J. Mol. Biol. (1981) **152**, 209–232

Control of Lysogenization by Phage P22 I. The P22 *cro* Gene

FRED WINSTON† AND DAVID BOTSTEIN‡

Department of Biology, Massachusetts Institute of Technology Cambridge, Mass. 02139, U.S.A.

(Received 10 November 1980, and in revised form 24 April 1981)

P22 *cro*$^-$ mutants were isolated as one class of phage P22 mutants (*cly* mutants) that have a very high frequency of lysogeny relative to wild-type P22. These mutants: (1) do not form plaques and over-lysogenize relative to wild-type P22 after infection of a wild-type *Salmonella* host; (2) are defective in anti-immunity; and (3) fail to turn off high-level synthesis of P22 *c2*-repressor after infection.

P22 *cro*$^-$ mutations are recessive and map between the P22 *c2* and *c1* genes. P22 *cro*$^-$ mutations are suppressed by clear-plaque mutations in the *c1* gene, one of which is simultaneously *cy*$^-$. They are also suppressed, but incompletely, by mutations in the *c2* (repressor) gene, especially those that do not completely abolish *c2* gene function.

Salmonella host mutants have been isolated that are permissive for the lytic growth of the P22 *cro*$^-$ mutants.

1. Introduction

The genomes of the temperate *Salmonella* phage P22 and the temperate coliphage λ share the same overall functional organization. They also share some common DNA sequences as evidenced by DNA–DNA hybridization (Skalka & Hanson, 1972) and by the ability of the two phages to recombine *in vivo* (Gemski *et al.*, 1972; Botstein & Herskowitz, 1974).

The immunity systems of the two phages differ in that P22 has two distinct regions that participate in immunity and repression (the *immC* and *immI* regions; Bezdek & Amati, 1968; Botstein *et al.*, 1975; Levine *et al.*, 1975), whereas λ has only one immunity region (Hopkins & Ptashne, 1971). If, however, the P22 *immI* region is deleted, the phage is still able to establish and maintain lysogeny normally. Furthermore, λ–P22 hybrid phage, which contain the *immC* region of P22 and the rest of λ, are able to establish and maintain lysogeny normally (Gemski *et al.*, 1972; Botstein & Herskowitz, 1974; Hilliker & Botstein, 1976).

The P22 *immC* region and the λ immunity region are structurally and functionally similar (Fig. 1). Each codes for a repressor necessary to maintain lysogeny (Levine,

† Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N.Y. 14853, U.S.A.

‡ To whom reprint requests should be addressed.

0022-2836/81/300209-24 $02.00/0

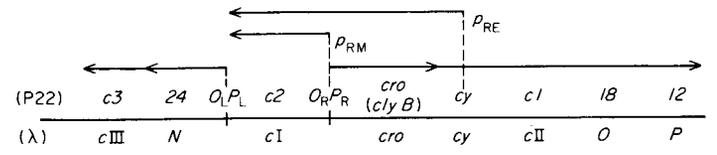


FIG. 1. Genetic map of the P22 *immC* and the λ immunity regions. P22 genes are above the line, λ genes are below the line. Lines above the map denote the transcription patterns. p_{RE} is the presumed promoter for the establishment mode transcription of the repressor gene (*c2* of P22, *c*I of λ; Jones *et al.*, 1979; Schmeissner *et al.*, 1980). p_{RM} is the promoter for the maintenance mode of transcription of the repressor genes (Yen & Gussin, 1973; A. R. Poteete, unpublished data). p_L and p_R are the promoters for the genes to the left and right, respectively (Kourilsky *et al.*, 1968; Roberts, 1969; Heinemann & Spiegelman, 1970; Hilliker & Botstein, 1975,1976).

1957; Kaiser, 1957) and the structure of the operator sites for repressor binding are similar for the two phages (Poteete *et al.*, 1980). In addition, both phages code for immunity-specific proteins necessary for high-level repressor synthesis during establishment of lysogeny: *c*II and *c*III for λ (Kaiser, 1957; Reichardt & Kaiser, 1971), and *c1* and *c3* for P22 (Levine, 1957; Gough & Tokuno, 1975).

The λ *cro* gene codes for an immunity-specific protein that is essential for lytic growth of λ after infection of a wild-type *Escherichia coli* host. The λ *cro* function has been studied in great detail. It has been shown to repress directly phage gene expression from the λ promoters p_{RM}, p_R and p_L (Reichardt & Kaiser, 1971; Reichardt, 1975*b*; Johnson *et al.*, 1978; Meyer *et al.*, 1980). It has also been shown to repress indirectly expression of the *c*I-repressor gene from the p_{RE} promoter by repressing expression of the *c*II and *c*III genes from p_R and p_L, respectively (Echols *et al.*, 1973; Reichardt, 1975*a*).

λ *cro*$^-$ mutants have several characteristic phenotypes: (1) they do not grow lytically after infection of a wild-type *E. coli* host; (2) they do not allow a defective prophage to become "anti-immune" (Calef & Neubauer, 1968; Eisen *et al.*, 1970; Calef *et al.*, 1971); and (3) they fail to turn off high-level repressor synthesis after infection (Reichardt & Kaiser, 1971). Due to the inability of λ *cro*$^-$ mutants to grow lytically after infection, virtually all λ *cro*$^-$ strains also carry the *c*I857 mutation, which specifies a thermolabile repressor. The *c*I857 allele suppresses the *cro*$^-$ defect at 37°C, but not at 30°C or 42°C (Eisen *et al.*, 1975).

Given the ability of the P22 *immC* region to substitute for the λ immunity region and given the fact that the λ *cro* protein is essential for lytic growth, it seemed logical to anticipate that P22 would code for a *cro* function. Suggestive evidence for a P22 *cro* function has been reported (Hilliker *et al.*, 1978) but a direct demonstration of such a function has been lacking.

In the course of isolation and analysis of P22 mutants that lysogenize at greatly increased frequency relative to wild-type P22 (P22 *cly* mutants; Hong *et al.*, 1971; Roberts *et al.*, 1976), we found three different classes of mutants, which we have named *clyA*, *clyB* and *clyC* (Winston, 1980; Winston & Botstein, 1981, accompanying paper). The P22 *cly* mutants studied by Hong *et al.* (1971) appear to fall into what we call the *clyA* class. P22 *clyB* mutants, unlike the other *cly* mutants, have the phenotypes expected of P22 *cro*$^-$ mutants. In addition, these mutants, like the previously identified P22 *clyA* mutants, lysogenize at very high

frequency and do not form plaques on a wild-type *Salmonella* host. They can form plaques on the *cly*-permissive hosts identified by Hong *et al.* (1971) and on new hosts reported here. In this paper, we shall refer to the P22 *clyB* mutants as P22 *cro*$^-$ mutants. The *Salmonella* hosts permissive for lytic growth by these and other classes of P22 *cly* mutants will be referred to as *cly*-permissive hosts.

In this paper we present evidence for a P22 *cro* function, the isolation and analysis of P22 *cro*$^-$ mutants, and the isolation and preliminary characterization of new hosts permissive for lytic growth of P22 *cro*$^-$ mutants. By this analysis we have found: first, that P22 *cro*$^-$ mutants do not grow lytically (and lysogenize at very high frequency) after infection of wild-type *Salmonella* hosts but can grow lytically after infection of certain mutant *Salmonella* hosts. By use of these *cly*-permissive *Salmonella* hosts, we have been able to study P22 *cro*$^-$ mutants without the presence of a conditional clear-plaque mutation, which otherwise would be required to suppress conditionally the Cro$^-$ phenotype. Second, we found that different *cly*-permissive mutations affect different host functions based on their phenotypes. Third, we found that a defective P22 *cro*$^+$ lysogen can become anti-immune and that a *cro*$^-$ mutation blocks the ability of a defective P22 lysogen to become anti-immune. Fourth, we found that a P22 *cro*$^-$ mutant fails to turn off high-level synthesis of *c2*-repressor and at least one other early phage-coded protein after infection of a wild-type host. These last two phenotypes are similar to the phenotypes of λ *cro*$^-$ mutants that are cited above.

2. Materials and Methods

(a) *Bacteria*

Bacterial strains are listed in Table 1. All *Salmonella typhimurium* strains are derivatives of LT2. DB7000 was actually used as the standard *su*$^-$ host. Most often, the *cly*-permissive hosts used contained the *rif*39 allele. The strains containing the *pclA*, *pclB* and *pclC* alleles were isolated as Tn*10* insertion mutants permissive for P22 *cly* mutants (see below).

DB147 contains a short (*c2*$^-$ *mnt*$^+$) deletion of the prophage, which begins outside the left end of the P22 prophage and extends into the *c1* gene; DB5201 contains a long prophage deletion (*c2*$^+$ *mnt*$^-$), which deletes everything between a Tn*10* insertion in the P22 *al* region and the *c1* gene (Chan & Botstein, 1972).

All lysogens (except DB6077) were made by selecting for ampicillin resistance since all the phage strains used to construct lysogens carried the Ap31 *pfr*1 deletion-substitution (see below).

(b) *Phage*

All bacteriophage strains used in this work are derivatives of wild-type P22. The alleles used are listed in Table 2. Phages constructed in this work contain various combinations of these alleles. The *cro*H100 allele was initially called *cly*17. *cly*N3 and *cly*N11 are the same as *cly*3 and *cly*11 (Hong *et al.*, 1971). The *cy*27 mutation is the same as the *c27* mutation previously described (Tokuno & Gough, 1976). We call it *cy*27 because of its similarity to *cy* mutations of phage λ.

(i) *Construction of P22* cro$^-$ *clear double mutants*

A large number of phage constructions in this work involved 2 classes of markers affecting the plaque morphology: clear (*c1*$^-$, *c2*$^-$ and *cy*$^-$) and *cro*$^-$. For construction of P22 *cro*$^-$ *c1*$^-$

TABLE 1

Bacterial strains

Strain	Genotype	Source/Reference
Non-lysogens		
DB21	LT2, prototroph	Botstein (1968)
DB47	*recA*	Wing *et al.* (1968)
DB4381	*hisG*46 Δ(*bio uvrB*)	B. Ames via G. Walker
DB5397	*cya*-408	B. Ames
DB7000	*leuA-am*414	D. Botstein
DB7001	*leuA-am*414 *rpsL*	Hilliker & Botstein (1975)
DB7154	*leuA-am*414 *hisC-am*527 *supD*10	Winston *et al.* (1979)
DB7155	*leuA-am*414 *hisC-am*527 *supE*20	Winston *et al.* (1979)
DB7156	*leuA-am*414 *hisC-am*527 *supF*30	Winston *et al.* (1979)
DB7157	*leuA-am*414 *hisC-am*527 *supJ*60	Winston *et al.* (1979)
DB7158	*leuA-am*414 *rif*39	Hong *et al.* (1971)
DB7521	*pclA* : : Tn*10*	This work
DB7524	*pclB* : : Tn*10*	This work
DB7525	*pclC* : : Tn*10*	This work
DB7533	*leuA-am*414 *supE*20 *rif*39	This work
DB7572	*leuA-am*414 *cya*408	This work
DB7590	*leuA-am*414 *rif*39 *recA*	This work
DB7658	*leuA-am*414 *rif*39 *cya*408	This work
Lysogens		
DB6077	*leuA-am*414 (P22 *int*3 *c2*$^-$*ts*29 *sieA*1 *m*3)	D. Botstein
DB7621	*leu-am*414 *rpsL* (P22 *12*$^-$*am*H342 *24*$^-$*am*S36 *ct*$^-$*ts*30 *int*$^-$*am*137Ap31*pfr*1)	This work
DB7639	*leuA-am*414 *rpsL* (P22 *12*$^-$*am*H342 *24*$^-$*am*S36 *c2*$^-$*ts*30 *cro*H100 *int*$^-$*am*137 Ap31*pfr*1)	This work
Prophage deletions		
DB147		Chan & Botstein (1972)
DB5201		Chan & Botstein (1972)

double mutants, the effect of these markers on plaque morphology was useful in detecting the desired recombinant phage. All of the P22 *cro*$^-$ *c1*$^-$ double mutants were initially identified as plaques that were of intermediate turbidity relative to the *c1*$^-$ and *cro*$^-$ parents. The same principle was applied to separate the mutations in P22 *cro*$^-$ clear double mutants.

The construction of P22 *cro*$^-$ *c2*$^-$ double mutants was accomplished in 2 different ways. The P22 *cro*H100 *c2*$^-$*am*O8 double mutant was constructed simply by crossing the 2 single mutants by each other, plating the progeny on a wild-type *su*$^-$ host, and then scoring the small clear plaques for plaque morphology on 2 amber suppressor hosts, DB7155 (*supE*) and DB7156 (*supF*). On DB7155, the P22 *cro*H100 *c2*$^-$*am*O8 double mutant makes a plaque that is more turbid than that of the *c2*$^-$*am*O8 parent and furthermore does not plate on DB7156, which is a better suppressor of the *c2*$^-$*am*O8 mutation.

Four-factor crosses were used to construct P22 *cro*$^-$*c2*$^-$*ts* double mutants. In every case one parent was *12*$^-$*am*H342 *c2*$^-$ and the other was *erf*1 *cro*$^-$. Crosses were done in a *rif*39 host (permissive for *cro*$^-$ mutants) and the outside markers *12*$^+$ and *erf*$^+$ were selected on a *recA*, *rif*39 host (DB7590). All constructions were tested by recovering the individual component mutations after a backcross to wild-type P22. The P22 *cro*$^-$ *c2*$^-$ double mutants were found by scoring the inside markers and looking for the recombinant class that was different from either parent or from wild-type. In one case (P22 *cro*H100 *c2*$^-$*ts*30) this double mutant was found as a class that grew more poorly on DB7000 at 40·5°C and for the other case (P22 *cro*H100 *c2-ts*29) this double mutant was found as a class that made a more turbid spot on DB7000.

TABLE 2

Phage alleles

Allele	Source/Reference
int$^-$*am*137	D. Botstein
24$^-$*am*S36	Hilliker & Botstein (1975)
12$^-$*am*H342	D. Botstein
5$^-$*am*H312	D. Botstein
8$^-$*am*H202	D. Botstein
Vx	Bronson & Levine (1971)
K5	Bronson & Levine (1971)
c2$^-$*am*O8	H. Prell
c2$^-$5	Levine (1957)
c1$^-$7	Levine (1957)
c1$^-$*ts*101	M. Levine
c1$^-$*ts*97	This work
c1$^-$*ts*2	This work
c1$^-$*am*8	This work
c1$^-$*am*26	This work
c1$^-$*am*59	This work
c1$^-$*am*214	This work
*cy*27	Levine (1957)
*cy*12	This work
Ap31 *pfr*1	This work
clyA alleles	
*cly*N3	Hong *et al.* (1971)
*cly*N11	Hong *et al.* (1971)
*cly*H102	This work
*cly*H109	This work
clyB (*cro*) alleles	
*cro*H100	This work
*cro*H101	This work
*cro*H104	This work
*cro*H105	This work
*cro*H106	This work
*cro*H107	This work
*cro*H108	This work
*cro*H110	This work
*cro*H111	This work
*cro*H116	This work
*cro*H124	This work
*cro*S100	This work
clyC alleles	
*cly*H112	This work
*cly*H125	This work

(ii) *Construction of phage strains used for the anti-immunity experiments*

Phage strains used for the anti-immunity experiments were constructed using the same general principles described above. In order to preserve the desired inside markers during construction, parent phages were constructed that contained all *cro* and clear alleles desired

in the final recombinant and carrying either the *12*$^-$*am*H342 or the *24*$^-$*am*S36 markers. Then these *12*$^-$ and *24*$^-$ derivatives were crossed with each other to produce the final recombinant.

(iii) *Construction of P22 Ap31*pfr*1*

In order to have a strong selection for lysogens, a P22 strain was constructed that contained the β-lactamase gene from Tn*1*, thereby allowing selection for lysogens by selecting for Amp^R colonies. This strain also contains a deletion of non-essential P22 material so that the phage DNA in particles will be terminally repetitious, a feature necessary for P22 to grow after single infection (Botstein & Matz, 1970).

The parent strain was P22 Ap31, a strain containing a Tn*1* insertion in the non-polar orientation in the carboxyl-terminal end of the P22 *ant* gene (Weinstock *et al.*, 1979). The non-polar orientation allows expression of the tail gene (gene *9*), which is downstream (with respect to transcription) of *ant*. The presence of the Tn*1* element results in a genome too large to fit inside the phage head. Therefore, the packaged DNA is not terminally repetitious and the Tn*1*-carrying phage cannot grow by single infection. Selection for plaque-forming revertants (pfrs) is therefore a selection for deletions.

pfrs were selected, purified and then scored for whether they still carried the β-lactamase gene by testing for formation of Amp^R lysogens. One pfr strain that carries the intact β-lactamase gene has been designated P22 Ap31*pfr*1.

Genetic tests showed that this phage had become *mnt*$^-$ (since the parent phage contained the *sieA*44 mutation (Susskind *et al.*, 1974), loss of the *sieA* gene function could not be scored). By electron microscopy of heteroduplex molecules, this phage has a deletion of approximately 5000 base-pairs beginning within the Tn*1* element and extending leftward (D. Koshland, unpublished data).

(c) *Growth of phage stocks*

Concentrated phage stocks were prepared either by infection or by ultraviolet induction of lysogens (Botstein & Matz, 1970). Since lysates made by u.v. induction are tail-deficient (Israel, 1967) they were treated with approx. 10^{12} phage equivalents/ml of P22 tails (provided by P. Berget) at 37°C for 1 h prior to concentration.

For growing high-titer stocks of some of the P22 *cro*$^-$ mutants by infection, a slightly different procedure was used. The permissive host was grown to 2×10^8/ml at 37°C, infected at a multiplicity of approximately 1 and grown for 90 min at 37°C. Several drops of chloroform were added to the cultures, which were then put back to shake slowly at 37°C for approx. 10 min. The lysis of phage stocks with chloroform seemed to be greatly aided by incubation of the lysates at 37°C rather than at room temperature. The lysates were then concentrated by the usual procedures.

(d) *Phage crosses*

(i) *Standard phage crosses*

The procedure used for standard P22 crosses was adapted from that of Gough & Levine (1968) and of Botstein & Matz (1970). Each parent was infected at a multiplicity of infection (m.o.i.) of 7.

For some crosses the m.o.i. was shifted (to 7 for one parent and 1 for the other parent) to help bias in favor of a particular marker. For example, to construct a *12*$^-$*am c1*$^-$*am* double mutant, the *c1* parent, whose plaque morphology is easily identifiable, would be infected at a multiplicity of 1 to increase the probability of recombination with a genome carrying the *12*$^-$*am* allele.

(ii) *Four-factor crosses*

Four-factor crosses were done by the standard cross procedure. The crosses were done in strain DB7533 (*rif*39 *supE*) for mapping *cly* mutations and in strain DB7155 (*supE*) for mapping clear mutations. For each set of crosses performed, the parents alone were infected in the identical procedure at a multiplicity equal to the total multiplicity in the coinfection.

All of the 4-factor crosses were done with outside markers in genes *12* (*12*$^+$ and *12*$^-$*am*H342) and *erf* (*erf*$^+$ and *erf*2). The progeny from the crosses were assayed on a permissive host (DB7533 and DB7155) and the non-permissive host DB47 (*recA su*$^-$), which only allows growth of the *12*$^+$ *erf*$^+$ recombinants. Once the titer of the *12*$^+$ *erf*$^+$ recombinants was determined, the crosses were replated on DB47 to examine a larger number of *12*$^+$ *erf*$^+$ recombinants for the frequency of clear and turbid (c^+) plaques.

The relative positions of the 2 inside markers in question were always determined by the relative frequencies of turbid progeny in reciprocal crosses where the *12* and *erf* markers were in combination with each inside marker.

(e) *Media*

Liquid media used were LB broth (Levine, 1957), M9 minimal medium and M9 supplemented with charcoal-clarified Casamino acids (M9CAA; Smith & Levine, 1964). Solid media used were LB plates and λ plates (Signer & Weil, 1968) and green indicator plates (Levine & Curtiss, 1961; Susskind *et al.*, 1971). Dilutions were made in dilution fluid (Botstein & Matz, 1970) or buffered saline (Botstein, 1968). Nutrient top agar (Levine, 1957) was used in all cases. Drugs were used at the following concentrations: tetracycline, 25 μg/ml and ampicillin, 25 to 100 μg/ml. When needed, amino acids were supplemented at 20 μg/ml.

(f) *Chemicals and radioisotopes*

The sources of chemicals were as follows: tetracycline, Calbiochem–Behring Corp. (La Jolla, CA) and Sigma Chemical Co. (St Louis, MO); ampicillin, Bristol Laboratories (Syracuse, N.Y.) and Sigma; hydroxylamine, J. T. Baker Chemicals (Phillisburg, N.J.); acrylamide, Eastman Kodak Co. (Rochester, N.Y.); bisacrylamide, Eastman and Bio-Rad Laboratories (Richmond, CA); Temed, Bio-Rad; ammonium persulfate, Bio-Rad and Mallinckrodt Inc. (St Louis, MO); and sodium dodecyl sulfate, BDH Chemicals. [^{35}S]methionine was purchased from Amersham (Arlington Heights, IL).

(g) *Hydroxylamine mutagenesis*

Hydroxylamine mutagenesis was adapted from the procedure of Hall & Tessman (1966). Phage were mutagenized *in vitro* for 20 to 24 h to a survival of approx. 1%. The frequency of clear-plaque mutants was generally near 1%.

(h) *Isolation of* cly *mutants by plaque morphology*

This procedure, adapted from that of Hong *et al.* (1971), was to plate a hydroxylamine-mutagenized P22 lysate for 100 to 200 plaques per plate on a *cly*-permissive host, either DB7160 (*rif*39) or DB7521 (*pclA*). Plates were incubated at either 30 or 37°C until the majority of the plaques on the plate began to have a faint turbid or bull's-eye center. At that time the plates were removed from the incubator and screened visually for fully turbid plaques. The turbid plaques were purified and tested for the *cly* phenotype of no plaque formation on a *cly*-non-permissive (wild-type) host by either streaking or plating out a single-plaque suspension. Those that grew on the *cly*-permissive host and did not grow on the *cly*-non-permissive host were grown into lysates for further study.

(i) *Isolation of* cly *mutants by lysogen selection*

This procedure selects for mutants that are able to lysogenize efficiently under conditions where lysogens are extremely rarely formed. Strain DB7158 (*rif*39) was grown in LB to a

concn of 5×10^8/ml. The cells were centrifuged and resuspended in buffered saline to the same concentration. The cells were then infected at a multiplicity of 0·1 with P22 Ap31*pfr*1. After adsorption for 20 min at room temperature, 0·1 ml of P22 anti-serum was added and the infected cells were incubated an additional 10 min at room temperature. The infected cells were then plated on green plates containing ampicillin; the plates were incubated at 37°C.

Colonies that grew on the plates after a day or two were purified on ampicillin plates and then on LB plates. After purification, these strains were tested initially for their immunity by cross-streaking (Susskind *et al.*, 1971). Those that were sensitive to P22 *ant*$^+$ and immune to P22 *ant*$^-$ were repurified and tested for spontaneous phage release by stabbing single colonies of the lysogens into lawns of *cly*-non-permissive and *cly*-permissive hosts. For those lysogens that warranted further testing, u.v.-induced lysates were grown.

(j) *Isolation of* cly*-permissive hosts by Tn*10 *insertion mutagenesis*

After Tn*10* mutagenesis of strain DB21 (adapted from Kleckner *et al.*, 1975), the Tet^R colonies were replica-plated onto green tetracycline plates and green tetracycline plates seeded with 10^6 P22 *cly*N3 phage (Hong *et al.*, 1971), which were then incubated at 37°C. The replica plates were then examined for those colonies that were nibbled by the *cly*N3 phage (Susskind *et al.*, 1971). These candidates were purified and retested for *cly*-sensitivity by cross-streaking and then by streaking-out *cly* mutants on lawns of the candidates. For those insertions isolated that did render DB21 *cly*-sensitive, the Tn*10* insertion mutation was moved by P22 generalized transduction (selecting Tet^R) into the original parental strain, DB21 (Kleckner *et al.*, 1975). One hundred per cent of the Tet^R transductants were *cly*-sensitive, demonstrating that the Tn*10* insertion mutation caused the *cly*-sensitivity.

(k) *Selection for revertants of P22* cro$^-$ *mutants*

Individual plaques on a *rif*39 *cly*-permissive host were picked and replated on a non-permissive host, either DB7000 (*su*$^-$) or DB7304 (*su*$^+$). This generally yielded between 5 and 100 revertant plaques/plate with an occasional jackpot (Luria & Delbruck, 1943).

(l) *Spot tests for identification of clear mutants*

In order to distinguish among clear mutants we used spot complementation tests similar to those developed by Levine (1957). These distinguish *c1* mutations from *c2*, *cy* and *K5* mutations.

A procedure to test for *K5* mutants was developed by Bronson & Levine (1971) and relies on the fact that neither a *Vx* (left-side virulent) nor a *K5* (right-side virulent) mutant alone can overcome immunity in a P22 *sieA* lysogen; however, a lysogen that is co-infected by the 2 phages will allow phage growth.

A spot test to distinguish between *c2* and *cy* mutants (both of which complement as *c2*$^-$) was adapted from the colony-sparing test developed by Susskind *et al.* (1974). In this test, one infects a strain that is lysogenic for P22 *c2*$^-$*ts*29 *sieA*44 at 40·5°C, a temperature non-permissive for growth of this lysogen. If the super-infecting phage carries a wild-type *c2* template, then wild-type *c2*-repressor can be made at the high temperature and the lysogen will survive. (The exact mechanism by which the lysogen is able to survive is not clear: presumably *c2*-repressor is being made from the good template in the maintenance mode.) If the super-infecting phage carries a defective *c2* allele, no functional repressor can be made and the temperature-sensitive lysogen will die.

For this test phage candidates that complement as *c2*$^-$ are spotted on a lawn of DB6077 (a P22 *c2*$^-$*ts*29 *sieA*44 lysogen) and the plates are incubated at 40·5°C. If a patch of survivors grows where the phage were spotted then the candidate is a *cy* mutant; if not, then the superinfecting phage carries a defective *c2* gene.

(m) *Non-permissive marker rescue from prophage deletions*

Non-permissive rescue was done as described by Chan & Botstein (1972). For rescue from DB147 an additional overlay of DB7000 was added to the plates to improve the plaque morphology.

(n) *Frequency of lysogeny measurements*

The frequency of lysogeny by wild-type P22 and by P22 *cro*$^-$ mutants was measured in the *cly*-non permissive (wild-type) host DB7000. Cells were grown in M9/CAA to 2×10^8/ml at 37°C, infected with the phage at the desired multiplicity and incubated for 15 min at 37°C. The infected cells were then diluted and spread on plates seeded with 5×10^8 P22 *c2*-5 and incubated at 37°C. Under these conditions only lysogens form colonies. The frequency of lysogeny is calculated from the number of infected cells and the number of lysogens formed.

(o) *Dominance tests*

The basic procedure for dominance tests was the same as for the standard cross with respect to growing the cells and the infection, growth and titering of the phage. Two different dominance tests were done. First, dominance tests for phage growth were done under conditions where each parent infected, at low multiplicity (<0·1), the *cly*-non-permissive (and *su*$^-$) host DB7000; each parent carried a complementing amber mutation in a P22 late gene. Thus one parent carried a *5*$^-$*am* mutation and the other parent carried an *8*$^-$*am* mutation. In this way, only cells which were co-infected by each parent would produce a burst of phage and these co-infected cells would virtually always be infected by no more than one of each parent.

Second, dominance tests for frequency of lysogeny were done at a high multiplicity of infection (5) in strain DB7158 (*rif*39). The cells were infected, incubated at 37°C and 20 min later plated for lysogens as described above.

(p) *Anti-immunity tests*

If one constructs a lysogen that is defective in early functions and carries a thermosensitive mutation in the *c2*-repressor, one can test for the ability of this lysogen to become anti-immune by incubating the lysogen at a temperature non-permissive for the *c2*$^-$ mutation and then returning the temperature to the permissive temperature. If the Cro function of the lysogen can be expressed, it will inhibit the restoration of immunity upon return to the permissive temperature, allowing indefinitely the continued expression of the Cro function instead of immunity (*c2*-repressor function). Anti-immunity can be observed conveniently by superinfecting with a phage that ordinarily would fail to grow because of the presence of repressor. Anti-immunity is a phenomenon unrelated to the ability of superinfecting phages that can synthesize anti-repressor to grow; anti-repressor directly inactivates repressor while Cro function interferes with its synthesis. In the case of P22, anti-immunity tests could be carried out in such a way as to allow plating of anti-repressor-producing phages to serve as a control, since all lysogens were deleted for the *imm1* region. In such lysogens, all *ant*$^+$ P22 phages can grow but *ant*$^-$ phages cannot grow unless the lysogen has lost its immunity (Botstein *et al.*, 1975).

Lysogens were tested for whether or not they could become anti-immune by growing them at 40·5°C (a temperature non-permissive for the *c2*$^-$*ts* mutation in the prophage) to a concentration of 2 to 5×10^8. Tester phage were plated using these cultures and the plates incubated at 35°C. The tester phages used were wild-type P22 and P22 Ap31*pfr*1; wild-type P22 serves as a control since it is *ant*$^+$ and, therefore, grows on all the lysogens tested regardless of their anti-immunity state. P22 Ap31*pfr*1 grows only on anti-immune hosts since its *ant* gene is partially deleted. Lysogens used in these tests carry the *rps*L allele to make the host more non-permissive for the P22 *24*$^-$*am*S36 allele.

(q) *Preparation of samples for gel electrophoresis*

Samples for gel electrophoresis were prepared as follows: strain DB4381 (*ΔuvrB-bio hisG46*) was grown in M9/glucose medium supplemented with histidine and biotin to approx. $2{\cdot}5 \times 10^8$/ml, counted in the Petroff–Hauser chamber to determine the exact cell titer, pelleted in the Sorvall centrifuge and resuspended in the same medium to a concentration of 5×10^8/ml. The cells were then u.v.-irradiated with a dose of 4000 ergs/mm^2 and infected by adding them to a flask containing the proper amount of the same medium (prewarmed) and the correct number of phage to give a final cell concentration of 2×10^8 and the desired m.o.i. of 7 phage per cell.

For ^{35}S-labeled extracts, each sample was prepared by adding 100 μl of infected cells to an Eppendorf tube containing approximately 50 μCi of [^{35}S]methionine (in a 20 μl vol.), incubating the mixture for 1 min and then ending the pulse by adding 60 μl of 3 × sample buffer and heating the sample at 95°C for 1 min. Sample buffer contains 10% glycerol, 5% β-mercaptoethanol, 3% sodium dodecyl sulfate, 0·0625 M-Tris (pH 6·8) and 0·002% bromophenol blue.

Polyacrylamide gels were run as slab gels (Studier, 1973) using the buffer system of Laemmli (1970). Gels were fixed in 50% trichloroacetic acid for 1 h and then rinsed, dried and exposed to Kodak XR-5 film at room temperature. For autoradiograms that were to be traced, pre-flashed film was used (Laskey & Mills, 1975). Autoradiograms were traced and the areas under the peaks were calculated using a Zeineh soft laser scanning densitometer (Biomed Instruments, Chicago, IL).

3. Results

(a) *Isolation of P22* cro$^-$ *mutants by plaque morphology*

We initially set out to isolate P22 mutants that over-lysogenize after infection of wild-type *Salmonella*. Such mutants had already been isolated by Hong *et al.* (1971). Those mutants, which they named *cly* mutants (for control of *ly*sogeny), have the properties that: (1) they do not make plaques and virtually always lysogenize after infection of wild-type *Salmonella*; and (2) they can grow lytically after infection of *Salmonella cya*$^-$, *crp*$^-$ or *rif*39 hosts and make turbid plaques on these hosts (on which wild-type P22 makes clear plaques). Hong *et al.* mapped these mutations between the P22 genes *c1* and *18*.

The initial method we used to isolate more mutants of this type was the same as that used by Hong *et al.* (1971): to screen for P22 mutants that make turbid plaques on a host permissive for *cly* mutants.

In this procedure, P22 stocks were mutagenized with hydroxylamine and plated on a *cly*-permissive host (*rif*39) to look for mutants that form turbid plaques. Among the candidates for *cly* mutants, the final criterion used was whether or not the phage was able to grow on the *cly* non-permissive (wild-type) host, *Salmonella* strain DB7000. The procedure yielded seven mutants in the 15,000 mutagenized plaques screened. The seven *cly* mutants isolated represent approximately 10% of the turbid plaque-formers tested. Most of the phage that formed more turbid plaques than P22$^+$ on the *cly*-permissive host also appeared to plate normally on DB7000 and were not analyzed further.

(b) *Isolation of* cly *mutants by lysogenization frequency*

This procedure selects for mutants that can lysogenize more often than P22$^+$ in a host in which P22$^+$ lysogenizes at an extremely low frequency. The host carries the

*rif*39 allele; the phage for this selection is the strain P22 Ap31*pfr*1, which carries a gene coding for β-lactamase (*amp*^{R}) and whose construction is described in Materials and Methods. P22 Ap31*pfr*1 lysogenizes at very low frequency after low multiplicity infection of a *rif*39 host (approx. 10^{-5} lysogens per infected cell), as expected since P22$^+$ makes clear plaques on this host.

cly mutants were selected using both unmutagenized and hydroxylamine-mutagenized phage stocks. Among 99 Amp^R colonies tested, 16 had a *cly* mutant as a prophage. The strength of the selection is indicated by the isolation of a spontaneous *cly* mutant. All 16 *cly* mutants isolated by this procedure were in the *clyB* or *clyC* class (see below).

(c) *Preliminary mapping of* cly *mutations and designation of* cro *mutants*

Preliminary mapping of the *cly* mutations by non-permissive rescue from two prophage deletion strains allows the *cly* mutations to be grouped into two sets.

The two prophage strains used, DB147 and DB5201, are deleted in from the left end and the right end of the prophage map, respectively, ending in the *c1* region. Chan & Botstein (1972) showed that the prophage deletion in DB147 includes the *c2* gene but not the *18*$^-$*am*100 allele and that the deletion in DB5201 does include *18*$^-$*am*100 but not the *c2* gene. We have shown further that the deletion in DB147 includes almost all markers in the *c1* gene (Winston & Botstein, unpublished results).

The results (Fig. 2) show that the *cly* mutants fall into two categories: those that can form *cly*$^+$ recombinants with the deletion prophage strain DB147 and those that cannot. All of the mutants can form *cly*$^+$ recombinants with the deletion prophage in strain DB5201.

These preliminary mapping results, in conjunction with the plating phenotypes of the *cly* mutants that show *cly*H125 and *cly*H112 to be in a different class from the other *cly* mutants based upon their host range (Winston & Botstein, 1981, accompanying paper), define three classes of P22 *cly* mutants: *clyA*, *clyB* and *clyC*. The one class of mutants that map under deletion DB147 and have the same host

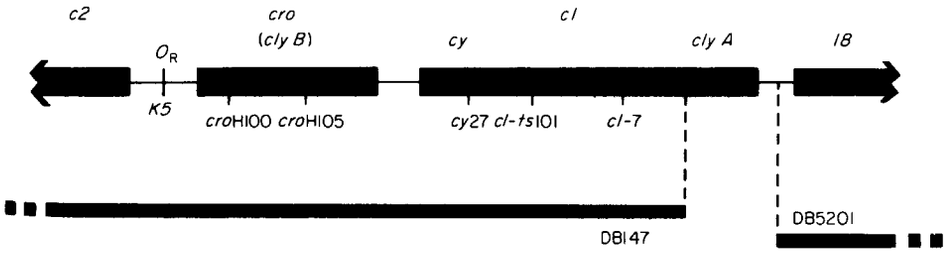


FIG. 2. Fine structure map of the P22 *cro-cy-c1* region. Map positions were determined by 4 factor crosses (Table 3) and non-permissive rescue from the prophage deletion strains DB147 and DB5201. The *clyA* mutants map between the endpoints of the 2 deletions and the *clyB* mutants map under the DB147 deletion. The *clyC* mutants also map under DB147 (not shown). The *cly* alleles mapped by non-permissive rescue are listed in Table 2.

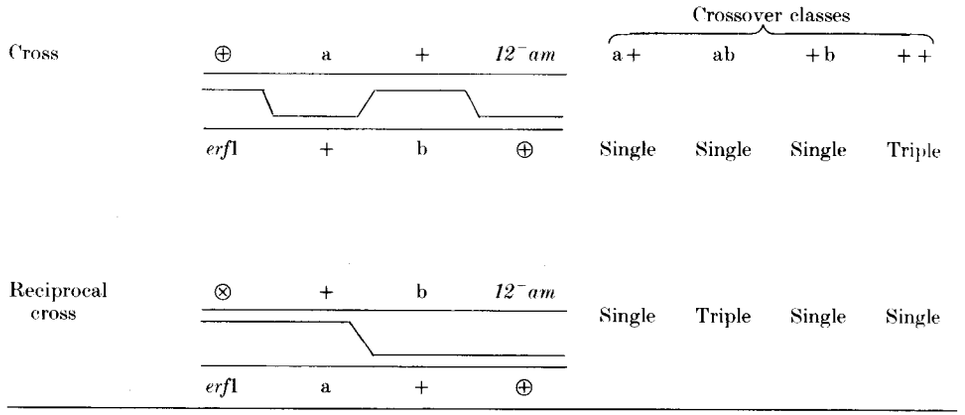
range as the H100 mutant (*clyB* mutants) will be discussed further here. The set of *clyB* mutants will hereafter be referred to as P22 *cro*$^-$ mutants.

(d) *Mapping P22* cro$^-$ *mutations by four-factor crosses*

In order to determine more precisely the map positions of the P22 *cro*$^-$ mutations, four-factor crosses were used to order *cro* mutations relative to known P22 markers. The results of mapping the *cro* mutations by four-factor crosses (Table 3) order the markers *cro*H100 and *cro*H105 relative to the P22 markers *K5*, *cy*27, *c1*$^-$7 and *c1*$^-$*ts*101 and to each other (Fig. 2). The four-factor crosses place

TABLE 3

Four-factor crosses for mapping cro$^-$ *mutations*



Depending upon the relative position of the inside unselected markers, either the cross or the reciprocal cross will require a triple crossover to generate wild-type progeny. For each pair of crosses listed below, the cross is listed first, the reciprocal cross second. The % c^+ refers to the frequency of wild-type plaques among progeny recombinant for the outside markers. For cross 5, frequencies were determined by directly plating for *cro*$^+$ recombinants. p.f.u., plaque-forming units.

	Unselected markers a	b	c^+ p.f.u.	%c^+	Deduced map order
1.	*cro*H100	*c1*$^-$*ts*101	34/295	11·5	*erf*– – –*cro*H100– – –*c1*$^-$– –*12*
	c1$^-$*ts*101	*cro*H100	392/870	45·1	
2.	*cro*H100	*c1*$^-$7	172/948	18·1	*erf*– – –*cro*H100– – –*c1*$^-$– –*12*
	c1$^-$7	*cro*H100	722/1620	44·6	
3.	*cro*H100	*K5*	332/1186	28·0	*erf*– – –*K5*– – –*cro*H100– –*12*
	K5	*cro*H100	20/680	2·9	
4.	*cro*H105	*cy*27	23/672	3·4	*erf*– – –*cro*H105– – –*cy*– – –*12*
	*cy*27	*cro*H105	96/534	18·0	
5.	*cro*H100	*cro*H105		0·02	*erf*– – –*cro*H100– – –*cro*H105– – –*12*
	*cro*H105	*cro*H100		0·16	
6.	*c1*$^-$7	*c1*$^-$*ts*101	26/1212	2·1	*erf*– – –*c1*$^-$*ts*101– – –*c1*$^-$7– – –*12*
	c1$^-$*ts*101	*c1*$^-$7	0/1924	<0·05	

*cro*H100 and *cro*H105 to the right of *K5*, which defines P22 O_R (Bronson & Levine, 1971; Poteete *et al.*, 1980), and to the left of the *cy* region. These crosses also definitively order *cro*H100 to the left of *cro*H105 thus demonstrating that not all the *cro* mutations are identical.

(e) *Frequency of lysogeny by P22* cro *mutants*

Measurements of the frequency of lysogeny by different P22 *cro*$^-$ mutants after infection of a *cro*-non-permissive host (Table 4) show that they greatly over-

TABLE 4

Frequency of lysogeny of P22$^+$ *and P22* cro$^-$ *mutants in DB7000*

Phage	Multiplicity of infection 0·1	10
P22$^+$	1·7	67
P22 *cro*H100	87	97
P22 *cro*H105	100	87
P22 *cro*H116	85	87
P22 *cro*H124	68	93

The *cro*-non-permissive (wild-type) host DB7000 was infected with phage at the indicated multiplicities. The frequency of lysogeny was determined as described in Materials and Methods. The numbers above indicate the percentage of infected cells that became lysogens.

lysogenize relative to wild-type P22 after low multiplicity infection. They also over-lysogenize after high multiplicity infection although the difference is not as great since wild-type P22 also lysogenizes more often at high multiplicity.

(f) *Dominance tests*

Dominance tests show that the P22 *cro*$^-$ mutants are recessive to wild-type P22 for both growth and high frequency of lysogeny.

(i) *Low multiplicity dominance tests for phage growth*

In order to determine the dominance or recessiveness of P22 *cro*$^-$ mutants under conditions where the number of infecting phage is guaranteed to be one of each parent per cell, low multiplicity dominance tests for phage growth were done as described in Materials and Methods.

These results (Table 5) show that the P22 mutants *cro*H100 and *cro*H105 are recessive to wild-type P22 for growth under these conditions. The burst size in the *cro*$^-$ + P22$^+$ infections, while reduced a few fold from the P22$^+$ single infection, is over fiftyfold larger than the burst of *cro*$^-$ infections alone. Also, in the mixed infection, approximately equal numbers of P22$^+$ and P22 *cro*$^-$ phage are produced. We conclude that the *cro*$^-$ mutants are missing a diffusible product; lack of this product results in over-lysogenization after infection.

TABLE 5

Low multiplicity dominance and complementation tests

5^-amH312	8^-amH202	Burst size
cro$^+$	*cro*H100	209
*cro*H100	*cro*$^+$	244
cro$^+$	*cro*H105	83·3
*cro*H105	*cro*$^+$	117
*cro*H100	*cro*H105	1·6
*cro*H105	*cro*H100	2·8
cro$^+$	*cro*$^+$	755
*cro*H100	*cro*H100	2·4
*cro*H105	*cro*H105	4·8

cro alleles are listed by whether they were on the same genome with the 5^-amH312 or the 8^-amH202 allele, Infections were done in the *cly*-non-permissive host DB7000 at 37°C.

Co-infection by P22 *cro*H100 and P22 *cro*H105 yields a burst size typical of either mutant parent alone, demonstrating lack of complementation between these two *cro*$^-$ mutations.

(ii) *High multiplicity dominance tests for frequency of lysogeny*

Since the P22 *cro*$^-$ mutants lysogenize at a much greater frequency than wild-type P22, a second type of dominance test is possible, i.e. one that measures dominance of the ability to lysogenize at high frequency in a *cro*-permissive host. These experiments were done in a *rif*39 host where the frequencies of lysogeny of wild-type P22 and P22 *cro*$^-$ mutants are significantly different from each other after a high multiplicity of infection.

These dominance test results (Table 6) confirm the results of the low multiplicity dominance tests: recessiveness of P22 *cro*H100 to wild-type P22.

TABLE 6

*High multiplicity dominance tests in DB7158 (*rif*39)*

Phage	% Lysogeny
P22$^+$ + P22 *cro*H100	3·6
P22$^+$	2·7
P22 *cro*H100	19·6

The procedure is described in Materials and Methods. Single parent infections were done at a multiplicity equal to the total multiplicity in the co-infections.

(g) *Demonstration of an anti-immunity function of P22*

Demonstration of an anti-immunity function for P22 was done in a manner similar to that used for phage λ by Calef & Neubauer (1968) and Eisen *et al.* (1970). We constructed the P22 lysogens DB7621 and DB7639 (see Materials and Methods), which are isogenic except for the *cro* alleles: the prophage in DB7621 is *cro*$^+$ and the prophage in DB7639 carries the *cro*H100 allele. Both prophages contain a $c2^-ts$ mutation and amber mutations in genes *24* and *12* so that the lysogen will not die after thermal induction. The prophages are also *int*$^-$ to prevent excision of the prophage and carry the Ap31*pfr*1 marker, which makes the prophages $sieA^-$ (which allows superinfecting P22 to inject their DNA; Susskind *et al.*, 1974) and provides a convenient marker (amp^R) to score for their presence. Calef & Neubauer (1968) and Eisen *et al.* (1970) have shown for the analogous λ *cro*$^+$ lysogen that after growth at high temperature and a shift to low temperature the lysogen did not regain immunity and furthermore directed all superinfecting phage into the lytic pathway of growth. The λ *cro*$^-$ lysogen was able to regain immunity.

The results of plating P22 on DB7621 and DB7639 grown under the same conditions (Table 7) demonstrate that P22 does have an anti-immunity function

TABLE 7

Plating of P22 on defective P22 lysogens

Phage	Host	Relevant prophage marker	Temp. at which host grown (°C)	Plating efficiency (relative to DB7621, 40·5°C)
P22 Ap31*pfr*1	DB7621	*cro*$^+$	30	$<1·5\times10^{-8}$
	DB7621	*cro*$^+$	40·5	1·0
	DB7639	*cro*H100	30	$<1·5\times10^{-8}$
	DB7639	*cro*H100	40·5	$<1·5\times10^{-8}$
P22 *cro*H100 Ap31*pfr*1	DB7621	*cro*$^+$	30	$<8·3\times10^{-9}$
	DB7621	*cro*$^+$	40·5	1·0
	DB7639	*cro*H100	30	$<8·3\times10^{-9}$
	DB7639	*cro*H100	40·5	$<8·3\times10^{-9}$

Plating efficiencies are calculated by titering the tester phage (first column) on the desired host (second column) at either 35 or 40·5°C and dividing this titer by the titer measured on DB7621 at 40·5°C (permissive conditions). The prophages in the host all contain the 12^-amH342, 24^-amS36, $c2^-ts$37, int^-am137 and Ap31*pfr*1 markers. They differ only by their *cro* alleles.

and that this function is defective in the P22 *cro*H100 mutant. After being grown at 40·5°C (a temperature non-permissive for the $c2^-ts$30 allele), and plated at 35°C (permissive for the $c2^-ts$ allele) the *cro*$^+$ lysogen (DB7621) fails to regain immunity as shown by the plating of the P22 Ap31*pfr*1 (*ant*$^-$) strain. The lysogen remains immune when grown at 30°C and plated at 35°C. The plaques that form on this strain after it has been grown at the high temperature are clear, indicating that no lysogens are formed and that the strain has become anti-immune. Furthermore, the anti-immune host is also permissive for P22 *cro*$^-$ mutants probably because it expresses *cro* function constitutively.

The *cro*$^-$ lysogen (DB7639) is deficient in expressing the anti-immunity function; after being grown at 40·5°C and plated at 35°C, this strain is immune, as evidenced by the failure of P22 Ap31*pfr*1 to form plaques. These results further confirm that the P22 *cro*$^-$ mutations define a gene similar to the *cro* gene of phage λ.

(h) *The pattern of P22 protein synthesis in P22 wild-type and P22* cro*-infected cells*

The rates of synthesis of P22 proteins in wild-type P22 and P22 *cro*H100 infections were analyzed by pulse-labeling infected cells at various times after infection and then examining the labeled products on sodium dodecyl sulfate/polyacrylamide gels. Results of those experiments (Figs 3 and 4) demonstrate: first, that wild-type P22 synthesizes *c2*-repressor at a high rate for a short time after infection and then reduces the rate of synthesis to a low level; second, that P22 *cro*H100 has the same initial kinetics of *c2* synthesis as wild-type P22 but continues to synthesize repressor at a high rate until a much later time after infection; third, that at least one other P22 early protein, the product of the *erf* gene, is also synthesized at a higher rate later in infection in the P22 *cro*H100 infection than in the P22 wild-type infection; and fourth, that the P22 *cro*H100 infection results in a lower rate of synthesis of the major capsid protein (gene 5) product than the wild-type P22 infection, indicating that expression of the P22 late genes is defective in the P22 *cro*$^-$ infection.

These results are all consistent with the idea that the P22 *cro* function normally serves to turn down expression of P22 early gene expression. This deficiency in turn-off of early gene expression in the P22 *cro*$^-$ infection is similar to the Tof$^-$ phenotype observed in a λ *cro*$^-$ infection (Radding, 1964; Eisen *et al.*, 1966; Pero, 1971). Harvey *et al.* (1979) also showed that this same P22 mutant overproduces *c2*-repressor.

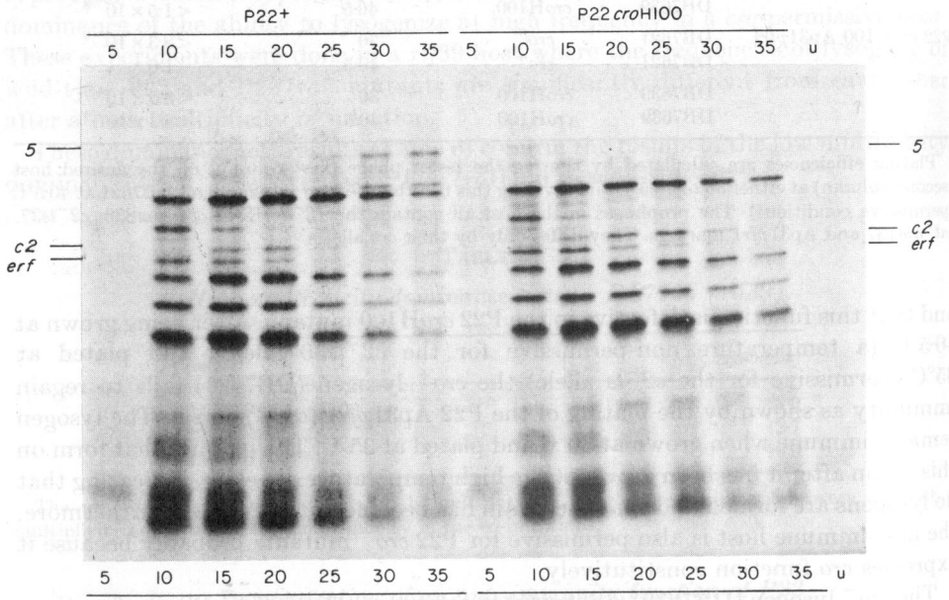


FIG. 3. Rates of protein synthesis after infection by wild-type P22 and P22 *cro*H100. Samples of infected cells were pulse-labeled with [^{35}S]methionine and run on sodium dodecyl sulfate/polyacrylamide gels as described in Materials and Methods. Lanes are labeled with the time the 1-min pulses were begun. Lanes labeled u are uninfected samples.

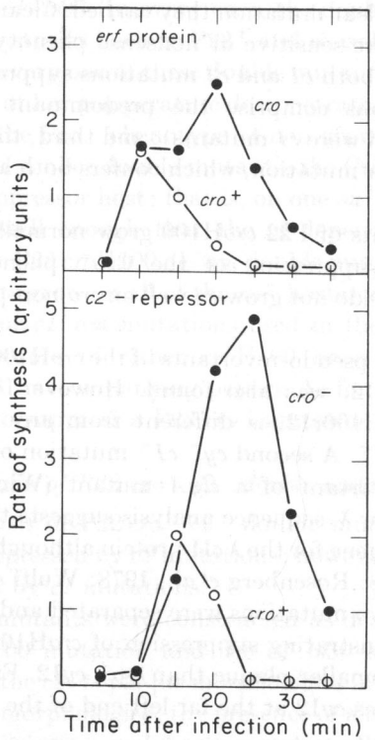


FIG. 4. Rates of synthesis of *erf* protein and *c2*-repressor after infection by wild-type P22 and P22 *cro*H100. Infections and pulse-labelings were performed as described in Materials and Methods. The curves indicate the relative rates of synthesis of *c2*-repressor and *erf* protein. Autoradiograms were traced and the areas were normalized to the amount of trichloroacetic acid-precipitable radioactivity in each gel slot.

(i) *Partial suppression of a* 24-am *mutation by the* cro*H100 mutation*

Franklin (1971) and Court & Campbell (1972) noted that λ *cro* mutations can often suppress λ *N*$^-$ mutations (Fed phenotype). We tested for this phenotype with P22 *cro*$^-$ mutants and found that the *cro*H100 mutation weakly suppresses a P22 *24*$^-$*am* mutation. The relative efficiency of plating (data not shown) of a P22 *cro*H100 *24*$^-$*am*S36 double mutant is approximately 0·2 on an *su*$^-$ host with respect to plating on an *su*$^+$ host. For the P22 *24*$^-$*am*S36 single mutant, the relative efficiency of plating is less than 10^{-6}. Since both phage strains used here carry the *c2*$^-$*ts*30 mutation, which suppresses the growth defect of the *cro*H100 mutation, these strains grow on *cly*-non-permissive hosts.

(j) *Isolation and characterization of pseudo-revertants*

Among revertants of P22 *cro*$^-$ phages, there is a high frequency of clear-plaque pseudo-revertants. Clear pseudo-revertants of the *cro*H100 mutant were selected, purified and tested by spot complementation tests (as described in Materials and

Methods) for the type of clear mutation they carried. Clear pseudo-revertants were also tested for temperature-sensitive or nonsense phenotypes. The results of this analysis show: first, that both *c1* and *c2* mutations suppress the Cro$^-$ phenotype; second, that *c1* mutations comprise the predominant class of clear pseudo-revertants (54 of 67 tested were *c1* mutants); and third, that one pseudo-revertant defines a new class of clear mutation, which confers both a *cy*$^-$ and *c1*$^-$ phenotype on the phage.

The *c1* pseudo-revertants of P22 *cro*H100 grow normally on *cly*-non-permissive hosts, indicating total suppression of the Cro$^-$ phenotype. The *c2* pseudo-revertants of P22 *cro*H100 do not grow as well on *cro*-non-permissive hosts, making smaller plaques.

In addition to *c1* and *c2* pseudo-revertants of the *cro*H100 mutant, one *cy* pseudo-revertant, P22 *cro*H100r12, was also found. However, the *cy*12 mutation, the *cy* mutation in P22 *cro*H100r12, is different from any previously identified in P22 or in λ as it is also *c1*$^-$. A second *cy*$^-$ *c1*$^-$ mutation of this type has also been isolated as a pseudo-revertant of a *clyA* mutant (Winston & Botstein, 1981, accompanying paper). For λ, sequence analysis suggests that some λ *cy* mutations are within the structural gene for the λ cII protein although all λ *cy* mutants are still cII$^+$ (Schwarz *et al.*, 1978; Rosenberg *et al.*, 1978; Wulff *et al.*, 1980).

For *cro*H100r12, the two mutations were separated and then the double mutant was reconstructed, demonstrating suppression of *cro*H100. The P22 *cro*H100*cy*12 double mutant makes a smaller plaque than *cro*$^+$ *cy*12. Fine structure mapping of the P22 *cy c1* region places *cy*12 at the far left end of the *c1* gene (Winston, 1980).

(k) *Construction and analysis of* cro-*clear double mutants*

Construction and analysis of *cro*-clear double mutants confirmed and extended what was inferred from the isolation and analysis of the *cro*$^-$ pseudo-revertants. First, *c1* mutations suppress P22 *cro*$^-$ mutations; second, a *c1* amber mutation suppresses *cro*H100 unless the *c1* amber mutation is well suppressed, in which case the Cro$^-$ phenotype returns; third, *c2* missense (including temperature-sensitive) mutations suppress *cro*$^-$ mutations; and fourth, a *c2* amber mutation suppresses *cro*H100, but the suppression is temperature-sensitive.

(i) *Construction and analysis of* cro$^-$ c1$^-$ *double mutants*

To construct a P22 *cro*$^-$ *c1*$^-$*ts* double mutant, *cro*H100 was crossed with *c1*$^-$*ts*2 and recombinants that would make a more turbid plaque than the *c1*$^-$*ts*2 parent at 37°C were found at low frequency. These recombinants made turbid plaques at 37°C and clear plaques at 40·5°C. The *c1*$^-$*ts*2 parent makes clear plaques at both temperatures. When the presumed P22 *cro*H100 *c1*$^-$*ts*2 double mutant was crossed by wild-type P22, both single mutations could be recovered, thereby confirming the genotype. These results confirm that a *c1* mutation will suppress a P22 *cro*$^-$ mutation.

The suppression of *cro*H100 by *c1* mutations is not allele specific for the particular *c1* mutation. P22 *cro*$^-$ *c1*$^-$*ts* double mutants were constructed using two different P22 *cro*$^-$ mutations (*cro*H100 and *cro*H105) and two other *c1*$^-$*ts* mutations, *c1*$^-$*ts*101 and *c1*$^-$*ts*97. In every case, the double-mutant genotype was confirmed by crossing the strains back to P22$^+$ and re-isolating the single parental mutations. The fact that every one of these double mutants is able to grow on a *cly*-non-permissive host even at low temperature demonstrates that a wild-type level of *c1* activity is crucial for the Cro$^-$ phenotype of non-growth on a wild-type host.

For two P22 *cro*H100 *c1*-amber double mutants, the Cro$^-$ phenotype is restored on a particular amber suppressor host; that is, on one *su*$^+$ host (*supF*), the *c1*$^-$*am* mutation is suppressed well enough that the *c1*-dependent Cro$^-$ phenotype is manifested. For two other P22 *cro*H100 *c1*$^-$*am* double mutants, this is not the case; rather, the double mutants grow on all of the *su*$^+$ hosts, plating clear on some and turbid on others. The four *c1*$^-$*am* mutations used in these experiments were all isolated as pseudo-revertants of either *cro*H100 or a *clyA* mutant, *cly*H109 (Winston & Botstein, 1981, accompanying paper). As for *c1*$^-$*ts* mutations, then, a *c1*$^-$*am* mutation is able to suppress a P22 *cro*$^-$ mutation.

(ii) *Construction and analysis of P22* cro$^-$ c2$^-$ *double mutants*

Construction and analysis of P22 *cro*$^-$ *c2*$^-$ double mutants demonstrates that *cro*$^-$ mutations can be suppressed by *c2* mutations; however, the suppression is not so efficient as suppression by *c1* mutations.

P22 *cro*$^-$ *c2*$^-$*ts* double mutants were constructed as described in Materials and Methods using the *cro*H100 mutation and the *c2*$^-$*ts*30 and *c2*$^-$*ts*29 alleles. The *cro*H100 mutation affects the *c2*$^-$*ts* plaque morphologies in a manner resembling its effect on the *c1*$^-$*ts* plaque morphologies; the presence of a *cro*$^-$ mutation makes the plaque more turbid than it is in a *cro*$^+$ background at all temperatures tested.

The P22 *cro*H100 *c2*$^-$*am*O8 double mutant has a temperature-sensitive phenotype, which is dependent upon the suppressor strain on which it is grown. As can be seen from the results in Table 8, on *su*$^-$ and *supF*, this phage is quite temperature-sensitive. On these two hosts the *c2*$^-$*am*O8 mutation, judging by the plaque morphology, is unsuppressed and well-suppressed, respectively. On the *supE* host, where the *c2*$^-$*am*O8 mutation is suppressed to an intermediate level, this phage shows only slight temperature sensitivity. These results suggest that the quantity or quality of the *c2* made is important in the temperature-sensitive

TABLE 8

Growth of P22 cro*H100* c2$^-$am*O8 on different hosts*

Host	Relevant genotype	Efficiency of plating at: 30°C	40·5°C
DB7158	*rif*39	0·4	$6·2 \times 10^{-7}$
DB7000	*su*$^-$	1·0	$3·2 \times 10^{-4}$
DB7155	*supE*	1·6	0·4
DB7156	*supF*	0·1	$1·6 \times 10^{-6}$

Phage dilutions were plated on the above hosts at 30 and 40·5°C. All strains were grown at 37°C. Under the same conditions the P22 *cro*H100 *c2*$^-$*ts* mutants grew at both temperatures.

phenotype. The non-growth at high temperature has not been investigated further but is reminiscent of the λ Tro phenotype (Eisen *et al.*, 1975; Georgiou *et al.*, 1979).

(l) *Isolation and characterization of new* cly*-permissive hosts*

After insertion mutagenesis of the wild-type *Salmonella* strain DB21 with the translocatable tetracycline resistance element, Tn*10*, approximately 30,000 colonies were tested for sensitivity to *cly*N3 by replica-plating onto plates seeded with this *cly* mutant and looking for "nibbled" colonies (Susskind *et al.*, 1971). Three *cly*N3-sensitive mutants were found and are the strains *pclA*, *pclB* and *pclC* (*pcl* for *p*ermissive for *cly*). These three hosts are permissive for lytic growth of all of the P22 *cro*$^-$ mutants tested.

These three strains have been tested for several characteristics and the following is known about them: they are *cya*$^+$ and *crp*$^+$, they are prototrophic and they are not u.v.-sensitive. Preliminary Hfr crosses (not shown) indicate that the *pclA*, *pclB* and *pclC* mutations map in different locations. The efficiency of plating of four P22 *cro*$^-$ mutants on the set of *Salmonella cly*-permissive hosts is shown in Table 9.

TABLE 9

Plating efficiency of P22 cro$^-$ *mutants on permissive and non-permissive hosts*

P22 *cro*$^-$ mutant	Host					
	*rif*39	*cya*408	*pclA*	*pclB*	*pclC*	Non-permissive
P22 *cro*H100	1·0	1·4	1·2	0·2	1·3	$3{\cdot}1 \times 10^{-4}$
P22 *cro*H105	1·0	1·0	0·7	0·4	3·4	$1{\cdot}4 \times 10^{-4}$
P22 *cro*H116	1·0	0·9	1·2	0·4	1·8	$5{\cdot}6 \times 10^{-4}$
P22 *cro*H124	1·0	1·5	1·7	0·1	3·9	$8{\cdot}6 \times 10^{-5}$

The efficiencies of plating were normalized to the titer on the *rif*39 host DB7158. Platings were all done at 37°C.

These hosts are all significantly more permissive for the P22 *cro*$^-$ mutants than the parent strain, DB21. Additionally, wild-type P22 makes a clearer plaque on all of the *cly*-permissive hosts so far identified, although the degree of clarity differs among the different hosts. Grodzicker *et al.* (1972) showed that an *E. coli cya*$^-$ host is more permissive for a λ *cro*$^-$ mutant than a *cya*$^+$ host.

We also tested the plating of P22 *cro*H100 on the set of permissive hosts at a range of temperatures and found this mutant to be quite temperature-sensitive for growth on one host (*rif*39 at 40·5°C), to have an intermediate temperature-sensitive phenotype on two hosts (*pclA* and *pclB*) and to not be temperature-sensitive on two other hosts (*pclC* and *cya*-408). These results suggest that different host loci are affected among the different *cro*-permissive hosts and that they may affect phage growth in different ways. However, they seem to affect all phage with the Cly phenotype in the same general way, i.e. by allowing lytic growth.

4. Discussion

The experiments presented here define the P22 *cro* gene and also demonstrate that certain mutant *Salmonella* hosts are able to suppress the Cro$^-$ phenotype in that they allow P22 *cro*$^-$ mutants to grow lytically.

(a) *Identification of the P22* cro *gene*

The P22 *cro* gene has been identified by an ensemble of several results: (1) demonstration of an anti-immunity state in P22 *c2*$^-$*ts cro*$^+$ lysogens; (2) demonstration that the P22 *cro*$^-$ mutants are defective in the anti-immunity function as well as in turning off at least some P22 early gene expression; (3) demonstrating that these mutations map in a region between the P22 O_R and *cy* regions; (4) demonstration that the P22 *cro*$^-$ mutations are recessive; and (5) demonstration that a P22 *cro*$^-$ mutation can weakly suppress a P22 *24*$^-$*am* mutation, a phenotype that is analogous to the Fed phenotype of λ *cro*$^-$ mutants (Franklin, 1971; Court & Campbell, 1972).

The simplest interpretation of these results is that the P22 *cro* gene codes for a protein that acts as the λ *cro* protein does, i.e. to repress early gene expression at the transcriptional level by binding to the operators, O_R and O_L. In a P22 *cro*$^-$ infection, then, early gene expression is not turned off and *c1* protein continues to be made, resulting in greater *c2*-repressor synthesis and a greater frequency of lysogenization. This interpretation is based on the postulate that the P22 Cro$^-$ phenotype is mediated by overproduction of the P22 *c1* protein. More direct evidence that the P22 *cro* protein binds to O_R and O_L to repress transcription must await purification of the P22 *cro* protein and demonstration of its function *in vitro*.

The virtual total suppression of P22 *cro*$^-$ mutations by *c1* mutations both supports the interpretation that the overproduction of *c1* protein causes the Cro$^-$ phenotypes and strengthens the analogy to λ *cro*$^-$ mutations that are suppressed by mutations in the analogous λ cII gene (Reichardt, 1975*a*).

The suppression of P22 *cro*$^-$ mutations by *c2* mutations is not straightforward. Both *c2*$^-$*ts* alleles used suppress the *cro*H100 mutation. However, suppression of *cro*H100 by the *c2*$^-$*am*O8 mutation is temperature-sensitive. For phage λ, a λ *cro*27 cI$^-$*ts*857 double mutant does not grow at 42°C (Tro phenotype; Eisen *et al.*, 1975), presumably because of overproduction of some λ gene products in the absence of any repression by either cI or *cro*. For the P22 *c2*$^-$*am*O8 *cro*H100 double mutant a similar argument can be made if the mutant *cro* protein made in a P22 *c2*$^-$*am*O8 *cro*H100 infection does not lose all activity until 40·5°C. This predicts that this infection should also have the other Tro phenotypes (Eisen *et al.*, 1975; Georgiou *et al.*, 1979).

Unlike λ *cro*$^-$ mutations, which are suppressed by λ cIII mutations, indirect evidence suggests that P22 *c3* mutations do not suppress P22 *cro*$^-$ mutations. Although a P22 *cro*$^-$ *c3*$^-$ double mutant has not been constructed, no *c3* mutations were obtained as pseudo-revertants of *cro*$^-$ mutations. In conjunction with the result that on the growth media generally used for P22 a P22 *c3* mutant makes a turbid plaque (Levine, 1957) and lysogenizes only two to threefold less well than wild-type P22 (Winston & Botstein, unpublished), this result probably reflects the

less than critical role of the P22 *c3* protein in establishment of lysogeny under these conditions.

(b) *The role of host functions in establishment of lysogeny*

The role of bacterial host functions in regulating establishment of lysogeny after P22 or λ infection is still largely obscure. Results of others (Hong *et al.*, 1971) and those presented here have indicated that at least six different *Salmonella* loci (*cya*, *crp*, *rif*, *pclA*, *pclB* and *pclC*) can be altered to lower the frequency of lysogenization by wild-type P22 and by P22 *cly* mutants (which include P22 *cro*$^-$ mutants). The steps at which these host mutations act to affect establishment are unknown. The fact that some of the host mutations could have multiple effects also makes any interpretation of their effect more difficult. For example, the *cly*-permissive *Salmonella rif*39 mutation could be affecting a step as direct as initiation of transcription at the P22 p_{RE} promoter or a factor as indirect as altering the host physiology in a general way resulting in lower establishment of lysogeny.

E. coli hfl mutants, which allow efficient establishment of lysogeny by a λ *c*III mutant (Belfort & Wulff, 1971,1974), are the best-characterized host mutants that affect establishment of lysogeny. Epp (1978) has shown that the λ *c*II protein is more stable after infection of an *E. coli hfl* host. Furthermore, Belfort & Wulff (1974) have shown that a λ *c*III mutant can establish lysogeny at normal frequencies in an *E. coli cya hfl* double mutant. From these results and in conjunction with the results that the λ *can1* mutation (which stabilizes the λ *c*II protein) suppresses a λ *c*III$^-$ mutation (Jones & Herskowitz, 1978; Epp, 1978), and that the λ *c*III protein appears to stabilize the λ *c*II protein, Jones & Herskowitz (1978) and Epp (1978) have put forth the model that the *E. coli hfl* product is at least partially responsible for the instability of the *c*II protein and that cyclic AMP and the *crp* protein positively regulate a protein, "Z", which is a negative regulator of *hfl*. By this model, a mutation that lowers or abolishes Z function or expression would be expected to over-produce *hfl* protein, lower λ *c*II stability, and therefore lower the frequency of establishment of lysogeny.

Assuming that there is an *hfl*-like function in *Salmonella* and knowing that *Salmonella cya* and *crp* mutations affect P22 lysogeny as *E. coli cya* and *crp* mutations affect λ lysogeny, it is logical to hypothesize that the *Salmonella pcl* mutations, isolated as permissive hosts for P22 *cly* mutants, may affect a *Salmonella* Z function. One could postulate that there are several Z functions to account for the different loci that affect establishment of lysogeny by λ and P22.

While it is attractive to think that all host mutations affecting establishment of lysogeny mediate their effect by affecting activity of the P22 *c1* protein or the λ *c*II protein, there is little evidence to support this belief. Host mutations that affect expression of the *cro* gene or stability of the *cro*-protein for either λ or P22 would also affect establishment of lysogeny. Possible examples of such host mutants are *E. coli* ER437 (Oppenheim *et al.*, 1974) and *Salmonella typhimurium pox1* (Tokuno & Gough, 1975).

We thank Carl Falco and Doug Koshland for many helpful discussions, Paula Grisafi for help in isolating some of the P22 mutants, and Russ Maurer for critical reading of the manuscript. We also thank Pamela Oppenheimer for help in preparing this manuscript. This work was supported by grants to D.B. from the National Institutes of Health (GM18973 and GM21253). F.W. was supported by a training grant to the Department of Biology from the National Institutes of Health (GM07287).

REFERENCES

Belfort, M. & Wulff, D. L. (1971). In *The Bacteriophage Lambda* (Hershey, A. D., ed.), pp. 739–742, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Belfort, M. & Wulff, D. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 779–782.
Bezdek, M. & Amati, P. (1968). *Virology*, **36**, 701–703.
Botstein, D. (1968). *J. Mol. Biol.* **34**, 621–641.
Botstein, D. & Herskowitz, I. (1974). *Nature (London)*, **251**, 584–589.
Botstein, D., Lew, K. K., Jarvik, V. & Swanson, C. A. Jr (1975). *J. Mol. Biol.* **91**, 439–462.
Botstein, D. & Matz, M. J. (1970). *J. Mol. Biol.* **54**, 417–440.
Bronson, M. J. & Levine, M. (1971). *J. Virol.* **7**, 559–568.
Calef, E. & Neubauer, Z. (1968). *Cold Spring Harbor Symp. Quant. Biol.* **33**, 765–767.
Calef, E., Avitabile, A., del Giudice, L., Marchelli, C., Menna, T., Neubauer, Z. & Soller, A. (1971). In *The Bacteriophage Lambda* (Hershey, A. D., ed.), pp. 609–620, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Chan, R. K. & Botstein, D. (1972). *Virology*, **49**, 257–267.
Court, D. & Campbell, A. (1972). *J. Virol.* **9**, 938–945.
Echols, H., Green, L., Oppenheim, A. B., Oppenheim, A. & Honigman, A. (1973). *J. Mol. Biol.* **80**, 203–216.
Eisen, H., Fuerst, C. A., Siminovitch, L., Thomas, R., Lambert, L., Pereira da Silva, L. & Jacob, F. (1966). *Virology*, **30**, 224–241.
Eisen, H., Brachet, P., Pereira da Silva, L. & Jacob, F. (1970). *Proc. Nat. Acad. Sci., U.S.A.* **66**, 855–862.
Eisen, H., Georgiou, M., Georgopoulos, C. P., Selzer, S., Gussin, G. & Herskowitz, I. (1975). *Virology*, **68**, 266–269.
Epp, C. (1978). Ph.D. dissertation, University of Toronto.
Franklin, N. C. (1971). In *The Bacteriophage Lambda* (Hershey, A. D., ed.), pp. 621–638, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Gemski, P. Jr, Baron, L. S. & Yamamoto, N. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 3110–3114.
Georgiou, M., Georgopoulos, C. P. & Eisen, H. (1979). *Virology*, **94**, 38–54.
Gough, M. & Levine, M. (1968). *Genetics*, **58**, 161–169.
Gough, M. & Tokuno, S.-I. (1975). *Mol. Gen. Genet.* **138**, 71–79.
Grodzicker, T., Arditti, R. R. & Eisen, H. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 366–370.
Hall, D. H. & Tessman, I. (1966). *Virology*, **29**, 339–345.
Harvey, A. M., Heil, J. & Prell, H. H. (1979). *Mol. Gen. Genet.* **167**, 337–339.
Heinemann, S. & Spiegelman, W. (1970). *Proc. Nat. Acad. Sci., U.S.A.* **67**, 1122–1129.
Hilliker, S. & Botstein, D. (1975). *Virology*, **68**, 510–524.
Hilliker, S. & Botstein, D. (1976). *J. Mol. Biol.* **106**, 537–566.
Hilliker, S., Adhya, S. & Gottesman, M. (1978). *Virology*, **86**, 37–47.
Hong, J.-S., Smith, G. R. & Ames, B. N. (1971). *Proc. Nat. Acad. Sci., U.S.A.* **68**, 2258–2262.
Hopkins, N. & Ptashne, M. (1971). In *The Bacteriophage λ* (Hershey, A. D., ed.), pp. 571–574, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Israel, V. (1967). *Virology*, **33**, 317–322.
Johnson, A., Meyer, B. J. & Ptashne, M. (1978). *Proc. Nat. Acad. Sci., U.S.A.* **75**, 1783–1787.
Jones, M. O. & Herskowitz, I. (1978). *Virology*, **88**, 199–212.
Jones, M. O., Fischer, R., Herskowitz, I. & Echols, H. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 150–154.
Kaiser, A. D. (1957). *Virology*, **3**, 42–61.
Kleckner, N., Chan, R. K., Tye, B. K. & Botstein, D. (1975). *J. Mol. Biol.* **97**, 561–575.

Kourilsky, P., Marcaud, L., Sheldrick, P., Luzzati, D. & Gros, F. (1968). *Proc. Nat. Acad. Sci., U.S.A.* **61**, 1013–1020.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Laskey, R. A. & Mills, A. D. (1975). *Eur. J. Biochem.* **56**, 335–341.
Levine, M. (1957). *Virology*, **3**, 22–41.
Levine, M. & Curtiss, R. (1961). *Genetics*, **46**, 1573–1580.
Levine, M., Truesdell, S., Ramakrishnan, T. & Bronson, M. J. (1975). *J. Mol. Biol.* **91**, 421–438.
Luria, S. E. & Delbruck, M. (1943). *Genetics*, **28**, 491–511.
Meyer, B. J., Maurer, R. & Ptashne, M. (1980). *J. Mol. Biol.* **139**, 163–194.
Oppenheim, A., Honigman, A. & Oppenheim, A. B. (1974). *Virology*, **61**, 1–10.
Pero, J. (1971). In *The Bacteriophage Lambda* (Hershey, A. D., ed.), pp. 599–608, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Poteete, A. R., Ptashne, M., Ballivet, M. & Eisen, H. (1980). *J. Mol. Biol.* **137**, 81–91.
Radding, C. M. (1964). *Proc. Nat. Acad. Sci., U.S.A.* **52**, 965–972.
Reichardt, L. (1975*a*). *J. Mol. Biol.* **93**, 267–288.
Reichardt, L. (1975*b*). *J. Mol. Biol.* **93**, 289–309.
Reichardt, L. & Kaiser, A. D. (1971). *Proc. Nat. Acad. Sci., U.S.A.* **68**, 2185–2189.
Roberts, J. W. (1969). *Nature (London)*, **224**, 1168–1174.
Roberts, J. W., Roberts, C. W., Hilliker, S. & Botstein, D. (1976). In *RNA Polymerase* (Losick, R. & Chamberlin, M., eds), pp. 707–718, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Rosenberg, M., Court, D., Shimatake, H., Brady, C. & Wulff, D. L. (1978). *Nature (London)*, **272**, 414–423.
Schmeissner, U., Court, D., Shimatake, H. & Rosenberg, M. (1980). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 3191–3195.
Schwarz, E., Scherer, G., Hobom, G. & Kossel, H. (1978). *Nature (London)*, **272**, 410–414.
Signer, E. & Weil, J. (1968). *J. Mol. Biol.* **34**, 261–271.
Skalka, A. & Hanson, P. (1972). *J. Virol.* **9**, 583–593.
Smith, H. O. & Levine, M. (1964). *Proc. Nat. Acad. Sci., U.S.A.* **52**, 356–363.
Studier, F. W. (1973). *J. Mol. Biol.* **91**, 165–174.
Susskind, M. M., Botstein, D. & Wright, A. (1974). *Virology*, **62**, 350–366.
Susskind, M. M., Wright, A. & Botstein, D. (1971). *Virology*, **45**, 638–652.
Tokuno, S. & Gough, M. (1975). *J. Virol.* **16**, 1184–1190.
Tokuno, S. & Gough, M. (1976). *Mol. Gen. Genet.* **144**, 199–204.
Weinstock, G., Susskind, M. M. & Botstein, D. (1979). *Genetics*, **92**, 685–710.
Wing, J. P., Levine, M. & Smith, H. D. (1968). *J. Bacteriol.* **95**, 1828–1834.
Winston, F. (1980). Ph.D. dissertation, Mass. Institute of Technology, Cambridge, MA.
Winston, F. & Botstein, D. (1981). *J. Mol. Biol.* **152**, 233–245.
Winston, F., Botstein, D. & Miller, J. H. (1979). *J. Bacteriol.* **137**, 433–439.
Wulff, D. L., Beher, M., Izumi, S., Beck, J., Mahoney, M., Shimatake, H., Brady, C., Court, D. & Rosenberg, M. (1980). *J. Mol. Biol.* **138**, 209–230.
Yen, K-M. & Gussin, G. N. (1973). *Virology*, **56**, 300–312.

Edited by M. Gottesman