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Multiple repressor binding sites in the genome of bacteriophage P1

(repressor protein/operator sequence and mutation/DNA cloning)

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ABSTRACT **After digestion of bacteriophage P1 DNA with *Eco*RI in the presence of P1 repressor, 6 repressor binding sites were identified in 5 of 26 *Eco*RI fragments. Binding sites were localized by the decreased mobility of DNA fragment–repressor complexes during electrophoresis and by DNase protection ("footprinting") analysis. The repressor binding sites, or operators, comprise a 17-base-pair-long consensus sequence lacking symmetrical elements. Three operators can be related to known genes, whereas the function of the others is still unknown. The mutant P1 *bac*, rendering *ban* expression constitutive, is identified as an operator-constitutive mutation of the *ban* operon.**

P1 is a temperate bacteriophage with a genome size of about 90 kilobases (kb). In the prophage state the proviral DNA is maintained as a plasmid, and the vegetative P1 functions are repressed. Repression is accomplished by a phage-specific repressor, the product of the *c1* gene, which is located at the far right side of the P1 genetic map in *Eco*RI restriction fragment 7 (P1:7) (1, 2). Partially purified P1 repressor binds *in vitro* to at least two regions near *c1* within *Bam*HI fragment 9 that itself is located within P1:7 (3, 4).

The binding sites close to the *c1* gene, however, are not the only region at which the P1 repressor acts. The latter can also repress *in vivo* the expression of the P1 *ban* gene, which is located in P1:3 (5, 6). Furthermore, P1:14 also contains a promoter repressible by the product of *c1* (7). Together these results reveal that the P1 *c1* repression system must differ from that of other temperate phages such as λ, P2, and P22, in which only promoters adjacent to the repressor gene are repressed (7).

During our studies on the regulation of phage P1 *ban* expression we have localized by indirect methods a region 5′ upstream of the *ban* gene within P1:3 at which the P1 repressor acts (8). Highly purified P1 repressor protein binds to this region *in vitro* (H.S., unpublished data). Now in a systematic search for other repressor binding sites in the phage genome, *Eco*RI-digested P1 DNA is incubated with repressor, and binding regions are identified by the decreased mobility of *Eco*RI fragment–repressor complexes during electrophoresis (9, 10). Thus binding regions were detected in P1:7 and P1:14, as expected and, in addition, in P1:9 and P1:11. Moreover, the results of DNA sequence and DNase protection analyses reveal an asymmetric 17-base-pair (bp)-long consensus sequence for the P1 repressor binding site.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *Escherichia coli* K-12 strains used included the following: C600, HB101 (*recA13*) (11), NY58 (*dnaB107, recA56*) (12), JM101 (13), N100 (*galK, recA*) (14), DW101 (*sup*$^+$), and DW103 (*supD*) (15). For the cloning of P1 DNA fragments containing the *ban* operon (8) the recipient bacteria C600, HB101, NY58, and JM101 carried the plasmid pKT101-P1:7 harboring the P1 repressor gene (8). Strains DW101 and DW103 were transformed by plasmid pBR325-P1:11 for marker rescue tests (2).

Phage used were M13mp8/9 (16) and P1 *vir am13* (15). The P1 *Eco*RI fragments (in brackets) to be cloned derived from P1Cm*c1.100* (17) [P1:3], P1 *bac crr* (18) [P1:3*bac*, P1:7, P1:11], and P1 *crr* (18) [P1:9, P1:14]. P1:3Δ was originally derived from a λ–P1:3 hybrid phage (2), and the P1:3 portion of the hybrid DNA had been recloned in pBR325. Recloning was done by selecting the recombinant pBR325 plasmid in NY58 in the absence of P1 repressor (8). For the overproduction of P1 repressor, a P1 DNA subfragment of pBR325-P1:7 (8) and pBR325-P1:7*c1.100* (obtained from N. Sternberg, Du Pont) was inserted into the expression vector pPLc28 (19) and pJF118EH (20), respectively. Other plasmids used were pBR325 (21), pKT101 (22), and the *galK* promoter selection vector pFD51 (23).

Plasmid Constructions. Following *Eco*RI digestion of P1 DNA the fragments were separated by 0.7% agarose gel electrophoresis, eluted as described (24), and inserted into the single *Eco*RI site of pBR325, pKT101, or pFD51. Recombinant plasmids containing pBR325 were selected in strains NY58, HB101, or C600 at 30°C or 37°C as *amp*^{r}- + *cm*^{s}-transformants, and those containing pKT101 or pFD51 were selected as *km*^{r} transformants. After appropriate restriction enzyme treatment, subfragments of the plasmids containing the repressor binding site were either directly inserted into vector M13mp8/9, or first inserted into the polylinker region of pJF118EH, excised by *Eco*RI/*Hin*dIII, and then inserted into vector M13mp8/9. Handling of restriction enzymes and DNAs was as recommended by the manufacturers and in reference 25.

P1 Repressor. Highly purified repressor protein (1–2 mg/ml) was stored in buffer A [20 mM Tris·HCl, pH 7.6/50 mM NaCl/1 mM dithiothreitol/0.1 mM EDTA/10% (vol/vol) glycerol] plus 50% (vol/vol) glycerol at −20°C or −70°C. DNA–repressor interactions were studied by incubating DNA fragments and repressor protein in buffer A plus 100 μg of bovine serum albumin per ml for 15 min at 30°C.

DNA Hybridization, Sequencing, and Protection by DNase "Footprinting". pBR325-P1:11 and pFD51-P1:14 were nick-translated using [α-^{35}S]dATP. The labeled DNAs were hybridized to *Bam*HI- and *Bgl* II-treated P1 *crr* DNA following electrophoretic separation and Southern transfer of DNA fragments to nitrocellulose (25).

The complementary strand of M13mp8/9 recombinant DNAs containing P1 repressor binding regions were sequenced by the dideoxy chain-termination method (26) using the M13 17-mer primer (20) and [α-^{35}S]dATP.

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Abbreviations: P1 *Eco*RI DNA fragment 3, 7, etc. are abbreviated P1:3, P1:7, etc; in recombinant plasmids carrying P1 *Eco*RI restriction fragments the vector DNA name precedes the fragment term, for example pBR325-P1:3; ss, single stranded; ds, double stranded; Op, operator.

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For "footprinting" analysis the same primer was ^{32}P end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (25), and joined to the M13 recombinant DNA. The complementary strand was synthesized using Pol I Klenow fragment. DNase protection experiments with the M13 recombinant double-stranded (ds) DNA were done essentially as described by Johnson *et al.* (27). To 25-μl-reaction volumes containing $\leq$1 nM operator DNA various amounts of repressor protein (10–100 nM final concentration) were added. After incubation for 5 min at 30°C DNase was added to a concentration of 50 ng/ml, and the mixture was again incubated at 30°C for 5 min. The protocol then follows again the procedure described (27).

RESULTS

Isolation of P1 Repressor Protein. A *Pvu* II–*Bcl* I subfragment of P1:7 containing the *c1*$^+$ repressor gene (Fig. 2) was inserted into pPLc28. After heat induction a protein of about 33 kDa was overproduced and purified. Purification to near homogeneity (Fig. 1, lane a) was achieved by ammonium sulfate (0.39 g/ml of cellular crude extract) precipitation, and heparin-Sepharose-, DEAE-Sephacel-, and CM-Sepharose chromatography (in that order). The 33-kDa protein binds to and is eluted from heparin-Sepharose and CM-Sepharose, but it does not bind to DEAE-Sephacel. The purified protein specifically binds to the *Bam*HI subfragment 9 of P1:7 as will be shown below. The same fragment is bound to nitrocellulose by a partially purified P1 repressor (3). A molecular mass of 33 kDa for the c1 repressor was indicated by others (29). Therefore, we consider the purified 33-kDa protein to be the product of the P1 *c1*$^+$ gene. Similarly a *Bal* I–*Bcl* I subfragment within P1:7*c1.100* was inserted into the *tac* expression vector pJF118EH. [The *Bal* I site is located 54 and 25 bp away from the *Bam*HI site and the beginning of the *c1* gene, respectively (Fig. 2).] By isopropyl β-D-thiogalactoside induction a 33-kDa protein was overproduced, purified, and shown to bind also to *Bam*HI fragment 9 (Fig. 1, lane c). After *in vivo* induction of c1$^+$ repressor, prolonged incubation of crude cellular extract at low temperature causes proteolysis of the repressor protein. A primary degradation product is a

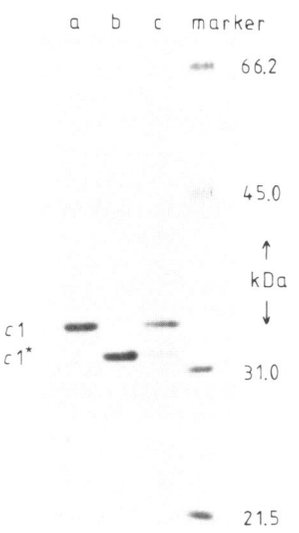

FIG. 1. NaDodSO$_4$/15% PAGE of P1 repressor protein. Samples of highly purified P1 repressor protein ($\approx$5 μg each) are subjected to gel electrophoresis (28). (Lane a) c1$^+$ wild-type repressor, (lane b) c1*, a proteolytic degradation product of c1$^+$, and (lane c) c1.100 repressor. Marker proteins are in descending order of molecular mass: bovine serum albumin, ovalbumin, carbonic anhydrase, and (soybean) trypsin inhibitor.

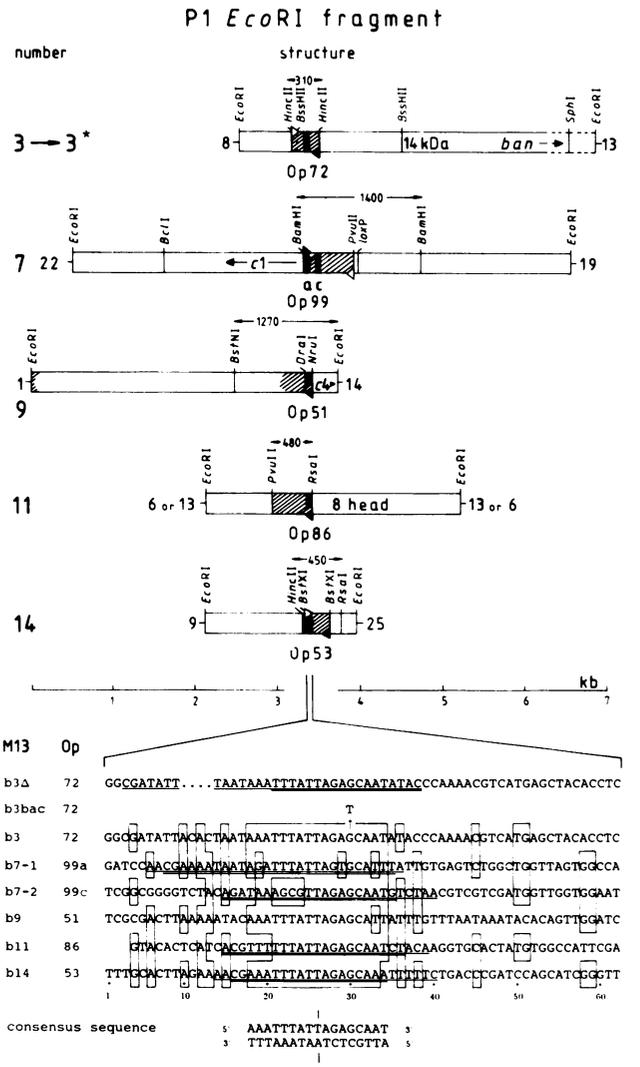

FIG. 2. P1 repressor binding sites in the genome of P1. (*Upper*): P1 *Eco*RI fragments that were inserted into the single *Eco*RI site of pBR325 are shown. P1:3 was further reduced in size by *Sph* I treatment and is called P1:3*; it contains an unknown gene coding for a 14-kDa protein and the *ban* gene in that order (8). The orientation of the diagrammed *Eco*RI fragments follows clockwise the genetic map of P1, and neighboring *Eco*RI fragments are numbered to the left and right (except for P1:11, the orientation of which is still undetermined). Each repressor binding site or operator (Op) sequence is assigned the integral number portion of its map position; letters a and c indicate the existence of more than one operator within a map unit (30, 31). The *Eco*RI fragments are aligned so that (with the exception of Op99c) operators are within a vertical black line comprising 62 bp. Only those genes, restriction enzyme sites, and other sites relevant for the repressor binding studies and the characterization of the fragments are shown. Subfragments to which repressor binds (see Fig. 3) are indicated by horizontal arrows between two restriction enzyme sites—the number referring to their size in bp. Subfragments that were cloned into M13mp8/9 are indicated by hatched areas between two such sites. The subfragment of P1:9 is a deletion derivative retaining the left *Eco*RI- and the *Nru* I site close to *c4*. Strands that have been sequenced are marked by white or black triangles, and the sequence shown in Fig. 2 (*Lower*) is the Op site following the black triangle. (*Lower*): Operator sequences. The M13 recombinant DNAs are named b3, b7, etc., according to their origin from P1:3, P1:7, etc. DNase protection experiments were done with b3Δ, b7, b11, and b14, and the sequences protected by repressor are underlined (with single line extensions where protection is not definite). Areas are boxed when 4–6 (67 to 100%) of the nucleotides at a given position are identical. Numbers 1–62 are arbitrary.

31-kDa protein, which we call c1* and which was also purified (Fig. 1, lane b). This protein also binds specifically to P1

*Bam*HI fragment 9, but the native, multimer form of the c1* molecule was found to be more labile than that of the c1+ protein. In all following experiments only the latter protein was used if not otherwise noted. The cloning, purification, and properties of the P1 repressor will be described in detail elsewhere (unpublished work).

Localization of Repressor Binding Regions in the P1 Genome. A 1.3-kb *Bss*HII subfragment of P1:3 that is 1.3–2.6 kb 5′ upstream of the beginning of the *ban* gene (Fig. 2) was identified as the target for the P1 repressor by *in vivo* experiments (8). Purified repressor binds to this subfragment *in vitro*, as was revealed by a decreased mobility of the subfragment–repressor complex during gel electrophoresis. To search for other repressor binding sites, we dissected the P1 genome with *Eco*RI because most of the recent studies on P1 phage functions had been done with cloned *Eco*RI fragments (2, 7, 32). In preliminary experiments we tested the sensitivity of the method; whereas a decrease in the mobility of P1:3 (9.5 kb) could not be detected, the mobility of an *Eco*RI–*Sph* I subfragment of P1:3 (5.3 kb) was visibly reduced with repressor. The *Eco*RI digest of P1 DNA (≈90 kb) yields 26 bands from 15 to 0.05 kb in size (33). When P1 DNA was digested with *Eco*RI in the presence of repressor, complex formation with P1:7 (6.0 kb), P1:9 (3.7 kb), P1:11 (3.1 kb), and P1:14 (1.8 kb) was revealed by the slower movement of only these fragments during electrophoresis. The fragments were cloned in pBR325, the recombinant DNAs were isolated, and repressor binding to the P1 *Eco*RI fragments again were confirmed after *Eco*RI digestion. The repressor binding regions were then delimited in tracing the subfragment–repressor complexes after appropriate restriction enzyme treatment of the recombinant DNAs. Finally, the binding regions were cloned in M13mp8/9, and the repressor binding sites were pinpointed by DNase protection experiments and DNA sequence analysis. This is described in detail for each restriction fragment-bearing plasmid in the following sections.

pBR325-P1:3*. P1:3 is characterized by its ability to express *ban* (2, 5, 8). Plasmid pBR325-P1:3* is obtained from pBR325-P1:3 by *Sph* I treatment and religation. The truncated plasmid retains 55% of P1:3 containing the *ban* gene (Fig. 2) and 70% of the vector DNA (8). In both plasmids, pBR325-P1:3 and -P1:3*, *ban* expression is brought under the control of P1 c1+ expressed from a separate plasmid, pKT101-P1:7, in the same cell (8). pBR325-P1:3* DNA was treated with *Hin*cII and electrophoresed with and without repressor. Five fragments are obtained of which the mobility of a 310-bp fragment located about 2.4 kb 5′ upstream of the *ban* gene (Fig. 2) is reduced with repressor (Fig. 3). The migration of a second 1510-bp *Hin*cII fragment containing the P1-pBR325 DNA junction 5′ upstream of the *ban* gene is also slowed (Fig. 3). This fragment was isolated, digested with *Eco*RI, and incubated with and without repressor. Neither the vector nor P1 DNA bound repressor (data not shown).

When pBR325-P1:3**bac* and pBR325-P1:3*Δ were treated with *Hin*cII and electrophoresed with and without repressor, the mobility of the 310-bp *Hin*cII fragment from both plasmids was likewise reduced with repressor (data not shown). The former plasmid corresponds to subfragment P1:3* but carries the *bac* mutation. It confers thermoresistance to an *E. coli dnaBts* strain due to the constitutive expression of *ban* (2, 8). In this respect it mimicks the property of the P1 *bac* prophage (34). P1:3*Δ also corresponds to P1:3* but was derived from a λ-P1:3 hybrid phage and was the only P1:3 derivative that had been cloned in the absence of P1 repressor; the significance of this detail will become apparent later.

pBR325-P1:7. Bacteria harboring pBR325-P1:7 (with P1:7 in either of two orientations inserted into pBR325) express P1 repressor. These bacteria can be lysogenized by P1Cm*c1.100* at 40°C (8). Following a *Bam*HI/*Eco*RI digestion of pBR325-P1:7, five fragments are obtained, of which the 1400-bp *Bam*HI fragment 9 binds repressor (Fig. 3).

pBR325-P1:9. P1:9 carries the genes of *c4* and *sim* (35, 36), and the recombinant plasmid was characterized by its ability to confer superimmunity to its bacterial host (35). Binding of repressor to P1:9 was first discovered by B. Baumstark (personal communication). When pBR325-P1:9 is digested with *Bst*NI/*Eco*RI, five P1 subfragments appear, of which the largest one binds repressor (Figs. 2 and 3). The binding site was further localized to a 77-bp *Dra* I–*Nru* I fragment (Fig. 2), the mobility of which is reduced by repressor (data not shown).

pBR325-P1:11. P1:11 carries gene 8, which is involved in head formation (15). Its DNA was characterized by hybridization to P1 *Bam*HI fragment 2 and by the ability of strain DW101(pBR325-P1:11) to rescue the *am*+ allele of P1 *vir am13* (2). No c1 repressor-controlled function has yet been identified in P1:11. Also no evidence for a promoter that directed transcription toward the neighboring P1:6 or P1:13 was seen when P1:11 was fused to pFD51 in either orientation. After digestion of pBR325-P1:11 with *Pvu* II/*Rsa* I,

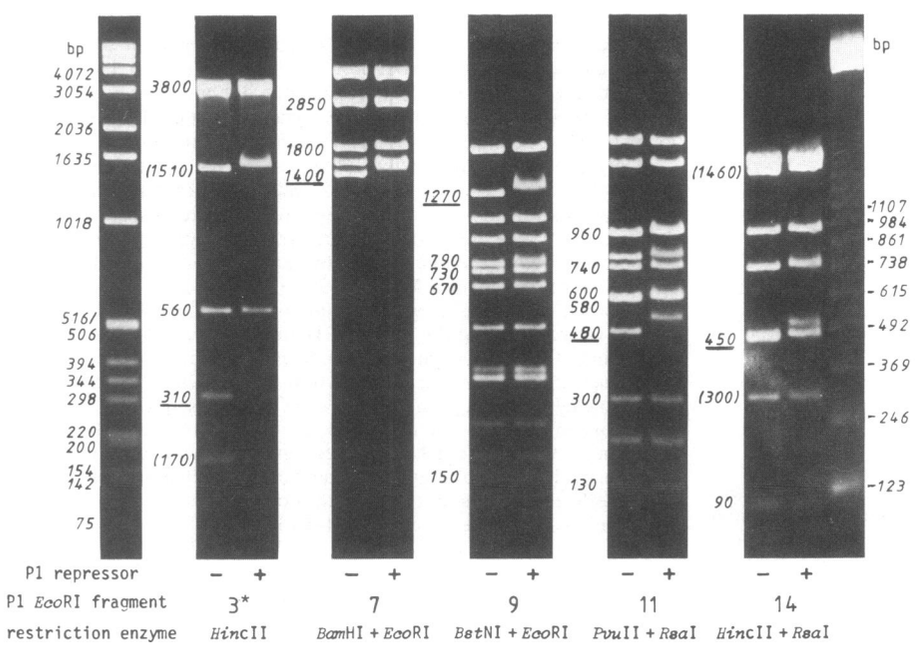

FIG. 3. Decreased mobility of P1 DNA fragment–repressor complexes. Plasmid DNA was treated with the restriction enzyme(s) indicated and subjected to 2% agarose gel electrophoresis after incubation with and without repressor as described. Only those subfragments that derive from P1 DNA and P1-pBR325 DNA junctions (in parentheses) are marked by size (in bp). Underlined numbers correspond to those fragments the mobility of which is reduced by repressor. The 1-kb (left lane) and 123-bp ladders (right lane) served as markers.

binding of repressor to a 480-bp fragment is seen, which was located centrally within P1:11 (Figs. 2 and 3).

pBR325-P1:14. P1:14 was characterized by hybridization to P1 *Bgl*II fragment 3. It contains a repressible promoter originally discovered by Sternberg (cited in reference 7), which is demonstrated as follows. Strain N100 (pFD51-P1:14) expresses galactokinase and yields red colonies on MacConkey–galactose plates if the *galK* gene had been fused to P1:14 at the P1:14–P1:25 junction (Fig. 2). When plasmid pKT101-P1:7 was introduced into such cells, repression of the promoter was shown by the appearance of white colonies. When pBR325-P1:14 is digested by *Hin*cII/*Rsa* I, binding of repressor to a 450-bp fragment is seen (Figs. 2 and 3).

A Consensus Sequence for P1 Repressor Binding Sites. Subfragments of P1:3, P1:7, P1:9, P1:11, and P1:14, as indicated by the hatched areas in Fig. 2, were cloned into vectors M13mp8 and/or M13mp9, and the single-stranded (ss) and ds M13 recombinant DNAs were isolated. The DNAs were named M13 b3, -b7, -b9, -b11, and -b14 according to their origin from P1:3, P1:7, etc. The presence of a repressor binding site was once more verified by the decreased mobility of the fragment–repressor complexes described above after excision of the P1 DNA subfragment from the M13 recombinant DNAs. Subsequently all subfragments were sequenced as indicated in Fig. 2, and the M13 ds DNA of b3Δ, b7, b11, and b14 were analyzed by DNase protection experiments. A repressor binding site is designated as an operator (Op) in accordance with suggested nomenclature (30, 31), although, with one exception, we do not yet have direct evidence whether the binding sites are true operators. [The one exception is the operator of the *ban* operon (8), Op72, which will be discussed.]

When M13 ds DNA b7 was incubated with increasing amounts of $c1^+$ repressor, two repressor binding sites, Op99a and Op99c, are easily detectable (Fig. 4). Because both are located within *Bam*HI fragment 9, they had escaped separate detection by gel retardation analysis (Fig. 3). Two additional repressor binding sites are indicated, presumably Op99d and Op99e (Fig. 4). Incubation of M13 ds DNA b11 with $c1^+$ repressor and of M13 ds DNA b3Δ with $c1^+$ or c1.100 repressor revealed the repressor binding sites Op86 and Op72, respectively (Fig. 5). The sequence data and repressor-protected regions are summarized in Fig. 2 (*Lower*). The operators contain a 17-bp A+T-rich consensus sequence of strong homology that is flanked by regions of less homology. The repressor protein not only covers the consensus sequence, but also protects sequences to the left and right differently. In the operator Op72 the sequence of b3*bac* differs from b3 (wild type) by only one-base-pair exchange, thus classifying P1 *bac* as an operator-constitutive mutation. In b3Δ, which derives from P1:3*Δ and still binds repressor, four base pairs adjacent to Op72 are deleted.

DISCUSSION

We have identified six repressor binding sites or operators in five P1 *Eco*RI fragments (Fig. 2). Op51 and Op53 were found independently by Baumstark *et al.* (4), and J. L. Eliason and N. Sternberg (Du Pont) (personal communication), respectively. Analysis of the *ref* gene (37) revealed two other putative binding sites, Op2a and b. Moreover, it became apparent that the *Bam*HI–*Pvu* II fragment in front of the *c1* gene (Fig. 2) contains a cluster of binding sites (Op99a to e). Op99a, d, and e were identified in using a synthetic palindromic oligonucleotide as an effective competitor for repressor binding *in vitro* (4), Op99a to e were identified by filter binding experiments, and Op99d and e were identified by DNase footprinting (Eliason and Sternberg, personal communication). The sequence of the *Bam*HI–*Pvu* II fragment has been determined (ref. 4; Eliason and Sternberg, personal communication; M.V. and H.S., unpublished data).

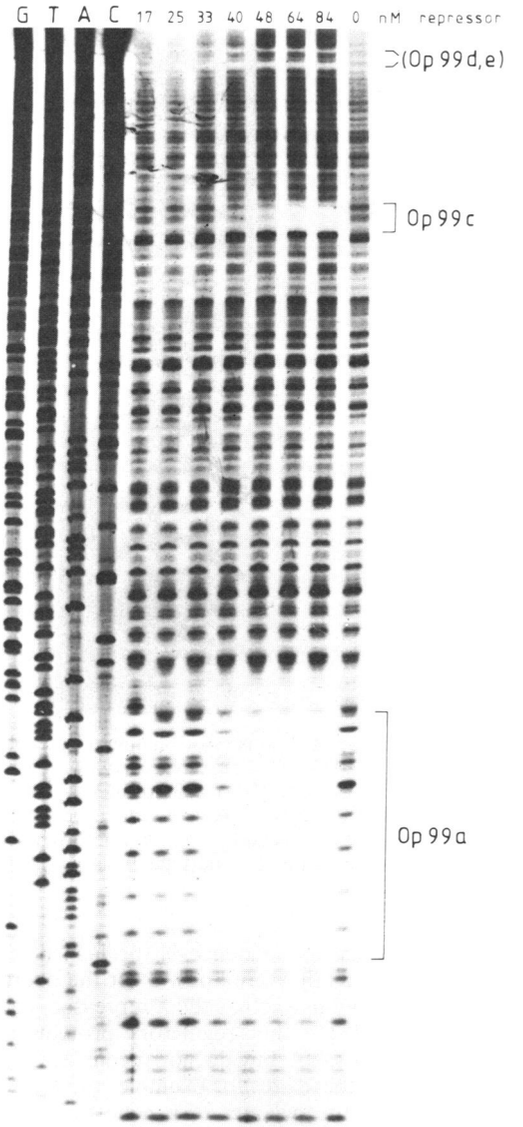

FIG. 4. Visualization of P1 repressor binding to operators 5′ upstream of the P1 *c1* gene. (Lanes 1 to 4) To the M13mp8 b7 ss DNA carrying the *Bam*HI–*Pvu* II fragment of P1:7 (Fig. 2) the (unlabeled) primer was joined, and the sequence of the complementary strand was determined by the dideoxy chain-termination method (26). Probes containing ddGTP, ddTTP, ddATP, and ddCTP are marked G, T, A, and C, respectively. (Lanes 5 to 12) To the M13mp8 b7 ss DNA the (5′ end-labeled) primer was joined, the complementary strand was synthesized, and the DNA was treated with DNase I in the presence of various amounts of repressor as described. Products were visualized by autoradiography after electrophoresis through a 6% polyacrylamide gel. The regions of the gel displaying fragments from cleavage within operators are named Op99a and Op99c, respectively. For the presumptive location of Op99d and Op99e see the *Discussion*.

Therefore the relative positions of the operators to one another are known, and the distances between Op99a, c, d, and e observed in our footprinting analysis (Fig. 3, and data not shown) agree with our data from sequence analysis and with that of others (see above). However, a fifth possible binding site, Op99b, which should overlap with Op99a (Eliason and Sternberg, personal communication), was not detectable by DNase footprinting (Fig. 4).

The efforts of several laboratories have identified a total of 11 operators with a common 17-bp-consensus sequence (Fig. 2) (31). Inverted or direct repeat structures are located within or overlapping the Op sequences. However, the striking

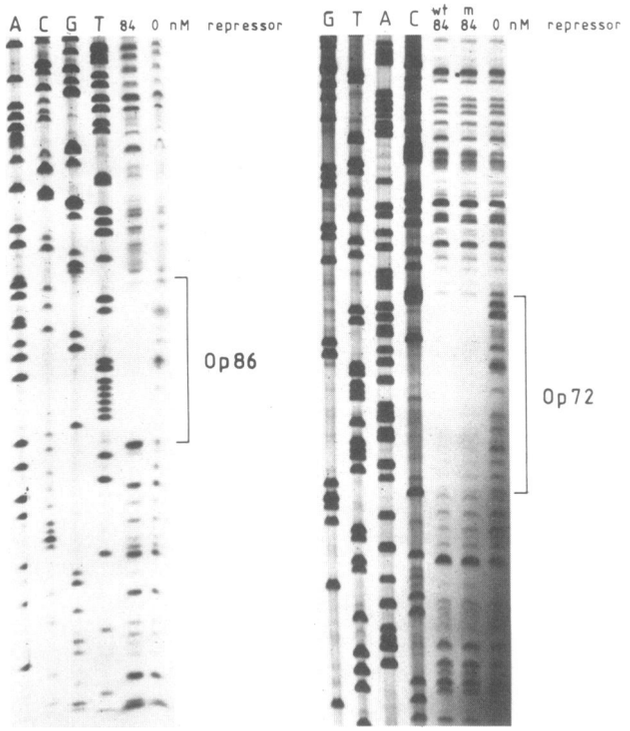

FIG. 5. Visualization of P1 repressor binding to the operator in P1:3Δ and P1:11. (*Left*) M13mp8 b11 ss DNA carrying the *Pvu* II–*Rsa* I fragment of P:11 (Fig. 2), and (*Right*) M13mp9 b3Δ ss DNA carrying the *Hin*cII fragment of P1:3Δ (Fig. 2) were sequenced and analyzed by DNase footprinting (see legend for Fig. 4). Footprinting analysis of M13mp9 b3Δ was done with $c1^+$ wild-type (wt) and c1.100 mutant (m) repressor.

feature of the consensus sequence is the lack of common 2-fold related sites, as they are known, for the operators of other regulatory proteins such as the λ repressor, λ cro, and the CAP protein (38). Other regulatory proteins are also known that bind to DNA but do not recognize symmetric sequences. Among these are, for example, the λ cII protein (39). Furthermore, the product of a gene involved in the regulation of lysogeny in the temperate *B. subtilis* phage φ105 was shown to interact with a 14-bp-long asymmetric operator site (P. Dhaese, personal communication).

The asymmetry of the Op sequences suggests a directionality of the repressor-controlled promoter. This assumption is supported by the facts that transcription of *ban* in P1:3, *c1* in P1:7, and that toward P1:25 in P1:14 always runs from right-to-left on the template DNA shown in Fig. 2 (*Lower*). If this also occurs for Op51 in P1:9, then transcription starting at Op51 would be directed toward the *c4* gene (Fig. 2).

The operator Op72 of the *ban* operon (8) was studied in more detail. In this situation an RNA polymerase binding site overlaps with the repressor binding site as revealed by the observations that (*i*) preincubation of an Op72-containing DNA fragment with repressor inhibits *in vitro* the binding of RNA polymerase to that site and (*ii*) P1:3*Δ containing a 4-bp deletion close to Op72 (Fig. 2) has lost the ability to bind RNA polymerase. Presumably, the promoter has thus become inactive and allowed selection of the P1:3Δ containing recombinant plasmid in the absence of P1 repressor (8). Classification of P1 *bac* as an operator-constitutive mutation appears justified for the following reasons. In comparison with P1:3* more repressor is needed for P1:3**bac*—firstly, to inhibit binding of RNA polymerase to Op72 and, secondly, to completely retard the Op72-containing *Hin*cII fragment during electrophoresis (Fig. 2) (R. Lurz, A. Heisig, M.V., R. Dobrinski, and H.S., unpublished data).

About half the P1 genome has thus far been analyzed for repressor binding sites by the methods described here. Regardless of whether additional binding sites are discovered, the scattering of such sites over the genome indicates the existence of independent repressible blocks of genes in P1, which is in contrast to the clustering of such genes in other temperate phages (31).

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