Délégation Générale à la Recherche Scientifique et Technique and the University of Paris VII.

1. Morse, M., Lederberg, E. & Lederberg, J. (1956) *Genetics* **41**, 121–142.
2. Jackson, D. A., Symons, R. H. & Berg, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2904–2909.
3. Cohen, S. N., Chang, A. C. Y., Boyer, H. W. & Belling, R. B. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3240–3244.
4. Chang, A. C. Y. & Cohen, S. N. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1030–1034.
5. Sussman, R. & Jacob, F. (1962) *C.R.H. Acad. Sci.* **254**, 1517–1520.
6. Parkinson, J. S. & Huskey, J. R. (1971) *J. Mol. Biol.* **56**, 369–384.
7. Beckwith, J. R. & Signer, E. R. (1966) *J. Mol. Biol.* **19**, 254–265.
8. Arber, W. & Linn, S. (1969) *Annu. Rev. Biochem.* **38**, 467–500.
9. Bachmann, B. J. (1972) *Bacteriol. Rev.* **36**, 525–557.
10. Yoshimori, R. N. (1971) Ph.D. Dissertation, University of California.
11. Arber, W. & Kühnlein, U. (1967) *Pathol. Microbiol.* **50**, 946–952.
12. Allet, B., Jeppensen, P. G. N., Katagiri, K. J. & Delius, H. (1973) *Nature* **241**, 120–123.
13. Davidson, N. & Szybalski, W. (1971) in *Bacteriophage Lambda* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.-Y.), pp. 45–82.
14. Eskin, B., Lautenberger, J. A. & Linn, S. (1973) *J. Virol.* **11**, 1020–1023.