Role of the Cro Repressor Carboxy-Terminal Domain and Flexible Dimer Linkage in Operator and Nonspecific DNA Binding†

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ABSTRACT: A series of mutations comprising single and multiple substitutions, deletions, and extensions within the carboxy-terminal domain of the bacteriophage λ Cro repressor have been constructed. These mutations generally affect the affinity of repressor for specific and nonspecific DNA. Additionally, substitution of the carboxy-terminal alanine with several amino acids capable of hydrogen-bonding interactions leads to improved specific binding affinities. A mutation is also described whereby cysteine links the two Cro monomers by a disulfide bond. As a consequence, a significant improvement in nonspecific binding and a concomitant reduction in specific binding are observed with this mutant. These results provide evidence that the carboxy terminus of Cro repressor is an important DNA binding domain and that a flexible connection between the two repressor monomers is a critical factor in modulating the affinity of wild-type repressor for DNA.

The fundamental importance of sequence-specific DNA binding proteins in the regulation of gene expression has resulted in a concentrated effort to understand how these proteins locate and interact with their DNA binding sites. As the interaction of a protein with DNA is generally believed to occur through hydrogen bonding as well as electrostatic and hydrophobic contacts (Pabo & Sauer, 1984), the challenge is to define the precise nature of these recognition processes for a particular protein.

The elucidation of the tertiary structure of λ Cro repressor by X-ray crystallography (Anderson et al., 1981) and its confirmation by 2-D NMR (Weber et al., 1985) have made it possible to predict the interactions that occur between this 66 amino acid protein and the operators to which it binds. In common with other DNA binding proteins whose crystal structures have been determined [including the catabolite gene activator protein (McKay & Steitz, 1981), tryptophan repressor from *Escherichia coli* (Schevitz et al., 1985), and the bacteriophage 434 cI repressor in complex with its operator (Anderson et al., 1987)], Cro repressor has a protruding bihelical unit which is postulated to provide most of the sequence-specific contacts with operator DNA. The model defining these contacts (Ohlendorf et al., 1982) requires that these bihelical regions interact with consecutive major groove domains located on one face of the operator. Site-directed mutagenesis studies support and modify this hypothesis by showing that certain amino acids within this domain are important contact sites (Eisenbeis et al., 1985; Hochschild & Ptashne, 1986; Caruthers et al., 1986, 1987).

On the basis of crystallographic and 2-D NMR studies, the five carboxy-terminal amino acids of Cro repressor do not assume a regular structure. The model suggests that a portion of the flexible carboxy-terminal arm lies in the minor groove of operator DNA and participates in sequence-independent interactions. Here we describe attempts, using site-directed mutagenesis, to probe the role of this carboxy-terminal domain in its binding to DNA. We show that this region contributes significantly to the stability of repressor–operator and repressor–nonspecific DNA complexes. Additional structural flexibility has been proposed as a result of the intersubunit pairing of Glu-54, Val-55, and Lys-56. A mutation is reported which reduces this flexibility and also significantly changes the relative affinity of the resulting repressor for both specific and nonspecific DNA sequences.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* strain MY202 (Δ lac-pro Spc^R/F′lac $I^q pro^+$) was transformed with derivatives of the plasmid pJS305 (Sutton, 1985). Plasmid pJS305 carried a nonfunctional *cro* gene under control of the *tac* promoter. Restoration of a functional *cro* gene was achieved by replacing the short *Bam*HI–*Sal*I linker fragment, which contained a unique *Xho*I site, with synthetic DNA defining the amino acid sequence between amino acid positions 50 and 66. Background, due to plasmids which still contained the linker after the ligation step, was eliminated by cutting with *Xho*I prior to transformation.

In order to introduce simultaneously a large number of mutations at one amino acid position within the *Bam*HI–*Sal*I gene region, a new *Bam*HI–*Sal*I segment was chemically synthesized so that all possible codons were positioned at a target amino acid (Caruthers, 1985). This was accomplished by introducing all four nucleotides, via a mixture of the deoxynucleoside phosphoramidites, at a target codon position within the new *Bam*HI–*Sal*I gene region. Theoretically, all possible amino acid substitutions at a defined codon could be generated by this procedure. For the remainder of mutations, synthetic DNA defining the specific amino acid change replaced the *Bam*HI–*Sal*I linker. The plasmids generated from this chemical mutagenesis procedure were designated pCTmut.

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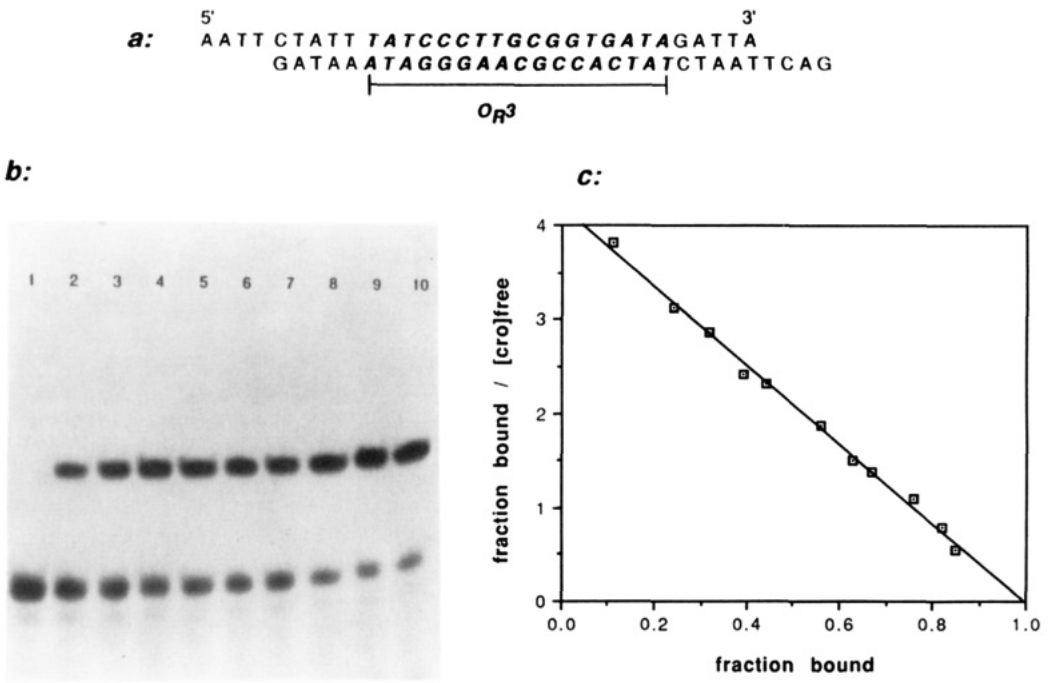

FIGURE 1: Determination of the relative specific equilibrium constants for Cro repressors. Chemically synthesized deoxyoligonucleotides shown in (a) were labeled using the Klenow fragment of DNA polymerase and deoxynucleotide [α-^{32}P]triphosphates (see text) to yield a 35 base pair O_R3 duplex. Various concentrations of Cro repressor and operator were incubated together on ice in binding buffer, dyes were added, and the total solution was loaded onto a polyacrylamide gel (b). Lane 1, free operator; lanes 2–10, operator with increasing amounts of repressor. Bands corresponding to operator and Cro repressor–operator complex were excised and counted. The concentration of active repressor was determined at $[O_R3] = 1 \times 10^{-8}$ M where all active protein was bound to operator. The equilibrium binding constants were determined at $[O_R3] = 5 \times 10^{-11}$ M. When $\nu/[\text{Cro}]_{\text{free}}$ was plotted versus ν as shown in (c), a straight line was obtained and used to calculate the equilibrium binding constant (slope = $-1/K_{ds}$); $\nu = [\text{Cro-O}_r3]/[\text{Cro-O}_R3] + [O_R3])$; $[\text{Cro}]_{\text{free}} = [\text{Cro}] - [\text{Cro-O}_R3]$.

Molecular Cloning. Isolation of plasmid, DNA cleavage with restriction endonucleases, isolation of DNA fragments, ligations with T4 DNA ligase, and transformations of *E. coli* were carried out as described by Maniatis et al. (1982).

Synthetic Gene Sequences. Gene sequences were confirmed by the method of Sanger et al. (1977) using reverse transcriptase (AMV, Life Sciences).

Cell Crude Lysates. These were produced by inoculating 5 mL of Luria broth with 10 μL of an overnight culture of *E. coli* strain MY202/pCTmut. The cells were grown to an OD_{600} of 0.3, induced with 10 mM IPTG for 3.5 h, pelleted, and resuspended in lysis buffer [50 mM potassium phosphate (pH 7.3), 0.1 mM EDTA, 100 mM KCl, 5% glycerol, 100 μg/mL phenylmethanesulfonyl fluoride, and 0.03% β-mercaptoethanol]. After sonication (W-225 Heat Systems ultrasonic processor, Ultrasonics Inc.), the cell debris was pelleted and the supernatant brought to 40% glycerol. Protein preparations were stored at −70 °C.

Protein Purification. A 10-mL overnight culture of *E. coli* strain MY202/pCTmut was used to inoculate 1 L of Luria broth containing 45 μg/mL ampicillin. The cells were grown to an OD_{600} of 0.3, induced with 10 mM IPTG for 3.5 h, and pelleted by centrifugation at 5000 rpm for 10 min at 4 °C. The cells were then resuspended in 10 mL of lysis buffer and ruptured by soniction, the cellular debris was removed by centrifugation, and the supernatant was applied to a DE-52 column preequilibrated with lysis buffer (packed volume = 2.5 × 6 cm^3). The column was washed with lysis buffer at a flow rate of 3 mL/min, and 1.5-mL fractions collected. The fractions containing Cro repressor, as determined from the coelectrophoresis assay (Figure 1), were pooled and dialyzed against 2 L of PE buffer–100 mM KCl [PE buffer is 50 mM phosphate (pH 6.8), 0.1 mM EDTA, 5% glycerol, 0.03% β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride] using Spectrapore 1 tubing. The dialysate was applied to a CM-Sephadex C-50 column preequilibrated with PE buffer–100 mM KCl (packed volume = 2.5 × 4 cm^3). The column was developed with 10 mL of PE buffer–100 mM KCl, 15 mL of PE buffer–150 mM KCl, 15 mL of PE buffer–400 mM KCl, and 20 mL of PE buffer–600 mM KCl (1.5-mL fractions). By use of the coelectrophoresis assay as outlined in Figure 1, Cro repressor starts to elute from the column during the PE buffer–400 mM KCl wash. After fractions containing Cro repressor were pooled, the protein was concentrated by ammonium sulfate precipitation and stored at −70 °C in standard binding buffer containing 50% glycerol and no BSA. Cro repressor purified by this procedure was homogeneous by SDS–polyacrylamide gel electrophoresis as only one band was observed (silver or Coomassie Brilliant Blue staining).

Specific Equilibrium Binding Constants. A 35 base pair, radioactively labeled O_R3 duplex was prepared by a combination of chemical synthesis of the deoxyoligonucleotide shown in Figure 1 followed by repair synthesis using the Klenow fragment of *E. coli* DNA polymerase (U.S. Biochemicals) and deoxynucleotide [α-^{32}P]triphosphates. Various concentrations of Cro repressor protein (either in purified form or from cell crude lysates) and labeled operator were incubated together on ice for 15 min in 20 μL of binding buffer [10 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM DTT, 20 mM KCl, and 50 μg/mL BSA], diluted with dye (3 μL of 80% glycerol containing 0.1% bromophenol blue), and loaded onto a 5% polyacrylamide gel using a 1 × TBE gel buffer system [1 × TBE is 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, and 2.5 mM disodium (ethylenedinitrilo)-tetraacetic acid]. The Cro repressor–operator complex moved more slowly than duplex DNA in this gel–buffer system. After autoradiography, the appropriate gel bands containing Cro repressor–operator complex and free operator were excised and counted. All mutant proteins were assumed to exist as dimers

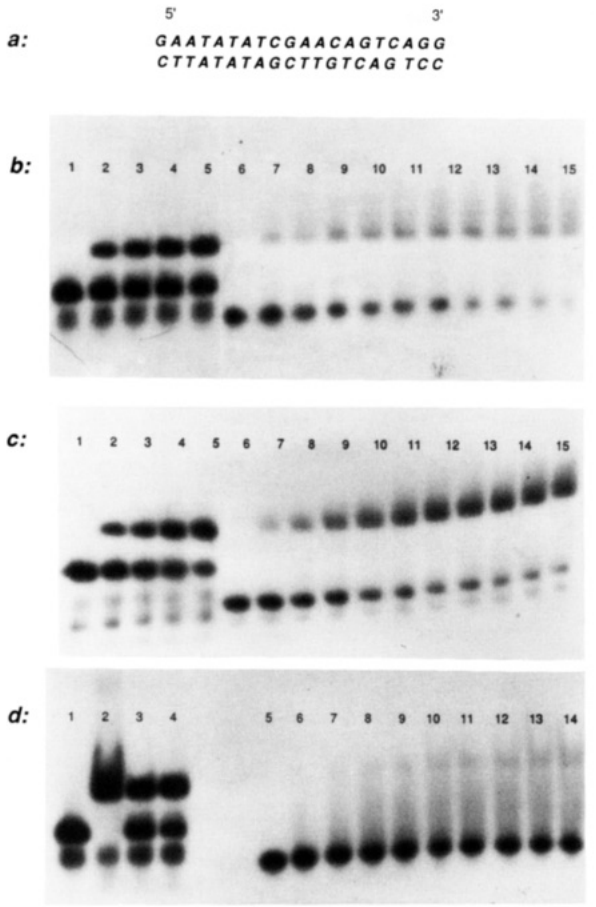

FIGURE 2: Determination of the relative nonspecific equilibrium constants for Cro repressors. A 20 base pair synthetic duplex was chemically synthesized (a) and labeled as described in the text. It was designed to be blunt-ended in order to avoid the possibility of Cro recognizing nonspecifically any single-stranded overhang as has been observed for RNA polymerase (Melancon et al., 1983). This duplex and various concentrations of either purified Cro repressor or mutant repressor were incubated together on ice in binding buffer before addition of dyes and then loaded onto polyacrylamide gels (see text). The concentration of active repressor was determined at $[O_R3] = 1 \times 10^{-8}$ M (tracks 1–5 in panels b and c; tracks 1–4 in panel d). Nonspecific DNA ($[DNA] = 1 \times 10^{-7}$ M usually or 1×10^{-8} M for mutants with higher nonspecific relative affinities) was incubated with increasing amounts of purified repressors. Results with various repressors were as follows: (b) wild-type repressor, lanes 6–15; (c) purified Lys-64, lanes 6–15; (d) purified ∂4, lanes 5–14. Bands corresponding to free and complexed DNA were excised and counted as described in the text. The data were interpreted in the same manner as for determining specific equilibrium binding constants.

in solution. Additionally, Scatchard independent binding site analysis was used to interpret the data as there is no evidence to suggest that Cro repressor exhibits cooperative binding nor have any effector molecules been identified (Johnson et al., 1979).

Nonspecific Equilibrium Binding Constants. A 20 base pair DNA duplex (Figure 2) having no sequence homology to any operators recognized by Cro repressor was chemically synthesized. The individual strands were separately labeled with ^{32}P (T4 kinase from U.S. Biochemicals and [γ-^{32}P]ATP), added to binding buffer, and annealed by heating together at 90 °C and slow cooling to 0 °C. Various concentrations of purified Cro repressor were incubated with the labeled, nonspecific DNA on ice for 15 min in 20 μL of binding buffer, and then 3 μL of 80% glycerol containing 0.1% bromophenol blue was added. The samples were loaded onto a polyacrylamide gel similar to that employed to measure specific binding except that the buffer system was 0.2 × TBE. Some representative autoradiograms from this assay are presented in Figure 2. For mutants whose nonspecific affinities were similar to or better than that of the wild-type repressor, the complex was seen as a tight band on the autoradiogram. However, for those mutants with much lower nonspecific relative affinities, the Cro repressor–DNA complex was a smear beginning at the point where the complex would be expected to migrate and then trailing to the free DNA. For this reason and in every experiment, the excised band of Cro repressor or Cro repressor mutant with DNA was the region from the complex to the free DNA. These data were interpreted in a manner similar to the specific binding results. Because the nonspecific interaction of Cro repressor with DNA is much more sensitive to salt than is the specific interaction, the ionic strength of the buffer system in the nonspecific binding assay was only 20% of that used to measure specific binding. As a consequence, the specific and nonspecific binding affinities cannot be compared directly. Instead, a comparison of these two equilibrium constants for a particular mutant repressor was made via affinities relative to wild-type Cro. This parameter is called the selectivity for a particular repressor and is the ratio of specific to nonspecific relative affinities (see Table IV).

Carboxy-Terminal Amino Acid Analysis. This analysis was performed for all the Cro repressors listed in Table IV. Purified protein (60 pmol) was digested with carboxypeptidase A (Boehringer Mannheim) in 100 mM Hepes buffer, pH 8, and 56 mM sodium dodecyl sulfate at 37 °C. Aliquots were removed at intervals, and the reaction was stopped by addition of an equal volume of 10% trichloroacetic acid in water. After centrifugation, the supernatant was recovered, and the released amino acids were reacted with *o*-phthaldehyde (Roth, 1971). These fluorescent derivatives were separated and identified by using an Adsorbosphere OPA-HS column (Alltech Associates, Inc.) utilizing buffers and procedures found in the column operating instructions. In every case, the amino acid first released was found to be the carboxy-terminal amino acid as predicted from the synthetic gene sequence.

RESULTS

A synthetic *cro* gene with new restriction endonuclease sites in the coding sequence has been constructed (Eisenbeis et al., 1985). These sites allow portions of the gene to be removed and replaced with DNA duplexes bearing desired mutations. This type of cassette mutagenesis, which is similar to an approach published concurrently (Wells et al., 1985), was used to alter the length of the carboxy terminus by amino acid extensions and deletions, introduce a variety of amino acid substitutions between positions 60 and 66, and link the two Cro monomers via a disulfide bond by substituting cysteine at position 55. The objectives were to probe whether amino acid substitutions near the carboxy terminus altered the specific and nonspecific interactions of Cro repressor with Cro operator and nonspecific DNA.

In order to quantitatively determine the affinities of the various Cro repressor mutants for operator and nonspecific DNA, a polyacrylamide gel coelectrophoresis system was used (Fried & Crothers, 1981; Garner & Revzin, 1981). The operator as O_R3, the λ operator to which Cro repressor binds with highest affinity (Johnson et al., 1978), was prepared as part of a 35 base pair duplex. It was generated by chemically synthesizing two deoxyoligonucleotides followed by repair synthesis with the Klenow fragment of *E. coli* DNA polymerase and used to obtain the relative equilibrium binding constants for mutant Cro repressors (Figure 1). The results from these studies were compared to the equilibrium binding constant of wild-type Cro repressor with the same duplex and

Table I: Specific Relative Affinities for Cro Repressors Having Deletions and Basic Amino Acid Mutations in the Carboxy-Terminal Seven Amino Acids

amino acid sequence from position 60	mutant[a]	specific relative affinity[b]
Ser-Asn-Lys-Lys-Thr-Thr-Ala	wild type	1.00
Ser-Asn-Lys-Lys-Thr-Thr	∂1	1.20
Ser-Asn-Lys-Lys-Thr	∂2	1.00
Ser-Asn-Lys-Lys	∂3	0.35
Ser-Asn-Lys	∂4	0.01
Ser-Asn	∂5	NP[c]
Ser-Asn-Lys-Gly-Thr-Thr-Ala	Gly-63	0.48
Ser-Asn-Gly-Lys-Thr-Thr-Ala	Gly-62	0.29
Ser-Asn-Gly-Gly-Thr-Thr-Ala	Gly-62,63	NP
Ser-Gly-Lys-Lys-Thr-Thr-Ala	Gly-61	0.41
Ser-Asn-Lys-Gly-Gly-Gly-Ala	Gly-63–65	0.43
Ser-Asn-Lys-Gly-Gly-Gly-Gly-Ala	Gly-63–66–Ala	0.92
Ser-Asn-Lys-Gly	Gly-63∂3	0.32
Ser-Asn-Thr-Lys-Thr-Lys-Ala	TKTKA	0.13
Ser-Asn-Thr-Thr-Lys-Lys-Ala	TTKKA	0.07
Lys-Asn-Lys-Lys-Thr-Thr-Ala	Lys-60	0.97
Ser-Lys-Lys-Lys-Thr-Thr-Ala	Lys-61	1.60
Ser-Asn-Lys-Lys-Lys-Thr-Ala	Lys-64	1.02
Ser-Asn-Lys-Lys-Lys-Lys-Ala	Lys-64,65	0.94
Ser-His-Lys-Lys-Thr-Thr-Ala	His-61	1.00
Ser-Asn-Arg-Lys-Thr-Thr-Ala	Arg-62	1.25
Ser-Asn-Lys-Arg-Thr-Thr-Ala	Arg-63	NP

[a] Mutant repressors are defined in the text. [b] The ratio of specific (s) equilibrium binding constants, K_{ds}[wild type]/K_{ds}[mutant]. Specific equilibrium binding constant for wild type = 2.4×10^{-10} M. [c] No protein.

Table II: Specific Relative Affinities for Cro Repressors Having Serine, Threonine, Asparagine, Tyrosine, and Glutamine Mutations among the Carboxy-Terminal Seven Amino Acids

amino acid sequence from position 60	mutant[a]	specific relative affinity[b]
Ser-Asn-Lys-Lys-Thr-Thr-Ala	wild type	1.00
Ser-Asn-Ser-Lys-Thr-Thr-Ala	Ser-62	0.36
Ser-Asn-Thr-Lys-Thr-Thr-Ala	Thr-62	0.23
Ser-Asn-Lys-Ser-Thr-Thr-Ala	Ser-63	1.38
Ser-Asn-Lys-Thr-Thr-Thr-Ala	Thr-63	1.54
Ser-Asn-Lys-Lys-Thr-Thr-Ser	Ser-66	3.50
Ser-Asn-Lys-Lys-Thr-Thr-Thr	Thr-66	4.00
Ser-Asn-Lys-Lys-Ser-Thr-Ala	Ser-64	0.43
Thr-Asn-Lys-Lys-Thr-Thr-Ala	Thr-60	0.83
Ser-Ser-Lys-Lys-Thr-Thr-Ala	Ser-61	0.50
Ser-Asn-Lys-Lys-Thr-Thr-Gln	Gln-66	2.00
Ser-Asn-Lys-Lys-Thr-Asn-Ala	Asn-65	4.00
Ser-Asn-Lys-Lys-Thr-Gln-Ala	Gln-65	2.45
Ser-Asn-Lys-Lys-Gln-Thr-Ala	Gln-64	1.20
Ser-Asn-Asn-Lys-Thr-Thr-Ala	Asn-62	0.17
Ser-Asn-Lys-Lys-Thr-Tyr-Ala	Tyr-65	1.0

[a] Mutant repressors are defined in the text. [b] The ratio of specific (s) equilibrium binding constants, K_{ds}[wild type]/K_{ds}[mutant]. Specific equilibrium binding constant for wild type = 2.4×10^{-10} M.

Table III: Specific Relative Affinities for Cro Repressors Having Proline, Glutamic Acid, Phenylalanine, Tryptophan, Leucine, Isoleucine, and Cysteine Mutations among the Carboxy-Terminal Amino Acids

amino acid sequence from position 60	mutant[a]	specific relative affinity[b]
Ser-Asn-Lys-Lys-Thr-Thr-Ala	wild type	1.00
Ser-Asn-Lys-Lys-Pro-Thr-Ala	Pro-64	0.85
Ser-Asn-Lys-Pro-Thr-Thr-Ala	Pro-63	0.46
Ser-Pro-Lys-Lys-Thr-Thr-Ala	Pro-61	0.53
Ser-Asn-Lys-Glu-Thr-Thr-Ala	Glu-63	NM[e]
Ser-Asn-Lys-Lys-Thr-Glu-Ala	Glu-65	1.0
Ser-Glu-Lys-Lys-Thr-Thr-Ala	Glu-61	0.25
Ser-Phe-Lys-Lys-Thr-Thr-Ala	Phe-61	0.38
Ser-Asn-Phe-Lys-Thr-Thr-Ala	Phe-62	NP[d]
Ser-Asn-Lys-Lys-Phe-Thr-Ala	Phe-64	0.30
Ser-Trp-Lys-Lys-Thr-Thr-Ala	Trp-61	1.10
Ser-Asn-Lys-Lys-Leu-Thr-Ala	Leu-64	1.10
Ser-Asn-Ile-Lys-Thr-Thr-Ala	Ile-62	0.30
Ser-Asn-Lys-Ile-Thr-Thr-Ala	Ile-63	0.51
Ser-Asn-Lys-Lys-Thr-Thr-Cys	Cys-66	0.5
Ser-Asn-Lys-Lys-Thr-Thr-Ala	Cys-55	0.32

[a] Mutant repressors are defined in the text. [b] The ratio of specific (s) equilibrium binding constants, K_{ds}[wild type]/K_{ds}[mutant]. Specific equilibrium binding constant for wild type = 2.4×10^{-10} M. [c] Not measurable. [d] No protein.

Table IV: Nonspecific Relative Affinities for Mutant Cro Repressors

mutant[a]	specific relative affinity[b]	nonspecific relative affinity[c]	selectivity[d]
wild type	1.00	1.00	1.00
Lys-64	1.02	7.40	0.14
Lys-64,65	0.94	9.70	0.10
TKTKA	0.13	0.90	0.14
TTKKA	0.07	NM[e]	>1.0
∂3	0.35	1.05	0.33
∂4	0.01	0.06	0.16
Pro-64	0.85	1.74	0.49
Pro-63	0.46	0.38	1.21
Ser-63	1.38	0.83	1.66
Cys-55	0.32	4.17	0.08

[a] Mutant repressors are defined in the text. [b] The ratio of specific (s) equilibrium binding constants, K_{ds}[wild type]/K_{ds}[mutant]. [c] The ratio of nonspecific (ns) equilibrium binding constants, K_{dns}[wild type]/K_{dns}[mutant]. Nonspecific equilibrium binding constant for wild type = 4.2×10^{-8} M. [d] The ratio of specific to nonspecific relative affinities. [e] Not measurable. No complex was observed using the assay described in Figure 2.

reported as a relative affinity for O_R3 (Tables I–III). Affinity to a 20 base pair blunt end, nonspecific DNA duplex (Figure 2) was also determined by using a similar coelectrophoresis system (Table IV). We found that specific equilibrium binding constants derived from purified protein with O_R3 were identical with those obtained from cell crude lysates. For this reason, the affinities of various mutant repressors to O_R3 were measured by using cell crude lysates. Nonspecific binding constants were obtained by using purified protein.

Deletion mutants were constructed in order to investigate the importance of the amino acids between positions 62 and 66 (mutants ∂1–∂5; Table I). Both alanine-66 and threonine-65 (∂1 and ∂2) could be removed from the carboxy terminus without a substantial effect on repressor affinity to O_R3. However, upon removal of the three carboxy-terminal amino acids (∂3), the stability of the complex decreased approximately 3-fold. An even more dramatic decrease was observed upon loss of lysine 64 (∂4) since only 1% of wild-type affinity to O_R3 remained. Unfortunately, upon deletion of the codons for the last five amino acids, including lysine-62, the presence of mutant repressor in the cell crude lysate could not be detected (Figure 3).

The role of a particular amino acid chain in operator–repressor interactions was evaluated by replacing the natural amino acid with glycine (Table I). The assumptions were that this substitution conserved protein backbone interactions with DNA and also minimally altered repressor secondary structure (especially within the carboxy-terminus domain where little secondary structure was expected). Modified repressors of this type included single and multiple replacements with glycine and a mutation extending the length of the carboxy-terminal region by one amino acid. Single substitutions of glycine for

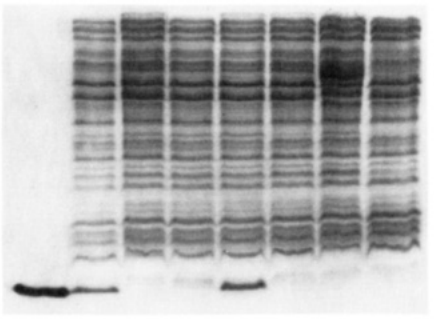

FIGURE 3: Protein gel of cell crude lysates. The SDS–polyacrylamide gel was stained with Coomassie Brilliant Blue. Crude lysates are shown in lanes 2–8. Lane 1, purified wild-type Cro repressor; lane 2, wild-type Cro repressor; lane 3, Phe-62; lane 4, Gly-62,63; lane 5, Glu-63; lane 6, Arg-63; lane 7, ∂5; lane 8, MY202.

asparagine at 61 (Gly-61) and lysine at 63 (Gly-63) led to approximately the same loss in affinity (41% and 48%, respectively). However, the substitution of glycine for lysine at position 63 was less detrimental than the analogous exchange at position 62 (29% and 48%, respectively). Unfortunately, simultaneous substitution of lysine-62 and -63 with glycine (Gly-62, -63) did not yield a protein of the expected size in the cell crude lysate (Figure 3), and further studies with this mutant were impossible. An additional mutant, Gly-63–65, was then prepared where the natural amino acids from 63 to 65 were replaced with glycine. Since results with the Gly-63 mutant indicated a 52% reduction in complex stability, this polyglycine mutant could be used to assess the additional affect of removing two threonine side chains from the carboxy terminus. These mutants (Gly-63 and Gly-63–65) were found to be comparable (0.48 and 0.43, respectively). When the carboxy terminus was further modified by replacing amino acids 63–66 with glycine and adding alanine (Gly-63–66–Ala), the binding affinity improved more than 50% relative to Gly-63–65. The importance of lysine at position 63 was also probed by designing the mutant Gly-63∂3 in which glycine replaced lysine and the remaining amino acids were deleted. The relative specific binding was then found to be similar to that of ∂3 where the terminal amino acid was lysine instead of glycine.

The significance of the ordering of amino acids within the carboxy terminus was studied with two mutant repressors containing amino acid transpositions. Mutant TKTKA, which was generated by exchange of lysine and threonine at positions 62 and 65, retained only 13% of wild-type affinity. Mutant TTKKA was prepared by simultaneous exchange of both lysines and threonines. This double transposition mutant lost 93% of its affinity for O_R3. In both mutations, the overall electrostatic charge on the protein was the same as for unmodified Cro repressor.

In an attempt to increase binding interactions between repressor and DNA, lysines were substituted for various other amino acids. Although a lysine insertion at position 61 increased the affinity by 60%, single substitutions at positions 60 and 64 and also a double replacement at positions 64 and 65 did not significantly alter binding to O_R3. Substitutions with other basic amino acids (His-61, Arg-62) had a similar minor effect (the exception to this was Arg-63 as no mutant repressor was observed in the crude lysate, Figure 3).

A series of repressor mutants were generated that carried either serine or threonine substitutions within the carboxy terminus (Table II). Whereas serine and threonine could not substitute effectively for lysine-62 (36% and 23%, respectively, of wild-type affinity), similar changes at position 63 gave repressors with improved binding (138% and 154%, respectively). Additional enhancement in repressor binding was observed for mutations where serine or threonine replaced alanine-66 (3.5- and 4-fold, respectively). It is also notable that serine does not substitute effectively for threonine-64 as the mutant retained only 43% of wild-type binding affinity. Reductions were also observed when threonine replaced serine-60 (83%) and serine replaced asparagine-61 (50%).

In addition to serine and threonine, other hydrogen-bonding amino acids (asparagine, glutamine, and tyrosine) were substituted at various sites in the carboxy terminus (Table II). As with insertion of serine or threonine, replacement with glutamine at position 66 led to improved repressor binding (2-fold). Also, replacement of threonine-65 with either glutamine or asparagine increased binding interactions 2.45- and 4-fold, respectively. A less marked improvement in repressor binding (1.2-fold) was seen when threonine-64 was substituted by glutamine. Conversely, only 17% of wild-type affinity remained when asparagine replaced lysine-62. Finally, tyrosine, although significantly more bulky, substituted effectively for threonine-65.

Another series of mutations involving the substitution of proline, aspartic acid, glutamic acid, or bulky hydrophobic amino acids were designed to disrupt the normal function of the carboxy terminus (Table III). The effects of mutations with proline were unexpected. Such substitutions, which place a kink within this domain, were predicted to distort or eliminate certain repressor–DNA interactions and therefore significantly reduce repressor–operator affinity. However, substitution with proline at position 64 had a negligible effect on repressor binding and at position 63 led to a protein that still had 46% retention of wild-type affinity. Even more unexpected was the small effect observed when proline was substituted for asparagine-61 (53% retention of wild-type affinity). Substitution of glutamic acid in the carboxy-terminal domain, however, did disrupt the interaction of Cro repressor with operator. For example, no DNA binding was observed for the mutant where glutamic acid replaced lysine-63 (a protein of the size expected was observed in the cell crude lysate as shown in Figure 3). Although substitution of glutamic acid for threonine at position 65 had no measurable effect upon binding, a similar substitution for asparagine-61 did reduce affinity 4-fold. For repressors where bulky hydrophobic phenylalanine replaced the natural amino acid (Phe-64 and Phe-61), binding affinity was 30% and 38% of wild-type, respectively (unfortunately, as shown in Figure 3, mutant Phe-62 did not yield detectable amounts of repressor in the cell crude lysates). Surprisingly, substitution of tryptophan for asparagine-61 did not significantly alter repressor affinity for O_R3. In addition, the effects of substitution with other less sterically bulky hydrophobic amino acids were examined. Whereas leucine replaced threonine-64 with only a small decrease in repressor binding, substitution of isoleucine for lysines-62 or -63 led to 30% and 51% of wild-type repressor affinity, respectively.

In an attempt to covalently link the two repressor monomers, cysteine was introduced (Cys-55) into the region where interactions between these monomers were proposed to stabilize the Cro repressor dimer (Anderson et al., 1981). This substitution led to the formation of a disulfide bridge between the two monomers (Figure 4). Thus, Cys-55 repressor migrated as a monomer under reducing conditions (3% β-mercaptoethanol) and as a dimer under nonreducing conditions when analyzed by SDS–polyacrylamide gel electrophoresis (Cys-55

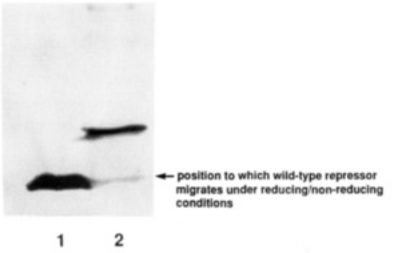

FIGURE 4: Protein gel comparing wild-type Cro repressor and the Cys-55 mutant under reducing and nonreducing conditions. The SDS–polyacrylamide gel was developed by silver staining. Lane 1, Cys-55 repressor + β-mercaptoethanol; lane 2, Cys-55 repressor.

repressor migrated as a dimer under conditions used for equilibrium binding studies). This disulfide linkage between the monomers caused a 68% reduction in specific binding affinity (Table III). An additional mutation carrying a cysteine substitution within the carboxy terminus (Cys-66) was found to reduce binding approximately 50%.

Because the model describing the interactions between Cro repressor and its operator placed the carboxy terminus within the minor groove of DNA, this domain was postulated to be involved in sequence-independent interactions (Anderson et al., 1981). If this were the case, we anticipated that some of the mutations described in Tables I–III would have a significant effect on nonspecific binding. In order to test this prediction, an assay was developed to measure nonspecific binding constants (Figure 2 and Materials and Methods). The results with a selection of mutants are presented in Table IV. The Cro repressors used in this assay were purified to homogeneity (as demonstrated by SDS–polyacrylamide gel electrophoresis and silver staining) by a two-column ion-exchange procedure. Exceptions to this degree of purity were the mutants Pro-63 and Cys-55 which were no more than 90% homogeneous. For these purified repressors, the carboxy terminus was examined by carboxypeptidase A digestion, derivatization of released amino acids with *o*-phthaldehyde, and HPLC analysis. The results from this assay demonstrated that the carboxy-terminal amino acid first released was the one expected from the gene sequence. Therefore, these repressors were not degraded either in vivo or during purification.

Whereas substitution of additional lysines into the carboxy terminus did not significantly improve the affinity of Cro repressor for O_R3, the effect on nonspecific binding was more apparent (Table IV). Replacement with lysine at position 64 improved nonspecific binding more than 7-fold, and an additional lysine substitution at position 65 (Lys-64, -65) increased the nonspecific interaction nearly 1 order of magnitude relative to wild-type repressor. For the mutation involving a single transposition of lysine and threonine (TKTKA), the effect upon nonspecific binding was found to be minimal (90% of wild-type affinity was retained) although the reduction in specific binding was much larger. However, the double transposition, mutant TTKKA, appeared to have significantly reduced binding to both specific and nonspecific DNA, with the latter being too low to measure by our assay. Removing the final three carboxy-terminal amino acids (∂3) did not affect nonspecific binding, but the next deletion mutant, ∂4, had only 6% of the wild-type repressor affinity for nonspecific DNA. Substitution of proline for threonine at position 64 enhanced nonspecific binding significantly although the analogous substitution at position 63 was detrimental and reduced nonspecific binding nearly 60% when compared to wild type. Unexpectedly, replacement of lysine-63 with serine only reduced nonspecific binding to 83% of wild-type, and mutant Cys-55 increased nonspecific binding more than 4-fold.

Discussion

The mutations described here should not adversely affect protein stability. This assumption is supported by several sets of related experimental data. One is the finding that the same carboxy-terminal sequence protects two different and unrelated proteins from proteolysis (Bowie & Sauer, 1989). This observation suggests that the same sequence at the carboxy terminus of two different proteins does not affect degradation by altering the physical properties specific to the protein in which it resides. Our assumption is that a similar result would be expected from amino acid replacements or deletions in the carboxy-terminal domain of Cro repressor. Furthermore, as the carboxy-terminal residues are added last in protein assembly, they are unlikely to affect Cro repressor folding and hence stability (Parsell & Sauer, 1989; Pakula et al., 1986). It has also been shown for a series of temperature-sensitive mutations of bacteriophage T4 lysozyme that solvent-accessible domains (as is the case for the carboxy-terminus amino acids in Cro repressor) are much less susceptible to detabilization by amino acid substitutions (Alber et al., 1987). Although these results encouraged us to expect that Cro repressors modified at the carboxy terminus would be stable toward endogenous proteolysis, prior to binding studies each mutant was analyzed as to mobility on polyacrylamide gels and, for several mutants as listed in Table IV, the carboxy-terminal amino acid analyzed. In all cases examined, this amino acid was found to be correct, and for all but the four mutants described in Figure 3, a protein was observed migrating at the expected position for a Cro repressor. It may be significant that these four mutants all contained either a substitution or a deletion of one or both carboxy-terminal lysines. Increased resistance to proteolysis has been observed for mutants in the Arc repressor of bacteriophage P22 where charged side chains were introduced within one or two residues of the carboxy terminus of the protein (Bowie & Sauer, 1989).

Cro and other repressor proteins locate their binding sites much faster than would be expected from a three-dimension, diffusion-controlled process (Takeda et al., 1977; Riggs et al., 1970). A two-step facilitated mechanism has been proposed to account for this observation with Cro repressor (Kim et al., 1987). Thus, Cro repressor is proposed to bind initially to an arbitrary site on DNA and form a nonspecific protein–DNA complex. As part of this binding process, the carboxy terminus of the Cro dimer is postulated to wrap around the DNA and constrain the movement of the protein to a one-dimension slide until its sequence-specific binding site is found (Ohlendorf et al., 1982). The process is reversed when Cro repressor dissociates from the operator. In common with other DNA–protein complexes with an observable ionic strength dependence, the interaction of Cro repressor with DNA is presumed to have a significant electrostatic component. The electrostatic potential surface surrounding a dimer of Cro repressor has been calculated (Ohlendorf et al., 1983) and is dominated by an elongated positive region that straddles the dimer 2-fold axis. This feature coincides both in position and in orientation with the presumed DNA binding site. Because the carboxy terminus is also a region of positive potential, the suggestion that these flexible arms are involved in initial nonspecific binding of the repressor to DNA is compelling. For this reason together with direct verification by ^{15}N NMR of the proposal that the carboxy terminus is involved in DNA binding (Leighton & Lu, 1987), we assume that the role of electrostatic interactions in Cro repressor–DNA binding (specific and nonspecific) can be evaluated by substituting or removing basic

amino acids located within the carboxy terminus.

We found it surprising that substitution of additional basic amino acids did not lead to significant increases in specific binding (Table I). It is assumed that the carboxy terminus lies in the minor groove of DNA and that the lysine residues at 62 and 63 in the wild-type repressor contribute to DNA binding via ionic contacts with phosphate (Ohlendorf et al., 1982). We predicted that it would be possible to create new ionic interactions between basic residues and phosphate groups further along the minor groove in an arrangement similar to that proposed for oligoarginine, oligolysine, and nucleoproteins with B-DNA (Feughelman et al., 1955; Wilkins, 1956). A possible explanation as to why basic amino acid substitutions did not significantly improve specific binding comes from model-building studies with basic peptides and B-DNA (Feughelman et al., 1955). This distance between the ends of successive protein side chains pointing in opposite directions is equal to the separation, in the helix axis direction, of the two phosphate helices in linear B-DNA. Therefore, the regular spacing of phosphate groups is necessary for effective combination with basic polypeptides, and, if the DNA is distorted, such efficient combination cannot take place. There is direct evidence from ^{1}H NMR that complexing Cro repressor with O_R3 leads to a structural distortion in the middle of the operator where the carboxy termini are believed to lie (Lee et al., 1987). Our results suggest that the distortion (bending) of DNA in the O_R3–repressor complex precludes lysines in the carboxy terminus (at positions other than 62 and 63) from interacting optimally with phosphate.

A comparison of amino acid replacements for lysines-62 and -63 indicates that the loss in specific binding affinity caused by substitutions at 62 is consistently greater than for the analogous changes at 63 (Tables I–III). Further support for this conclusion comes from comparing mutants $\partial 3$ and Gly-63$\partial 3$. These mutants are different because Gly-63$\partial 3$ replaces lysine with glycine as the carboxy-terminal amino acid. Their affinities for O_R3 were found to be similar, which suggests that the contribution made by lysine-63 to DNA binding is not significant. Again, it is possible to invoke DNA bending within this complex in order to explain the different relative contributions of these lysines to specific binding. Experiments have been performed by us (results not shown) using a polyacrylamide gel mobility shift assay (Crothers, 1987) in attempts to detect differences in DNA bending when mutant repressors form complexes with O_R3 embedded in the center of a 331 base pair duplex. The differences in gel mobility for the various complexes were not significant.

A comparison of specific relative affinities for mutant repressors carrying additional lysines in the carboxy terminus (Table I) with their nonspecific relative affinities (Table IV) demonstrates that placing additional lysines into the carboxy terminus has an additive effect on nonspecific binding. Thus, mutant Lys-64,65 shows approximately a 10-fold improvement in nonspecific binding relative to wild type, and even one additional lysine in the carboxy terminus (mutant Lys-64) leads to a 7.4-fold enhancement. However, since the interaction of these mutants with O_R3 is unchanged, the selectivity for operator is only 10–15% of wild-type Cro repressor. It is possible to apply the argument used to explain the small effect caused by additional lysines on specific binding to the more significant effect on nonspecific binding. We then conclude that in the nonspecific complex DNA is bent to a lesser extent than in the specific complex. These suggestions are further supported by comparing the specific and nonspecific relative affinities for mutant TKTKA which carries the transposition of a single threonine and lysine (Tables I and IV). Whereas the specific binding affinity is reduced approximately 8-fold, nonspecific binding remains similar to wild type. However, it is unclear why the nonspecific affinity for mutant TTKKA (in which both carboxy-terminal threonines are exchanged with lysines) is too low to be measured by our assay.

For the series of deletion mutants ($\partial 1$–$\partial 4$; Table I), it is apparent that three amino acids can be removed from the carboxy terminus with the resulting repressor still retaining 35% of wild-type affinity for O_R3. However, further deletion of lysine-63 (mutant $\partial 4$) is much more disadvantageous. Comparison of mutant $\partial 4$, which retains only 1% of wild-type specific affinity, with mutant Gly-63, whose specific relative affinity is 0.48 (both mutations have one lysine at 62), indicates that the severe loss in binding affinity observed for $\partial 4$ is not simply due to loss of lysine at 63. We consider that reduction in length of the carboxy terminus with concomitant loss of possible hydrogen bonding between amide nitrogens of the peptide backbone and phosphate groups on DNA could be significant. Similar interactions are suggested by the crystal structure for the repressor–operator complex of bacteriophage 434 (Anderson et al., 1987). Although the side chains of threonine residues at 64 and 65 in mutant Gly-63 could participate in hydrogen bonding with the minor groove of operator, we discount this possibility upon comparison of specific binding affinities for mutant Gly-63 with mutant Gly-63–65 (both carboxy-terminal threonines are replaced by glycine) as they are found to be similar. Confirmation of the importance of the carboxy-terminal backbone in binding to DNA is found by comparing specific binding affinities for mutants Gly-63–65 and Gly-63–66–Ala. These mutants differ essentially in that the latter has an additional amino acid. This extension of the carboxy-terminal backbone improves the specific binding affinity by almost 50% when compared to mutant Gly-63–65. Among the single-site substitutions at positions 62 and 63, it is notable that specific binding increased for mutant repressors in which serine or threonine replaced lysine at 63. It is possible that these substitutions introduced a new hydrogen-bonding interaction into the carboxy terminus between the hydroxyl function of serine or threonine and the 2-amino group of a guanine located at the bottom of the minor groove of the operator. Inspection of the model where Cro repressor is complexed with O_R3 supports this proposition as position 63 is located in a region of G-C-rich DNA (Ohlendorf et al., 1982).

The only single-site mutations that led to significant improvement in repressor binding to operator where those where the replacement amino acid could contribute hydrogen-bonding interactions near the end of the carboxy terminus (Table II). For example, replacement of alanine-66 with glutamine, serine, or threonine increased binding of the mutant repressor to O_R3 at least 2-fold. Similarly, replacement of threonine-65 with either glutamine or asparagine also significantly improved repressor binding. Surprisingly, substitution at position 65 with a sterically bulky tyrosine or a negatively charged glutamic acid had no effect on binding, but replacement with serine led to more than a 50% reduction in affinity. Thus, the mutations introduced at position 65 suggest an unusual pattern. Removal of wild-type threonine ($\partial 2$) and introduction of a bulky tyrosine or a negatively charged glutamic acid had no effect on repressor affinity, but substitution with hydrogen-bonding amino acids (Gln and Asn) significantly enhanced affinity for O_R3. Presumably this is a very flexible region of the protein where a potentially unfavorable contact, such as with glutamic acid, is avoided whereas favorable interactions can be made if these

are available, such as with glutamine or asparagine. Cysteine substitution for alanine at position 66 led to a 50% reduction in binding. This was unexpected as both are small hydrophobic amino acids (the probability of a proportion of mutant Cys-66 having carboxy termini linked by a disulfide bond is not relevant as the equilibrium binding assay is based upon the concentration of active protein). Even more unexpected was the small effect upon binding due to proline substitution at various positions within the carboxy terminus. Perhaps the conformational flexibility of the carboxy terminus accommodates distortion in the α-carbon backbone due to proline.

Other mutations anticipated to disrupt binding are found in Table III. Even though an intact Cro repressor was found in the cell crude lysate (Figure 3), mutant Glu-63 was not observed to bind to O_R3 as measured by our assay. This was probably due to unfavorable charge interactions introduced at a site presumed to interact through contacts with DNA phosphate. Glutamic acid and phenylalanine substitutions at position 61 reduced specific binding by 75% and 62%, respectively. However, replacement with tryptophan did not affect binding affinity to operator even though it is a bulky hydrophobic amino acid. The results at position 61 are therefore difficult to rationalize relative to protein–DNA interactions as diverse groups of amino acids lead to both 50–60% reductions in affinity (Glu, Phe, Pro, Ser, Gly) and no change in binding (His, Lys, Trp). Although these data do not address the issue, perhaps these results relate to changes in the overall protein structure rather than to alterations in specific Cro repressor–operator contacts.

Mutant Cys-55 was prepared in an attempt to covalently link the Cro monomers in a region of repressor where interactions between subunits are known to stabilize the dimer. The results demonstrate that indeed a disulfide bond is generated between monomers under the nonreducing conditions as used in binding assays (Figure 4) and that specific binding is only 32% of the wild-type affinity (Table III). It is also striking that nonspecific binding for mutant Cys-55 is improved more than 4-fold while its selectivity is reduced to less than 10% of wild type (Table IV). Because contacts between Cro monomers involve hydrophobic and hydrogen-bonding interactions, it has been postulated that this protein has considerable intramolecular motion and flexibility. As a consequence, Cro repressor can efficiently search for its operator site and more easily change conformations to accommodate either sequence-specific or nonspecific interactions with DNA (Ohlendorf & Matthew, 1985). Perhaps introduction of a disulfide bridge restricts flexibility and motion between monomers and therefore inhibits the protein recognition elements (the helix–turn–helix region of Cro) from efficiently contacting the major groove of O_R3. Presumably as well, reduced flexibility stabilizes Cro repressor in a conformation that more favorably recognizes nonspecific DNA.

It is probable that the carboxy terminus modulates the selectivity of Cro repressor for its different operators and that the wild-type carboxy-terminus sequence and length reflect this function rather than that of ensuring the tightest possible DNA binding domain. The mutagenesis data and the conclusions we have drawn are consistent with the recently solved crystal structure for Cro repressor in complex with its 17 base pair operator (B. W. Matthews, personal communication).

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micF RNA Binds to the 5′ End of *ompF* mRNA and to a Protein from *Escherichia coli*†

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ABSTRACT: *micF* RNA regulates the levels of outer membrane protein F (OmpF) in *Escherichia coli* in response to temperature increase and other stress conditions by decreasing the levels of *ompF* mRNA (Andersen et al., 1989). A 93-nucleotide *micF* RNA was synthesized in vitro directly from polymerase chain reaction generated DNA which was designed to contain a functional T7 RNA polymerase promoter upstream of the *micF* RNA gene and an appropriate restriction site for transcription termination. A transcript (150 nucleotides) containing the ribosomal binding domain of *ompF* mRNA messenger was synthesized in vitro from the *ompF* gene cloned into a T7 expression vector. A stable duplex was formed between *micF* RNA and the 150-nucleotide 5′ transcript of *ompF* mRNA after incubation at 37 °C in a physiological buffer. The melting curve of the duplex formed by *micF* RNA and 150-nucleotide transcript revealed a T_m of 56 °C and a ΔT_m that spans about 20 °C; both are consistent with the proposed structure for the *micF*/*ompF* duplex. In addition, as determined by competition studies and UV cross-linking/label-transfer analyses, an *E. coli* protein was found to bind specifically to *micF* RNA. The protein also bound weakly to the 150-nucleotide *ompF* transcript. The data are the first to demonstrate the complex between *micF* RNA and the 5′ end of *ompF* mRNA and suggest that in vivo a *micF* ribonucleoprotein (RNP) particle may participate in the destabilization *ompF* mRNA during thermoregulation of OmpF porin.

The outer membrane protein OmpF, a major porin protein of *Escherichia coli*, is regulated in response to changes in temperature, osmolarity, and other stress conditions during growth [Lugtenberg et al., 1976; Hall & Silhavy, 1981; Andersen et al., 1989; see Forst and Inouye (1988) for a review]. It has been shown that chromosomally derived 4.5S *micF* RNA plays an essential role in the thermal regulation of OmpF (Andersen et al., 1989). The 93-nucleotide *micF* RNA represses OmpF synthesis by decreasing the levels of *ompF* mRNA (1.1 kb) in response to temperature increase as well as to the other stress-related factors (Andersen et al., 1989). While *micF* RNA is necessary for the observed decreased levels of *ompF* MRNA, it is not sufficient; another factor, possibly a cognate protein, was deduced to participate with *micF* RNA in the regulation of the messenger's levels (Andersen et al., 1989).

Although the *micF* RNA gene is at 48′ on the *E. coli* chromosome and distal from the *ompF* gene at 21′ (Inokuchi et al., 1982; Mizuno et al., 1983; Bachmann, 1987), *micF* RNA is believed to function as a natural antisense RNA against the mRNA for OmpF. Consistent with its hypothesized role as an antisense RNA, the suppression of OmpF via *micF* RNA has been shown to occur at a posttranscriptional level (Misra & Reeves, 1987; Cohen et al., 1988), and *micF* RNA can significantly inhibit translation of *ompF* mRNA if overexpressed (Mizuno et al., 1983, 1984; Andersen et al., 1989). A model of *micF* RNA/*ompF* mRNA has been proposed (see Figure 1). In this model, the primary sequence of *micF* RNA shows extensive complementarity with the 5′ end of *ompF* mRNA in and around its ribosome binding domain; however, the complementarity is imperfect since there are several looped-out positions and non-Watson–Crick base pairing in the proposed duplex (Mizuno et al., 1984; Andersen et al., 1987).

Since *micF* RNA is found in low levels in the cell (Andersen et al., 1987), it is difficult to obtain in sufficient quantities for in vitro studies. In a novel approach, polymerase chain reaction (PCR) was used to generate DNA from which bona fide *micF* RNA could be synthesized by T7 RNA polymerase. Using T7-synthesized RNAs, we show that *micF* RNA hybridizes to the 5′ end of *ompF* mRNA and forms a stable duplex. We also show the *micF* RNA binds specifically to a protein from *E. coli*, suggesting that a *micF* RNP particle may regulate the stability of *ompF* mRNA.

Experimental Procedures

Polymerase Chain Reaction Generated DNA. The PCR technique (Mullis & Faloona, 1987) was used to introduce a T7 RNA polymerase promoter upstream of the *micF* RNA gene and to create a restriction site for run-off transcription at the end of the gene (Figure 2A). Due to paucity of restriction sites around the *micF* RNA gene, this novel method was chosen in lieu of conventional cloning to position the

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