The c1 Repressor of Bacteriophage P1

ISOLATION AND CHARACTERIZATION OF THE REPRESSOR PROTEIN*

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The c1 repressor gene of bacteriophage P1 is located on P1 DNA EcoRI fragment 7 (Sternberg, N. (1979) Virology 96, 129–142). Subfragments of P1 DNA EcoRI fragment 7 were cloned into expression vectors, and the c1 repressor protein from P1 wild-type phage and a revertant of a temperature-sensitive repressor mutant were overproduced in Escherichia coli and purified to near-homogeneity. The decreased electrophoretic mobility of P1 DNA BamHI fragment 9 in the presence of appropriate protein fractions was used as an assay for the repressor protein.

Highly purified repressor migrates as a single polypeptide on denaturing sodium dodecyl sulfate-polyacrylamide gels, corresponding to a molecular weight of about 33,000. A molecular weight of about 63,000 for the native repressor molecule was calculated from determinations of the sedimentation coefficient, which was 2.6 s, and the Stokes radius, which was 55 Å. Cross-linking the protein with glutaraldehyde yielded two bands. These data and a high frictional coefficient (2.1) suggest that the native repressor exists in solution as an asymmetric dimer molecule.

P1 is a temperate phage which, in the prophage state, is maintained as a plasmid with the vegetative functions being repressed. Originally, it was suggested that the c1 gene of P1 codes for a repressor that prevents the expression of lytic functions indirectly, possibly by repressing an operator whose product(s) is required for the transcription of genes required for phage production (1). The c1 gene is located at the far right side of the P1 genetic map in EcoRI:7 (2, 3). Partially purified P1 repressor protein binds in vitro to at least two regions near c1 within BamHI:9, which itself is located within EcoRI:7 (1, 4). This protein was found to be absent from nonlysogenic bacteria infected with a P1 c1 amber mutant. Furthermore, the binding activity of a protein which derived from a c1 temperature-sensitive mutant was found to be thermolabile in vitro (1). These findings identify the repressor protein as the product of the c1 gene.

Additional repressor-binding sites have been found in the meantime (5, 6). We have previously localized a region 5' upstream of the P1 ban gene at which the repressor acts using indirect methods (7). This result confirmed earlier findings (5). In order to localize this binding site more precisely by in vitro studies, we have cloned the c1 gene of P1 wild-type phage and of a revertant of a c1 temperature-sensitive mutant in expression vectors. The repressor protein was overproduced, purified to near-homogeneity, and characterized. These procedures as well as the characterization of the repressor protein are described in this paper. In previous analyses (8), we have already used this purified repressor to look systematically for repressor-binding sites in P1 DNA. Our results and results obtained by others (9, 10) allowed the identification of seven regions widely scattered throughout the P1 genome which interact with the repressor. Multiple repressor-binding sites characteristic of P1 indicate that its repression system differs considerably from that of other temperate phages, in which only promoters adjacent to the repressor gene are repressed (10).

EXPERIMENTAL PROCEDURES†

RESULTS

Strategy of Cloning the c1 Repressor Gene

Recombinant plasmid DNAs being composed of EcoRI:7 of P1 wild-type or P1 repressor mutant strains and pBR325 DNA were used as starting material for subcloning the c1 gene (Fig. 1). Subcloning was performed with the intention: (i) to insert the c1 gene into inducible expression vectors in order to optimize the expression of the c1 gene (for that purpose, vectors pPLc28 (under the control of the heat-inducible λcl857 repressor) and pJP115EH (under the control of the lac repressor inducible by IPTG) were used); and (ii) to study the effect of the control region Op99 (9, 25) on the expression of the c1 gene. For that purpose, this region was either retained in the plasmid (pBD2 and pMV1, Fig. 1) or deleted from it (pBD3, pMV2, and pMV2-B, Fig. 1). The subcloning procedure is described in the legend to Fig. 1.

The presence of P1 repressor was tested by the ability of the c1 gene-containing recombinant plasmid to promote lysogenization at 40 °C of the bacterial cell by the phage P1Cmbac c1-1.00. (The presence of the bac mutation was a prerequisite to measure lysogenization of Escherichia coli dnaB mutants.) By that means, it was verified that plasmid pHS7-100-B, as expected, contains a c1 mutant gene coding for a thermolabile repressor (Table 2). In the same way, it

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§ The abbreviations used are: EcoRI:7, for example, P1 DNA EcoRI fragment 7; IPTG, isopropyl-β-D-thiogalactopyranoside; bp, base pairs; kb, kilobase; RF, replicative form; Op, operator; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

18 U.S.C. Section 1734
was discovered that, in plasmid pHS7-100, the same cl mutant had undergone an (as yet unknown) alteration which had reversed the thermostable character (Table 2).

The amount of repressor protein in crude cellular extracts was roughly estimated by 15% SDS-PAGE and staining of the gel with Coomassie Blue. It was found that the ptac-promoted expression of cl had undergone an (as yet unknown) alteration which had reversed the thermostable character (Table 2).

When cells of C600 (pBD2, pC1857) are induced by heat treatment, a polypeptide with molecular weight of 33,000 is overproduced as shown by electrophoresis in a denaturing gel (Fig. 2). The same is true, when HB101 (pMV2) and C600 (pMV2-B) are induced by IPTG (data not shown). Purification of Repressor

When cells of C600 (pBD2, pC1857) are induced by heat treatment, a polypeptide with molecular weight of 33,000 is overproduced as shown by electrophoresis in a denaturing gel (Fig. 2). The same is true, when HB101 (pMV2) and C600 (pMV2-B) are induced by IPTG (data not shown). Purification of this protein, which was considered to be the cl repressor, was monitored in the initial steps by tracing the overproduced polypeptide electrophoretically and later on by complex formation with BamHI:9 (1, 4). As ε typical result, about 5 mg of repressor (Fraction V) was obtained from 17.5 g of C600 (pBD2, pC1857) wet cell paste. Based on the total number of bacteria, this amount corresponds to about 18,000 native repressor protein molecules/cell.

The most effective purification is achieved by heparin-Sepharose chromatography. More than 90% of the proteins of Fraction II are removed by this step. Typical of a DNA-binding protein, the repressor protein is eluted from the column by high salt. In crude extracts, the 33-kDa protein of pBD2 (wild-type repressor) and of pMV2 (mutant repressor) is not stable and is slowly converted to a 31-kDa protein. As an example, this is shown in a heparin-Sepharose elution profile of a pMV2 mutant repressor extract. Elution of a 31-kDa protein precedes and overlaps the elution of the 33-kDa protein (Fig. 3).

That the 31-kDa protein is a proteolytic degradation product of the 33-kDa protein is shown in two ways. (i) A prolonged incubation at 0 °C of crude extracts and/or a prolonged dialysis of Fraction I from induced cells of C600 (pBD2, pC1857) results in complete conversion of the larger to the smaller protein. The latter is gradually degraded further and finally disappears completely. (ii) Crude fractions of both the 33- and 31-kDa proteins form a complex with BamHI:9 equally well, as can be seen by a comparison of Figs. 3 and 4. Titration of the repressor-containing fraction 79 indicates that the minimal amount sufficient to retard BamHI:9 is 0.1 μl. The same amount of fraction 74 is equally efficient even though the amount of the 33-kDa protein is about 10-fold lower in fraction 74 compared to fraction 79. Furthermore, highly purified, equivalent fractions of the 31- and 33-kDa proteins bind equally well to BamHI:9 (data not shown).

The lability of the repressor in crude extracts is most pronounced with the repressor protein from pMV2-B. The protein can be overproduced by IPTG induction. But neither
Protein binds to BarnHI:9 only at low temperature.5

Type repressor protein (Fraction V). The 33- or 31-kDa polypeptide band (Fig. 3) nor a BarnHI-binding activity can be recovered from heparin-Sepharose if protein is quickly degraded in cellular crude extracts. How-

**Fig. 2.** Induction and purification of repressor. A cellular crude extract and purified fractions of C600 (pBD2, pCH57) were subjected to 15% SDS-PAGE and stained with Coomassie Blue. Lane a, 30 μl of crude extract, uninduced culture; lane b, 30 μl of crude extract, induced culture; lane c, 10 μl of Fraction II; lane d, 40 μl of Fraction III; lane e, 5 μl of Fraction IV; lane f, 2.5 μl of Fraction V. Mobilities are calculated relative to a standard of bromphenol blue protein to BarnHI:9 had already been observed by retention of the DNA-repressor complex on nitrocellulose filters (1, 4).

As can be seen in Fig. 5, wild-type and pMV2 mutant repressor proteins (Fraction V each) bind to BamHI:9 specifically and about equally well at 47°C. Preincubation of the repressor protein for 1 min at 63°C only marginally reduces the DNA binding of the mutant repressor compared to the wild-type protein. Preincubation at 58°C abolishes the DNA-binding ability of both repressor proteins. The fact that the temperature dependence of DNA binding of mutant and wild-type repressors is nearly identical is in accordance with the finding that bacteria containing pMV2 or wild-type repressor cannot be lysogenized at 40°C by P1Cmbac c1-100 with about equal efficiency as at 30°C (Table 2). Thus, both the in vitro and in vivo data support the assumption that pMV2 contains the c1 gene of a temperature-resistant revertant of P1 c1-100. On the other hand, bacteria containing pMV2-B repressor can be lysogenized at 40°C (Table 2).

**Other P1 Operators**—In a systematic search for repressor-binding sites in the genome of P1 using the electrophoretic retardation assay of DNA-repressor complexes, six such sites have previously been found (8). Fragments containing these V each, 1–2 mg/ml) are at least 90% pure. The preparations are stable for at least 1 year, if kept at –70°C. Repeated thawing and freezing do not affect their ability to form a complex with BarnHI:9, but results in the gradual conversion of the 33-kDa to the 31-kDa protein.

**DNA-Repressor Complex Formation**

P1 c1 Operator—The specificity of complex formation of the 33-kDa protein with P1 DNA is a characteristic feature and gives further evidence of the identity of this protein with the repressor. In vitro binding of a partially purified repressor protein to BarnHI:9 had already been observed by retention of the DNA-repressor complex on nitrocellulose filters (1, 4).

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3 The 33- or 31-kDa polypeptide band (Fig. 3) nor a BarnHI9-binding activity can be recovered from heparin-Sepharose if the protocol described under "Experimental Procedures" is followed. Apparently, the thermolabile pMV2-B repressor protein is quickly degraded in cellular crude extracts. However, if freshly prepared Fraction I is directly mixed with heparin-Sepharose, an overproduced 35-kDa protein is adsorbed to and can be eluted from the solid material. This protein binds to BarnHI:9 only at low temperature.6

Wild-type and pMV2 mutant repressor proteins (Fraction

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6 J. Heinrich, unpublished data.
sites had been cloned in M13mp8/9 and M18 (see "Experimental Procedures," Table 1, and Ref. 8). Complex formation with wild-type repressor protein was tested here again by excising the P1-specific DNA fragment from the recombinant M13 RF DNAs and incubating these DNA probes with repressor.

As shown in Fig. 6, the repressor binds to: (i) a 310-bp HincII fragment of the recombinant M13 RF DNA b3 (this fragment is located 5' upstream of the ban gene in EcoRI3 (7)); (ii) a 600-bp PvuII-BamHI fragment of b7, which is located in front of the c1 gene (Fig. 1) (binding of repressor to this fragment had been observed before (4)); (iii) a 500-bp Rsal-PvuII fragment of b11 (this fragment is contained in EcoRI:11 in the neighborhood of a P1 head gene (26)); and (iv) a 320-bp BstXI fragment of b14. This fragment is part of EcoRI:14. Furthermore, the repressor also binds to EcoRI:9 (8), as was discovered originally by Baumstark. Repressor-controlled gene functions have been found located in or starting from the P1 DNA part of b5 (6, 7), b7 (4), and b14 (6). The controllable P1 functions in b9 and b11 are not known yet.

Physical Properties of P1 Repressor

Gel Electrophoresis—The single band of highly purified wild-type repressor observed on denaturing 15% SDS-PAGE has a molecular weight of 33,000 (Fig. 2), and the same value was found for the pMV2 mutant repressor (data not shown). A molecular weight of 33,000 for the c1 repressor has already been indicated by others (27).

Gel Filtration—The apparent molecular weight of the native wild-type repressor was determined by analytical Sephacryl S-200 chromatography. A comparison of the elution volume of the repressor with those of reference proteins indicated an apparent molecular weight of the repressor of about 230,000 (Fig. 7a). The Stokes radius of the repressor was estimated to be about 55 Å (Fig. 7b).

Glycerol Gradient Centrifugation—Sedimentation of the repressor protein in a 15-35% glycerol gradient leads to a sedimentation coefficient of 2.6 s based on comparison to sedimentation properties of standard proteins (Fig. 8). The low s value indicates a much lower molecular weight than that derived from gel filtration analysis.

Apparent Partial Specific Volume—A value of 0.74 ml/g was calculated from the amino acid composition (28), which in turn was deduced from the DNA sequence of the c1 gene. Molecular Weight and Frictional Coefficient—A molecular weight of 63,000 for the native repressor protein was calculated from the equation: $M_w = 6\pi n N_a / (1 - \nu P)$. The frictional coefficient ($ff_0$) was calculated to be 2.1 from the equation: $ff_0 = a(3\pi M_w / 4\pi N)^{1/3}$. In these equations, $a = 0.4$ Stokes radius, $s$ = sedimentation coefficient, $\nu = $ partial specific volume, $n = $ viscosity of medium (0.01002 poise), $P = $ density of medium (1.00), and $N = $ Avogadro's number (23).

These data suggest that the native repressor protein ($M_w = 63,000$) is composed of two subunits ($M_w = 33,000$ each). The
high frictional coefficient indicates that the repressor molecule is asymmetric (25).

**Cross-linking Reaction**—Support for the existence of a dimeric repressor molecule comes from cross-linking studies. Treatment of the repressor protein with glutaraldehyde slowly converts the repressor monomer to a product which has a molecular weight of about 66,000 (Fig. 9). The diffuse nature of the dimer and monomer molecules most probably is due to combinations and mixtures of reacted and unreacted monomers as well as to the presence of a small amount of degradation products of the repressor.

**DISCUSSION**

Cloning of the P1 c1 repressor gene, overproduction, and purification of the c1 gene product yielded a protein with a molecular weight of 33,000 under denaturing conditions. Both the molecular weight determination of the native molecule and the result of cross-linking experiments indicate that the repressor exists as a dimer in solution. Moreover, the high frictional coefficient suggests that the shape of the molecule is asymmetric.

Cloning of the repressor gene was traced by an *in vivo* assay for the presence of a functionally active repressor. The resolution of a temperature-sensitive repressor mutation shown here proves the requirement for a reliable test to follow the results of the cloning procedures. Analysis of the gene product guarantees that the subcloning procedure itself does not alter the gene which is being investigated. In this connection, it is worth mentioning that the *Bal*—*Bcl* subfragment from BamHI:7*" (Fig. 1) used for the induction of PMV2 and PMV2-B repressors must contain the intact c1 reading frame for the following reasons. Cloning the P1 c1 region by similar methods had indicated that the c1 reading frame is contained within BamHI:7* (4). In addition, the c1 region had been sequenced, and an open reading frame coding for a protein of 283 amino acids has been found that starts 26 bp downstream of the *Bal* site. Moreover, deletion analysis establishes the starting point of repressor translation at that site. In contrast, c1 repressor is not expressed by cloned BamHI:7* unless a heterologous promoter (ptac) is fused to the 5′ end of that fragment (4). This indicates that at least part of the c1 promoter is located on BamHI:9 (Fig. 1). The ability of plasmids pBD3 and PMV2 to promote lysisogenization by a P1Cmbac c1-100 phage (Table 2) must therefore be due to the leakiness of the ptac promoter.

Purification of enriched repressor protein fractions was monitored by complex formation of the repressor with a P1Cmbac c1-100 phage (Table 2) must therefore be due to the leakiness of the ptac promoter. The mode of interaction of the repressor protein with its operator is not yet understood. The most convincing evidence that binding of the repressor to an operator site affects gene expression comes from studies with the operator of the *ban* operon, Op72, the sequence of which matches the consensus sequence. First, the *in vitro* binding of RNA polymerase to the Op72 region is inhibited by the repressor. Second, the prophage mutant P1bac, which constitutively expresses *ban* in vivo (30), was found to be an Op72 operator constitutive mutation. Furthermore, binding of the repressor protein to Op72 is stronger with P1 wild-type than with P1 bac DNA (34).

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**REFERENCES**


Continued on next page.
Bacteria and Phage: The E. coli strain listed below was used for the following experiments: DH10B (originated from ref. 11) for the preparation of the plasmid DNA; AD1007 and AD1007 (in parentheses) for the preparation of DNA from plasmids P128 and P122, respectively (Fig. 1). D1007 (and the K12 hybrid strain O101 [11] for subcloning plasmids 6897-100 and 8875-100 (Fig. 1). D1007 and D1007(ElD1-17) were used for the propagation of Mlsp6 in recombinant phase (8, 14). Phase P1280 and P1280 (15) were used for the isolation of P1 repressor (Table 2). Phase M1880 and M1880 were used for cloning PI operator (16, 17).

Recombinant PI DNA: Plasmid pH1 (Fig. 1) contains the EcoRI site of P128 and was isolated as described (17). Provirus (Fig. 1) was isolated from the recombinant plasmid p1280-1280(El1-17) (8). The K12 derivative of the PI gene product was transduced as described in this reference. The source of these two repressors was the MD gene which was cloned into the K12 strain AD1007 (11). The recombinant plasmid was constructed by inserting EcoRI of 1210(El1-17) into p1280. This recombinant plasmid was used to express temperature-sensitive active PI repressor in the recipient strain. The plasmid was transformed by electroporation (12, 13).

Recombinant Phase DNA: Strain M1880 is grown in TY medium at 37°C. At a cell density of approximately 10^9/ml, 100 ml of PI-lysate (prepared by addition of 20 ml of 1 M NaCl) was added. After incubation for 15 min at 30°C, the mixture was subjected to 15% SDS-PAGE, and the gel was stained with Coomassie blue. The gel was scanned, and the volume of the native repressor was determined using a Bio-imaging analyzer (Bio-rad). The amount of PI repressor used in each experiment was determined as described above.

Physical Measurements: The following marker proteins (with Stokes radius in parentheses) were mixed with 8 pg of repressor protein (Fig. 1) as described above: 2.0, 1.5, 1.0, 0.5, 0.25, 0.125, and 0.0625 of BSA (1.0), 2.0, 1.0, 0.5, and 0.25 of ovalbumin (2.6; 4.6), and 0.5 of bovine serum albumin (6,4). The mixture was layered onto 0.1 ml of a 0.8-30% linear sucrose gradient in Buffer A and centrifuged in the Beckman 45Ti rotor. The mixture was centrifuged at 30,000 rpm at 4°C for 1 h. The mixture was then subjected to 15% SDS-PAGE, and the gel was stained with Coomassie blue. The gel was scanned, and the volume of the native repressor was determined using a Bio-imaging analyzer (Bio-rad).

Other Methods: Protein Determination: Protein concentrations were determined according to Miller (19). The repressor concentrations were determined in the following way: serial dilutions of repressor and BSA (known concentrations) were subjected to 10% SDS-PAGE, stained with Coomassie blue G250, and scanned at 595 nm. The absorbance of the repressor was then determined. The absorbance of the repressor was then determined by subtracting the absorbance of BSA at the same concentration.

Results: The following marker proteins (with Stokes radius in parentheses) were mixed with 8 pg of repressor protein (Fig. 1) as described above: 2.0, 1.5, 1.0, 0.5, 0.25, 0.125, and 0.0625 of BSA (1.0), 2.0, 1.0, 0.5, and 0.25 of ovalbumin (2.6; 4.6), and 0.5 of bovine serum albumin (6,4). The mixture was layered onto 0.1 ml of a 0.8-30% linear sucrose gradient in Buffer A and centrifuged in the Beckman 45Ti rotor. The mixture was centrifuged at 30,000 rpm at 4°C for 1 h. The mixture was then subjected to 15% SDS-PAGE, and the gel was stained with Coomassie blue. The gel was scanned, and the volume of the native repressor was determined using a Bio-imaging analyzer (Bio-rad).
Table 2

<table>
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<th>Plasmid</th>
<th>40°C/30°C ratio of chloramphenicol-resistant colonies</th>
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<th>Rifampicin×10^6</th>
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<td>0.1</td>
<td>0.3</td>
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<td>2×10^-3</td>
<td>3×10^-6</td>
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Plasmid-containing bacteria (2×10^8/ml) were infected with PlmBac cl-100 (well 1) in T1 medium containing 9 mG CaCl_2. After incubation for 15 min at 30°C, serial dilutions of the infected bacteria were spotted in duplicate on agar plates containing 25 µg/ml of chloramphenicol. Plates were incubated overnight at 30°C and 40°C and the number of chloramphenicol-resistant colonies counted. (--) not determined.

Fig. 9 SDS-PAGE of repressor cross-linked with glutaraldehyde
Repressor protein was treated with glutaraldehyde at 22°C for the indicated times as described in "Experimental Procedures". The protein was denatured with SDS, subjected to 10% SDS-PAGE, and the gel stained with Coomassie blue. Markers in descending order are phosphorylase b, BSA, ovalbumin, and chymotrypsinogen a.

Fig. 4 Binding of wild type- and pMV2 repressor to the Pl BamHI-Y fragment
Wild type repressor protein (fraction Y) in Buffer A supplemented with 100 µg/ml of BSA, and pMV2 repressor protein (heparin-sepharose fractions, see Fig.3) were incubated for 15 min at 30°C with 0.2 µg of pBR322 DNA which had been treated with EcoRI and BamHI (11 µl total volume). The probes were then subjected to 0.8% agarose gel electrophoresis. Fragments named by the letters a and b, and the numbers 7', 8', and 9 are explained in Fig.1. Marker: Mixture of λDNA/HindIII- and φX174 RF DNA/HaeIII fragments.