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The study of the C-terminal region of the cyclic AMP receptor protein

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THE STUDY OF THE C-TERMINAL REGION OF THE CYCLIC AMP RECEPTOR PROTEIN

by

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ABSTRACT

THE STUDY OF THE C-TERMINAL REGION OF THE CYCLIC AMP RECEPTOR PROTEIN

By

Zhaohui Yang

Adviser: Joseph S. Krakow

The cAMP receptor protein, or CRP (CAP), is an allosteric protein which regulates transcription of more than 20 genes in *E. coli*. Transcription regulation is achieved by binding of cAMP/CRP to a specific promoter-related DNA sequence. A truncated form of CRP, CRP^{CY}, was prepared by treating CRP with carboxypeptidase Y. CRP^{CY}, which has lost seven C-terminal amino acids, binds cAMP with an affinity similar to that of wild-type CRP. Unlike the wild-type CRP, it is sensitive to endoproteolytic cleavage in the absence of cAMP. CRP^{CY} does not bind DNA or support transcription from *lac* P^+ by RNA polymerase. Substitution and deletion mutations at the C-terminal arginine or in the C-terminal region have been made using site-directed mutagenesis and the mutant proteins purified. The mutant CRPs bound cAMP with an affinity similar to that of the wild-type CRP. The cleavage patterns of the mutant proteins by the proteases were similar to those of the wild-type CRP. Some of the mutants showed weak binding to a DNA fragment containing

the *lac* P^+, while others bound with affinities similar to wild-type CRP. DNase I footprinting showed that binding to *lac* P^+ by some of the mutant CRPs was enhanced in the presence of RNA polymerase, suggesting that there was interaction between the two proteins and that such interaction stabilized the binding of both proteins to the DNA. The mutant proteins generally retained 15 to 30% activity of the wild-type CRP in supporting abortive initiation from *lac* P^+ by RNA polymerase. The results show that the C-terminal arginine as well as the other amino acids studied in the C-terminal region play a role in the function of CRP.

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Abbreviations used:

ApA: adenylyl (3', 5') adenosine

BSA: bovine serum albumin

cAMP: 3', 5'-cyclic AMP

cGMP: 3', 5'-cyclic GMP

CRP: cyclic AMP receptor protein

Da: daltons

DTNB: dithionitrobenzoic acid

DTT: dithiothreitol

kDa: kilodaltons

lac: lactose operon

lac P^+: wild-type *lac* promoter

mA: milliampere

mAb: monoclonal antibody

NEN: N-ethylmaleimide

PBS: phosphate buffered saline

PEG: polyethylene glycol

PMSF: phenylmethanesulfonylfluoride

RP_o: open promoter complex

SDS: sodium dodecyl sulfate

ssDNA: single-stranded DNA

INTRODUCTION

The cAMP receptor protein, or CRP (also known as catabolite activator protein, CAP), is involved in the regulation of the expression of more than 20 genes in *E. coli* (de Crombruggh *et al.*, 1984). CRP activates the transcription of certain genes while repressing the transcription of others. In each case, transcription regulation is dependent upon binding of the cAMP/CRP complex to a specific DNA sequence at or near the promoter region of the gene in question. No cellular role for cAMP and CRP unrelated to transcription has been discovered in *E. coli* (Adhya and Garges, 1982).

CRP is a dimeric protein (MW 47,238) of two identical 209 amino acid subunits. The amino acid sequence of CRP has been deduced from the nucleotide sequence of the cloned *crp* gene (Aiba *et al.*, 1982). The crystal structure of CRP complexed with cAMP has been determined at 0.29 nm and further refined at 0.25 nm resolution (McKay and Steitz, 1981; Weber and Steitz, 1987). It shows that each CRP subunit folds into two domains: the larger N-terminal domain extends from residue 1 to residue 129 and the smaller C-terminal domain from residue 139 to residue 209. The two domains are connected by a "hinge" region which runs from residue 130 to residue 138. In the crystal structure, the two subunits have different conformations: in the subunit in the "closed" conformation, the C-terminal and N-terminal domains lie closer together than in the "open" conformation. The two cAMP molecules in the anti conformation are bound to CRP. The atoms of cAMP make specific interactions with the protein. The charged phosphate of cAMP makes ionic interaction with Arg-82 from β-strand 7. Oxygens on the ribose and phosphate form hydrogen bonds with

Gly-71, Glu-72 and Ser-83. The 6-amino group on the adenine ring forms hydrogen bonds with the OH of Thr-127 of the C α-helix in the same subunit and the OH group of Ser-128 of the C α-helix in the adjacent subunit. Interdomain hydrogen bonds occur mainly between residues of β-strand 5 and residues of the E α-helix. In both subunits there are hydrogen bond interactions between the NH of Ile-60 and C=O of Gln-174, and between the side-chains of Glu-58 and Gln-174. Additional hydrogen bonds form only in the "closed" subunit between the side-chains of Tyr-63 and Glu-171, and between the side-chains of Asn-133 and Ile-145. When cAMP is bound, the effects can be transmitted along the C α-helices to the hinge region via the intersubunit hydrogen bonds, and also to the E α-helix via interdomain hydrogen bonds (Weber and Steitz, 1987). The D α-helix seems to be on the route of the allosteric change, since many of the mutations that produce a cAMP-independent phenotype lie on this helix (Aiba *et al.*, 1985; Garges and Adhya, 1985; Weber *et al.*, 1987). Thus far crystal structure information is available for only CRP-$(cAMP)_2$. However, it is believed that the physiologically relevant active form of CRP is CRP-$(cAMP)_1$ (Takahashi *et al.*, 1989). At concentrations between 0 and 200 μM cAMP, the major complex is CRP-$(cAMP)_1$ (Heyduk *et al.*, 1989). The physiological concentration range of cAMP is from 0 to 10 μM. In addition, in both CRP-*gal* or CRP-*lac* complexes the stoichiometry of CRP to cAMP is 1:1. Undoubtedly, a great deal of the understanding of the mechanism by which cAMP activates CRP could be obtained from the analysis of CRP and CRP-$(cAMP)_1$ crystal structures which are not available at the present time. CRP functions as a transcription control element in a dimer form. The crystal structure of the cAMP/CRP complex indicates that the bound

cAMP molecules interact with amino acid residues from both subunits as discussed earlier (Weber and Steitz, 1987). This could account for the stabilizing effect of cAMP on the dimeric structure of CRP which has been reported by Brown and Crothers (1989).

The crystal structure of cAMP/CRP indicates that the molecule of cAMP is deeply buried within the CRP dimer (Weber and Steitz, 1987). Binding of cAMP to the N-terminal domain of CRP elicits conformational changes in the C-terminal domain (Eilen and Krakow, 1977a; Eilen *et al.*, 1978). For example, the unliganded CRP is relatively resistant to attack by several proteases but is readily digested in the presence of cAMP (Eilen *et al.*, 1978). Another example of cyclic AMP induced conformational change is provided by crosslinking CRP with dithionitrobenzoic acid (DTNB). The intersubunit linking of CRP requires cAMP (Eilen and Krakow, 1977b). Binding studies on the products of proteolytic digestion (Eilen *et al.*, 1978; Aiba and Krakow, 1981) suggest that there is a separate function for each domain as has also been observed for other gene regulatory proteins such as the *lac* repressor (Platt *et al.*, 1973; Files and Weber, 1976; Geisler and Weber 1975) and λ repressor (Pabo *et al.*, 1979). The larger N-terminal domain binds cAMP and shows homologies in amino acid sequence with the regulatory subunit of the mammalian cAMP-dependent protein kinase (Weber *et al.*, 1982, 1987). The smaller C-terminal domain of CRP is involved in binding to DNA and shows structural and sequence homologies with several other gene regulatory proteins such as *cro* repressor, cI, *lac* repressor (Steitz *et al.*, 1982; Matthews *et al.*, 1982; Sauer *et al.*, 1982; Weber *et al.*, 1982; Steitz and Weber, 1984).

A class of mutant CRP proteins, termed CRP*, has been isolated. In contrast to wild-type CRP, they do not require cAMP to activate the promoters which are normally under the control of cAMP/CRP. One of these mutants, CRP*598 (Arg-142 to His, Ala-144 to Thr), has been well characterized. The unliganded CRP*598 exhibits the behavior of a cAMP/CRP complex (Ren *et al.*, 1988). In the absence of cAMP, it is sensitive to cleavage by trypsin, chymotrypsin, subtilisin, and *Staphylococcus aureus* V8 protease, properties observed with the wild-type CRP only in the presence of cAMP. The fragments produced from the digestion of CRP*598 were identical to those produced from the digestion of the wild-type cAMP/CRP complex (Ren *et al.*, 1988). While unliganded CRP is resistant to digestion by carboxypeptidase Y at 37°C, CRP*598 was readily digested by the enzyme to yield a core similar to that produced by digestion of cAMP/CRP. Amino acid analysis showed that the same number (seven) of the C-terminal amino acids have been removed from CRP*598 as from the wild-type cAMP/CRP complex (Yang *et al.*, 1991). These results suggested that the unliganded CRP*598 assumed a conformation similar to that of cAMP/CRP complex. Ren *et al.* (1988) showed that, at high concentrations, the unliganded CRP*598 was as active as cAMP/CRP in supporting abortive initiation from *lac* P^+ by RNA polymerase, indicating that the mutation in CRP*598 has biological significance. The authors further provided evidence that there is protein-protein interaction between CRP and RNA polymerase during promoter binding. CRP*598 but not wild-type CRP is activated by cGMP in abortive initiation assays (Ren *et al.*, 1988). The residues 130-138 in CRP have been termed the "hinge" region of the protein connecting the cAMP binding domain (the N-terminal domain) to the

DNA binding domain (the C-terminal) domain (Weber and Steitz, 1987). It is thought that the hinge region regulates the relative orientation of the two domains of CRP and that it is responsible for the transmittance of conformational changes elicited by cAMP from the N-terminal domain to the C-terminal domain. The two amino acid substitutions of CRP*598 are located near the hinge region. Adhya and coworkers recently reported that residues 138 and 141 must be polar in order to achieve cAMP independence (i.e., CRP* activity), which suggested that there is an interaction causing inward motion between the C and D α-helices, releasing the F α-helix from the proximity of the D α-helix. This rearrangement makes the F α-helix available for DNA recognition (Kim *et al.*, 1992). The authors further demonstrated that at position 144 in the D α-helix and within interaction distance of the F α-helix, substitution of alanine by an amino acid with a larger side-chain, regardless of its nature, allows cAMP independence (CRP* activity). This suggested that pushing against the F α-helix may be a way of making the helix available for DNA binding. The authors proposed that the cAMP-induced allosteric change involved similar hinge reorientation to adjust the C and D α-helices allowing outward movement of the F α-helix.

Comparison of a number of cAMP/CRP binding sites shows that the complex covers at least 22 base pairs and suggests the consensus sequence is 5'-aaaTGTGAtct*agaTCACAttt-3', where upper and lower case letters denote strongly and weakly conserved nucleotides, respectively, and "*" indicates the center of symmetry (Gaston *et al.*, 1989). The nucleotide sequence of the CRP binding site in *lac* P^+ is 5'-taaTGTGAgtt*gctTCACTcat-3', where underlined bases are different

from those predicted as the consensus sequence. It is noted that the sites to which CRP binds are not found at the same distance from the transcription start point in all activated promoters. In the *lac* promoter, for example, cAMP/CRP binds to a site centered at 61.5 bp upstream from the *lac* transcription start point (Simpson, 1980; Schmitz, 1981). In contrast, the CRP binding site in the galactose operon is centered at 41.5 bp upstream of the *gal* P1 start point (Taniguchi *et al.*, 1979; Busby *et al.*, 1982). In a third example, the *malT* promoter, cAMP/CRP binds to a site centered at 70.5 bp upstream of the transcription start point (Chapon and Kolb, 1983). The *gal* promoter sequence is 5'-aagTGTGAcat*ggaATAAAtta-3', the underlined bases are those which differ from the consensus sequence (de Crombrugghe *et al.*, 1984).

Transcription initiation involves the three overall steps shown schematically below (Chamberlin, 1974; McClure, 1985):

$$R + P \underset{K_B}{\rightleftarrows} RP_c \xrightarrow[k_f]{} RP_o \xrightarrow[\text{promoter clearance}]{\text{NTPs}} RNA$$

where RNA polymerase (R) binds to the promoter (P) with a binding constant of K_B, to form a competitor-sensitive complex, termed the "closed complex" (RP_c) which subsequently isomerizes with a rate constant of k_f to a transcriptionally active complex, termed the "open complex" (RP_o). RNA polymerase then clears the promoter after a certain length of RNA has been synthesized. A CRP-dependent promoter may be limited at a different step in the transcription initiation process and CRP activates transcription by relieving the step(s) in which RNA polymerase is trapped. For example, in *lac*, RNA polymerase binds to the non-functional *lac* P2

promoter in the absence of cAMP/CRP; CRP increases the binding of RNA polymerase to the *lac* P1 promoter as well as the isomerization of the closed complex to the open complex (Straney *et al.*, 1989). In *gal* P1, CRP accelerates the isomerization of the initial closed complex without affecting the initial binding of RNA polymerase to the promoter (Herbert *et al.*, 1986). In the *mal* T promoter, CRP functions by helping the escape of RNA polymerase from the open complex into the actively transcribing species without affecting the rates of initial binding or isomerization (Menendez *et al.*, 1987).

Cooperative binding to specific DNA sites by CRP and RNA polymerase has been demonstrated by footprinting analyses (Li and Krakow, 1985; Ren *et al.*, 1988). In the *lac* UV5 promoter, the promoter region has been mutated so that its RNA polymerase binding site contains the consensus sequence. The *lac* L8UV5 is a double mutant of *lac* promoter in which the CRP binding site has also been mutated. cAMP/CRP formed a stable complex with *lac* L8UV5 only in the presence of RNA polymerase but not in the absence of RNA polymerase (Li and Krakow, 1987). Neither unliganded CRP*598 nor RNA polymerase could form a stable complex with *lac* P^+; however, when both proteins were incubated with *lac* P^+, they formed a stable complex which isomerized to generate a transcriptionally active complex (Ren *et al.*, 1988). In addition, RNA polymerase in its primary complex with cAMP/CRP and *lac* P^+ stabilizes the promoter binding of CRP against dissociation compared with the cAMP/CRP-*lac* P^+ complex lacking RNA polymerase. The extra stabilization by RNA polymerase is lost if half a helical turn of DNA is inserted between the CRP binding site and RNA polymerase binding site and is partially restored if a full DNA turn is

introduced. Presumably the half helical turn distorts the angular orientation necessary for contact between bound CRP and RNA polymerase and a full helical turn restores the orientation (Straney *et al.*, 1989). Fluorescence polarization experiments demonstrated that CRP and RNA polymerase can interact in the absence of DNA (Pinkney and Hoggett, 1988). The results of the studies mentioned above strongly favor the argument that CRP directly contacts RNA polymerase and that such contacts play an important role in the activation of *lac* P^+.

E. coli strains producing mutant CRP proteins which bind to specific DNA sites normally but fail to activate transcription have been isolated and collectively called positive control mutants (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Irwin and Ptashne, 1987). It is believed that these positive control mutants have lost the contact sites necessary for interaction with RNA polymerase. Another important line of evidence supporting protein-protein interaction between CRP and RNA polymerase comes from the study of RNA polymerase. The α subunit of RNA polymerase consists of 329 amino acids. Two truncated α subunits, one terminated at residue 256 and the other at residue 235, formed holoenzyme which transcribed CRP-independent promoters normally but failed to function at *lac* P^+, suggesting that the C-terminal region of this subunit of RNA polymerase is responsible for interaction with CRP (Igarashi and Ishihama, 1991). Complicating the story is the finding that these α deletions did not affect activation by CRP at *gal* P1 (Igarashi *et al.*, 1991). Of note is that the distance between CRP binding site and transcription start site is 41.5 bp in *gal* P1 promoter while it is 61.5 bp in *lac* P1 promoter. The distance between CRP binding site and transcription start site may affect the way

CRP interacts with RNA polymerase.

CRP contains a helix-turn-helix DNA binding motif (McKay and Steitz, 1981; Weber and Steitz, 1987). Hence, CRP belongs to the superfamily of gene regulatory proteins which recognize and bind specific DNA sequences with the helix-turn-helix structure (Jordon and Pabo, 1988; Anderson, *et al.*, 1987; Pabo and Sauer, 1984). The E α-helix turn F α-helix motif is situated in the C-terminal domain of CRP and spans amino acid residues 168 to 191. Following the helix-turn-helix motif are β-strands 11 and 12 (residues 195 to 205) and a segment of four C-terminal amino acid residues (206 to 209), which in the crystal structure of cAMP/CRP are unstructured (Weber and Steitz, 1987). The F α-helices, which are the DNA recognizing helices, protrude from the surface of CRP dimer and run approximately parallel to each other at a distance of 3.4 nm (Mckay and Steitz, 1981; McKay *et al.*, 1982). It has been proposed that The N-terminal part of the F α-helix enters two successive major grooves of DNA in a way similar to the interaction of helix 3 of the λ repressor (Pabo and Lewis, 1982; Steitz *et al.*, 1982). For the λ repressor the orientation of helix 3 is thought not to be exactly parallel to the path of the major groove (Pabo and Lewis, 1982). The symmetrical helices 3 of the cI dimer cannot fit completely in two successive major grooves, because the C-terminal part of these helices do not protrude enough from the surface of the protein. In the case of CRP a similar arrangement would allow interactions for about 8 to 9 bp in a region of 14 bp, shorter than the CRP binding site. A bend in the DNA would, however, provide for additional contacts with the DNA. Several lines of evidence have indicated that CRP induces bending in its DNA binding site (Porschke *et al.*, 1984; Wu and Crothers,

1984; Kolb *et al.*, 1983; Liu-Johnson *et al.*, 1986; Schultz *et al.*, 1991). Bending the DNA allows for interactions with a 28-bp segment which is required for full activity (Liu-Johnson *et al.*, 1984). Model building showed that a bend of 100° to 160° is necessary in order to place the sugar-phosphate backbone in contact with large region of positive electrostatic potential present on the surface of CRP (Warwicker *et al.*, 1987). Analysis of the mobility of CRP-DNA complexes in polyacrylamide gels puts the estimation of the extent of bending at 90° (Liu-Johnson and Crothers, 1986). The crystals of a cAMP-CRP-DNA complex have now been obtained and analyzed (Schultz *et al.*, 1991). The 30-bp DNA in the crystals contained the *lac* promoter sequence. The crystal structure of the tertiary complex clearly shows that the DNA has been bent by 90°. The bending results almost entirely from two 40° kinks that occur in the conserved TGTGA sequence, between TG/CA bp at positions 5 and 6 on each of the CRP half site. The kinks derive from ionic and hydrogen bond interactions between residues of CRP and DNA bases and phosphates (Schultz *et al.*, 1991).

How DNA bending induced by CRP participates in transcription activation is unclear. It is assumed that DNA bending facilitates additional protein-DNA or protein-protein contacts required for the initiation of transcription by RNA polymerase. Schultz *et al.* (1991) proposed that the bend induced by CRP might facilitate contacts between RNA polymerase and DNA upstream from the CRP binding site to form a hair-pin structure. Such a model could serve to explain why CRP could activate transcription from such a variety of positions since the size of the loop could vary (Schultz *et al*, 1991). Alternatively, DNA bending by CRP may create

the proper geometry needed for a direct contact between CRP and RNA polymerase essential for activation.

The last decade or two has witnessed major advances in our understanding of the mechanisms of transcription control in procaryotic as well as eukaryotic systems. CRP has been one of the prime transcription factors which serve as a model for the study of protein-DNA and protein-protein interactions. Despite the large amount of information on this protein, questions regarding the mechanism by which CRP activates transcription remain open. The allosteric effect of cAMP on CRP is still not completely understood due to the lack of the crystal structure of unliganded CRP. The interaction of CRP with RNA polymerase remains to be defined in detail in order to understand the activation of transcription by CRP.

This dissertation reports on a study of CRP using as probes a truncated form of CRP and mutant CRPs generated by site-directed mutagenesis. The function of the roles that the C-terminal amino acids may play in the activation process by CRP is addressed.

MATERIALS and METHODS

1.Reagents

Reagents were obtained as follows. cAMP, cAMP-agarose (Cat. #: A 0144), cGMP, adenylyl (3', 5') adenosine, calf thymus DNA, yeast tRNA, bovine serum albumin, Tris base, boric acid, trypsin, chymotrypsin, subtilisin, *Staphylococcus aureus* V8 protease, pepstatin, carboxypeptidase Y, PMSF, ß-mercaptoethanol, NEM, dithionitrobenzoic acid, Coomassie brilliant blue and bromophenol blue, Sigma; Sephacryl S200, S-Sepharose Fast Flow, ATP, UTP, Pharmacia; sodium dodecyl sulfate, ammonium persulfate, N,N,N'N'-tetramethylethylenediamine, Bio-Rad; acrylamide, bisacrylamide, urea, Serva; restriction endonucleases, DNA polymerase I Klenow fragment, DNA sequencing reagents, Boehringer Mannheim; T4 DNA ligase, T4 polynucleotide kinase, BRL; ^{32}P-α-dATP, ^{32}P-α-UTP, ^{3}H-cAMP, scintillation fluid, ICN; ZetaChrom SP100 capsule, CUNO, Inc. (Meriden. CT); DNase I, Promega; Polymin P, Gallard Schlessinger (Carle Place, NY); polyethylene glycol, Merck, Inc.; Tween, J. T. Baker Chemical Co.; Spin columns, Clontech.

2. Bacterial strains and plasmids

A *crp*$^-$ strain, XE64.2 and a *crp*$^-$*cya*$^-$ strain, CA8445, were kindly given by Dr. Richard Ebright (Rutgers University, New Jersey). pHA7, a pBR322 derivative containing the *crp* gene, was kindly given by Dr. Hiroji Aiba (University of Tsukuba, Japan). pHW104, a plasmid containing four segments of *lac* P$^+$ cloned within *Eco*RI sites, was kindly given by Dr. Donald M. Crothers (Yale University). pAA121, a

plasmid which contains the consensus CRP binding site, was kindly provided by Dr. Steven Busby (University of Birmingham, Birmingham, U.K.). pBR322 was purchased from Boehringer Mannheim. M13mp18 was obtained from BRL.

3. Protein purification

Wild-type or mutant CRP, was purified from large preps (10 liter cultures) according to Eilen *et al.* (1977) or small preps (1 liter preps) by a modified method of Ghosaini *et al.* (1988). For purification from the small cultures, the cells were lysed as described (Eilen *et al.*, 1977). After passing the lysate through a 10 x 2.5 cm cAMP-agarose affinity column, CRP was eluted by passing buffer containging 5 mM cAMP through the colume. The fractions containing CRP band were pooled and subjected to a 50 x 1 cm Sephacryl S200 gel filtration column and a 5 x 1.5 cm S Sepharose Fast Flow cation-exchange column according to Eilen *et al.* (1977) to further purify the protein.

RNA polymerase was isolated from *E. coli* K12 by a modification of the method of Burgess and Jendrisak (1975). Protein concentrations were determined using the extinction coefficients: CRP, $E^{1\%}_{280nm}$ = 8.8 (Aiba and Krakow, 1981); RNA polymerase holoenzyme, $E^{1\%}_{280nm}$ = 6.7 (Levine *et al.*, 1980).

4. DNA fragment purification

DNA fragments were excised from the vectors with the appropriate restriction endonucleases and separated from the vector DNA by 0.8% agarose gel electrophoresis. The appropriate bands were cut out of the gel and purified using

Gene Clean (US BioScience). The DNA fragments were separated by eletrophoresis on 0.8% agarose gel in 0.5x TAE buffer. The relevant bands were cut out and weighed. After addition of 4x volumes of 6 M NaI solution, the agarose pieces were heated at 55°C for 5 min to melt the agarose. The DNA solution was then incubated with glassmilk (US BioScience) at 0°C for 5 min followed by centrifugation at room temperature. After washing with 50% cold ethanol (-20°C), the DNA was eluted with H_2O and ready for use.

5. Labeling of DNA fragments

^{32}P-*lac* P$^+$ and ^{32}P-*gal* AA'were prepared by labeling with [α-^{32}P]dATP using DNA polymerase I Klenow fragment. The reaction mixture contained (final volume 50 μL): 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1mM DTT, 40 μCi [^{32}P]dATP (3000 mCi/mmol), 4 μg *lac* P$^+$ or *gal* AA' fragment and 5 units of DNA polymerase I Klenow fragment. After incubating for 15 minutes at room temperature, 10 μL of 0.65 mM dATP was added and the incubation was continued for an additional 15 minutes. For gel shift assay, the DNA fragments were ethanol precipitated, washed with 70% ethanol, and resuspended in H_2O. For footprinting assays, the doubly end-labeled *lac* P$^+$ fragment was digested with *Pvu*II to generate a promoter fragment uniquely labeled on the upper strand. After *Pvu*II digestion, the smaller fragment was removed using a spin filter (Clontech).

6. cAMP binding assay

Binding assays were performed in a reaction mixture containing (final volume 100 μL): 40 mM Tris-HCl (pH 8.0), 0.4 M KCl, 1 mM DTT, 1 mM EDTA, 50 μg casein

and varying amounts of CRP protein and [^{3}H]cAMP (Anderson *et al.*,1972). The mixtures were incubated for 30 minutes at 0°C followed by addition of 500 μL of saturated ammonium sulfate (pH8.0). After incubation at 0°C for 15 minutes, the mixtures were centrifuged and the supernatants removed. The precipitated proteins were redissolved in 500 μL of H_2O, mixed with 5 ml of Ecolume and counted.

7. Carboxypeptidase Y digestion of CRP

Mixtures contained (final volume, 40 μL): 10 mM MES (4-morpholine ethanesulfonic acid) (pH 5.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 8 μg CRP, 0.24 μg carboxypeptidase Y, 0.024 μg pepstatin, and where indicated 1 mM cAMP or cGMP were incubated at the indicated temperatures for the indicated time periods; the reactions were terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. The samples were then subjected to SDS-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel with a 4.75% stacking gel.

Large scale preparation of CRP^{CY}: CRP (500μg/mL) in 10 mM MES (pH 5.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 1.5 μg/mL pepstatin and 1.5 μg/mL carboxypeptidase Y was incubated at 47°C for 40 min; the CRP^{CY} was kept at 0°C until used. For incubations with CRP*598 or cAMP-CRP the reactions were carried out at 37°C with 10 mM Tris (pH 8.0) replacing the MES buffer.

8. Proteolytic cleavage of CRP by endoproteases

Reaction mixtures contained (final volume, 50 μL): 10 mM Tris-HCl (pH 8.0), 20

mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.2 mg/ml of CRP or CRP^{CY} or mutant CRP as indicated, 0.1 mM cAMP or 0.1 mM cGMP, and the indicated amounts of a protease. The mixtures were incubated at 37°C for the indicated time periods and the reactions were stopped by addition of PMSF to a final concentration of 0.8 mM followed by heating for 2 min at 100°C in SDS-Gel loading buffer (0.5 M Tris-HCl, pH 8.0, 5% SDS, 1.43 M β-mercaptoethanol, 50% glycerol, and 1 mg/ml bromophenol blue). The resultant cleavage products were resolved by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on a 15% polyacrylamide gel with a 4.75% stacking gel.

9. Intersubunit crosslinking

Reaction mixtures contained (final volume, 40 μL): 20 mM 1,3-bis[tris(hydroxy-methyl)-methylamino]propane (pH8.0), 4 μg CRP or CRP^{CY} and 0.1 mM cAMP or cGMP where indicated. The mixtures were preincubated at 30°C for 15 min, followed by addition of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to a final concentration of 20 μM and the incubation was continued for 15 min at 30°C. After the addition of 0.1 mM N-ethylmaleimide, aliquots were added to SDS-Gel loading buffer lacking ß-mercaptoethanol and heated at 100°C for 2 min prior to electrophoresis.

10. Gel retardation assays

A modified method of Garner and Revzin (1981) was used. Mixtures contained (final volume, 15 μL): 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM cAMP, 2 nM ^{32}P-*lac* P^+, 90 nM CRP, CRP^{CY}, or mutant CRP

proteins and where indicated, 80 nM RNA polymerase and 0.5 μg tRNA. The mixtures were incubated for 15 min at 37°C and binding was assayed by 7.5 % polyacrylamide gel electrophoresis with 1x TBE buffer (45 mM Tris-borate, 10 mM EDTA). The DNA bands were visualized by autoradiography at -70°C using Kodak XAR-5 film and a Cronex H-Plus intensifying screen.

11. Amino acid analysis

Incubation of CRP with carboxypeptidase Y was carried out as described above. The samples to be used for determination of the released amino acids were dried in pyrolyzed and siliconized 6x50 mm Corning glass tubes and redissolved in 100μL of fresh derivitization buffer (ethanol:triethylamine:H_2O = 15:2:3) and dried under vacuum. 100μL of derivitization buffer plus 1 μL PITC was added to each tube and the reaction was carried out for 5 min at room temperature. The samples were dried under vacuum and dissolved in 100 μL of a solution containing 49 mM sodium acetate, pH 5.4, plus 28% acetonitrile and loaded on an Applied Biosystems C-18 PTC-amino acid column for analysis.

12. Abortive initiation assay

A modified method of Malan and McClure was used to determine the ability of mutant CRP proteins to support abortive transcription initiation (Malan and McClure, 1984). Reaction mixtures contained (final volume 50 μL): 40 mM Tris-HCl (pH_ϕ8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM cAMP, 5% glycerol, 2 nM RNA polymerase holoenzyme and the indicated concentration of native or

modified CRP. After preincubation at 37°C for 10 minutes, 0.5 mM ApA and 50 nM [^{3}H]UTP (360 cpm/pmol) were added. The reaction was allowed to proceed for 15 minutes at 37° C then terminated by addition of 10 μL of 0.5 M EDTA. The radioactive products were resolved by paper chromatography in WASP solvent (for 100 mL, 18 mL of H_2O, 80 mL of saturated ammonium sulfate pH 8.0, and 2 mL of isopropanol) (McClure *et al.*, 1978). After chromatography, the segment containing the product ApApUpU was cut and the amount of ApApUpU synthesized was determined by counting the appropriate segments in Ecolume (ICN).

13. DNase I footprinting assay

Experimental conditions for DNase I footprinting assays were similar to those for abortive initiation assays. The mixtures contained (final volume 50 μL): 40 mM Tris-HCl (pH8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM cAMP, 5% glycerol, 3 nM [^{32}P]*lac* P$^+$ fragment, 20 nM RNA polymerase holoenzyme (where indicated) and the indicated concentration of CRP or mutant CRP. After formation of the complexes at 37°C for 30 minutes, 4 μL of a solution containing 0.25 units/μL of DNase I (Promega) was added to the mixture. (Note: the enzyme was supplied as 1 unit/μL and diluted to 0.25 units/μL in ice-cold TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) just before use.) Digestion was performed for 30 seconds at 37°C. The reaction was terminated by addition of 100 μL of phenol-chloroform (1:1) mixture and vortexing. After centrifugation at room temperature for 5 minutes, 4 μL of the aqueous phase was removed and added to 6 μL of formamide solution. Samples were loaded on an 8% denaturing sequencing gel according to Maxam and Gilbert

(Maxam and Gilbert, 1980). After electrophoresis, the gel was autoradiographed at -70°C using Kodak XAR-5 film and a Cronex H-Plus intensifying screen.

14. Phosphorylation of oligonucleotides

Reaction mixtures contained (final volume, 30μL): 0.1 M Tris, pH 8.0, 10 mM $MgCl_2$, 7 mM dithiothreitol, 1 mM dATP, 1.7 μM oligonucleotide, and 2 units of T4 polynucleotide kinase. The mixtures were incubated at 37°C for 15 minutes then heated at 70°C for 10 minutes to inactivate the kinase. The phosphorylated oligonucleotides were kept at 0°C before use or -20°C for storage.

15. Site-directed mutagenesis

CRP mutants were made according to the method of Eckstein and coworkers (Nakamaye and Eckstein, 1986) using a kit supplied by Amersham. Briefly,the *crp* gene was excised from plasmid pHA7 with *EcoR*I and *Hind*III and cloned into the same sites of M13mp18 RF DNA. Single-stranded DNA (ssM13mp18crp$^+$) was prepared in *E. coli* XL-1 Blue cells according to the procedure described in *Current Protocols* and used as the template for mutagenesis and DNA sequencing. Oligonucleotides to generate the desired mutations were synthesized using the Applied Biosynthesis System and purified using Nensorb columns. After phosphorylation with T4 polynucleotide kinase, the mutant oligonucleotides were annealed to the single-stranded template by heating at 70°C for 5 minutes followed by incubation at 37°C for 30 minutes. Polymerization and ligation were carried out by addition of DNA polymerase I (Klenow fragment), T4 DNA ligase, dATP, dGTP,

dTTP, and dCTPαS. The reaction mixtures were incubated at 14°C for 16 hours. Single-stranded DNA and non-incorporated nucleotides were removed by passing the ligation mixture through a nitrocellulose filter. The double-stranded DNA was then precipitated by addition of 1/10 volume of 3 M NaOAc and 2.5 volumes of cold ethanol (-20°C). After 15 min incubation at room temperature the DNA was precipitated by centrifugation at 4°C for 30 min, rinsed with 70% ethanol and redissolved in buffer composition not given by Amersham). The doubled-stranded DNA was then subjected to nicking by the restriction endonuclease, *NciI*, which would cut the wild-type strand but not the newly synthesized mutant strand because the latter contained the thionucleotide dCTP-αS. Exonuclease III was then introduced into the system to degrade the nicked, wild-type strand. The condition was such that the exonuclease was allowed to digest the parental DNA through the site of the mutation. The digested DNA was then repolymerized and ligated, with the mutant sequence as the template so that the proportion of phage particles containing mutant *crp* sequence was greatly enhanced. The ligation mixtures were then used to transform *E. coli* XL-1 Blue cells and individual plaques were obtained. Single-stranded DNA was again purified and sequencing analysis confirmed that mutants were isolated. Then the mutant *crp* genes were reintroduced into pBR322, the plasmid used for cloning the wild-type *crp*$^+$ gene.

16. DNA sequencing

Materials for this procedure were obtained for Boehringer Mannheim. Single-stranded DNA was prepared by the method used for site-directed mutagenesis. The

sequencing procedure was carried out according to that described in Boehringer Mannheim's M13 Sequencing Kit.

17. Western blotting

Proteins were separated on a 15% SDS polyacrylamide gel following the procedure of Laemmli (1970). The proteins were then transferred at 2.5 mA/cm^2 for 30 to 45 minutes to a nitrocellulose membrane using MiniBlot-SDE (Millipore). Identification of antigens on the nitrocellulose membrane was performed according to the method of Johnson *et al.* (1984). The nitrocellulose membrane was air-dried and blocked with 5% non-fat dried milk (Carnation, Inc.) in Tris buffered saline (TBS: 20 mM Tris, pH 7.4, 0.9% NaCl) at room temperature for 2 hours followed by washing 3 times with the same buffer. Anti-CRP monoclonal antibodies dissolved in 1% non-fat milk (in TBS) to a final concentration of 3 μg/ml were incubated with the membrane for 1 hour at room temperature followed by 3 washes with 1% non-fat milk in TBS. Goat-anti-mouse IgG conjugated with phosphatase was diluted 500 times in 1% non-fat milk and incubated with the membrane for 1 hour at room temperature followed by 3 washes with 1% non-fat milk in TBS. The substrate (0.3 mg/ml NBT and 0.15 mg/ml BCIP in 0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$) was then added and allowed to develop at room temperature. Color development was terminated by rinsing the membrane with deionized water.

18. in vitro run-off assay

Mixtures contained (final volume 50 μL): 50 mM Tris-HCl, (pH 8.0), 3 mM

magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, 25 μg/ml BSA, 20 μg/ml heparin, 3 nM *lac* P^+, 20 nM RNA polymerase and 15 or 30 nM CRP as indicated were preincubated at 37°C for 1 hour. The substrate (0.16 mM ATP, 0.16 mM GTP, 0.16 mM CTP, and 0.05 mM UTP and 2 μCi of ^{32}P-UTP) was added and the reaction was allowed to proceed for 5 min then stopped by addition of 50 μL of stop solution (40 mM EDTA and 300 μg/ml of *E. coli* tRNA. The RNA was ethanol precipitated and resuspended in 15 μL of DNA sequencing sample buffer (Boehringer Mannheim). Samples were heated at 90°C for 2 min and placed on ice for 5 min. RNA was resolved on 8% polyacrylamide gel containing 8 M urea.

RESULTS

Characterization of CRP^{CY}:

Previous studies showed that unliganded CRP is relatively resistant to attack by a variety of endoproteases (Krakow and Pastan, 1973; Eilen *et al.*, 1978). In contrast cAMP/CRP is digested resulting in N-terminal cores which bind CRP and retain the dimeric structure of native CRP. The results obtained for carboxypeptidase Y digestion of CRP indicate that at 37°C, cAMP/CRP is rapidly attacked by carboxypeptidase Y yielding a slightly smaller subunit fragment (Figure 3). At 37°C the C-terminal arm of unliganded CRP is folded in a manner preventing attack by carboxypeptidase Y. Even after 80 minutes at 42°C only a trace amount of the CRP^{CY} core is produced after incubation of unliganded CRP with carboxypeptidase Y (Figure 3). When incubated at 47°C the C-terminal domain melts allowing access to carboxypeptidase Y; by 40 minutes complete conversion to CRP^{CY} results. In contrast, cGMP/CRP is attacked at a much slower rate so that even after 80 minutes at 47°C residual intact CRP subunit is still evident (Figure 3).

The apparent impediment to continued exoproteolytic hydrolysis made the determination of the number of the C-terminal amino acid residues removed straightforward. Following incubation of CRP with carboxypeptidase Y for 40 minutes at 47°C, the released amino acids were determined by the PITC method. The data in Table I indicate that seven residues were removed; CRP^{CY} terminates at Thr-202. Similar results were obtained after analysis of the amino acids released from cAMP/CRP and unliganded CRP*598 following incubation with carboxypeptidase Y

at 37°C.

In the closed structure of unliganded CRP, it appears that the C-terminal domain must interact with a proximal segment of CRP. Trypsin was used to probe whether the loss of the C-terminal oligopeptide segment affects CRP^{CY} conformation. The results shown indicate that in contrast to CRP, unliganded CRP^{CY} is sensitive to trypsin (Figure 4). Native CRP complexed with cAMP is attacked by trypsin with the formation of a 19,000 Da intermediate and the accumulation of a 14,000 Da core. The cAMP/CRP^{CY} shows a similar pathway for tryptic attack but appears to be more sensitive than cAMP/CRP. CRP^{CY} in the presence of cGMP is attacked by trypsin at a rate similar to that seen for unliganded CRP^{CY}. However, there does not appear to be an accumulation of the 19,000 Da intermediate; the final product appears to be identical to that formed from cAMP/CRP or cAMP/CRP^{CY}. The unliganded CRP^{CY} is further degraded yielding smaller fragments. The results indicate that the conformation of CRP^{CY} differs markedly from that of native CRP.

Reaction of the two available sulfhydryl groups (Cys-178) of cAMP/CRP with DTNB results in the formation of an intersubunit bond within the CRP dimer. Under the conditions used, CRP and cGMP/CRP do not show the DTNB-mediated subunit crosslinking (Figure 5). In contrast, the subunits of CRP^{CY} were crosslinked in the presence or absence of cyclic nucleotide. The results provide additional evidence for an altered conformation of the C-terminal domain of CRP^{CY}.

The CRP^{CY} is prepared by incubation at pH 5.5 with carboxypeptidase at 47°C. The data presented in Figure 6 indicate that the incubation temperature and pH used do not affect cAMP binding by CRP. The CRP^{CY} prepared under these conditions

retains cAMP binding activity.

The CRP core formed after cutting with the *Staph. aureus* V8 protease at Glu-171 is unable to support *lac* P^+-directed abortive initiation (unpublished results). The data presented in Figure 7 demonstrate that the truncated CRP^{CY} which terminates at Thr-202 also does not activate RNA polymerase in the abortive initiation reaction.

It is possible that the observed inactivity of CRP^{CY} in supporting abortive initiation is a consequence of the loss of the contact site or conformation required for interaction with RNA polymerase or that CRP^{CY} is unable to bind to the CRP site on the *lac* P^+ promoter. The data presented in Fig. 8 show that CRP^{CY} does not bind to the *lac* P^+ fragment. Virtually all of the DNA migrates as the free *lac* fragment in the presence of cAMP/CRP^{CY} (Figure 8, lanes e, f). The incubation of cAMP/CRP^{CY} plus RNA polymerase with the *lac* fragment results in a complex which is dissociated by tRNA. The pattern obtained is comparable to that observed with RNA polymerase in the absence of CRP.

Characterization of C-terminal CRP mutants:

Substitution of the C-terminal arginine results in mutant CRPs which are less active than wild type CRP in supporting abortive initiation by RNA polymerase from *lac* P^+ (Table II). Of the four mutants tested, CRP209RE which has the C-terminal arginine replaced by glutamic acid retains 14% of wild type activity. In those mutants which retain the C-terminal arginine while having other C-proximal amino acid replacements the CRP207GV is relatively inactive. It would appear that the flexibility conferred by glycine at this position is important for CRP activity. Progressive C-

terminal truncation of CRP results in loss of activity. This is consistent with the apparently critical role of arginine at the C-terminus.

The results of single round transcription from the *lac* promoter show that CRP is required for initiation from *lac* P1 (Figure 9). In the presence of CRP a shorter runoff transcript is made. The C-terminal replacement mutants are impaired to varying degree (Figure 9) with the CRP209RD being the most affected. The internal substitution mutants (Figure 10) show activity comparable to wild type CRP. These results are comparable to those obtained for the abortive initiation reaction (Table II). In marked contrast are the results obtained for the C-terminal truncation mutants (Figure 11A, B) in which all are able to support initiation from *lac* P1 to an extent similar to that of wild type CRP, with the exception of CRP207GV, which appeared to be less active than the other mutants.

Transcription requires the binding of CRP to its site on the *lac* promoter and its interaction with RNA polymerase. Figures 12-14 show retardation assays for binding of CRP mutants to the *lac* P^+ DNA fragment. Removal of the C-terminal arginine results in marked loss in DNA binding activity. The results indicate that the mutants which retain the C-terminal arginine show relatively normal DNA binding activity although binding by CRP207GV is evidently weaker than that shown by wild type CRP or CRP208TI (Figure 13). The truncated CRPs show progressively weaker binding as more of the C-terminal amino acids are removed (Figure 14A, B). Loss of the C-terminal arginine in CRP209RO results in a partial loss of *lac* P^+ binding activity; comparable binding is seen for CRP208TO. However, removal of 3 or 5 amino acids results in truncated CRPs which retain little *lac* DNA binding activity.

This correlates with the loss of ability to effectively support abortive initiation (Table II).

Figure 15 shows the binding to *lac* P^+ and *gal*AA' of CRP mutants in which the C-terminal arginine has been changed to leucine (CRP209RL) or aspartic acid (CRP209RD). Binding of these mutants to *gal*AA' which contains a symmetric CRP binding site (Gaston *et al.*, 1988) is less affected by the alterations in the C-terminal arginine. CRP209RL retains high affinity binding for *gal*AA' while binding to the *lac* P^+ with a slightly lower affinity than wild type CRP. Replacing the C-terminal arginine with aspartic acid markedly lowers the affinity of CRP209RD for *lac* P^+ while still showing some decrease in affinity for the symmetrical *gal*AA' site. The results indicate that the C-terminal arginine is important but not absolutely essential for site specific DNA binding by CRP.

Substitution of one amino acid at the C-terminus of CRP is not expected to cause substantial change in cAMP binding since the cAMP binding site is located in the N-terminal domain. The mutant CRP proteins bound cAMP with an affinity similar to that of the wild-type CRP (data not shown), indicating that substitution of the C-terminal arginine does not cause significant conformational change in the N-terminal domain of CRP. In the absence of cAMP, the C-terminal mutant CRP proteins were as resistant to proteolytic cleavage as wild-type CRP. In the presence of cAMP, the digestion patterns for the mutant CRP proteins were similar to that of the wild-type CRP (Figure 16).

DNase I footprinting provides information on the interaction of CRP both with its site on the *lac* promoter and with RNA polymerase. CRP binds to the -80 to -50

segment of the [^{32}P]*lac* P^{+} fragment, the characteristic open promoter pattern is obtained upon addition of RNA polymerase with protection seen in the region spanning -50 to +20. Indicative of binding by RNA polymerase is the pronounced enhancement at about -25. The relative binding by the four C-terminal substitution mutants (Figure 17) is consonant with the abortive initiation results. All are relatively defective in binding to the -80 to -50 region. In the presence of RNA polymerase there is some increased binding of the mutant CRPs to the CRP site and a reciprocal formation of the open complex. Relative to the pattern shown by the wild type CRP the footprints indicate that CRP209RD and CRP209RL bind weakly to the CRP site and poorly support binding of RNA polymerase to the promoter. CRP209RE, which shows the lowest activity of this set in supporting abortive initiation is similarly impaired for binding to the CRP site and the ability to effect RNA polymerase binding.

Footprints for the CRP mutants which retain the C-terminal arginine (CRP208TI, CRP207GV, CRP206F) are shown in Figure 18. Of this set of CRP mutants, CRP206YF shows the strongest activity in establishing the open complex in the presence of RNA polymerase. This correlates with its ability to support abortive initiation. Binding of CRP207GV and CRP208TI to the *lac* promoter are similar, complex formation in the presence and absence of RNA polymerase are comparable at a CRP to DNA ratio of 5 to 1. When the ratio of CRP to DNA is increased to 10 to 1 interaction of each of these mutants is similar to that shown by wild type CRP. The results do not correlate well with the ability to support abortive initiation, CRP207GV is relatively impaired for abortive initiation but not for open complex

formation.

Footprints for C-terminal truncations (Figure 19) indicate that all show weaker binding to the CRP site both in the presence and absence of RNA polymerase. CRP207GO which terminates at Tyr-206 shows the weakest binding and ability to establish the open complex pattern in the presence of RNA polymerase. CRP207GO also showed weak binding in the retardation assay (Figure 14). Removal of two additional amino acids with the truncated CRP205VO terminating at Val-204 restored binding activity to an extent comparable to that of CRP209RO and CRP208TO. Although CRP206YO was constructed the truncated CRP could not be isolated apparently due to its *in vivo* instability. Western blots of lysates containing this mutant using an anti-CRP monoclonal antibody did not show any CRP derivative (data not shown).

MacConkey lactose agar plates were used to test the activities of the mutants in supporting β-galactosidase synthesis *in vivo*. All of the mutants examined in this study were able to support RNA polymerase in the synthesis of β-galactosidase in a *crp*$^-$ cell line, XE64.2, as indicated by the red color of the colonies (Table III). KC1071 is a *crp*$^-$ *E. coli* cell line which contained a consensus CRP binding site replacing the operator in the *lac* UV5 operon; therefore, the cells were red when grown on MacConkey plates. Binding of CRP in KC1071 will prevent *lac* expression and the synthesis of β-galactosidase, yielding white colonies. When the mutants were tested in KC1071 cells, they all gave white colonies (except for CRP206YO), indicating that these mutant CRP proteins were able to bind to the DNA site. Of interest was the unstable mutant CRP206YO. It was able to support β-galactosidase synthesis in

XE64.2 (*crp*$^-$) cells, indicating that it could transiently bind to the CRP binding site in the *lac* promoter *in vivo*. Yet the mutant protein failed to bind to the CRP binding site in KC1071 cells. A possible explanation for this observation would be that CRP206YO could bind transiently to the CRP binding site in the *lac* promoter *in vivo* to form the RP_o. In addition, low levels of CRP which would support low level of synthesis of β-galactosidase could give a positive MacConkey result in XE64.2 (*crp*$^-$) cells whereas relatively high DNA binding activities of CRP would be required to constantly block the synthesis of β-galactosidase by RNA polymerase in order to get a negative MacConkey result in KC1071 (*lac* UV5) cells. Western blotting assays indicated that CRP206YO was unstable in both of the cell lines discussed above.

DISCUSSION

Limited proteolysis has proven to be a useful probe of the conformation of CRP and CRP* mutants. Unliganded CRP is resistant to a variety of endoproteases (Krakow and Pastan, 1973; Eilen, *et al.*, 1978). Conversion to a protease-sensitive state can be effected in several ways. cAMP/CRP is degraded to yield N-terminal cores which retain cAMP-binding activity and the dimeric structure of CRP (Eilen *et al.*, 1978; Tsugita *et al.*, 1982). The DNA-CRP complex formed at low ionic strength is attacked by trypsin while remaining resistant to attack by subtilisin, chymotrypsin and the *Staph. aureus* V8 protease (Angulo and Krakow, 1986). Two fragments are formed after incubation of DNA-CRP with trypsin and cAMP binding activity is lost. The CRP within the monoclonal antibody 64D1-CRP complex becomes sensitive to attack by all four of the endoproteases used (Li and Krakow, 1987). The 9,000 Da fragment formed by tryptic hydrolysis of the mAb 64D1-CRP complex appears to be identical to the N-terminal fragment formed after tryptic attack on the DNA-CRP complex. The results obtained from the study using carboxypeptidase Y indicates that facile attack of CRP at 37°C by carboxypeptidase Y occurs in the presence of cAMP. Unliganded CRP is resistant to carboxypeptidase Y at 37°C. At 47°C the C-terminal arm appears to melt allowing for attack by carboxypeptidase Y. The scanning calorimetric study of the thermal unfolding of CRP by Ghosaini *et al.* (1988) indicates that CRP structure is not markedly affected at temperatures below 60°C. In contrast to the insensitivity of unliganded CRP, CRP* mutants are inherently sensitive to attack by both endoproteases (Ren *et al.*, 1988; Harman and Dobrogosz, 1983;

Harman *et al.*, 1986) and carboxypeptidase Y (Yang *et al.*, 1991). The effect of the mutation near the hinge region results in a general effect on the conformation of the C-terminal domain which extends to the C-terminus of CRP*598.

The results indicate that the C-terminus of CRP is folded in a manner which prevents attack by carboxypeptidase Y. Hydrolysis by carboxypeptidase Y results in a core, CRP^{CY}, which terminates at Thr-202. CRP^{CY} is formed from cAMP-CRP at 37°C or CRP at 47°C. Weber and Steitz (1987) proposed that interaction between the β 11 (residues 195-199) and β 12 (residues 201-205) strands may occur in cAMP-CRP. This should not result in a conformational impediment preventing continued hydrolysis by carboxypeptidase Y. An alternative hypothesis is that the block is due to an unfavorable amino acid sequence which prevents further hydrolysis by carboxypeptidase Y. The resistance of unliganded CRP to attack by carboxypeptidase suggests that stabilization of the C-terminal region is effected by interaction with a proximal region of CRP.

As was shown previously with much shorter N-terminal cores (Krakow and Pastan, 1973; Eilen *et al.*, 1978), the CRP^{CY} retains cAMP-binding activity. Removal of the 7 amino acid residues from the C-terminus results in the complete loss of the ability of CRP^{CY} to activate *lac* P^+ transcription and to bind to the *lac* DNA fragment. This is probably not due to the loss of amino acid residues directly involved in DNA binding beyond the intact helix-turn-helix region. In an analysis of the binding of CRP to a consensus DNA site for CRP, Ebright *et al.* (1989) proposed that Lys-201 is one of the residues of CRP involved in ion-pair formation with DNA. Based on this model, CRP^{CY} should retain DNA-binding activity.

The N-terminal seven amino acid arm of the λ repressor is considered to be involved in making contacts with the operator site. The N-terminal arm of the λ repressor is accessible to attack by trypsin suggesting that this region may be less compactly folded than the rest of the N-terminal domain (Pabo *et al.*, 1982). The crystal structures indicate that the C-terminal arm of cAMP-bound CRP and the N-terminal arm of the λ repressor should be flexible in solution (Weber and Steitz. 1987; Pabo and Lewis, 1982). The accessibility of the C-terminus of cAMP-CRP to attack by carboxypeptidase Y and the N-terminal arm of the λ repressor to attack by trypsin are in keeping with these regions being less tightly folded. The truncated CRP^{CY} is sensitive to attack by endoproteases and subject to intersubunit crosslinking mediated by DTNB in the absence of cAMP. These properties are also seen with the conformationally relaxed CRP*mutants which, however, are active for DNA binding and transcription activation (Ren *et al.*, 1988; Harman and Dobrogosz, 1983; Harman *et al.*, 1986). The loss of the C-terminal arm results in a major effect on the conformation of the resultant CRP^{CY}. This appears to be a consequence of the ensuing disruption of the small β-sheet present in the C-terminal domain of CRP (Weber and Steitz, 1987). Loss of critical amino acid residues in the C-proximal β-strand 12 would prevent hydrogen bonding with the β11 and β10 strands. The consequent effects on the conformational orientation of the helix-turn-helix domain would explain the observed loss of DNA binding by CRP^{CY}. In order for CRP to bind DNA its helix-turn-helix structure must assume and maintain the "proper" conformation. Amino acids that flank the helix-turn-helix region are likely to be involved in orienting the helix-turn-helix motif in the proper conformation.

Site specific DNA binding requires the appropriate conformation of the helix-turn-helix region present in the C-terminal domain of CRP (Weber and Steitz, 1987). The ß-sheet structure involving interactions between ß-strands 9, 10, 11 and 12 is involved in establishing this conformation in cAMP/CRP. A truncated form of CRP produced by digestion with carboxypeptidase Y lacking the seven C-terminal amino acids is unable to bind DNA (Yang and Krakow, 1991) presumably as a consequence of the damage to ß-strand 12. With the exception of CRP205VO all of the other mutations studied lie beyond ß-strand 12 and presumably would not affect the formation of the ß-sheet structure. Alteration or removal of the C-terminal arginine produces an obvious effect on the CRP-dependent assays used in this study. DNA binding is weakened and abortive initiation by RNA polymerase is inhibited to varying extent. Replacement of C-proximal residues while maintaining the C-terminal arginine is less deleterious consonant with an important role for Arg-209. Replacement of arginine with an acidic amino acid produces a less active CRP mutant. Progressive deletion of C-terminal amino acids produces an incremental decline in both DNA binding activity and the ability to support abortive initiation. Part of this loss in activity can be ascribed to the removal of Arg-209. The effect of replacing the C-terminal arginine with other amino acids is less pronounced when DNA binding is assessed using the symmetric *gal*AA' than with *lac* P^+. If the C-terminal arginine is involved in DNA binding it can only contribute a relatively small effect.

The N-terminal arm of the λ repressor shows some similarities with the C-terminal arm of cAMP/CRP. The crystal structures indicate that the C-terminal

arm of cAMP-bound CRP (Weber and Steitz, 1987) and the N-terminal arm of the λ repressor (Pabo and Lewis, 1982) should be flexible in solution. In addition to the interaction of its helix-turn-helix motif with the operator the N-terminal arm of the λ repressor provides additional DNA contact(s). The crystal structure for the cAMP/CRP-DNA complex (Schultz *et al.*, 1992) does not indicate that the C-terminal arm of CRP is involved in DNA binding.

It is interesting that while abortive initiation appears to be sensitive to truncations of the C-terminal arm of CRP, single round transcription is not. Abortive initiation activity represents the sum of many cycles of initiation and release of product from RNA polymerase. The enzyme may maintain its contact with CRP during this reaction and the lower activity seen for the mutant CRPs may be a consequence of a weakened interaction with RNA polymerase.

As expected, none of the mutant CRPs show changes in their binding for cAMP. The crystal structure of CRP/cAMP complex predicts that the last four C-terminal amino acids are not part of a ß-strand or involved in H-bonding with other residues in the protein. While no crystal structure of unliganded CRP is available at this time, our results from the carboxypeptidase Y digestion experiments suggest that this region is structured since it is inaccessible to carboxypeptidase Y in unliganded CRP at 37°C. The results presented in this study also indicate that this region, albeit outside the helix-turn-helix and at the end of the protein, plays a role in CRP function. It is not clear what role the positive charge on the arginine residue may play on the structure in its vicinity. Replacement of the positive charge with a neutral amino acid residue causes a dramatic reduction in the ability of the mutant CRP to

support abortive initiation, while changing the positive charge to a negative charge produces a profound effect on specific DNA binding as well as in the ability to support abortive initiation seen in CRP209RD and CRP209RE. Replacing Gly-207 with valine, which eliminates the flexibility provided by glycine, causes a substantial reduction in the activity of CRP in supporting abortive initiation. This decrease in supporting abortive initiation parallels with the decrease in DNA binding of the protein, as detected by footprinting assays, suggesting that the last two amino acids in the C-terminus of CRP reorient in the interaction of CRP with DNA. The fact that CRP207GV is a more active mutant than CRP207GO suggests the possibility that maintaining the original length of the CRP-terminal region is more important to the structure and function of CRP than keeping the original amino acids. In summary, the results from the studies of the C-terminal mutations indicate that the C-terminal arginine as well as the other four C-terminal amino acids studied are important in the function of CRP.

SUMMARY

Incubation of CRP with carboxypeptidase Y at 47°C results in formation of a CRP core termed CRP^{CY}. The resistance of CRP to cleavage by carboxypeptidase Y suggests that at 37°C the C-terminal arm of unliganded CRP is folded in manner preventing attack by the exoprotease. At 47°C the C-terminal arm melts allowing access to carboxypeptidase Y and by 40 minutes complete conversion to CRP^{CY} results. Amino acid analysis indicates that seven amino acids have been removed from the C-terminal region of CRP at 47°C or from the C-terminal of cAMP/CRP at 37°C. Trypsin was used to probe whether the loss of the C-terminal oligopeptide segment affects CRP^{CY} conformation. The results indicate that in contrast to CRP, unliganded CRP^{CY} is sensitive to trypsin. Reaction of the two available sulfhydryl groups (Cys-178) of cAMP/CRP with DTNB results in the formation of an intersubunit bond within the CRP dimer. Under the conditions used, CRP and cGMP/CRP do not show the DTNB-mediated subunit crosslinking. In contrast, the subunits of CRP^{CY} were crosslinked in the presence or absence of cyclic nucleotide. The results from the two assays indicate that the conformation of CRP^{CY} differs markedly from that of native CRP. The CRP core formed after cutting with the *Staph. aureus* V8 protease at Glu-171 is unable to support *lac* P^+-directed abortive initiation (unpublished results). The data presented in Figure 7 demonstrate that the truncated CRP^{CY} which terminates at Thr-202 also does not activate RNA polymerase in the abortive initiation promoter. The data presented in Fig. 8 show that CRP^{CY} does not bind to the *lac* P^+ fragment.

Substitution of the C-terminal arginine results in mutant CRPs which are less

active than wild type CRP in supporting abortive initiation by RNA polymerase from *lac* P^+. In those mutants which retain the C-terminal arginine while having other C-proximal amino acid replacements the CRP207GV is relatively inactive. It would appear that the flexibility conferred by glycine at this position is important for CRP activity. Progressive C-terminal truncation of CRP results in loss of activity. This is consistent with the apparently critical role of arginine at the C-terminus. All of the mutants were able to support single-round transcription by RNA polymerase from *lac* P^+, with the exception of CRP206GO, which appeared to be less active than the other mutants. DNA binding assays show that replacing the C-terminal arginine with an acidic amino acid causes substantial loss of DNA binding activity of CRP. Such loss in DNA binding by CRP is partially restored in the presence of RNA polymerase. Binding of CRP207GV to the *lac* promoter is similar to that shown by wild-type CRP. The fact that CRP207GV is impaired for abortive initiation suggests that the flexibility conferred by glycine at this position may be important for normal interaction of CRP with RNA polymerase. Removal of three or more amino acids from the C-terminal region of CRP causes marked loss of activities both in DNA binding and in supporting abortive initiation as seen for CRP207GO and CRP205VO. Another deletion mutant, CRP206YO, is unstable in the cells. All mutants studied including CRP206YO are able to support ß-galactosidase synthesis *in vivo* in the *crp*- strain, XE64.2. CRP206YO is the only mutant which fails to block RNA polymerase from synthezing ß-galactosidase in KC1071 cells. The results suggest that can CRP206YO bind transiently to the CRP binding site in the *lac* promoter *in vivo* to form RP_o.

Table I. Amino acids released from the C-terminus of CRP after incubation with carboxypeptidase Y.

Amino acid Released	CRP (47°C) (pH 5.5)	cAMP-CRP (37°C) (pH 8.0)	CRP*598 (37°C) (pH 8.0)
		pmol	
Arg	291	277	236
Thr	350	295	360
Gly	368	397	287
Tyr	331	323	311
Val	584	582	508
Ile	418	402	303
Lys	40	12	0
His	0	0	0

Determination of the amino acids released following incubation of 330 pmol CRP or CRP*598 with carboxypeptidase Y was carried out as indicated in Experimental Procedures. The result shown for CRP (47°) is the average of three experiments. The C-terminal sequence of CRP (5) is shown below:

199 200 201 202 203 204 205 206 207 208 209

CRP--His-Gly-Lys-Thr-Ile-Val-Val-Tyr-Gly-Thr-Arg-COOH

Table II. Ability of CRP and mutants to support abortive initiation from the *lac* P^+ promoter.

	% activity by mutant or wild type CRP
CRP	100
CRP209RD	24
CRP209RE	14
CRP209RL	31
CRP209RN	24
CRP208TI	52
CRP207GV	25
CRP206YF	78
CRP209RO	33
CRP208TO	20
CRP207GO	2
CRP205VO	10
No CRP	6

Reaction mixtures contained (final volume, 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM cAMP, 2 nM *lac* P^+ fragment, 40 nM RNA polymerase, 1% glycerol and 20 nM CRP or mutant protein. The mixtures were incubated for 15 min at 37°C; after addition of 500 μM ApA and 50 μM [^{3}H]UTP the incubation was continued for 15 min at 37°C. 100% activity = 52 pmol of [^{3}H]UMP incorporated.

Table III. MacConkey plate test on the activity of mutant CRP proteins.

	XE64.2	KC1071
pHA7 (wild-type)	red	white
pZY209RD	red	white
pZY209RE	red	white
pZY209RL	red	white
pZY209RN	red	white
pZY209RO	red	white
pZY208TO	red	white
pZY207GO	red	white
pZY206YO	red	red
pZY205VO	red	white
pZY208TI	red	white
pZY207GV	red	white
pZY206YF	red	white
pBR322	white	red

Figure 1. The amino acid sequence of DNA sequence of CRP (from Aiba, *et al.*, 1982).

CRP

```
          12              24              36              48              60              72              84
ATG GTG CTT GGC AAA CCG CAA ACA GAC CCG ACT CTC GAA TGG TTC TTG TCT CAT TGC CAC ATT CAT AAG TAC CCA TCC AAG AGC
TAC CAC GAA CCG TTT GGC GTT TGT CTG GGC TGA GAG CTT ACC AAG AAC AGA GTA ACG GTG TAA GTA TTC ATG GGT AGG TTC TCG
Met Val Leu Gly Lys Pro Gln Thr Asp Pro Thr Leu Glu Trp Phe Leu Ser His Cys His Ile His Lys Tyr Pro Ser Lys Ser
     1           4           7          10          13          16          19          22          25

          96             108             120             132             144             156             168
ACG CTT ATT CAC CAG GGT GAA AAA GCG GAA ACG CTG TAC TAC ATC GTT AAA GGC TCT GTG GCA GTG CTG ATC AAA GAC GAA GAG
TGC GAA TAA GTG GTC CCA CTT TTT CGC CTT TGC GAC ATG ATG TAG CAA TTT CCG AGA CAC CGT CAC GAC TAG TTT CTG CTT CTC
Thr Leu Ile His Gln Gly Glu Lys Ala Glu Thr Leu Tyr Tyr Ile Val Lys Gly Ser Val Ala Val Leu Ile LYS Asp Glu Glu
28          31          34          37          40          43          46          49          52          55

          180             192             204             216             228             240             252
GGT AAA GAA ATG ATC CTC TCC TAT CTG AAT CAG GGT GAT TTT ATT GGC GAA CTG GGC CTG TTT GAA GAG GGC CAG GAA CGT AGC
CCA TTT CTT TAC TAG GAG AGG ATA GAC TTA GTC CCA CTA AAA TAA CCG CTT GAC CCG GAC AAA CTT CTC CCG GTC CTT GCA TCG
Gly Lys Glu Met Ile Leu Ser Tyr Leu Asn Gln Gly Asp Phe Ile Gly Glu Leu Gly Leu Phe Glu Glu Gly Gln Glu Arg Ser
       58          61          64          67          70          73          76          79          82

          264             276             288             300             312             324             336
GCA TGG GTA CGT GCG AAA ACC GCC TGT GAA GTG GCT GAA ATT TCG TAC AAA AAA TTT CGC CAA TTG ATT CAG GTA AAC CCG GAC
CGT ACC CAT GCA CGC TTT TGG CGG ACA CTT CAC CGA CTT TAA AGC ATG TTT TTT AAA GCG GTT AAC TAA GTC CAT TTG GGC CTG
Ala Trp Val Arg Ala Lys Thr Ala Cys Glu Val Ala Glu Ile Ser Tyr Lys Lys Phe Arg Gln Leu Ile Gln Val Asn Pro Asp
     85          88          91          94          97          100         103         106         109

          348             360             372             384             396             408             420
ATT CTG ATG CGT TTG TCT GCA CAG ATG GCG CGT CGT CTG CAA GTC ACT TCA GAG AAA GTG GGC AAC CTG GCG TTC CTC GAC GTG
TAA GAC TAC GCA AAC AGA CGT GTC TAC CGC GCA GCA GAC GTT CAG TGA AGT CTC TTT CAC CCG TTG GAC CGC AAG GAG CTG CAC
Ile Leu Met Arg Leu Ser Ala Gln Met Ala Arg Arg Leu Gln Val Thr Ser Glu Lys Val Gly Asn Leu Ala Phe Leu Asp Val
112         115         118         121         124         127         130         133         136         139

          432             444             456             468             480             492             504
ACG GGC CGC ATT GCA CAG ACT CTG CTG AAT CTG GCA AAA CAA CCA GAC GCT ATG ACT CAC CCG GAC GGT ATG CAA ATC AAA ATT
TGC CCG GCG TAA CGT GTC TGA GAC GAC TTA GAC CGT TTT GTT GGT CTG CGA TAC TGA GTG GGC CTG CCA TAC GTT TAG TTT TAA
Thr Gly Arg Ile Ala Gln Thr Leu Leu Asn Leu Ala Lys Gln Pro Asp Ala Met Thr His Pro Asp Gly Met Gln Ile Lys Ile
       142         145         148         151         154         157         160         163         166

          516             528             540             552             564             576             588
ACC CGT CAG GAA ATT GGT CAG ATT GTC GGC TGT TCT CGT GAA ACC GTG GGA CGC ATT CTG AAG ATG CTG GAA GAT CAG AAC CTG
TGG GCA GTC CTT TAA CCA GTC TAA CAG CCG ACA AGA GCA CTT TGG CAC CCT GCG TAA GAC TTC TAC GAC CTT CTA GTC TTG GAC
Thr Arg Gln Glu Ile Gly Gln Ile Val Gly Cys Ser Arg Glu Thr Val Gly Arg Ile Leu Lys Met Leu Glu Asp Gln Asn Leu
    169         172         175         178         181         184         187         190         193

          600             612             624
ATC TCC GCA CAC GGT AAA ACC ATC GTC GTT TAC GGC ACT CGT TAA
TAG AGG CGT GTG CCA TTT TGG TAG CAG CAA ATG CCG TGA GCA ATT
Ile Ser Ala His Gly Lys Thr Ile Val Val Tyr Gly Thr Arg
196         199         202         205         208
```

Figure 2. Drawing of the CRP dimer (from de Crombrugghe *et al.*, 1984). The N-terminal domain consists of α-helix A, β-sheets 1 to 8 and α-helices B and C. The DNA binding C-terminal domain consists of α-helices D, E and F, and the residues connecting these secondary structures. The two F helices, which clearly protrude from the surface of the dimer, are thought to provide many interactions with DNA. All of the interactions between the two subunits are provided by the large N-terminal domain and the majority of these are provided by the two long C helices that lie together in the center of the dimer. The two subunits are not exactly related by a perfect dyad symmetry.

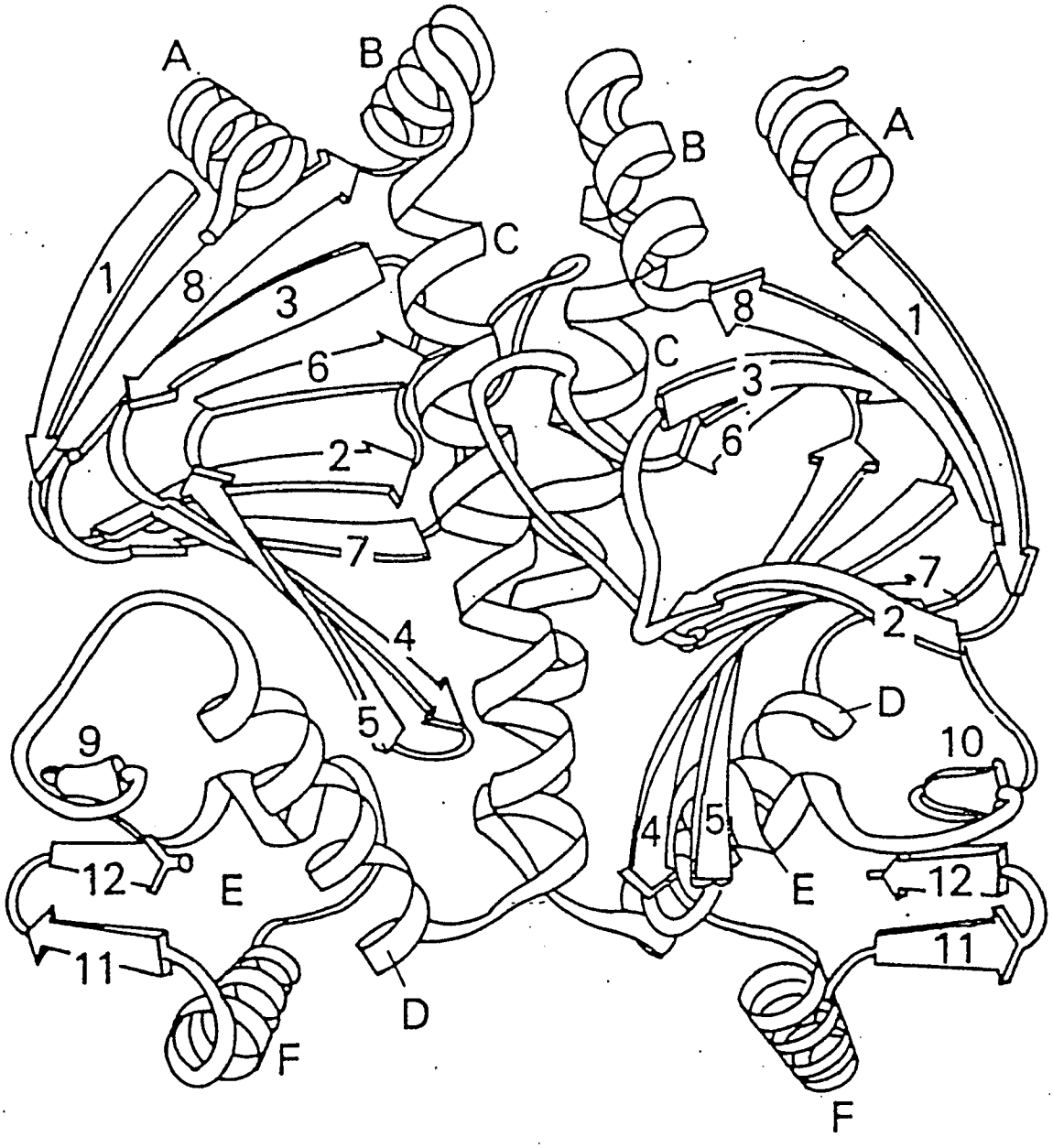

A
B
C
1
8
3
6
2
7
4
5
9
12
E
11
D
F
B
A
C
8
1
3
6
7
2
D
10
4
5
E
12
11
F

Figure 3A. Time course of digestion of CRP by carboxypeptidase Y. The reaction conditions are described in "Materials and Methods". The reactions were run at 37°C for the indicated times and then terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. Lanes a, b, c, and d incubated for 10, 20, 40, and 80 min respectively in the absence of cyclic nucleotide; lanes e, f, g, and h incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cAMP; lanes i, j, k, and l incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cGMP.

37°C

a b c d e f g h i j k l

Figure 3B. Time course of digestion of CRP by carboxypeptidase Y. The reaction conditions are described in "Materials and Methods". The reactions were run at 42°C for the indicated times and then terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. Lanes a, b, c, and d incubated for 10, 20, 40, and 80 min respectively in the absence of cyclic nucleotide; lanes e, f, g, and h incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cAMP; lanes i, j, k, and l incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cGMP.

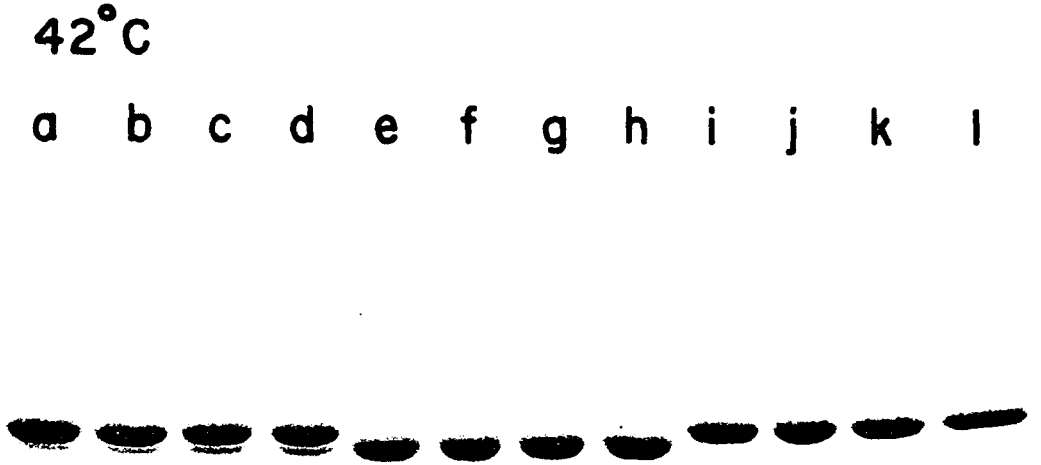

42°C
a b c d e f g h i j k l

Figure 3C. Time course of digestion of CRP by carboxypeptidase Y. The reaction conditions are described in "Materials and Methods". The reactions were run at 47°C for the indicated times and then terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. Lanes a, b, c, and d incubated for 10, 20, 40, and 80 min respectively in the absence of cyclic nucleotide; lanes e, f, g, and h incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cAMP; lanes i, j, k, and l incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cGMP.

47°C

a b c d e f g h i j k l

Figure 4A. Sensitivity of CRP to trypsin attack in the presence and absence of cyclic nucleotide. Conditions for protease digestion assays are described in "Materials and Methods". CRP + trypsin incubated for: lane a, 10 min; lane b, 20 min; lane c; 40 min; lane d, + cAMP 10 min; lane e, + cAMP 20 min; lane f; + cAMP 40 min; lane g, + cGMP 10 min; lane h, + cGMP 20 min; lane i, + cGMP, 40 min; lane j, omit trypsin.

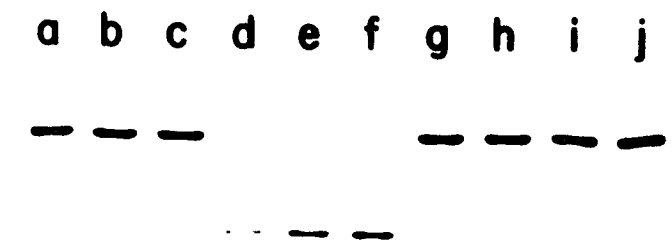

a b c d e f g h i j

Figure 4B. Sensitivity of CRP^{CY} to trypsin attack in the presence and absence of cyclic nucleotide. CRP^{CY} was prepared by carboxypeptidase Y digestion of CRP at 47°C. Conditions for protease digestion assays are described in "Materials and Methods". CRP^{CY} incubated for: lane a, 5 min; lane b, 10 min; lane c, 20 min; lane d, + cAMP 5 min; lane e, + cAMP 10 min; lane f; + cAMP 20 min; lane g, + cGMP 5 min; lane h, + cGMP 10 min; lane i, + cGMP, 20 min; lane j, omit trypsin.

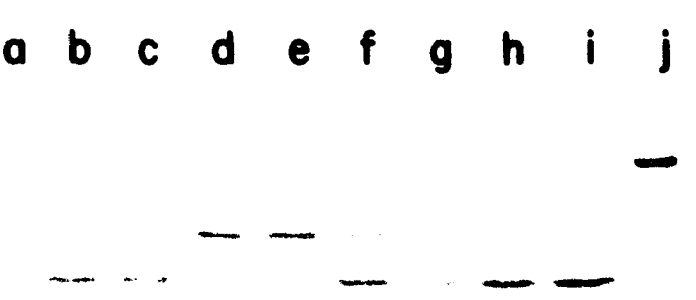

a b c d e f g h i j

Figure 5. Effect of DTNB on intersubunit crosslinking of CRP and CRP^{CY}. CRP^{CY} was prepared by incubation with carboxypeptidase Y at 47°C. Conditions for crosslinking assays are described in "Materials and Methods". Lane a, CRP + DTNB; lane b, CRP + cAMP + DTNB; lane c, CRP + cGMP + DTNB; lane d, CRP; lane e, CRP^{CY}; lane f, CRP^{CY} + DTNB; lane g, CRP^{CY} + cAMP + DTNB; lane h, CRP^{CY} + cGMP + DTNB.

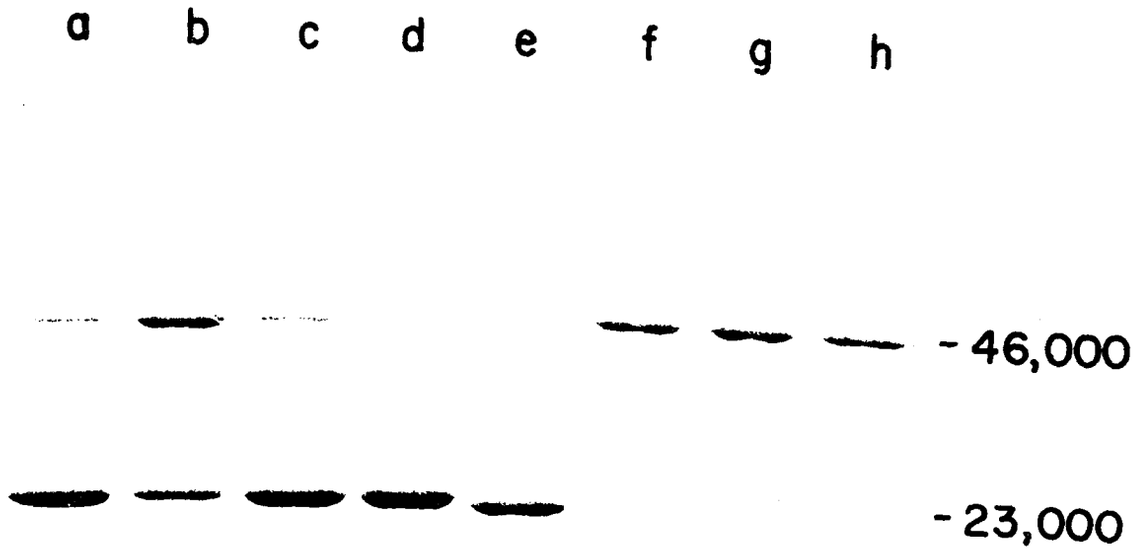

a
b
c
d
e
f
g
h
-46,000
-23,000

Figure 6. Binding of cAMP by CRP and CRP^{CY}. Conditions for binding assays are described in "Materials and Methods". CRP, ●-●; CRP preincubated at 47°C, o-o; CRP^{CY}, ■-■.

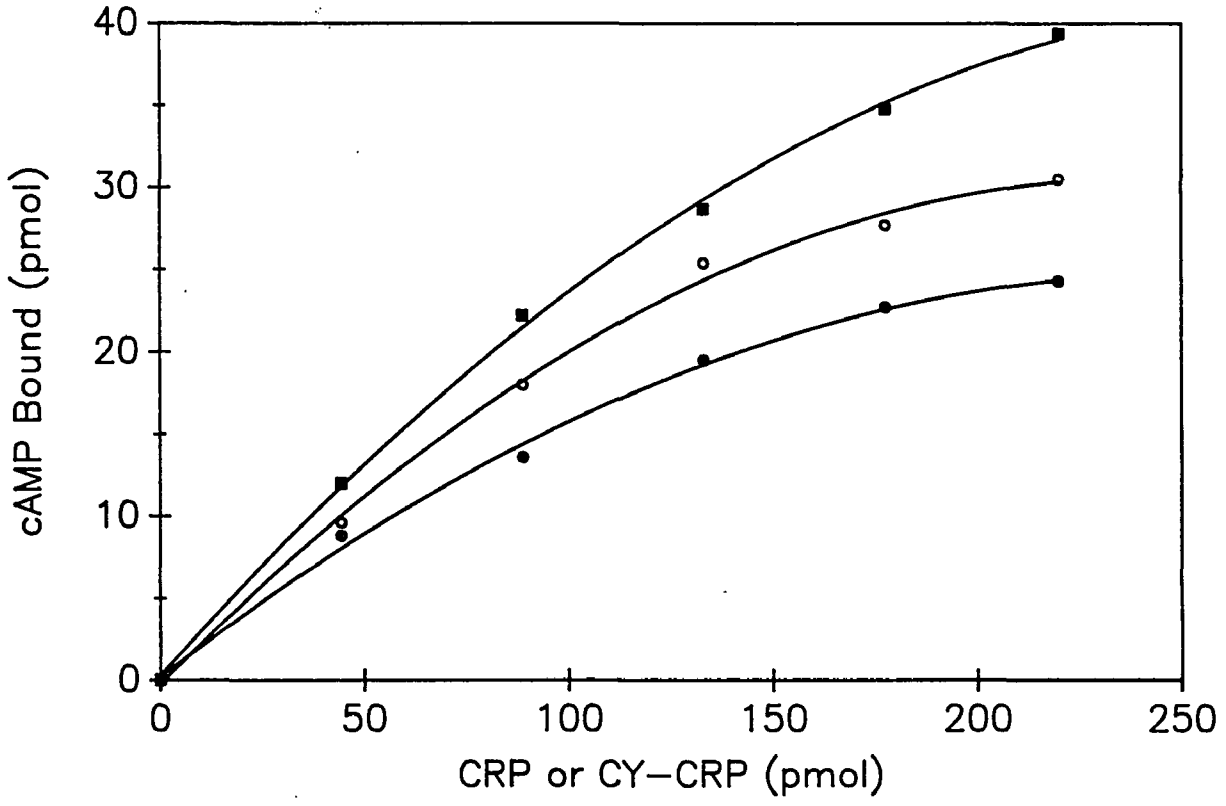

40
30
20
10
0
cAMP Bound (pmol)
0
50
100
150
200
250
CRP or CY–CRP (pmol)

Figure 7. Ability of CRP and CRP^{CY} to support abortive initiation from the *lac* P^+ promoter. Conditions for abortive initiation assays are described in "Materials and Methods" using 2 nM *lac* P^+ fragment, 40 nM RNA polymerase, 1% glycerol and the indicated amount of CRP or CRP^{CY} (prepared by incubation at pH 8.0 of cAMP-CRP with carboxypeptidase Y at 37°C). The mixtures were incubated for 15 min at 37°C; after addition of 500 μM ApA and 50 μM [^{3}H]UTP the incubation was continued for 15 min at 37°C.

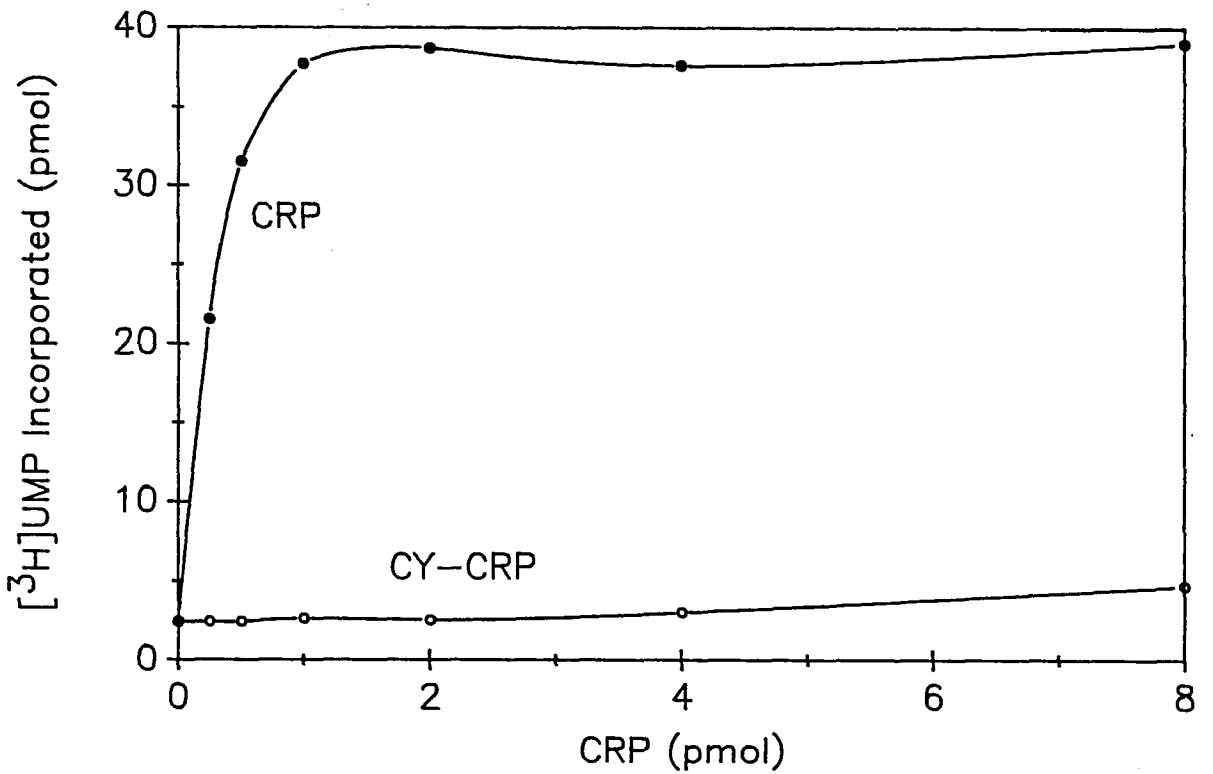

40
30
20
10
0
[3H]UMP Incorporated (pmol)
CRP
CY–CRP
0
2
4
6
8
CRP (pmol)

Figure 8. Binding of CRP and CRP^{CY} to the *lac* P^+ fragment. Conditions for gel retardation assays are described in "Materials and Methods" using 2 nM ^{32}P-labeled *lac* P^+ fragment (0.1 μg), 80 nM RNA polymerase and 90 nM CRP or CRP^{CY}. The mixtures were incubated for 15 min at 37°C. Lane a, free *lac* P^+; lane b, CRP; lane c, CRP + tRNA; lane d, CRP + RNA polymerase; lane e, CRP + RNA polymerase + tRNA; lane f, CRP^{CY}; lane g, CRP^{CY} + tRNA; lane h, CRP^{CY} + RNA polymerase; lane i, CRP^{CY} + RNA polymerase + tRNA; lane j, RNA polymerase.

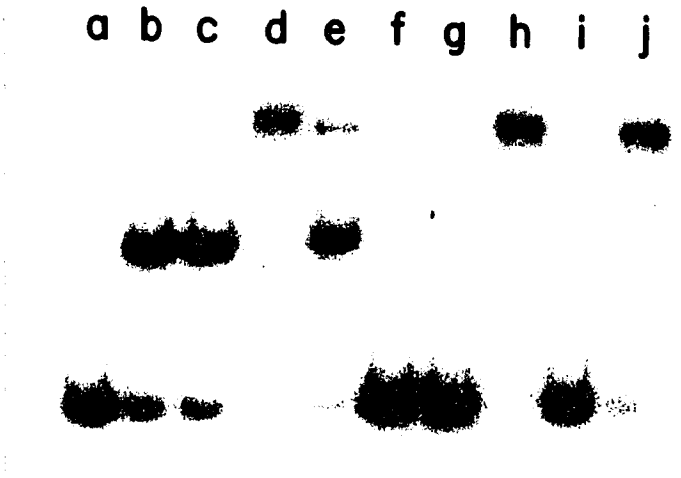

a b c d e f g h i j

Figure 9. Activity of mutant CRPs in which the C-terminal arginine is replaced by aspartic acid, leucine or asparagine to support single-round transcription by RNA polymerase from *lac* P^+. Conditions for run-off assays were as described under " Materials and Methods" using 3 nM *lac* P^+, 15 nM (lanes A-D) or 30 nM (lanes E-H) CRP or mutant CRP, and 20 nM RNA polymerase. Lane A, CRP; lane B, CRP209RD; lane C, CRP209RL; lane D, CRP209RN; lane E, CRP; lane F, CRP209RD; lane G, CRP209RL; lane H, CRP209RN; lane I, no CRP.

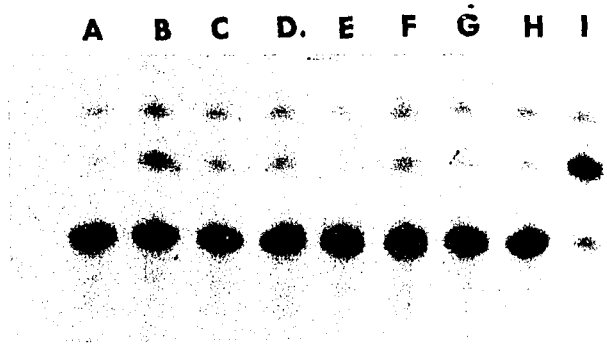

A
B
C
D.
E
F
G
H
I

Figure 10. Activity of CRP mutants with C-proximal replacements to support single-round transcription by RNA polymerase from *lac* P^+. Conditions for run-off assays were as described under " Materials and Methods" using 3 nM *lac* P^+, 15 nM (lanes A-D) or 30 nM (lanes E-I) CRP or mutant CRP, and 20 nM RNA polymerase. Lane A, CRP; lane B, CRP207GV; lane C, CRP208TI; lane D, CRP206YF; lane E, CRP; lane F, CRP207GV; lane G, CRP208TI; lane H, CRP206YF; lane I, no CRP.

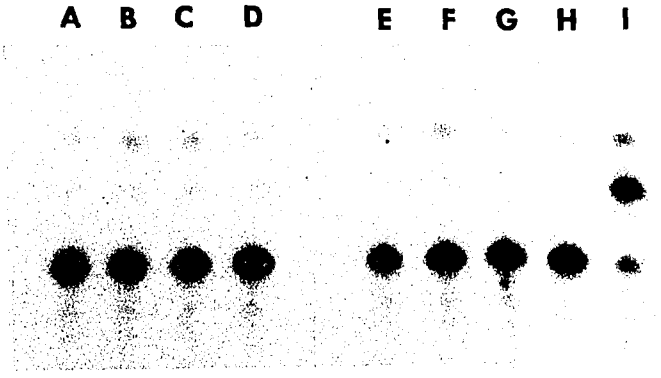

A B C D E F G H I

Figure 11A. Activity of CRP truncation mutants to support single-round transcription by RNA polymerase from *lac* P^+. Conditions for run-off assays were as described under " Materials and Methods" using 3 nM *lac* P^+, 15 nM CRP or mutant CRP, and 20 nM RNA polymerase. Lane A, CRP; lane B, CRP209RO; lane C, CRP208TO; lane D, CRP207GO; lane E, CRP205VO; lane F, no CRP.

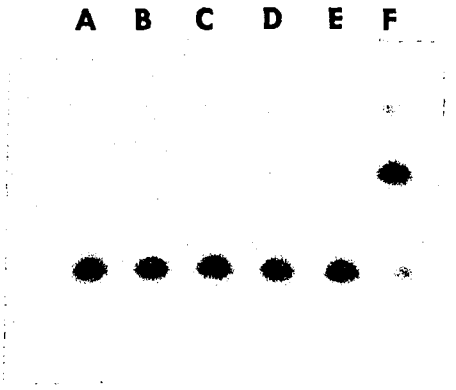

A B C D E F

Figure 11B. Activity of CRP truncation mutants to support single-round transcription by RNA polymerase from *lac* P^+. Conditions for run-off assays were as described under " Materials and Methods" using 3 nM *lac* P^+, 30 nM CRP or mutant CRP, and 20 nM RNA polymerase. Lane A, CRP; lane B, CRP209RO; lane C, CRP208TO; lane D, CRP207GO; lane E, CRP205VO; lane F, no CRP.

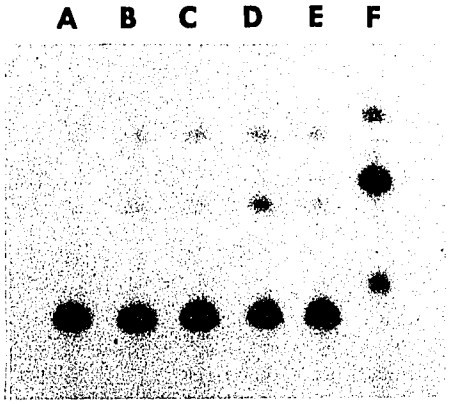

A
B
C
D
E
F

Figure 12. Binding of CRP and CRP mutants in which the C-terminal arginine is replaced by aspartic acid or glutamic acid to the *lac* P$^+$ fragment. Conditions for gel retardation assays are described in "Materials and Methods" using 0.5 nM ^{32}P-labeled *lac* P$^+$ fragment, 10 nM (lanes A, D and G), 5 nM (lanes B, E and H), or 2.5 nM (lanes C, F and I) CRP protein. Lanes A-C, CRP; lanes D-F, CRP209RD; lanes G-I, CRP209RE; lane I, no CRP.

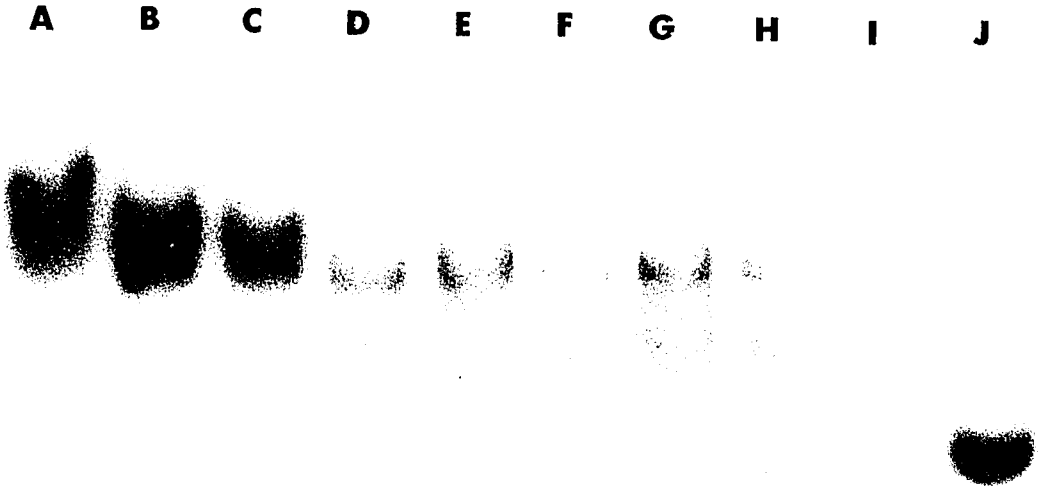

A
B
C
D
E
F
G
H
I
J

Figure 13. Binding of CRP mutants with internal C-proximal replacements to *lac* P^+. Conditions for gel retardation assays are described in "Materials and Methods" using 0.5 nM ^{32}P-labeled *lac* P^+ fragment, 5 nM (lanes B-D), 2.5 nM (lanes E-G), or 1.25 nM (lanes H-J) CRP protein. Lane A, no CRP; lanes B, E and H, CRP; lanes C, F, and I, CRP207GV; lanes D, G and J, CRP208TI.

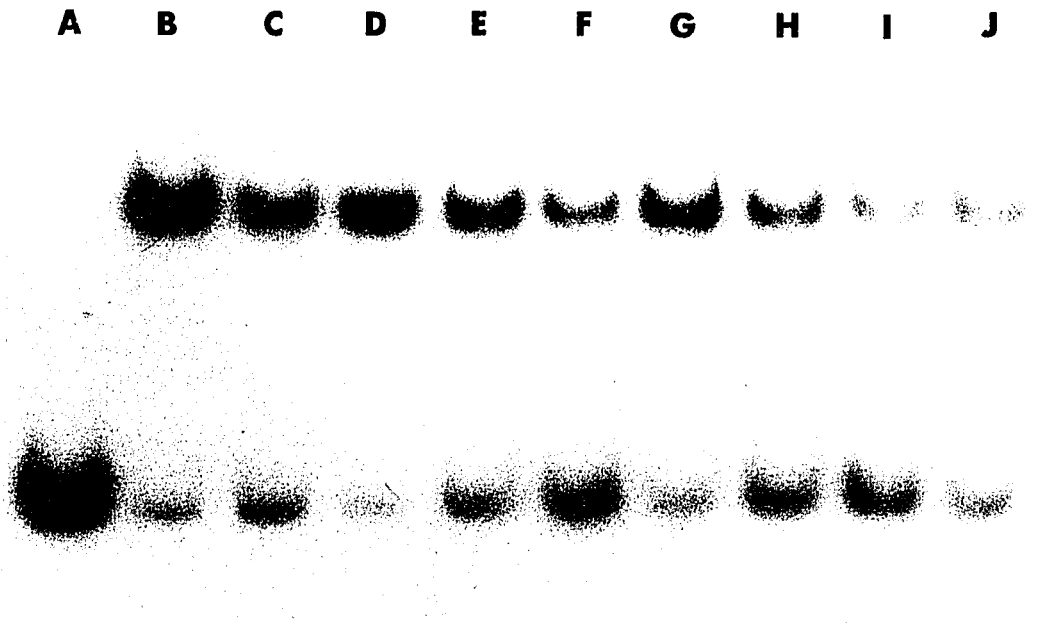

A
B
C
D
E
F
G
H
I
J

Figure 14A. Binding of CRP truncation mutants to *lac* P^+. Conditions for gel retardation assays are described in "Materials and Methods" using 0.5 nM ^{32}P-labeled *lac* P^+ fragment, 5 nM (lanes A-C), 2.5 nM (lanes D-F), or 1.25 nM (lanes G-I) CRP protein. Lanes A, D and G, CRP; lanes B, E, and H, CRP209RO; lanes C, F and I, CRP208TO; Lane J, no CRP.

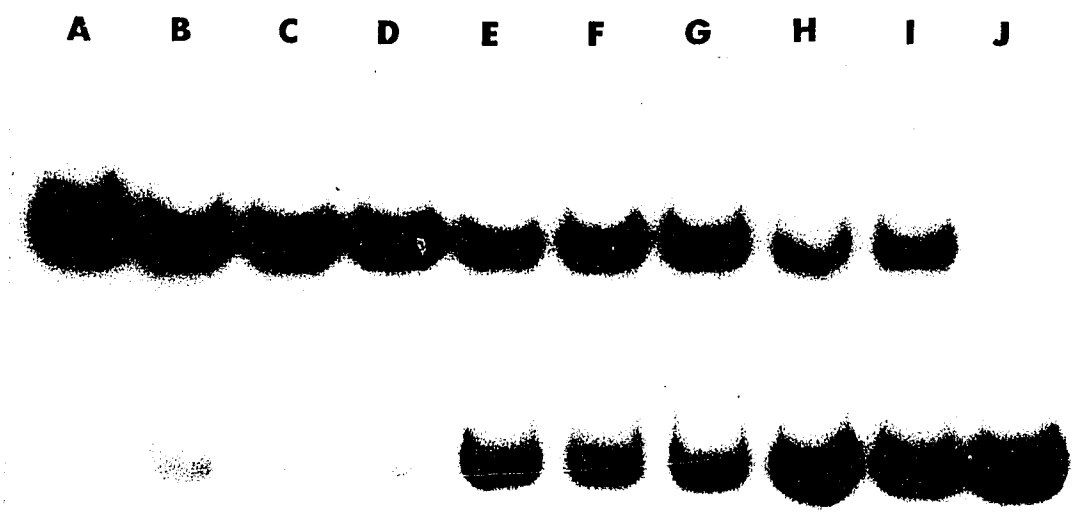

A
B
C
D
E
F
G
H
I
J

Figure 14B. Binding of CRP mutants with internal C-proximal replacements to *lac* P$^+$. Conditions for gel retardation assays are described in "Materials and Methods" using 0.5 nM ^{32}P-labeled *lac* P$^+$ fragment, 5 nM (lanes B-D), 2.5 nM (lanes E-G), or 1.25 nM (lanes H-J) CRP protein. Lane A, no CRP; lanes B, E and H, CRP; lanes C, F, and I, CRP207GO; lanes D, G and J, CRP205VO.

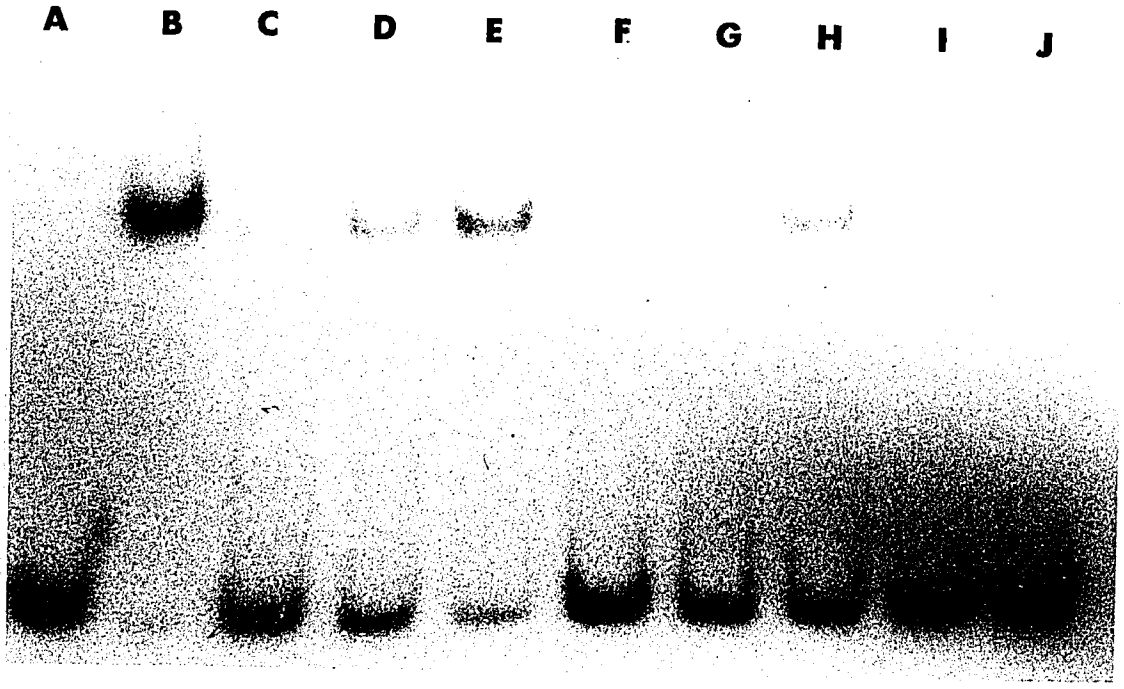

A
B
C
D
E
F
G
H
I
J

Figure 15. Binding of *lac* P^+ and *gal*AA' by CRP mutants in which the C-terminal arginine is replaced by leucine or aspartic acid. Conditions for gel retardation assays are described in "Materials and Methods" using 0.5 nM [^{32}P]-labeled DNA fragment and 5 nM CRP protein. Lanes a-e, *gal*AA', lanes f-j, *lac* P^+. Lanes a and f, CRP209RL; lanes b and g, CRP209RD; lanes c and h, CRP.

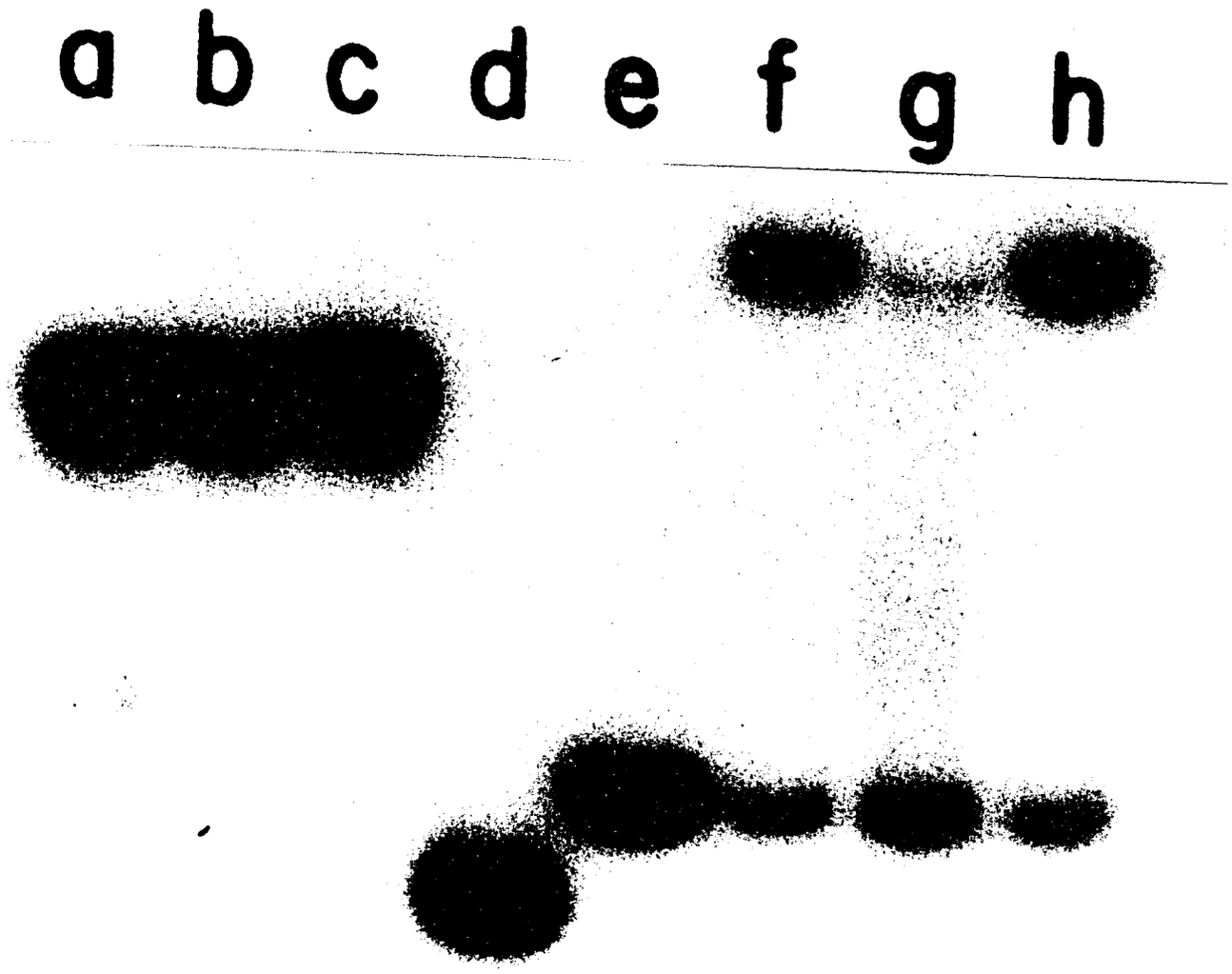
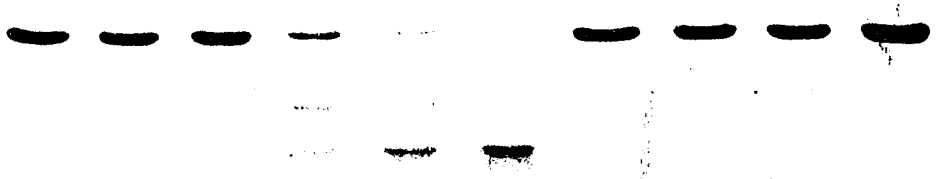

a b c d e f g h

Figure 16. Sensitivity of CRP mutants to trypsin attack in the presence and absence of cyclic nucleotide. Conditions for protease digestion assays are described in "Materials and Methods". Lane a, 5 min; lane b, 10 min.; lane c, 20 min; lane d, + cAMP 5 min; lane e, + cAMP 10 min; lane f, + cAMP 20 min; lane g, + cGMP 5 min; lane h, + cGMP 10 min; lane i, + cGMP 20 min; lane j, 0 min.

Arg

a b c d e f g h i j



Asp

a b c d e f g h i j

Leu

a b c d e f g h i j

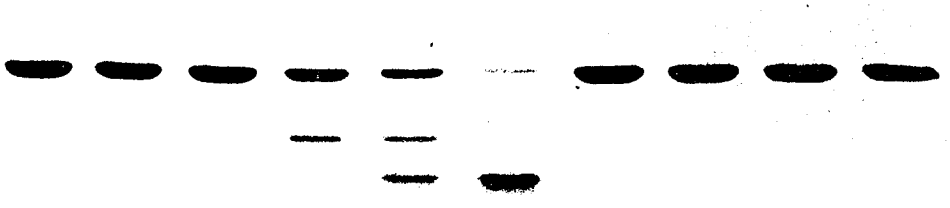


Figure 17. Binding of CRP mutants in which the C-terminal arginine is replaced by leucine, glutamic acid or aspartic acid to *lac* P^+. Conditions for DNase I footprinting assays were as described under "Materials and Methods" using 3 nM *lac* P^+, 15 nM (lanes C-L) or 30 nM (M-V) CRP or mutant CRP and where indicated, 20 nM RNA polymerase. Lane A, no CRP; lane B, RNA polymerase; lane C, CRP; lane D, CRP + RNA polymerase; lane E, CRP209RD; lane F, CRP209RD + RNA polymerase; lane G, CRP209RE; lane H, CRP209RE + RNA polymerase; lane I, CRP209RL; lane J, CRP209RL + RNA polymerase; lane K, CRP209RN; lane L, CRP209RN + RNA polymerase; lane M, CRP; lane N, CRP + RNA polymerase; lane O, CRP209RD; lane P, CRP209RD + RNA polymerase; lane Q, CRP209RE; lane R, CRP209RE + RNA polymerase; lane S, CRP209RL; lane T, CRP209RL + RNA polymerase; lane U, CRP209RN; lane V, CRP209RN + RNA polymerase.

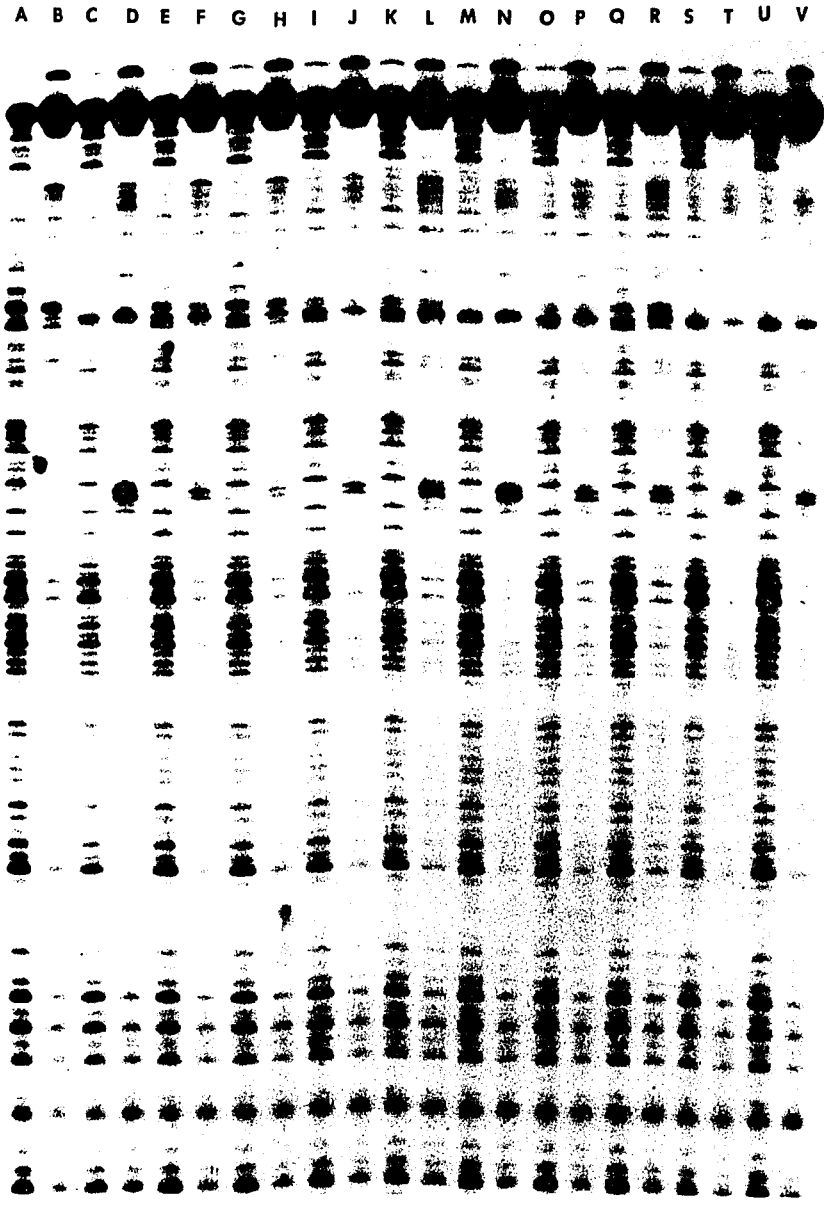

A B C D E F G H I J K L M N O P Q R S T U V

Figure 18. Binding of CRP mutants with internal C-proximal replacements to *lac* P^+. Conditions for DNase I footprinting assays were as described under "Materials and Methods" using 3 nM *lac* P^+, 15 nM (lanes C-J) or 30 nM (lanes K-R) CRP or mutant CRP and where indicated, 20 nM RNA polymerase. Lane A, no CRP; lane B, RNA polymerase; lane C, CRP; lane D, CRP + RNA polymerase; lane E, CRP207GV; lane F, CRP207GV + RNA polymerase; lane G, CRP208TI; lane H, CRP208TI + RNA polymerase; lane I, CRP206YF; lane J, CRP206YF + RNA polymerase; lane K, CRP; lane L, CRP + RNA polymerase; lane M, CRP207GV; lane N, CRP207GV + RNA polymerase; lane O, CRP208TI; lane P, CRP208TI + RNA polymerase; lane Q, CRP206YF; lane R, CRP206YF + RNA polymerase.

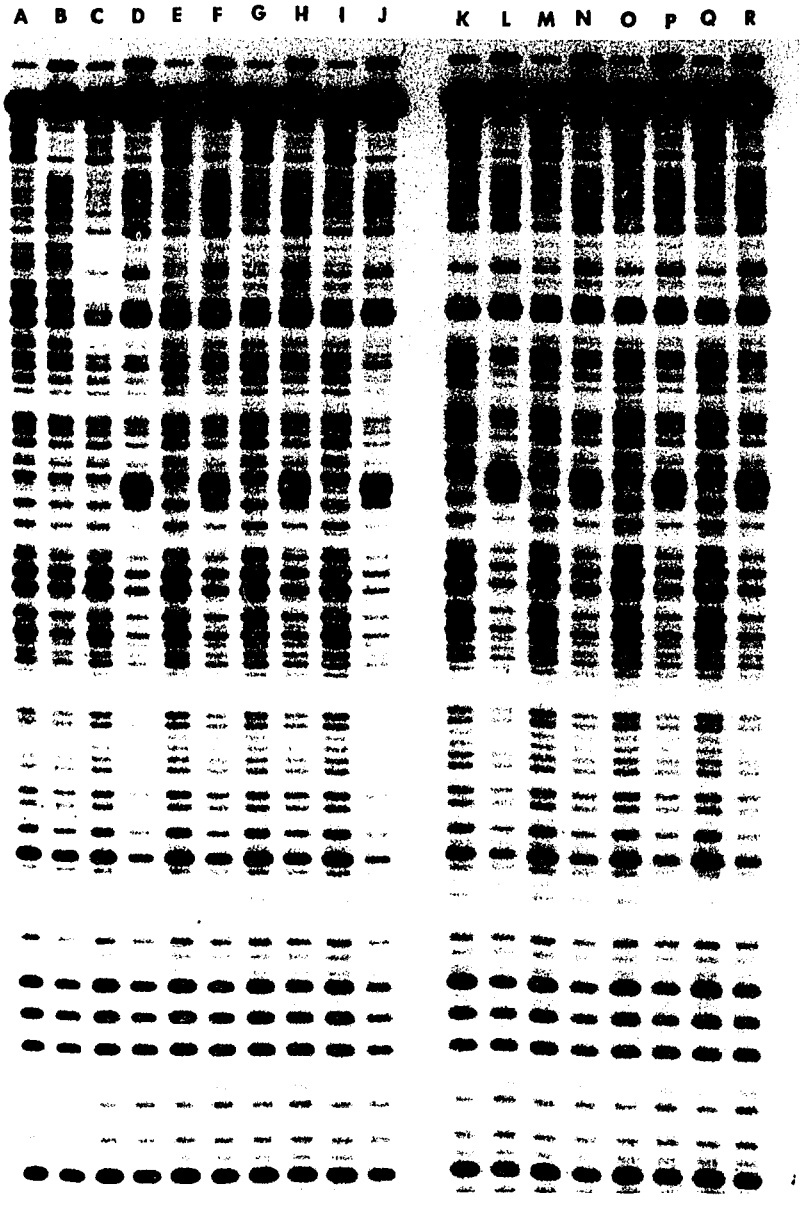
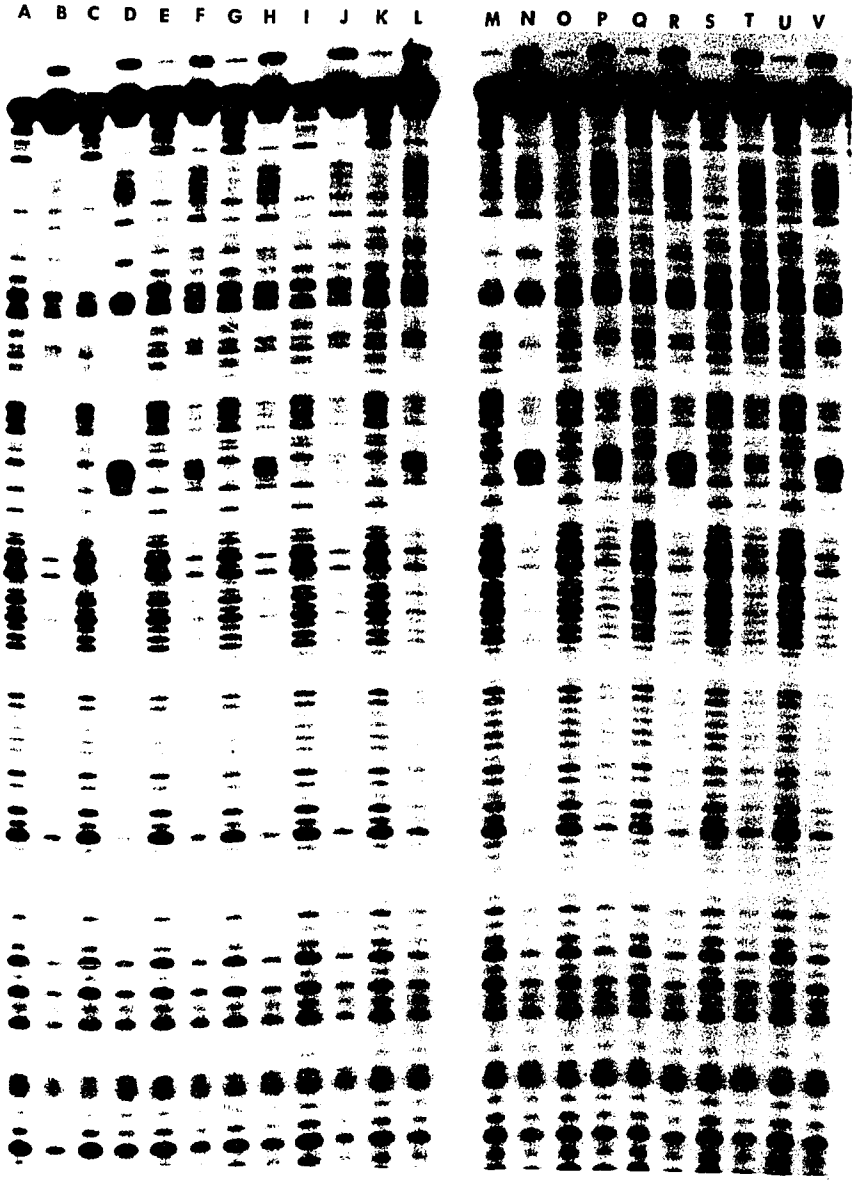

A B C D E F G H I J K L M N O P Q R

Figure 19. Binding of CRP truncation mutants to *lac* P^+. Conditions for DNase I footprinting assays were as described under "Materials and Methods" using 3 nM *lac* P^+, 15 nM (lanes C-L) or 30 nM (lanes M-V) CRP or mutant CRP and where indicated, 20 nM RNA polymerase. Lane A, no CRP; lane B, RNA polymerase; lane C, CRP; lane D, CRP + RNA polymerase; lane E, CRP209RO; lane F, CRP209RO + RNA polymerase; lane G, CRP208TO; lane H, CRP208TO + RNA polymerase; lane I, CRP207GO; lane J, CRP207GO + RNA polymerase; lane K, CRP205VO; lane L, CRP205VO + RNA polymerase; lane M, CRP; lane N, CRP + RNA polymerase; lane O, CRP209RO; lane P, CRP209RO + RNA polymerase; lane Q, CRP208TO; lane R, CRP208TO + RNA polymerase; lane S, CRP207GO; lane T, CRP207GO + RNA polymerase; lane U, CRP205VO; lane V, CRP205VO + RNA polymerase.


A B C D E F G H I J K L
M N O P Q R S T U V

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