THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 3, Issue of January 25, pp. 1391-1397, 1988
Printed in U.S.A.

The *c*1 Repressor of Bacteriophage P1

ISOLATION AND CHARACTERIZATION OF THE REPRESSOR PROTEIN*

(Received for publication, July 8, 1987)

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The *c*1 repressor gene of bacteriophage P1 is located on P1 DNA *Eco*RI fragment 7 (Sternberg, N. (1979) *Virology* 96, 129–142). Subfragments of P1 DNA *Eco*RI fragment 7 were cloned into expression vectors, and the *c*1 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 *Bam*HI 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 *c*1 gene of P1 codes for a repressor that prevents the expression of lytic functions indirectly, possibly by repressing an operon whose product(s) is required for the transcription of genes required for phage production (1). The *c*1 gene is located at the far right side of the P1 genetic map in *Eco*RI:7 [1] (2, 3). Partially purified P1 repressor protein binds *in vitro* to at least two regions near *c*1 within *Bam*HI:9, which itself is located within *Eco*RI:7 (1, 4). This protein was found to be absent from nonsuppressing bacteria infected with a P1 *c*1 amber mutant. Furthermore, the binding activity of a protein which derived from a *c*1 temperature-sensitive mutant was found to be thermolabile *in vitro* (1). These findings identify the repressor protein as the product of the *c*1 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 *c*1 gene of P1 wild-type phage and of a revertant of a *c*1 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)[2,3] 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[4]

RESULTS

Strategy of Cloning the c1 Repressor Gene

Recombinant plasmid DNAs being composed of *Eco*RI:7 of P1 wild-type or P1 repressor mutant strains and pBR325 DNA were used as starting material for subcloning the *c*1 gene (Fig. 1). Subcloning was performed with the intention: (i) to insert the *c*1 gene into inducible expression vectors in order to optimize the expression of the *c*1 gene (for that purpose, vectors pPLc28 (under the control of the heat-inducible λ*c*I857 repressor) and pJF118EH (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 *c*1 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 *c*1 gene-containing recombinant plasmid to promote lysogenization at 40 °C of the bacterial cell by the phage P1Cm*bac c*1·100. (The presence of the *bac* mutation was a prerequisite to measure lysogenization of *Escherichia coli dnaB* ts mutants.) By that means, it was verified that plasmid pHS7·100-B, as expected, contains a *c*1 mutant gene coding for a thermolabile repressor (Table 2). In the same way, it

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[1] The abbreviations used are: *Eco*RI:7, for example, P1 DNA *Eco*RI 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.

[2] Sternberg, N., and Eliason, J. L. (1987) *J. Mol. Biol.*, in press.

[3] B. R. Baumstark, personal communication.

[4] Portions of this paper (including "Experimental Procedures," Figs. 4 and 9, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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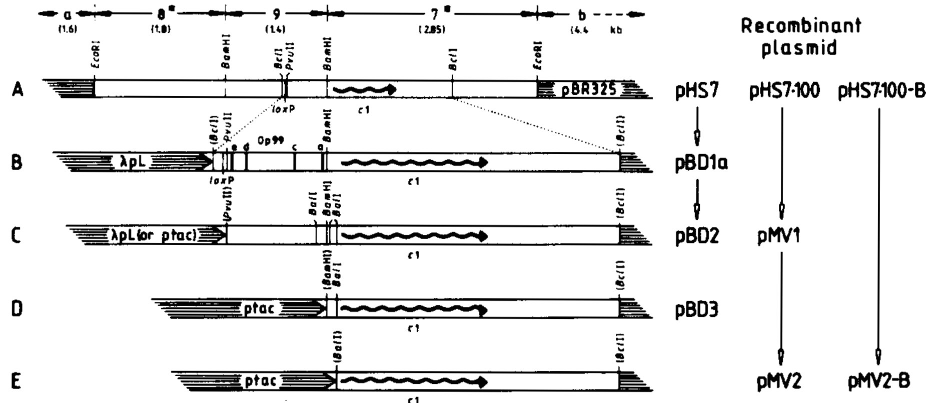

FIG. 1. **Subcloning of the c1 gene from P1 wild-type and mutant repressor strains.** *A*, the recombinant plasmid DNA contains the *Eco*RI:7 fragment (*open box*) from P1 wild-type (pHS7) or P1 repressor mutant (pHS7·100 or pHS7·100-B) strains which is inserted into the single *Eco*RI site of pBR325 (*hatched box*). *Eco*RI:7 (≈6 kilobases (*kb*)) is divided schematically into the three subfragments 8 *, 9, and 7 * by the *Bam*HI sites (31). The numbers in parentheses represent the size (in kilobases) of the *Bam*HI and *Bam*HI-*Eco*RI fragments of P1 and pBR325 DNA (*a* and *b* are not drawn to scale). *Eco*RI:7 was inserted into pBR325 in either orientation. Only one type of recombinant DNA is shown because the orientation of the *Eco*RI:7 insert is irrelevant for subcloning steps *B–E*. The position of *lox*P is taken from Hoess *et al.* (32). The position and direction of transcription of the *c*1 gene are shown by an *arrow-headed wavy line*. *B*, a *Bcl*I fragment of pHS7 (2.35 kilobases) was inserted into the single *Bam*HI site of pPLc28 to yield pBD1a. The *Pvu*II-*Bam*HI fragment in front of the *c*1 gene contains the repressor-binding sites or operators Op99 a, c, d, and e (see the text for an explanation). *C*, the *lox*P site was deleted from the recombinant plasmid by: (i) digestion of pBD1a with *Eco*RI and *Pvu*II (the sticky end of the *Eco*RI site was then filled up with the Klenow fragment of DNA polymerase I, and the plasmid was religated to yield pBD2); and (ii) isolation of the *Pvu*II-*Bcl*I fragment from pHS7·100 (insertion of the fragment into the polylinker region of pJF118EH, which had been treated with *Sma*I and *Bam*HI; this procedure yields pMV1). *D*, pBD3 was derived from the *Bam*HI fragment of pHS7 which contains the *c*1 gene. The sticky ends of the *Bam*HI sites were filled up with the Klenow fragment of DNA polymerase I; the fragment was then cut with *Bcl*I, and the *Bam*HI-*Bcl*I subfragment containing the *c*1 gene was inserted into pJF118EH, which had been treated with *Bam*HI and *Sma*I. *E*, pMV2 was obtained by treatment of pMV1 with *Eco*RI and *Bal*I; the sticky end of the *Eco*RI site was then filled up with the Klenow fragment of DNA polymerase I, and the plasmid was religated. For the construction of pMV2-B, the *Bcl*I fragment of pHS7·100-B was isolated and treated with *Bal*I. The *Bal*I-*Bcl*I subfragment was then inserted into pJF118EH, which had been treated with *Bam*HI and *Sma*I.

was discovered that, in plasmid pHS7·100, the same *c*1 mutant had undergone an (as yet unknown) alteration which had reversed the thermolabile 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 p*tac*-promoted expression of repressor at 30 °C is higher with pMV2 and pMV2-B in comparison to pBD3, pBD2 (*tac*), and pMV1. On the other hand, the amount of repressor found by heat induction of pBD2 was comparable to that of pMV2 and pMV2-B (data not shown). Apparently, the presence of the control region Op99 does not affect significantly the expression of *c*1 by inducible, heterologous promoters. Therefore, plasmids pBD2 and pMV2 were used for the preparation of wild-type and mutant repressor proteins.

Purification of Repressor

When cells of C600 (pBD2, pcI857) 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 *c*1 repressor, was monitored in the initial steps by tracing the overproduced polypeptide electrophoretically and later on by complex formation with *Bam*HI:9 (1, 4). As a typical result, about 5 mg of repressor (Fraction V) was obtained from 17.5 g of C600 (pBD2, pcI857) 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, pcI857) 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 *Bam*HI: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 *Bam*HI: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, equimolar amounts of the 31- and 33-kDa proteins bind equally well to *Bam*HI: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

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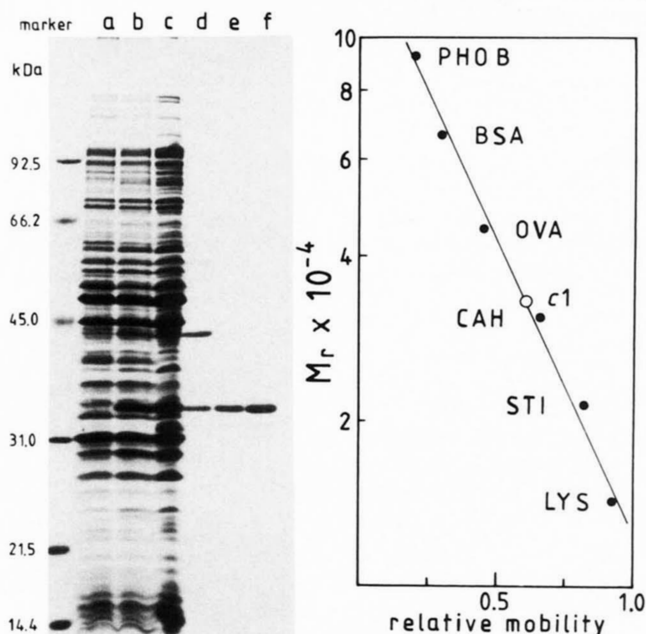

FIG. 2. **Induction and purification of repressor.** A cellular crude extract and purified fractions of C600 (pBD2, pcI857) were subjected to 15% SDS-PAGE and stained with Coomassie Blue. *Lane a*, 30 µl of crude extract, uninduced culture; *lane b*, 30 µl of crude extract, induced culture; *lane c*, 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 for the proteins: phosphorylase *b* (*PHOB*), bovine serum albumin (*BSA*), ovalbumin (*OVA*), carbonic anhydrase (*CAH*), soybean trypsin inhibitor (*STI*), and lysozyme (*LYS*).

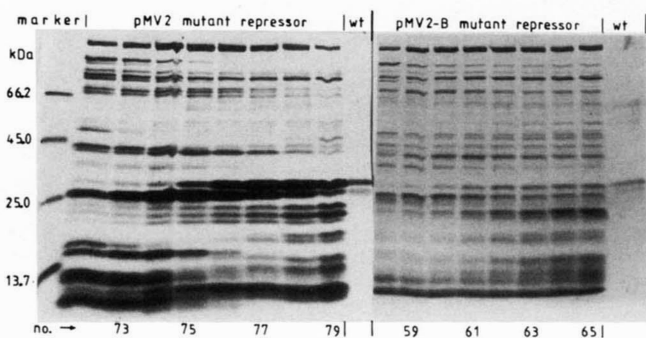

FIG. 3. **Heparin-Sepharose chromatography of repressor mutants.** Fraction II of an induced culture of HB101 (pMV2) and C600 (pMV2-B) was obtained from 5 and 9.5 g of wet cell paste, respectively. It was loaded onto a heparin-Sepharose column. Protein fractions (0.5–1% of each fraction) eluting with 450 to 650 mM NaCl were subjected to 15% SDS-PAGE, and the gel was stained with Coomassie Blue. Markers in descending order are bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase A. *wt*, wild-type repressor protein (Fraction V).

the 33- or 31-kDa polypeptide band (Fig. 3) nor a *Bam*HI:9-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 33-kDa protein is adsorbed to and can be eluted from the solid material. This protein binds to *Bam*HI:9 only at low temperature.[5]

Wild-type and pMV2 mutant repressor proteins (Fraction 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 *Bam*HI:9, but results in the gradual conversion of the 33-kDa to the 31-kDa protein.

[5] J. Heinrich, unpublished data.

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 *Bam*HI: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 *Bam*HI:9 specifically and about equally well at 47 °C. Preincubation of the repressor protein for 1 min at 53 °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 can be lysogenized at 40 °C by P1Cm*bac 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 cannot 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

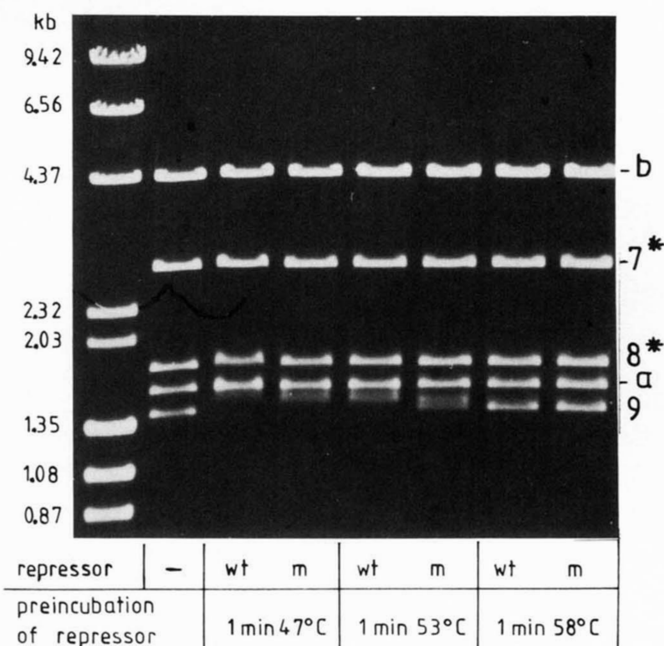

FIG. 5. **Dependence of binding of repressor to *Bam*HI:9 on temperature.** Samples of repressor protein were preincubated as indicated. Wild-type (*wt*) and pMV2 mutant (*m*, Fraction V each) repressors were then incubated for 15 min at 47 °C with 0.2 µg of pHS7 DNA, which had been treated with *Eco*RI and *Bam*HI (75 nM repressor monomer, 25-µl total volume). The probes were subsequently subjected to 0.7% agarose gel electrophoresis. Fragments marked by *a* and *b*, and *7* *, *8* *, and *9* are explained in Fig. 1. *kb*, kilobases.

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sites had been cloned in M13mp8/9 and sequenced (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 *Hinc*II fragment of the recombinant M13 RF DNA b3 (this fragment is located 5′ upstream of the *ban* gene in *Eco*RI:3 (7)); (ii) a 600-bp *Pvu*II-*Bam*HI fragment of b7, which is located in front of the *c*1 gene (Fig. 1) (binding of repressor to this fragment had been observed before (4)); (iii) a 500-bp *Rsa*I-*Pvu*II fragment of b11 (this fragment is contained in *Eco*RI:11 in the neighborhood of a P1 head gene (26)); and (iv) a 320-bp *Bst*XI fragment of b14. This fragment is part of *Eco*RI:14. Furthermore, the repressor also binds to *Eco*RI:9 (8), as was discovered originally by Baumstark.[3] Repressor-controllable gene functions have been found located in or starting from the P1 DNA part of b3 (6, 7), b7 (4),[2] 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 *c*1 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 for the repressor of about 230,000 (Fig. 7*a*). The Stokes radius of the repressor was estimated to be about 55 Å (Fig. 7*b*).

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 *c*1 gene.[3]

Molecular Weight and Frictional Coefficient—A molecular weight of 63,000 for the native repressor protein was calculated from the equation: $M_r = 6\pi\eta Nas/(1 - \nu\rho)$. The frictional coefficient (f/f_0) was calculated to be 2.1 from the equation: $f/f_0 = a/(3\nu M_r/4\pi N)^{1/3}$. In these equations, a = Stokes radius, s = sedimentation coefficient, ν = partial specific volume, η = viscosity of medium (0.01002 poise), ρ = density of medium (1.00), and N = Avogadro's number (23).

These data suggest that the native repressor protein (M_r = 63,000) is composed of two subunits (M_r = 33,000 each). The

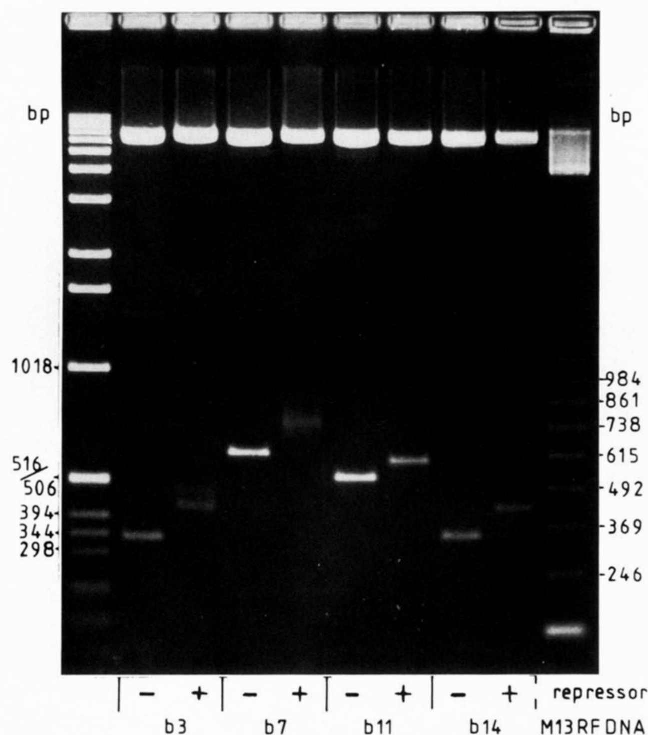

FIG. 6. **Binding of wild-type repressor to different P1 operator DNAs.** The recombinant M13 RF DNAs (1 μg each) were treated with *Eco*RI and *Hin*dIII for 1 h at 37 °C. After inactivation of the enzymes (10 min at 65 °C), the probes were incubated with and without repressor (500 nM) in Buffer A supplemented with 100 μg/ml bovine serum albumin for 15 min at 37 °C (25-μl total volume). The probes were then subjected to 1.5% agarose gel electrophoresis. Markers are the 1-kilobase ladder (*left*) and the 123-bp ladder (*right*).

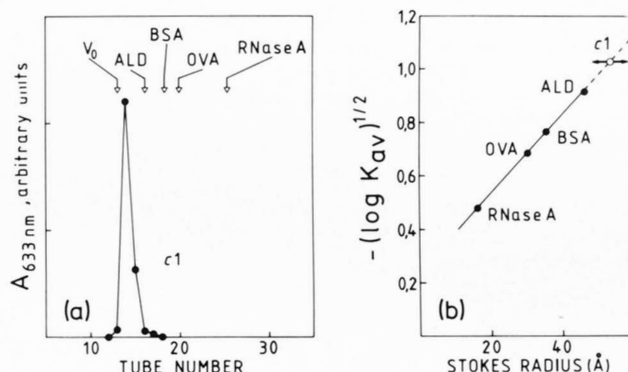

FIG. 7. **Sephacryl S-200 filtration (*a*) and Stokes radius estimation (*b*) of repressor protein.** The zonal method of Andrews (33) was used for molecular weight estimation by gel filtration. The Stokes radius was estimated by the procedure of Siegel and Monty (23). *c*1, position of repressor. The protein markers are aldolase (*ALD*), bovine serum albumin (*BSA*), ovalbumin (*OVA*), and RNase A. Details are described under "Experimental Procedures."

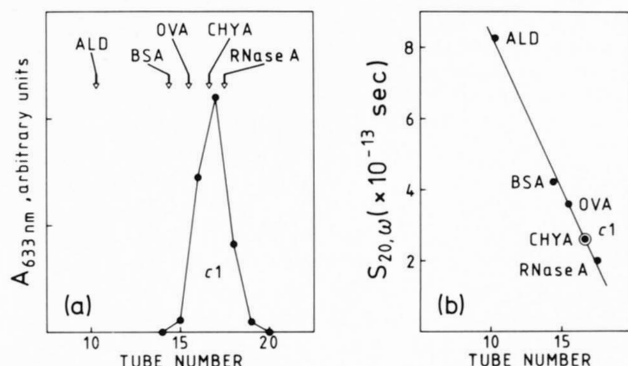

FIG. 8. **Glycerol gradient sedimentation (*a*) and sedimentation coefficient estimation (*b*) of repressor protein.** The sedimentation coefficient was determined by glycerol gradient centrifugation. The protein markers are aldolase (*ALD*), bovine serum albumin (*BSA*), ovalbumin (*OVA*), chymotrypsinogen A (*CHYA*), and RNase A. Details are described under "Experimental Procedures."

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high frictional coefficient indicates that the repressor molecule is asymmetric (29).

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 *c*1 repressor gene, overproduction, and purification of the *c*1 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 reversion 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*I-*Bcl*I subfragment from *Bam*HI:7 * (Fig. 1) used for the induction of pMV2 and pMV2-B repressors must contain the intact *c*1 reading frame for the following reasons. Cloning the P1 *c*1 region by similar methods had indicated that the *c*1 reading frame is contained within *Bam*HI:7 * (4). In addition, the *c*1 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*I site.[3] Moreover, deletion analysis establishes the starting point of repressor translation at that site.[2] In contrast, *c*1 repressor is not expressed by cloned *Bam*HI:7 * unless a heterologous promoter (p*tac*) is fused to the 5′ end of that fragment (4). This indicates that at least part of the *c*1 promoter is located on *Bam*HI:9 (Fig. 1). The ability of plasmids pBD3 and pMV2 to promote lysogenization by a P1Cm*bac c*1·100 phage (Table 2) must therefore be due to the leakiness of the p*tac* promoter.

Purification of enriched repressor protein fractions was monitored by complex formation of the repressor with *Bam*HI:9. The latter turned out to be the most appropriate substrate because it was only found recently that it contains at least four repressor-binding sites or operators named Op99 a, c, d, and e (4, 8).[2] In the meantime, a total of 11 repressor-binding sites which are widely scattered throughout the P1 genome have been found by the efforts of several laboratories (reviewed in Ref. 9). From these sites, a consensus sequence 5′-ATTGCTCTAATAAATTT-3′ lacking dyad symmetry has been derived (8, 9).

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 P1*bac*, 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).

Acknowledgments—We are much indebted to N. Sternberg for recombinant DNA pBR325-P1:7*c*1·100. We thank him, B. Baumstark, and M. Yarmolinsky for providing data prior to publication. We thank J. Heinrich for the large-scale preparation of P1 *c*1 repressor protein and D. Vogt for the preparation of plasmid DNA. The excellent technical assistance of I. Severin and M. Warmuth is gratefully acknowledged.

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Supplementary Material to:

"The c1 Repressor of Bacteriophage P1. Isolation and Characterization of the Repressor Protein"

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EXPERIMENTAL PROCEDURES

Materials: The following materials were used (manufacturer in parentheses): ammonium sulfate (Baker), ATP and streptomycin sulfate (Boehringer, Mannheim), Brij 58 (Serva, Heidelberg), glycerol (≈87%, Merck), dithiothreitol (RSA Corp., Ardsley, New York), glutaraldehyde (Fluka), IPTG (Sigma), molecular weight marker proteins (Boehringer, Mannheim), Klenow fragment of DNA polymerase I (Biolabs), 123 bp ladder, 1 kb ladder, λ DNA/HindIII-, and ΦX174 RF DNA/HaeIII fragments (BRL), CM-Sepharose, DEAE-Sephacel, heparin-Sepharose, and Sephacryl S-200 SF (Pharmacia).

Buffers: During purification and characterization of the repressor the following buffers were used: Buffer A, 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol; Buffer B, 20 mM Tris-acetate, pH 6.5, 1 mM dithiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol; Buffer C, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% (w/v) Brij 58.

Bacteria and Phage: The E.coli K12 strains listed below were used for the following experiments: GM82dam (cited in ref. 11) for the preparation of the recombinant plasmid DNAs pHS7, pHS7·100, and pHS7·100-B because subcloning of the c1 repressor gene required the use of BclI (see below and Fig.1); C600(λ) and C600(pcI857) for subcloning plasmid pHS7, and for the controlled expression of the c1 gene from plasmids pBD1a and pBD2, respectively (Fig.1); C600, NY58dnaBts (12) and the K12xB hybrid strain HB101 (13) for subcloning plasmids pHS7·100 and pHS7·100-B (Fig.1). JM101 and JM101(pKT101-P1:7) for the propagation of M13mp8/9 recombinant phage (8,14). Phage P1Cmbac-1 c1·100 (15) was required for the in vivo assay of P1 repressor (Table 2). Phage M13mp8 and M13mp9 were used for cloning P1 operator DNAs (8,14).

Recombinant Plasmid DNA: Plasmid pHS7 (Fig.1) contains the EcoRI:7 of P1bac crr and was isolated as described (7). pHS7·100 (Fig.1) was derived from the recombinant plasmid pBR325-P1:7c1·100 (8); the expected thermolabile character of its c1 gene product had been reverted accidentally to thermoresistance as judged from the in vivo properties of pHS7·100 (Table 2). pHS7·100-B (Fig.1) was constructed by inserting EcoRI:7 of P1Cmc1·100 (16) into pBR325. This recombinant plasmid conferred the expected temperature-sensitive character of the c1 immunity to the recipient cell (Table 2).

Plasmid pcI857 is specified by resistance to kanamycin and carries the λcI857 allele coding for a λts repressor protein (17). The λp_L vector pPLc28 (18) and the tac vector pJF118EH (19) were used for the expression of the P1 c1 repressor gene; repressor protein synthesis was induced by heat and IPTG, respectively.

Recombinant Phage DNA: Strain JM101 is grown in TY medium at 37°C. At a cell density of about 2×10^8/ml, bacteria were infected with M13mp8 and M13mp9 phage. Growth was continued overnight. Bacteria were pelleted by centrifugation, and M13mp8/9 RF DNAs were extracted by the method of Holmes and Quigley (20). The construction of the recombinant M13mp8/9 RF DNAs is described in Table 1.

Repressor Assay:

in vivo - Bacteria harboring a recombinant plasmid carrying a c1 repressor gene were infected with P1Cmbac-1 c1·100. Serial dilutions of the infected bacteria were plated on agar plates containing chloramphenicol at 30° and 40°C. Only those bacteria containing a temperature-resistant repressor are able to grow as chloramphenicol-resistant colonies at both 30° and 40°C.

in vitro - Repressor-containing protein fractions or purified repressor (Fraction V) were incubated with EcoRI- and BamHI-digested pHS7 plasmid DNA in Buffer A supplemented with 100 µg/ml of BSA. The conditions are specified in "Results". The presence of repressor is indicated by complex formation of the protein with the P1 BamHI:9 fragment. Complex formation is detected by the decreased mobility of the fragment to which repressor remains bound during electrophoresis (21,22).

Overproduction of P1 Repressor:

Thermoinduction - C600(pBD2, pcI857) was grown in 2 liters of TY medium at 30°C. At a cell density of $6\text{-}8 \times 10^8$ cells/ml 2 liters of prewarmed TY medium (54°C) were added which quickly raises the temperature to 42°C. After incubation at 42°C for 15 min the temperature was lowered to 37°C and the culture shaken for 3 hours at 37°C. Subsequently bacteria were pelleted from the culture by centrifugation (10 min at 4,200 x g, 4°C, Sorvall GS-3 rotor), and the wet cell paste (16 to 20 g) resuspended in 200 mM NaCl - 2.5 mM EDTA - 20 mM spermidine (5 ml/g of wet cell paste). This material was frozen in liquid nitrogen and stored at -70°C.

IPTG Induction - HB101(pMV2) and C600(pMV2-B) were grown at 30°C. At a cell density of $2\text{-}3 \times 10^8$ cells/ml IPTG (2 mM final concentration) was added and incubation continued for 2 hours at 30°C. Bacteria were subsequently harvested as described above.

Repressor Purification:

Preparation of Extracts - All operations were performed at 2° to 6°C unless stated otherwise. Frozen bacteria (17.5 g of wet cell paste) were thawed, adjusted to 120 ml with (final concentrations) 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 3.5% (w/v) sucrose, 0.1% (w/v) Brij 58, 2 mM EDTA, 15 mM spermidine, and 0.5 mg/ml of lysozyme. After incubation for 30 to 60 min at 0°C the suspension was warmed up to 30°C for 5 min, cooled down again, adjusted to 1 M NaCl, and centrifuged for 60 min at 70,000 x g in a Beckman 45Ti rotor. The supernatant (125 ml) was diluted 1:1 with Buffer A (Fraction I, 250 ml).

Streptomycin Sulfate Step - One-tenth volume of 30% (w/v) streptomycin sulfate was slowly added to Fraction I, and the mixture was stirred for 20 min. The precipitate was collected by centrifugation at 70,000 x g (Beckman 45Ti rotor) for 30 min. The pellet was discarded and the supernatant (275 ml) collected.

Ammonium Sulfate Precipitation - Solid ammonium sulfate was slowly added to 60% saturation (0.39 g/ml) and the mixture was stirred for 30 min. After 10-15 hours the precipitate was collected by centrifugation (30 min at 70,000 x g, Beckman 45Ti rotor), washed once with 60% ammonium sulfate in Buffer A, centrifuged again, and dissolved in 60 ml of Buffer A. The solution was subsequently dialyzed for 3 h against Buffer A (Fraction II, 65 ml).

Heparin-Sepharose Chromatography - Fraction II was loaded onto a heparin-Sepharose column (2 cm² x 15 cm) previously equilibrated with Buffer A. The flow rate was 60 ml/h. Then the column was washed with 150 ml of Buffer A at the same flow rate. The repressor binds to heparin-Sepharose. Proteins were eluted with a 450 ml-linear gradient of 50-800 mM NaCl in Buffer A at a flow rate of 30 ml/h. 7.5-ml fractions were collected, the repressor-containing fractions (450-650 mM NaCl) pooled, and dialyzed for 3 h against Buffer A (Fraction III, 115 ml).

DEAE-Sephacel Chromatography - Fraction III was loaded onto a DEAE-Sephacel column (2.5 cm² x 4.5 cm) equilibrated with Buffer A. The flow rate was 20 ml/h. Then the column was washed with 100 ml of Buffer A. Since the repressor does not bind to the column the flow-through fractions containing the repressor were collected and pooled. Solid ammonium sulfate was slowly added to 60% saturation. The mixture was stirred for 1 h, and subsequently stored overnight. Then the precipitate was collected by centrifugation (30 min at 70,000 x g, Beckman 45Ti rotor), and the pellet dissolved in 7 ml of Buffer B. The solution was dialyzed for 3 h against Buffer B (Fraction IV, 8.5 ml).

CM-Sepharose Chromatography - Fraction IV was loaded onto a CM-Sepharose column (1.2 cm² x 3.4 cm) which had been equilibrated with Buffer B. The flow rate was 5 ml/h. The column was subsequently washed with 45 ml of Buffer B. The repressor which binds to CM-Sepharose was eluted with a 50 ml-linear gradient of 20-600 mM Tris-acetate, pH 6.5, in Buffer B. The flow rate was 4 ml/h. 0.9-ml fractions were collected. Repressor-containing fractions (300-450 mM Tris-acetate) were pooled, the protein precipitated by ammonium sulfate (60% saturation) as described above, and dissolved in Buffer A. Finally the solution was dialyzed for 3 h against Buffer A, diluted 1:1 with glycerol, and kept frozen at -70°C (Fraction V, 2.5 ml).

Physical Measurements:

Molecular Weight of the Denatured and Native Repressor Protein - The polypeptide molecular weight was estimated from data obtained by 15% SDS-PAGE using phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) as molecular weight markers (Fig.2). The molecular weight of the native repressor was calculated from determinations of the sedimentation coefficient, the Stokes radius and the apparent partial specific volume (23).

Sedimentation Coefficient - The following marker proteins (with s values in parentheses) were mixed with 8 µg of repressor protein (Fraction V as described above) in a total volume of 0.1 ml: 11 µg of aldolase (8.27), 11 µg of ovalbumin (3.66) and 8 µg of RNase A (2.0). The mixture was layered onto a 3.8-ml linear, 15 to 35% glycerol gradient in Buffer C supplemented with 20 µg/ml of BSA. In a separate tube 5 µg of BSA (4.22) and 6 µg of chymotrypsinogen A (2.58) were mixed and applied to a gradient in Buffer C. Sedimentation of both tubes was for 24 h at 225,000 x g in a Spinco SW60 rotor at 3°C. Fractions containing 0.19 ml were collected from the bottom of the gradient. Aliquots of each fraction were subjected to 15% SDS-PAGE, and the gel stained with Coomassie blue. Sedimentation of repressor and marker proteins was traced by scanning the gel.

Stokes Radius - The following marker proteins (with Stokes radius in Å in parentheses) were mixed with 0.12 mg of repressor protein (Fraction V as described above) in a total volume of 0.4 ml: 0.6 mg of aldolase (48.1), 0.5 mg of ovalbumin (30.5), and 0.6 mg of RNase A (16.4). The mixture was applied to a Sephacryl S-200 column (1.6 cm² x 86 cm) which had been equilibrated at 4°C with Buffer C supplemented with 20 µg/ml of BSA. In a separate experiment the same marker proteins and 4 mg of BSA were applied to the Sephacryl S-200 column which had been equilibrated with Buffer C. Blue dextran and ATP were added in both experiments for the calibration of the column. The flow rate was 14 ml/h and 4-ml fractions were collected. Migration of the proteins was traced as described above. Gel filtration data are presented in terms of $K_{av} = V_e - V_o / V_t - V_o$, where V_e = elution volume of a given protein, V_o = void volume of the column, and V_t = total volume of the gel bed (23).

Cross-linking of Protein Subunits - Repressor protein (50 µg/ml) in 5 mM natrium phosphate buffer, pH 7.0, 150 mM NaCl, and 0.1% (w/v) of Brij 58 was incubated in 3 mM glutaraldehyde at 22°C. The reaction was terminated by the addition of 0.5 volume of 1 M Tris-HCl, pH 7.0. After 10 min at 22°C 0.5 volume of 2 M trichloroacetic acid was added. After 15 min at 0°C the solution was centrifuged (20 min at 27,000 x g, 4°C, Sorvall SS34 rotor), and the pellet dissolved in 100 mM natrium phosphate buffer, pH 7.0, and 1% SDS, and 1% β-mercaptoethanol. The sample was then subjected to 15% SDS-PAGE.

Other Methods:

Protein Determination - Protein concentrations were determined according to Miller (24). Repressor protein concentrations were determined in the following way: serial dilutions of repressor and BSA (of known concentrations) were subjected to 15% SDS-PAGE, stained with Coomassie blue R250 and scanned by the laser densitometer Ultroscan XL (A_{633} nm, LKB). All other methods used are described elsewhere (7,8,12).

Table 1
Cloning of P1 operator DNA in M13mp8/9 RF DNA

P1 EcoRI fragment no	subfragment	M13 vector (restriction enzyme site) used	Resulting recombinant M13 RF DNA
3	HincII-HincII[a]	mp9 (SmaI)	b3
7	EcoRI-BamHI[b]	mp8 (EcoRI + BamHI)	b7
11	RsaI-PvuII[a]	mp8 (SmaI)	b11
14	BstXI-BstXI[a]	mp8 (SmaI)	b14

Subfragments of P1 EcoRI fragments which contain a repressor binding site were inserted into the polylinker region of M13mp8/9 RF DNA as indicated.
[a]Subfragments are inserted into the vector by blunt end ligation. The sticky ends of the BstXI fragment are filled up with Klenow fragment of DNA polymerase I before. [b]The EcoRI-BamHI subfragment was derived from plasmid pBD2, in which the EcoRI site of the vector pPLc28 is preserved (Fig.1). The P1 specific DNA can be excised again from the recombinant M13 RF DNAs by EcoRI and HindIII.

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Table 2
P1 repressor gene-containing plasmids promote lysogenization by P1Cm*bac* *c*1·100

Plasmid	40°/30°C ratio of chloramphenicol-resistant colonies	
Strain	C600*dnaB*+	NY58*dnaB*ts
pHS7	1.0	0.1
pHS7·100	-	0.3
pHS7·100-B	-	$< 3 \times 10^{-6}$
pBD2(*tac*)	0.9	-
pMV1	1.1	-
pBD3	0.2	-
pMV2	1.1	-
pMV2-B	$< 2 \times 10^{-3}$	$< 8 \times 10^{-4}$

Plasmid-containing bacteria ($\approx 2 \times 10^8$/ml) were infected with P1Cm*bac* *c*1·100 (moi ≥ 2) in TY medium containing 5 mM $CaCl_2$. After incubation for 15 min at 30°C, serial dilutions of the infected bacteria were spotted in duplicates on agar plates containing 25 μg/ml of chloramphenicol. Plates were incubated overnight at 30° and 40°C and the number of chloramphenicol-resistant colonies counted. (-) = not determined.

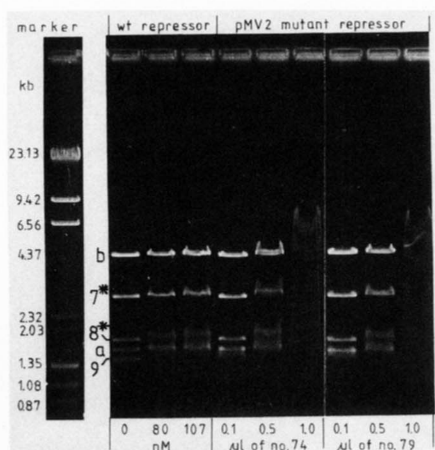

Fig. 4 Binding of wild type- and pMV2 repressor to the P1 BamHI:9 fragment
Wild type repressor protein (Fraction V) in Buffer A supplemented with 100 μg/ml of BSA, and pMV2 repressor protein (heparin-Sepharose fractions, see Fig.3) were incubated for 15 min at 30°C with 0.2 μg of pHS7 DNA which had been treated with EcoRI and BamHI (11 μl total volume). The probes were then subjected to 0.7% 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 λ DNA/HindIII- and ΦX174 RF DNA/HaeIII fragments.

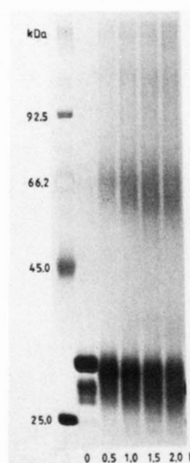

Fig. 9 SDS-PAGE of repressor cross-linked with glutaraldehyde
Repressor protein was treated with glutaraldehyde at 22°C for the indicated times as described in "Experimental Procedures". The protein was denatured with SDS, subjected to 10% SDS-PAGE, and the gel stained with Coomassie blue. Markers in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

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