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Genetics

Construction of plasmids carrying the *c*I gene of bacteriophage λ

(repressor/DNA cloning/operon construction)

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ABSTRACT **By techniques of recombination *in vitro*, we have constructed a plasmid bearing the repressor gene (*c*I) of bacteriophage λ fused to the promoter of the *lac* operon. Strains carrying this plasmid overproduce λ repressor. This functional *c*I gene was reconstituted by joining DNA fragments bearing different parts of that gene. Flush end fusion techniques, involving no sequence overlap, were necessary for the construction; in certain cases, the abutting of the DNA molecules bearing ends generated by different restriction endonucleases creates a sequence at the junction which is recognized by one of the restriction endonucleases.**

We have constructed a plasmid in which the promoter of the *lac* operon has been placed adjacent to the repressor gene (*c*I) of bacteriophage λ, by techniques of recombination *in vitro*. *Escherichia coli* strains carrying this plasmid overproduce repressor because transcription of *c*I originates mainly at the *lac* promoters and because each cell contains multiple copies of the plasmid. Certain novel aspects of our construction may be applied to the construction *in vitro* of other hybrid operons. Other λ repressor overproducing strains have been created by Gronenborn and Müller-Hill (1), who have isolated *lac* promoter-*c*I fusions *in vivo*.

MATERIALS AND METHODS

E. coli K12 strain 294 (endo I^-, B_1^-, r_K^-, m_K^+; obtained from M. Meselson) was the host used in most of these experiments. A *lac* repressor overproducing strain (2), V2000 [F′ *pro lac* $i^{Q1}Z^-{}_{U118}/\Delta$(*lac-pro*) Sm^R(λ*imm*^{21} *plac*5)], was constructed and used in some experiments. The plasmid vectors pCR11 (3) and pMB9 (4) were used. Plasmid DNA was prepared by the method of Clewell and Helinski (5). DNA fragments generated by restriction endonucleases were prepared by electrophoresis on polyacrylamide gels as described by Maniatis *et al.* (6). *Eco*RI, *Hin*dIII, and *Hae*III digestions were performed in *Hin* buffer (7) and *Hpa*II digestions were performed in *Hpa* buffer (7). Reactions using *E. coli* DNA polymerase I (DNA nucleotidyltransferase, deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) (a gift of W. McClure) were performed in 50 mM Tris·HCl at pH 7.8, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 μg/ml of bovine serum albumin plus 2 μM each of dATP, dCTP, dGTP, and dTTP for 1 hr at 15° (8). T4 polynucleotide ligase [polynucleotide synthetase, poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase(AMP-forming), EC 6.5.1.1] reactions were performed in 6.6 mM Tris·HCl at pH 7.4, 6.6 mM $MgCl_2$, 6.6 mM 2-mercaptoethanol, 100 μM ATP at 4°; DNA fragments were present at 100–300 μg/ml. For joining DNA fragments bearing flush ends (9), 5-fold higher concentrations of ligase were used than were required to join an equivalent number of staggered ends, and the reactions were incubated at room temperature for 4 hr. Cells were transformed with DNA as described by Cohen *et al.* (10). Drug resistant transformants were selected on drug supplemented agar plates, and λ immune transformants were selected on agar plates seeded with 10^9 λKH54 (11) and 10^9 λKH54h_{80}; in some cases, both selections were performed simultaneously. The immunity of plasmid containing strains was further tested by streaking single colonies across a streak of phage on an agar plate. Colonies of transformants which constitutively synthesized β-galactosidase appeared blue on agar plates containing a chromogenic, noninducing substrate, 5-chloro-4-bromo-3-indolyl-β-D-galactoside (40 μg/ml). Assays of λ repressor (12), β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (13), and the isolation of operator containing DNA fragments on nitrocellulose filters (14) have been described previously. Purified λ repressor was a gift of R. Sauer and purified *lac* repressor was a gift of A. Maxam. Experiments were carried out in a P1 (EK1) facility.

RESULTS

Preliminary considerations

We wished to proceed in two steps; first, to clone *c*I flanked by as little extraneous phage DNA as possible on a plasmid and then to insert a DNA fragment bearing the *lac* promoter near the beginning of *c*I. Two problems arose. No single restriction endonuclease cleaves λ DNA just outside the ends of *c*I without also cleaving within it, and no restriction endonuclease site suitable for inserting the *lac* promoter exists in λ DNA near the beginning of *c*I. We adopted a strategy based on the following considerations: gene *c*I can be neatly isolated on two DNA fragments, one of which bears two *Hin*dIII ends and the other of which bears one *Hin*dIII end and one *Hae*III end (see Fig. 1). Proper joining of these fragments reconstitutes *c*I on a larger fragment bearing one *Hin*dIII end and one *Hae*III end. *Hin*dIII ends are staggered and readily anneal to each other, whereas *Hae*III ends are flush. Staggered ends which anneal to each other can be joined by T4 polynucleotide ligase (15), and flush ends can be joined to other flush ends by that enzyme (9). To clone our *c*I fragment, we sought a plasmid which could be opened so as to produce one *Hin*dIII end and one flush end. The plasmids we used, pCR11 and pMB9, each have a single *Hin*dIII site and a single *Eco*RI site. Although *Eco*RI produces staggered ends, we anticipated that these ends could be converted to flush ends by treatment with DNA polymerase I and the four deoxyribonucleotide triphosphates, because the recessed 3′ end can be extended by copying the protruding 5′ end of the complementary strand (a process we hereafter refer to as filling-in). Precise joining of a filled-in *Eco*RI end to a *Hae*III end should produce a molecule recognized by *Eco*RI at the junction (see Fig. 2). This regenerated *Eco*RI site near the beginning of *c*I could then be used as a site to insert the *lac* promoter.

The plasmids pCR11 and pMB9 are derived from Col E1, which is normally present in about twenty copies per cell (16). pCR11 and pMB9 carry drug resistance determinants (kanamycin and tetracycline, respectively).

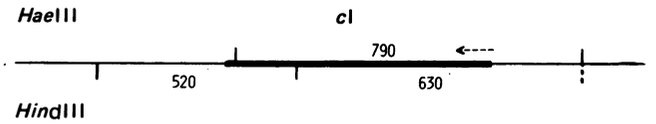

FIG. 1. Restriction endonuclease sites in the vicinity of the *c*I gene of λ. *Hae*III cleavage sites are indicated above, and *Hin*dIII sites below, the map of the region including and flanking *c*I. (The extreme right *Hae*III site is also indicated below the line.) Distances in base pairs between various cleavage sites are given. The extent of *c*I is indicated by the heavy line. The extreme right *Hae*III site is in the *cro* gene, and the extreme left *Hin*dIII site is in the gene which lies to the left of *c*I, *rex*. The arrow indicates the direction of transcription of *c*I.

Cloning *c*I

With these considerations in mind, we proceeded as follows (see Fig. 2): plasmid pCR11 DNA was digested with *Eco*RI and the protruding single-stranded ends converted to double-stranded flush ends by filling-in with DNA polymerase I. The product was then digested with *Hin*dIII to produce a vector DNA molecule bearing one *Hin*dIII end and one flush end. This procedure removes at least part of the kanamycin resistance gene(s). Two DNA fragments bearing portions of *c*I were produced as follows (see Fig. 1): λ DNA was digested with *Hae*III, and a 790 base pair fragment was isolated that carries the right portion of *c*I plus about 150 base pairs to the right of *c*I. This fragment was further digested with *Hin*dIII to produce a fragment 630 base pairs long. Separately, λ DNA was digested with *Hin*dIII and a 520 base pair fragment bearing the left portion of *c*I was isolated. Together these two fragments span the entire *c*I gene. Gene *c*I was then simultaneously reconstituted and inserted in the plasmid by treating a mixture of the specially prepared plasmid and the fragments which span *c*I with T4 polynucleotide ligase (see Fig. 2). Five λ immune clones were isolated from bacteria transformed with the products of this reaction, and their plasmids were analyzed by restriction endonuclease digestion. One isolate, pKB155, produced three fragments (5000, 630, and 520 base pairs long) when cleaved with *Eco*RI and *Hin*dIII, as predicted by the construction shown in Fig. 2 (E and F). In particular, the fused *Hae*III–*Eco*RI junction to the right of *c*I was cleaved again by *Eco*RI.

The *c*I gene was transferred from pKB155, which bears no drug resistance marker, to the plasmid pMB9, which carries a tetracycline resistance gene, as follows: an 1150 base pair piece of DNA bearing the entire *c*I gene was excised from pKB155 by complete digestion with *Eco*RI and partial digestion with

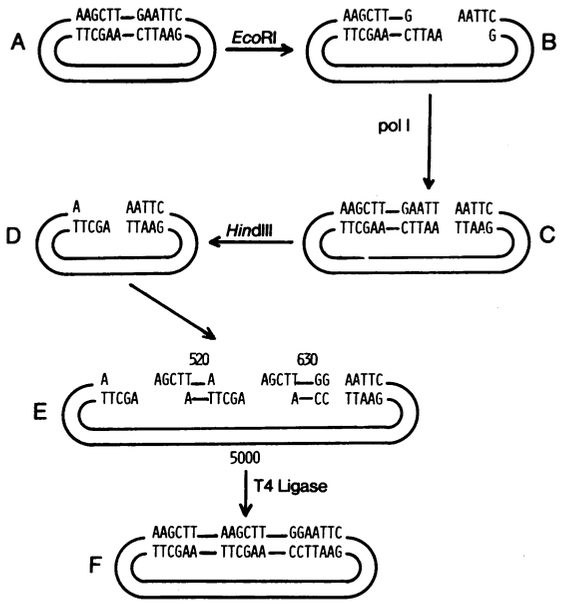

FIG. 2. Cloning the *c*I gene. Gene *c*I, carried on two DNA fragments, was inserted into the plasmid pCR11 as diagrammed (see *text*). The plasmid pCR11 (A) was digested with *Eco*RI (B), and the protruding 5′ ends were filled in (see *text*) with DNA polymerase I (pol I) (C); the product was digested further with *Hin*dIII (D). The *Hae*III 790 base pair fragment digested with *Hin*dIII and the *Hin*dIII 520 base pair fragment (see Fig. 1) were added to the prepared plasmid (E). No attempt was made to remove the extra pieces of DNA generated by the *Hin*dIII digestions of the plasmid and of the *Hae*III 790 fragment. The mixture was treated with polynucleotide ligase, and the desired recombinant (F) was selected as described in the *text*.

*Hin*dIII. Plasmid pMB9 was digested with *Eco*RI and *Hin*dIII, which removes a nonessential 350 base pair fragment; the 1150 base pair *c*I gene fragment was added, and the mixture was treated with ligase. Bacteria were transformed with the products of ligation, and tetracycline resistant lambda immune clones were readily isolated. Restriction endonuclease analysis of several isolates (not shown), typical of which is pKB158, confirmed the structure shown in Fig. 3.

Construction of a plasmid fusing the *lac* promoter to the *c*I gene

A plasmid bearing a (nominally) 205 base pair fragment of DNA which carries the *lac* UV5 promoter-operator region was constructed by F. Fuller. He used a 203 base pair fragment of known sequence generated by *Hae*III. The *Hae*III ends of this

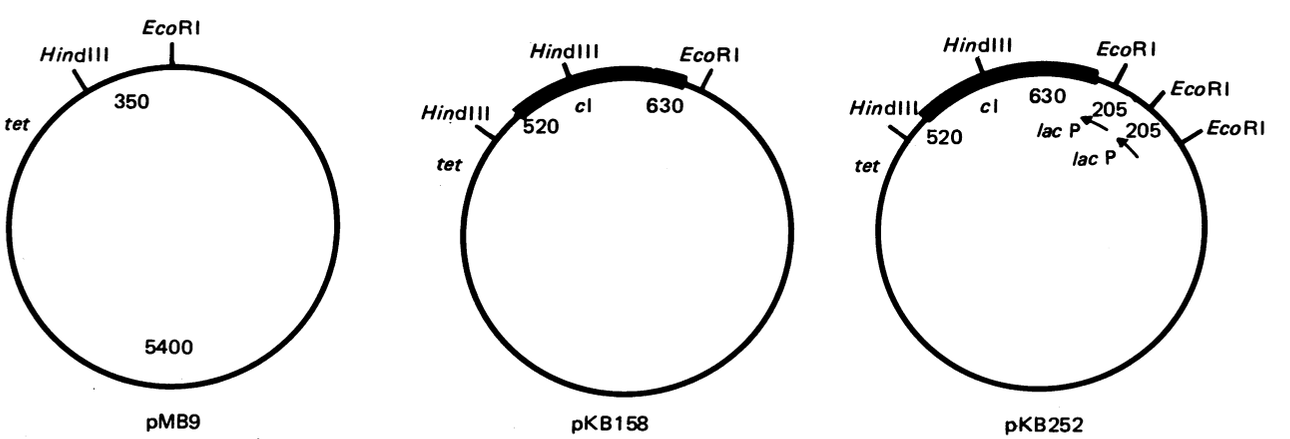

FIG. 3. The structure of pMB9, pKB158, and pKB252. The *Eco*RI and *Hin*dIII sites, the sizes of the cleavage products, and the location of *c*I in the various plasmids are shown. The location and orientation of the *lac* promoters in pKB252 are indicated.

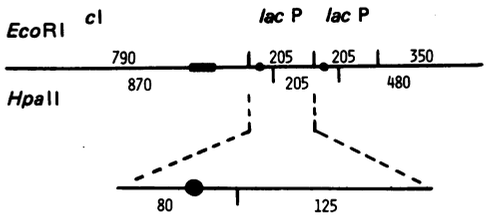

FIG. 4. Structure of pKB252 in the *lac* promoter-cI gene region. The location of *Eco*RI and *Hpa*II cleavage sites are shown, respectively, above and below the map of the 1550 base pair fragment produced by *Hae*III digestion of pKB252. The locations of the *lac* and lambda operators on this fragment are indicated by the solid circles and solid box, respectively. Distances between various endonuclease sites and the position of the *Hpa*II site in the *lac* promoter are given in base pairs.

fragment were converted to *Eco*RI ends by joining the fragment to filled-in *Eco*RI ends on a plasmid by using the methods described above. Colonies harboring plasmids which carry the *lac* promoter-operator were identified by their constitutive synthesis of β-galactosidase, which renders them blue on agar plates containing 5-chloro-4-bromo-3-indolyl-β-D-galactoside. The *lac* promoter fragment serves as a "portable promoter" with an *Eco*RI site 65 base pairs from the startpoint of transcription (corresponding to the ninth amino-acid residue of the *lac z* gene). This fragment was excised from its carrier with *Eco*RI and inserted near the beginning of *c*I at the single *Eco*RI site in pKB158. About one half of the tetracycline resistant clones which were isolated carried the *lac* promoter fragment on the plasmid, as manifested by their constitutive synthesis of β-galactosidase. All of these clones were immune to λ*vir* (unlike the strain carrying pKB158) and sensitive to λ*imm*^{434}*c*I^{-} in cross-streak tests, although many clones exhibited some cell death at the junction of the cross-streak with λ*vir*. Three clones which exhibited no apparent sensitivity to λ*vir* were selected for further study. Typical of the plasmids in these clones is pKB252.

Structure of pKB252

An *Eco*RI plus *Hin*dIII digest of pKB252 yielded the 205 base pair promoter fragment as well as the fragments produced by a similar digestion of pKB158. When pKB252 was digested with *Hae*III, a 1550 base pair fragment was produced. This fragment, which extends from the *Hae*III site in *c*I to a *Hae*III site which is 350 base pairs beyond the *Eco*RI site in the plasmid DNA (17), was expected to contain the *lac* promoter. Analysis of this 1550 base pair fragment confirmed the structure of pKB252 as shown in Fig. 3. The products of *Hpa*II digestion of this fragment were 205, 480, and 870 base pairs long (see the diagram of Fig. 4). Because the *c*I region of λ does not have any *Hpa*II sites (11), and the *lac* promoter fragment does have a *Hpa*II site about 85 bases from one end, this pattern of fragments suggested the structure shown in Fig. 4. In particular, the 205 base pair product suggested the presence of multiple *lac* promoters. If only one promoter were present, then no such piece would have been produced. Moreover, all such promoters must have the same orientation; if any two promoter regions were oppositely oriented, then *Hpa*II digestion would have produced a 160 or a 250 base pair fragment.

The exact number of *lac* promoters in pKB252 was determined as follows: uniformly ^{32}P labeled plasmid DNA was digested with *Hae*III and the 1550 base pair fragment was isolated. This fragment was digested with *Eco*RI and the products separated on a polyacrylamide gel. The resulting fragments (205, 360, and 790 base pairs) were cut from the gel and the radioactivity in each fragment was determined (Table 1). The 205 base pair fragment was present in twice molar quantities compared to the 360 and 790 base pair fragments which indicated that there are two *lac* promoters in pKB252.

Table 1. The number of *lac* promoter fragments in pKB252

Fragment size in base pairs	Radioactivity ^{32}P counts per minute	Stoichiometry
790	3110	1
360	1345	1
205	1350	2

Stoichiometry is calculated by comparing the ratios of radioactivity/fragment size.

To prove that the *lac* promoters transcribe toward *c*I, we took advantage of the fact that *Hpa*II cuts near the *lac* operator in the *lac* promoter. The *Hae*III 1550 fragment was digested with *Hpa*II and the fragments produced were mixed with either *lac* or lambda repressor and passed through nitrocellulose filters. The fragments retained on the filters were eluted and analyzed on a polyacrylamide gel. It was found that the 870 base pair fragment was retained by both *lac* and λ repressors, the 205 base pair fragment was retained only by *lac* repressor, and the 480 base pair fragment was not retained by either repressor. These results are consistent only with the orientation shown in Fig. 4 in which the *lac* promoters transcribe toward *c*I.

Control of repressor synthesis *in vivo*

Cell extracts of strains containing various plasmids were assayed for λ repressor and, in some cases, an aliquot of the culture was assayed for β-galactosidase. The results are listed in Table 2. Strains carrying plasmids bearing *c*I but not the *lac* promoter (pKB155 and pKB158) contained about five times more repressor than did single lysogens. Strains carrying pKB252, which has two *lac* promoters, produced 35 times more repressor than did single lysogens.

Synthesis of lambda repressor in pKB252 strains is regulated by *lac* repressor (Table 2). A strain bearing a wild-type *lac* operon does not make enough *lac* repressor to repress significantly the synthesis of λ repressor from the *lac* promoters on pKB252 (Table 2). When this plasmid was transferred to a strain which

Table 2. Repressor synthesis in plasmid containing strains

Strain	Repressor level × 10^{-3}	β-Galactosidase level
28(λ)	0.3	N.D.
294/pKB155	1.0	N.D.
294/pKB158	1.5	3
294/pKB252	9.7	2.8×10^3
294/pKB252 (+IPTG)	10.7	2.8×10^3
V2000/pKB252	1.3	10
V2000/pKB252 (+IPTG)	2.9	1.9×10^3

Various strains were grown overnight at 37° in M9 salts and glucose medium, and were assayed for lambda repressor and β-galactosidase. The results are given in units of repressor per milligram of protein. One unit is the amount of repressor which gives half-maximal binding in the nitrocellulose filter binding assay and corresponds to approximately 0.5 ng of repressor. β-Galactosidase results are given in the units of Miller (13). A single lysogen, 28 (λ), is included for comparison purposes. Strain 294 makes wild-type *lac* repressor levels, whereas strain V2000 over-produces *lac* repressor about 100-fold (2). ITPG, isopropyl-thiogalactoside. N.D., not determined.

Table 3. Use of the filling-in method with various restriction endonucleases

Restriction endonuclease	Recognition sequence	Requirement for site regeneration	Subsite	Subsite Recognition sequence
*Eco*RI	↓ GAATTC / CTTAAG ↑	5′ C	*Eco*RI*	↓ AATT / TTAA ↑
*Eco*RII	↓ CCAGG / GGTCC ↑	None	*Eco*RII	↓ CCAGG / GGTCC ↑
*Hin*dIII	↓ AAGCTT / TTCGAA ↑	5′ T	*Alu*I	↓ AGCT / TCGA ↑
*Bam*I	↓ GGATCC / CCTAGG ↑	5′ C	*Mbo*I	↓ GATC / CTAG ↑
*Bgl*II	↓ AGATCT / TCTAGA ↑	5′ T	*Mbo*I	↓ GATC / CTAG ↑

Several endonucleases which produce 5′ protruding ends are listed with the DNA sequence which they recognize (20–24, R. J. Roberts, G. Wilson, and F. E. Young, submitted for publication). After filling in these ends with DNA polymerase I, the original recognition sequence can be regenerated by joining the filled-in end to a flush ended DNA molecule which meets the requirement listed in the third column. When joined to a flush ended fragment not meeting that requirement, the filled-in end generates a sub-specificity, which is described in the fourth and fifth columns (20, 25, R. E. Gelinas, P. A. Myers, K. Murray, and R. J. Roberts, unpublished). The top line of each recognition sequence reads from the 5′ to the 3′ end; the bottom line reads in the opposite direction.

overproduces *lac* repressor, the synthesis of λ repressor was reduced to the basal (i.e., pKB158) level. IPTG (isopropyl-thiogalactoside), an inducer of the *lac* operon, partially induces β-galactosidase synthesis in cells which overproduce *lac* repressor. Table 2 shows that isopropyl-thiogalactoside also partially induces λ repressor synthesis when such cells carry pKB252.

DISCUSSION

We have described the use of techniques of DNA recombination *in vitro* to construct a plasmid on which the λ repressor gene (*cI*) is transcribed largely from the promoter of the *lac* operon. This plasmid has two copies of the *lac* promoter which transcribe toward *cI*. As predicted by this arrangement, the synthesis of λ repressor in strains bearing this plasmid is regulated by the *lac* repressor. The *lac* promoter fragments in pKB252 contain the startpoint of translation of the *lac z* gene; however, several translational stop signals immediately precede *cI* (18), and repressor synthesized in strains carrying pKB252 is not a fusion product initiated at the *lac z* translational startpoint. Strains carrying this plasmid produce about 35 times more repressor than do single lysogens, as measured by DNA binding activity; however, these levels are variable and are often as much as 3-fold higher. The source of this variation is not understood. These repressor levels are 10- to 25-fold lower than would be expected on the basis of the known strength of the *lac* promoter (19). This inefficient expression might occur because transcription beginning at the *lac* promoter attenuates frequently before transcribing *cI*, because the progress of RNA polymerase is blocked by λ repressor bound to the operator just to the right of *cI*, or because the mRNA produced is inefficiently translated. The level of repressor was not higher when plasmid pKB252 was carried by a strain bearing an *SuA* mutation. Nevertheless, the high levels of repressor made by strains carrying pKB252 have greatly facilitated structural studies of repressor.

Strains carrying plasmids in which *cI* is transcribed from its own promoter produce about five times more repressor than does a single lysogen, even though these plasmids should be present in about 20 copies per cell (16). Gene *cI* is known to regulate its own synthesis (18), and these strains are exemplary of such autogenous regulation.

The expression of tetracycline resistance in strains carrying pKB158 and pKB252 may be under the control of the promoter for *cI* (P_{RM}) and the *lac* promoter. H. W. Boyer (personal communication) has found evidence suggesting that the *Hin*dIII site in pMB9 is within the promoter of the tetracycline resistance gene. In our experiments, tetracycline resistant λ immune clones were readily isolated which were missing the pMB9 specific material between the *Eco*RI and *Hin*dIII sites; presumably the expression of tetracycline resistance in these cases results from an extension of the *cI* mRNA.

Our construction required the use of new techniques which are generally applicable. We have assembled a hybrid operon from separate pieces; some junctions involved sequence overlap, some did not. Furthermore, the DNA polymerase I filling-in technique allowed us to join precisely DNA fragments which could not be joined directly by treatment with ligase. These techniques greatly expand the spectrum of restriction endonucleases which are useful in cloning experiments. An especially important feature of these techniques is the ability to regenerate endonuclease recognition sites at junctions involving filled-in ends. We demonstrate this explicitly in one case (the joining of a filled-in *Eco*RI end to a *Hae*III end), and available sequence data suggest that it will be a general feature of the method (see Table 3).

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1. Gronenborn, B. & Müller-Hill, B. (1976) *Mol. Gen. Genet.*, in press.
2. Müller-Hill, B., Fanning, T., Geisler, N., Gho, D., Kania, J., Kathman, P., Meissner, H., Schlotmann, M., Schmitz, A., Treisch, I. & Beyreuther, K. (1975) in *Protein Ligand Interactions*, eds. Sund, H. & Blauer, G. (Walter de Gruyter, Berlin), pp. 211–224.
3. Covey, C., Richardson, D. & Carbon, J. (1976) *Mol. Gen. Genet.* **145**, 155–158.
4. Rodriguez, R. L., Bolivar, F., Goodman, H. M., Boyer, H. W. & Betlach, M. (1976) "Molecular mechanisms in control of gene expression," eds. Nierlich, D. P., Rutter, W. S. & Fox, C. F., in *Proceedings of the ICN-UCLA Symposia on Molecular and Cellular Biology* (Academic Press, San Francisco, California), Vol. 5, in press.
5. Clewell, D. B. & Helinski, D. R. (1970) *Biochemistry* **9**, 4428–4440.
6. Maniatis, T., Jeffrey, A. & van deSande, H. (1975) *Biochemistry* **14**, 3787–3794.

7. Maniatis, T., Ptashne, M. & Maurer, R. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38,** 857–868.
8. Maniatis, T., Jeffrey, A. & Kleid, D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1184–1188.
9. Sgaramella, V., van deSande, J. & Khorana, H. G. (1970) *Proc. Natl. Acad. Sci. USA* **67,** 1468–1475.
10. Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2110–2114.
11. Blattner, F. R., Fiandt, M., Hass, K. K., Twose, P. A. & Szybalski, W. (1974) *Virology* **62,** 458–471.
12. Chadwick, P., Pirrotta, V., Steinberg, R., Hopkins, N. & Ptashne, M. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 283–294.
13. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)
14. Maniatis, T. & Ptashne, M. (1973) *Nature* **246,** 133–136.
15. Sgaramella, V. & Khorana, H. G. (1972) *J. Mol. Biol.* **72,** 427–444.
16. Clewell, D. B. & Helinski, D. R. (1972) *J. Bacteriol.* **110,** 1135–1146.
17. Maniatis, T., Kee, S. G., Efstratiadis, A. & Kafatos, F. C. (1976) *Cell* **8,** 163–182.
18. Ptashne, M., Backman, K., Humayun, M. Z., Jeffrey, A., Maurer, R., Meyer, B. & Sauer, R. T. (1976) *Science,* **194,** 156–161.
19. Cohn, M. (1957) *Bacteriol. Rev.* **21,** 140–168.
20. Polisky, B., Greene, P., Garfin, D., McCarthy, B., Goodman, H. & Boyer, H. W. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3310–3314.
21. Bigger, C. H., Murray, K. & Murray, N. E. (1973) *Nature New Biol.* **244,** 7–10.
22. Boyer, H. W., Chow, L. T., Dugaiczyk, A., Hedgpeth, J. & Goodman, H. M. (1973) *Nature New Biol.* **244,** 40–43.
23. Old, R., Murray, K. & Roizes, G. (1975) *J. Mol. Biol.* **92,** 331–339.
24. Pirrotta, V. (1976) *Nucleic Acids Res.* **3,** 1747–1760.
25. Roberts, R. J., Myers, P. A., Morrison, A. & Murray, K. (1976) *J. Mol. Biol.* **102,** 157–165.