JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 6907–6914
0021-9193/94/$04.00+0

Vol. 176, No. 22

Expression, Purification, and Functional Characterization of the Carboxyl-Terminal Domain Fragment of Bacteriophage 434 Repressor

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Received 16 June 1994/Accepted 21 August 1994

The repressor protein of bacteriophage 434 binds to DNA as a dimer of identical subunits. Its strong dimerization is mediated by the carboxyl-terminal domain. Cooperative interactions between the C-terminal domains of two repressor dimers bound at adjacent sites can stabilize protein-DNA complexes formed with low-affinity binding sites. We have constructed a plasmid, pCT1, which directs the overproduction of the carboxyl-terminal domain of 434 repressor. The protein encoded by this plasmid is called CT-1. Cells transformed with pCT1 are unable to be lysogenized by wild-type 434 phage, whereas control cells are lysogenized at an efficiency of 1 to 5%. The CT-1-mediated interference with lysogen formation presumably results from formation of heteromeric complexes between the phage-encoded repressor and the plasmid-encoded carboxyl-terminal domain fragment. These heteromers are unable to bind DNA and thereby inhibit the repressor's activity in promoting lysogen formation. Two lines of evidence support this conclusion. First, DNase I footprinting experiments show that at a 2:1 ratio of CT-1 to intact 434 repressor, purified CT-1 protein prevents the formation of complexes between 434 repressor and its O_R1 binding site. Second, cross-linking experiments reveal that only a specific heterodimeric complex forms between CT-1 and intact 434 repressor. This latter observation indicates that CT-1 interferes with 434 repressor-operator complex formation by preventing dimerization and not by altering the conformation of the DNA-bound repressor dimer. Our other evidence is also consistent with this suggestion. We have used deletion analysis in an attempt to define the region which mediates the 434 repressor–CT-1 interaction. CT-1 proteins which have more than the last 14 amino acids removed are unable to interfere with 434 repressor action in vivo.

The *cI* gene of the lambdoid bacteriophage 434 encodes a DNA-binding protein called the repressor (18). Similar to the case with the repressors of the other lambdoid phages (20), each 209-amino-acid-long monomer of 434 repressor can be structurally and functionally divided into two domains (2). The amino-terminal domain (R1-69) is composed of the first 69 amino acids and contains a helix-turn-helix structural motif. This unit is responsible for mediating all specific and nonspecific contacts between repressor and DNA (25). The carboxyl-terminal domain encompasses the last 115 amino acids of the repressor polypeptide. This domain stabilizes the formation of repressor dimers and mediates the formation of DNA-bound repressor tetramers (2). The remaining ~15% of the protein's sequence forms a linker between the amino- and carboxyl-terminal domains (18).

Several observations highlight the importance of carboxyl-terminal domain-mediated repressor oligomerization in regulating 434 repressor's tight binding to operator DNA. First, separating the repressor into amino- and carboxyl-terminal domains by proteolytic cleavage reduces the affinity of the DNA-binding domain for operator by at least 100-fold (14, 24). Furthermore, the isolated amino-terminal domain is less able to discriminate between synthetic 434 operators of various strengths than is intact protein (14). For example, the intact protein binds 60-fold better to a synthetic reference operator than it does to mutant operator, whereas the isolated amino-terminal domain prefers the reference over the mutant site by only 12-fold (14). A similar decrease in the ability of the amino-terminal domain to differentiate among the naturally occurring operators has been observed (24). These results show that the carboxyl-terminal domain serves to enhance both repressor's affinity for specific sequences and its ability to distinguish between specific sites of various strengths.

In addition to the effects of carboxyl-terminal domain-mediated dimerization on DNA affinity, the cooperative interactions between two DNA-bound repressor dimers also mediated by this domain influence repressor's operator preferences. Repressor displays different orders of preference for the sites in O_R, depending on whether the measurements are made with single, isolated sites or with intact O_R (24). On independent sites, 434 repressor binds with lowest affinity to O_R2, twofold more tightly to O_R3, and with yet a sixfold-higher affinity to O_R1. In intact O_R, repressor binds O_R1 and O_R2 with almost equal affinities and subsequently binds O_R3 with an eightfold-lower affinity. This demonstrates that repressor dimers do not bind independently to the three sites in intact O_R; rather, they form DNA-bound tetramers which cooperatively bind to two adjacent sites while binding the third site independently. The cooperative binding of repressor to O_R1 and O_R2 requires the carboxyl-terminal domain; cooperative binding is not displayed by the isolated amino-terminal domain (24). Since maintenance of the lysogenic state requires occupancy of O_R2, this cooperatively is a crucial feature of the lysis-lysogeny switch (18).

The study of the bacteriophage 434 repressor protein-DNA interaction, together with studies of other protein-DNA complexes, has yielded intimate details of how amino acids and base pairs can interact. While these investigations point out the importance of base-specific contacts, they have also made it

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clear that binding specificity is not determined solely by these contacts. X-ray crystallographic and biochemical examinations of the 434 repressor-operator complex indicate that the geometry of the protein-protein interactions in the DNA-bound repressor dimer indirectly influences its operator specificity (1, 12a, 13). Despite the critical role that carboxyl-terminal domain-mediated repressor oligomerization has in regulating 434 repressor DNA binding, little is known about either the nature of the protein-protein interfaces or the structure of the domain itself. In this paper we report the results of our initial investigations of the structure and function of 434 repressor's carboxyl-terminal domain.

MATERIALS AND METHODS

Media and growth conditions. Liquid cell cultures were grown in Luria broth (17). Plate assays were performed on Luria agar (17). Both the Luria broth and Luria agar were supplemented with the antibiotic tetracycline at 10 μg/ml.

Bacterial strains, phages, and plasmids. *Escherichia coli* X90 (6) was used in all lysogen interference assays, plasmid preparations, and protein purifications. RW102, a Dam^- derivative of JM101, was used to prepare plasmid DNA used in deletion mutagenesis (23). To construct a plasmid which directs the high-level expression of 434 repressor's carboxyl-terminal domain, we modified the plasmid pRW198 (24). pRW198 normally directs the synthesis of high levels of intact 434 repressor. The expression of this gene is under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter. pCT1 was created by cleaving pRW198 with *Asp* 718 and *Hin*dIII, repairing the recessed ends by Klenow fragment of DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs), and subsequent recircularization. The resulting plasmid, pCT1, has 434 repressor amino acids 26 to 93 deleted and encodes a polypeptide containing the first 25 amino acids of the amino terminus of 434 repressor fused to the last 116 amino acids of the protein. pRW218-Bgl, obtained from R. Wharton (Duke University, Durham, N.C.), is another derivative of pRW198 in which its 250-bp *Eco*RI-*Hin*dIII fragment, which encodes almost the entire DNA-binding domain of 434 repressor, has been replaced with the 30-bp *Eco*RI-*Hin*dIII polylinker fragment of the plasmid piVX (21). Control experiments show that this plasmid does not produce any functional 434 repressor protein fragments, and therefore it served as a control in the in vivo assays. The bacteriophages λ*imm*^{434} Meselson (i^4m) and wild-type λ (10^9 PFU/ml) were propagated and maintained as described previously (7).

Plasmid DNA containing a synthetic 434 O_R1 was obtained from A. Bell of our laboratory and was labelled for DNase I digestion studies as described previously (4).

Bal 31 nuclease deletions were performed essentially as described by Sambrook et al. (19). The plasmid pCT1 was digested with *Bcl*I, which cuts in the stop codon of the plasmid-encoded protein. The linearized plasmid was resected with *Bal* 31 at 37°C, and the reactions were quenched by phenol extraction at various times following addition of enzyme. Following ethanol precipitation, the ends were repaired by using dNTPs and the Klenow fragment of DNA polymerase I. A linker which encodes a stop codon in all three frames was ligated onto the blunt ends, and the circularized plasmid DNA was transformed into X90 cells. Defective deletion mutants were selected in the lysogen interference assay (see below).

To facilitate discussion of the results of the deletion mutagenesis, we number amino acids of the carboxyl-terminal fragment starting with the position of the RecA cleavage site as amino acid 1. The actual positions of the amino acids in the sequence of the intact repressor are given in the legend to Fig. 2. The amino-terminal sequence of the purified carboxyl-terminal domain fragment is given in Results and Discussion.

In vivo assays of carboxyl-terminus function. A lysogen interference assay was utilized to ascertain the function of the carboxyl-terminal protein (CT-1) or its derivatives in vivo. X90 cells transformed with either pCT1, its deletion derivatives, or pRW218-Bgl were plated on Luria agar plates containing either 0.5 mM IPTG or 0.5 mM IPTG plus 10^8 i^4m phage. After 48 h of growth at 37°C, the plates were scored for the formation of 434 lysogens. Cells bearing unmutagenized pCT1 always failed to form any colonies in the presence of i^4m, whereas approximately 1% of cells bearing the control plasmid pRW218-Bgl formed stable 434 lysogens under identical conditions.

Purification of CT-1. Strain X90 bearing pCT1 was grown with aeration at 37°C in 3 liters of Luria broth plus 10 μg of tetracycline per ml to an A_{600} of approximately 0.6 to 0.8. IPTG was added to a final concentration of 5×10^{-4} M, and growth at 37°C was continued for 4 h. The cells were harvested by centrifugation and resuspended in 20 ml of lysis buffer (200 mM KCl, 50 mM EDTA, 100 mM Tris-HCl, pH 7.4) along with the protease inhibitors phenylmethylsulfonyl fluoride (final concentration, 0.3 mg/ml), leupeptin, benzamidine, *N*-tosyl-L-phenylalanine chloromethyl ketone, and pepstatin (all present at 7.5×10^{-3} mg/ml), and aprotinin (75 Kallikrein units per ml). The cells were lysed by two passages through a French pressure cell (18,000 lb/in^2) at 4°C. The lysate was diluted with 100 ml of cold 20 mM $NaPO_4$ (pH 6.8) plus 200 mM NaCl. All subsequent steps were performed at 4°C. The progress and purity of the preparation was monitored by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE), and the proteins were visualized by Coomassie blue staining. The diluted lysate was centrifuged for 10 min at $10,000 \times g$. Sufficient 10% polyethyleneimine was added to the resulting supernatant to give a final concentration of 0.6%. This mixture was stirred for 10 min and then centrifuged for 10 min at $10,000 \times g$. The proteins in the supernatant were precipitated by adding 30 g of ammonium sulfate per 100 ml. The resulting pellet was dissolved in 20 ml of 20 mM $NaPO_4$ (pH 6.8) plus the protease inhibitors. The protein was dialyzed against two 1-liter changes of the same buffer. The resulting solution was clarified by centrifugation for 20 min at $12,000 \times g$. The proteins were loaded onto a quaternary amine ion-exchange column (Bio-Rad) equilibrated with 20 mM $NaPO_4$ (pH 6.8). Following a wash with the same buffer, the proteins were eluted from the column with a linear NaCl gradient. Column fractions were analyzed on an SDS–15% PAGE gel. The CT-1 protein eluted from the column between 0.14 and 0.25 M NaCl. The CT-1-containing fractions were pooled and precipitated with 50% ammonium sulfate. The precipitated protein was dissolved in 50 mM $NaPO_4$ (pH 6.8) and dialyzed against 1 liter of 50 mM $NaPO_4$ (pH 6.8) plus 10% glycerol (storage buffer). Protein used for spectroscopic and cross-linking analysis was further purified on a Sephacryl S-200 column (1.5 by 30 cm) which was equilibrated with storage buffer. Fractions containing the protein were pooled, frozen, and stored at −80°C. The concentration of CT-1 protein was determined by A_{280} measurements with an estimated extinction coefficient.

Other proteins. P22 repressor and 434 repressor were prepared in this laboratory according to the procedures described in references 2 and 8, respectively. R1-69 was the kind gift of M. Drottar and S. C. Harrison (Harvard University, Cambridge, Mass.). The concentrations of these proteins

quoted elsewhere in this paper denote the concentrations of active species.

Characterization of CT-1. The amino-terminal 15 amino acids of purified CT-1 and derivatives were determined at the CAMBI Protein Sequencing Facility at the State University of New York, Buffalo. Circular dichroism (CD) spectra of intact 434 repressor, 434 amino-terminal domain, and CT-1 were obtained at 4°C in storage buffer without glycerol by using a JASCO spectropolarimeter equipped with a constant-temperature device. Several scans of each spectrum were obtained, and the averaged spectra are presented.

DNase I footprinting. Footprinting was performed essentially as described in reference 12. Operator DNA (<1 nM), 2×10^{-8} M 434 repressor, and various amounts of purified CT-1 or its deleted derivatives were incubated in buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 1 mM $MgCl_2$, 100 μg of bovine serum albumin (BSA) per ml, and 2.5 μg of sonicated chicken blood DNA per ml at 23°C for 10 min. Under these conditions, the K_D of 434 repressor monomers for O_R1 is 5×10^{-9} M. Sufficient DNase I was added to give, on average, one cleavage per molecule in 5 min of additional incubation. The digested samples were ethanol precipitated, dissolved in a mixture containing 90% formamide and tracking dyes, and fractionated on a denaturing gel (30 by 40 cm) containing 7.5 M urea, 89 mM Tris-HCl (pH 8.9), 89 mM borate, and 1 mM EDTA. The cleavage products were visualized by exposure of the gel to Kodak XAR-5 film in the absence or presence of an intensifying screen at −80°C.

Cross-linking analysis. Cross-linking was performed as described by Finger and Richardson (9). Dimethyl suberimidate (DMS) buffer (160 mM triethanolamine-HCl [pH 8.0], 20 mM magnesium acetate, 2 mM EDTA, 0.2 mM dithiothreitol, 0.2 M NaCl) and cross-linking buffer (60 mM triethanolamine-HCl [pH 8.0], 7.5 mM magnesium acetate, 0.75 mM EDTA, 0.75 mM dithiothreitol, 75 mM NaCl, 20% [vol/vol] glycerol) were prepared and placed on ice. Ten micrograms of protein was added to a total volume of 24 μl of cross-linking buffer. Thirty milligrams of DMS was added to the DMS buffer, titrated to pH 8.5 with 50% (wt/vol) NaOH, and mixed quickly, and 6 μl was placed into each cross-linking reaction mixture. Following a 50-min incubation at 23°C, the reactions were quenched by the addition of 3 μl of 1.4 M ethanolamine. An equal volume of Laemmli's SDS-PAGE gel loading buffer was added, and the reactions products were resolved on an SDS–15% PAGE gel and visualized by Coomassie blue staining.

Immunoblotting. Immunoblots were used to assess the relative levels of carboxyl-terminal protein and its deletion mutants in cell extracts. Antibodies to intact 434 repressor were prepared by inoculating female rabbits with highly purified repressor preparations according to standard procedures. Protein samples were resolved on an SDS–15% PAGE gel. The proteins were subsequently transferred to a 0.1-μm nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), and the membrane was washed, blocked, and then exposed to the primary 434 repressor antibody at a 1:5,000 dilution. Following a wash, the membrane was exposed to the secondary ImmunoGold goat anti-rabbit antibody (Life Technologies, Grand Island, N.Y.) and developed with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) (Life Technologies).

434 repressor dissociation kinetics. Measurements of dissociation rate were performed essentially as described previously (13). Approximately 1 nM labelled, O_R1-containing DNA was incubated with 434 repressor at a concentration sufficient to half-maximally bind the labelled input DNA in a buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM KCl, and 100 μg of BSA per ml. Following a 5-min incubation at room temperature, either buffer or a 20-fold molar excess of CT-1 was added along with a 1,000-fold molar excess of cold competitor DNA. At various times after this addition, portions of each reaction mixture were filtered through a nitrocellulose filter. The filters were dried, the radioactivity was counted, and the data were plotted as ln (percentage of initial counts remaining) versus time. Rate constants were determined by linear least-squares analysis of these plots.

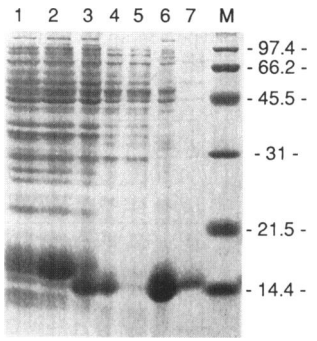

FIG. 1. Purification of CT-1. CT-1 samples taken at various stages of purification are shown. Lanes: 1, whole-cell lysate of uninduced X90 cells bearing pCT1; 2, whole-cell lysate of IPTG-induced X90 cells bearing pCT1; 3, soluble proteins extracted from lysed, induced cells; 4, supernatant following treatment of soluble protein extract with 0.6% polyethyleneimine; 5, supernatant from 20% ammonium sulfate precipitation of polyethyleneimine-treated extract; 6, pellet fraction of 20% ammonium sulfate precipitation; 7, previously purified CT-1; M, molecular weight standards (thousands).

RESULTS AND DISCUSSION

Direct expression of the carboxyl-terminal domain. As a first step in defining the regions of 434 repressor's carboxyl-terminal domain, we constructed pCT1, a plasmid which directs the overproduction of this domain. The pCT1-encoded repressor fragment, CT-1, contains the first 25 amino acids of 434 repressor fused to the final 116 amino acids of the protein. When cells bearing this plasmid are induced with IPTG, the cells produce ~10% of total cellular protein as CT-1 (Fig. 1). As determined by SDS-PAGE, the molecular mass of this 434 repressor derivative closely corresponds to that predicted from the plasmid's DNA sequence (Fig. 1, lane 2). This protein product cross-reacts with an antibody raised against the intact 434 repressor on immunoblots (data not shown). This result confirms the conclusion that the band migrating at 14.5 kDa on the SDS-PAGE gel corresponds to the 434 repressor carboxyl-terminal domain.

CT-1-mediated negative dominance. Control experiments established that the CT-1 protein is unable to bind DNA (data not shown). We reasoned that if CT-1 is capable of oligomerizing with intact 434 repressor, it should act as a negative dominant repressor mutant. If this is true, then the pCT1-encoded C-terminal domain will prevent phage lysogeny. Thus, a 434 phage which infects a cell bearing pCT1 will develop lytically. Table 1 shows that cells bearing pCT1 are incapable of forming any colonies when infected by wild-type 434 phage, whereas cells bearing the control plasmid, pRW218-Bgl, form lysogens at the expected frequency. The specificity of lysogen interference by CT-1 was examined by using wild-type λ phage to infect the cells. As shown in Table 1, cells bearing the control plasmid or pCT1 formed λ lysogens at equal frequen-

TABLE 1. Effect of CT-1 expression on lysogenization frequencies of wild-type 434 and λ phages

Strain	No. of colonies[a] (% lysogeny) in the presence of infecting phage:		
	None	λ	λ *imm*^{434}
X90(pRW218-Bgl)	930	18 (2)	15 (1.6)
X90(pCT-1)	976	20 (2)	0 (0)

[a] Number of colonies appearing on Luria agar-tetracycline plates treated as described in Materials and Methods. Values are averages from three typical experiments. Standard deviations are ±10%.

cies. This indicates that CT-1 specifically interferes with lysogen formation by 434 phage by oligomerizing with 434 repressor.

To better characterize the mechanism of CT-1 negative dominance, the properties of this protein were studied in vitro. As described in Materials and Methods, CT-1 was purified from *E. coli* following IPTG induction. The protein was found in the soluble fraction of the cell extract, and the average yield was 10 mg per liter. Figure 1 characterizes the products of the purification steps through the anion-exchange chromatography step. Peak fractions from this column were further purified by size exclusion chromatography. Overall, the method used to purify these proteins gives products which are judged to be >95% pure from analysis of Coomassie blue-stained SDS-PAGE gels (see also Fig. 6). The molecular weight of the purified CT-1 is lower than that of the protein found in whole-cell extracts. Amino-terminal sequencing of the purified protein reveals that the decrease in molecular weight results from a proteolytic removal of the first 19 amino acids of the protein. Since the gene encoding CT-1 was constructed by deleting an internal fragment of 434 repressor-coding sequence, the residues removed correspond to the first 19 amino acids of intact 434 repressor. Hence, the purified CT-1 contains amino acids 20 to 26 fused to the final 116 amino acids of 434 repressor. The sequence is shown in Fig. 2.

Figure 3 compares the CD spectra of intact 434 repressor, the amino-terminal DNA-binding domain of 434 repressor (R1-69, composed of amino acids 1 to 69 of repressor), and CT-1 protein. As expected from high-resolution structural studies, the CD spectrum of R1-69 has the characteristics of that of an entirely α-helical protein, with minima at 207 and 224 nm. By contrast, the CD spectrum of purified CT-1 displays a single minimum at 205 nm. This suggests that the secondary structure of CT-1 is composed largely of β-sheet, an observation consistent with predictions made from computer analysis of its amino acid sequence (10). Figure 3 shows that the spectrum of intact repressor has the combined characteristics of the spectra of the two isolated domains. Consistent with this assertion, the spectrum of the intact repressor can be

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CT-1  Leu Ala Gln Lys Val Gly Thr
          1                   5                  10                  15                  20
434   Gly Ser Trp Cys Glu Ala Cys Glu Pro Tyr Asp Ile Lys Asp Ile Ala Glu Trp Tyr Asp
P22   Gly Gln Trp Met Glu Ala Val Glu Pro Tyr His Lys Arg Ala Ile Glu Asn Trp His Asp
λ     Gly Met Phe Ser Pro Glu Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu Arg Trp Val Ser

                        25                  30                  35                  40
434   Ser Asp Val Asn Leu Leu Gly Asn Gly Phe Trp Leu Lys Val Glu Gly Asp Ser Met Thr
P22   Thr Thr Val Asp Cys Ser Glu Asp Ser Phe Trp Leu Lys Val Gln Gly Asp Ser Met Thr
λ     Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu Glu Val Glu Gly Asn Ser Met Thr

                        45                  50                  55                  60
434   Ser Pro Val Gly Gln --- --- Ser Ile Pro Glu Gly His Met Val Leu Val Asp Thr Gly
P22   Ala Pro Ala Gly Leu --- --- Ser Ile Pro Glu Gly Met Ile Ile Leu Val Asp Pro Glu
λ     Ala Pro Thr Gly Ser Lys Pro Ser Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu

                        65                  70                  75                  80
434   Arg Glu Pro Val Asn Gly Ser Leu Val Val Ala Lys Leu Thr Asp Ala Asn Glu Ala Thr
P22   Val Glu Pro Arg Asn Gly Lys Leu Val Val Ala Lys Leu Glu Gly Glu Asn Glu Ala Thr
λ     Gln Ala Val Glu Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp --- Glu Phe Thr

                        85                  90                  95                  100
434   Phe Lys Lys Leu Val Ile Asp Gly Gly Gln Lys Tyr Leu Lys Gly Leu Asn Pro Ser Trp
P22   Phe Lys Lys Leu Val Met Asp Ala Gly Arg Lys Phe Leu Lys Pro Leu Asn Pro Gln Tyr
λ     Phe Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn Pro Gln Tyr

                        105                 110                 115                 120
434   Pro Met Thr Pro Ile Asn Gly Asn Cys Lys Ile Ile Gly Val Val Val Glu Ala Arg Val
P22   Pro Met Ile Glu Ile Asn Gly Asn Cys Lys Ile Ile Gly Val Val Val Asp Ala Lys Leu
λ     Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly Lys Val Ile Ala Ser Gln Trp

                        125
434   Lys Phe Val
P22   Ala Asn Leu Pro
λ     Pro Glu Glu Thr Phe Gly
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FIG. 2. Sequence comparison of the carboxyl-terminal domains of the repressors of bacteriophages 434, P22, and λ (20). The sequences are aligned at their RecA cleavage sites and are numbered beginning from this amino acid. This site corresponds to amino acid 112 in λ repressor, 89 in 434 repressor, and 95 in P22 repressor. The locations of mutations in P22 (22) and/or λ (3, 5, 11, 26) repressors which alter their ability to bind cooperatively are underlined. The arrow indicates the site at which amino acids 20 to 26 of 434 repressor are fused to CT-1. The sequences of these amino acids in CT-1 are given. The boxed regions denote the positions of amino acid identity in the three proteins.

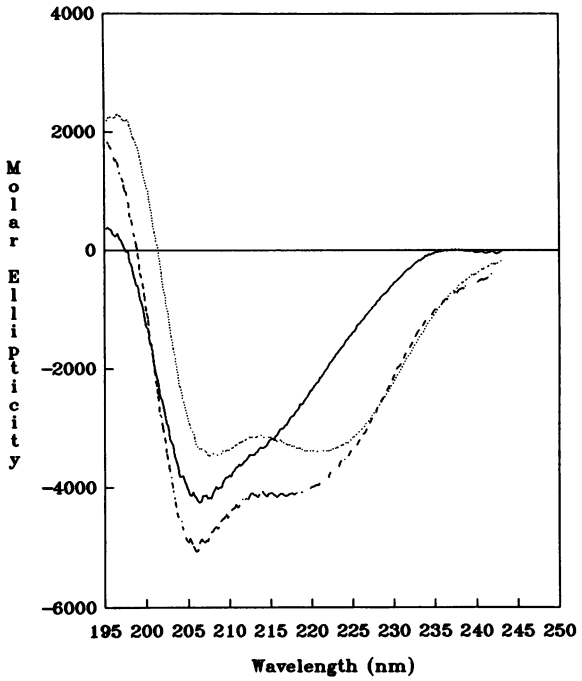

FIG. 3. CD spectra of CT-1 (solid line), R1-69 (dotted line), and 434 repressor (dashed line). Spectra were recorded as described in Materials and Methods.

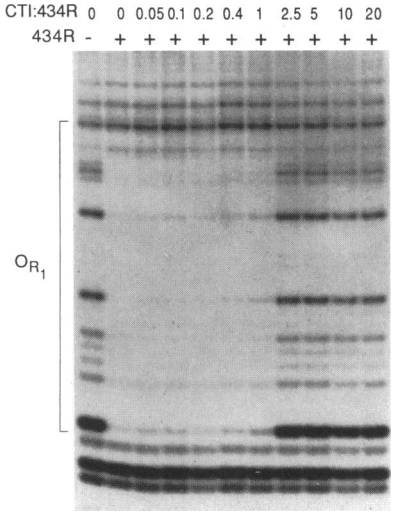

FIG. 4. Effect of CT-1 on the binding of 434 repressor to O_R1. Shown are the DNase I digestion patterns of an O_R1-containing DNA fragment in the absence (−) or presence (+) of saturating concentrations of intact 434 repressor with increasing amounts of CT-1. The numbers denote the molar ratio of CT-1 protein added to total amount of added repressor. The repressor concentration (20 nM) remained constant in all + lanes and is expressed as the concentration of repressor monomers active in DNA binding. The concentration of CT-1 was determined spectrophotometrically.

simulated by adding the R1-69 and CT-1 spectra together (not shown). These observations indicate that the structures of the carboxyl-terminal domain in the intact repressor and isolated fragment are similar.

The ability of purified CT-1 to interfere with the formation of intact 434 repressor-DNA complexes was examined by DNase I footprinting. Figure 4 shows that adding a 2.5:1 molar ratio of CT-1 to a mixture of 434 O_R1 and intact repressor completely prevents 434 repressor from forming a specific protein-DNA complex at this operator. Under identical conditions, addition of a 20-fold molar excess of CT-1 to a P22 repressor-P22 operator complex did not interfere with P22 repressor-DNA complex formation. This shows that CT-1 interference with 434 repressor DNA binding is due to a specific effect on 434 repressor and not to a general effect of CT-1 addition on protein stability. Together these results show that CT-1 interferes with lysogen formation in vivo by preventing the binding of 434 repressor to operator DNA.

Mechanism of CT-1-mediated interference. An unusual aspect of the experiments shown in Fig. 4 is that CT-1-mediated interference with 434 repressor binding appears to exhibit threshold, or all-or-none, behavior. If the association constants for repressor homodimer and repressor–CT-1 heterodimer formation are equal, we would expect that CT-1 addition would titrate 434 repressor off the DNA in a stepwise fashion. We therefore attempted a further characterization of the mechanism of interference.

CT-1-mediated interference with the formation of 434 repressor-operator complexes could be hypothesized to occur by either of two mechanisms. In the first case, CT-1 can be imagined to interact with a DNA-bound repressor dimer. Such a heteromeric interaction could catalyze a conformational change in DNA-bound 434 repressor which is incompatible with stable protein-DNA complexes. In this case, CT-1 would increase the dissociation rate of the 434 repressor dimer. Figure 5 shows that the addition of CT-1 does not significantly increase the dissociation rate of 434 repressor-O_R1 complexes. In both the absence and presence of CT-1, 434 repressor dissociates at a rate of 0.016 s^{-1}. Hence, CT-1 does not decrease the stability of these complexes.

Alternatively, CT-1 may interfere with the formation of 434

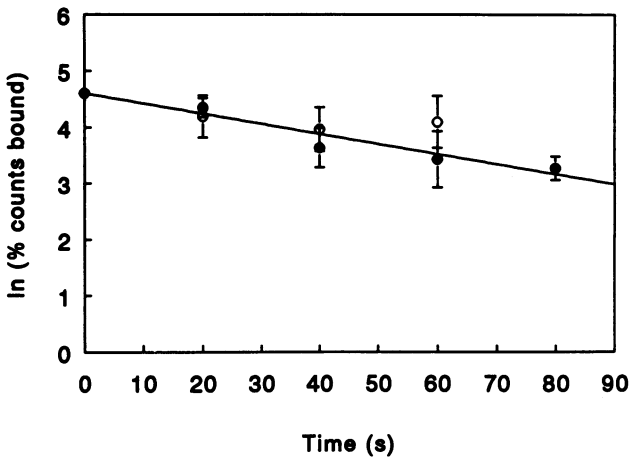

FIG. 5. Effect of CT-1 on the dissociation kinetics of 434 repressor-operator complexes. Plotted is the ln (percentage of initial counts remaining) as a function of time after addition of a 1,000-fold molar excess of cold competitor DNA to a preformed complex between 434 repressor and O_R1. Open circles, reactions performed in the absence of CT-1; closed circles, reactions performed in the presence of a 20-fold molar excess of CT-1 to total added repressor. The two values determined in the absence or presence of CT-1 at 0 and 80 s are identical and therefore overlap. The line represents a linear least-squares fit to the data; the same least-squares fitted line describes both data sets.

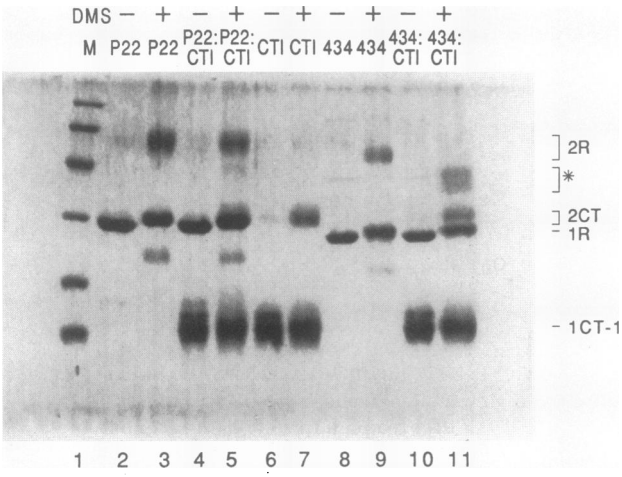

FIG. 6. Cross-linking analysis of protein-protein complexes. The products of cross-linking reactions performed with various mixtures of CT-1, 434 repressor, and P22 repressor are shown. Lane 1 shows molecular weight markers. For lanes 2 to 11, even-numbered lanes contain proteins incubated in buffer in the absence of DMS cross-linker, and odd-numbered lanes contain proteins incubated in the presence of cross-linker. Lanes 2 and 3, P22 repressor; lanes 4 and 5, equimolar amounts of P22 repressor and CT-1; lanes 6 and 7, CT-1; lanes 8 and 9, 434 repressor; lanes 10 and 11, equimolar amounts of 434 repressor and CT-1. The positions of CT-1 monomer (1CT-1), P22 and 434 repressor monomers (1R), CT-1 homodimer (2CT), P22 and 434 repressor homodimers (2R), and 434 repressor–CT-1 heterodimer (*) are indicated on the right. The band appearing at ~23 kDa in lanes 3, 5, and 9 is of unknown origin. Immunoblots probed with anti-434 repressor or anti-P22 repressor antibodies show that this band is not derived from either of these proteins or CT-1.

repressor-operator complexes by forming a heterodimeric complex with one monomer of intact 434 repressor. This heterodimer would contain only one DNA-binding domain. Since 434 repressor binds DNA specifically only as a dimer (12a), the repressor–CT-1 heterodimer would be unable to form a specific complex with DNA. To test this idea, the types of complexes formed in solutions of 434 repressor, CT-1, and their mixtures were examined by chemical cross-linking. Adding cross-linker to a solution of purified CT-1 results in the formation of a species with a molecular mass of 28 kDa, which is similar to that expected for a CT-1 homodimer (Fig. 6, lanes 6 and 7). No higher-molecular-mass species were detected. Under similar conditions, 434 repressor forms a cross-linked species with a molecular mass of 45 kDa (compare lanes 8 and 9 in Fig. 6). This indicates that at this concentration, 434 repressor also forms only homodimeric complexes. A comparison of lanes 10 and 11 of Fig. 6 shows that when a mixture of CT-1 and 434 repressor is incubated with cross-linker, a new species with a molecular mass distinct from that of either of the homodimeric products is formed. The apparent molecular mass of this species corresponds to that of a 434 repressor–CT-1 heterodimer. To confirm that the observed 434 repressor–CT-1 interaction detected by cross-linking is specific, these experiments were repeated with mixtures of P22 repressor and CT-1. As shown in Fig. 6 (lanes 2 to 5), no species indicative of a P22 repressor–CT-1 heterodimer is observed. This result is consistent with the observation that added CT-1 does not interfere with the stability of P22 repressor-DNA complexes (data not shown). Taken together, these data strongly suggest that CT-1 interferes with the formation of 434 repressor-DNA complexes by forming heterodimeric complexes with unbound 434 repressor.

A comparison of lanes 9 and 11 in Fig. 6 shows that when CT-1 and 434 repressor are incubated together, the amount of repressor homodimer is decreased and the yield of CT-1 homodimer remains constant. These observations indicate that the stabilities of CT-1 homodimers and 434 repressor–CT-1 heterodimers are similar and exceed that of the 434 repressor homodimers. That the CT-1 homodimer complex is more stable than the intact repressor homodimer is consistent with the observation that intact repressor elutes from gel filtration columns as a monomer at concentrations lower than 1 μM, while CT-1 chromatographs as a dimer at concentrations at least as low as 10 nM (data not shown).

The suggestion that repressor homodimers are significantly weaker than either CT-1 homodimers or CT-1–intact repressor heterodimers helps to explain the threshold behavior of CT-1-mediated interference with 434 repressor-DNA complex formation seen in Fig. 4. A numerical simulation in which the equilbria

$$2\,R \underset{}{\overset{K_R}{\leftrightarrows}} R^2$$

$$2\,CT\text{-}1 \overset{K_C}{\leftrightarrows} CT\text{-}1^2$$

$$R + C \overset{K_{RC}}{\leftrightarrows} RC$$

$$R^2 + O_R1 \overset{K_D}{\leftrightarrows} R^2 \cdot O_R1$$

occur simultaneously showed that when the dimer dissociation constant (K_R) of intact 434 repressor (R) is set at 1 μM and the dissociation constants for CT-1 dimers (K_C) and CT-1–repressor heterodimers (K_{RC}) are assumed to be 1 nM, use of the known dissociation constant (K_D) for repressor-O_R1 complexes (here ~10^{-12} M), predicts that increasing CT-1 concentrations would abruptly interfere with the repressor-O_R1 complex at a CT-1/repressor ratio of about 2:1. This is because in this range of CT-1 concentration, the concentration of intact repressor dimer, the active DNA-binding species, would be reduced by an amount greater than the amount of CT-1 added. In agreement with experimental observation, the simulation also predicts that the concentration of CT-1 needed to compete with repressor would be relatively independent of DNA concentration (data not shown).

Deletion analysis of CT-1. The ability of the carboxyl-terminal domain to interfere with lysogen formation provides the basis of a screen for loss-of-function mutations in the carboxyl-terminal domain. Under normal growth conditions, cells bearing nonfunctional mutant CT-1 will be lysogenized by phage 434 at a frequency of 1 to 5% and will therefore form colonies on plates seeded with wild-type 434 phage, whereas cells producing functional CT-1 do not form colonies in the presence of λ*imm*^{434} phage (Table 1).

Deletion analysis was used as a first step towards determining which part of the pCT1-encoded carboxyl-terminal domain is responsible for mediating the interference with intact repressor function. Figure 7 shows that deletion of the last nine amino acids of CT-1 (115 to 123) does not diminish the ability of CT-1 to interfere with lysogeny. Removal of the next three amino acids (Val-114, Gly-113, and Ile-112) results in a loss of the ability of CT-1 to interfere with lysogen formation and partially destabilizes CT-1. Additional derivatives bearing deletions of amino acids more amino terminal than Lys-110 accumulate to levels in the cell similar to those of the full-

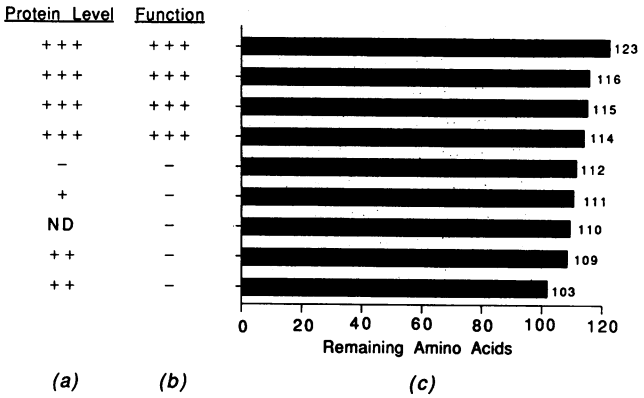

FIG. 7. Summary of the effects of C-terminal deletion on CT-1 protein level in vivo (a), lysogen interference (b), and sequence (c). Protein levels were determined from SDS-gel electrophoretic analysis of whole-cell extracts. Function was assayed by the ability of cells bearing the derivatized constructs to form lysogens on plates seeded with wild-type 434 bacteriophage. +++, no lysogens were formed (see Materials and Methods for details). For protein level, +++, ++, +, and − indicate that ~10%, 7%, 2%, and none of the total cell protein was produced as CT-1, respectively. The sequence of the protein is inferred from the DNA sequences of the deleted plasmids. ND, not determined.

length CT-1 and are also unable to interfere with lysogen formation. This result indicates that the impaired function of these deleted proteins is probably not due to misfolding, since proteins bearing this type of defect are expected to accumulate to significantly lower intracellular levels or not at all (15, 16). These observations indicate that amino acids 110 to 114 lie in the dimerization surface of the carboxyl-terminal domain or that a critical part of the dimer interface is formed by a structure which is stabilized by these amino acids.

Extensive mutagenesis and biochemical studies of the repressors of the related lambdoid bacteriophages λ and P22 have located the amino acids which are part of the cooperativity interfaces of these two proteins (3, 5, 11, 22, 26). As demonstrated by cooperativity "swap" experiments between P22 and λ repressor (26), the critical part of this interface lies between amino acids 86 and 94 (Fig. 2). To date, similarly detailed studies on the location of the dimerization interface of either λ, P22, or 434 repressor have not been reported. Our deletion analysis provides evidence that a critical part of the dimerization interface in 434 repressor's carboxyl-terminal domain lies beyond amino acid Lys-110 of CT-1. This conclusion is consistent with the results that mutation of Ser-228 to Asn/Arg or of Thr-234 to Lys in λ repressor (amino acids 118 and 124 in Fig. 2) decreases its dimerization constant (3, 5, 11). It is interesting that deletion of the region which affects dimerization does not overlap the part of the sequence which has been implicated to be critical for cooperativity in the homologous λ and P22 repressor proteins. This observation suggests that the cooperativity and dimerization interfaces in the carboxyl-terminal domains of the phage repressors are segregated into two distinct, but nearby, regions of the proteins' primary structures. This conclusion is supported by the observation that substituting amino acids 183 to 209 (96 to 123 in Fig. 2) of intact 434 repressor with those of the homologous region of P22 repressor eliminates CT-1's ability to block binding of the chimeric protein to DNA, whereas protein-DNA complexes between 434 operator and a 434 repressor hybrid bearing P22 repressor amino acids 171 to 183 (80 to 96 in Figure 2) do not (5a). Further work is required to confirm that dimerization and cooperativity functions are segregated in the primary sequence and to determine whether these interfaces display similar segregation in the tertiary structures of these domains.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM42138 from the National Institutes of Health.

We thank the members of our laboratory, and P. Gollnick, and E. Brody for helpful suggestions. We also thank the reviewers of an earlier version of the manuscript, whose comments increased the clarity of the manuscript.

REFERENCES

1. **Aggarwal, A., D. W. Rodgers, M. Drottar, M. Ptashne, and S. C. Harrison.** 1988. Recognition of a DNA operator by the repressor of phage 434: a view at high resolution. Science **242**:899–907.
2. **Anderson, J. E., M. Ptashne, and S. C. Harrison.** 1984. Co-crystals of the DNA-binding domain of phage 434 repressor and a synthetic 434 operator. Proc. Natl. Acad. Sci. USA **81**:1307–1311.
3. **Beckett, D., D. S. Burz, G. K. Ackers, and R. T. Sauer.** 1993. Isolation of lambda repressor mutants with defects in cooperative operator binding. Biochemistry **32**:9073–9079.
4. **Bell, A. C., and G. B. Koudelka.** 1993. Operator sequence context influences amino acid-base-pair interactions in 434 repressor-operator complexes. J. Mol. Biol. **234**:542–553.
5. **Benson, N., C. Adams, and P. Youderian.** 1994. Genetic selection for mutations that impair the co-operative binding of lambda repressor. Mol. Microbiol. **11**:567–579.

5a. **Carlson, P. A., and G. B. Koudelka.** Unpublished results.

6. **Coulondre, C., and J. H. Miller.** 1977. Genetic studies of the lac repressor. III. Additional correlation of mutational sites with specific amino acid residues. J. Mol. Biol. **117**:525–567.
7. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **DeAnda, J., A. R. Poteete, and R. T. Sauer.** 1983. P22 c2 repressor-domain structure and function. J. Biol. Chem. **258**:10536–10542.
9. **Finger, L. R., and J. P. Richardson.** 1982. Stabilization of the hexameric form of Escherichia coli protein rho under ATP hydrolysis conditions. J. Mol. Biol. **156**:203–219.
10. **Genetics Computer Group.** 1991. Program manual for the GCG package, 7th ed. University of Wisconsin, Madison.
11. **Hochschild, A., and M. Ptashne.** 1988. Interaction at a distance between lambda repressors disrupts gene activation. Nature (London) **336**:353–357.
12. **Johnson, A. D., B. J. Meyer, and M. Ptashne.** 1979. Interaction between DNA-bound repressors govern regulation by the lambda repressor. Proc. Natl. Acad. Sci. **76**:5061–5065.

12a. **Koudelka, G.** Unpublished results.

13. **Koudelka, G. B., and P. Carlson.** 1992. DNA twisting and the effects of non-contacted bases on affinity of 434 operator for 434 repressor. Nature (London) **355**:89–91.
14. **Koudelka, G. B., S. C. Harrison, and M. Ptashne.** 1987. Effect of non-contacted bases on the affinity of 434 operator for 434 repressor and Cro. Nature (London) **326**:886–888.
15. **Lim, W. A., D. C. Farruggio, and R. T. Sauer.** 1992. Structural and energetic consequences of disruptive mutations in a protein core. Biochemistry **31**:4324–4333.
16. **Lim, W. A., and R. T. Sauer.** 1991. The role of internal packing interactions in determining the structure and stability of a protein. J. Mol. Biol. **219**:359–376.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Ptashne, M.** 1986. A genetic switch. Blackwell Press, Palo Alto, Calif.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis, and C. O. Pabo.** 1982. Homology among DNA-binding proteins suggests use

of a conserved supersecondary structure. Nature (London) **298:** 447–451.
21. **Seed, B.** 1983. Purification of genomic sequences from bacteriophage libraries by recombination and selection in vivo. Nucleic Acids Res. **11:**2427–2445.
22. **Valenzuela, D., and M. Ptashne.** 1992. P22 repressor mutant deficient in cooperative binding and DNA loop formation. EMBO J. **8:**4345–4350.
23. **Wharton, R. P.** 1986. Ph.D. thesis. Harvard University, Cambridge, Mass.
24. **Wharton, R. P., E. L. Brown, and M. Ptashne.** 1985. Substituting an α-helix switches the sequence specific DNA interactions of a repressor. Cell **38:**361–369.
25. **Wharton, R. P., and M. Ptashne.** 1985. Changing the binding specificity of a repressor by redesigning an α-helix. Nature (London) **316:**601–605.
26. **Whipple, F., N. H. Kuldell, L. A. Cheatham, and A. Hochschild.** 1994. Specificity determinants for the interaction of lambda and P22 repressor dimers. Genes Dev. **8:**1212–1223.