IN VITRO CHARACTERIZATION OF cAMP RECEPTOR PROTEIN MUTATED AT POSITION 127

by

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ABSTRACT

The cyclic adenosine 3′,5′ monophosphate (cAMP) receptor protein (CRP) complexed with cAMP binds a region upstream of the lactose operon promoter (*lac*P) to facilitate RNA polymerase (RNAP) recognition of *lac*P. The DNA binding and *lac*P activation characteristics of four mutant forms of CRP were investigated. These proteins contained a single amino acid substitution (either cysteine [C], glycine [G], isoleucine [I] or serine [S]) for threonine (T) at position 127. All but the T127G CRP mediated *lac*P activation in the presence of cAMP. CRP:cAMP-mediated *lac*P activity differed for the T127C, T127I and T127S forms of CRP. The level of *lac*P activation observed for T127C CRP was comparable to wild-type (WT) CRP. The levels mediated by the T127I CRP or the T127S CRP were about one-half that of WT CRP. Transcription reaction mixtures that contained each of these forms of CRP differed in the rate of formation of the *lac*P:RNAP open complex.

DNase I footprint analysis of complexes formed between *lac*P DNA and the position 127 CRP mutants showed the following. First, stable DNA:CRP:cAMP complexes formed only in reactions that contained WT CRP or T127C CRP. Second, stable DNA:CRP:cAMP:RNAP complexes formed in reactions that contained the WT, T127C, T127G, T127I or T127S CRP. Protection of the *lac*P CRP-binding site by the T127G, T127I or T127S CRP was less complete than for WT or T127C CRP. Third, stable, non-specific DNA:CRP complexes formed at high CRP concentrations (in the absence of cAMP) in reactions that contained the T127C or the T127G CRP; cAMP addition produced site-specific DNA binding for T127C CRP but not for T127G CRP. Non-specific DNA binding was not observed for the WT, T127I or T127S forms of CRP. The

results of this study show that position 127 amino acid substitutions in CRP differentially affect cAMP-mediated changes in CRP structure that: (1) minimize non-specific DNA affinity and introduce high affinity site-specific DNA binding in the absence of RNAP and (2) establish the productivity of CRP:RNAP interactions at *lac*P. Importantly, these data also show that these position 127 amino acid substitutions have little effect on CRP:RNAP:*lac*P binding cooperativity.

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LIST OF ABBREVIATIONS

ANS	N-acetylaminoethyl-1-naphthylamine-5-sulfonate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5' monophosphate
CM-Sephadex	carboxymethyl-sephadex
CTP	cytosine triphosphate
CRP	cAMP receptor protein
DNA	deoxyribonucleic acid
DTNB	5:5'-dithiobis (2-nitrobenzoic acid)
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
GTP	guanosine triphosphate
HTP	hydroxylapatite
*lac*P	lactose promoter
LB	Luria-Bertani
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonylfluoride
RNA	ribonucleic acid
RNAP	RNA polymerase
SDS	sodium dodecyl sulfate
TTP	thymine triphosphate
UTP	uridine triphosphate
WT	wild-type

CHAPTER I

INTRODUCTION

The lactose operon

CRP regulates transcription of several catabolite sensitive operons in *Escherichia coli* such as those involved in the metabolism of lactose (*lac*), galactose and maltose (1,2). The *lac* operon is one of the better characterized operons in *E. coli* consisting of a control region and three structural genes, *lac*Z, *lac*Y and *lac*A, that encode the enzymes; β-galactosidase, *lac* permease and thiogalactoside transacetylase, respectively (3). The control region located upstream of *lac*Z includes a promoter, a binding site for the CRP:cAMP complex, and a binding site for the repressor (Fig 1.1).

Regulation of *lac* gene transcription initiation is mediated by both positive and negative control factors. The *lac* operon is negatively regulated by a repressor, encoded by the *lac*I gene. Repressor binding to the operator region blocks transcription initiation. In the presence of β-galactoside, the repressor no longer binds to the operator and transcription of the *lac* structural genes can proceed. Positive control of the *lac* operon is mediated by cyclic adenosine 3',5'- monophosphate (cAMP) and the cAMP receptor protein (CRP) (4), also called the catabolite gene activator protein (CAP) (2). The CRP:cAMP complex binds to a region located upstream of the promoter. CRP:cAMP binding aids RNA polymerase (RNAP) recognition of the *lac* promoter and dramatically increases the rate of transcription initiation.

The expression of the *lac* operon structural genes is regulated by the presence of available carbon sources in the environment. Glucose is used in preference to other sugars. When *E. coli* is grown in the presence of glucose

and lactose, it metabolizes glucose and not lactose. This results from decreased expression of lactose operon genes, an effect called catabolite repression. The effect represents a coordinating mechanism that shows a preference for glucose by preventing the expression of genes for sugars of alternative metabolite pathways. In *E. coli*, the cAMP and CRP concentrations determine the form of CRP that predominates under various growth conditions. The synthesis of cAMP is catalyzed by adenylate cyclase in the cell. Strains that lack a functional adenylate cyclase, encoded by *cya*, cannot activate CRP mediated transcription of *lac* operon (5). Expression of catabolite-regulated operons shows an inverse relationship with the level of cAMP (6,7). Catabolite repression results from glucose transport-mediated reduction of cAMP (from four- to over ten-fold reduction) in the cell (8) and the downregulation of CRP (up to two-and-one-half-fold reduction) in expression of *crp* gene (9). The fluctuation in the level of CRP* (a CRP mutant which activates CRP-mediated transcription in the absence of cAMP) may play a role of catabolite repression in *cya* deficient cells (10).

Structure of cAMP receptor protein

CRP, a dimer protein, contains two identical subunits of molecular weight 23,619 daltons with 209 amino acid residues in each subunit (11,12). Each subunit can bind one molecule of cAMP (4). There are three forms of CRP *in vitro*: free CRP, CRP:$(cAMP)_1$ and CRP:$(cAMP)_2$ (13). For wild-type (WT) CRP, when the cAMP concentration is at μM levels, CRP:$(cAMP)_1$ forms; when the cAMP concentration is at mM levels, CRP:$(cAMP)_2$ forms. From *in vivo* evidence, that the physiological concentration of cAMP in cell is 0 to 10 μM (14) and *in vitro* evidence, that one equivalent of CRP:$(cAMP)_1$

binds to one equivalent of *lac*P, CRP:$(cAMP)_1$ seems to be the relevant active form of the protein (15).

cAMP binding to CRP changes the conformation of the protein. WT CRP is resistant to protease in the absence of cAMP and exhibits little if any DNA binding, while the CRP:cAMP complex is sensitive to protease in the presence of cAMP and demonstrates sequence-specific DNA binding. A global structural change in CRP upon binding cAMP was observed in ANS fluorescence probe and protease experiments (13), DTNB-mediated crosslinking experiments (16), X-ray scattering experiments (17) and by Raman spectral differences between CRP and CRP:$(cAMP)_2$ (71). The major effect of the cAMP-induced allosteric change may be to expose and reposition the F helices to make contact with the two adjacent major grooves of the DNA (18,19). A comprehensive understanding of the allosteric mechanism by which cAMP binds CRP will clearly benefit from the analysis of both the unliganded CRP and CRP:$(cAMP)_1$ crystal structures (20).

A 2.5 Å structure of CRP:$(cAMP)_2$ has been determined from X-ray crystallographic studies (21). In CRP:$(cAMP)_2$ co-crystals, each subunit is folded into two domains (Fig 1.2). The amino-proximal domain contains extensive β–sheet structure, forms the cAMP binding pocket, and contains many of the subunit-subunit contacts of the CRP dimer. Analysis of CRP:$(cAMP)_2$ crystal structure identified five specific amino acid-ligand contacts that are likely to be important in cAMP binding to CRP in solution and/or in mediating cAMP activation of CRP (21). The charged phosphate of cAMP is aligned with arginine (R) 82 to form a salt bridge, and specific hydrogen bond interactions occur between (1) the axial phosphate oxygen atom of the 3', 5'-cyclic phosphate ring and serine (S) 83, (2) the 2' hydroxyl of ribose and glutamate (E) 72 and (3) the N^6 amino group of adenine and

threonine (T) 127 of one subunit and S128 of the other subunit (21). The effects of specific amino acid substitutions at these positions (E72, R82, S83, T127 and S128) in CRP were studied (22,23). The carboxyl-proximal domain contains 3 α-helices (termed D, E and F) connected by short stretches of β-sheet that forms the DNA binding surface of the protein (1).

Model of CRP:DNA interaction

In the absence of cAMP, CRP shows non-specific and low binding affinity to DNA (31-33). When cAMP complexes with CRP, the non-specific DNA binding is changed to specific and high affinity binding (31-33). CRP binds to right-handed B-DNA and uses the F-helices in contacting consecutive major grooves of the DNA (34,35). The F helix protrudes from the surface of the CRP dimer and provides the main sites for CRP-DNA interaction. CRP has a helix-turn-helix DNA binding motif located in the C-terminal domain. The E helix fits above the groove near the DNA backbone and F helix fits into the major groove (Figure 1.3) (24). Three amino acids located in the N-proximal region of F helix in CRP appear to hydrogen bond directly and sequence-specifically to three bases located in the major groove of the DNA (25). These include: (1) R180 whose guanidium group hydrogen bonds to the O^6 and $N^7$of guanine on the seventh position of the consensus sequence (shown below) as proposed by Ebright et al. (24,26,27); (2) E180 whose carboxylate group interacts with the N^4 of cysteine on the fifth position of the consensus sequence as proposed in mutagenic studies by Ebright et al. (27,28); and 3) R185 interacts with either the O^6 or the N^7 of guanine on the fifth position and the O^4 of thymine on the sixth position of the consensus sequence. However, the role of R185 has been questioned (29). The C-proximal and N-proximal domains are connected covalently by a hinge

region. It is proposed that the allosteric conformation change upon binding cAMP are transmitted along the C helices to the hinge region via intersubunit hydrogen bonds (30). In this way, the conformation of CRP is altered, thus inducing CRP transcriptional control activity. However, the structural basis of the activation is still unsettled and requires information of the relative positioning of CRP, RNAP, and DNA during closed complex formation.

A 22 bp consensus sequence (shown below) was derived from the analysis of a number of CRP-dependent promoters (36).

AAATGTGATCT AGATCACATTT

TTTACACTAGA TCTAGTGTAAA

The consensus sequence is the ideal DNA site for CAP binding from affinity binding (37) and DNA base pair substitution studies (38). The CRP binding pattern is consistent with CRP binding to one face of DNA, the same face that is bound by RNAP (39). However, distal sequences also play a role in determining the affinity of CRP to a specific site on DNA (40,41), the integrity of the spacer DNA between the CRP and RNAP binding sites must be maintained for transcription activation from phasing studies (39).

The CRP DNA binding site in *lac* extends from position -72 to position -52 relative to the start point of transcription (42). CRP binds to DNA and bends the DNA. This has been shown from analysis of the mobility of CRP:DNA complexes in polyacrylamide gels (43,44). From a 30 bp DNA fragment which contains the 22 bp consensus sequence, a 3 Å crystal structure of CRP:$(cAMP)_2$:DNA shows that DNA is bent by 90 degrees resulting mainly from two 40 degree bends, on either side of the dyad axis of the complex (25). The CRP in CRP:$(cAMP)_2$:DNA is a symmetric dimer of closed subunits

rather than an asymmetric dimer with one open and closed subunits as in the CRP:$(cAMP)_2$ structure (45).

The binding of CRP to DNA leads to bending of the DNA and activates the transcription of *lac* operon promoter (*lac*P). Gartenberg proposed two models to explain the CRP-mediated activation of transcription in terms of DNA bending (46). One model proposed that the DNA bend itself provides upstream DNA-RNAP contacts or relieves the energy barrier to initiate transcription resulting from the constraints imposed by DNA superhelicity *in vivo*. Another model proposed that bending of DNA could position CRP in an optimal configuration for CRP-RNAP contact.

CRP-dependent promoter activation

The CRP:cAMP complex specifically binds to DNA sequences upstream of the promoter DNA and enhances RNAP binding to the promoter region to form an ternary complex (closed complex) that rapidly isomerizes to an open complex (1,47). The CRP:cAMP complex activates *lac*P through closed complex formation followed by a series of isomerizations, leading to open complex formation (47,48). A model of transcription initiation involves three overall steps shown schematically below (48):

$$R + P \underset{K_b}{\longleftrightarrow} RPo \xrightarrow[k_f]{} RPc \xrightarrow[\text{clearance}]{\text{promoter}} RNA$$

This scheme involves the initial binding of RNAP (R) and the promoter (P) with a binding constant K_b to form the closed complex (RP_c). The closed complex subsequently isomerizes with a rate constant k_f to form the

transcriptionally active open complex (RP_o). CRP is found dispensable after the formation of an open complex (49).

There are two classes of promoter. At one class of promoter, known as constitutive, RNAP (RNAP) recognizes the -10 and -35 hexamers in DNA and binds DNA with high affinity. Another class of promoter is factor-dependent in that additional information is required for RNAP recognition. For example, *lac*P, a weak promoter which does not fit well to the consensus promoter sequence requires CRP:cAMP complex binding to upstream of *lac*P to provide additional recognition point for RNAP. The CRP:cAMP complex functions as a positive control element in *lac* operon transcription.

There are class I and class II CRP-dependent promoters (50). For a class I promoter, DNA site for CRP is immediately upstream of the -35 region. The prototype class I CAP-dependent promoter is *lac*P (DNA site for CRP is centered at position - 61.5). For a class II CRP-dependent promoter, the DNA site for CRP replaces the -35 determinants for binding to RNAP. The prototype class II CRP-dependent promoter is the *gal*P1 promoter (DNA site for CRP centered at position -41.5).

Protein-protein contact is important in CRP-mediated transcription activation (51). The CRP and RNAP make direct protein-protein contact which is required for *lac* operon transcription activation (52,53). Footprinting analysis demonstrated that RNAP stabilizes the interaction of CRP on the DNA (54,55). Loop 156-162 of CRP is suggested to be the primary site for CRP contact of RNAP (50,52,56-59). Zhou et al. have designated amino acids 156-162 of CRP the "activating region" (57). There is evidence to support the formation of CRP:cAMP:RNAP contacts: (1) fluorescence polarization showed that CRP:cAMP interacts with RNAP in the absence of promoter DNA while a CRP mutant (Ala 158) defective in transcription activation did not interact

with RNAP (52); (2) photocrosslinking showed that a site on RNAP is in proximity to the activating region of CRP in the complex of *lac*P:CRP:cAMP:RNAP (53). The α subunit of RNAP is important in this CRP-RNAP interaction. This has been shown in a carboxyl terminal deletion of α subunit in RNAP which fails in *lac* operon transcription in the presence of CRP yet is competent in transcribing several CRP-dependent promoters (61). The σ subunit of RNAP is important in promoter recognition through nested deletion studies (60). The CRP and RNAP contact requires the activating region of the promoter-proximal subunit of CRP in transcription activation at the *lac* promoter (62) and the activating region of the promoter-distal subunit of CRP in transcription activation at the *gal* promoter (63).

Mutant forms of CRP

There are four classes of CRP mutants: (1) CRP^{+}, which are phenotypically WT CRP, (2) CRP^{*}, which activate transcription in the absence of cAMP and frequently show relaxed cyclic nucleotide specificity (72, 73), (3) CRP^{Pc}, which are defective in transcription activation but not defective in DNA binding and bending, and (4) CRP^{-}, which do not activate transcription under any conditions.

Analysis of CRP cAMP binding pocket mutants at positions 72, 82, 83, 127, 128 was conducted (22,23). Protease assay, cAMP binding assay and β-galactosidase assays were used to assess the role of these amino acids on CRP activation. Among these mutants, 72 and 82 position mutants were not active *in vivo* and had large cAMP binding affinity differences compared to WT CRP. While 83, 128 position mutants were active *in vivo*; they were sensitive to protease in the presence of cAMP, and their cAMP binding affinities were similar to that of WT CRP. T127 position mutants with the exception of

T127G CRP were active *in vivo* and were sensitive to protease in the absence of cAMP, similar to WT CRP:cAMP; In addition, their cAMP binding affinity was similar to that of WT CRP. The results indicated that E72 and R82 are important in cAMP binding to CRP, while S83, T127 and S128 are important in CRP activation by cAMP but not in cAMP binding itself (22,23).

Purpose of the work

Residue127 located on C α-helix (Fig 1.4) has been implicated by Weber and Steitz to be important in contacting cAMP and/or mediating cAMP activation of CRP (21). This 127 residue was proposed to play an important role in establishing the CRP hinge region conformation in the absence of cAMP, and also to play a role in the transmission of allosteric signal after cAMP binding (23). According to Lee et al. (23), mutants at position 127 (threonine was substituted to serine, cysteine, glycine, or isoleucine) displayed, in the absence of cAMP, protease sensitivity similar to that of the WT CRP:cAMP complex. These results demonstrate that threonine 127 plays an important role in maintaining the protease-resistant structure of CRP. Different T127 mutants have different characteristics. The T127 mutants display protease sensitivity in the absence of cAMP similar to that of WT CRP:cAMP and are either fully competent (T127C) or limited (T127G, T127I, T127S) in *lac*P activation in the presence of cAMP. In the absence of cAMP, there was no transcription activation promoted by any of these mutants. None of position 127 CRP mutants have a significant effect on the affinity of CRP for cAMP. But whether these mutants bind to *lac*P DNA is not known. This lead to further investigation of mechanism of transcription activation by CRP 127 mutants.

The objective of the work reported here was: to evaluate the stage at which the 127 (I, S, G) substituted CRP's are limited in *lac* transcription activation. The methods used were: (1) gel shift and DNase I footprinting assays to assess the DNA binding characteristics of T127 CRP mutants and (2) *in vitro* transcription assays to assess the effect of different T127 mutant CRP on *lac*P transcription activation.

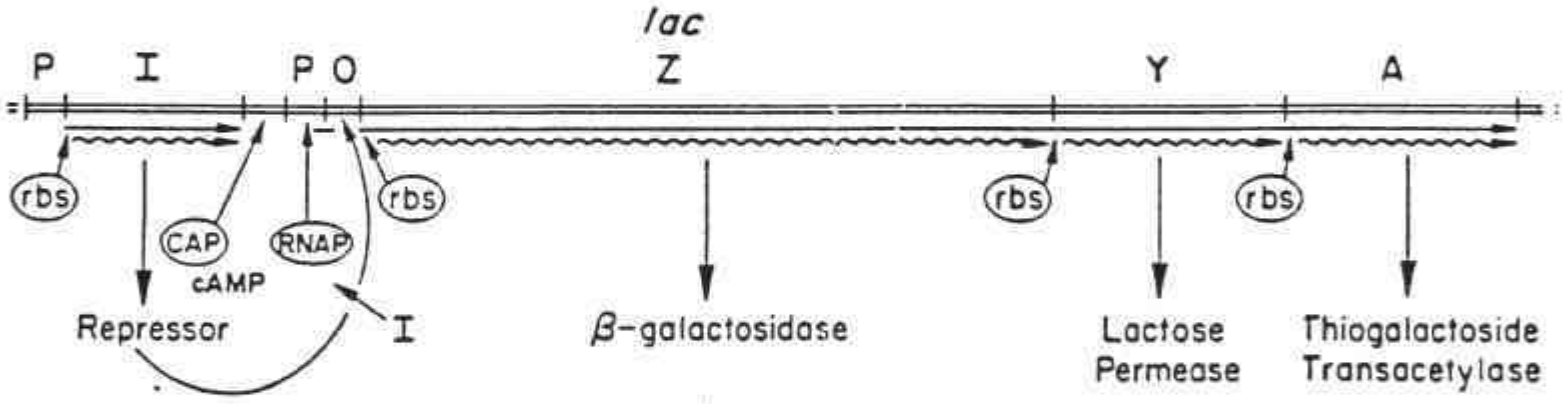


Figure 1.1. The lactose operon (adapted from Reznikoff et al., 1985). The transcription of the DNA *lac*Z, Y and A genes results from transcription initiation by RNAP (RNAP) at *lac*P. This event is positively regulated by CRP when it is complexed with cAMP and negatively regulated by repressor, the *lac*I gene product. In the presence of inducer, the repressor will no longer bind to operator. Translation (indicated by the wavy arrows) of each of the gene products results from ribosome (rbs) binding to the mRNA translation signal for each gene.

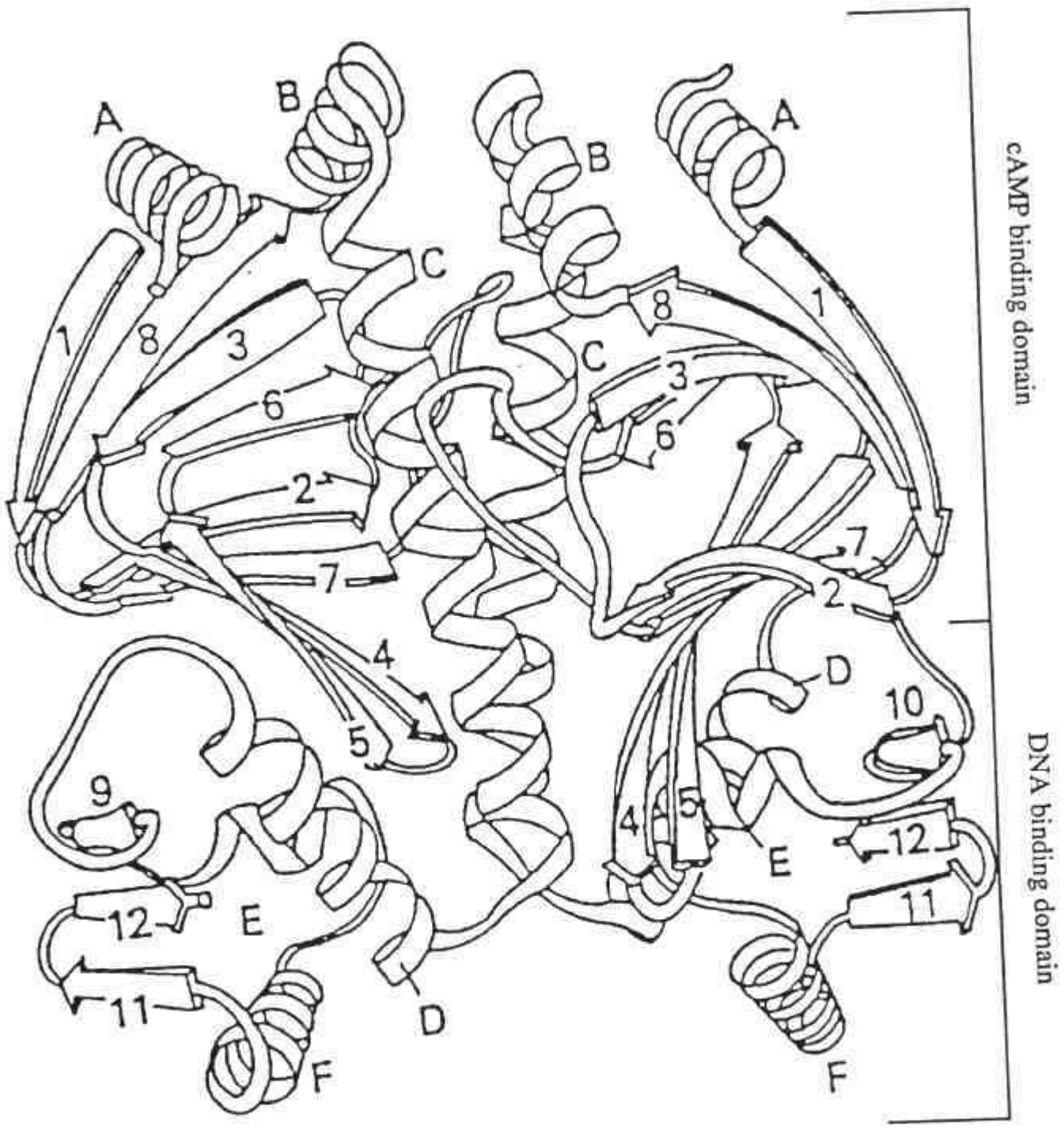


Figure 1.2. Schematic representation of the CRP dimer (adapted from de Crombrugghe et al., 1984). The amino-proximal domain consists of α-helix A, β-sheets and α-helices B and C. The DNA binding carboxyl terminal domain consists of α-helices D, E, F and the residues connecting these helices. The two subunits are not exactly related by a perfect dyad axis of symmetry.

```
     2  4  6  8 10 12 14 16 18
5'  ATGTGAGTTAGCTCACTC  3'
3'  TACACTCAATCGAGTGAG  5'
```

Figure 1.3. Sequence of the CRP binding site in *lac*P. The number indicates the residue number on the 18 bases of *lac*P fragment.

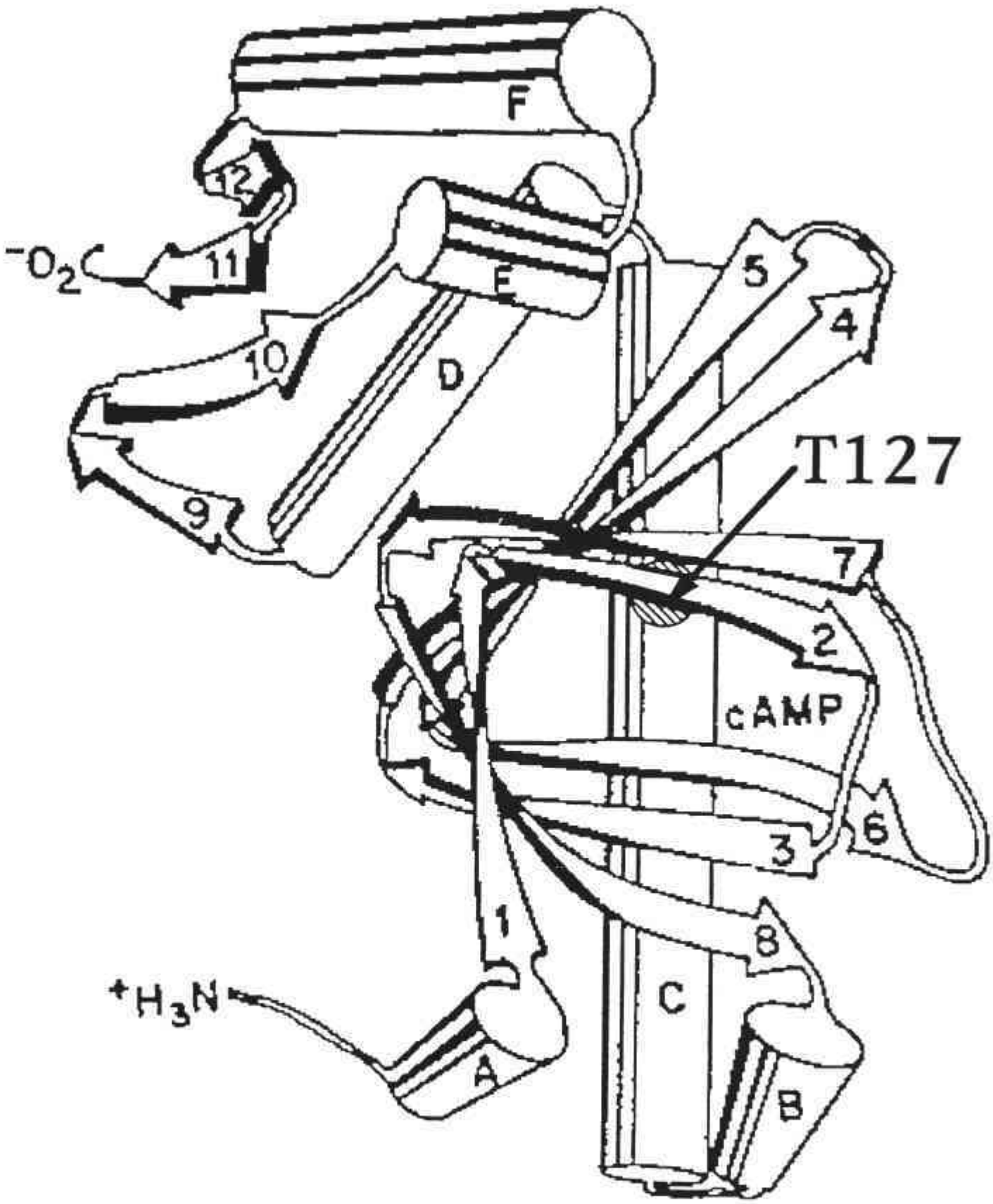


Figure 1.4. Schematic representation of CRP monomer secondary structure (Modified from McKay et al., 1982). The regions of the polypeptide that are in α helical conformation are presented as tubes and are lettered A through F. The regions that are in a β-sheet conformation are represented as arrows number 1 to 12. The two domains are connected by a hinge region between the C and D α-helices. The 127 position amino acid residue is shown on this figure.

CHAPTER II

MATERIALS AND METHODS

Materials

Bacterial strains and plasmids

E. coli strains MZ1 (*his, ilv, rsp*L, *gal*Kam, *pg*/Δ8 (bio-uvrB),*ΔH*1) (64) and AG1 (F^-, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rk^-,mk^+), *sup*E44, *rel*A1, λ^-). pRK248cI^{ts}, which encodes a temperature-sensitive λ cI repressor and a tet^r determinant, was the gift of Dr. Helinski (65). CRP position 127 mutants were the gift of Dr. Lee (23). The plasmid pHW104 was the gift of Dr. Crothers (43). pKL201 was the gift of Dr. Harman (66).

Enzymes and other materials

Restriction enzymes, bacteriophage lambda (l) BstE II digested size standards, Klenow fragment of DNA polymerase I were from New-England Biolabs. Ammonium persulfate, Bio-Rex 70 resin, hydroxylapatite resin, Bio-Gel A-1.5 m resin and Bio-Rad protein assay kits were obtained from Bio-Rad laboratories. Electrophoresis-grade acrylamide, shrimp phosphatase, ammonium sulfate, PMSF and EDTA were purchased from United States Biochemical Corp, Inc. N, N'-bisacrylamide, electrophoresis-grade agarose and TEMED were obtained from International Biotechnologies, Inc. α-^{32}P-labeled UTP, γ-^{32}P-labeled ATP were purchased from DuPont New England Nuclear. Nucleoside triphosphates (ATP, CTP, GTP, UTP) and deoxynucleoside triphosphates (dTTP, dGTP, dATP) were purchased from Pharmacia. RNase ZAP, phenol:chloroform:isoamyl alcohol (25:24:1) were obtained from Ambion. X-ray film (XAR-5, BMR-2) were obtained from Eastman Kodak. T4

polynucleotide kinase megalabel kit was purchased from Panvera Corporation. Bacto-tryptone, casamino acid and yeast extract were purchased from Difco laboratory. 3':5' cAMP, dithiothretiol, bovine pancreas ribonuclease A, bovine serum albumin, CM-Sephadex resin, protease (type XXIV), double strand DNA-cellulose resin, single strand DNA-cellulose resin and b-mercaptoethanol were purchased from Sigma Chemical Company. Nitrocellulose membrane filters (0.22 μm and 0.45 μm pore sizes) were purchased from Microfiltration systems. Synthetic oligonucleotides were synthesized by the Texas Tech Biotechnology Institute Core Facility. Fisherbiotech Scintiverse^{TM} BOA was purchased from Fisher Scientific. Common salts were reagent grade or better.

Growth media

Luria-Bertani (LB) contains 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl per liter. The pH was adjusted to 7.4 with the addition of NaOH. LB agar plates contained 15 g bacto-agar per liter LB.

Methods

Purification of RNAP

The purification of RNA polymerase (RNAP) was accomplished through sequential double strand DNA-cellulose, high salt Bio-Gel A-1.5m and single strand DNA-cellulose chromatography as described by Burgess and Lowe (67,68).

AG1 cells were grown in rich medium (LB+1% glucose) at 37°C to $OD_{600} = 1.0$. The cells were harvested by centrifugation at 7 K RPM for 10 minutes and stored as a frozen paste at -20°C. Frozen cells were broken into relatively small pieces (2 cm diameter) and placed in a Waring blender along

with 3 volumes (v/w wet cell paste) of lysis buffer (0.05 M Tris-HCl [pH 7.9], 5% glycerol, 2 mM EDTA, 0.1 mM dithiothretiol, 1.0 mM β-mercaptoethanol, 0.233 M NaCl, 130 mg/ml lysozyme, 23 mg/ml PMSF). Cells were blended at low speed for 2-3 minutes. The blender was placed in a 20°C water bath and stirred with a glass rod until the cell suspension reached 5°C. Cells were maintained at this temperature for 30 minutes. Sodium deoxycholate was added to a final concentration of 0.05% and the mixture was blended at low speed for 30 seconds. After 20 minutes, the cell mixture was blended at high speed for 60 seconds to shear the DNA. One volume of TGED (0.01 M Tris-HCl [pH 7.9], 5% glycerol [v/v], 0.1 mM EDTA, 0.1 mM dithiothretiol) + 0.2 M NaCl was added to the mixture and the mixture was blended at low speed for 30 seconds. The cell extract was centrifuged at 8 K RPM for 45 minutes at 4°C. The amber supernate was decanted and titrated with a 10% (v/v) solution of polymin P, with stirring, to a polymin P concentration of approximately 0.35%. Stirring was continued for 5 minutes. The mixture was then centrifuged at 6 K RPM for 15 minutes. The pellet was drained and resuspended in 500 ml of TGED + 0.5 M NaCl with gentle stirring. The solution was centrifuged at 6 K RPM for 15 minutes and the pellet was resuspended in TGED + 1.0 M NaCl with gentle stirring for 5 minutes. The supernate was decanted and brought to 50% saturation with the addition of solid ammonium sulfate. The solution was stirred for 20 minutes and centrifuged at 8 K RPM for 45 minutes. The drained pellet was dissolved in TGED+0.15 M NaCl and dialyzed against the same buffer for chromatography.

Double strand DNA-cellulose chromatography

The protein solution was loaded onto a double strand DNA-cellulose column (2.5 cm x 10 cm) equilibrated in TGED + 0.15 M NaCl at a rate of 42

ml/hour. The column was washed with 2 column volumes of TGED + 0.15 M NaCl. OD_{280} of the column eluate determine the point at which washing was complete. Bound protein was eluted in a series of 2 ml fractions in a linear salt gradient made from 50 ml TGED + 0.15 M NaCl and 50 ml TGED + 0.5 M NaCl. Column fractions were run on 15% SDS-PAGE to monitor the components of the fractions. The fractions that contain the most RNAP (peak fractions) were pooled and concentrated with the addition of solid ammonium sulfate to 50% saturation. The pooled solution was centrifuged at 8 K RPM for 30 minutes and dissolved in TGED for Bio-Gel A 1.5 m chromatography.

High salt Bio-Gel A 1.5 m chromatography

The sample was added to the Bio-Gel A 1.5m column (2.5 cm x 100 cm) equilibrated with TGED + 0.5 M NaCl. The eluate was collected in 3.5 ml fractions at 25 ml/hour. The fractions were run on 15% SDS-PAGE and peak fractions were pooled for single strand DNA-cellulose chromatography.

Single strand DNA-cellulose chromatography

The most pure fractions from Bio-Gel 1.5m column were pooled and diluted to 0.25 M NaCl with the addition of one volume of TGED. The protein was applied to a single strand DNA-cellulose column (1.5 cm x 10 cm) equilibrated in TGED + 0.25 M NaCl at 35 ml/hour. The column was washed with one volume of TGED + 0.25 M NaCl. OD_{280} of the column eluate was monitored to determine the point at which washing was complete. The column was eluted with 50 ml TGED + 1.0 M NaCl at 20 ml/hour for holoenzyme. The fractions were ran on a 15% SDS-PAGE. The fractions were pooled separately and dialyzed against storage buffer (10 mM Tris-HCl [pH

7.9], 50% glycerol, 0.1 mM EDTA, 0.1 mM dithiothretiol, 0.1 mM NaCl). RNAP was shown to contain a full complement of subunits ($\alpha_2\beta\beta'\sigma$) as determined by SDS-PAGE. The final enzyme concentration was determined spectrophotometrically using a extinction coefficient of E_{280} (1%) = 6.5 for RNA polymease (69).

Purification of WT CRP and mutant forms of CRP

Hyperexpression of *E. coli* pRE2/*crp* gene

E. coli MZ1 was used for overproduction of the CRP. Although this strain is crp^+, CRP only represents 0.1% soluble protein (0.1 mg/ml wet weight) present in wild-type *E. coli* (4).

pRE2/*crp* clones were transformed into MZ1 cells by the $CaCl_2$ method (70). Transformed cells were plated onto LB agar medium that contained 50 µg/ml ampicillin and incubated at 30°C overnight. One clone that contained the plasmid encoding each mutant *crp* gene was used to inoculate a 1.5 ml culture. 30 ml overnight cell cultures were used to inoculate 1 l LB at OD_{600} = 0.1. Cells were grown at 30°C for approximately 3 hours until the OD_{600} reached 0.5 - 0.6. The temperature of the cultures was then shifted to 42°C to inactivate the temperature sensitive λ cI repressor produced by MZ1 cells. Growth at 42°C was maintained for 2 hours to allow expression of the *crp* gene. Cells were harvested by centrifugation in a Beckman JA-17 rotor at 7 K RPM for 15 minutes at 4°C and the cell pellet was frozen at -20°C.

Purification of WT CRP protein

WT CRP protein was purified by sequential chromatography on Bio-Rex 70 and hydroxylapatite (HTP) as described by Harman (66). Frozen cell pellets were thawed and suspended in PC buffer (50 mM potassium

phosphate, 2 mM EDTA, 1 mM β-mercaptoethanol, 5% [v/v] glycerol, 100 mM KCl). Cells were ruptured with two passages through French pressure cell at 500 psi and centrifuged at 12 K RPM for 30 minutes. The supernant fractions were subsequently centrifuged at 100,000 X g for 90 minutes to remove ribosomes and insoluble material. The crude extract was dialyzed against PC buffer + 0.1 M KCl. The dialyzed crude extract was batch loaded onto equilibrated Bio-Rex 70 resin with periodically mixing at 4°C for one hour. The resin and extract were added to a 1 cm X 10 cm column. The column was washed with PC + 0.1M KCl until the eluate OD_{280} was less than 0.1. CRP was eluted in PC + 0.4 M KCl and collected in 5 ml fractions. The fractions were monitored by OD_{280}. Those fractions that contained CRP were pooled and dialyzed against PC buffer + 0.2 M KCl. The dialyzed extract was loaded into a HTP column (1.5 cm x 20 cm) at 20 ml/hour, washed with PC + 0.2 M KCl for one hour and eluted as 2 ml fractions in a linear gradient of 50 ml PC + 0.2 M KCl and 50 ml PC + 0.2 M KCl + 0.33 M K_2HPO_4/KH_2PO_4, pH 7.5. Those fractions were collected and monitored by using OD_{280}. Samples of each fraction were run on a 10% SDS-PAGE gel and those fractions that contained the most CRP were pooled. The pooled protein fractions were dialyzed against storage buffer. WT CRP was greater than 95% pure as judged by electrophoresis on 10% SDS-polyacrylamide gels. CRP concentrations were determined using an extinction coefficient for CRP of $3.5 \times 10^4\ M^{-1}\ cm^{-1}$(4).

Purification of mutant CRP protein

Mutant CRP preparations were further purified over CM-Sephadex column.

The pooled protein fractions from HTP column were dialyzed against CM-sephadex buffer (50 mM potassium phosphate [pH 8.0], 100 mM NaCl, 1

mM EDTA). The dialyzed protein fractions were loaded onto a CM-Sephadex column (1.5 cm X 20 cm), washed with one column volume of CM buffer and eluted as 2 ml fractions at 10 ml/hour in a linear gradient of CM buffer + 0.2 M NaCl and CM buffer + 1.0 M NaCl. Fractions were collected and monitored by using OD_{280}. The fractions were run through a 10% SDS-PAGE. Those fractions that contained the most CRP were pooled and dialyzed against storage buffer. Mutant CRP was greater than 95% pure as judged by electrophoresis on 10% SDS-polyacrylamide gels. CRP concentrations were determined using an extinction coefficient for CRP of $3.5 \times 10^4 M^{-1} cm^{-1}$ (4).

Preparation of chromatographic materials

Preparation of Bio-Rex 70 resin

Bio-Rex 70 resin was equilibrated as follows: five grams of Bio-Rex 70 resin was suspended in a 100 ml of PC buffer + 0.1 M KCl (pH 7.43) for 30 minutes at room temperature. The suspension was decanted and resuspended in equal volume PC buffer. This was repeated several times until the pH of the supernant stayed stabilized at pH 7.43. Excess buffer was decanted and the equilibrated resin was suspended in PC buffer + 0.1 M KCl as a 50 % (v/v) resin slurry.

Preparation of HTP resin

Five grams of HTP resin was thoroughly resuspended in 100 ml PC + 0.2 M KCl. After 5 minutes, the buffer was decanted to remove fines and fresh buffer was added. The procedure was repeated. The equilibrated resin was made 50% (v/v) in PC + 0.2 M KCl. Half of the resin was poured onto a 1.5 cm x 20 cm column for column packing.

Preparation of CM-Sephadex resin

Five grams of CM-Sephadex resin was added to 100 ml CM-Sephadex buffer. After 5 minutes, the buffer was decanted and fresh buffer was added and the procedure was repeated. The equilibrated resin was made a 50% (v/v) slurry in CM-Sephadex buffer. Half of the resin was poured onto a 1.5 cm x 20 cm column for column packing.

In vitro characterization of CRP position 127 mutants

In vtro transcription assay

Transcription reactions were carried out in a final volume of 10 ml in transcription buffer (30 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 100 mM KCl, 500 μg/ml BSA). The template pKL201 (2.52 nM) (Figure 2.1), CRP position 127 mutant protein (250 nM) and RNAP (100 nM) were incubated for 30 minutes at 37°C in the absence or presence of cAMP. A solution of nucleoside triphosphate (final concentration: 0.25 mM ATP, GTP CTP, 5 nM UTP and 0.05 mM α-^{32}P-UTP) and heparin (0.1 mg/ml) was added to the mixture to initiate a single round of transcription from pre-formed open complexes. The elongation reaction was allowed to proceed for 15 minutes at 37°C and was terminated with the addition of a equal volume of a solution that contained 10 M urea and 0.04% (w/v) bromophenol blue and heated to 75 °C for 5 minutes. RNA was resolved on a 6% polyacrylamide (19:1, acrylamide: methylene bis-acrylamide) gels that were 7 M in urea. RNA was visualized by autoradiography and analyzed by densitometry. The *lac* and *rep* RNA bands were quantitated from the peak areas.

Gel shift assay

Isolation of 203 bp *lac*P DNA fragment

Plasmid pHW104 containing five copies of 203 bp *lac*P fragment was digested with EcoRI restriction endonuclease and the *lac*P fragment was separated from the vector through a 1% agarose gel. The *lac*P DNA band was cut from the gel using a razor blade. DNA was eluted from the gel piece by electroelution at 25 V for 3 minutes. TE (10 mM Tris, pH 8.0, and 1 mM EDTA) - saturated isobutanol was used to extract ethidium bromide from the DNA solution. The DNA was run through a 5% polyacrylamide gel and visualized by UV shadowing. The DNA was eluted from a gel piece in 0.3 M sodium acetate. The DNA was precipitated with ethanol, washed with 70% ethanol and dried. The DNA was dissolved in TE buffer and its concentration was determined using OD_{260} (1 $A_{260} = 50\ \mu g/ml$).

End labeling of 203 *lac*P DNA fragment

DNA was dephosphorylated using shrimp alkaline phosphatase. The dephosphorylated DNA solution was exchanged into TE by ethanol precipitation. The DNA was end-labeled with γ-^{32}P ATP (10 mCi/ml; specific activity: 3000 Ci/mmole) using polynucleotide kinase at 37°C for 30 minutes. Polynucleotide kinase was heat-inactivated at 75°C for 10 minutes. The labeled DNA solution was passed through a push column previously equilibrated with 1 x STET buffer (100 mM NaCl, 20 mM Tris-HCl [pH 7.8]) and eluted with 1x STET buffer to remove unincorporated ATP. 1 µl of labeled DNA was counted using standard scintillation techniques in a Beckman LS 5000 TD liquid scintillation counter to determine its radioactivity.

Formation of CRP:DNA complexes

Formation of CRP:*lac*PDNA complexes was performed by incubating various concentrations of CRP in the absence or presence of 100 μM cAMP in transcription buffer at 37 °C for 30 minutes. The total reaction volume was 20 ml. One tenth volume of 50% glycerol, 0.2 mg/ml bromophenol was added and the reaction mixtures and loaded onto a 5% nondenaturing polyacrylamide gel (acrylamide:bis = 29:1). The gel was run at 150 volts for 30 minutes. Dried gels were used to expose Kodak XAR-5 films.

DNase I footprinting assay

DNA fragment production from polymerase chain reaction

PCR primers for amplifying *lac*P fragments and random sequence fragments (that does not contain CRP-binding site) were prepared by the Texas Tech Biotechnology Core Facility. The sequences of individual primers were shown in Figure 2.2.

A master mix was prepared that contained 100 μl 10 X buffer (Idaho Technology, 20 mM $MgCl_2$ with Ficoll/tartrazine), 10 μl 20 mM dNTP mix, 8 μl Taq DNA polymerase, 1 μl pfu DNA polymerase and brought to 700 μl using HPLC-grade water. In a 50 μl reaction, 0.5 mM of each primer, 50 fg/μl pKL201 and 35 μl master mix were combined. Fifteen μl mineral oil was layered on top of the reaction mixture. The reaction tube was put into a thermal cycler (Perkin Elmer Cetus) for 25 cycles. Each cycle had a 30 second denaturation step at 94°C, a 30 second annealing step at 59°C followed by a 15 second elongation step at 72°C.

Purification of the DNA fragment

The DNA from PCR was run on a 1.2% agarose to monitor the success of the reaction. The DNA was purified over a Qiagen column (Qiagen purification kit) and further purified through a 5% nondenaturing acrylamide gel. The DNA was visualized by UV-shadowing and the DNA was cut from the gel and eluted in 0.3 M sodium acetate at 37°C. The DNA was ethanol precipitated, washed with 70% ethanol and dried. The DNA was dissolved in TE buffer and its concentration was determined using OD_{260} (1 $A_{260} = 50\ \mu g/ml$).

Labeling and digestion of the DNA fragments

The purified DNA fragments (Figure 2.3) were end-labeled with γ-^{32}P ATP (10 mCi/ml; specific activity: 3000 Ci/mmole) using polynucleotide kinase at 37°C for 1 hour. Polynucleotide kinase was heat-inactivated at 75°C for 30 minutes. The labeled DNA was passed through a Qiagen nucleotide removal column (Qiagen nucleotide removal kit) to eliminate the unincorporated γ-^{32}P ATP. The *lac*P was then digested with *Hin*d III to give a 199 bp top strand-labeled, *lac*P fragment. The random sequence fragment was digested with *Dde* I to give a 190 bp top strand-labeled DNA fragment. Completion of digestion was monitored using a 5% nondenaturing acrylamide gel.

Binding of the protein to the DNA and digestion of DNA:CRP complexes by DNase I

Single stranded labeled DNA fragment was added to reaction mixtures (100 µl) that contained WT or mutant CRP at the indicated concentration, 100 µM cAMP, 5% glycerol and 5 mM $CaCl_2$ in 1 X transcription buffer at 37°C for

5 minutes. 100 nM RNAP was then added and the reaction was incubated at 37°C for 30 minutes. DNase I (prepared in 20 mM potassium phosphate [pH 6.8], 1 mM EDTA, 50% glycerol) was added to each tube to a final concentration of 50 μg/ml. The tube was incubated for one minute at room temperature. The reaction was terminated with the addition of 25 μl stop solution (1.5 M sodium acetate, 20 mM EDTA, 100 μg/ml tRNA) and the reaction tube was put on ice. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to extract the proteins from the reaction mixture. The aqueous phase was withdrawn and the DNA was precipitated with 100% ethanol, washed with 70% ethanol and air dried. The DNA was dissolved in loading buffer (7 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol FF in 1 X TBE) and loaded onto a 10% sequencing gel to separate single strand-labeled fragments. The gel was run at 40 watts until the bromophenol blue dye front was at the bottom of the gel. The gel was soaked in 5% glacial acetic acid and 15% methanol for 5 minutes and then dried. The dried gel was exposed to Kodak BMR-2 X-ray film.

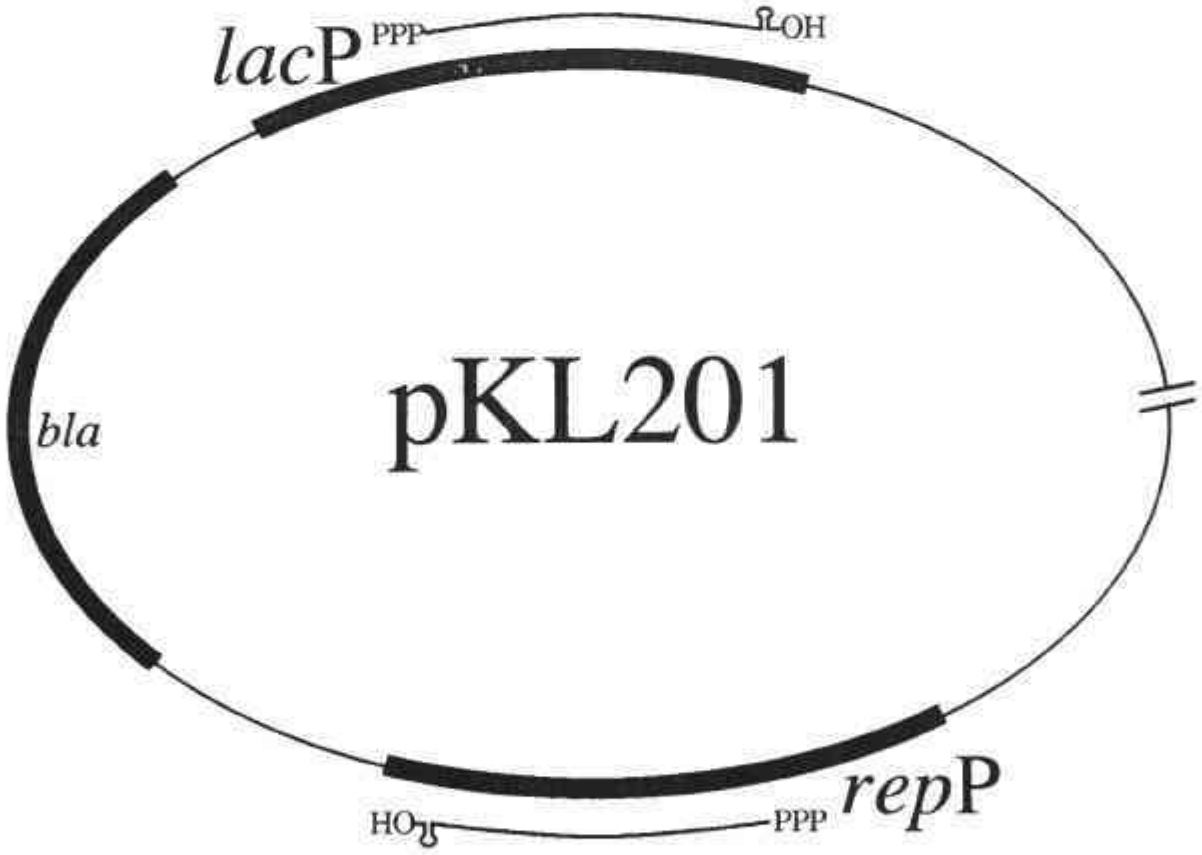


Figure 2.1. Structure of pKL201. The plasmid contains: the AMP^r determinant *bla*; *rep*P, promoting the synthesis of a 106/107 base RNA that terminates at $t_{rep;lac}$P, promoting the synthesis of a 136/137 base RNA that terminates at t_o; the ColE1 origin of replication and a cryptic *E. coli gal*K sequence.

Two primers for *lac* fragments:

5' CTGAGCCCGGCCAAGCTTACTCCCCAT 3'

and

5' ACTGGAGGGAATTCGTAATCATGGTCATAGC 3'

Two primers for random sequence fragments:

5' TACTCAACCAAGTCATTCTGAGAA 3'

and

5' AAGTAAAAGATGCTGAAGATCAGTTGG 3'

Figure 2.2. Sequence of primers used in PCR reactions for amplifying *lac*P fragments and random sequence fragments.

Sequence of *lac* fragment:

```
CTTATCATCGATAAGCTTACTCCCCATCCCCCTGGCACGACA
              HindIII
GGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTA

ATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACT
  CRP-binding site                    -35
TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGAT
                      -10
AACAATTTCACACAGGAAACAGCTATGACCATGATTACGAAT

TCCCTCCAGT
```

Sequence of random sequence fragments:

```
TACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG
                      DdeI
AGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCA

CATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCT

TCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC

AGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCA

TCTTTTACTT
```

Figure 2.3. Sequences of *lac* fragments and random sequence fragments used in DNase I footprinting assays.

CHAPTER III

RESULTS

Introduction

Wild-type (WT) CRP is a regulator of a number of catabolite-sensitive operons, including the *lac* operon. The *lac* operon is negatively regulated by a repressor, encoded by the *lac*I gene. Repressor binding to the operator region blocks transcription initiation. Positive control of the *lac* operon is mediated by the CRP:cAMP complex. The CRP:cAMP complex binds to a region located upstream of the *lac* promoter (*lac*P). CRP:cAMP binding aids RNAP (RNAP) recognition of *lacP* and dramatically increases the rate of transcription initiation.

The structure of CRP:$(cAMP)_2$ has been determined from X-ray crystallographic studies (21). Analysis of CRP:$(cAMP)_2$ crystals identified five specific amino acid-ligand contacts (E72, R82, S83, T127 and S128) that are likely to be important in cAMP binding to CRP and/or in mediating cAMP activation of CRP (21). The effects of specific amino acid substitutions at these positions in CRP were recently studied (22,23). Position 127 CRP mutants are unique in that: (1) they showed protease sensitivity in the absence of cAMP, indicating that threonine 127 plays a role in maintaining the protease-resistant structure of WT CRP; (2) all bound cAMP with affinities similar to that of WT CRP; (3) cAMP was required for *lac* transcription activation; and (4) all exhibited differences in their ability to activate *lac*P.

The goal of this project was to investigate position 127 mutant DNA binding and *lac*P activation characteristics *in vitro*. The first aspect of this study was to evaluate these mutants in promoting *lac*P mRNA synthesis

using *in vitro* transcription assay. The second aspect of the study was to evaluate the DNA binding characteristics of these mutants using gel shift and DNase I footprinting assays. The gel shift assay allows visualization of CRP:DNA complexes after electrophoretic separation of the free DNA and protein-bound forms of DNA in a polyacrylamide gel. The DNase I footprinting assay allows evaluation of the binding of CRP and RNAP on *lac*P fragment where protein:DNA complexes protect regions of DNA from DNase I attack.

Protein purification

Most of the components for the chemically-defined assays utilized in this study were commercially available. The DNA template, CRP and RNAP preparations were prepared as described in Materials and Methods. The WT CRP and position 127 mutant forms of CRP were expressed in *Escherichia coli* and purified using conventional column chromatography (Figure 3.1). The homogeneity of the CRP preparations was better than 95% according to densitometric analysis of coomassie blue-stained gels. RNAP was isolated from *E. coli* AG-1 cells using conventional column chromatography. Figure 3.2 shows the stages of purification of RNAP; the preparation contained a full complement of subunits (2α, β, β' and σ).

In vitro transcription assay

WT CRP and four position 127 CRP mutants were utilized in an assay to promote *lac* RNA synthesis using as template, the plasmid pKL201. These mutants were designated T127C CRP, T127G CRP, T127I CRP, T127S CRP and contained, respectively, either cysteine, glycine, isoleucine or serine for threonine at position 127. The complete transcription reaction mixtures

contained CRP, RNAP, cAMP and supercoiled pKL201. The plasmid pKL201 directed the synthesis of a 106/107 nucleotide RNA (*rep* RNA) that originated at *rep*P (a CRP-independent promoter) and terminated at t_{rep} and a 136/137 nucleotide RNA (*lac* RNA) that originated at *lac*P (a CRP-dependent promoter) and terminated at λt_o. Through the course of this study, transcription reaction products were resolved on 6% polyacrylamide-7 M urea gels. Synthesis of the *lac* RNA was CRP and cAMP dependent; synthesis of *rep* RNA was CRP and cAMP independent. The *rep* RNA served as an internal control for individual transcription reactions. Densitometric analysis of the *lac* and *rep* RNA band intensities allowed quantitation of the assay. Under the appropriate reaction conditions, the *lac* to *rep* ratio was utilized to display data in a manner that eliminated sample loading differences between samples of a reaction series.

Figure 3.3 shows a typical set of *in vitro* transcription reaction products. This figure shows that in the presence of cAMP, there were different levels of transcription activation for reaction mixtures that contain each of the four position 127 CRP mutants. T127C CRP showed a similar level of *lac* transcript compared to the reaction that contained WT CRP. Reaction mixtures that contained T127G CRP produced little *lac* RNA whereas those reactions that contained either T127I or T127S CRP produced *lac* RNA at 50% and 25% of the level observed in reactions that contained WT CRP. In the absence of cAMP, *lac* RNA was not produced (not shown). To optimize the transcription assay system, titrations of the individual components were conducted in reactions that contained WT CRP.

The effect of varying template DNA concentration on the *in vitro* system was assessed; the results are shown in Figure 3.4. A plot of *rep* RNA expressed as a percentage of the maximal value shows that *rep*P activity

increased with increased DNA concentration. At 2 nM and 20 nM DNA reaction sconditions, there was a 2-fold increase in the *rep* transcript. A parallel response was observed for the *lac* RNA (not shown); a plot of the *lac/rep* ratio versus DNA concentration shows the *lac/rep* ratio showed no significant deviation from linearity.

The effect of varying CRP concentration on the *in vitro* system was assessed by titrating WT CRP in the presence of cAMP; the results are shown in Figure 3.5. The bottom panel shows that *rep* P activity was not affected by changes in CRP concentration. Therefore, CRP concentration effects on the *lac/rep* ratio were due to changes in *lac*P activity. Figure 3.5 shows that *lac*P activity was CRP concentration-dependent. The level of transcription activation reached a maximum value at 250 nM CRP, then showed a 30% decrease between the CRP concentrations between 250 nM and 1.5 mM.

The effect of varying RNAP concentration on the *in vitro* system was assessed by conducting an RNAP titration series in reactions that contained WT CRP and cAMP; the results are shown in Figure 3.6. The *lac* to *rep* ratio showed two phases. The first one occurred at RNAP concentrations below 20 nM where there was no transcript synthesis. The second phase occurred at RNAP concentrations between 40 and 200 nM where a linear increase in *lac/rep* with increased RNAP was observed. The *lac* and *rep* RNA band intensities showed a similar response to increased RNAP concentration that appeared to saturate at 160-200 nM RNAP. The results presented in Figure 3.6 indicate that RNAP concentration affected the *in vitro* transcription system and that *lac*P and *rep*P responded similarly to changes in RNAP.

The effect of varying cAMP concentration on the *in vitro* system was assessed by conducting a cAMP titration series in transcription reactions that contained WT CRP; the results are shown in Figure 3.7. Changes in cAMP

concentration had little if any effect on *rep*P activity. In the absence of cAMP, *lac*P activity was low; between cAMP concentrations of 0.5 and 25 μM, the *lac*/*rep* ratio was high. A similar, high, *lac*/*rep* ratio was observed at cAMP concentrations of 50 and 100 μM (data not shown). These data indicate that *lac*P transcription was cAMP-dependent but not cAMP concentration-dependent between the range of 0.5 μM and 100 μM cAMP.

The effect of varying cAMP concentration on the *in vitro* system was assessed by conducting a cAMP titration series in transcription reactions that contained either the T127C, T127G, T127I or T127S forms of CRP; the results are shown in Figure 3.8. This figure shows that the activity of *rep* P was not significantly affected by either the CRP form used or the concentration of cAMP used in the transcription reactions. These data demonstrate that, in the absence of cAMP, none of the mutant proteins supported significant levels of *lac*P activity. The *lac*/*rep* response of T127C CRP showed a pattern in cAMP titration similar to that seen in parallel reactions that contained WT CRP. The *lac* transcription level increased immediately at 0.5 μM cAMP and remained at essentially the same level at increased cAMP concentrations. T127G CRP failed to active *lac*P at any concentration of cAMP. Reactions that contained T127I CRP showed increased *lac*P activity with increased cAMP concentration from 0 to 5 μM reaching saturation at 5 μM cAMP. Reactions that contained T127S CRP showed increased *lac*P activity with increased cAMP concentration from 0 to 10 μM reaching saturation at 10 μM cAMP.

The rate of formation and stability of the open complexes formed between RNAP and *lac*P DNA is shown in Figure 3.9. Control reactions containing no CRP established the levels of *lac*P activity when RNAP was incubated with DNA for a period of 30 seconds to 60 minutes prior to the addition of nucleoside triphosphates and heparin. Reactions that contained

the T127G CRP showed background levels of *lac*P activity. Reactions that contained WT CRP showed rapid open complex formation to levels that were 14 times that observed in the control. These levels were maintained for approximately 20 minutes and then decreased with increased incubation time. Similar results were observed in reactions that contained the T127C CRP. For reactions that contained either the T127I CRP or T127S CRP, the *lac /rep* ratio increased over a period of 15 minutes, maintained a value twice that of background for at least 15 minutes, and then decreased with increased incubation time. The data presented in this figure indicate that productive open complex formation for WT and T127C required 0.5 to 1 minute, whereas productive open complex formation for T127I and T127S mutants required 15 minutes. Figure 3.10 shows the response of the *rep* transcript, expressed as a percent of the maximal value, to changes in the time allowed for open complex formation. The level of *rep* did not change significantly with time.

Gel shift assay

Gel shift assay was used to separate free DNA and CRP:DNA complexes formed between different forms of CRP (WT CRP, T127C CRP, T127G CRP, T127I CRP or T127S CRP). The 203 bp *lac*P fragments were labeled with ^{32}P and incubated with CRP in the presence or absence of cAMP. CRP:DNA complexes were separated from unbound DNA on 5% non-denaturing gels.

The effect of varying CRP concentration in reactions that contained WT CRP is shown in Figure 3.11. The results show that in the absence of cAMP, no WT CRP:DNA complex formed. In the presence of 100 μM cAMP, there were different levels of DNA binding for WT CRP at various concentrations of CRP. In the presence of cAMP, a WT CRP:cAMP:DNA complex was clearly observed at concentrations of 50, 150 and 450 nM. This

figure indicates that WT CRP interaction with DNA was dependent on both cAMP and the concentration of WT CRP.

As was observed in reactions that contained WT CRP, no CRP:DNA complexes formed between *lac*P DNA and any of the position 127 CRP mutants in the absence of cAMP (data not shown). Figure 3.12 shows that in the presence of cAMP, a T127C CRP:cAMP:DNA complex was formed in reactions that contained CRP at 50, 150 or 450 nM; a pattern similar to that observed in assays that contained WT CRP. In contrast, no CRP:cAMP:DNA complexes were formed at any concentration of T127G CRP tested. Similarly, no distinct CRP:cAMP:DNA complexes were observed in assay mixtures that contained either T127I CRP or T127S CRP (Figure 3.13). This figure showes a smear that was only formed in reactions that contained the highest concentrations of CRP. These results suggest that the CRP:cAMP:DNA complexes formed between the T127I and T127S forms of CRP:cAMP complex and *lac*P-containing DNA are less stable than those formed by WT CRP:cAMP complex.

DNase I footprinting assay

DNase I attacks the phosphodiester bonds of the DNA double helix. The phosphodiester bonds that interact with bound protein are protected from DNase I attack. The region protected by protein yields bands of reduced intensity when the DNA is resolved on sequencing gels. This assay provides a direct visualization of the interaction of CRP, RNAP with their binding sites on the *lac* promoter.

The effect of varying cAMP concentration on DNA, CRP and RNAP interaction was evaluated by conducting a cAMP titration series reactions that contained either WT CRP alone or WT CRP and RNAP (Figure 3.14). No CRP

protection was observed in the absence of cAMP. CRP protection was observed at 5 μM cAMP and the protection was more complete at increased cAMP concentrations up to 100 μM. In reactions that contained RNAP, CRP protection was observed at a cAMP concentration of 1 μM and was more complete throughout the reactions series compared to reactions that did not contain RNAP. These results are consistent with cooperative binding of CRP and RNAP at *lac*P (54,55).

The *lac*P fragment from PCR reactions used in the DNase I footprinting experiments showed the major ^{32}P-labeled *lac*P DNA band and two additional ^{32}P-labeled bands. The nature of the two additional bands are unknown. It is known that these two extra bands were not double strand DNA in that they survived DNase I treatment; they were not single strand DNA in that they survived Nuclease VII treatment; they were not RNA in that they survived RNase treatment (data not shown).

Figure 3.15 shows the results from reactions that contained either WT CRP, T127C, T127G, T127I or T127S at 100 nM in the absence of cAMP (group I), in the presence of cAMP (group II) or in the presence of cAMP and RNAP (group III). Group I reactions showed no CRP protection. Group II reactions showed complete CRP-mediated protection of the *lac*P CRP binding site only in reactions that contained either the WT CRP or the T127C CRP. Group III showed CRP-mediated protection of both the CRP binding site and the RNAP binding site in all reactions. The degree of protection observed in reactions that contained the T127G, T127I or T127S CRP was lower than that observed in reactions that contained the WT CRP. The results from this series of experiments indicate that cAMP alone was sufficient to promote CRP binding to *lac*P for reactions that contain the WT CRP or the T127C CRP. A unique finding is that while cAMP alone did not to promote T127G CRP, T127I CRP

or T127S CRP binding to *lac*P; however, cAMP in combination with RNAP did promote protection of the DNA. The results of experiments conducted with RNAP alone showed that protection of *lac*P was strictly dependent upon cAMP(Figure 3.16).

This reaction series (Figure 3.15) was repeated at a CRP concentration of 500 nM. The results of these experiments are shown in Figure 3.17. For those reactions that contained WT CRP at 500 nM the group I (no cAMP), II (cAMP) and III (cAMP and RNAP) results are comparable to those displayed in Figure 3.15. Similarly, the results for those reactions that contained the T127I or T127S CRP are comparable to those displayed in Figure 3.15.

Clear differences between the two experiments (Figure 3.15 and Figure 3.17) were observed in reactions that contained the T127C and T127G forms of CRP. Group I reactions that contained T127C and T127G showed virtually complete protection of the DNA indicating that these two forms of CRP have high non-specific DNA sequence affinity in the absence of cAMP. This characteristic was maintained for T127G CRP in reactions that contained cAMP (group II) and in reactions that contained cAMP and RNAP (group III). In contrast, the addition of cAMP to reactions that contained the T127C CRP induced CRP-mediated protection of the *lac*P CRP-binding site. The addition of cAMP and RNAP completed the conversion of T127C CRP from a high affinity, sequence non-specific DNA binding protein to a sequence specific DNA binding protein.

The binding characteristics of the WT and mutant froms of CRP to DNA that lacked a CRP binding site were evaluated. The DNase I footprinting reactions contained CRP at 100 nM (category A) or 500 nM (category B) in the absence of cAMP (group I) or in the presence of cAMP (group II) (Figure 3.18). In reactions that contained 100 nM CRP DNase I-mediated DNA digestion

was similar for all five forms of CRP and was unaffected by cAMP. In reactions that contained 500 nM CRP, a similar pattern of DNase I digestion were observed for WT CRP, T127I CRP and T127S CRP. Reactions that contained either the T127C CRP or the T127G CRP showed protection of the entire DNA fragment in the presence and in the absence of cAMP. The results presented in Figures 3.17 and 3.18 indicate that the substitution of C or G for T at position 127 resulted in increased CRP non-specific DNA binding. Cyclic AMP binding to T127C CRP induced site-specific DNA binding without eliminating the non-specific DNA binding characteristic of T127C CRP.

Variation of the incubating time of *lac*P DNA and CRP prior to the addition of DNase was used to assess DNase I footprinting in reactions that contained either 500 nM WT CRP or 500 nM CRP in the absence (Figure 3.19) or presence (Figure 3.20) of cAMP. Reactions that contained WT CRP, DNA protection did not occur at any time interval. Reactions that contained T127C CRP or T127G CRP showed complete protection of the *lac*P fragment at all time intervals. Reactions that contained T127I or T127S CRP showed protection of DNA only at 15 minutes incubation time. These results indicate the binding of CRP to DNA was established within 15 minutes and was stable for a period of 60 minutes. There was no evidence that the WT CRP, the T127I CRP or the T127S CRP had affinity for DNA in the absence of cAMP. Clearly, the T127C CRP and T127G CRP exhibited stable, non-specific DNA binding in the absence of cAMP; T127I CRP and T127S CRP exhibited unstable, non-specific DNA binding in the absence of cAMP, and this unstable non-specific DNA binding decreased with increased incubating time.

Reactions that contained WT CRP, the T127C CRP, the T127I CRP or the T127S CRP and cAMP showed protection of the *lac*P fragment CRP-binding site at all time intervals. The binding of these CRP:cAMP complexes

to DNA was stable for a period of 60 minutes. Reactions that contained T127G CRP and cAMP showed generalized DNA protection at the 15, 30 and 60 minutes intervals but not at the 45 minutes interval. This suggests that while cAMP binding did not induce site-specific DNA binding for this CRP mutant, it may destabilize, to a degree, the non-specific complexes formed between T127G:cAMP and DNA.

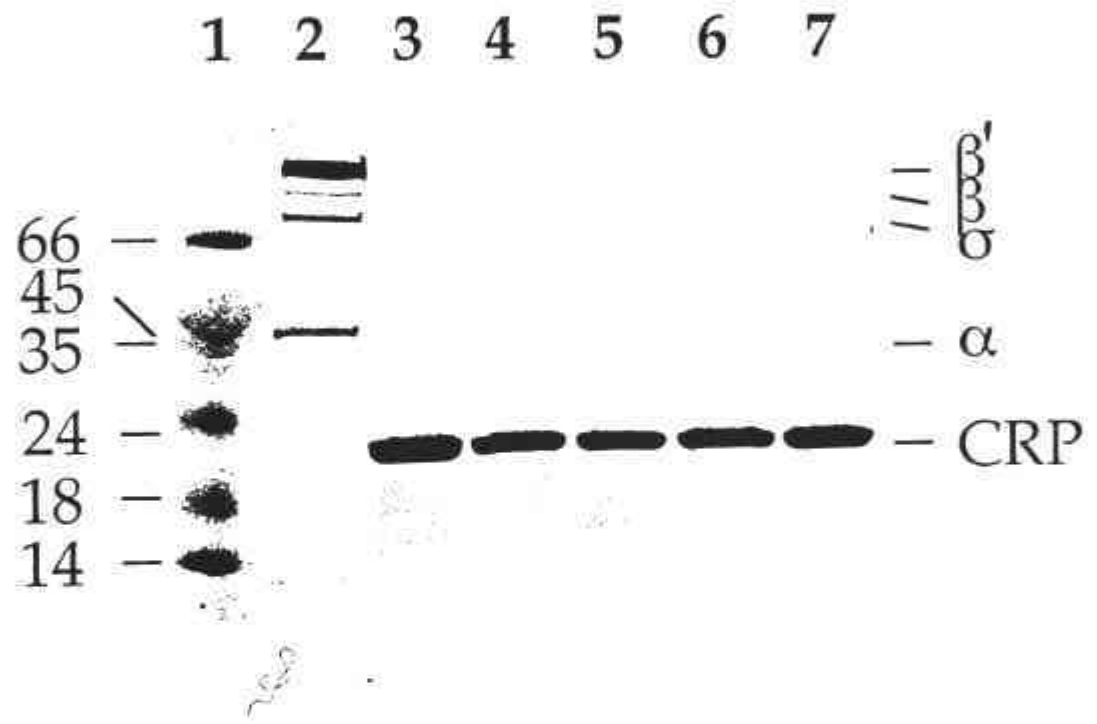


Figure 3.1. RNAP and CRP preparations used in this study. Each lane was loaded with the indicated protein preparation. Lane designations are: lane 1, mass standards (bovine serum albumin, 66 kDal), (egg albumin, 45 kDal), (pepsin, 35 kDal), (trypsinogen, 24 kDal), (β-lactoglobulin, 18.4 kDal), (lysozyme, 14.3 kDal); lane 2, RNAP, 5 μg; lane 3, WT CRP, 5 μg; lane 4, T127C CRP, 5 μg; lane 5, T127G CRP, 5 μg; lane 6, T127I CRP, 5 μg; lane 7, T127S CRP, 5 μg.

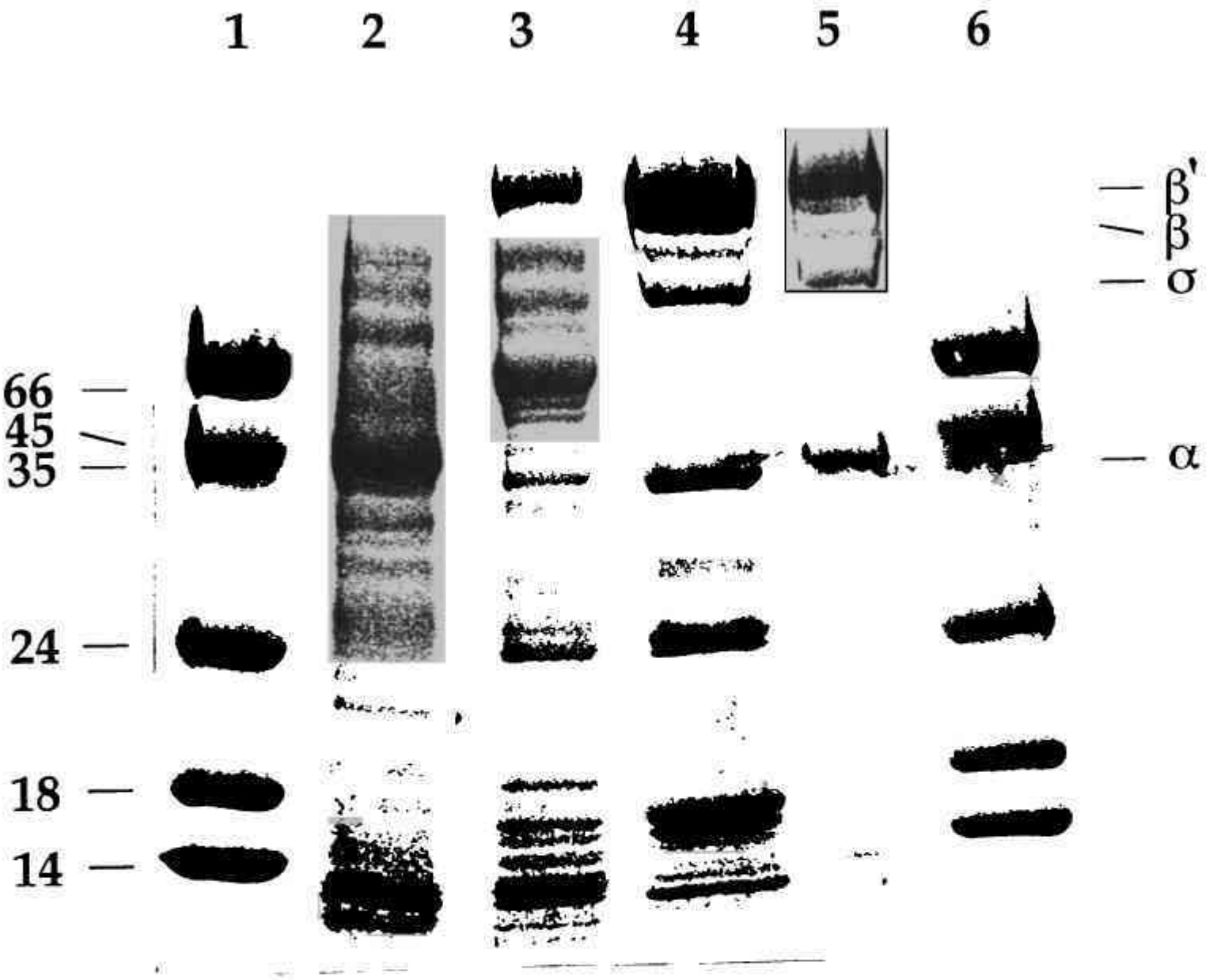
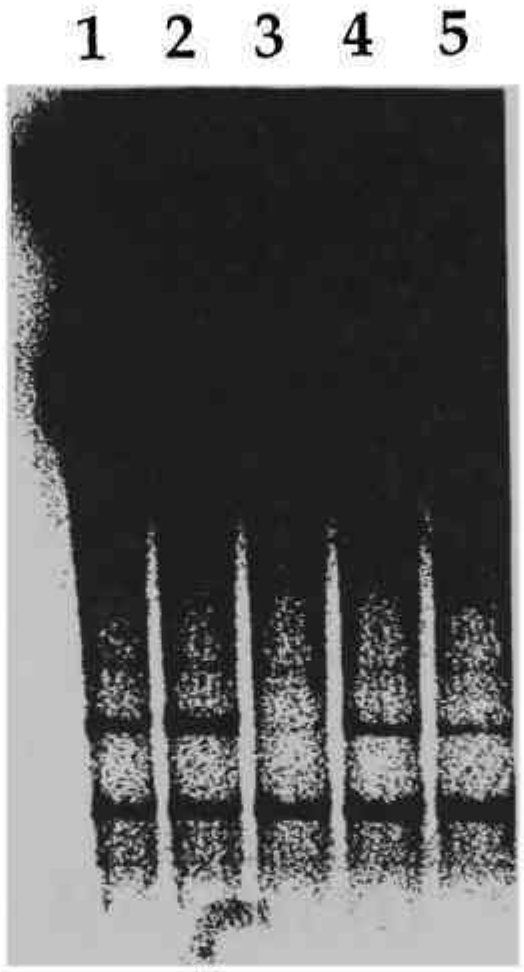
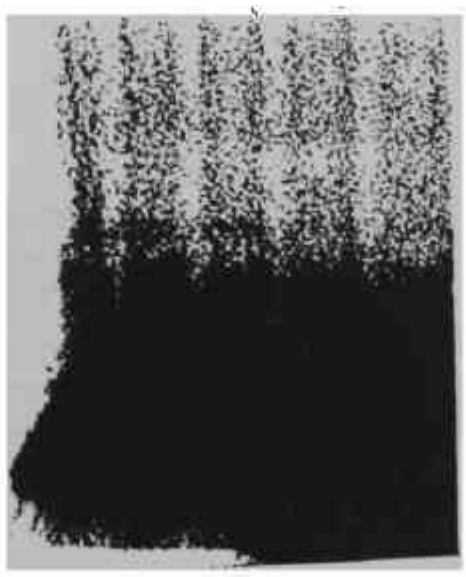


Figure 3.2. RNAP purification from *E. coli* AG1 cells. Lane designations are: lane 1, mass standards (bovine serum albumin, 66 kDal), (egg albumin, 45 kDal), (pepsin, 35 kDal), (trypsinogen, 24 kDal), (β-lactoglobulin, 18.4 kDal), (lysozyme, 14.3 kDal); lane 2, crude cell extract, 50 μg; lane 3, protein fraction pool after double strand cellulose column chromatography, 25 μg; lane 4, protein fraction pool after Bio-Gel A 1.5 m column chromatography, 25 μg; lane 5, protein fraction pool after single strand cellulose column chromatography, 10 μg; lane 6, mass standards.


1 2 3 4 5
– Lac
– Rep



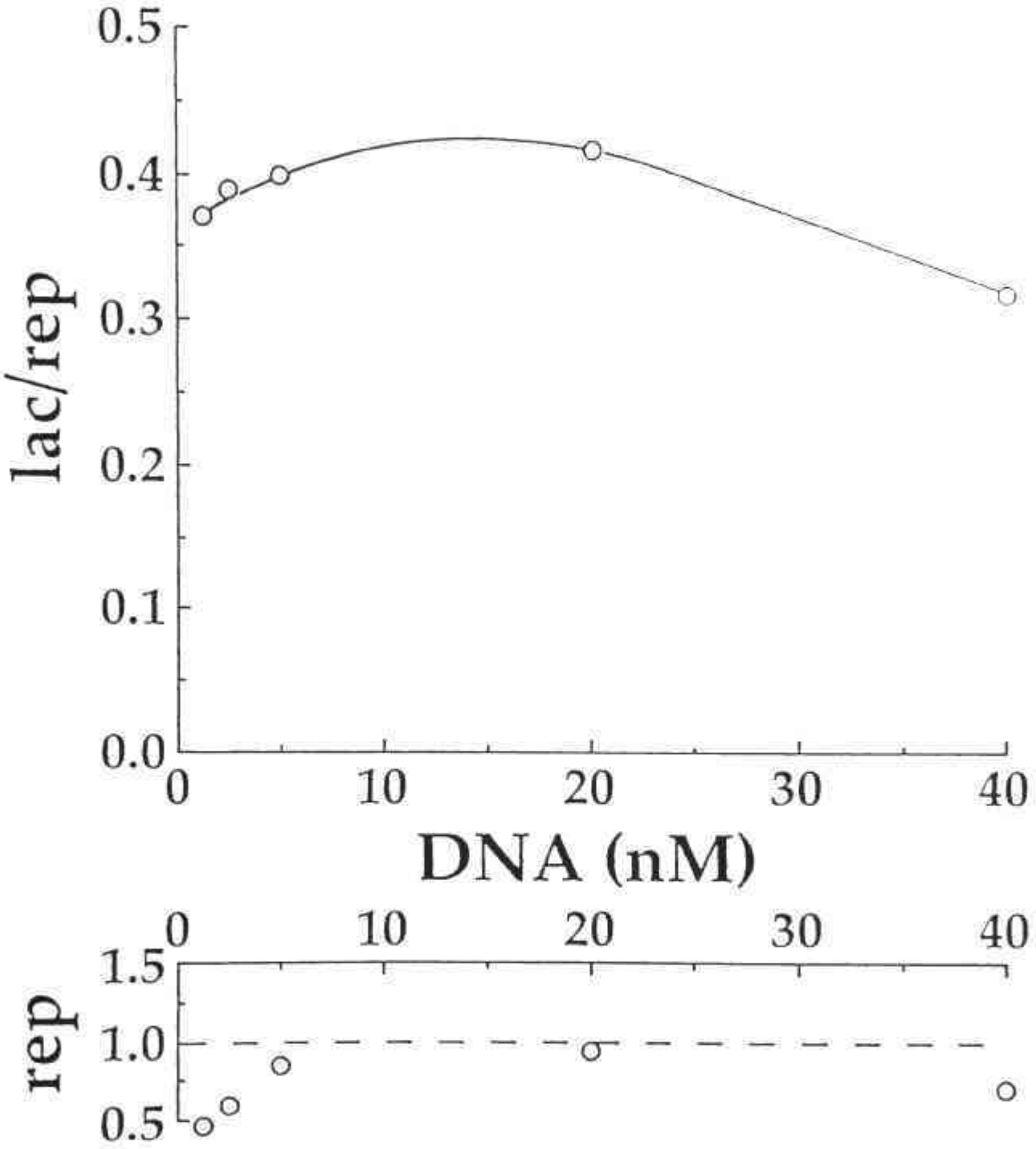


Figure 3.4. *In vitro* transcription; template DNA titration. Reactions contained the indicated concentration of pKL201, WT CRP at 250 nM, cAMP at 100 μM and RNAP at 100 nM. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. Top panel showed *lac/rep* ratio, bottom panel showed *rep* RNA percentage of *rep* maximal value in the reaction set.

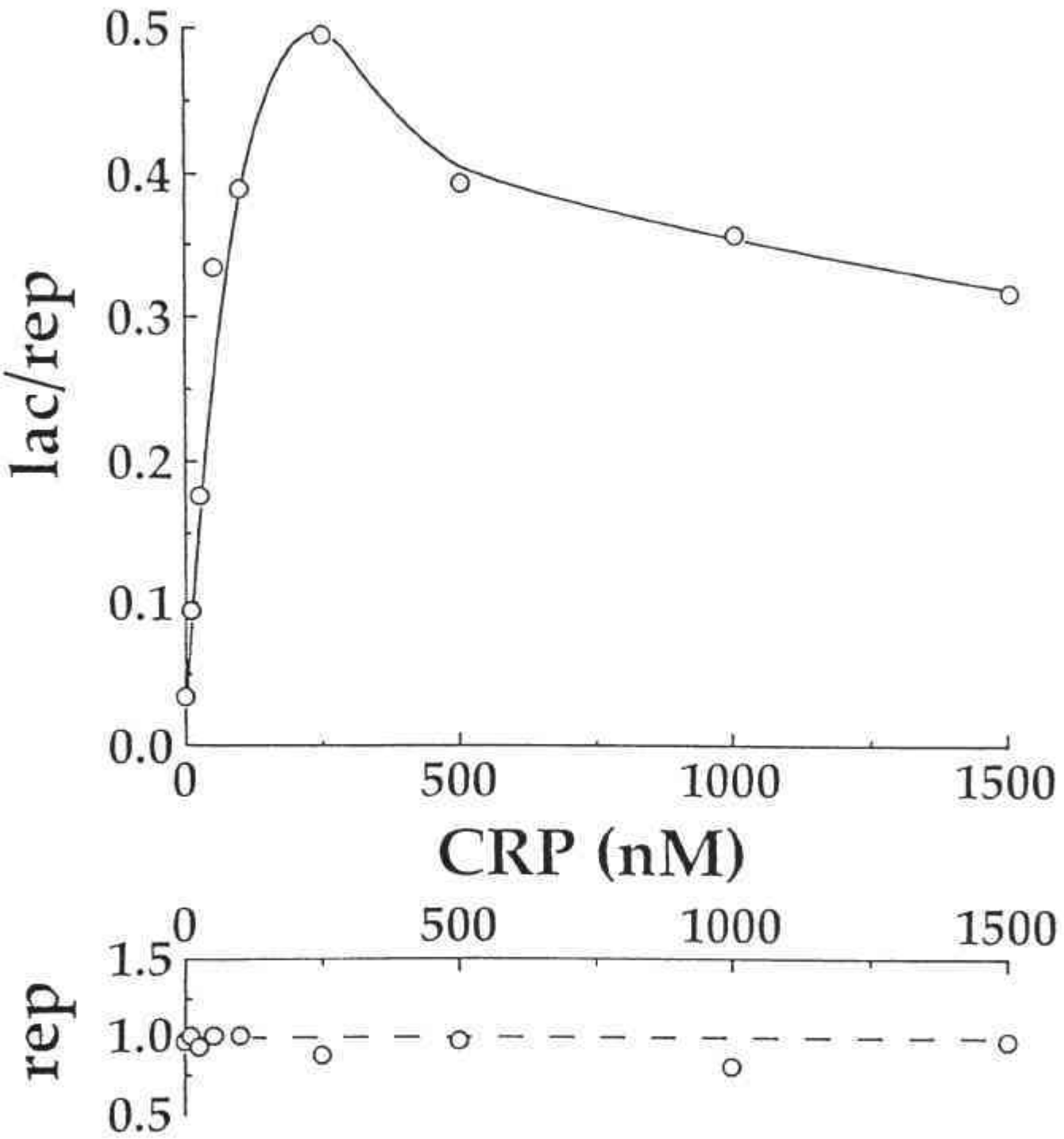
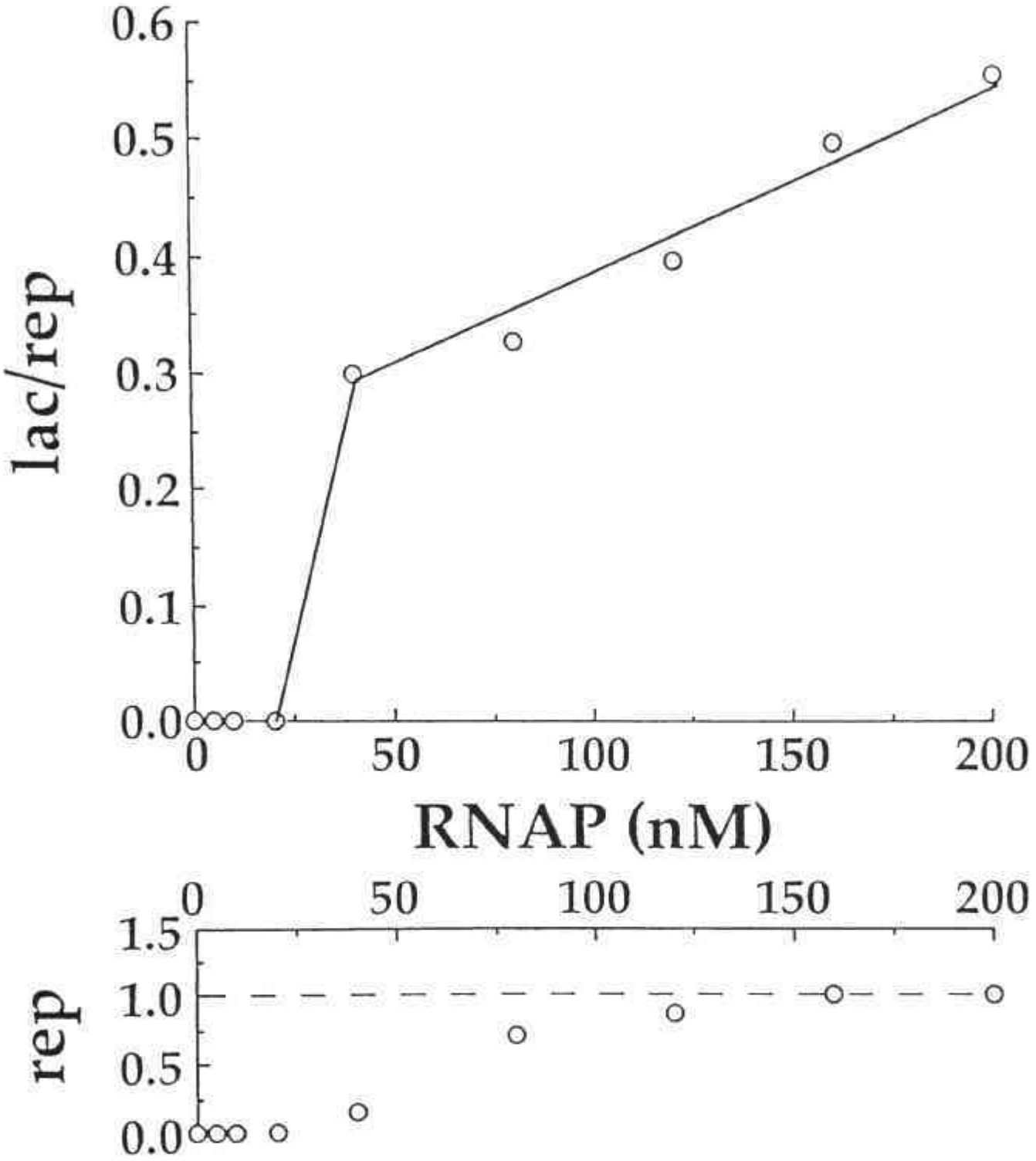


Figure 3.5. *In vitro* transcription; CRP titration. Reactions contained the indicated concentration of WT CRP, pKL201 at 2.52 nM, cAMP at 5 μM and RNAP at 100 nM. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. Top panel showed *lac*/*rep* ratio, bottom panel showed *rep* RNA percentage of *rep* maximal value in the reaction set.



rigure 3.6. *In vitro* transcription; RNAP titration. Reactions contained the indicated concentration of RNAP, pKL201 at 2.52 nM, cAMP at 5 μM and WT CRP at 1 μM. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. Top panel showed *lac/rep* ratio, bottom panel showed *rep* RNA percentage of *rep* maximal value in the reaction set.

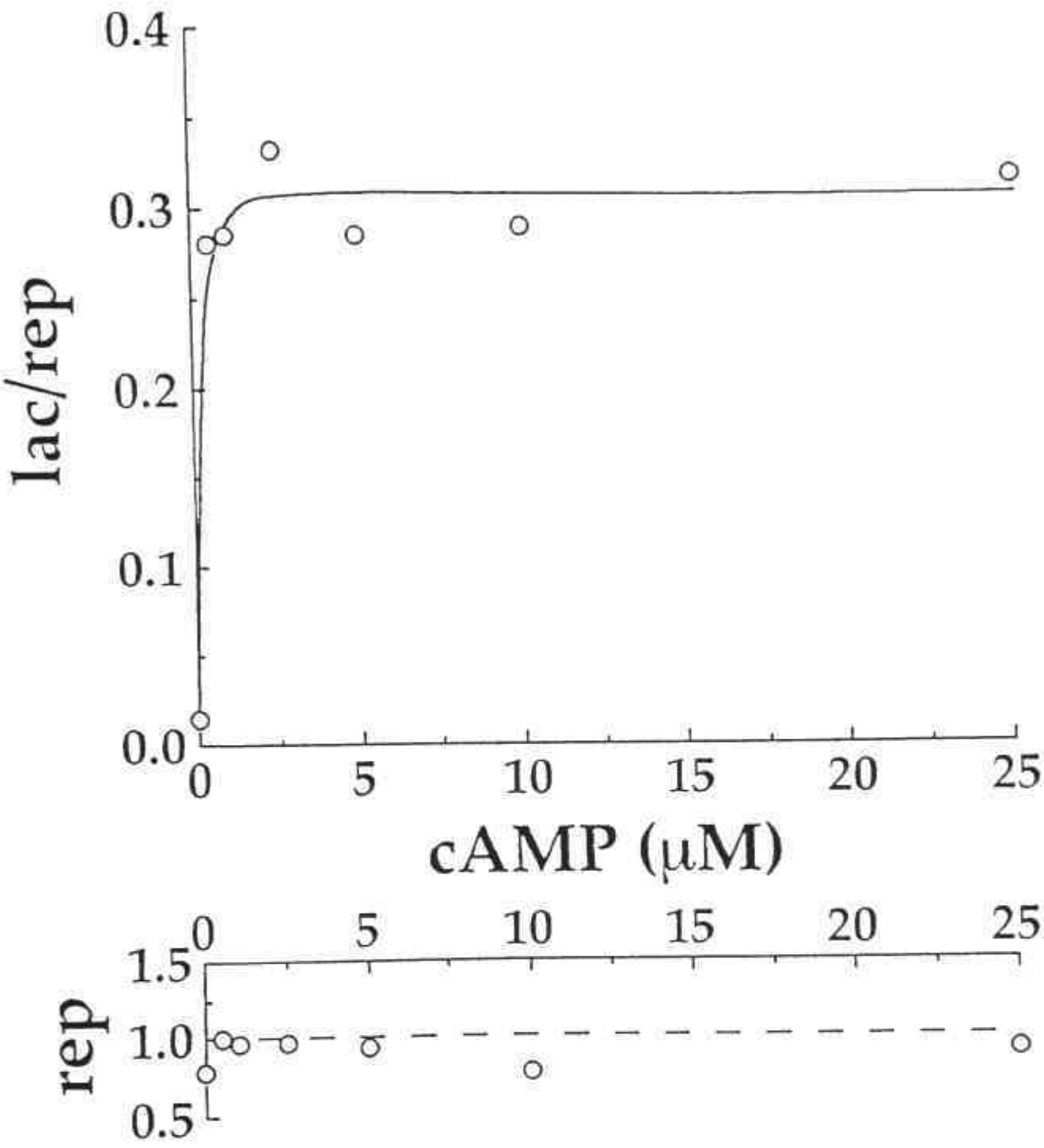


Figure 3.7. *In vitro* transcription; cAMP titration. Reactions contained the indicated concentration of cAMP, pKL201 at 2.52 nM, WT CRP at 1 μM and RNAP at 100 nM. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. Top panel showed *lac/rep* ratio, bottom panel showed *rep* RNA percentage of *rep* maximal value in the reaction set.

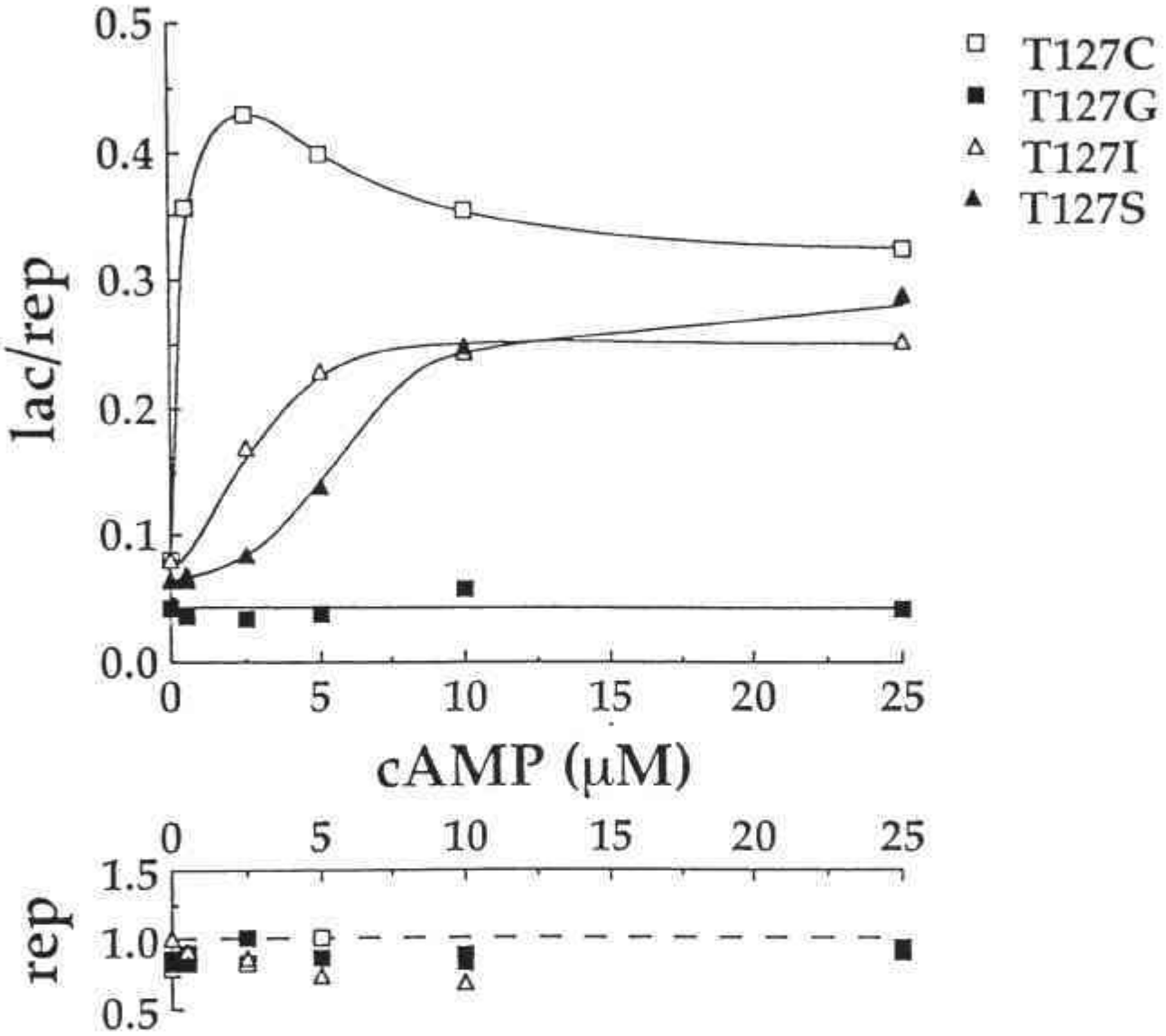


Figure 3.8. *In vitro* transcription; cAMP titration in reactions that contained mutant CRP. Reactions contained the indicated concentration of cAMP, the indicated mutant CRP at 250 nM, pKL201 at 2.52 nM, and RNAP at 100 nM. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. Top panel showed *lac/rep* ratio, bottom panel showed *rep* RNA percentage of *rep* maximal value in the reaction set.

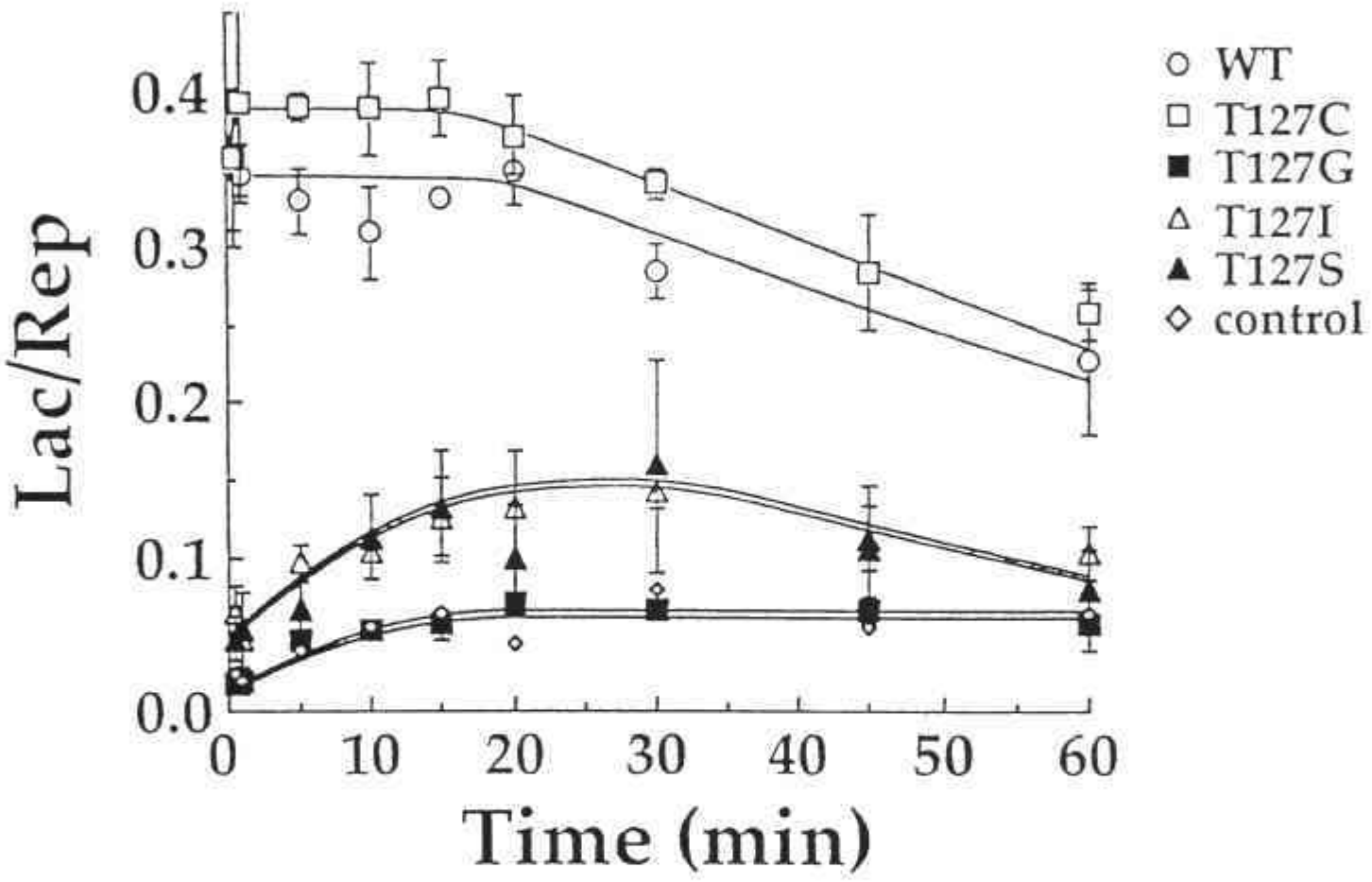


Figure 3.9. Open complex formation at *lac*P. Reactions contained pKL201 at 2.52 nM, cAMP at 5 μM, the indicated CRP at 250 nM and RNAP at 100 nM. Reactions are incubated at 37°C for the indicated time prior to the addition of nucleoside triphosphates and heparin. The autoradiograph of RNA products separated on a 6% polyacrylamide-7 M urea gel was scanned on a densitometer, and the *lac* and *rep* band areas were quantitated. The error bars represent the standard deviation of three independent experimental data for each reaction condition applied.

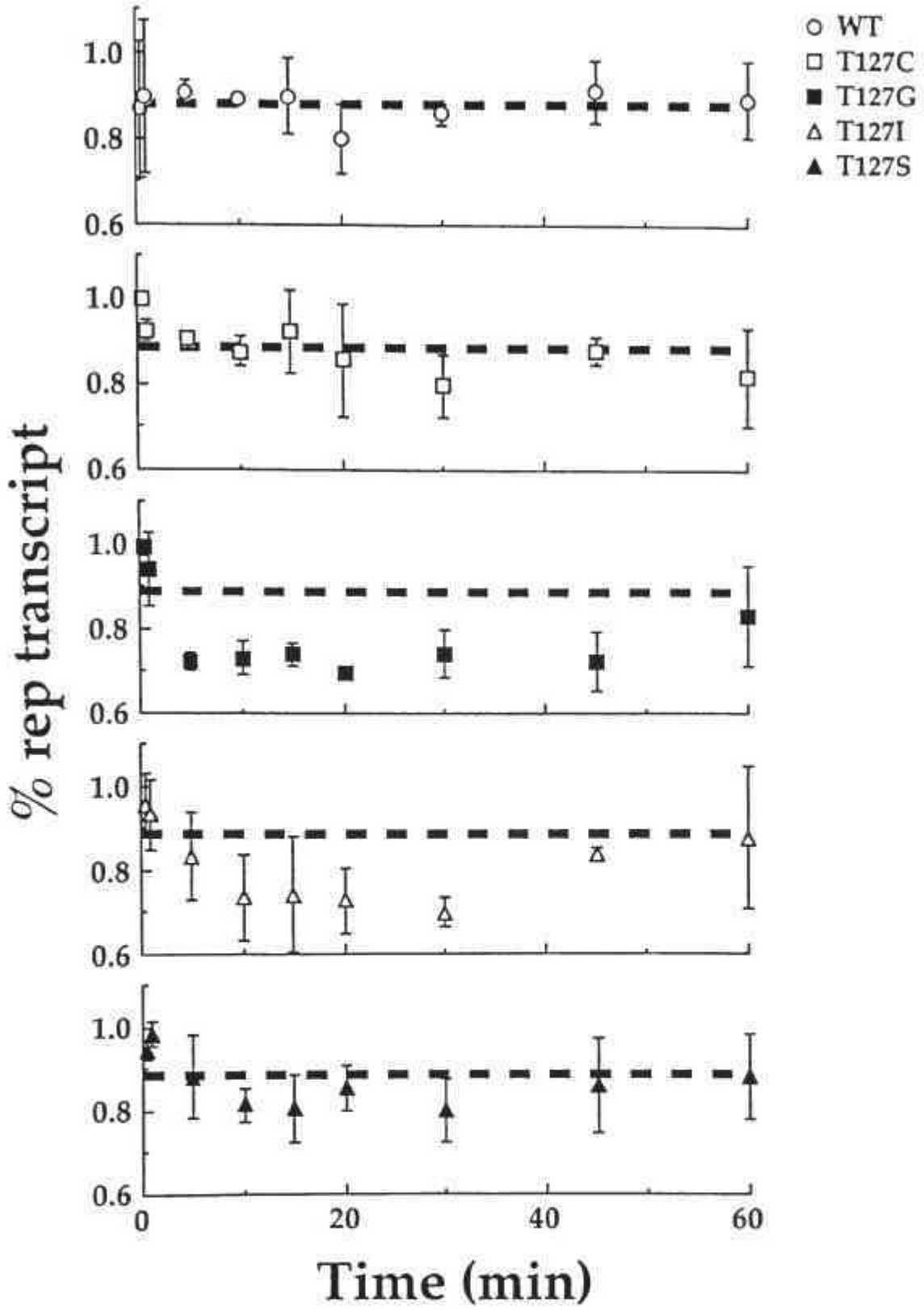


Figure 3.10. Time course of individual *rep* area in transcription reactions. Display of the areas of the *rep* RNA from the experiment in Figure 3.9. The error bars represent the standard deviation of three independent experimental data for each reaction condition applied.

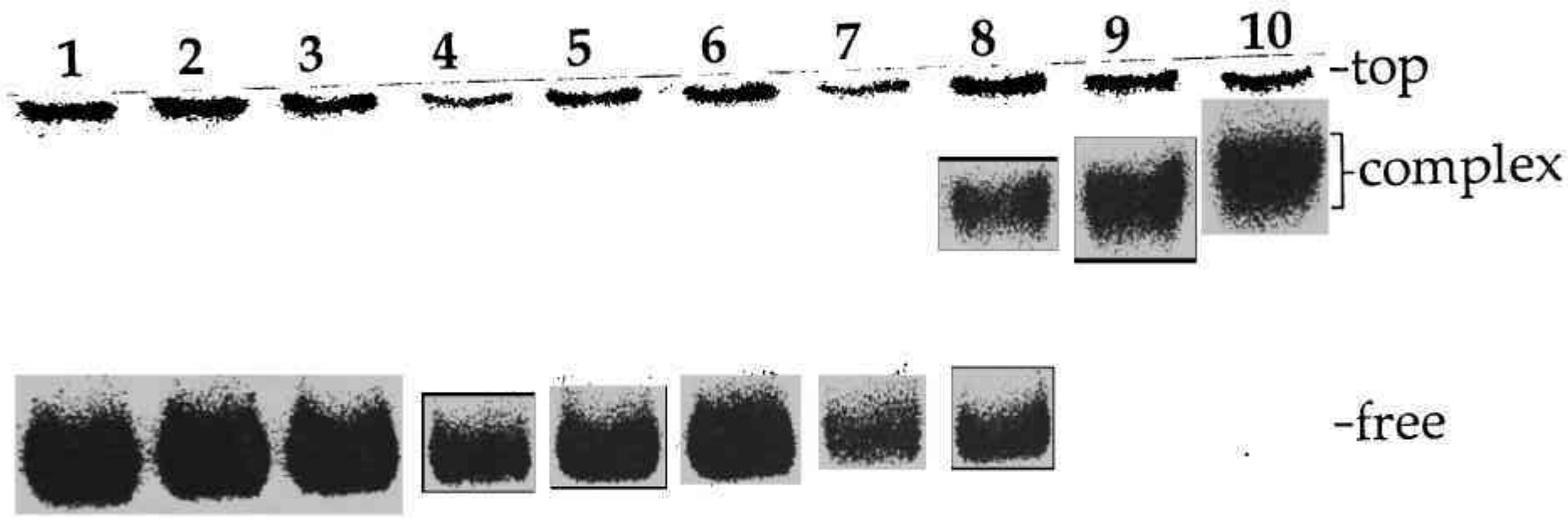


Figure 3.11. Gel shift assay of the 203 bp *lac*P fragment in the presence of WT CRP. Lane designations are: lanes 1 and 6, no CRP; lanes 2 and 7, CRP at 10 nM; lanes 3 and 8, CRP at 50 nM; lanes 4 and 9, CRP at 150 nM; lanes 5 and 10, CRP at 450 nM. Lanes 1 through 5 were run on reaction mixtures that contained no cAMP. Lanes 6 through 10 were run on reaction mixtures that contained cAMP at 100 μM.

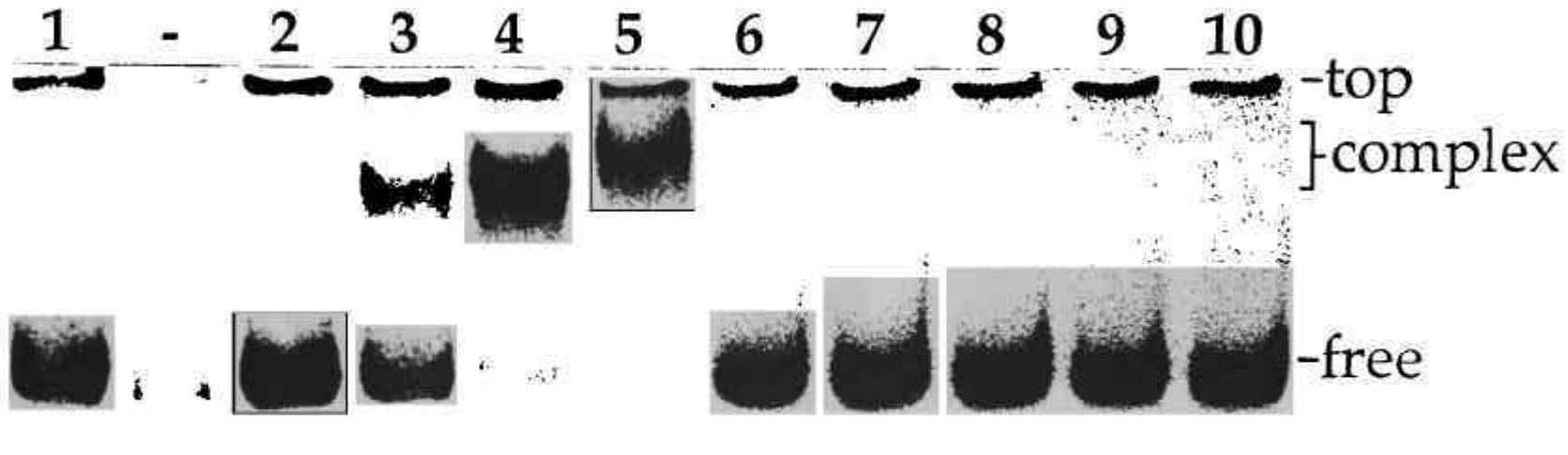


Figure 3.12. Gel shift assay of the 203 bp *lac*P fragment in the presence of either T127C CRP or T127G CRP. Lane designations are: lanes 1 and 6, no CRP; lanes 2 and 7, CRP at 10 nM; lanes 3 and 8, CRP at 50 nM; lanes 4 and 9, CRP at 150 nM; lanes 5 and 10, CRP at 450 nM. Lanes 1 through 5 contained T127C CRP. Lanes 6 through 10 contained T127G CRP. All reaction mixtures contained cAMP at 100 μM.

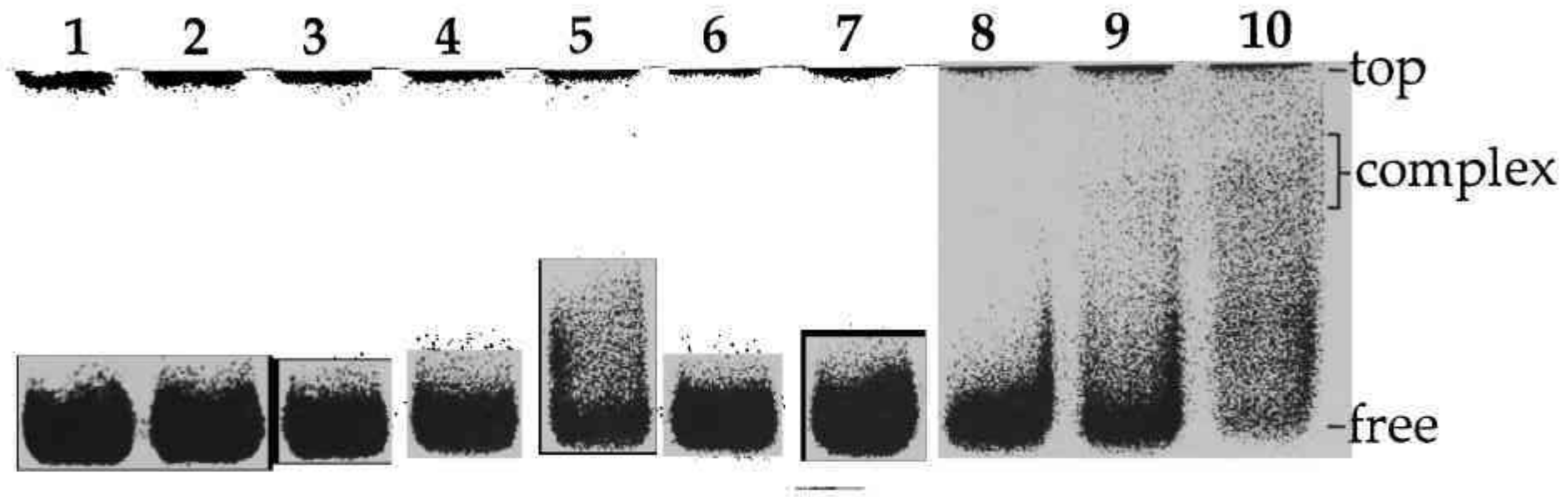
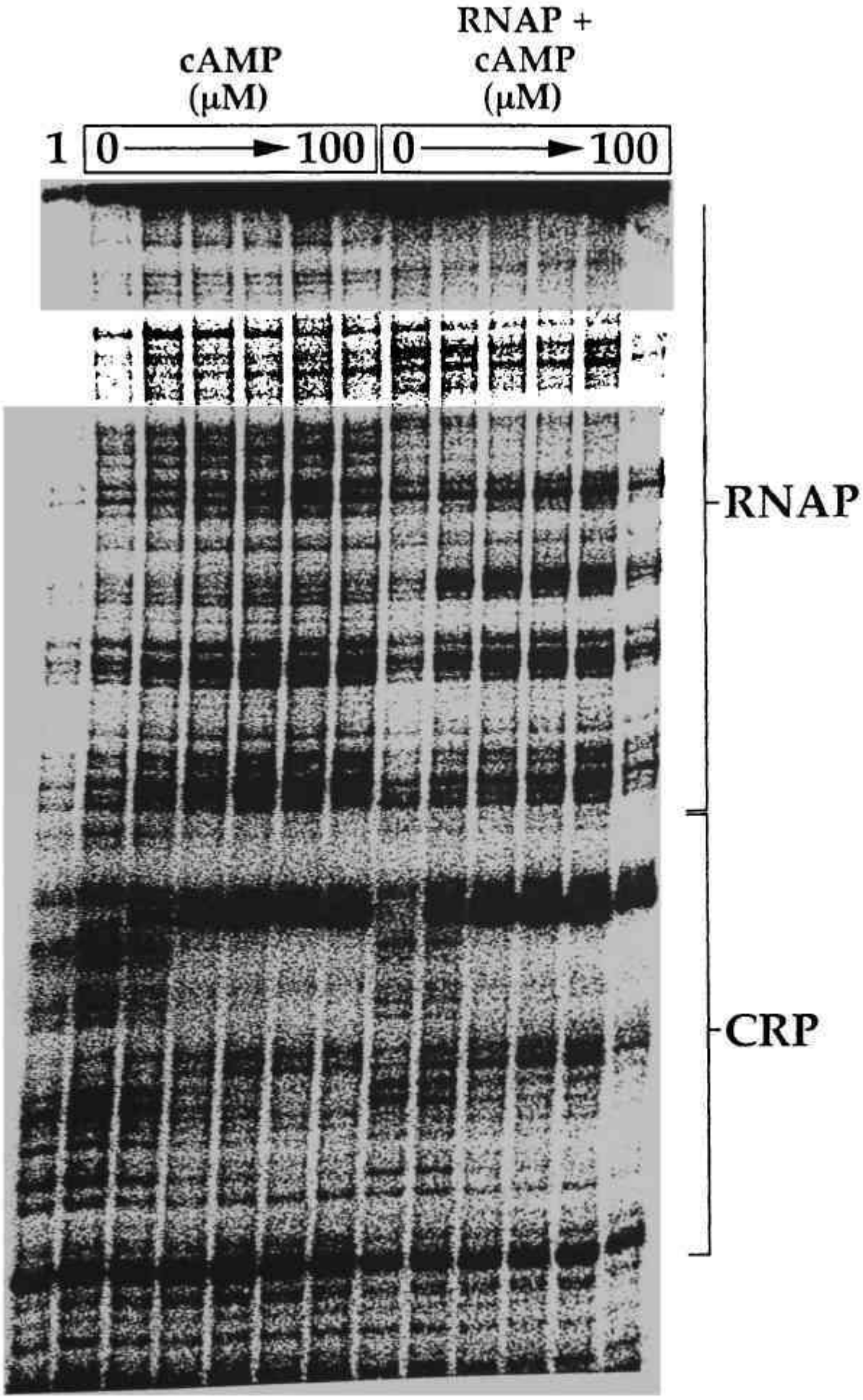
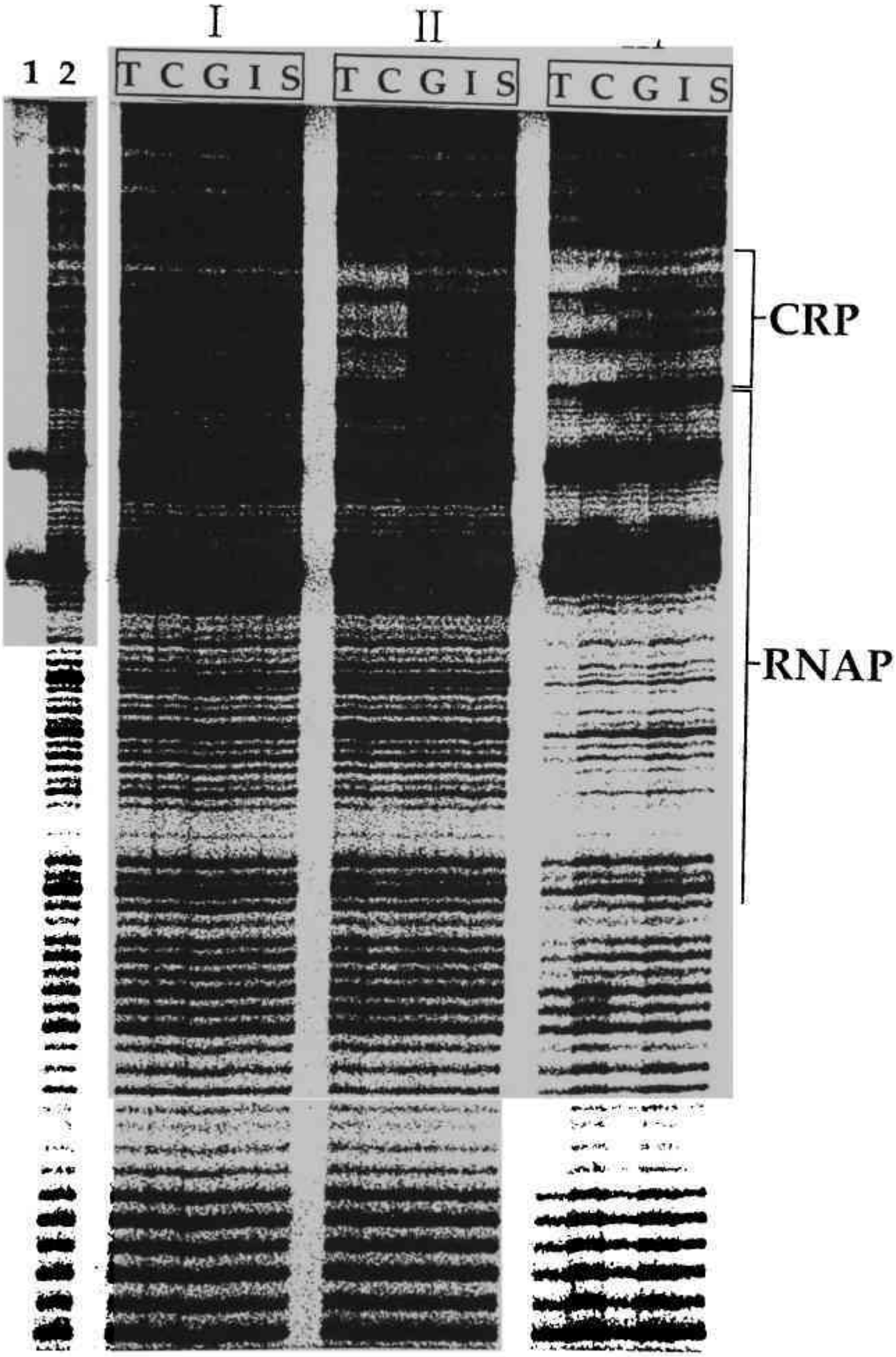
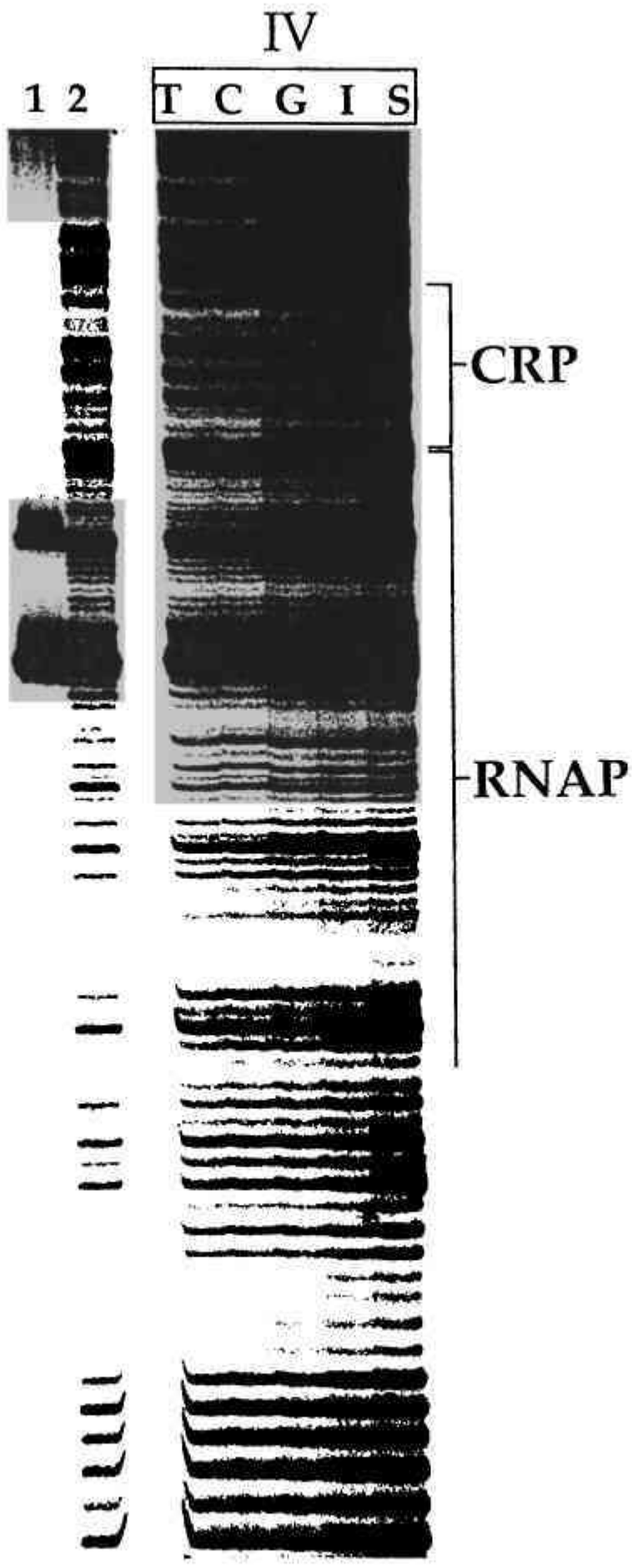
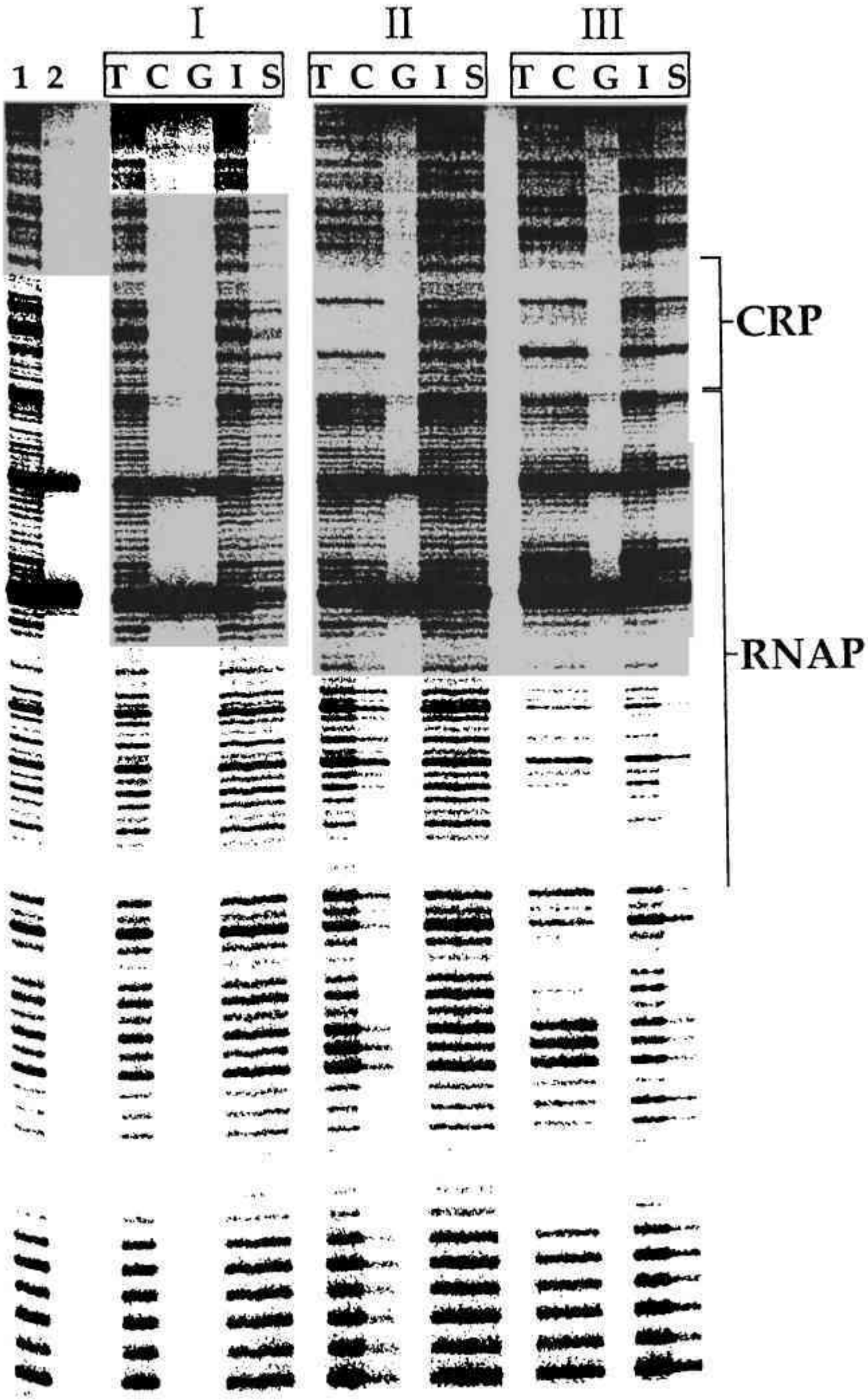


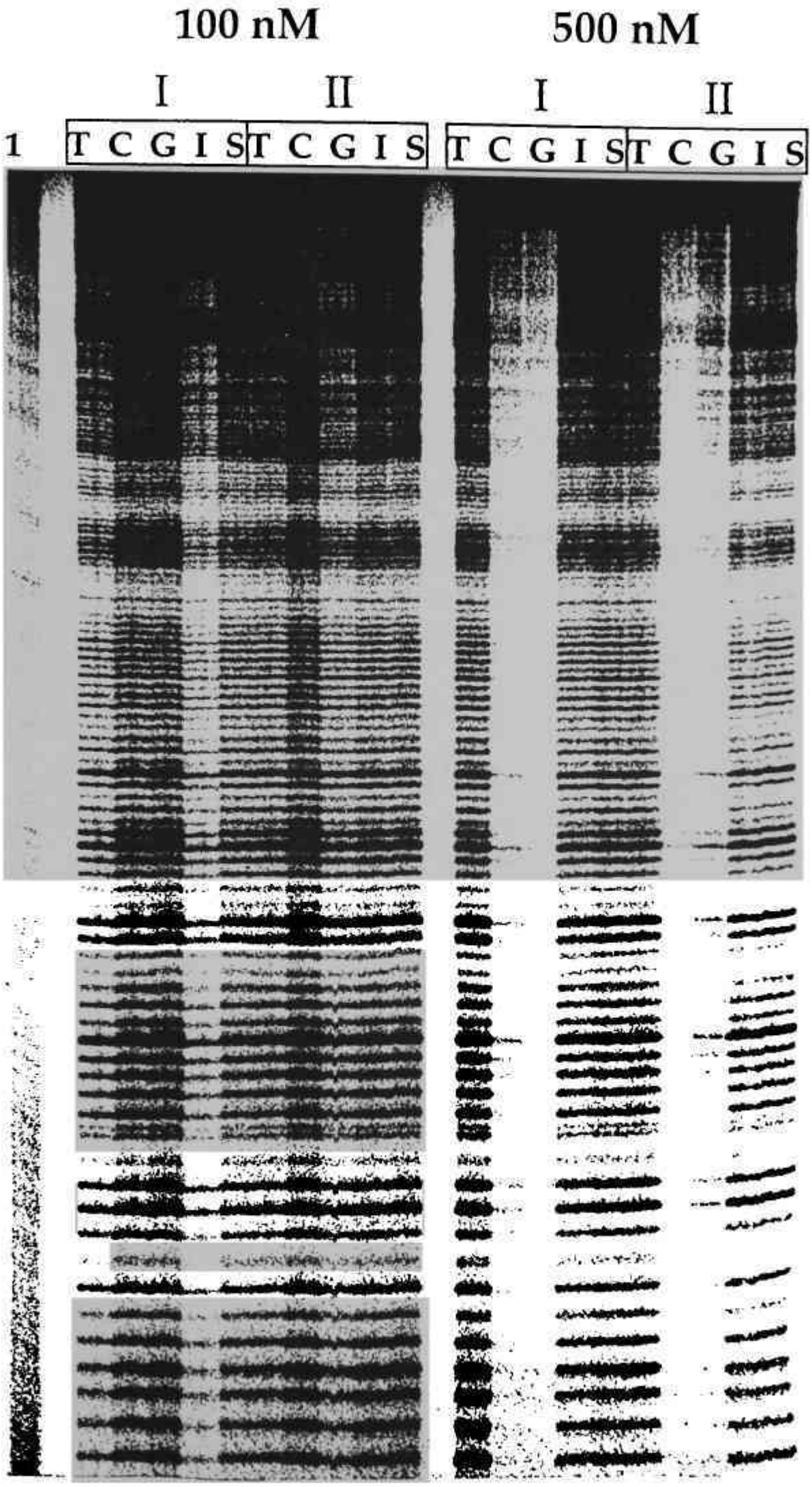
Figure 3.13. Gel shift assay of the 203 bp *lac*P fragment in the presence of either T127I CRP or T127S CRP. Lane designations are: lanes 1 and 6, no CRP; lanes 2 and 7, CRP at 10 nM; lanes 3 and 8, CRP at 50 nM; lanes 4 and 9, CRP at 150 nM; lanes 5 and 10, CRP at 450 nM. Lanes 1 through 5 contained T127I CRP. Lanes 6 through 10 contained T127S CRP. All reaction mixtures contained cAMP at 100 μM.

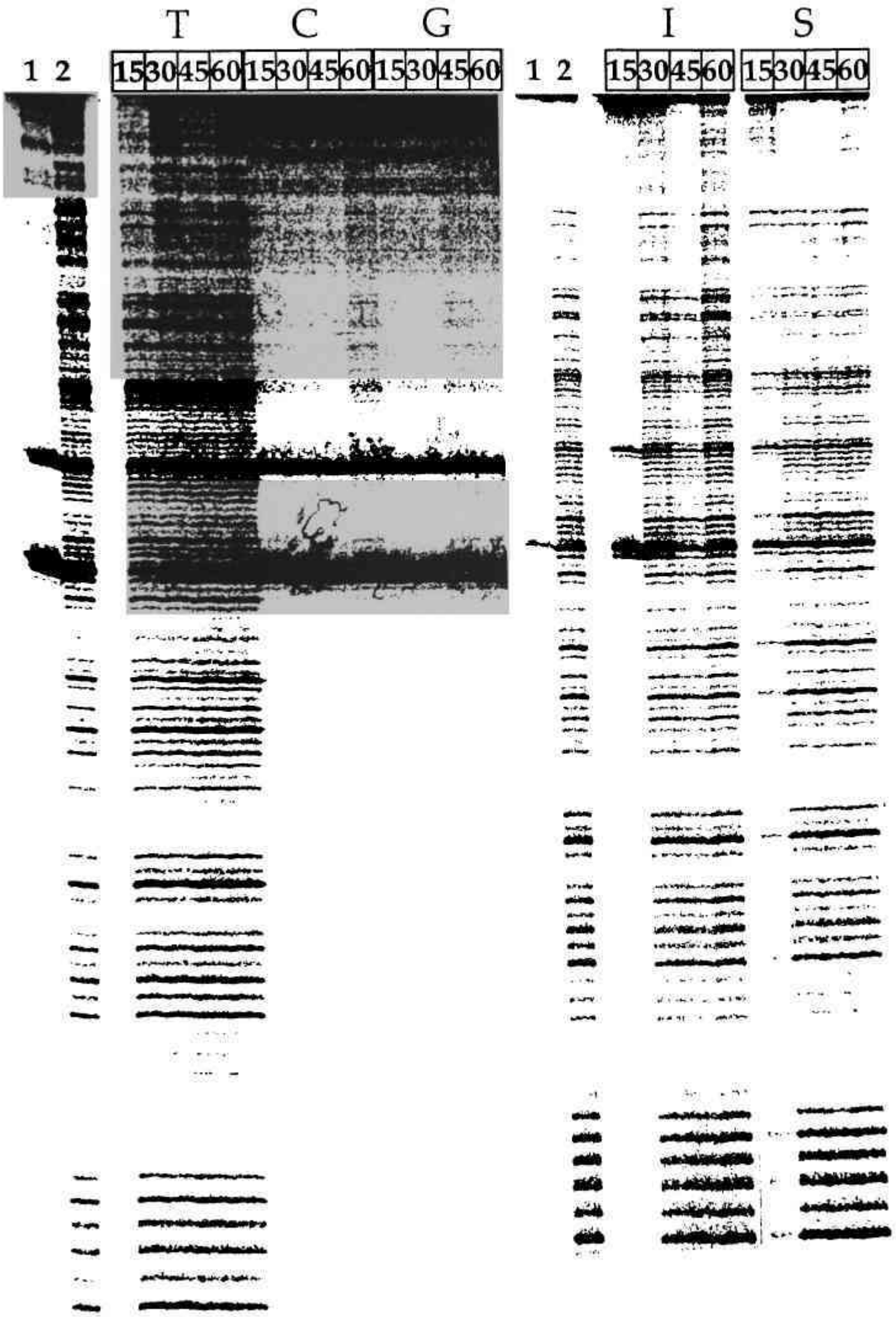

RNAP +
cAMP
(μM)
cAMP
(μM)
1
0
100
0
100
RNAP
CRP

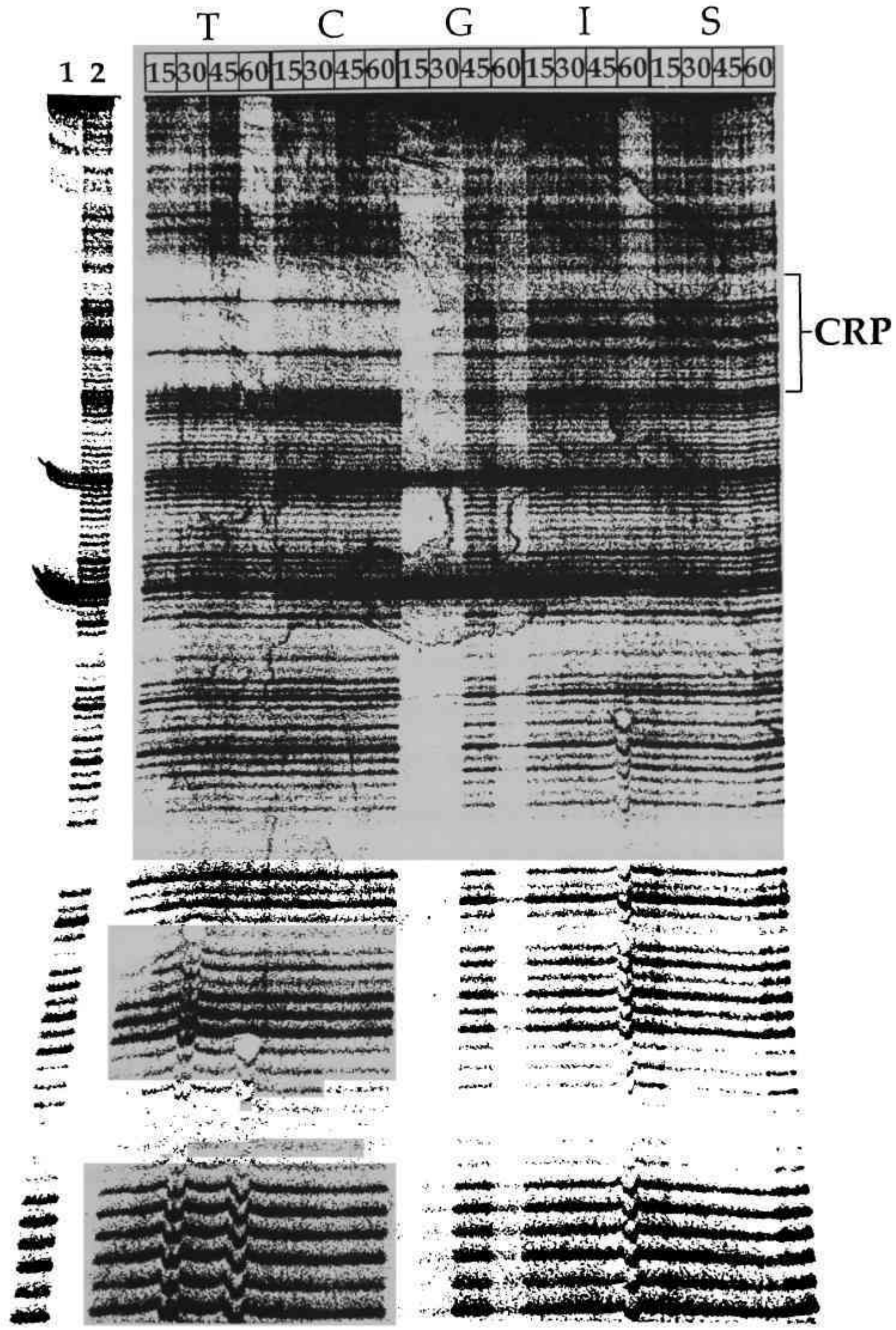

I
II
1 2
T C G I S
T C G I S
T C G I S
CRP
RNAP


IV
1 2
T C G I S
CRP
RNAP


I
II
III
1 2
T C G I S
T C G I S
T C G I S
CRP
RNAP


100 nM
500 nM
I
II
I
II
1
T C G I S
T C G I S
T C G I S
T C G I S


T
C
G
I
S
1 2
15 30 45 60 15 30 45 60 15 30 45 60
1 2
15 30 45 60
15 30 45 60


T C G I S
1 2
15 30 45 60 15 30 45 60 15 30 45 60 15 30 45 60 15 30 45 60
CRP

CHAPTER IV

DISCUSSION

Analysis of CRP:$(cAMP)_2$ crystal structure identified five specific amino acid-ligand contacts that are important in contacting cAMP and/or mediating transcription activation of *lac* operon; E72, R82, S83, T127 and S128 (21). The effects of specific amino acid substitutions at these positions in CRP were studied (22,23). Position 72 and 82 mutants were not active *in vivo* in the presence of cAMP and had low cAMP binding affinities compared to WT CRP (22). Position 83, 128 mutants were active *in vivo* in the presence of cAMP and had cAMP binding affinities similar to WT CRP (23). Position 127 mutants activated *lac*P at different levels *in vivo* in the presence of cAMP and their cAMP binding affinities were not significantly different from that of WT CRP (23). Substitutions made at CRP position 127 had unique structural effects on CRP in that they produced proteins which, in the absence of cAMP, displayed protease sensitivities similar to that of the WT CRP:cAMP complex. This indicated that T127 plays a role in maintaining the protease-resistant structure of CRP (23).

The objective of the work described here was to evaluate the stage at which position 127 substituted forms of CRP (T127C, T127G, T127I and T127S) are limited in *lac* transcription activation. The methods used were: (1) gel shift and DNase I footprinting assays to assess T127 CRP mutant binding to DNA; and (2) transcription assays to assess T127 CRP mutant function in mediating *lac*P transcription activation *in vitro*. The results of experiments conducted on the T127 mutant forms of CRP in the presence or absence of

cAMP are compared to those obtained for WT CRP or the WT CRP:cAMP complex. The following set of conclusions have been drawn.

1. WT CRP in the absence of cAMP:
   - has no apparent affinity for non-specific DNA sequences (37; Figure 3.18 of this work),
   - has no apparent affinity for the *lac*P CRP-binding sequence (37; Figures 3.11, 3.14, 3.15, 3.17, 3.19 of this work),
   - does not interact with RNAP in solution in the absence of DNA (52),
   - does not promote the interaction of RNAP and *lac*P DNA (54, 55; Figures 3.15, 3.16, 3.17 of this work),
   - does not active *lac*P (23; Figure 3.7 of this work).
2. WT CRP in the presence of cAMP:
   - has no apparent affinity for non-specific DNA sequences (37; Figure 3.18 of this work),
   - has high affinity for the *lac*P CRP-binding sequence (37; Figures 3.11, 3.14, 3.15, 3.17, 3.19 of this work),
   - interacts with RNAP in solution in the absence of DNA (52),
   - promotes the interaction of RNAP and *lac*P DNA that results in the rapid formation of open complexes *in vitro* (54, 55; Figures 3.9, 3.15, 3.17 of this work),
   - activates *lac*P to high levels (23; Figures 3.3, 3.7, 3.9 of this work).

In contrast to the results obtained with WT CRP, the position 127 substituted forms of CRP showed different characteristics with respect to DNA binding and *lac*P activation. These results are sumarized below.

1. T127C CRP in the absence of cAMP:
   - has affinity for non-specific DNA sequences (Figure 3.18),

- has no apparent specific affinity for the *lac*P CRP-binding sequence (Figures 3.15, 3.17),
- does not promote the interaction of RNAP and *lac*P DNA (Figure 3.16),
- does not active *lac*P *in vivo* (23) or *in vitro* (Figure 3.8).

2. T127G CRP in the absence of cAMP:
   - has affinity for non-specific DNA sequences (Figure 3.18),
   - has no apparent specific affinity for the *lac*P CRP-binding sequence (Figures 3.15, 3.17),
   - does not promote the interaction of RNAP and *lac*P DNA (Figure 3.16),
   - does not active *lac*P *in vivo* (23) or *in vitro* (Figure 3.8).
3. T127I CRP and T127S CRP in the absence of cAMP:
   - have no apparent affinity for non-specific DNA sequences (Figure 3.18),
   - have no apparent specific affinity for the *lac*P CRP-binding sequence (Figures 3.15, 3.17),
   - do not promote the interaction of RNAP and *lac*P DNA (Figure 3.16),
   - do not active *lac*P *in vivo* (23) or *in vitro* (Figure 3.8).
4. T127C CRP in the presence of cAMP:
   - has affinity for non-specific DNA sequences (Figure 3.18),
   - has high specific affinity for the *lac*P CRP-binding sequence (Figures 3.15, 3.17)
   - promotes the interaction of RNAP and *lac*P DNA that results in the rapid formation of productive open complexes *in vitro* (Figures 3.9, 3.15, 3.17),
   - activates *lac*P to high levels *in vivo* (23) and *in vitro* (Figures 3.3, 3.8, 3.9).
5. T127G CRP in the presence of cAMP:
   - has affinity for non-specific DNA sequences (Figure 3.18),

- has no apparent affinity for the lacP CRP-binding sequence (Figures 3.15, 3.17)
- promotes the interaction between RNAP and lacP DNA but does not promote productive open complex formation in vitro (Figures 3.9, 3.15, 3.17),
- does not activate lacP in vivo (23) or in vitro (Figures 3.3, 3.8, 3.9).

6. T127I CRP and T127S CRP in the presence of cAMP:
   - have no apparent affinity for non-specific DNA sequences (Figure 3.18),
   - have no apparent affinity for the lacP CRP-binding sequence (Figures 3.15, 3.17),
   - promote the interaction of RNAP and lacP DNA that results in a slow and relatively non-productive formation of open complexes in vitro (Figures 3.9 3.15, 3.17),
   - do not activate lacP to high levels in vivo (23) or in vitro (Figures 3.3, 3.8, 3.9).

Phenotypically, there are three classes of mutant CRP: (1) those that are CRP^+ and activate CRP-dependent promoters in the presence of cAMP, (2) those that are CRP^- and fail to activate CRP-dependent promoters in the presence of cAMP, and (3) those that are CRP* and activate CRP-dependent promoters in either the absence or presence of cAMP. Members of the CRP* class of CRP mutant frequently show relaxed cyclic nucleotide specificity in promoter activation (20, 47).

As a group, position 127 CRP mutants do not fit any of the CRP mutant classes. T127C CRP may be classified as a CRP^+; however, this mutant protein showed the altered effector specificity of a CRP* (23). T127G CRP clearly showed a CRP^- phenotype while the T127I CRP and T127S CRP had phenotypic characteristics (i.e., altered effector specificity, reduced cAMP-

dependent activity [23; Figure 3.9]) that do not readily fit the CRP$^+$, CRP$^-$ or CRP* classification.

Ebright and co-workers recently succeeded in isolating a fourth class of CRP mutant that is in many respects similar to CRP$^-$ (57). These mutants failed to activate a CRP-dependent promoter in the presence of cAMP yet repressed the synthesis of mRNA from a promoter that contained a downstream CRP binding site (57). *In vitro* studies showed that these CRP mutants bound cAMP, bound to DNA that contained a CRP binding site in the presence of cAMP yet failed to interact with RNAP (50, 52, 56-59). These mutants were classified as CRP^{pc} where pc denotes a defect in the positive control of transcription. In many respects the position 127 CRP mutants are a type of CRP^{pc}.

CRP activation is a complex process involving not only the binding of cAMP to CRP but also the interaction of CRP with DNA and RNAP. Two changes in CRP structure must occur upon cAMP that lead to CRP activation; exposure or reorientation of the site-specific DNA binding helicies (30) and exposure or reorientation of the RNAP interaction domain (19). The original CRP^{pc} mutants isolated by Zhou et al. (57) were shown to contain amino acid substitutions in a carboxy-proximal surface loop of CRP that specifically interacts with RNAP. This loop is necessary for CRP:RNAP interaction and CRP-dependent promoter activation. The changes in CRP^{pc} structure upon binding cAMP induce apparently normal site-specific DNA binding and, perhaps, normal exposure or reorientation of a defective RNAP interaction domain.

Position 127 is located in the cAMP binding pocket. Substitutions at position 127 affect CRP structure but do not in and of themselves lead to the activation of CRP in the absence of cAMP. The results presented in Figures

3.17 and 3.18 illustrate that the substitution of threonine 127 by either cysteine or glycine (but not isoleucine or serine) produced forms of CRP that showed non-sequence specific affinity for DNA.

When bound by cAMP the position 127 mutants undergo a conformation change that lead to the reorientation of the DNA binding helicies in a manner that was dependent upon the nature of the mutation; T127C CRP:cAMP complex bound *lac*P whereas the T127G, I and S forms of CRP:cAMP complex did not (Figures 3.15, 3.17). Without exception, cAMP binding to the position 127 mutants lead to the exposure or reorientation of the RNAP interaction domain which, in turn, was sufficient to promote the formation of ternary complexes at *lac*P in the presence of cAMP and RNAP (Figures 3.15, 3.17); However, RNAP alone with 127 position mutants did not promote formation of ternary complex (Figure 3.16). The activity of these complexes was again dependent upon the nature of the mutation at position 127; complexes that contained T127C CRP were fully active, complexes that contained either the T127I CRP or the T127S CRP were only partially active, and complexes that contained T127G were inactive. One or more component(s) important to the *lac*P activation process at the ternary complex stage of promoter activation was either completed for ternary complexes that contained the T127C CRP:cAMP complex, was only partially completed for ternary complexes that contained either the T127I or T127S CRP:cAMP complexes, or was not completed for ternary complexes that contained the T127G CRP:cAMP complex. Considering all of the data presented here it appears that the position 127 CRP mutants represent a new category of CRP^{pc} mutant.

One additional conclusion can be drawn from the results presented here. Allosteric changes induced in CRP structure upon the binding of cAMP

include specific reorientation of the DNA binding helicies and exposure or reorientation of the RNAP interaction domain. Clearly the amino acid at position 127 affects the proper reorientation of the DNA binding helicies while it does not affect functional exposure or reorientation of the RNAP interaction domain. This suggests that one or more of the interactions between cAMP and E72, R82, S83, and S128 are sufficient for functional exposure or reorientation of the CRP RNAP interaction domain.

CHAPTER V

SUMMARY

The results of the characterization of position 127 CRP mutants are summarized as follows: (1) In the absence of cAMP, the WT CRP did not show non-specific DNA interaction; the T127C CRP and the T127G CRP showed stable non-specific DNA interactions; the T127I CRP showed unstable non-specific DNA interaction; a non-specific DNA interaction was not observed in reactions that contained the T127S CRP; (2) The presence of cAMP induced specific CRP-binding site recognition in reactions that contained the WT CRP and the T127C CRP but not in reactions that contained either the T127G CRP, the T127I CRP or the T127S CRP; (3) The presence of cAMP and RNA polymerase caused allosteric changes in CRP that matured the DNA and RNA polymerase recognition surfaces of the T127G CRP, the T127I CRP and T127S CRP; (4) In the presence of cAMP, the WT CRP and the T127C CRP fully activated *lac*P; whereas the T127I CRP and the T127S CRP activated *lac*P at an intermediate level; the T127G CRP did not active *lac*P.

Three conclusions were drawn from this study. First, the amino acid residue at position 127 affects the reorientation of the DNA binding helicies. Second, the amino acid residue at position 127 does not affect functional exposure or reorientation of the RNAP interaction domain. Third, one or more of the interactions between cAMP and E72, R82, S83, and S128 are sufficient to promote the functional exposure or reorientation of the CRP RNAP interaction domain.

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