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DNA sequences of the repressor gene and operator region of bacteriophage P2

(overlapping transcription/DNA-binding proteins/biotin operator)

ELISABETH LJUNGQUIST, KERSTIN KOCKUM*, AND L. ELIZABETH BERTANI†

Department of Microbial Genetics, Karolinska Institutet, S-10401 Stockholm, Sweden

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ABSTRACT **The nucleotide sequence of the repressor gene *C* of the temperate phage P2 has been determined. It codes for a nonbasic polypeptide, 99 amino acids long. Twelve repressor-defective mutants have been mapped. All but one are located within the presumed coding part of the gene. There is a strong promoter sequence and an 8-base-pair inverted repeat preceding the gene. The P2 repressor protein shows structural similarity to other DNA-binding proteins. The operator region for the early replication functions was located by sequencing the DNA of three virulent mutants. The sequence indicates that there are two repressor-binding sites. In addition, one of the sites shows sequence homology with part of the operator region of the biotin operon of *Escherichia coli*.**

In prokaryotes, the control of gene expression by a repressor system is quite common. With the possible exception of the tumor antigen of simian virus 40 (1), no well-characterized repressors have been described, as yet, for eukaryotic systems. In temperate phages, however, repressor control is the key mechanism for establishing and maintaining the lysogenic state.

Among temperate phages, most of our knowledge of repressor structure and function comes from studies on phage λ and closely related phages. In phage λ, the main repressor molecule is a polypeptide of 236 amino acids that has two domains and binds as a dimer to three individual sites in each of two operator regions (2). In addition, phage λ has a second repressor of 66 amino acids, which is coded for by a different gene but binds to the same sites in the operator regions (2).

Little is known about phages that are not related to phage λ. Some features of the immunity region of phage Mu have been inferred from a cloned-DNA sequence (3), and a protein with the expected DNA-binding specificity has been isolated from phage P1 (4).

Bacteriophage P2 is the most thoroughly described representative of a group of temperate, noninducible phages unrelated to phage λ (for review, see ref. 5). The phage P2-specific repressor, coded for by the *C* gene, binds to one operator region, which regulates the expression of the early genes: *cox*, *B*, and *A* (Fig. 1). There is no evidence, so far, for an antirepressor function in phage P2 (6) or for genes comparable to the genes *N*, *CII*, or *CIII* of phage λ. Thus, regulation in P2 may be simpler than in the other temperate phages that have been studied.

The phage P2 repressor has been partially purified, and its specific DNA-binding has been measured (7). To extend our knowledge of the P2 repressor system still further, we have determined the complete nucleotide sequence of the *C* gene and of the operator region.

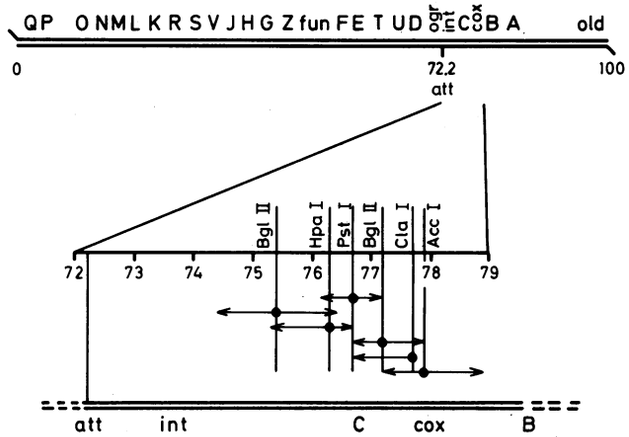

FIG. 1. Genetic map of phage P2 and of the region sequenced. (*Upper*) The gene sequence is shown above the top line. (*Lower*) The relevant region is enlarged. Locations and directions of the DNA segments sequenced are indicated by arrows, with the labeled ends symbolized by filled circles.

MATERIALS AND METHODS

Chemicals and Enzymes. [γ-^{32}P]ATP was purchased from New England Nuclear and cordycepin 5′-[α-^{32}P]triphosphate was from Amersham. Restriction endonucleases were from New England Biolabs or Boehringer Mannheim; calf intestinal alkaline phosphatase (EC 3.1.31), from Boehringer Mannheim; T4 polynucleotide kinase (EC 2.7.1.78), from New England Nuclear; and terminal deoxynucleotidyl transferase, from Amersham.

Bacteriophages. The phage P2 mutants used are listed in Table 1. The amber *C* mutants were isolated from wild-type P2 after mutagenesis with hydroxylamine as described (13). Mutants forming clear plaques on the nonpermissive indicator, C-85, were picked and spotted on strains CA 154 (su_0) and CA 266 (su_1) (14). Of the mutants isolated, *c11* and *c13* were more efficiently suppressed than *c10* and *c12*. At isolation, *c12* formed abnormally small plaques. This phenotype was presumably the result of a second mutation induced by the hydroxylamine treatment and was shown to segregate in crosses to P2 *amA127*. The *c12* isolate used here is a normal-plaque-size recombinant from such a cross.

DNA Preparation. All DNA sequencing was done with phage DNA. Phages were prepared as described (15), concentrated with polyethyleneglycol, and purified by band centrifugation in a step gradient of CsCl in standard saline citrate (0.15 M NaCl/0.015 M Na citrate, pH 7.0). DNA was extracted from the phage particles with phenol, which was

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*Present address: Department of Microbiology, University of Stockholm, S-10691 Stockholm, Sweden.
†Present address: Biology Division, California Institute of Technology, Pasadena, CA 91125.

Table 1. P2 mutants used

Mutant	Pertinent features	Origin or ref.
c1	Spontaneous, clear plaque-forming mutant. Lysogenizes at reduced frequency, but its lysogens are stable and immune to superinfection.	8
c5, *c6*, *c7*, *c8*, *c9*	Obtained after UV irradiation. Form clearer plaques at high temperature.	9
c10, *c11*, *c12*, *c13*	Obtained after hydroxylamine mutagenesis. Amber (suppressor-sensitive) mutations.	This work
vir1	Spontaneous mutant. "Inducing virulent." Capable of limited multiplication in lysogens, although it gives no plaques on lysogenic indicators.	8
vir3, *vir24*	Spontaneous mutants. Form plaques on lysogens. Mapped between genes *C* and *B* (10). No immunity-sensitive recombinants were observed in crosses between the two mutants (10), and both were indistinguishable from wild type in particle buoyant density and thermal stability (11).	9
vir6	Spontaneous mutant. Gives plaques on lysogens with variable efficiency, dependent on the immunity level of the host.	9
sly1	Obtained after mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. Lysogenizes normally, but the lysogens are unable to liberate phage spontaneously. The mutation was located in the repressor gene by genetic mapping (unpublished data).	12*

*Originally classified as "*cox1*."

later removed by extensive dialysis at 4°C against 10 mM Tris/1 mM EDTA, pH 7.5.

Mapping with Restriction Enzymes *Acc* I and *Cla* I. The locations of the restriction sites for several restriction enzymes on phage P2 DNA are known (16). By using similar methods, the cleavage sites for two other restriction enzymes, *Acc* I and *Cla* I, were determined. *Acc* I has eight cleavage sites in P2, located at 5.2, 10.2, 37.5, 41.0, 57.0, 66.1, 78.0, and 86.6% from the left end of the P2 genetic map. *Cla* I has four cleavage sites, located at 49.7, 77.7, 87.7, and 91.6% from the left end. The *Cla* I site at 77.7 is partially resistant. This has also been noted by P. Palm (personal communication).

DNA Sequence Determination. The DNA was cleaved with an appropriate restriction enzyme, and the 5′ ends generated (except in the case of *Pst* I) were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The *Pst* I-generated 3′ ends were labeled with cordycepin 5′-[α-^{32}P]triphosphate and terminal deoxynucleotidyl transferase. The labeled fragments were then cleaved with another restriction enzyme, and the fragments were separated by gel electrophoresis. The labeled fragments to be sequenced were located on the gel by autoradiography. The corresponding gel sections were cut out, sliced, and eluted by diffusion in 10 mM Tris/0.3 M NaCl/1 mM EDTA, pH 7.5, at 37°C for 24–48 hr. The DNA was collected on a DEAE column, eluted, and concentrated by precipitation with ethanol. The DNA sequencing was done with the method of Maxam and Gilbert (17), and the products were separated on 6%, 8%, or 20% polyacrylamide gels, 0.4 × 230 × 1000 mm in size. The gels were subsequently autoradiographed with Kodak Xomat XRPI film.

RESULTS

Sequencing Strategy. The structural gene for the phage P2 repressor is known, from cloning experiments, to be contained on the *Bgl* II restriction fragment that covers the region 75.4–77.2% from the left end of the P2 chromosome (18) (see Fig. 1). This fragment cloned into pBR322 expresses the repressor gene in one orientation only, as if, in this case, the gene were under the control of a plasmid promoter. This orientation would correspond to a right-to-left direction of transcription for the gene *C*, relative to the P2 map, and would predict that the phage promoter for the repressor gene would be to the right of 77.2%. Using the technique of Maxam and Gilbert (17), we sequenced the 75–78% region of P2 DNA from the restriction enzyme cleavage sites indicated in Fig. 1. The sequence obtained is presented in Fig. 2.

The DNA Sequence of the Repressor Gene. To determine the location and reading frame of the repressor gene, we determined the DNA sequences from several amber *C* mutants of phage P2. The mutations *c11* and *c13* were identical: the replacement of the C at position 205 by a T (Fig. 2) forms the nonsense codon UAG. This establishes the correct reading frame as indicated in Fig. 2. DNA from the *c10* or *c12* mutants was not cleaved by *Pst* I at the 76.7 cleavage site, suggesting that these mutations lie in the *Pst* I recognition sequence. Since the mutations were obtained by mutagenesis with hydroxylamine, they probably involve a C-to-T change at position 160 (Fig. 2).

The reading frame thus established is open from position −12 to +297. The first in-frame methionine codon is located at +1, and this would result in a polypeptide 99 amino acids long. There is a second methionine codon 11 amino acids further down. However, the first methionine codon is preceded by a hypothetical ribosome binding site (indicated by a wavy line in Fig. 2), complementary to the 3′ end of 16S RNA (19) and, therefore, is the more likely candidate for the initiation codon. The stop codon, *ochre*, is located in the *Hpa* I site at 76.3.

Past the repressor gene, the sequence contains stop codons in all reading frames, several inverted repeats (indicated by arrows in Fig. 2), and three stretches of six or seven continuous adenines. In addition, the sequence C-A-A-T-C-A-A, which is common to some *rho*-dependent terminators (20), is found about 120 bases after the stop codon (last line in Fig. 2). Thus, the transcriptional terminator may be *rho* dependent.

Eight phage P2 mutations (*c1*, *c5*, *c6*, *c7*, *c8*, *c9*, *vir1*, and *sly1*), known to affect the immunity repressor (see Table 1 for their features) were examined by DNA sequence analysis. All, except *c1*, are located in the coding part of the *C* gene (see Fig. 2) and give rise to triplets coding for different amino acids compared with the wild type. The *c1* mutation is located at position −23 in what would be the control region for transcription of the repressor gene.

The location of the *c5* and *vir1* mutations agrees with the previous finding (21) that neither could be rescued by recombination from the cloned 62.6–76.6% *Pst* I fragment—i.e., they should lie to the right of site 76.7. In similar experiments with the mutants *c11* and *c13*, the c^+ alleles were found with frequencies of 0.1–0.2% among the D^+ recombinants, confirming that these mutations do lie on the *Pst* I fragment—i.e., to the left of site 76.7.

Location of the Possible Promoter for the Repressor Gene. By comparing the sequences of several promoter sites recognized by the *Escherichia coli* RNA polymerase, a promoter prototype was constructed (22). In the phage P2 DNA sequence preceding the repressor gene, a −10 (Pribnow) heptamer, T-A-T-A-A-T-G, identical to the prototype can be seen (Fig. 2). In addition, the hexamer preceding the Pribnow sequence is identical to the corresponding hexamer in

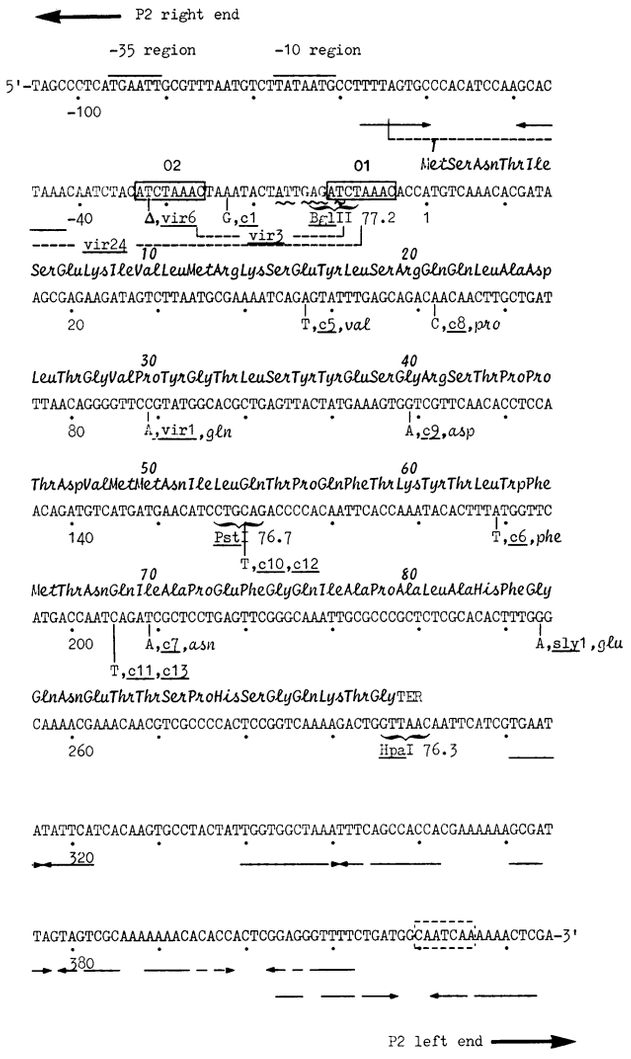

FIG. 2. DNA base sequence of the phage P2 wild-type repressor and early operator region. The predicted amino acid sequence for the repressor is also given. The solid bars above the base sequence (top row) indicate the −10 and −35 regions proposed to interact with RNA polymerase. The wavy underlining (second row) indicates the (transcript) sequence that is proposed to bind the ribosome. The relevant restriction sites, with their map positions and the examined mutational base changes with their corresponding amino acid changes are indicated below the sequence. The arrows indicate inverted repeats. The presumed repressor binding sites are within the boxes labeled 01 and 02. The broken line box (bottom row) shows the presumed *rho*-dependent terminator. Note that the beginning of the sequence, as shown, points toward the right end of the P2 chromosome and the *cox* gene, while the end of the sequence points to the left end of the P2 chromosome and the *int* gene. In this representation, the repressor gene is transcribed left-to-right, whereas in the standard P2 map orientation it would be transcribed right-to-left. Position 1 was chosen to correspond to the first base of the first codon of the repressor.

the fdV promoter. A −35 region, A-T-G-A-A-T, also can be identified (Fig. 2), although the homology with the consensus sequence, T-T-G-A-C-A, is not as strong. Assuming that the mRNA is initiated 4–7 base pairs after the Pribnow sequence, the repressor mRNA should have a leader sequence of about 60 base pairs.

Amino Acid Composition of the Phage P2 Repressor. By assuming that the repressor gene codes for a polypeptide 99 amino acids long and that the protein is not processed, the amino acid composition inferred from the DNA sequence is shown in Table 2. It has only seven basic amino acids, which is slightly lower than an "average" protein (23). It has a high threonine content and lacks cysteine.

Table 2. The amino acid composition of the phage P2 repressor

Ala	5	(8.6)	Leu	8	(7.4)
Arg	3	(4.9)	Lys	4	(6.6)
Asn	4	(4.3)	Met	5	(1.7)
Asp	2	(5.5)	Phe	4	(3.6)
Cys	0	(2.9)	Pro	7	(5.2)
Gln	8	(3.9)	Ser	9	(7.0)
Glu	5	(6.0)	Thr	12	(6.1)
Gly	7	(8.4)	Trp	1	(1.3)
His	2	(2.0)	Tyr	5	(3.4)
Ile	5	(4.5)	Val	3	(6.6)

Tabulation of the occurrences of each amino acid in the repressor polypeptide, as inferred from the DNA base sequence. The average percentage of amino acids in proteins is given within the parenthesis (23).

Identification of the Operator. Genetic evidence suggests that there is only one operator region in phage P2. It controls the early genes *A* and *B* and is defined by several virulent mutations that make the phage insensitive to the immunity of lysogenic hosts. Two such mutations, *vir3* and *vir24*, have been mapped by genetic recombination experiments and found to lie between genes *C* and *B* (10). In addition, when these studies were begun, it was found that neither the DNA of *vir3* nor *vir24* could be cut by the restriction enzyme *Bgl* II at site 77.2, indicating that the recognition site for this enzyme had been altered by the mutations.

These two mutations plus a third, *vir6*, were sequenced and found to consist of small deletions of 1, 17, and 57 base pairs for *vir6*, *vir3*, and *vir24*, respectively (Fig. 2). The exact positions of the *vir3* and *vir24* deletions cannot be established because their ends coincide with short repeats of 5 and 2 base pairs, respectively. The *vir6* deletion (at position −32) lies within an 8-base-pair sequence, A-T-C-T-A-A-A-C (designated 02 in Fig. 2), that appears again (01 in Fig. 2) just before the start of the repressor gene. Part of one of these repeated sequences is removed in *vir3*, and all of one and part of the other in *vir24* (Fig. 2). It seems most likely, therefore, that they are involved in the binding of phage P2 repressor.

By including the six nucleotides to the left of each of the repeated sequences, two partially homologous, 14-base-pair structures with some internal symmetry can be constructed. Additional information on the regions of DNA protected by phage P2 repressor is needed to determine the actual size of the P2 operator sites.

The sequence C-T-A-A-A-C found in 01 and 02, is repeated once again at positions −46 to −41 and might represent part of a third binding site. This region is also part of an 8-base-pair inverted repeat, indicated by arrows in Fig. 2.

The pentamer C-T-A-A-A occurs four times over a sequence of about 40 bases in this region, whereas it is found only once elsewhere (at position +347 to +351) in the 500-base-long sequence given in Fig. 2. The *vir3* deletion could well have arisen through recombination within two such pentamers. The *vir24* deletion, on the other hand, could have originated through splicing, involving inverted repeats of the same pentamer. Short repeating-sequences also have been noted in the regulatory region of phage Mu (3).

DISCUSSION

The Repressor Gene. According to the DNA sequence, the repressor gene of phage P2 codes for a polypeptide 99 amino acids long. This gives a molecular mass of 11 kilodaltons, which fits with the values obtained by gel filtration of partially purified repressor (7). The phage P2 repressor is smaller than the *cI* or *cII* repressors of phages λ or P22, respectively,

and also differs from the *cro* repressors of phages λ and 434, which are still smaller and very basic (24–27).

Out of the 12 repressor mutants sequenced, 11 are located within the coding part of the gene, while the mutation *c1* is located 23 bases before the presumed initiation codon and is contained in the leader sequence (see Fig. 2). The *c1* mutant has two known characteristics: it forms lysogens with reduced frequency compared to wild-type phage P2, and the lysogens that are formed exhibit decreased immunity to superinfecting phages (8). Both characteristics could be explained if the mutants did not produce as much repressor as wild-type P2. From its location, the *c1* mutation does not seem to be in the *C* gene promoter but might instead affect ribosome binding, as the "Shine–Dalgarno" sequence is located only four bases further down.

Regulation of the Repressor Gene. Rather little is known about regulation of the level of repressor in a phage P2 lysogen. There is no evidence either for a regulation based on a *cro*-like gene product (6) of the type observed in phage λ and related phages or for autoregulation by the *C* gene product itself.

The presumed repressor binding sites, 01 and 02, lie some 30 base pairs downstream to the T-A-T-A-A-T-G heptamer sequence that is thought to be the promoter for the *C* gene. It seems unlikely, therefore, that the binding of repressor to sites 01 and 02 could affect the initiation of transcription at the heptamer, although the inverted repeat, which is found in the leader sequence, might play an as yet undefined regulatory role.

Even though the repressor gene would seem to have a strong promoter, it may be weakly expressed due to a low efficiency of translation. It has been suggested that rare codons that correspond to minor tRNAs in *E. coli* (so-called modulating codons) may be used to regulate the elongation rate during translation (28). Strongly expressed genes seem to have a low percentage of modulating codons (0.6%), while weakly expressed genes have a higher percentage (4.7%). The phage P2 repressor has a total of 7 modulating codons out of 99, which would indicate a slow elongation rate.

Structural Similarities Between Phage P2 Repressor and Other DNA Binding Proteins. The three-dimensional structures of three proteins that regulate gene expression have been determined: the *cro* repressor of phage λ (29), the NH_2-terminal end of the *cI* repressor of phage λ (30), and the CAP protein of *E. coli* (31). Each protein binds specifically to a different DNA sequence, but they show structural similarities in their DNA-binding domains. The structure of the DNA-binding region of other DNA-binding regulatory proteins has been inferred from the homologies in their amino acid sequences with those of cro, cI, and CAP (29, 32, 33). It was of interest, therefore, to compare the amino acid sequence of the phage P2 repressor with those of other DNA-binding proteins. In Fig. 3, the NH_2-terminal sequences of six proteins that bind to specific regions of DNA are aligned to give maximal homology (29). In the phage λ cro structure, α_1 and α_2 are structural α helices, whereas α_3 is the DNA recognition α helix that has been postulated to lie in the major groove of the B-form DNA. Line 7 of Fig. 3 shows NH_2-terminal amino acids 18–45 of the P2 repressor aligned to maximize the homology with the other proteins. As can be seen, the Gly-24 of λ cro, present in the bend between α_2 and α_3, is conserved in all proteins, and the Val-25, in five out of six proteins. They are both present in the P2 repressor. Another well conserved amino acid is the Ala-20, which is present in five of the six proteins as well as in the P2 repressor. This shows that the NH_2-terminal region of the P2 repressor has structural features in common with other repressors and suggests that amino acids 18–40 are involved in the DNA binding. In fact, three of the repressor mutations, *c8*, *c9*, and *vir1*, are located in this region (Fig. 2). Thus, the P2 repressor may well be related to other proteins with similar function.

The Operator Structure. The operator region has been defined by the location of the operator mutants *vir3*, *vir6*, and *vir24*. The region contains two 8-base-pair repeated sequences, 01 and 02, that most likely constitute at least part of the repressor-binding sites.

The extent of the deletions found in the mutants correlates well with the previously observed phenotypes: *vir6*, which lacks only 1 base-pair from site 02, is unable to multiply well in lysogens carrying more than one prophage; *vir3*, which has lost one of the repeats entirely, can multiply also in strains carrying two or three prophages; whereas, *vir24*, which has lost all of one repeat and part of the other, is the only one of the three that can plate on a host containing a multicopy pBR322 derivative into which an active phage P2 *C* gene has been cloned (18).

The relative binding of phage P2 repressor to sites 01 and 02 is uncertain. It has been reported that *vir3* DNA does not bind P2 repressor *in vitro*, even though it has an intact repeat (7). The phenotype of *vir3*, however, suggests that there must be some binding at high repressor concentrations.

Another consequence of the deletions in *vir3* and *vir24* is to reduce the length of the leader sequence for the *C* gene and also to remove the assumed ribosome binding site (Fig. 2). It is not known if this results in reduced translation of the *C* gene product in these mutants.

Regulation of the Early Functions. The first gene of the early operon, which is regulated by the repressor, is *cox*. It is located on the phage P2 map to the right of the operator re-

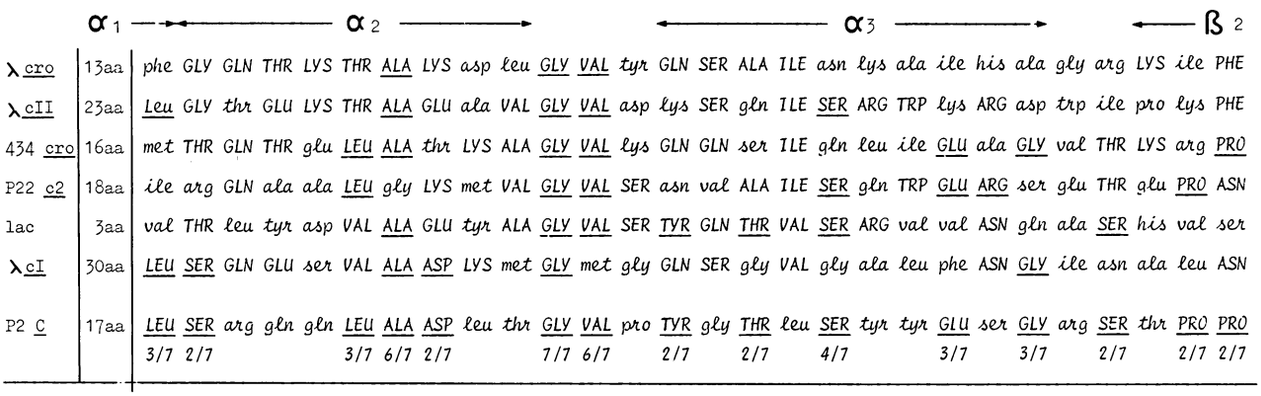

FIG. 3. Comparison of the NH_2-terminal amino acid sequence of phage P2 repressor with those of six other DNA-binding proteins, aligned as suggested by Matthews *et al.* (29). The residues that are common to two or more proteins are capitalized, and those that are common to P2 repressor and one or more of the other proteins are underlined. The designations α and β show the extensions of the α helices and the β-sheet strands of the phage λ cro protein.

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5' . . . T A T T G A G A T C T A A A C A C C A T . . . 01

5' . . . A A T C T A C A T C T A A A C T A A A T . . . 02

5' . . . A A T C G A C T T G T A A A C C A A A T . . . bio
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FIG. 4. Comparison of sequences found in the operator regions of phage P2 and the biotin operon (33). The repressor-binding sites 01 and 02 of P2 and one of the inverted repeats in the biotin operator are underlined.

gion and is transcribed left-to-right in relation to the map—i.e., in the opposite direction from the repressor gene. The sequencing of P2 DNA is being extended in the direction of *cox* (unpublished data). A possible promoter for the *cox* gene appears to span site 01. A repressor bound to 01 would then prevent the RNA polymerase from binding to the early promoter. Moreover, if this assignment for the early promoter were correct, it would imply overlapping of transcription of the early and repressor genes.

Sequence Homology with a Bacterial Operator Region. The repressor-binding sites 01 and 02 of phage P2 do not have any obvious homology with the right operator regions of the lambdoid phages (2). However, they do show some sequence homology with part of the operator region of the divergently transcribed biotin operon of *E. coli*. The biotin operator consists of two inverted repeats of 12 base-pairs each, separated by a stretch of 16 nucleotides (34). A comparison of P2 02 with one of the inverted repeats, including some of the DNA flanking these regions (Fig. 4), shows only four mismatches in a stretch of 20 nucleotides. Although it is unlikely that the P2 and biotin operator regions will prove to be functionally interchangeable, they might nevertheless be derived from some common ancestral sequence.

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