A Novel Antivirulence Element in the Temperate Bacteriophage HK022

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Lysogens of the temperate lambdoid phage HK022 are immune to superinfection by HK022. Superinfection immunity is conferred in part by the action of the HK022 CI repressor at the O_R operators. In this work, we have identified an additional regulatory element involved in immunity. This site, termed O_{FR} (operator far right), is located just downstream of the *cro* gene, more than 250 nucleotides distant from O_R . The behavior of phage containing a mutation in O_{FR} suggests that the wild-type site functions as an antivirulence element. HK022 O_{FR}^- mutants were able to form turbid plaques indistinguishable from those of the wild type. However, they gave rise to virulent derivatives at a far higher frequency than the wild type ($\approx 10^{-5}$ for O_{FR}^- versus about 10^{-9} for the wild type). This frequency was so high that cultures of HK022 O_{FR}^- lysogens were rapidly overgrown by virulent derivatives. Whereas virulent mutants arising from a wild-type O_{FR}^+ background contained mutations in both O_{R1} and O_{R2} , virulent derivatives of the O_{FR}^- mutant phage contained a single mutation in either O_{R1} or O_{R2} . We conclude that the wild-type O_{FR} site functions to prevent single mutations in O_R from conferring virulence. The mechanism by which O_{FR} acts is not yet clear. Both CI and Cro bound to O_{FR} and repressed a very weak rightward promoter (P_{FR}). It is unlikely that repression of P_{FR} by CI or Cro binding to O_{FR} can account in full for the antivirulence phenotype conferred by this element, since P_{FR} is such a weak promoter. Other models for the possible action of O_{FR} are discussed.

Temperate phages are able to set up a stable association with their bacterial host. To stabilize this lysogenic state, regulatory mechanisms must operate to block the lytic pathway. One consequence of this regulation is that lysogens are immune to superinfection by the same virus. In λ and related phages, superinfection immunity is conferred by the action of an immunity repressor termed CI, which binds to specific operator sites on the DNA of the prophage or the incoming phage, preventing expression of lytic genes (44).

Rare variants of a temperate phage can arise that have acquired the ability to grow on an immune host (22, 23, 44). These mutants are termed virulent, and they have escaped the mechanism by which lytic growth of a superinfecting wild-type phage is controlled. Their location and properties can provide useful clues as to the mechanism of immunity. In lambdoid phages, virulent mutants contain mutations in repressor binding sites that weaken or prevent repressor binding, leading to expression of the lytic genes that the repressor ordinarily controls.

The frequency with which virulent mutants arise in phage λ is very low (22, 23). Indeed, if they arose too frequently, it would preclude the lysogenic life-style, since spontaneously arising virulent mutants would be able to grow on the host that spawned them and then go on to infect neighboring cells and eventually to destroy the culture. The λ operators are 17 bp in size, a small mutational target; nonetheless, λ must suffer mutations in both O_L and O_R , since expression from both P_L and P_R is necessary for phage growth. Many virulent mutants, such as the classical λvir , contain two mutations in O_R (15, 22).

In this work, we have studied the mechanism of virulence in the lambdoid phage HK022. Like λ , this phage undergoes a lysis-lysogeny decision upon infecting a sensitive cell. The

immunity region of HK022 is organized similarly to that of λ (37). HK022 has its own *cI* and *cro* genes, operators in O_L and O_R (8, 37), and promoters P_R , P_{RM} , and P_L , which appear to be regulated by the CI repressor in a fashion similar to that seen with λ (7). However, HK022 differs from λ in that expression of phage genes from P_L is not required for lytic growth, and mutations in O_L are not required for virulence (8, 48). Among a collection of virulent HK022 mutants, almost all contained two mutations in the HK022 O_R region, one each in O_{R1} and O_{R2} (8). We hypothesized that two mutations in O_R are required because HK022 CI repressor binds O_{R1} and O_{R2} with a high degree of cooperativity (8). Consequently, a single mutation in O_{R1} or O_{R2} that weakens binding to that site by a factor of x would weaken binding to the two sites O_R by only the square root of x , an effect that might not suffice to allow derepression of P_R . The work described here suggests an alternative explanation of the need for two O_R mutations.

A few of the HK022 virulent mutants contained only a single mutation in O_R (8). We believe it likely that these mutants contain one or more additional mutations; if a single O_R mutation sufficed to allow virulence, the overwhelming majority of isolates would be of this type. The nature of these additional mutations was not known, but one possibility was that mutations in other operators outside O_R might contribute to virulence. We identified one such mutation, located just downstream of the *cro* gene, in a site which we named O_{FR} (operator far right).

In the present study, we have characterized the O_{FR} mutation in more detail. Our evidence suggests that an O_{FR} mutation, of itself, does not confer a detectable phenotype. However, it can give rise to virulent derivatives at such high rates that cultures of lysogens rapidly become overgrown by virulent phages. Although our evidence does not indicate the mechanism by which O_{FR} acts, its properties indicate that O_{FR} acts as an antivirulence element, in some way preventing single operator mutations from sufficing for virulence.

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TABLE 1. Bacterial strains, bacteriophage, and plasmids used in this study

Bacterial strain, plasmid, or phage	Relevant genotype or description	Reference or source
<i>E. coli</i> strains		
N99	W3102 <i>rpsL</i> <i>sup</i> ⁰	Max Gottesman
JL2442	MV1190 $\Delta(lac-proAB)$ <i>supF</i> <i>thi</i> $\Delta(srl-recA)306::Tn10/F'$ <i>traD36</i> <i>proAB</i> ⁺ <i>lacI</i> ^a <i>lacZ</i> Δ M15	8
JL794	GE2265 $\Delta(lacIPOZYA)169$ <i>suA211</i> <i>rpsL</i>	28
JL1144	W3101 <i>rpsL</i> ⁺ / <i>F'</i> <i>lacI</i> ^a <i>lacZ</i> Δ M15::Tn9	27
JL1877	N99 (HK022 <i>I</i> ::Tn10)	Max Gottesman
JL3248	GE2265/ <i>F'</i> <i>lacI</i> ^a <i>lacZ</i> Δ M15::Tn9	JL794 $\times$ JL1144 ^a
JL3274	JL3248 (HK022 <i>cI</i> ^{ts12})/pNGC45	This work
JL3276	JL3248 (HK022 <i>cI</i> ^{ts12})/pNGC47	This work
Phages		
M13-KO7	M13 helper phage for isolation of single-stranded DNA	8
HK022	Wild type	R. Weisberg
HK022 <i>cI</i> ^{ts12}	<i>cI</i> ^{ts12}	14
HK022 L1-1	Single-site <i>O</i> _{FR} mutant	This work
HK022 L5-1	Single-site <i>O</i> _{FR} mutant (separate isolate from HK022 L1-1)	This work
HK022 VW5-6	Virulent mutant	8
Plasmids		
pBS(+)	Cloning and sequencing vector	Stratagene
pGB2	Low-copy-number cloning vector	10
pRS415	<i>lacZ</i> operon fusion vector	50
pNGC11	Expression vector for T7 promoter::HK022 <i>cI</i>	8
pNGC24	<i>lacUV5</i> promoter::HK022 <i>cro</i> on pGB2 vector	This work
pNGC25	<i>lacUV5</i> promoter::HK022 <i>cI</i> on pGB2 vector	This work
pNGC42	HK022 insert with <i>O</i> _R , <i>cro</i> and <i>O</i> _{FR} ⁺ in pBS	This work
pNGC44	HK022 insert with part of <i>cro</i> and <i>O</i> _{FR} ⁻ in pBS	This work
pNGC45	Wild-type <i>O</i> _{FR} :: <i>lacZ</i> , rightward promoter	This work
pNGC46	Wild-type <i>O</i> _{FR} :: <i>lacZ</i> , leftward promoter	This work
pNGC47	Mutant <i>O</i> _{FR} :: <i>lacZ</i> , rightward promoter	This work
pNGC48	Mutant <i>O</i> _{FR} :: <i>lacZ</i> , leftward promoter	This work

^a Selection for resistance to streptomycin and chloramphenicol.

MATERIALS AND METHODS

Chemicals, enzymes, and proteins. [³²P]dATP (1,000 Ci/mmol) and γ -[³²P]ATP (7,000 Ci/mmol) were purchased from ICN. Deoxynucleotides for polymerase chain reaction (PCR) were from Perkin-Elmer Cetus. Nucleotides for DNA sequencing were from U.S. Biochemicals. Restriction enzymes were from New England Biolabs and Promega. T7 DNA polymerase (Sequenase version II) was from U.S. Biochemical Corp. *Taq* DNA polymerase was from Stratagene. DNase I was from Sigma Biochemicals. Acrylamide was from National Diagnostics. Chemicals were reagent grade or better and were from Sigma, Mallinckrodt, or Fisher. HK022 repressor was purified and its concentration was determined as described elsewhere (8).

Bacterial strains, phage strains, and bacterial growth. Some of the pertinent bacterial strains used are listed in Table 1; plasmid-bearing derivatives were made by transformation (1). LB, λ broth (tryptone broth), and 2 $\times$ YT broth were prepared as described previously (34) and supplemented with antibiotics as indicated elsewhere (1), except that ampicillin was used as 100 μ g/ml. HK022 phage was grown as described elsewhere (8). HK022 strains are listed in Table 1. Plasmids are listed in Table 1; their construction is described below. pGB2 and its derivatives are compatible with all the other plasmids listed, which were derived from pBR322.

Plasmids for Cro and CI repressor synthesis. pNGC11 contained the *cI* coding region starting from the initiation codon, extending to the end of the gene, and an additional 564 nucleotides 3' to the *cI* termination codon (positions 3006 to 1816 from the sequence of Oberto et al. [37]), cloned into the

NdeI site of the T7 expression vector pET3a (8). To make pNGC14, which contained the T7 promoter fused to the HK022 *cro* gene, a segment of DNA spanning this gene was cloned into M13 mp9; an *NdeI* site was introduced by site-directed mutagenesis; and a 260-bp fragment from the initiation codon of *cro* to a *HaeIII* site 30 bp past the termination codon in *cro* (positions 3086 to 3346 [37]) was cloned into pET3a (51) that had been cut with *NdeI* at the site adjacent to the *NdeI* site (partial digest), filled in, and cut with *NdeI*. This construction was verified by DNA sequence analysis. pNGC25 carried a *lacUV5* promoter::*cI* fusion and was derived from pNGC11 by a three-piece ligation of a 95-bp *EcoRI*-*PvuII* fragment from pLJ3 (25) containing the *lacUV5* promoter, a fragment from pNGC11 with a filled *XbaI* site (which cuts between the T7 promoter and the ATG start codon) and a downstream *BamHI* site, and the low-copy-number vector pGB2 (10) cut with *EcoRI* and *BamHI*. pNGC24 contained a *lacUV5* promoter::HK022 *cro* operon fusion and was made by a similar three-piece ligation using a 330-bp *cro*-containing fragment from pNGC14.

***O*_{FR}-containing plasmids.** Both wild-type and mutant versions of *O*_{FR} were cloned by first using PCR with primers P-192 (TGGATCCCAGGCCAGCGCAA), of which the 3' portion hybridizes beginning at position 2,954 (37), and P-647 (CTG GATCCATAGGTAAATAATTTGGG), of which the 3' portion hybridizes beginning at position 3427, to amplify a fragment which was then digested with *BamHI* and cloned into the *BamHI* site on pBS(+), giving pNGC42 and pNGC44, containing wild-type *O*_{FR} and the VW5-6 mutant version of *O*_{FR}, respectively. pNGC44 carried a spontaneous deletion of *O*_R

and most of the *cro* gene (leaving HK022 sequence corresponding to coordinates 3230 to 3430 on the sequence of Oberto et al. [37]), with 95 and 91 nucleotides flanking O_{FR} to the left and right, respectively. We surmise that an O_{FR} plasmid corresponding to pNGC42 would be lethal or detrimental to cell growth, leading to selective pressure for this deletion.

Plasmids designed to test for promoters near O_{FR} . The plasmids in the series pNGC45 to pNGC48 were constructed by digesting pNGC42 (for wild-type O_{FR}) and pNGC44 (for the mutant O_{FR}) with *ScaI*, which cuts at a site in *cro*, and either *EcoRI* or *BamHI* to generate fragments of 185 or 163 bp, respectively. These fragments were subsequently cloned into pRS415 (50) which had been digested with either *SmaI* and *BamHI* (to detect a rightward promoter), yielding pNGC45 and pNGC47, or with *SmaI* and *EcoRI* (to detect a leftward promoter), yielding pNGC46 and pNGC48 (see Fig. 5 below).

DNase I footprinting. DNase I footprinting was performed as described elsewhere (8). Templates were pNGC42 and pNGC44 digested with *EcoRI*, end labeled as described, and digested with *ScaI*. In this 180-bp fragment, 58 and 107 bp flanked the left and right sides of O_{FR} , respectively. The concentration of the O_{FR} -containing fragment in footprinting reactions was estimated to be ≤ 50 pM. Footprinting buffer contained 20 mM Tris-HCl (pH 7.4), 10% (wt/vol) sucrose, 1 mM EDTA, 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% (wt/vol) bovine serum albumin, 200 mM KCl, 4.3 μ g of calf thymus DNA per ml, and various concentrations of HK022 CI repressor (8). Fractional occupancy and dissociation constants were determined as described elsewhere (8) by using a Betagen betascope to quantify the distribution of radioactivity in gels of footprinting reactions.

DNA sequencing of HK022 mutants. Phage mutants were sequenced from asymmetric PCR products generated by using single plaque eluants as PCR templates as described previously (8) with primer P-192 and limiting amounts of primer P-647; the regions spanning O_{FR} and O_R were sequenced with primers P-647 and P-193 (CCCGGGAGAACTCGAACGG, which anneals to positions 3134 to 3119 [37]), respectively.

Revision of *cro* sequence. During the course of sequencing the region near O_{FR} , we found that the sequence initially reported (37) for the *cro* gene was in error since C3245 is actually not present; the proper sequence is A(3240)AG ATC GCC. The authors of this report (37) agree with this change (36). This deletion changes the reading frame for the distal portion of the *cro* gene; the conceptual translation (in one-letter code) of the corrected *cro* gene is MNEVTFGEVIKS VRVSVVADVCGLTTPKAIYKWLERGSLPRTEFTGETEY ADKIAKASGGKYSAQIRIRIGKQQFVM, with the altered sequence shown in boldface type. This correction does not affect any of the comparisons drawn in Fig. 8 of reference 37, but it extends the open reading frame 19 bp further downstream towards the O_{FR} site. This corrected sequence is incorporated into the figures in this paper.

Genetic cross of the O_{FR} mutation onto wild-type HK022. To cross the O_{FR} mutation onto wild-type HK022, a plate lysate (49) of wild-type HK022 was made on strain JL2442 carrying pNGC44. To identify isolates containing the O_{FR} mutation, this lysate was plated on *Escherichia coli* N99 and $\approx 10^6$ plaques were screened by plaque hybridization (29) with the oligonucleotide probe ATGTGCTTTTGTACC complementary to the O_{FR} mutation (the position of the mutation is underlined); this oligonucleotide was labeled for 2 h at 37°C with T4 polynucleotide kinase (Promega) and γ -[32 P]ATP and purified by electrophoresis on a 20% acrylamide–8.5 M urea gel. Bacterial lawns containing HK022 plaques were overlaid with nitrocel-

lulose paper (Schleicher & Schuell) and subjected to lysis as described elsewhere (29). Filters were baked under a vacuum at 80°C for 2 h and then prehybridized in a solution containing $6\times$ SSC (900 mM NaCl plus 90 mM Na citrate, pH 7.0), $10\times$ Denhardt's solution, and 0.2% sodium dodecyl sulfate at 67°C for 1 h and exposed to the labeled probe. Filters were washed in $6\times$ SSC at 37°C and exposed to Kodak XAR5 X-ray film. Subsequent washes in $6\times$ SSC were performed at 43°C. Ten plaques were picked which hybridized with the 32 P-labeled probe, and these isolates were purified and reprobed as described above. Two isolates, L1-1 and L5-1, were then sequenced for both O_R and O_{FR} regions and found to contain no mutations in O_R and only the single mutation in O_{FR} .

Determination of frequency of virulent mutants arising from O_{FR} mutants and wild-type phage. From single plaques for the wild type, L1-1, and L5-1 grown on N99, three separate plate lysates of each strain were prepared on N99. Titers were determined on strains N99 and JL1877, and the frequency of virulent mutants was taken as the ratio of titers on JL1877 and N99. For almost all cases, approximately 300 to 1,400 plaques were counted per plate; the frequency of virulent mutants arising from wild-type HK022 was so low that, even with 9×10^8 PFU per plate, only a few plaques were observed (ranging from 0 to 4 plaques on the three lysates tested). Therefore, our estimate of this frequency, 4×10^{-9} , is only an approximation. Both isolates of the single-site O_{FR} mutant gave an average of 3.4×10^{-5} (from triplicate lysates) for the frequency of virulent mutants; the values for each of the separate lysates were within a factor of 1.5 of the average value.

Assay for promoters near O_{FR} . Derivatives of strain JL3248 were made containing two plasmids: (i) pGB2 or a derivative encoding CI or Cro and (ii) one of several reporter plasmids (pRS415 or pNGC45, -46, -47, or -48). Cells were grown to exponential phase in L broth supplemented with the appropriate antibiotics and other components as listed for each experiment. The level of β -galactosidase from each culture was measured (with duplicate samples) as described previously (34). To test whether phage-encoded factors increased the activity of the weak rightward promoter, P_{FR} , cI^{ts12} lysogens JL3274 and JL3276 were grown to early exponential phase in L broth at 32°C (a permissive temperature for cI^{ts12}) and shifted to 42°C to allow prophage induction. Cells were removed just prior to and every 30 min after the shift in temperature. At each time point, the cells were lysed in the lysis buffer (34) and stored on ice until the final time point was taken (2 h after thermal induction); β -galactosidase activity was then measured.

RESULTS

CI binding site downstream of *cro*. Inspection of the DNA sequence from the immunity region of HK022 revealed a 15-bp region located 6 nucleotides 3' to the termination codon of the *cro* gene, a region which shared homology with known CI binding sites located in O_R and O_L (8). Other CI binding sites have dyad symmetry, and we have defined a consensus half-site on the basis of the sequences of these sites. In the sequence of the site distal to *cro*, one half-site matches this consensus and the other differs from it by one nucleotide (Fig. 1).

To test whether this sequence was a binding site for the HK022 CI repressor, DNase I footprinting analysis was done with purified repressor protein and a 32 P-labeled DNA fragment containing this site. As seen in Fig. 2, purified CI repressor protected this region from DNase I digestion. No other binding sites were detected near this site (designated O_{FR} , for operator far right), and the apparent affinity was

FIG. 1. Sequence of O_{FR} and half-operators. (a) The region around O_{FR} is shown; the O_{FR} operator is shown by the boxed region, and the end of the *cro* gene is indicated by an arrow. (b) The sequence for both half-operators from O_{FR} is shown; the sequence labeled WT tabulates the number of times particular nucleotides appear at each of the half-operators in O_{R1} , O_{R2} , O_{R3} , O_{L1} , O_{L2} , O_{L3} , and O_{FR} (8), and the consensus half-operator is shown below. The mutation in O_{FR} from VW5-6 is indicated by a small capital letter. This change affects one of the most highly conserved positions in the operator; A→G transitions at this position were the most frequent changes recovered in a set of sequenced virulent HK022 mutants (8) (see also Fig. 4).

determined in other experiments (data not shown) to be $K_d = 300$ pM. The affinity of CI for O_{FR} is considerably greater than for any of the individual operators in O_R (8); for comparison, the most tightly binding operator in O_R , O_{R1} , showed a value of $K_d = 6$ nM.

The role of O_{FR} in virulence of HK022. The biological relevance of O_{FR} was assessed by asking whether this site is involved in immunity control. Among the virulent mutants of HK022 sequenced, almost all contained two mutations in O_R , with single mutations in O_{R1} and O_{R2} (8). However, a few virulent mutants contained only a single mutation in O_R (either in O_{R1} or O_{R2}) and seemed likely candidates to contain other types of mutations. Since HK022 does not require mutations in O_L for virulence (see Discussion), we tested whether the three single-site O_R mutants contained a second mutation in O_{FR} . DNA sequence analyses in this region showed that one of the mutants tested (VW5-6) contained a mutation in O_{FR} (Fig. 3); second-site changes in the other two virulent mutants have not yet been identified. The O_{FR} mutation in VW5-6 was in a highly conserved position in the consensus half-operator (Fig. 1). DNase I protection studies with purified CI repressor and a DNA fragment containing the mutant O_{FR} revealed that this mutation decreased the affinity for the repressor by about 250-fold (data not shown).

Phenotype of a single-site O_{FR} mutant. To test whether a single mutation in O_{FR} by itself conferred a phenotype, the O_{FR} mutation was crossed onto wild-type HK022 phage from a

FIG. 2. DNase I footprinting analysis of the O_{FR} region with purified CI repressor protein. (a) Autoradiogram of polyacrylamide gel electrophoresis (PAGE) analysis of footprinting reactions. DNase I footprinting was done with purified CI repressor at the concentrations (in nM units) listed above each lane and a 32 P-labeled DNA fragment containing O_{FR} (the top strand was labeled). After the footprinting reactions were done, the DNA fragments were fractionated by PAGE and autoradiography was performed. Maxam and Gilbert purine and pyrimidine sequencing reactions (33) were used as sequence markers. At the highest repressor concentration, a periodic pattern of protections and enhancements was observed above and below O_{FR} ; this pattern, termed phasing, was previously seen with single O_{R1} and O_{R2} operators (8). (b) Sequence of the protected region. The sequence of O_{FR} and surrounding regions is shown. The region protected from DNase I digestion by CI repressor when the top strand was labeled is indicated by a shaded bar above the line; ambiguities due to a lack of DNase digestion in free DNA are indicated by the line extending from the bar. The 15-bp inverted repeat comprising O_{FR} is boxed, and the 3' end of the *cro* coding sequence is indicated by an arrow along with the putative -35 and -10 regions (bars below the sequence) of the weak (P_{FR}) promoter (see text).

plasmid (pNGC44) that contained the mutant O_{FR} . Single-site O_{FR} mutants from the cross were identified by plaque hybridization as those isolates that hybridized at higher stringency with a 32 P-labeled oligonucleotide complementary to the O_{FR} mutation (see Materials and Methods). Two HK022 isolates were identified that contained only the O_{FR} mutation (isolates L1-1 and L5-1). In each case, DNA sequence analysis of both the O_R and O_{FR} regions confirmed that only the O_{FR} mutation was present.

When these isolates were plated on the nonlysogenic indicator strain N99, both formed turbid plaques that were phenotypically indistinguishable from plaques of wild-type HK022

FIG. 3. Location of mutations in the virulent mutant VW5-6. The top line is a schematic representation of the portion of the HK022 genome spanning the *cro* gene. Operators are depicted as boxes and labeled as either O_{R1} , O_{R2} , O_{R3} , or O_{FR} . Coding regions for the *cro* gene and the beginning of the *cI* gene are indicated with arrows, and the promoter P_{RE} , located to the right of *cro*, is also shown. The lower lines depict the mutations in O_{R1} and O_{FR} in VW5-6.

on this strain. On a lysogenic indicator strain (JL1876), neither L1-1 nor L5-1 was virulent. However, both of these isolates gave rise to virulent mutants at a far higher rate than wild-type HK022. The spontaneous frequency for virulent mutants arising from wild-type HK022 was roughly 4×10^{-9} . By contrast, both L1-1 and L5-1 gave rise to virulent mutants at a frequency of $\approx 3 \times 10^{-5}$. This high frequency resembles that shown by the λ derivative $\lambda 11$ (23), which is not virulent but carries two operator mutations ($v2$ and $v3$ in O_L and O_R , respectively) and gives rise to virulent mutants at a similarly high frequency (22, 23) by acquiring another mutation in O_R (22).

By analogy, virulent mutants derived from O_{FR} mutants would be expected to have acquired a second mutation. To test this possibility, spontaneous virulent mutants were obtained from L1-1 and DNA sequence analyses were performed on both the O_{FR} and O_R regions. None of these virulent mutants had any additional changes in O_{FR} , and each had acquired a single mutation in either O_{R1} or O_{R2} (Fig. 4).

The high frequency of virulent mutants arising from L1-1 and L5-1 also caused lysogens of these mutant phage to form ragged-looking colonies that contained many tiny plaques. When cultures of these lysogens were grown to high density, considerable cell lysis ensued, yielding high titers of virulent phage. We infer that spontaneous O_{R1} or O_{R2} mutations

FIG. 4. Mutations conferring virulence obtained from the O_{FR} mutant L1-1. The mutations in O_{R1} or O_{R2} for virulent mutants obtained from L1-1 were identified by DNA sequence analysis and are indicated.

created virulent mutants that then grew in the initial host, and, upon lysis, in other cells in the culture.

Regulatory role for O_{FR} . The properties of the O_{FR} mutant phage suggest that O_{FR} acts in some way to prevent single changes in O_R from allowing virulence (see Discussion). How might it do so? One possibility, which we have investigated, is that repressor bound to O_{FR} controls the expression of a promoter overlapping O_{FR} . To test for potential promoters near O_{FR} , a 165-bp DNA fragment spanning this site was linked to a *lacZ* reporter gene in the operon fusion vector pRS415 (Fig. 5). Identical constructions were done for both wild-type and mutant O_{FR} operators. When the region containing O_{FR} was inserted in the orientation to detect a leftward promoter (pNGC46 and pNGC48), only a slight increase in β -galactosidase levels was observed relative to the vector, suggesting that there was no significant promoter directed leftward. A more substantial increase in the level of β -galactosidase was observed with the rightward orientation (pNGC45 and pNGC47). With this orientation, expression was diminished with wild-type O_{FR} (pNGC45) when either CI or Cro repressor, supplied in *trans* from a plasmid, was present; by contrast, the mutant O_{FR} construct (pNGC47) was relatively insensitive to repression by either protein.

This rightward promoter, designated P_{FR} (for promoter far right), appeared to be only a weak promoter. These operon fusions were present on a multicopy vector (see Materials and Methods) and gave relatively small amounts of β -galactosidase expression. Because of this relatively weak promoter strength of P_{FR} , it is not clear whether control of this promoter accounts for the entire mechanism of protection against virulence mediated by O_{FR} (see Discussion).

One possible explanation for the weak activity of P_{FR} with these operon fusions is that this promoter requires a phage-encoded activator for efficient expression as seen, for example, at the λP_{RM} and P_{RE} promoters. By this model, in a superinfecting O_{R1}^- (or O_{R2}^-) O_{FR}^- phage, partial derepression of P_{FR} by an O_{R1}^- or O_{R2}^- mutation would lead to expression of an activator, which would then act at P_{FR} , greatly increasing rightward transcription. We expected, therefore, that growth of HK022 in the absence of CI might also allow expression of

FIG. 5. Promoter activity of *O_{FR}::lacZ* operon fusions. A 165-bp fragment of HK022 DNA containing *O_{FR}* was cloned into the vector pRS415 in front of the *lacZ* gene. On the left is a schematic representation for each operon fusion, and the name of each plasmid is indicated. The region from HK022 is shown by a thin line, and *O_{FR}* is depicted as a box. An arrow indicates the 3' end of the *cro* coding sequence, and the *lacZ* gene is shown as a solid bar. The shaded bar depicts flanking vector sequences. The mutation in *O_{FR}* is shown by an X. To the right of each map are listed the β -galactosidase values obtained with each operon fusion in the presence of either no repressor (in cells containing pGB2), CI repressor (provided by pNGC25), or Cro repressor (provided by pNGC24); in each case, the indicated plasmids were present in JL3248. Synthesis of each repressor was under the control of the *lacUV5* promoter. Cro synthesis was increased in strains containing pNGC45 and pNGC47 by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a concentration of 10^{-5} M. The slight increase in β -galactosidase expression observed with pNGC46 and pNGC48 was repressed by CI; since CI cannot bind to *O_{FR}* in pNGC48, we believe it unlikely that this repression results from action of CI at *O_{FR}*.

such an activator. It was not possible to test this directly by measuring the β -galactosidase expression level after infection with HK022 phage because of the poor efficiency of adsorption by HK022 to cells (14). Instead, this possibility was tested by measuring β -galactosidase levels in strains after thermal induction of a prophage with the temperature-sensitive *cI* gene (*cI^{ts12}*). Lysogens containing either the wild-type (pNGC45) or mutant (pNGC47) *O_{FR} P_{FR}::lacZ* operon fusions were grown at 32°C and shifted to the nonpermissive temperature (42°C) to induce the prophage. The level of β -galactosidase expression for the fusion containing the mutant *O_{FR}* remained about the same, in the range of 200 to 300 units, for cells grown at 32°C and at all times up to 2 h after the temperature shift (data not shown). With the *O_{FR}⁺* template, the level of β -galactosidase increased from 30 units to about 80 units after the temperature shift; we attribute this increase to the loss of repression by CI. Although Cro protein was presumably expressed after induction, the finding that β -galactosidase levels increased suggests that repression by Cro was less efficient than that conferred by CI in the lysogen. We conclude from the result with the *O_{FR}* mutant template that no phage-encoded factor which would be made during prophage induction stimulated *P_{FR}* expression from this template.

DISCUSSION

In this work we identified a novel control element in the lambdoid phage HK022. This control element, *O_{FR}*, was lo-

cated 3' to the *cro* coding sequence and was a binding site for both the HK022 CI and Cro repressors. Binding of either protein repressed a weak rightward promoter (*P_{FR}*). Mutations in *O_{FR}* did not make the phage virulent and conferred no obvious phenotype. However, combination of an *O_{FR}* mutation with a single mutation in either *O_R1* or *O_R2* resulted in a virulent phenotype.

By themselves, single mutations in *O_R1* or *O_R2* do not confer virulence. Among a large collection of virulent mutants sequenced previously (8), almost all contained two mutations, one each in *O_R1* and *O_R2*. If a single mutation sufficed for virulence, we would expect that a vast majority of isolates would be of this type. The rate at which *O_R* mutations arise can be estimated from the rate at which virulent derivatives of the *O_{FR}* mutant phage arose, roughly 10^{-5} , a frequency much higher than seen with wild-type HK022. We infer, therefore, that wild-type *O_{FR}* acts in some way to prevent a single *O_R* mutation from causing virulence.

Mechanism of *O_{FR}* action. How can *O_{FR}* function so that a single *O_R* mutation is not virulent in an *O_{FR}⁺* background but is virulent in an *O_{FR}⁻* background? Binding of CI (or Cro) to *O_{FR}* must somehow prevent lytic growth of a superinfecting phage which contains a single mutation in *O_R1⁻* or *O_R2⁻*, despite the weak binding of CI to *O_R* relative to that in the wild type. Although we do not know whether it is binding of CI or of Cro that is important for this action of *O_{FR}*, CI seems a more plausible candidate, since a superinfecting phage would be

confronted with CI but not Cro. Although we cannot rule out the possibility that Cro binding to O_{FR} would somehow antagonize lytic growth, for example by acting as a sink for limiting amounts of Cro, we will assume for the sake of discussion that the crucial event is binding of CI to O_{FR} .

The many possibilities for the mechanism of O_{FR} action can broadly be divided into two classes. In the first class are mechanisms in which repressor binding to O_{FR} has a local effect on some process required for lytic growth; that is, CI bound to O_{FR} interacts with other molecules bound to sites near O_{FR} . In the second class are mechanisms in which repressor molecules bound to O_{FR} interact with other sites or with proteins bound elsewhere; of these, the most likely candidates are other CI molecules bound to O_R . We discuss these possibilities in turn.

We tested one plausible model for a local action by CI at O_{FR} and found that O_{FR} controls expression of a weak rightward promoter, P_{FR} . This promoter is so weak, however, that it seems unlikely to account for the entire mechanism by which O_{FR} acts. We may compare the relative strengths of P_{FR} and the HK022 P_R promoter: a $P_{R::lacZ}$ fusion derived from pRS415 gives about 1,000 units of β -galactosidase when present in single copy (31); using the data in Fig. 5 and a ratio of about 30 for expression from plasmids with a structure like those studied here to that seen with single-copy derivatives (50), we calculate that P_R is roughly 100 times stronger than P_{FR} . Accordingly, in an HK022 O_{FR}^- lysogen, expression of the rightward operon should be at roughly 1% of the fully derepressed level. Since such lysogens were stable, this level of expression did not markedly destabilize the lysogenic state; moreover, since this phage could lysogenize efficiently, expression of P_{FR} did not prevent HK022 from following the lysogenic pathway.

It seems likely that a single O_R mutation would lead to derepression of the rightward transcript to a level at least as high as that supported by a fully derepressed P_{FR} . Yet neither the O_{FR}^- phage nor, apparently, an O_R^- single-site mutant is virulent; both mutations are required. It seems implausible that the additive effects of these two types of mutations would make a difference in the level of rightward transcription significant enough to allow virulence, unless transcripts initiating from these two promoters are qualitatively different in a property such as antitermination. We conclude that the effect of the O_{FR} mutation in allowing virulence is probably not mediated through derepression of P_{FR} .

The other likely mechanism for the action of O_{FR} is that CI repressor bound to O_{FR} acts by interacting with other sites or with proteins bound to such sites. It is unlikely that such sites are nearby CI-binding sites. Footprinting analyses did not reveal any other operators near O_{FR} which would allow for the high degree of cooperative binding found when repressor is bound to adjacent operators in O_R .

The simplest mechanism for long-range interactions would be an interaction with CI bound to O_R . There are numerous precedents for effects of distantly separated sites (reviewed in references 21, 32, and 47). In this view, CI molecules bound to O_{FR} and either O_R1 or O_R2 would interact and would maintain repression of P_R on a template with a single mutation in either O_R1 or O_R2 . The high degree of cooperativity shown by HK022 CI in binding to adjacent operators suggests that formation of such a loop might be favorable. Mutations in O_{FR} would presumably not allow this interaction to occur, and phage carrying such mutations would be much more sensitive to mutations in either O_R1 or O_R2 . By analogy with Lac repressor tetramer, which can bind simultaneously to two distantly spaced operators (38, 55), if HK022 repressor is a tetramer it could also occupy O_{FR} and some other DNA site at the same time. This possibility is difficult to assess, since we do not know

the oligomeric state of this protein. A more complex model is also possible, in which another protein serves as an intermediary to bridge CI dimers bound to the two sites, as recently suggested for Gal repressor (9).

The center-to-center distances from O_{FR} to O_R1 , O_R2 , and O_R3 are 267, 291, and 324 nucleotides, respectively. It is not known whether cooperative binding can occur with HK022 CI repressor over these distances or whether these operators would have to be positioned on the same side of the DNA helix to allow cooperative binding (as is seen with λ CI and AraC). We have found (30), however, that HK022 repressor can bind cooperatively to templates with a variety of small spacing changes between adjacent operators, suggesting that it has a good deal of flexibility, so that the exact spacing between sites ca. 300 bp apart is probably not a major constraint. In addition, two features of the sequence between O_{FR} and O_R may contribute to DNA looping. A poly(A) tract lies next to O_{FR} (Fig. 2) and may facilitate DNA bending. Also, between O_{FR} and O_R lie three sequences which share some similarity with known integration host factor binding sites (not shown); if integration host factor can bind to these sites, it could also help bend the DNA (see references 17 and 18).

We are currently testing whether the presence of wild-type O_{FR} can influence the occupancy of O_R by CI. In a preliminary experiment using linear DNA fragments (data not shown), we compared the affinities of CI for linear DNA molecules carrying an O_R1^- or O_R2^- mutation and either a wild-type or a mutant O_{FR} site and saw no difference between the affinities of CI for O_R on templates with wild-type and mutant O_{FR} . However, on the basis of results with Lac repressor (4) and other systems (21, 32, 47) and results of recent computer simulations that suggest that supercoiling can raise the effective local concentration of two sites separated by this distance some 2 to 3 orders of magnitude (53), it is likely that supercoiling would greatly favor such an interaction.

How might O_{FR} -bound repressor work in a way that does not involve long-range interactions? Binding of CI to O_{FR} might act as a roadblock to impede elongation of the P_R transcript by RNA polymerase (13, 16, 20). This effect might be more pronounced at high repressor concentrations, when protection of adjacent nonspecific regions is observed, a pattern we term phasing (as seen in Fig. 2, lane 4). The same effect has been observed with binding of repressor to single operators in O_R (8). However, we would expect a blocking mechanism to be significant only at high rates of transcription, whereas the apparent need for O_{FR} would be to prevent low-level transcription from a largely repressed P_R , so that we believe that a blocking model is unlikely to explain the effects of O_{FR} .

Finally, we note two other issues that may bear on the mechanism of O_{FR} action. First, if repressor binding to O_{FR} in fact proves to be noncooperative, the binding curve for O_{FR} (fractional occupancy versus concentration of CI) would be less steep than for O_R (24). This would make occupancy of O_{FR} less sensitive to relatively small changes in CI concentration. Second, the affinity of CI for O_{FR} is far greater than for either O_R1 or O_R2 separately and is about the same as that seen for cooperative binding to O_R1 and O_R2 ($K_d = 0.3$ nM). Accordingly, when CI occupies O_R1 and O_R2 , O_{FR} should also be at least partially occupied.

Possibility of O_{FR} sites in other lambdoid phages. We examined the sequences of λ and the lambdoid phages $\phi 80$, 434, and P22 near the termination codon in the *cro* genes for likely operators that showed agreement with their respective consensus operator sequences. Only two of the phages, 434 and P22, showed partial matches to their consensus operator sequences (Fig. 6); however, these sequences contain mis-

FIG. 6. Potential *O*_{FR} operators in 434 and P22 phages. The sequences of potential *O*_{FR}s (boxed sequences) near the end of the *cro* genes (arrows) from P22 (41) and 434 (19) are shown in the upper portions of the figure for each phage. The half-operators for each *O*_{FR} are compared with the respective consensus half-operator (derived from a consensus of operators in *O*_R [42, 54]). The mismatches in the conserved positions of the half-operator are underlined. Lowercase letters indicate nucleotides that are less highly conserved.

matches in residues critical for specific repressor-operator interaction, so that it is unlikely that these are binding sites for their respective repressors. We have not tested for binding of 434 and P22 repressors to these sites. If *O*_{FR} acts at a distance, however, it is entirely possible that an *O*_{FR} analog in another lambdoid phage might be located in a different position.

Biological role of *O*_{FR}. As discussed above, the binding of CI (or conceivably of Cro) to *O*_{FR} is essential for HK022 to pursue the lysogenic lifestyle. Lysogens of an *O*_{FR}⁻ phage quickly generate virulent variants that overgrow the colony or culture. We may compare the requirements for virulence between HK022 and λ . In λ , three operator mutations must be combined to lead to virulence (15, 22), so that virulent derivatives are very rare. Full virulence in λ requires gene expression both from *P*_L and from *P*_R; interestingly, virulence can be obtained either with two *O*_R mutations and one *O*_L mutation or with one *O*_R and two *O*_L mutations (15). The implication is that full derepression of either operon can set in motion events that eventually lead to breakdown of immunity, given weakened CI binding to the other operator.

Unlike λ , HK022 does not require expression of *P*_L for virulence, probably because it does not have any analog to the *N* gene from λ . In λ , *N* protein is synthesized from *P*_L and is required for antitermination of transcription to allow expression of downstream genes (12). Antitermination does occur in HK022 but does not appear to require a phage-encoded factor (35). Accordingly, immunity in HK022 is maintained by mechanisms that probably do not involve *O*_L. Whatever the mechanism by which *O*_{FR} contributes to this regulatory circuitry, our work suggests that gene regulation in HK022 differs substantially from that in λ .

Single-step virulence in other phages. Certain other phages appear to be able to mutate to virulence by a single step and presumably must have mechanisms to prevent such virulent phage from overgrowing cultures of lysogens. Phage P1 utilizes an antisense RNA called the *c4* RNA to repress the *ant* gene, which encodes an antagonist to the *c1* immunity repressor. Single mutations in the target for the *c4* RNA can lead to virulence (3, 11). Two factors appear to curtail the spread of such virulent phages: virulent phages have small burst sizes, and P1 adsorption requires Ca⁺⁺, so that lysogens are not infected in the absence of this cation (2, 45). Phage P2 has only one early promoter, controlled by the C repressor; repressor apparently binds to two directly repeated sequences. In this case, most virulent mutants studied (46) had deletions of various sizes that spanned these repeats; a mutant with a deletion of a single base pair in one repeat, *vir1*, could not plate on a P2 lysogen, while a deletion mutant lacking one of the repeats could not plate on a strain with three P2 prophages. Hence it is unclear from published data whether virulent mutants of P2 that can overgrow cultures can arise at sufficiently high frequencies to make lysogens unstable. A hybrid between phage 434 and λ , termed λ imm⁴³⁴ (Meselson), can also give virulent derivatives in one step (5, 43), although the mutation frequency at which this occurs has not been published. Repressor levels in λ imm⁴³⁴ (Meselson) lysogens appear to be low, relative to those in λ lysogens. A derivative termed λ imm⁴³⁴ (Tomizawa) makes repressor levels comparable to those of λ ; this derivative carries an *O*_R3 mutation (39) which reduces negative autoregulation of *cI* (5). Neither hybrid can give rise in a single step to virulent derivatives that plate on λ imm⁴³⁴ (Tomizawa) lysogens. We speculate that, during the course of passaging the original *imm*⁴³⁴ hybrid in the laboratory, this property was selected as a matter of experimental convenience. It is not known whether the original 434 has properties similar to those of λ imm⁴³⁴ (Meselson); it is possible (6) that a site analogous to *O*_{FR} was separated from the immunity region during the recombination events that created this hybrid (26). Finally, a derivative of P22 carrying a mutation in the *sieA* (superinfection exclusion) gene can mutate to virulence in a single step by a mutation in the Mnt binding site in the *ant* promoter (40, 52); wild-type P22 excludes superinfecting phage by the SieA gene product. Accordingly, P22 also ensures immunity to superinfection by acting at a level different from that of gene regulatory circuitry. The diversity reflected among all these examples indicates that nature can use a wide range of means to achieve the same regulatory end.

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REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, et al. 1987. Current protocols in molecular biology. Wiley Interscience, New York.
2. Baumstark, B. Personal communication.
3. Biere, A. L., M. Citron, and H. Schuster. 1992. Transcriptional control via translational repression by *c4* antisense RNA of bacteriophages P1 and P7. *Genes Dev.* 6:2409-2416.
4. Borowiec, J. A., L. Zhang, S. Sasse-Dwight, and J. D. Gralla. 1987. DNA supercoiling promotes formation of a bent repression loop in *lac* DNA. *J. Mol. Biol.* 196:101-111.
5. Bushman, F. D. 1993. The bacteriophage 434 right operator. Roles of *O*_R1, *O*_R2 and *O*_R3. *J. Mol. Biol.* 230:28-40.
6. Bushman, F. D. Personal communication.

7. Cam, K., J. Oberto, and R. A. Weisberg. 1991. The early promoters of bacteriophage HK022: contrasts and similarities to other lambda-doid phages. *J. Bacteriol.* **173**:734–740.
8. Carlson, N. G., and J. W. Little. 1993. Highly cooperative DNA binding by the coliphage HK022 repressor. *J. Mol. Biol.* **230**:1108–1130.
9. Choy, H. E., and S. Adhya. 1992. Control of *gal* transcription through DNA looping: inhibition of the initial transcribing complex. *Proc. Natl. Acad. Sci. USA* **89**:11264–11268.
10. Churchward, G., D. Belin, and Y. Nagamine. 1984. A pSC101-derived plasmid which shows no sequence homology to other commonly used cloning vectors. *Gene* **31**:165–171.
11. Citron, M., and H. Schuster. 1990. The *c4* repressors of bacteriophages P1 and P7 are antisense RNA's. *Cell* **62**:591–598.
12. Das, A. 1992. How the phage lambda *N* gene product suppresses transcription termination: communication of RNA polymerase with regulatory proteins mediated by signals in nascent RNA. *J. Bacteriol.* **174**:6711–6716.
13. Deuschle, U., R. Gentz, and H. Bujard. 1986. *lac* repressor blocks transcribing RNA polymerase and terminates transcription. *Proc. Natl. Acad. Sci. USA* **83**:4134–4137.
14. Dhillon, T. S., and E. K. Dhillon. 1976. Temperate coliphage HK022. Clear plaque mutants and preliminary vegetative map. *Jpn. J. Microbiol.* **20**:385–396.
15. Flashman, S. M. 1978. Mutational analysis of the operators of bacteriophage lambda. *Mol. Gen. Genet.* **166**:61–73.
16. Flashner, Y., and J. D. Gralla. 1988. Dual mechanism of repression at a distance in the *lac* operon. *Proc. Natl. Acad. Sci. USA* **85**:8968–8972.
17. Friedman, D. I. 1988. Integration host factor: a protein for all reasons. *Cell* **55**:545–554.
18. Goodman, S. D., and H. A. Nash. 1989. Functional replacement of a protein-induced bend in a DNA recombination site. *Nature (London)* **341**:251–254.
19. Grosschedl, R., and E. Schwarz. 1979. Nucleotide sequence of the *cro-clf* region of bacteriophage 434 DNA. *Nucleic Acids Res.* **6**:867–881.
20. He, B., and H. Zalkin. 1992. Repression of *Escherichia coli purB* is by a transcriptional roadblock mechanism. *J. Bacteriol.* **174**:7121–7127.
21. Hochschild, A. 1991. Detecting cooperative protein-DNA interactions and DNA loop formation by footprinting. *Methods Enzymol.* **208**:343–361.
22. Hopkins, N., and M. Ptashne. 1971. Genetics of virulence, p. 571–574. In A. D. Hershey (ed.), *The bacteriophage lambda*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
23. Jacob, F., and E. L. Wollman. 1954. Étude génétique d'un bactériophage tempéré d'*Escherichia coli*. I. Le système génétique du bactériophage λ . *Ann. Inst. Pasteur (Paris)* **87**:653–673.
24. Johnson, A. D., A. R. Poteete, G. Lauer, R. T. Sauer, G. K. Ackers, and M. Ptashne. 1981. λ Repressor and *cro*—components of an efficient molecular switch. *Nature (London)* **294**:217–223.
25. Johnsrud, L. 1978. Contacts between *Escherichia coli* RNA polymerase and a *lac* operon promoter. *Proc. Natl. Acad. Sci. USA* **75**:5314–5318.
26. Kaiser, A. D., and F. Jacob. 1957. Recombination between related temperate bacteriophages and the genetic control of immunity and prophage localization. *Virology* **4**:509–521.
27. Lin, L. L., and J. W. Little. 1989. Autodigestion and RecA-dependent cleavage of Ind[−] mutant LexA proteins. *J. Mol. Biol.* **210**:439–452.
28. Little, J. W., and S. A. Hill. 1985. Deletions within a hinge region of a specific DNA-binding protein. *Proc. Natl. Acad. Sci. USA* **82**:2301–2305.
29. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Mao, C., N. G. Carlson, and J. W. Little. Cooperative DNA-protein interactions: effects of changing the spacing between adjacent binding sites. *J. Mol. Biol.*, in press.
31. Mao, C., and J. W. Little. Unpublished data.
32. Matthews, K. S. 1992. DNA looping. *Microbiol. Rev.* **56**:123–136.
33. Maxam, A. M., and W. Gilbert. 1980. Sequencing end labeled DNA with base specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
34. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. Oberto, J., M. Clerget, M. Ditto, K. Cam, and R. A. Weisberg. 1993. Antitermination of early transcription in phage HK022. Absence of a phage-encoded antitermination factor. *J. Mol. Biol.* **229**:368–381.
36. Oberto, J., and R. A. Weisberg. Personal communication.
37. Oberto, J., R. A. Weisberg, and M. E. Gottesman. 1989. Structure and function of the *nun* gene and the immunity region of the lambda-doid phage HK022. *J. Mol. Biol.* **207**:675–693.
38. Oehler, S., E. R. Eismann, H. Krämer, and B. Müller-Hill. 1990. The three operators of the *lac* operon cooperate in repression. *EMBO J.* **9**:973–979.
39. Pirrotta, V. 1979. Operators and promoters in the O_r region of phage 434. *Nucleic Acids Res.* **6**:1495–1508.
40. Poteete, A. R. Personal communication.
41. Poteete, A. R., K. Hehir, and R. T. Sauer. 1986. Bacteriophage P22 Cro protein: sequence, purification, and properties. *Biochemistry* **25**:251–256.
42. Poteete, A. R., M. Ptashne, M. Ballivet, and H. Eisen. 1980. Operator sequences of bacteriophages P22 and 21. *J. Mol. Biol.* **137**:81–91.
43. Ptashne, M. 1971. Repressor and its action, p. 221–237. In A. D. Hershey (ed.), *The bacteriophage lambda*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. Ptashne, M. 1992. A genetic switch: phage λ and higher organisms, 2nd ed. Cell Press and Blackwell Scientific Publications, Cambridge, Mass.
45. Rosner, J. L. Personal communication.
46. Saha, S., B. Lundqvist, and E. Haggård-Ljungquist. 1987. Auto-regulation of bacteriophage P2 repressor. *EMBO J.* **6**:809–814.
47. Schleif, R. 1992. DNA looping. *Annu. Rev. Biochem.* **61**:199–223.
48. Shean, C. S., and M. E. Gottesman. Personal communication.
49. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. Simons, R. W., F. Houman, and N. Kleckner. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**:85–96.
51. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60–89.
52. Susskind, M. M., and D. Botstein. 1978. Molecular genetics of bacteriophage P22. *Microbiol. Rev.* **42**:385–413.
53. Vologodskii, A. V., S. D. Levene, K. V. Klenin, M. Frank-Kamenetskii, and N. R. Cozzarelli. 1992. Conformational and thermodynamic properties of supercoiled DNA. *J. Mol. Biol.* **227**:1224–1243.
54. Wharton, R. P., E. L. Brown, and M. Ptashne. 1984. Substituting an α -helix switches the sequence-specific DNA interactions of a repressor. *Cell* **38**:361–369.
55. Wu, H.-Y., K. Lau, and L. F. Liu. 1992. Interlocking of plasmid DNAs due to *lac* repressor-operator interaction. *J. Mol. Biol.* **228**:1104–1114.