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Structure and function of a mutant of the cyclic AMP receptor protein: CRP*598

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STRUCTURE AND FUNCTION OF A MUTANT OF THE CYCLIC AMP RECEPTOR PROTEIN: CRP'598

by

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ABSTRACT

STRUCTURE AND FUNCTION OF A MUTANT OF THE CYCLIC AMP RECEPTOR PROTEIN: CRP*598

by

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The cAMP receptor protein (CRP) acts to modulate the expression of a large number of *E. coli* genes. Binding of cAMP involves a conformational change in CRP with a resultant increase in the affinity and specificity for specific promoter-associated sites; unliganded CRP binds nonspecifically to DNA. CRP is composed of two identical subunits. The CRP monomer has a two-domain structure in which the large N-terminal domain is responsible for cAMP binding and subunit-subunit interaction and the smaller C-terminal domain is involved in DNA binding. One CRP mutant, CRP*598 (Arg-142 to His, Ala-144 to Thr), has been characterized with regard to its conformational properties and ability to bind to and support abortive initiation from the *lac* promoter. In the absence of cAMP, CRP*598 shows a more open conformation than CRP, as indicated by its sensitivity to proteolytic attack and by 5,5'-dithiobis(2-nitrobenzoic acid)-mediated subunit crosslinking. CRP*598 can activate *lac* P^+-directed abortive initiation in the presence of cAMP and less

efficiently in the presence of cGMP or in the absence of cyclic nucleotide. DNase I protection indicates that cAMP-CRP*598 and cGMP-CRP* form a stable complex with the [^{32}P]*lac* P^{+} fragment only in the presence of RNA polymerase, showing cooperative binding of two heterologous proteins. This cooperative binding provides strong evidence for a contact between CRP and RNA polymerase for activation or transcription. The binding of cAMP and cGMP to CRP and CRP*598 have been determined. The results indicate that the affinity of CRP and CRP*598 for cGMP is relatively unchanged while the affinity of CRP*598 for cAMP is approximately twenty times greater than that shown by CRP. Binding of cAMP by CRP and cGMP by CRP or CRP*598 exhibits slight negative cooperativity. The major difference seen is that CRP*598 binds cAMP with strong positive cooperativity. The positive cooperativity observed for binding of cAMP by CRP*598 may be a consequence of effects modulated by altered subunit contacts and/or interdomain contacts. Three anti-CRP*598 mAbs have been characterized. The dissociation constants for anti-CRP and anti-CRP*598 mAbs to CRP and CRP*598 are determined in the presence and absence of cyclic nucleotides. The results indicate that N^{6}-butyryl-cAMP induces conformational change of CRP/CRP*598 different from cAMP.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations used:

ApA: adenylyl (3', 5') adenosine

BB: blocking buffer

BSA: bovine serum albumin

cAMP: 3', 5'-cyclic AMP

cGMP: 3', 5'-cyclic GMP

CRP: Cyclic AMP receptor protein

Da: daltons

DMEM: Dulbecco's Modified Eagle's Medium

DTNB: dithionitrobenzoic acid

DTT: dithiothreitol

EDTA: (ethylenedinitrilo) tetra-acetic acid

ELISA: enzyme-linked immunosorbent assay

kDa: kilo-daltons

lac P^+: *lac* wild type promoter

lac: lactose operon

mAb: monoclonal antibody

NEM: N-ethylmaleimide

PBS: phosphate buffered saline

PEG: polyethylene glycol

PMSF: phenylmethanesulfonylfluoride

RP_o: open initiation complex

SDS: sodium dodecyl sulfate

TCA: trichloroacetic acid

Tris: Tris-(hydroxymethyl)aminomethane

WB: washing buffer

INTRODUCTION

The mechanism of transcription activation has been one of the most predominant questions for the last fifty years since the finding of catabolism inhibition by glucose (Epps & Gale, 1942; Monod, 1947). The cyclic AMP receptor protein (CRP), also referred to as the catabolite gene activator protein (CAP), is one of the regulatory proteins involved in the regulation of transcription in *E. coli.* The following is a study about the relation of structure to function of CRP.

CRP is a dimer of 45,000 Da composed of two identical subunits with 209 amino acid residues in each (Anderson *et al.*, 1971; Aiba *et al.*, 1982; Cossart & Gicquel-Sanzey, 1982). The complete amino acid sequence has been deduced from the nucleotide sequence of the cloned CRP gene (Figure 1; Aiba *et al.*, 1982). Like several other DNA binding regulatory proteins, the CRP monomer has a two-domain structure. The larger N-terminal domain is responsible for cAMP binding. It 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 is involved in DNA binding (Krakow & Pastan, 1973; McKay *et al.*, 1982). It shows structural (Steitz *et al.*, 1982) and sequence (Matthew *et al.*, 1982; Sauer *et al.*, 1982; Weber *et al.*, 1982) homologies with several other gene regulatory proteins such as *cro* repressor, cI, and *lac* repressor.

The structure of the CRP-cAMP complex has been determined at 2.5 Å resolution (Figure 2; Weber & Steitz, 1987). The crystals of the CRP-cAMP complex

show an asymmetric conformation of the two subunits. In each subunit, the large domain extends from amino acid 1 to 129 and the smaller domain continues from amino acid 139 to 209. The two domains are connected by a "hinge" region which comprises amino acids 130 to 138. The relative orientation of the two domains differs in each subunit. The two domains are further apart in one subunit than in the other, resulting in a more "open" conformation for one subunit and a more "closed" conformation for the other. The major feature of each N-terminal domain of CRP is the antiparallel ß roll structure that includes residues 19 to 99. This is a compact structure with very few excursions from the eight hydrogen-bonded ß-strands.

cAMP is bound between the two long C α-helices and the pocket formed by residues from the ß-roll. The two molecules of cAMP are deeply buried within the CRP dimer in an anti conformation. Each part of the cAMP molecule makes specific interactions with the protein and is stabilized by hydrogen bonding and nonpolar interactions. The phosphate group of cAMP interacts with Arg-82 and Ser-83; the ribose 2'- OH forms hydrogen bond interactions with Glu-72 and Gly-71. The adenine 6-amino group forms hydrogen bonds with the OH of Thr-127 from the C helix in the same subunit, and the OH of Ser-128 from the C helix in the other subunit (Weber & Steitz). This arrangement provides an opportunity for a cooperative binding of 2 cAMP molecules per CRP dimer (Takahashi *et al.*, 1980). The interactions between CRP and cAMP are also in good agreement with those from the binding of various cAMP analogs (Anderson *et al.*, 1972; Scholubbers *et al*, 1984).

Many intersubunit interactions occur between the two long C helices, because they are in contact with each other for most of their length. There are three hydrogen bonds formed symmetrically between the two subunits. Two of them are between the 6-amino groups of the two cAMP molecules and the side-chains of Ser-128 of the adjacent subunits; and the other one is between the two Ser-117 side-chains. The interaction between cAMP and both C helices suggests that subunit interactions may play an important role in the allosteric effect of cAMP binding.

The "hinge" region has distinctly different conformations in the two subunits. There are hydrogen bonds between: Leu-134 C=O and Arg-142 NH_2 of the D helix, and Asn 133 NH_2 and Gln 145 OH of the D helix. The side-chains of the D helix and the hinge tend to "pin" the D helix closer to the hinge. There are also other non-covalent interdomain interactions between ß-5 and helix E.

The most interesting characteristic of the DNA binding domain of CRP is a helix-turn-helix structure which has been observed in a large number of bacterial and viral gene regulatory proteins (Matthew *et al.*, 1982; Sauer *et al.*, 1982; Weber *et al.*, 1982). The F α-helices are exposed on the surface of the CRP dimer and their axes run approximately parallel to each other. Several polar side-chains from the F α-helices are available for interaction with DNA. Arg 169 from the E helix and Arg 180, Arg 185 and Lys 188 from the F helix, are positively charged and contribute to the electrostatic attraction of CRP and DNA.

Although the conformation of CRP in the absence of cAMP has not been determined, various lines of evidence indicate that CRP undergoes a conformational

transition on binding cAMP (Krakow & Pastan, 1973; Eilen *et al.*, 1978; Wu *et al.*, 1978; Kumar *et al.*, 1980; Eilen & Krakow, 1977; Ebright *et al.*, 1985). The sensitivity of CRP to proteases is much higher in the presence of cAMP (Eilen *et al.*, 1978); dithiobis-(2-nitrobenzoic acid) mediated cross-linking between Cys 178 of each subunit is remarkably enhanced in the presence of cAMP (Eilen & Krakow, 1977). Small-angle X-ray scattering experiments detect a decrease of 4.0 Å in the radius of gyration of CRP upon binding to cAMP (Kumar *et al.*, 1980). In the crystal structure, cAMP is bound deeply within the CRP dimer. Therefore it cannot enter its binding site without rearrangement of the protein.

Another change of CRP elicited by the binding of cAMP is an increased affinity for specific DNA sites (Weber & Steitz, 1984; Takahashi *et al.*, 1979) which will be discussed later.

A group of cAMP analogs has been identified with regard to binding to CRP, induction of the conformational charge in CRP, and activation of transcription (Ebright *et al.*, 1985). These analogs with monosubstitutions at the N-6 or C-2 positions in the purine bind to CRP with a comparable affinity as cAMP and produce the same effects on proteolysis and disulfide cross-linking. However, they fail to stimulate binding to DNA and to activate transcription *in vitro*. cAMP must therefore induce additional conformational changes which this group of analogs is unable to produce.

In *E. coli*, CRP acts to modulate the expression of a large number of *E. coli* genes (Adhya & Garges, 1982; Ullman and Dauchin, 1983; and de Crombrugghe *et*

al., 1984) with a resultant increase in the affinity and specificity for specific promoter-associated sites. Unliganded CRP binds nonspecifically to DNA. The specific DNA sites bound by the CRP-cAMP complex are located at or near a transcription start site and acts to modulate transcription initiation. The different CRP binding sites on promoters which are regulated by CRP have been characterized (Crombrugghe *et al.*, 1984). The CRP binding regions are located upstream of the transcription start point. The size of the DNA fragment protected by CRP against deoxyribonuclease attack is approximately 25 base pairs (Simpson, 1980; Taniguchi *et al.*, 1979; Ogden *et al.*, 1980; Lee *et al.*, 1981). The distance between the transcription start point and the CRP binding site is different for different promoters (Crombrugghe *et al.*, 1984). At the *lac* promoter, CRP binds the sequence between -50 to -75 base pairs. Within the CRP binding sites, the 5'TGTGA 3' sequence is critical and highly conserved between different sites. In the case of *lac*, a second sequence, less well conserved, containing an inverted repeat of the TGTGA motif is located 6 bp downstream of the TGTGA motif, generating a twofold symmetry in the DNA sequence. Because one CRP dimer binds at each *lac* promoter (Garner & Revzin, 1982) it is likely that the subunits of the dimer are arranged in such a way that they each recognize one of the two symmetrically arranged elements at each binding site.

CRP belongs to a superfamily of DNA binding proteins which recognize a DNA sequence using the helix-turn-helix motif. In this way, the E helices are perpendicular to the DNA backbone. The F helices protrude out in parallel, being separated by about 34 Å, and penetrate two successive turns of the major groove of

the right handed ß-DNA on the same surface. A model for the DNA-CRP complex first was proposed by McKay *et al.* (1981,1982). It was suggested that because the relative orientation of the two F helices is complementary to two successive major grooves of left-handed ß-DNA, CRP binds to left-handed ß-DNA. If the DNA at the CRP target site was left-handed, the binding of cAMP-CRP to closed circular DNA should greatly change its linking number when the DNA is nicked. In fact, this was not found (Kolb & Buc, 1982). Therefore, an alternative model was constructed in which right handed ß-DNA was oriented on the helix-turn-helix of CRP by electrostatic complementarity (Steitz *et al.*, 1982; Weber & Steitz, 1984). This model proposed that the N-terminal part of the F α-helices enters two successive major grooves of the right handed-DNA in a way that is similar to the interaction of helix "3" of the lambda repressor. For the lambda cI repressor the orientation of helix "3" is thought not to be exactly parallel to the path of the major groove. The two symmetrical helices "3" of the cI dimer cannot be fit completely in two successive major grooves, mainly because the carboxyl-terminal part of these helices does 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. However, a bend in the DNA would provide an opportunity for additional contacts with the DNA backbone. Evidence for CRP-cAMP induced DNA bending in the binding site has been obtained (Kolb *et al.*, 1983; Wu & Crothers, 1984; Liu-Johnson *et al.*,1986). Since a CRP binding site of at least 28 bp is required for full affinity, it was considered that DNA is bent between 90° and

180° and CRP has a strong distal binding domain (Liu-Johnson *et al.*, 1986). Other models suggest that CRP may bend its DNA binding site by as much as 150° (Warwicker *et al.*, 1987). Most recently, co-crystallization of cAMP-CRP with 29 and 30 bp DNA sequences has been obtained (Schultz *et al.*, 1990). More details about the interaction between DNA and CRP-cAMP are expected to be forthcoming.

An interaction between CRP and DNA may change the conformation of CRP as well. For example , in the presence of calf thymus DNA or polydeoxynucleotides, CRP without cAMP becomes sensitive to trypsin (Angulo & Krakow, 1986); in contrast, the CRP-cAMP-DNA complex becomes resistant to trypsin, *Staph. aureus* V8 protease, and subtilisin (Angulo & Krakow, 1985). Takahashi *et al.* (1980) demonstrated by equilibrium dialysis that calf thymus DNA changes cAMP binding from negative cooperativity to positive cooperativity and increases the affinity of cAMP for CRP. These experiments suggest that DNA binding to the C-terminal domain could induce an allosteric effect on CRP. It is assumed that there is a second conformational change when CRP-cAMP binds to DNA. This change may be required for CRP to activate transcription (Adhya & Garges, 1990).

The mechanism of the transcription activation caused by the binding of CRP-cAMP to DNA is not yet clear. However, the following effects are considered to be related to CRP-cAMP binding and transcription initiation: first, in the presence of the CRP-cAMP complex, the affinity of RNA polymerase to the *lac* promoter is increased by 20-fold, in terms of the K_B, the association constant of the formation of the closed promoter, than in the absence of the CRP-cAMP complex (Malan *et al.*,

1984). the CRP-cAMP complex also increases the occupancy of RNA polymerase on the promoter by blocking competing and overlapping promoter sites (Hawley *et al.*, 1982; McClure *et al.*, 1982); second, CRP-cAMP induces bending in DNA; third, a physical interaction between the CRP-cAMP complex and RNA polymerase occurs when they act on the *lac* promoter. The interaction between the two heterologous proteins results in cooperative binding that has been demonstrated as following: I, In a mutant *lac* promoter (L8, UV5), in which the CRP binding site is weakened but formation of the RP_o can occur in the absence of CRP, CRP-cAMP forms a stable complex with DNA only in the presence of RNA polymerase (Li & Krakow, 1988). II, In the wild type *lac* promoter, neither RNA polymerase nor a cAMP-independent CRP* mutant (CRP*598) binds to DNA separately, but both bind normally when present together (Ren *et al.*, 1988). III, RNA polymerase in its primary complex with CRP-cAMP and the *lac* promoter confers stabilization of CRP against dissociation from the DNA compared with the CRP-cAMP-*lac* promoter complex lacking RNA polymerase. Straney *et al.*(1989) showed that the extra stabilization by RNA polymerase is lost if half a helical DNA turn is inserted between the CRP binding site and RNA polymerase binding site and is partially restored if a full DNA turn is introduced. Presumably a half-helical turn distorts the angular orientation between bound CRP and RNA polymerase favorable for contact whereas a full turn restores the contact (Straney *et al.*, 1989). A direct interaction between CRP and RNA polymerase has been suggested by co-sedimentation during ultracentrifugation (Blazy *et al.*, 1980); the binding K_D between CRP-cAMP and RNA polymerase is about 3

x 10^{-6} M under physiological conditions without DNA present shown by using of fluorescein-labeled CRP (Pinkney & Hoggett, 1988).

Construction of CRP mutants is another approach to study the relation of structure to function of CRP. CRP* mutants which allow the mutant CRP to function in the absence of endogenous cAMP have been isolated and characterized by several groups (Sander & McGeoch, 1973; Dessein *et al.*, 1978; Harman & Dobrogoz, 1983; Garges & Adhya, 1985; Aiba *et al.*, 1985; Blazy & Ullmann, 1986). Since most of these mutations are at the interface between the two domains of the CRP monomer, Garges and Adhya (1985) proposed that domain-domain reorientation is part of the conformational change caused by cAMP binding. cAMP aligns the subunits of a CRP dimer by contacting the large domains of both subunits at the middle of α-helix C. These contacts transmit a change to residues at the end of α-helix C which interact with residues at the amino terminus of α-helix D in the small domain. This alters the relative orientation of the large and small domains by changing the angle between α-helices C and D. This combination of motions ultimately orients the two α-helices F to form the proper specific DNA binding surface. The CRP* mutant partially mimic this conformation, because of the introduction of bulkier side chains of the residues on α-helix D that reorient the α-helices in approximately the right way. The enhancement of the activity of CRP* by cAMP or cGMP suggests that a further conformational change is necessary to complete the allosteric transition.

One of the CRP* mutants, CRP*91, in which Ala-144 is substituted by Thr-144 has been crystallized with cAMP under the conditions as used to crystallize the wild

type CRP-cAMP complex (Weber *et al.*, 1987). Analysis of X-ray diffraction data of CRP*91 at 2.4 Å resolution indicated that the structures with bound cAMP of both wild type and mutant are very similar overall. However, small changes associated with the DNA-binding domains have been found. Concerted movements occur in the residues of the hinge between the two domains of the closed subunit, in the adjacent loop of ß-4 to ß-5 in the open subunit, and in the turn between the DNA-binding E and F helices in both subunits. Furthermore CRP*91 exhibits half-maximal activation of transcription at a concentration of cAMP that is approximately 40-fold lower than required by wild type (Harman *et al.*, 1986). Unlike wild type CRP, CRP*91 also can be activated by adenosine although the concentration of adenosine required is 20,000 times greater than that of cAMP (Vaney *et al.*, 1989). The crystal structure of CRP*91 with one adenosine substituting for one of the two cAMP binding sites (CRP*91A) has been determined by Vaney *et al.* (1989). Differences observed between CRP*91 and CRP*91A are of the same magnitude as the differences between CRP*91 and wild type CRP (Weber *et al.*, 1987).

The mutations exhibiting a CRP* phenotype occur widely in different parts of the CRP molecules. Substitutions in the 4, 5, 6, 7, 11 ß-strand and C, D, E α-helix have been reported (Garges & Adhya, 1985; Aiba *et al.*, 1985; Harman *et al.*, 1986). Many occur in the D-helix (residues 140-151), such as residues 141, 142, 144, and 148, suggesting that this helix is apparently important for activation of CRP by cAMP. The location of D-helix near the hinge also suggests that the D-helix plays a role in allosteric change when cAMP binds to CRP, since the Ser-128 and Thr-127 in C-

helices that precede the hinge form hydrogen bonds with cAMP. More interestingly, when other mutations (Thr-127 to Ala or Arg-169 to Cys, Glu-171 to Gly) are introduced into CRP* 141 or CRP* 144 mutants, these cAMP-independent mutants become cAMP-dependent (Garges & Adhya, 1988).

Irwin and Ptashne (1987) have isolated another group of CRP mutants (Gln-170 to Lys; Glu-171 to Lys; Glu-171 to Gln) that are analogous to the λ repressor positive control (pc) mutants. These CRP mutants bind DNA but are defective in stimulating transcription at the *gal* P_1 promoter. These mutants are also altered in positive control at the *lac* and *malT* promoters. Like λ repressor, they suggested, the surface of CRP that would most likely contact RNA polymerase at the *gal* P_1 promoter is in the E α-helix. The contact of the two proteins would facilitate formation of RP_o.

Mutations in the cAMP binding site or DNA binding site of CRP have also been studied (Gronenborn *et al.*, 1988; Ebright *et al.*, 1987; 1990; Zhang & Ebright, 1990). The Arg-82 → Leu mutant does not bind cAMP at all; Ser-83 → Ala, Ser-83 → Lys, Thr-127 → Ala or Ser-128 → Ala mutants bind cAMP similarly to the wild type; Ser-67 → Ala mutant binds cAMP better than the wild type (Gronenborn *et al.*, 1988). This study gives information about functional amino acids in the cAMP binding "pocket". Mutation of Glu-181 (Ebright *et al.*, 1987) and Arg-180 (Zhang & Ebright, 1990) in the F α-helix alters the DNA sequence specificity of CRP. Lys-188 does not directly contact the DNA binding site (Ebright *et al.*, 1990).

Although CRP has been studied extensively based on biochemical, genetic, and X-ray crystallographic analyses, an understanding of the relation between the

structure of cAMP-CRP-promoter complex and mechanism of transcription activation are still lacking. The molecular nature of cAMP induced activation of CRP remains a question since the crystal structure of unliganded CRP or CRP mutants are not available. It is assumed that DNA bending participates in transcription activation by facilitating additional protein-DNA or protein-protein contacts required for the initiation of transcription or that bending provides an additional DNA site that could contact with RNA polymerase to stimulate transcription (Plaskon & Wartell, 1987; Bracco *et al.*, 1989). However, these hypotheses need to be proved.

The study of cAMP induced transcription activation involves a variety of basic topics, such as protein-protein interactions, protein-DNA interaction, protein conformation, and DNA conformation. For each step of transcription initiation, there are still many biochemical details to be explored.

In this dissertation, one CRP mutant, CRP*598 (Arg-142 to His, Ala-144 to Thr) was characterized with regard to its conformational properties, ability to bind to and support abortive initiation from the *lac* promoter, and the nature of cyclic nucleotide binding. Effects of anti-CRP or anti-CRP*598 monoclonal antibodies on functions of both type of CRP were investigated. The relation of conformation and function is discussed.

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: cAMP, cGMP, adenosine, 8-bromo cAMP, 8-methylamino cAMP, N^6-butyryl cAMP, N^6,$O^{2'}$-dibutyryl cAMP, cIMP, cCMP, 2'-deoxy cAMP, 2'-deoxy cGMP, 5'-AMP, ATP, Adenylyl(3'-5')adenosine, bovine serum albumin, Proteases, calf thymus DNA, 4-methylumbelliferyl ß-D-galactopyranoside, 5-bromo-4-chloro-3-indolyl-phosphate, heparin, pronase, nitro blue tetrazolium, PMSF, ß-mercaptoethanol, N-ethylmaleimide, dithionitrobenzoic acid, lysozyme, p-nitrophenylphosphate, toluidine blue, DNase I and Freund's complete and incomplete adjuvant, Sigma; [^{3}H]-cAMP, [^{3}H]-cGMP, [^{3}H]-UTP, [^{32}P]dATP, and Ecolume, ICN; DNA polymerase I Klenow fragment, *Eco*RI and *Pvu*II restriction endonucleases, Boehringer Mannheim; ZetaChrom 100 capsule SP, CUNO, Inc. (Meriden. CT); Sephacryl 200, S Sepharose Fast Flow, Pharmacia; polymin P, Gallard Schlessinger (Carle Place, NY); N,N,N'N'-teramethylethylenediamine, and bisacrylamide, Bio-Rad; acrylamide, Serva (Heidelberg); goat-anti-mouse-IgG-ß-galactosidase, Hyclone; goat-anti-mouse-IgG-phosphatase, Kirkegaard & Perry Laboratories Inc; non-fat milk, Carnation Company; Tween 80, J. T. Baker Chemical Co.; fetal calf serum, Sterile Systems Inc.; Dulbecco,s modified Eagle's medium, GIBCO; PEG 4000, E. Merck Inc.; Protein A-Sepharose, Pharmacia; PolyBlot, ABN; MicroFluoro™ "B" flat-bottom plates, Dynatech Laboratories, Inc; mice were obtained from Jackson Laboratories.

Proteins. CRP was purified from an *E. coli* strain containing the recombinant plasmid pHA7 (Aiba *et al.*, 1982) donated by H. Aiba (University of Tsukaba, Ibaraki, Japan). CRP*598 was purified from an *E. coli* strain containing the recombinant plasmid pZ598 (Garges & Adhya, 1985). CRP and CRP*598 were purified by the method of Eilen *et al.* (1978) with the following modifications. After lysis, polymin P addition and centrifugation, the supernatant is adjusted to pH 6.5 with 1 M acetic acid and loaded onto a ZetaChrom SP-100 capsule (equilibrated with 50 mM sodium phosphate (pH 6.5), 0.1 M DTT, 0.1 mM PMSF and 5% glycerol). After washing the capsule with the equilibration buffer, CRP or CRP*598 is eluted with 300 mL of 50 mM sodium phosphate (pH 7.5), 0.5 M NaCl, 0.1 mM DTT, 0.1 mM PMSF, 5% glycerol. Fractions of 10 mL are collected and assayed for [^{3}H]cAMP binding. The most active fractions are pooled and precipitated by addition of ammonium sulfate to 60% saturation at pH 6.8. After 30 minutes (or overnight) the protein is collected by centrifuging at 12,000 rpm for 20 minutes and the precipitate is dissolved in 5 mL of 20 mM sodium phosphate (pH 6.8), 0.1 mM DTT and 5% glycerol. Chromatography on Sephacryl S-200 is carried out as given in Eilen *et al.* (1978). Fractions containing CRP are pooled and precipitated with ammonium sulfate (60% saturation at a pH of 6.8-7.0). After 30 minutes (or overnight) the protein is collected by centrifuging at 12,000 rpm for 20 minutes. The precipitate is dissolved in 100 mL of buffer A: 20 mM sodium phosphate (pH 6.8), 0.1 mM DTT, 1 mM EDTA and 5% glycerol and loaded onto a column of S-Sepharose Fast Flow (bed volume = 60 mL) equilibrated with Buffer A. After washing the column with

100 mL of Buffer A, a linear gradient is run with 400 mL Buffer A and 400 mL Buffer A + 0.5 M NaCl. Before pooling, the purity of the fractions containing CRP or CRP* is assessed by SDS-polyacrylamide gel electrophoresis. The concentration of CRP and CRP* was determined using the following extinction coefficient: $A^{1\%}_{278nm}$ = 9.2 (Takahashi *et al.*, 1980).

RNA polymerase was isolated form *E. coli* K12 by a modification of the method of Burgess and Jendrisak (1975). RNA polymerase holoenzyme concentration was determined using the following extinction coefficient: $E^{1\%}_{280nm}$ = 6.7 (Levine *et al.*,1980)

DNA Fragments. *E. coli* containing the *lac* P^+ promoter cloned into pMB9 by Dr. S. Fuller was obtained from Dr. A. Revzin (Michigan State University, East Lansing). Plasmid DNA was prepared by a modification of the method of Marko *et al.* (1982). The 203 bp fragment containing the *lac* P^+ promoter was excised by digestion with *Eco*R1 and purified by polyacrylamide gel electrophoresis. DNA concentrations were determined fluorometrically using ethidium bromide by the method of Le Pecq and Paoletti (1966).

Preparation of Labeled *lac* P^+ DNA. The reaction mixture contained (final volume 50 μL): 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 100 μCi [^{32}P]dATP (600 mCi/mmol), 4 μg *lac* P^+ DNA fragment and 5 units of DNA polymerase I Klenow fragment. After 15 minutes at room

temperature, 10 μL of 0.65 mM dATP was added and the incubation was continued for an additional 15 minutes. The reaction was terminated by addition of 200 μL of a solution which contained 3 M ammonium acetate and 30 mM EDTA. The labeled DNA was precipitated by addition of two volumes of 95% ethanol and the [^{32}P]*lac* DNA was dissolved in 50 μL of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol. The labeled fragment was then restricted with 35 units of *Pvu*II to cut the DNA at -123 yielding promoter fragments uniquely labeled on the upper strand. The restricted [^{32}P]*lac* P$^+$ DNA was precipitated with ethanol and dissolved in 140 μL of TE buffer.

Abortive Initiation Assay. A modification of the abortive initiation assay of Malan *et al.* (1984) was used to determine the effect of CRP* on CRP-dependent transcription from the *lac* promoter. The reaction mixture contained (final volume 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, the indicated concentration of cAMP or cGMP, 2 nM *lac* P$^+$ DNA fragment and 40 nM RNA polymerase holoenzyme to which was added 20 nM CRP or CRP*. After incubation at 37°C for 10 minutes, 0.5 mM ApA and 50 nM [^{3}H]UTP (9200 cpm/pmol) were added. The reaction was allowed to proceed for 15 minutes at 37°C when it was terminated by addition of 10 μL 0.5 M EDTA. The radioactive products were resolved by paper chromatography in WASP solvent (McClure *et al.*, 1978). After cutting the chromatography strip into 1 cm segments the amount of ApApUpU synthesized was determined by counting appropriate segments in Ecolume.

Assays for Cyclic Nucleotide Binding. Binding of cAMP or cGMP was measured by equilibrium dialysis and ammonium sulfate precipitation methods.

(A) **Equilibrium Dialysis.** The experiments were performed essentially as described in Takahashi *et al.* (1980) using a Hoefer EMD101 apparatus. The binding assays were performed in a mixture containing: 40 mM Tris-HCl (pH 8.0), 0.4 M KCl, 1 mM DTT and 1 mM EDTA and 4 to 6 μM CRP or CRP*598. The concentration of [^{3}H]cAMP (125 cpm/pmol) was varied from 2 x 10^{-6} M to 4 x 10^{-4} M for CRP and from 2.5 x 10^{-7} M to 5 x 10^{-5} M for CRP*598. The concentration of [^{3}H]cGMP (200 cpm/pmol) was varied from 4 x 10^{-6} M to 4 x 10^{-4} M for CRP and CRP*598. The dialysis membranes (Spectro/Por 2) were boiled for 5 min in 5% (w/v) sodium bicarbonate containing 50 mM EDTA and then rinsed with deionized water. [^{3}H]cAMP solution (0.2 mL) was introduced into one half-cell and CRP or CRP* solution (0.2 mL) into the other cell. Dialysis was allowed to occur at 4°C for 12 h. Two samples (20 μL) from each half-cell were added to scintillation vials containing 5 mL of Ecolume and counted.

(B) **Ammonium Sulfate Precipitation.** The 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 2 μM CRP or CRP*598. The concentration of [^{3}H]cAMP (283 cpm/pmol) was varied from 5 x 10^{-7} M to 1 x 10^{-4} M for CRP and from 1 x 10^{-7} M to 2.5 x 10^{-5} M for CRP*598. The concentration of [^{3}H]cGMP was varied from 1 x 10^{-6} M to 1.5 x 10^{-4} M for CRP and CRP*598. After 30 min at 0°C, 0.6 mL of a solution of saturated ammonium sulfate

(pH 8.0) was added and after 30 min at 0°C the samples were centrifuged at 10,000 rpm for 5 min. The supernatant was removed by aspiration and the pellets were dissolved in 500 μL of water. Radioactivity was determined by counting in 5 mL of Ecolume. Blanks lacking CRP or CRP* were run at all cyclic nucleotide concentrations and the values were subtracted from the test samples.

(C) **Assay for examining effects of the mAbs.** The CRP/CRP*598 and antibodies were preincubated in a reaction mixture containing (final volume 50 μL): 40 mM Tris-HCl (pH 8.0), 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 100 μg casein, 0.5 μM CRP or CRP*598 and the indicated concentrations of mAbs. After 15 min at 37°C, 50 μL of mixture containing 40mM Tris-HCl (pH 8.0), 0.1 M KCl, 1mM dithiothreitol, 1 mM EDTA and 4 μM [^{3}H]-cAMP (266 cpm/pmol) was added. After 30 min at 0°C, 0.6 mL of a solution of saturated ammonium sulfate (pH 8.0) was added and after 30 min at 0°C the samples were centrifuged at 10,000 rpm for 5 min. The supernatant was removed by aspiration and the pellets were dissolved in 500 μL of water. Radioactivity was determined by counting in 5 mL of Ecolume.

Competitive Binding Assay. The incubation mixtures contained (final volume 100 μL): 40 mM Tris-HCl (pH 8.0), 400 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 μg casein, 2 μM CRP or CRP*598, 2 μM [^{3}H]cGMP (284 cpm/pmol) and the indicated analog over an appropriate concentration range. After 30 min at 0°C 600 μL of saturated ammonium sulfate was added. After an additional 10 min at 0°C the mixture was centrifuged at 10,000 rpm for 5 min. The pellet was dissolved in

500 μL H_2O and the radioactivity determined in Ecolume. The nucleotide concentrations were varied as follows: cAMP, 8-bromo cAMP, 8-methylamino cAMP, N^6-butyryl cAMP, N^6-$O^{2'}$-butyryl cAMP from 1×10^{-7} M to 1×10^{-4} M; adenosine 1×10^{-5} M to 5×10^{-3} M; 2'-deoxy cAMP, 2'-deoxy cGMP, 5'-AMP and ATP, 2×10^{-2} M.

DNase I Footprinting. DNase I footprinting was carried out using incubation conditions similar to those used for the transcription assay. The standard binding mixture contained (final volume 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol (the glycerol was added because it gave clearer protection patterns; in order to be consistent this concentration of glycerol was also included in the abortive initiation assay), 3 nM [^{32}P]*lac* P^+ fragment, 120 nM RNA polymerase holoenzyme and the indicated concentration of cAMP or cGMP and CRP or CRP*. After formation of the complexes for 10 minutes at 37°C, 1 μL of a solution containing 20 ng/mL DNase I in 20 mM potassium phosphate (pH 6.0), 1 mM EDTA and 50% glycerol was added and incubated for 30 seconds at 37°C. The reaction was terminated by added 200 μL of a solution containing 3.1 M ammonium acetate (pH 7.6), 25 mM EDTA and 63 μg/mL tRNA followed by phenol extraction, ethanol precipitation and reprecipitation. After drying the pellets under vacuum, 10 μL of loading buffer containing 80% deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol was added. The resuspended samples were loaded on an 8% denaturing sequencing gel according to Maxam and Gilbert (1980). After electrophoresis, the gel was autoradiographed

at -70°C using Kodak XAR-5 film and Cronex H-Plus intensifying screen.

Proteolytic Cleavage of CRP and CRP*598. Mixtures contained (final volume 50 μL): 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 7.5 μg CRP or CRP*598 plus 0.5 mM cAMP or cGMP as indicated. Following addition of the indicated protease, the mixtures were incubated at 37°C for the times indicated. The reactions were terminated by addition of 2.5 μL 20 mM phenylmethanesulfonyl fluoride. The resultant cleavage products were resolved by SDS-polyacrylamide slab gel electrophoresis (Laemmli, 1970) on a 15 % polyacrylamide gel with a 4.75% stacking gel.

Inter-Subunit Crosslinking. Mixtures contained (final volume 50 μL): 20 mM Bis-Tris-Propane (pH 8.0), 0.01 mM dithionitrobenzoic acid, 10 μg CRP or CRP* plus 0.1 mM cAMP or cGMP as indicated. The mixtures were incubated for 15 minutes at 30°C. After the addition of 0.1 mM N-ethylmaleimide, aliquots were added to sample buffer lacking mercaptoethanol and heated at 100°C for 2 minutes prior to electrophoresis.

Gel retardation for detecting protein-DNA Complexes. A modified gel electrophoresis method of Garner and Revzin (1981) was applied. The samples containing: 40 mM Tris-HCl (pH 8.0), 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 100 μM cAMP, 0.1 pmol ^{32}P-labelled *lac* P$^+$ and the indicated amount of CRP/CRP*598 and mAb in a volume of 15 μL were incubated for 10 minutes at 37°C.

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Prior to loading on the polyacrylamide gel 5 μL of dye mix (two parts 50% glycerol: one part 0.01% bromphenol blue in water) was added. Electrophoresis was carried out on a 7.5% polyacrylamide slab gel with a 4% stacking gel. The buffer used was: 90 mM Tris base, 90 mM boric acid, 2 mM EDTA at pH 8.0. After 1 hour electrophoresis at room temperature, DNA bands were visualized by autoradiography.

Western blotting. CRP*598 and its fragments resolved by SDS polyacrylamide gel electrophoresis were transferred electrophoretically to nitrocellulose membrane (Svendsen *et al.*, 1980) using a PolyBlot apparatus purchased from ABN. A strip was cut from the nitrocellulose membrane for staining with 0.02% toluidine blue to locate the fragment positions. Before blocking, the membrane was incubated in 0.01% heparin for 10 min and washed twice with washing buffer. The identification of antigens on the nitrocellulose membrane was performed according to the method of Johnson *et al.* (1984).

Determination of dissociation constants of antigen-antibody complexes in solution by indirect ELISA. The conditions for the determination of dissociation constants of antigen-antibody complexes were originally described by Friguet *et al.* (1985). ELISA plates (MicroFluoro "ß" flat-bottom plates) were coated with 2 μg of core enzyme in PBS overnight at 4°C. Remaining protein binding sites were blocked by incubation with 200 μL/well of blocking buffer (PBS containing 0.2% bovine serum albumin,

0.02% NaN_3) for 90 minutes at room temperature. The plates were then washed three times with the washing buffer (PBS, 0.2% BSA, 0.05% Tween 80, 0.02% NaN_3). The antigen (CRP or CRP*598) at the concentrations indicated was incubated in solution with constant concentration of the antibody at 37°C for 1 hour at 4°C overnight to reach the equilibrium. 100 μL aliquots were removed from these mixtures for binding to the plate-immobilized antigen. After the incubation of these mixture on plates for 1 hour at 37°C, the plates were washed for four times with the washing buffer. 100 μL of ß-galactosidase-coupled goat-anti-mouse immunoglobulin (1:1000 dilution in blocking buffer) was added to each well and incubated for 45 min at 37°C. After being washed for three times with the washing buffer, 100 μL of a substrate solution containing 1 mg/mL of 4-methylumbelliferyl-ß-D-galactopyranoside, 1 mM $MgCl_2$, 10mM potassium phosphate (pH 7.5) was added and the fluorescence of the product was determined with a Dynatech MicroFluor Reader.

Production of Monoclonal Antibodies. SJL/J female mice were injected with 100 μg CRP emulsified in Freund's complete adjuvant. this was followed by three booster shots of 50 μg CRP in Freund's incomplete adjuvant administered at approximately 15 day intervals. Spleens from two mice were removed three days after the final injection. Fusion of spleen cells and p3x63Ag8.653 myeloma cells (Kearney *et al.*, 1979) was carried out using a modification of the method of Oi and Herzenberg (1980). Cells fused with PEG 4000 were distributed into 96-well Costar plates containing 2 x 10^4 mouse macrophage cells per well. The microcultures were

maintained in DMEM containing 20% fetal calf serum plus hypoxanthine, aminopterin and thymidine. The production of antibodies to CRP was determined by ELISA (see below). Positive antibody-producing cultures were subcloned three times by limiting dilution in DMEM containing 20% fetal calf serum and 2 x 10^4 mouse macrophage cells per well.

Antibodies were prepared from spent media of expanded cultures (200-500 mL) grown to stationary phase. Cells were removed by centrifugation at 400xg for 10 minutes. Immunoglobulin was concentrated by precipitation with 50% saturated ammonium sulfate, pH 7. After dialysis against 50 mM Tris-HCl (pH 8.6), 150 mM NaCl, 0.02% NaN_3, antibody was purified by chromatography on Protein A-Sepharose (Ey *et al.*, 1978). Immunoglobulin-containing fractions were concentrated to a volume of about 1 mL by negative pressure dialysis (Bio-Molecular Dynamics) against 50 mM potassium phosphate (pH 7.5), 150 mM KCl, 0.05% NaN_3 (KPK buffer) and stored in KPK buffer at 0°C. Immunoglobulin concentration was determined using the extinction coefficient: $E^{1\%}_{280nm}$ = 14.6 (Ey *et al.*, 1978).

Solid Phase ELISA. Costar 96-well EIA polystyrene plates were coated with 0.5 μg CRP or the indicated fragment in 50 μL PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) by a 3 hour incubation at 37°C followed by incubation overnight at 4°C. The remaining protein-binding sites were blocked by incubation with 200 μL wash buffer (WB: PBS, 2mg/mL BSA, 0.05% Tween 80, 0.02% NaN_3) per well for 90 minutes at 37°C. The plates were then washed twice with WB. Each well then

received 50 μL of culture supernatant or 0.1 μg mAb in 50 μL PBS plus 2 mg/mL BSA and the plate was incubated for 60 minutes at 37°C. After washing three times with WB 50 μL of phosphatase-coupled goat anti-mouse immunoglobulin (1/200 dilution in PBS + 1 mg/mL BSA) was added and incubated for 60 minutes at 37°C. After washing three times with WB 100 μL of a solution containing 1 mg/mL p-nitrophenylphosphate in 0.1 M diethanolamine (pH 9.0) plus 2.5 μM $MgSO_4$ was added and incubated for 30 to 60 minutes at 37°C. After addition of 100 μL 1 M NaOH the absorbance at 410 nm in each well was determined using a Dynatech Microelisa Reader.

RESULTS

Characterization of CRP*598:

Activation of abortive initiation by RNA polymerase with the *lac* P^+ promoter requires CRP complexed with cAMP (Table I). In the absence of added cyclic nucleotide or in the presence of cGMP, wild type CRP does not affect the basal level of ApApUpU synthesized in the absence of CRP. In the presence of cAMP, maximal levels of abortive synthesis are affected by 40 nM CRP and 40 nM CRP*598. The amount of ApApUpU synthesized decreased in the reactions containing cAMP plus 400 nM CRP or 400 nM CRP*598. It is possible that this effect seen with the higher cAMP-CRP or cAMP-CRP* concentration used may be due to binding to the low affinity CRP Site 2 which overlaps the *lac* operator with a consequent inhibition of promoter binding by RNA polymerase. In the complete absence of cAMP, CRP*598 was able to effect a low level activation of *lac* expression *in vivo* (Garges & Adhya, 1985). The data presented in Table I show that unliganded CRP*598 at both a low (40 nM) and high concentration (400 nM) is able to support the abortive initiation reaction. In the presence of cGMP, a further stimulation of CRP*598 activity is obtained. At a concentration of 400 nM CRP*598, abortive synthesis in the absence of cyclic nucleotide or in the presence of cGMP approaches that of the maximal level seen in the presence of cAMP. The results demonstrate that CRP*598 differs from CRP in its ability to function in the unliganded state or as a cGMP-CRP*598 complex. Similar results have been reported for other CRP* mutants by

Harman *et al.* (1986) and Blazy and Ullmann (1986).

DNase I footprinting can provide a direct visualization of the interaction of CRP and CRP*598 with the CRP binding site in the *lac* P^+ promoter. CRP*598 and CRP bind to the -80 to -50 bp segment of the [^{32}P]*lac* P^+ fragment in the presence of 100 uM cAMP (Figure 3, lanes c and g). The characteristic open promoter pattern is obtained with both cAMP-CRP and cAMP-CRP*598 on the further addition of RNA polymerase (Figure 3, lanes d and h). Unliganded CRP at a concentration of 400 nM does not show binding to the [^{32}P]*lac* P^+ fragment even in the presence of RNA polymerase (Figure 3, lane f) or in the presence of cGMP plus RNA polymerase (data not shown).

The data presented in Table I indicate that CRP*598 supports abortive initiation in the absence of cAMP or cGMP. The footprint data presented in Figure 4 indicate that CRP*598 at a concentration of 320 nM does not form a stable complex with the [^{32}P]*lac* P^+ fragment in the absence of cyclic nucleotide (Figure 4, lane h) or in the presence of cGMP (Figure 4, lane c). In the presence of cAMP, the characteristic footprint pattern is effected by CRP*598 (Figure 4, lane b). As the concentration of CRP*598 is raised from 40 nM to 320 nM, the presence of RNA polymerase stabilizes binding of unliganded CRP*598 to the [^{32}P]*lac* P^+ fragment (Figure 4, lanes i to 1). A similar effect of RNA polymerase in stabilizing binding of cGMP-CRP*598 to the [^{32}P]*lac* P^+ fragment can also be seen (Figure 4, lanes d to g). cGMP-CRP*598 shows a somewhat higher affinity than does the unliganded CRP*598 for RNA polymerase-mediated [^{32}P]*lac* P^+ fragment binding. In the presence of

CRP*598, there is a concomitant stabilization of RNA polymerase binding to its site in the *lac* P^+ promoter.

Previous studies demonstrated that sensitivity to proteolytic attack is a useful approach for assessing the conformation of CRP (Eilen et al., 1978; Ebright et al., 1985). Unliganded CRP and cGMP-CRP are resistant to a variety of proteases; cAMP-CRP is attacked generating N-terminal cores which retain cAMP binding activity (Eilen et al., 1978). The data presented in Figure 5 show that CRP*598 differs markedly from CRP in its sensitivity to proteolytic attack in the absence of cyclic nucleotides and in the presence of cGMP. The protease-resistant fragments generated from cAMP-CRP and cAMP-CRP*598 are identical. cGMP-CRP*598 is attacked by chymotrypsin (Figure 5, lane b) and subtilisin (Figure 5, lane f), resulting in the formation of a major core fragment along with a slightly smaller fragment. Unliganded CRP*598 is more sensitive to attack by chymotrypsin (Figure 5, lane d) and subtilisin (Figure 5, lane h) and smaller fragments are produced than those formed in the presence of cAMP or cGMP. We suggest that the initial cutting sites appear to be identical to those which are accessible in the cAMP-CRP and cAMP-CRP*598 and more so in unliganded CRP*598. Earlier results showed that the alpha core formed by subtilisin digestion of cAMP-CRP was in a conformational state which was sensitive to further attack in the absence of cAMP or cGMP (Eilen & Krakow, 1977).

Trypsin digestion of unliganded CRP*598 results in the accumulation of a fragment smaller than that formed after digestion of cGMP-CRP*598 or cAMP-CRP

(Figure 6). It is interesting to note that the cAMP-CRP*598 is more resistant to trypsin than the cGMP-CRP*598 complex. Assay for [^{3}H]cAMP binding activity after digestion of unliganded CRP*598 (Figure 7) shows that the CRP*598 fragment formed does not bind cAMP. A similar loss of cAMP binding activity was found following trypsin digestion of DNA-CRP (Angulo & Krakow, 1986).

In the presence of cAMP, reaction of the two available sulfhydryl groups (Cys-178) with dithionitrobenzoic acid results in the formation of a disulfide bond linking the two subunits within the CRP protomer (Eilen & Krakow, 1977b). The conformational change in CRP elicited by cAMP binding is required to bring the two cysteine residues into proximity. As shown in Figure 8, dithionitrobenzoic acid-mediated formation of the intersubunit disulfide bond occurs in cAMP-CRP (Figure 8, lane e) but not in cGMP-CRP (Figure 8, lane f) or unliganded CRP (Figure 8, lane g). In contrast, CRP*598 shows significant crosslinking in the absence (Figure 8, land d) or presence of cGMP (lane c) or cAMP (Figure 8, lane b). The data are consonant with an altered conformation in CRP*598 allowing the Cys-178 present between the E and F helices to approach within a distance required for the dithionitrobenzoic acid mediated intersubunit disulfide interchange reaction. Since the crosslinking has been demonstrated to occur within the CRP protomer, the results also indicate that the CRP*598 mutation does not affect the stability of the major subunit contacts in the N-terminal domain of CRP.

Characteristic of binding of cyclic nucleotides to CRP*598:

Formation of the open complex with the *lac* P^+ promoter by RNA polymerase requires the coincident binding of cAMP-CRP to its promoter-associated site. The response of CRP and CRP*598 to cAMP concentration in the abortive initiation reaction is shown in Figure 9. The data indicate that both forms of CRP respond to increasing cAMP concentration. However the concentration of cAMP required to achieve a half-maximal response for supporting abortive initiation by RNA polymerase differs by about 11 fold: CRP, 3.2×10^{-6} M cAMP; CRP*598, 2.8×10^{-7} M cAMP.

In order to determine whether the apparent increased affinity for cAMP is an intrinsic property of CRP*598, direct cAMP binding assays were carried out using equilibrium dialysis and ammonium sulfate precipitation procedures. Scatchard plots for binding of cAMP by CRP*598 (Figure 10) obtained by both methods are indicative of strong positive cooperativity. Under the same conditions wild type CRP shows a deviation from linearity indicative of negative cooperativity. Takahashi *et al.* (1980) found that at low salt concentration CRP bound cAMP with negative cooperativity; as the salt concentration was increased the cooperativity became progressively positive. The binding mixture used in the present study contains 0.4 M KCl where cooperativity was not observed by equilibrium dialysis (Takahashi *et al.*, 1980). The value of 1.9×10^4 M^{-1} for the intrinsic affinity constant, K, for binding of cAMP by CRP (Table II) is comparable to that obtained by Takahashi *et al.* (1980): $K = 3.9 \times 10^4$ M^{-1}. The data obtained for CRP*598 indicate a higher affinity for cAMP, $K = 3.8 \times 10^5$ M^{-1}. In addition, the strong positive cooperativity found for the

binding of cAMP by CRP*598 is in distinct contrast with the binding properties of wild type CRP.

Donoso-Pardo *et al.* (1987) have presented evidence indicating that the addition of the ammonium sulfate solution does not markedly disturb the binding equilibrium. We have found, using ammonium sulfate precipitation, that the high ionic strength and aggregation alter the binding properties of CRP. The apparent affinity for cAMP is increased to a value similar to that observed with CRP*598. This increased affinity for cAMP by CRP is not paralleled by a concomitant change in cooperativity. Under the same assay conditions the binding of cAMP by CRP*598 shows strong positive cooperativity.

The differential response seen for cAMP binding by CRP and CRP*598 is not observed for cGMP binding. Binding of cGMP by both forms of the protein showed negative cooperativity and similar values for K were obtained (Figure 11) using either equilibrium dialysis or ammonium sulfate precipitation. The results obtained for the binding of cAMP and cGMP by CRP and CRP*598 are summarized in Table II.

It has been reported that high concentrations of adenosine were able to support *in vitro* transcription from the *lac* P^+ promoter by another CRP* (Harman *et al.*, 1986). The binding of cGMP to CRP or CRP*598 is similar. This provides a convenient assay for a comparison of the ability of cAMP analogues to bind to CRP and CRP*598. The data presented in Figure 12 and Figure 13 show the results of assays for the ability of cAMP and adenosine to displace cGMP from CRP and CRP*598. The results indicate that displacement of cGMP from CRP*598 occurs at

lower concentrations of cAMP than are required for CRP (Figure 12). In a similar experiment using adenosine as the competitor the results indicate a much greater differential affinity of adenosine for CRP*598 versus CRP. The results establish the importance of the adenine moiety for the apparent enhanced affinity for CRP*598.

The binding properties of a series of cAMP analogues for CRP and CRP*598 are shown in Table III. The only ligands which show a greater apparent affinity for CRP*598 relative to CRP are cAMP and adenosine. Vaney *et al.* (1989) showed that 1 mM adenosine can support *in vitro lac* transcription by another CRP* mutant, CAP91. The differential binding is not a function of the intrinsic affinity of the ligand for CRP or CRP*598 since 8-bromo cAMP, 8-methylamino cAMP and N^6-butyryl cAMP show apparent affinities which are comparable to that observed for cAMP. It is of interest that N^6-butyryl cAMP which has been shown to elicit a conformational change in CRP (Ebright *et al.*, 1985) does not show any differential affinity for CRP*598.

Characterization of anti-CRP*598 mAbs:

Nine anti-CRP mAbs have been characterized before (Li & Krakow, 1985). For further conformational and functional study of CRP*598, anti-CRP*598 mAbs were produced and characterized. Figure 14 shows the results of Western blotting of proteolytic fragments of CRP*598. Digestion by trypsin yields two N-terminal fragments with molecular weights of 14,000 and 10,000 Da. Only mAb A517A1 is able to bind to these two fragments (lane A), indicating that the epitope for mAb

A517A1 is located within the N-terminal 10,000 Da fragment while epitopes for mAb A54D3 and A521C6 are not. Digestion of CRP*598 by *Staph. aureus* V8 protease produces a N-terminal 18,800 Da fragment which is bound by mAb A521C6 but not by mAb A54D3 (lane E, D). Therefore, the epitope for mAb A521C6 should be located within the region from 14,000 Da to 18,800 Da. Apparently, the mAb A54D3 epitope is within the fragment degraded by the *Staph. aureus* V8 protease. It is not present in the C-terminal seven amino acid sequence, because mAb A54D3 is still able to bind the fragment after carboxypeptidase Y digestion (lane G).

The functional properties of these three anti-CRP*598 mAbs were examined with regard to their effect on cAMP binding, *lac* P^+ DNA binding and abortive initiation. The results are summarized in Table IV. The effects of the mAbs on cAMP binding were determined by the ammonium sulfate precipitation method. CRP or CRP*598 were preincubated with the mAbs before cAMP was added. mAb A54D3 and A521C6 do not inhibit cAMP binding. In fact, both of them showed increased cAMP binding with increased mAb concentration (data not shown). mAb A517A1 inhibits cAMP binding partially at the cAMP concentration used (2 μM). The effects of the anti-CRP*598 mAbs on binding of cAMP-CRP or cAMP-CRP*598 complexes to *lac* P^+ DNA was determined by gel retardation assay (Garner & Revzin, 1981). The presence of mAb A54D3 and A521C6 resulted in a complete inhibition of *lac* DNA binding for both CRP and CRP*598 when the molar ratio of mAbs to CRP or CRP*598 are ten to one (Figure 15, lane d, e, h, i). In contrast, mAb A517A1 decreases the mobility of the cAMP-CRP-*lac* DNA or cAMP-CRP*598-

lac DNA complex (Figure 15, lane c, g), indicating that this mAb binds to the cAMP-CRP-*lac* DNA or cAMP-CRP*598-*lac* DNA complex. This effect is similar to that of anti-CRP mAb 66C3 and mAb 115D5 on CRP (Li & Krakow, 1988). All three of the anti-CRP*598 mAbs are inhibitors of abortive initiation under the experimental conditions used. There are no apparent differences between CRP and CRP*598 in terms of the effects of anti-CRP*598 mAbs.

In order to examine whether the binding of mAb A517A1 to cAMP-CRP/CRP*598-*lac* DNA complex is able to enhance the binding of cAMP-CRP to *lac* DNA, the following assays were executed: I, in the presence of cAMP, one CRP mutant, CRP209RD (Arg-209 → Asp), shows a weak binding to *lac* DNA. The anti-CRP mAb 66C3, which has been demonstrated to increase the affinity of CRP for *lac* DNA (Li & Krakow, 1988), strongly enhances the weak binding by the Arg-209 → Asp mutant (Figure 16, lane f, g), while mAb A517A1 does not give an obvious enhancement (Figure 16, lane c, d, e); II, the *lac* P^+ fragment is cut by *Hpa*II at -20 bp separating CRP site 1 and site 2 (-10 to +10). Figure 17 shows that cAMP-CRP binds to site 1 very well but hardly binds to the Site 2 fragment (lane b). The cAMP-CRP-mAb A517A1 forms a complex with all of the Site 1 fragment and only little of the Site 2 fragment (lane c, d, e, f). In contrast, cAMP-CRP-mAb 66C3 can form a complex with most of the Site 2 fragment (lane g, h, i, j). These results indicate that mAb A517A1 only slightly increases the affinity of CRP for its binding sites.

Determination of the dissociation constants for CRP or CRP*598 to anti-CRP or anti-CRP*598 mAbs:

The dissociation constants were determined by the method of Friguet *et al.* (1985). Under the experimental conditions, antigen and antibody complexes are formed in solution. Table V presents the dissociation constants of anti-CRP mAbs for CRP or CRP*598. These mAbs were selected from nine anti-CRP mAbs (Li & Krakow, 1985). In the absence of any cyclic nucleotide, the affinities of the mAbs for CRP are different from those for CRP*598 with the exception of mAb 64D1. In the presence of cAMP, affinities for both types of CRP are almost same. mAb 64B4 belongs to a class of mAbs which binds to denatured and native CRP (Li & Krakow, 1985). It has been shown that its epitope is located at the C helix and binds weakly to CRP in solution (Li & Krakow, 1985). The K_d for mAb 64B4 to CRP is (4.8 ± 0.23) x 10^{-6} M. In contrast, The K_d for CRP*598 is (3.3 ± 0.24) x 10^{-7} M, 10 times lower than that of CRP. Another distinction between CRP and CRP*598 is that mAb 64B4 does not inhibit cAMP or cGMP binding to CRP but does inhibit binding by CRP*598 (Figure 18, 19). This suggests that mAb 64B4 acts on CRP*598 in a way different from CRP. In the presence of cAMP or N^6-butyryl-cAMP, The K_d of mAb 64B4 for both of CRP and CRP*598 are over 10^{-5}, indicating that cyclic nucleotide binding decreases the availability of the epitope.

Table VI presents the dissociation constants of anti-CRP*598 mAbs to CRP or CRP*598. Similar to the results presented in Table V, CRP and CRP*598 show different affinities for anti-CRP*598 mAbs in the absence of cyclic nucleotides with

virtually the same affinities in the presence of cAMP.

Compared with the K_ds of cAMP-CRP/CRP*598 for both anti-CRP and anti-CRP*598 mAbs, N^6-butyryl-cAMP-CRP/CRP*598 shows different affinities for these mAbs, indicating that effects of N^6-butyryl-cAMP on CRP/CRP*598 differ from that of cAMP. It is interesting to note that for mAb 62D6, mAb 66C3, mAb A54D3 and mAb A521C6 which have epitopes located in the C-terminal domain, N^6-butyryl-cAMP does not cause any apparent change of their affinities for CRP and only changes the affinity of mAb A521C6 for CRP*598. Figure 20, 21, 22, 23 show the comparision of binding of mAb 62D6, mAb 66C3, mAb A54D3 and mAb A521C6 to CRP or CRP*598 in the absence or presence of cyclic nucleotides. Among these C-terminal binding mAbs, mAb 62D6 is the one whose K_d to CRP is not changed and the K_d for CRP*598 is changed in the presence of cAMP compared with the K_d in the absence of cyclic nucleotide.

DISCUSSION

CRP*598 is a representative of a set of CRP mutants which were predicted to show a destabilization of the interaction between the large and small domains of CRP (Garges & Adhya, 1985). Unliganded CRP is in a conformation which is unfavorable for site specific DNA binding. A conformational change occurs in cAMP-CRP which can be monitored by an increased susceptibility to proteolytic attack (Krakow & Pastan, 1973; Eilen *et al.*, 1978). The conformation established in cAMP-CRP enables binding to specific promoter-associated sites and the concomitant activation of transcription from CRP-dependent promoters. The CRP* mutants differ in their ability to activate transcription under conditions where CRP is inactive. CRP* can activate *in vivo lac* operon expression in the absence of endogenous cyclic nucleotides and in the presence of cGMP (Harman & Dobrogosz, 1983; Aiba *et al.*, 1985; Garges & Adhya, 1985; Blazy & Ullmann, 1986; Harman *et al.*, 1986). The CRP*598 mutations have been mapped within the hinge region of CRP (Garges & Adhya, 1985). Based on the chymotrypsin digestion patterns, the substitution in the D α helix of histidine for arginine at position 142 and threonine for alanine at position 144 in the CRP*598 appears to alter the stability within the N-terminal and C-terminal domains. The results obtained by using CRP*598 are comparable to those described by Harman *et al.* (1986) with the related NCR91 CRP* which has a single amino acid change: Ala-144 to Thr. The structure of the NCR91 CRP* crystallized with cAMP is similar but not identical to that of cAMP-CRP (Weber *et al.*, 1987c). Small

changes in the mutant CRP were noted which include concerted shifts in the small domains, in the hinge joining the two domains and in an adjacent loop between ß strands 4 and 5. The distortion resulting from the amino acid substitution proximal to the hinge region apparently disturbs the interaction between the two domains in unliganded CRP*598, thereby, opening up sites within each domain to attack by proteases. Unliganded CRP is relatively resistant to proteolytic attack. The proteases used generate N-terminal fragments from cAMP-CRP of differing sizes, indicating the presence of resistant folded regions of cAMP-CRP. In contrast, unliganded CRP*598 is sensitive to proteolysis and the limit digests generated under the conditions used in this study result in smaller fragments than those arising from cAMP-CRP or cAMP-CRP*598. The digestion patterns are indicative of the loss of important contacts between the large and small domains in unliganded CRP*598. Based on the protease study, it would appear that the conformations established in cAMP-CRP and cAMP-CRP*598 are similar. The binding of cGMP to CRP is without apparent effect while cGMP-CRP*598 adopts a conformation which is similar to but not identical with that established in cAMP-CRP*598.

A similar conclusion obtains from the results of the dithionitrobenzoic acid mediated disulfide crosslinking experiment. Crosslinking of the CRP subunits requires that the small domains adopt a conformation allowing the Cys-178 residues to move within a distance required for the formation of the disulfide bond. The Cys-178 is present in the small linker joining the E and F α helices present in the C-terminal domain. In wild type CRP, the subunit crosslinking reaction mediated by

dithionitrobenzoic acid is not favored in the absence of cAMP. In contrast, subunit crosslinking occurs in unliganded CRP*598, cGMP-CRP*598 and cAMP-CRP*598. The results indicate that the small domains of CRP*598 are less conformationally constrained than in the wild type CRP. cAMP is bound to a site in the large domain resulting in interaction with amino acid side chains from both subunits of the CRP dimer. McKay *et al.* (1982) stress the important role of the 6-amino group of cAMP in the allosteric activation of CRP. In the cAMP-CRP complex, hydrogen binding is believed to occur to both the Thr-127 and the Ser-128 of the other subunit. It was proposed that the binding of cAMP alters the relative orientation of the two subunits thereby affecting the shape of the DNA binding sites. The absence of a 6-amino group in cGMP accordingly would not allow for the trans-subunit allosteric transition evoked by cAMP binding. The lower protease resistance found for cGMP-CRP*598 may reflect the lack of second subunit contacts by bound cGMP and a consequent lower stability of subunit-subunit interactions.

The data clearly indicate that the conformations of unliganded CRP*598 and cGMP-CRP*598 differ from that established in cAMP-CRP*598. Each of these conformational variants of CRP*598 is able to activate *lac* P^+ abortive initiation, whereas, in the absence of RNA polymerase, only cAMP-CRP*598 can bind to the CRP site in the *lac* promoter. DNase I footprinting demonstrates that on addition of RNA polymerase the characteristic open promoter pattern is established with cAMP-CRP*598 and higher concentrations of cGMP-CRP*598 or unliganded CRP*598. The fact that unliganded CRP*598 and cGMP-CRP* bind to *lac* P^+ only

when RNA polymerase is present indicates a cooperative binding of heterologous proteins. Although other explanations are possible, the simplest interpretation of such cooperativity is a direct contact between the two DNA-bound proteins. With the *lac* L8UV5 promoter mutant, cAMP-CRP forms a stable complex at the L8 site only in the presence of RNA polymerase (Li & Krakow, 1988). Thus, these results provide strong evidence in support of a model whereby activation of transcription from CRP-dependent operons involves contact between CRP and RNA polymerase. RNA polymerase alone does not bind to the *lac* P^+ (referred to as *lac* P1; Malan & McClure, 1984) site and CRP*598 does not bind to the CRP site in the absence of cAMP. The conformation of CRP*598 and cGMP-CRP*598 is not optimal for site specific binding to the *lac* P^+. According to the model proposed by Malan and McClure (1984), RNA polymerase binds to an upstream promoter, *lac* P2, in the absence of cAMP-CRP. Addition of cAMP-CRP results in the coordinate repression of *lac* P2 and activation of *lac* P1. The coordinate repression-activation must also be effected by unliganded CRP*598 and cGMP-CRP*598. Since CRP*598 and cGMP-CRP*598 cannot by themselves bind optimally to the *lac* CRP site, the displacement of RNA polymerase present in the RP_o complex at *lac* P2 may first involve CRP* contact with the bound RNA polymerase followed by movement of the RNA polymerase into the P1 site. Accompanying this shift into the *lac* P1 site would be the concomitant binding of CRP* to the CRP site in the *lac* promoter.

Hwang and Gussin (1988) have shown that a similar situation of cooperativity between heterologous proteins exists in the bacteriophage λ *prm* promoter. In that

system, formation of open complexes at *prm* by RNA polymerase enhances binding at O_R by λ repressor, which functions as an activator of *prm*.

Another property of CRP* mutants is that the addition of exogenous cAMP or cGMP further stimulates the utilization of a variety of sugars. Subsequent characterization of the biochemical properties of the CRP* proteins demonstrated *in vitro* responses comparable to those seen *in vivo*. High concentrations of the CRP*598 are able to support *lac* P+ transcription in the absence of cyclic nucleotide. At protein concentrations where cAMP-CRP effectively supports abortive initiation from the *lac* P+ promoter the concentration of cAMP required by CRP*598 is approximately 9% of that required by CRP. The properties described for CRP*598 (Ren *et al.*, 1988) and other CRP* forms (Harman & Dobrogosz, 1983; Aiba *et al.*, 1985; Blazy & Ullmann, 1986; Harman *et al.*, 1986) indicate that this class of protein has an intrinsically different conformation than that seen for CRP. Unliganded CRP is resistant to attack by a variety of proteases, cAMP-CRP is attacked with the resultant formation of an N-terminal core whose length varies with the protease used (Eilen & Krakow, 1977). In contrast CRP* forms are sensitive to protease attack in the absence of cAMP. The CRP*598 which functions in the absence of cAMP or in the presence of low concentrations of cAMP appears to already exist in a more open conformation than that evinced by cAMP-bound CRP.

The available data do not allow one to distinguish whether the ability of CRP* to support transcription at low cAMP levels is directly attributable to an inherent property of the mutated protein or whether this is due to a secondary consequence

of the interaction of CRP* with its site on the *lac* P^+ promoter and/or contact with RNA polymerase. The data presented in this study demonstrate that CRP* differs from CRP in its mode of cAMP binding. Binding of cAMP by CRP has been shown by Takahashi *et al.* (1980) to vary from negative to positive cooperativity depending on the ionic strength of the binding buffer. Under the conditions used the binding of cAMP by CRP is negatively cooperative. In contrast, the binding of cAMP by CRP*598 shows strong positive cooperativity. Binding of cGMP by CRP and CRP*598 is virtually identical showing negative cooperativity and comparable association constants. Takahashi *et al.* (1980) found that the binding of CRP to double stranded DNA resulted in an increased affinity for cAMP together with the reversal of cooperativity from negative (in the absence of DNA) to positive. In a detailed study on the effect of DNA on cAMP binding by CRP, Takahashi *et al.* (1989) showed that the affinity of CRP for cAMP increases with NaCl concentration between 0.01 M and 0.2 M while cooperativity progressively changes from positive to negative. It appears likely that the CRP* mutant is shifting the equilibrium to favor the CRP conformation active in DNA binding away from that of the unliganded CRP. CRP bound to DNA at 10 mM NaCl becomes sensitive to trypsin attack with the formation of a N-terminal 9.7 kD fragment (Angulo & Krakow, 1986). Tryptic attack on CRP*598 results in an apparently identical fragment (Ren *et al.*,1988) suggesting that the CRP* dimer may be inherently unstable, in the same way CRP-DNA is unstable. Brown and Crothers (1989) found that under low ionic strength conditions DNA destabilizes the CRP dimer while cAMP has an opposite effect on dimer stability.

CRP is able to tolerate large substitutions at the N^6 position of cAMP without adverse effect on either binding or conformation. Ebright *et al.* (1985) identified several analogues, including N^6-butyryl cAMP, that are able to elicit conformational change in CRP but are unable to activate transcription. CRP*598 is able to bind N^6-butyryl cAMP with an apparent affinity similar to that observed for cAMP. However this analogue does not show the enhanced binding to CRP*598 shown by cAMP and adenosine. Furthermore, N^6-butyryl cAMP does not support abortive transcription from the *lac* P^+ promoter under conditions where CRP*598 is stimulated by cAMP (data not shown). Enhanced binding of cAMP and adenosine by CRP*598 requires the unsubstituted N^6 position of the adenine moiety. The N^6 of cAMP interacts with both subunits of CRP (Weber & Steitz, 1987). Ebright *et al.* (1985) have proposed that there must be an event taking place in proximity to the N^6 atom of cAMP which is required for CRP (or CRP*598) to bind to DNA.

The CRP*598 mutation results in amino acid replacements in the D α helix close to the hinge connecting the large and small domains of the subunit. The CRP*598 mutant shows an altered conformation, activates transcription at high CRP* concentration in the absence of cAMP, activates transcription at low CRP* concentration in the presence of a much lower cAMP concentration then required by CRP, and binds to *lac* P^+ DNA in the absence of cAMP in a RNA polymerase-dependent mode (Ren *et al.*, 1988). The property of positive cooperativity for the binding of cAMP by CRP*598 can be added to this list. The mutations in CRP*598 lie close to the hinge region and relatively far from the cAMP binding site. It is clear

that there is an effect on the conformation of the CRP*598 C-terminal domain based on its sensitivity to protease cleavage in the absence of cAMP. Binding of cAMP has been proposed to alter the conformation of CRP by altering the intersubunit contacts between the two large C α helices and also by affecting interdomain contacts (Weber & Steitz, 1987; Eilen *et al.*, 1978). The positive cooperativity observed for binding of cAMP by CRP*598 may be a consequence of effects modulated by altered subunit contacts and/or interdomain contacts.

Three anti-CRP*598 monoclonal antibodies were selected from a group of anti-CRP*598 mAbs based on results of ELISA assay (data not shown). mAbs A54D3 and A521C6 showed strong binding to CRP*598 and very weak binding to CRP by the ELISA plate assay while mAb A517A1 did not show any difference between the two types of CRP protein. Localization of the epitopes for these mAbs using proteolytic fragments of CRP*598 by Western blotting showed that mAb A517A1, mAb A521C6 and mAb A54D3 bind within the same region as the epitope for the anti-CRP mAb 115D5, 66C3 and 62D6 (Figure 24; Li & Krakow, 1985). The interesting location and the different effects of the two types of CRP of these mAbs make them potentially useful as probes for the study of conformation and function of CRP or CRP*598.

The three anti-CRP*598 mAbs have been characterized with regard to the region of CRP or CRP*598 in which the epitopes reside, and their effect on cAMP binding, lac DNA binding and abortive initiation. The approximate region bound by mAb A54D3 includes the F α-helix and part of the E α-helix. The region bound by

mAb A521C6 covers the D α-helix and part of the E α-helix. Since their effects on CRP or CRP*598 are very similar, it is possible that the epitopes for these two mAbs are very close to each other. In contrast, the effect of mAb A521C6 differs from that of mAb 66C3 even though the epitope of mAb 66C3 is also located within the region spanning the D α-helix to the E α-helix (Figure 24). If they are really next to each other both of the epitopes should be close to the E α-helix. As we know, the E α-helix is part of helix-turn-helix motif that belongs to a group of site specific DNA binding proteins, therefore, it is not surprising that mAb A54D3 and mAb A521C6 inhibit abortive initiation as well as *lac* DNA binding.

As mentioned before, mAb A517A1 is bound to the N-terminal 10 K Da fragment, the same region as bound by mAb 115D5. Although both mAb A517A1 and mAb 115D5 can bind to the CRP-*lac* DNA complex, mAb 115D5 does not affect cAMP binding (Li & Krakow, 1985), while mAb A517A1 shows a weak inhibition of cAMP binding; mAb 115D5 inhibits abortive initiation partially (Li & Krakow, 1988) while mAb A517A1 inhibits initiation (Figure 25).

Li and Krakow (1988) proposed that the binding of mAb 115D5 or mAb 66C3 to the cAMP-CRP complex increases the affinity of CRP for the *lac* P^+ CRP Site and induces CRP binding at the -10 to +10 region known as CRP Site 2. Binding to Site 2 would act like a repressor blocking RNA polymerase binding. For mAb 115D5, the enhancement to CRP Site 2 is weaker than that for mAb 66C3 and it shows only partial inhibition of transcription initiation. mAb A517A1 is a stronger inhibitor of the transcription initiation but only weakly enhances the Site 2 binding. Thus the

transcription inhibition by mAb A517A1 does not appear to involve *lac* Site 2 binding.

The study of the mechanism of transcription involves many interesting aspects, such as protein-protein interaction, protein-DNA interaction, protein conformation and DNA conformation. Several lines of evidence have shown that direct contact between CRP and RNA polymerase on the *lac* promoter (Li & Krakow, 1988; Ren *et al.*, 1988; Straney *et al.*, 1989) is involved in forming the open promoter complex. It has been mentioned in the introduction that the cAMP-CRP complex can cause bending of the *lac* DNA. DNA bending may participate in transcription activation by facilitating additional protein-DNA or protein-protein contacts required for the initiation (Plaskon & Wartell, 1987; Bracco *et al.*, 1989). The binding of mAb A517A1 to the cAMP-CRP complex may cause a steric hindrance that blocks the interaction between CRP and RNA polymerase or additional CRP-DNA or DNA-RNA polymerase contacts.

Previous experiments have shown that the mutations at the hinge region (Arg-142 → Lys-142, Ala 144 → Thr-144) of CRP*598 cause conformational and functional changes (Ren *et al.*, 1988). These changes are reflected by its sensitivity to proteolytic digestion and the ability to activate *lac* transcription in an unliganded form. The cAMP binding of CRP*598 also shows remarkable differences from that of wild type CRP. In this study, examination with anti-CRP or anti-CRP*598 mAbs to both type of CRP gives additional information about the conformational distinction between liganded and unliganded forms of CRP*598 and CRP. CRP*598 has been

assumed to partially mimic the conformation of cAMP-CRP complex (Garges & Adhya, 1985). Data presented here shows that most of the reactions of CRP*598 or CRP with anti-CRP*598 or anti-CRP mAbs are not identical, indicating they do not have the same conformation. Instead, liganded CRP*598 and CRP have similar affinities for both anti-CRP mAbs or anti-CRP*598 mAbs, implying they share similar local conformations. These results are consistent with those obtained by other assays (Ren *et al.*, 1988) and those described by Harman *et al.* (1986) with the related NCR 91 CRP*, which has a single amino acid change: Ala-144 → Thr-144.

mAb 62D6, 66C3, A54D3 and A521C6 are C-terminal domain-binding antibodies. Except for mAb 62D6, each of the others shows similar affinities for CRP*598 in the presence and absence of cAMP, suggesting conformational similarities to that of cAMP-CRP. This has been confirmed by DTNB cross linking (Ren *et al.*, 1988), *Staph. aureus* V8 protease and carboxypeptidase Y digestion (data not shown). cAMP increases the affinity of 62D6 to CRP*598 about 25-fold. The part of CRP*598 that is located at or close to F α-helix could be less exposed in the absence of cAMP. The F helix probably does not protrude from the protein surface or is not properly orientated so that CRP*598 forms an unstable complex with *lac* DNA.

In the N-terminal domain, the major difference between CRP*598 and CRP is that the two subunits of CRP*598 are more separated than those in CRP. This is suggested by the following, the epitope of mAb 64B4 located at the interface of the two subunits is almost inaccessible (Li & Krakow, 1988) while mAb 64B4 binds to

CRP*598 much better. This is also indicated by the inhibition of cAMP or cGMP binding to CRP*598 in the presence of mAb 64B4 but not to CRP. The binding of mAb 64B4 seems to result in a steric hindrance that prevents cAMP or cGMP from entering the cAMP binding "pocket". As shown before (Ren *et al.* 1990), positive cooperativity occurs for cAMP and CRP*598 binding, while a negative cooperativity is seen for cAMP and CRP binding. Previous experiment showed that N^6-butyryl-cAMP does not cause positive cooperativity for cAMP binding, suggesting that the unsubstituted N^6 position is probably required for this activity. However, in the case of cAMP, even if the N^6 position is not substituted, CRP still does not behave like CRP*598 when cAMP is bound, indicating there must be something more within CRP*598. The data here clearly show the difference between CRP and CRP*598 within cAMP-binding domain. The altered domain conformation of CRP*598 may also be responsible for the positive cooperativity of cAMP binding.

N^6-butyryl-cAMP belongs to a group of cAMP analogs which can elicit similar conformational change as cAMP as probed by protease digestion, but cannot stimulate *lac* transcription (Ebright *et al.*, 1985). cAMP must provide extra effects on CRP to change its conformation from an "inactive" form to an "active" form. The different effects of cAMP and N^6-butyryl-cAMP on CRP or CRP*598 are reflected by the K_ds of mAbs to CRP/CRP*598. For those C-terminal binding mAbs, N^6-butyryl-cAMP does not markedly change their affinities for CRP/CRP*598 compared with the affinities in the absence of any nucleotide. Another experiment (data not shown) suggests that the N^6-butyryl-cAMP-CRP complex is less sensitive to

carboxypeptidase Y than the cAMP-CRP complex. Therefore, The conformation induced by cAMP differs from that induced by N^6-butyryl-cAMP. The changes shown here which occur in the presence of cAMP but not N^6-butyryl-cAMP may be critical for CRP activation.

SUMMARY AND SUGGESTIONS

Garges and Adhya (1985) proposed that CRP*598 (Arg-142 to His, Ala-144 to Thr) assumes a conformation normally evoked only on binding of cAMP, one in which the relative orientation of three α-helices (C,D,and F) in both domains is altered. The experiments presented in this study demonstrate that the conformation of CRP*598 differs from that of CRP in the absence of cAMP. Although CRP*598 can activate *in vitro lac* transcription, in the presence of cAMP or cGMP as well as in the absence of added cyclic nucleotide, stable binding to CRP site in *lac* P^+ by unliganded CRP*598 or cGMP-CRP*598 is seen only in the presence of RNA polymerase. Various experiments show that the conformation of CRP*598 is not identical but may partially mimic that of the cAMP-CRP complex. Most of the similarities between CRP*598 and cAMP-CRP are likely to be located at DNA binding domain. The mutations at or close to the hinge region alter the conformation of the C-terminal domain to a state similar to the one induced by cAMP. The reorientation of the C-terminal domain consequently changes the domain-domain and/or the subunit-subunit interaction, resulting in a destabilization of the N-terminal domain. cAMP is able to stabilized the N-terminal domain. In the presence of cAMP, the conformations of CRP and CRP*598 are similar.

Equilibrium studies of the CRP-DNA interaction shows one cAMP molecule per *lac* promoter complex (Fried & Crothers, 1984). However, the crystallographic structure of cAMP-CRP complex shows two cAMP molecules in the complex.

Heyduk & Lee (1989) proposed that CRP-$(cAMP)_2$ complex may represent an inactive form of the complex. In the case of CRP*598, strong positive cooperativity occurs when cAMP is bound, suggesting two cAMP molecules are present in the complex. Therefore, the two cAMP complex is also a possible active form. For positive cooperativity, two requirements should be considered: first, there must be enough space between the two subunits of CRP to allow two cAMP molecules to enter the cAMP binding-"pockets"; second, a 6-NH_2 group in cAMP must exist (the importance of 6-NH_2 group has been stressed by McKay *et al.*, 1982). Lack of either of the two requirements will result in loss of positive cooperativity of cAMP binding.

Under physiological conditions, there is a large amount of DNA in the same compartment with CRP and cAMP. Possible interactions among CRP, cAMP and DNA could be as following: i, one molecule of cAMP is bound to CRP and elicits a conformational change which is involved in specific DNA binding; ii, one molecule of cAMP binds to CRP with high affinity and a second cAMP molecule consequently binds with lower affinity; iii, nonspecific interactions between CRP and DNA result in positively cooperative binding of cAMP to CRP similar to that of CRP*598. The question is which pathway is true *in vivo*. Probably there are differences between one cAMP and two cAMP forms regarding DNA binding or transcription activity. It would be interesting to quantify DNA binding or transcription in both forms of cAMP-CRP complex.

Monoclonal antibodies raised against both CRP and CRP*598 are useful tools to study structure and function of CRP protein. Some of them show interesting

effects on DNA binding and/or transcription. However, steric hindrance is a problem in interpretation. If the Fc fragment of the mAb can be removed, it will help to clarify the effect of these mAb. Particularly, mAb A54D3 shows certain interesting behaviors: it can increase cAMP binding activity by 50 percent; after preincubation with this mAb, wild type CRP becomes sensitive to trypsin and subtilisin in the absence of cAMP but remains insensitive to *Staph. aureus* V8 protease and carboxypeptidase Y in the presence of cAMP. These effects are similar to that resulting from binding of non-specific DNA (Angulo & Krakow, 1986). Under the same conditions, mAb A54D3 does not show any apparent effect on CRP*598. Since the epitope of mAb A54D3 is within the DNA binding fragment, it would be interesting to see how this mAb affects domain-domain interaction.

Table I. Effect of cyclic nucleotides on CRP and CRP* in *lac* P^+-directed abortive initiation

	Concentration of CRP or CRP*598 ([^{3}H]UMP incorporated, pmol)	
CRP	40nM	400nM
CRP*598	46	128
CRP*598 + cGMP	87	153
CRP*598 + cAMP	163	115
CRP	14	11
CRP + cGMP	13	11
CRP + cAMP	180	166

Conditions for the abortive initiation reaction were described under "Materials and Methods". Incorporation of [^{3}H]UMP in the absence of CRP or CRP*598 was 13 pmol.

Table II. Parameters for binding of cAMP and cGMP to CRP and CRP*598

	K	α	n_H
Equilibrium Dialysis Method			
cAMP			
CRP	(1.9 ± 0.23) x 10^4 M^{-1}	0.84 ± 0.22	0.82 ± 0.07
CRP*598	(3.8 ± 0.56) x 10^5 M^{-1}	3.74 ± 1.11	1.94 ± 0.26
cGMP			
CRP	(2.9 ± 0.13) x 10^4 M^{-1}	0.61 ± 0.13	0.97 ± 0.02
CRP*598	(2.7 ± 0.35) x 10^4 M^{-1}	1.00 ± 0.25	0.94 ± 0.01
Ammonium Sulfate Method			
cAMP			
CRP	(6.8 ± 0.53) x 10^5 M^{-1}	0.45 ± 0.06	0.9 ± 0.06
CRP*598	(8.4 ± 1.2) x 10^5 M^{-1}	6.9 ± 1.9	1.4 ± 0.08
cGMP			
CRP	(1.7 ± 0.13) x 10^4 M^{-1}	0.45 ± 0.06	0.71 ± 0.07
CRP*598	(1.9 ± 0.13) x 10^4 M^{-1}	0.32 ± 0.04	0.79 ± 0.06

The intrinsic association constant K and the cooperativity parameter α were determined by the method of Takahashi *et al.* (1980). The Hill coefficient n_H was calculated using the EZ-FIT program (Perrella, 1988).

Table III. Displacement of [^{3}H]cGMP binding by cyclic nucleotides with CRP or CRP*598.

	Concentration of cNMP resulting in 50% inhibition of [^{3}H]cGMP binding	
Competitor	CRP	CRP*598
	μM	
cAMP	3.2	1.5
adenosine	3300	320
8-bromo cAMP	3.2	4.2
8-methylamino cAMP	2.8	2.4
N^6-butyryl cAMP	2.8	2.4
N^6,$O^{2'}$-dibutyryl cAMP	42	56
cIMP	63	63
cCMP	23	28
2'-deoxy cAMP	NE	NE
2'-deoxy cGMP	NE	NE
5' AMP	NE	NE
ATP	NE	NE

The conditions used for the assays are presented in Materials and Methods. The concentration required to give the 50% displacement was determined graphically. NE: no effect seen at the highest concentration used (20 mM).

TABLE IV. SUMMARY OF FUNCTIONAL PROPERTIES OF ANTI-CRP*598 MABS

mAb	Location	cAMP Binding	Inhibition of *lac* P+ Binding	Abortive Initiation
A54D3	E, F	-	+	+
A521C6	D, E	-	+	+
A517A1	N-10K	+/-	-	+

D, E, F represent α-helices; N-10k represents the N-terminal 10 K fragment (Figure 24). -, no inhibition; +, inhibition; +/-, partial inhibition (The molar ratio of mAb to CRP are 0, 0.5, 1, 2 and 4. The cAMP binding are 41 pmol, 27.5 pmol, 17 pmol, 17 pmol and 21.7 pmol for CRP*598; 17 pmol, 13 pmol, 8.3 pmol, 8.8 pmol and 9.3 pmol for CRP).

TABLE V. DISSOCIATION CONSTANTS FOR BINDING OF ANTI-CRP MABS TO CRP AND CRP*598

mAb	-	cAMP	N^6-Butyryl-cAMP
CRP			
62D6	$(9.7 \pm 0.42) \times 10^{-7}$	$(9.4 \pm 0.86) \times 10^{-7}$	$(1.36 \pm 0.09) \times 10^{-6}$
66C3	$(2.4 \pm 0.05) \times 10^{-8}$	$(3.3 \pm 0.16) \times 10^{-7}$	$(2.4 \pm 0.1) \times 10^{-8}$
64D1	$(2.6 \pm 0.35) \times 10^{-7}$	ND	ND
115D5	$(1.6 \pm 0.12) \times 10^{-6}$	$(4.5 \pm 0.29) \times 10^{-6}$	$(2.86 \pm 0.07) \times 10^{-6}$
64B4	$(4.8 \pm 0.29) \times 10^{-6}$	$> 10^{-5}$	ND
CRP*			
62D6	$(1.57 \pm 0.06) \times 10^{-5}$	$(6.1 \pm 0.39) \times 10^{-7}$	$(1.1 \pm 0.03) \times 10^{-5}$
66C3	$(2.7 \pm 0.52) \times 10^{-7}$	$(3.8 \pm 0.37) \times 10^{-7}$	$(2.2 \pm 0.19) \times 10^{-7}$
64D1	$(2.7 \pm 0.02) \times 10^{-7}$	ND	ND
115D5	$> 10^{-5}$	$(3.3 \pm 0.23) \times 10^{-6}$	ND
64B4	$(3.3 \pm 0.24) \times 10^{-7}$	$> 10^{-5}$	$(1.1 \pm 0.05) \times 10^{-5}$

The method of Frigret *et al.* (1985) was used to determine the K_d.

Table VI. DISSOCIATION CONSTANTS FOR BINDING OF ANTI-CRP*598 MABS TO CRP AND CRP*598

mAb	-	cAMP	N^6-Butyryl-cAMP
CRP			
A54D3	$(1.33 \pm 0.31) \times 10^{-6}$	$(1.97 \pm 0.1) \times 10^{-7}$	$(2.3 \pm 0.23) \times 10^{-6}$
A521C6	$(1.24 \pm 0.1) \times 10^{-6}$	$(8.5 \pm 0.78) \times 10^{-7}$	$(2.4 \pm 0.17) \times 10^{-6}$
A517A1	$(5.49 \pm 0.3) \times 10^{-6}$	$(2.11 \pm 0.05) \times 10^{-5}$	$(1.05 \pm 0.06) \times 10^{-5}$
CRP*598			
A54D3	$(1.15 \pm 0.02) \times 10^{-7}$	$(2.19 \pm 0.2) \times 10^{-7}$	$(1.3 \pm 0.21) \times 10^{-7}$
A521C6	$(1.39 \pm 0.47) \times 10^{-7}$	$(4.6 \pm 0.35) \times 10^{-7}$	$(1.95 \pm 0.07) \times 10^{-6}$
A517A1	$(1.21 \pm 0.1) \times 10^{-5}$	$(1.64 \pm 0.09) \times 10^{-5}$	$(6.41 \pm 0.54) \times 10^{-6}$

The method of Friguet *et al.* (1985) was used to determine the K_d.

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

CRP

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                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 GTG 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, ß-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 helices. The two F helices, which clearly protrude from the dimer, are thought to provide many of the 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 axis of symmetry.

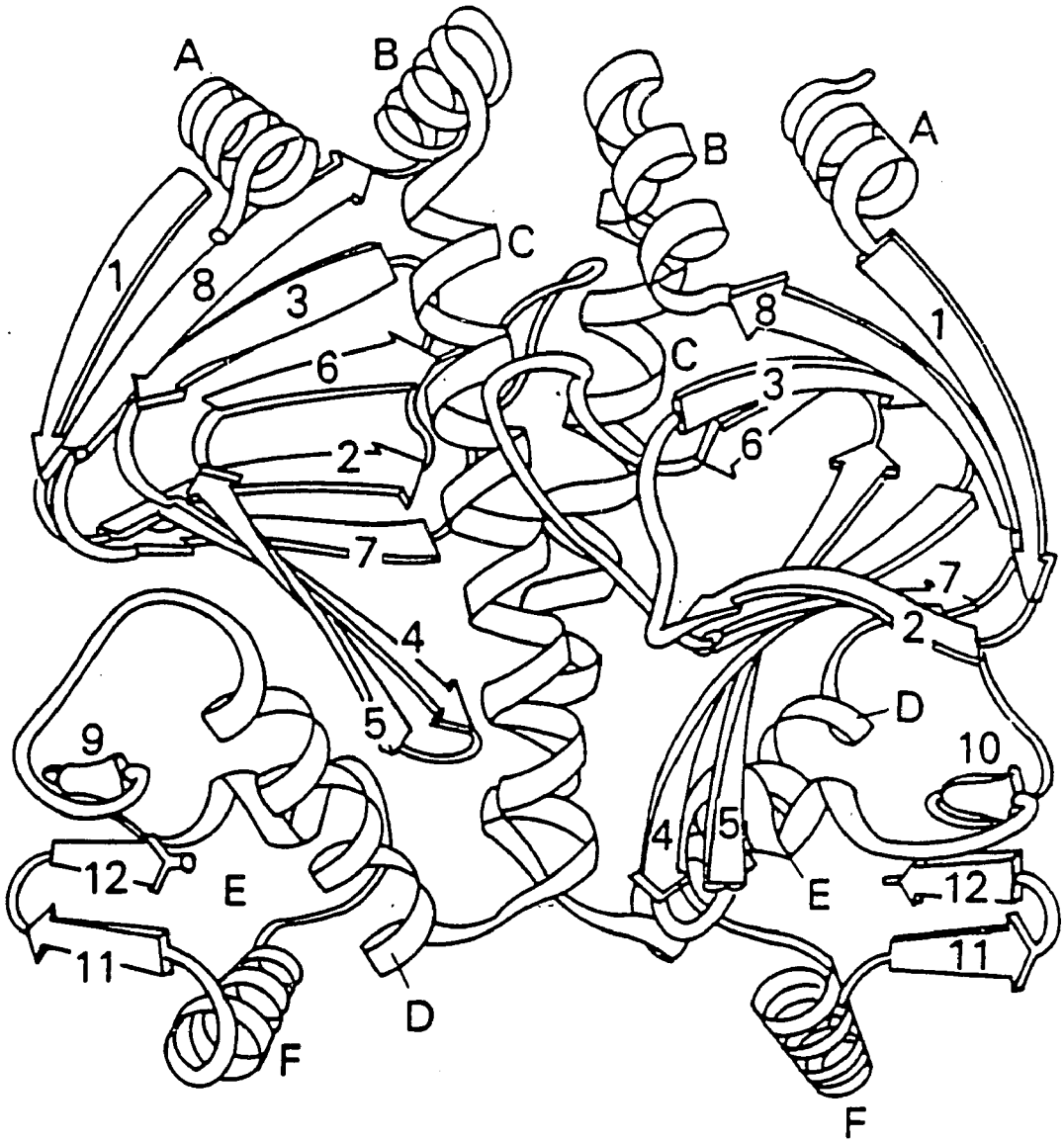

A
B
C
1
8
3
6
2
7
4
5
9
12
E
11
D
F
B
A
C
8
1
3
6
7
2
D
10
4
5
E
12
11
F

Figure 3. Binding of CRP and CRP*598 to *lac* P^+ in the presence of cAMP. Conditions are described under "Materials and Methods" using 3 nM [^{32}P]*lac* P^+ fragment and where indicated 120 nM RNA polymerase, 100 μM cAMP and the specified concentration of CRP or CRP*598. Lane a, [^{32}P]*lac* P^+; lane b, [^{32}P]*lac* P^+ + RNA polymerase; lane c, [^{32}P]*lac* P^+ + cAMP-CRP*598 (40 nM); lane d, [^{32}P]*lac* P^+ + cAMP-CRP*598 (40 nM) + RNA polymerase; lane e, [^{32}P]*lac* P^+ + CRP (400 nM); lane f, [^{32}P]*lac* P^+ + CRP (400 nM) + RNA polymerase; lane g, [^{32}P]*lac* P^+ + cAMP-CRP (40 nM); lane h, [^{32}P]*lac* P^+ + cAMP-CRP (40 nM) + RNA polymerase.

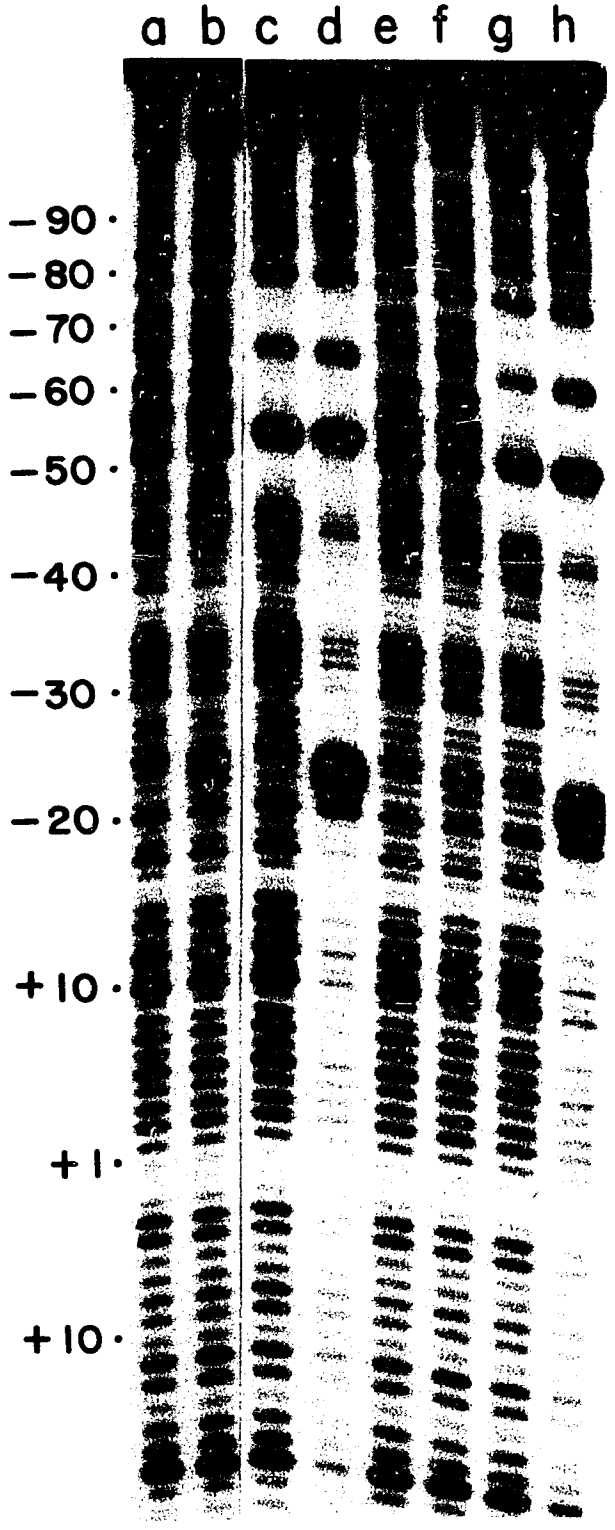

a b c d e f g h
−90
−80
−70
−60
−50
−40
−30
−20
+10
+1
+10

Figure 4. Effect of CRP*598 concentration on open promoter formation in the absence of cAMP. Conditions are described under "Materials and Methods" using 3 nM [^{32}P]*lac* P$^+$ fragment and where indicated 120 nM RNA polymerase, 1 mM cGMP or 0.1 mM cAMP and the specified concentration of CRP*598. Lane a, [^{32}P]*lac* P$^+$ + cAMP-CRP*598 (40 nM) + RNA polymerase; lane b, [^{32}P]*lac* P$^+$ + cAMP-CRP*598 (40 nM); lane c, [^{32}P]*lac* P$^+$ + cGMP*598 (320 nM); lane d, [^{32}P]*lac* P$^+$ + RNA polymerase + cGMP-CRP*598 (320 nM); lane e, [^{32}P]*lac* P* + RNA polymerase + cGMP-CRP*598 (160 nM); lane f, [^{32}P]*lac* P$^+$ + RNA polymerase + cGMP-CRP*598 (80 nM); lane g, [^{32}P]*lac* P$^+$ + RNA polymerase + cGMP-CRP*598 (40 nM); lanes h through l are identical to lanes c through g except that cGMP is absent; lane m, [^{32}P]*lac* P$^+$ + RNA polymerase; lane n, [^{32}P]*lac* P$^+$.

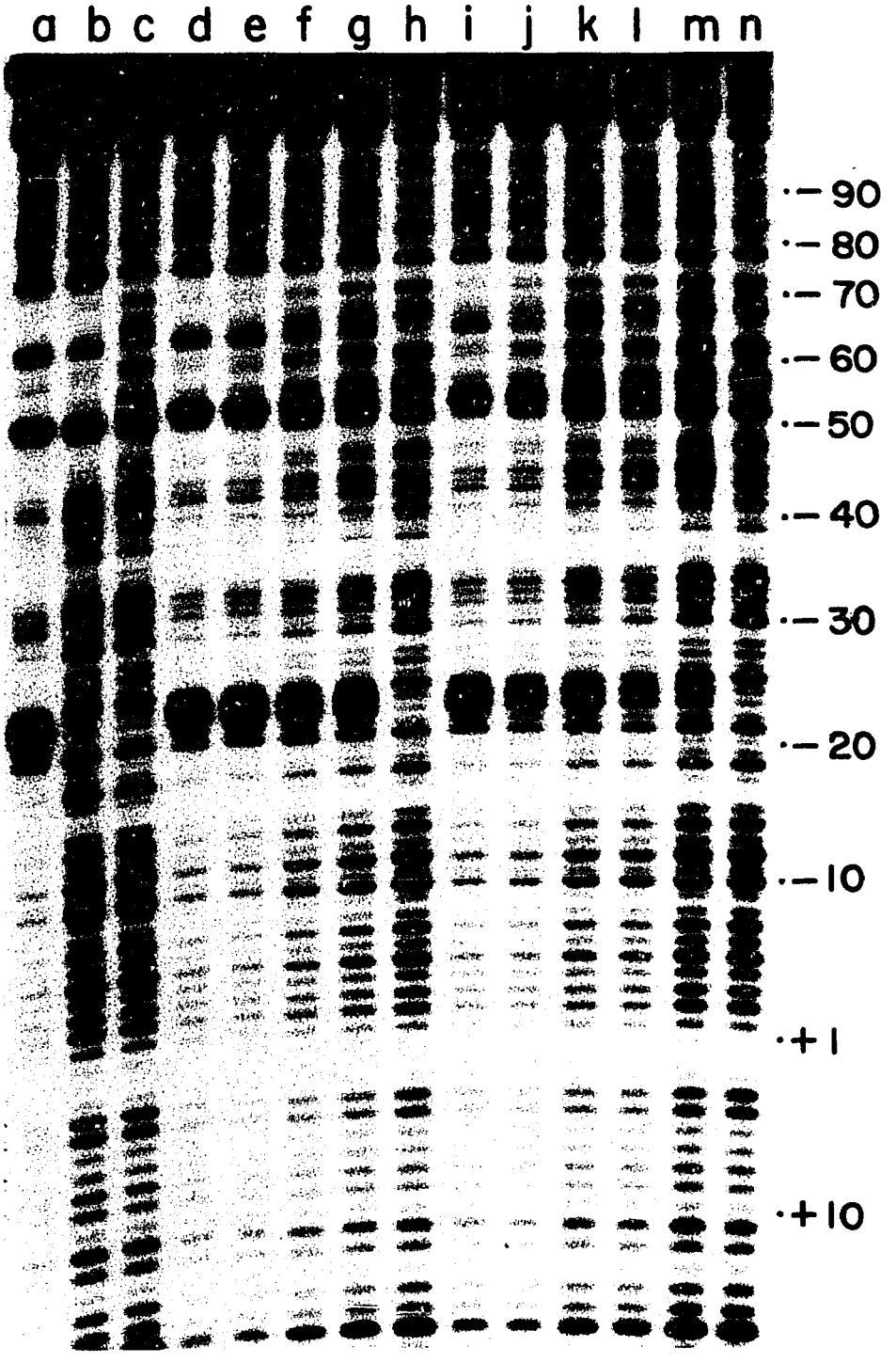

a b c d e f g h i j k l m n
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
+10

Figure 5. Sensitivity of CRP*598 to proteolytic attack in the presence and absence of cyclic nucleotide. Conditions are described under "Materials and Methods". Lane a, cAMP-CRP + chymotrypsin (0.1 μg); lane b cGMP-CRP*598 chymotrypsin; lane c, cAMP-CRP*598 + chymotrypsin; lane d, CRP*598 + chymotrypsin; lane e, cAMP-CRP + subtilisin (0.1 μg); lane f, cGMP-CRP*598 + subtilisin; lane g, cAMP-CRP*598 + subtilisin; lane h, CRP*598 + subtilisin; lane i, cAMP-CRP + *Staph. aureus* V8 protease (0.5 μg); lane j, cGMP-CRP*598 + *Staph. aureus* V8 protease; lane k, cAMP-CRP*598 + *Staph. aureus* V8 protease; lane l, CRP*598 + *Staph. aureus* V8 protease. The molecular weight markers are myoglobin, ctyochrome C and bovine trypsin inhibitor.

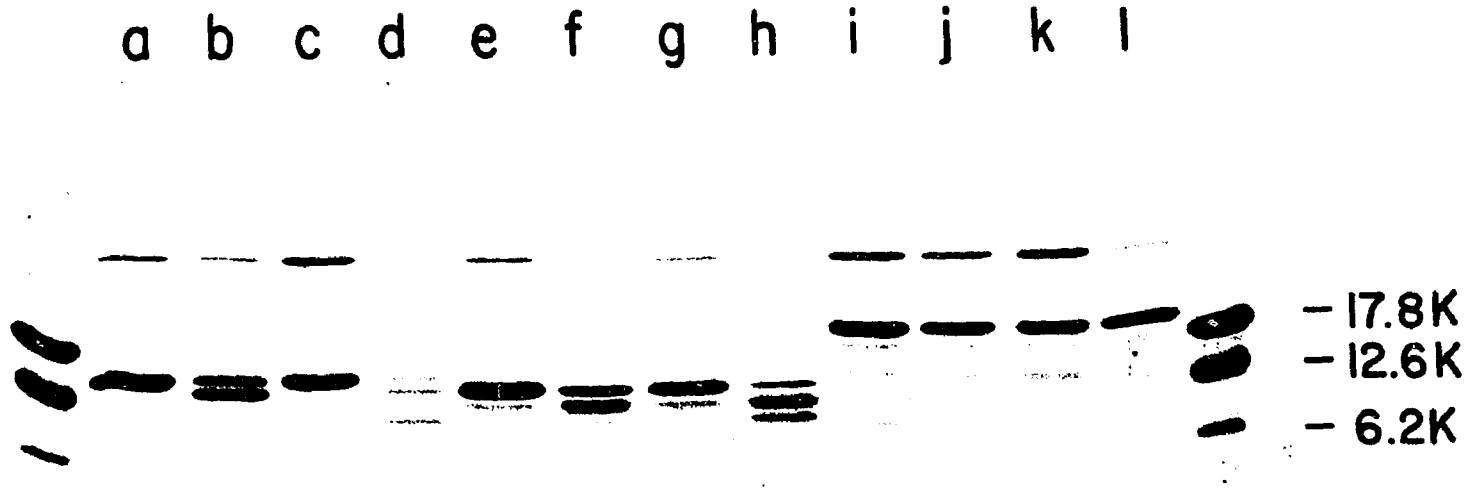

a b c d e f g h i j k l
17.8K
12.6K
6.2K

Figure 6. Time course of trypsin digestion of CRP*598 in the presence and absence of cyclic nucleotide. Conditions are described under "Materials and Methods". Lane a, CRP*598, lanes b, c, d, CRP*598 + trypsin (0.1 μg) incubated at 37°C for 10, 20 and 40 minutes; lanes e, f, g. cAMP-CRP*598 + trypsin (0.1 μg) incubated at 37°C for 10, 20 and 40 minutes; lanes h, i, j, cGMP-CRP*598 + trypsin (0.4 μg) incubated at 37°C for 10, 20 and 40 minutes; lanes k, l, m, cAMP-CRP + trypsin (0.4 μg) incubated at 37°C for 10, 20 and 40 minutes. The molecular weight markers are myoglobin and cytochrome C.

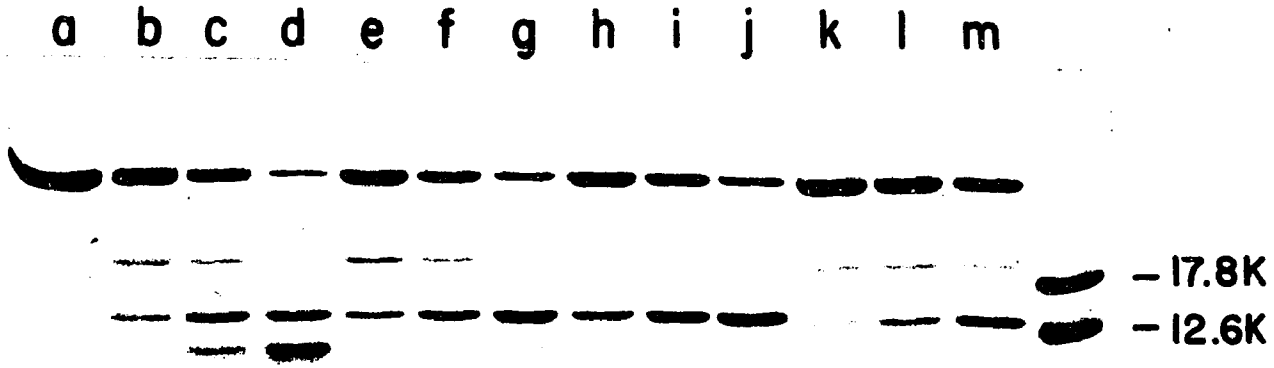

a b c d e f g h i j k l m
17.8K
12.6K

Figure 7. Loss of cAMP binding activity after trypsin digestion of unliganded CRP*598. Reaction mixtures contained (final volume 500 μL): 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 75 μg CRP or CRP*598 and 1 μg trypsin. After incubation at 37°C for the times indicated, aliquots (50 μL) were removed and the reaction terminated by addition of 2.5 μL of 20 mM phenylmethanesulfonyl fluoride. Binding of [^{3}H]cAMP was assayed using 20 μL of the sample removed at each time point.

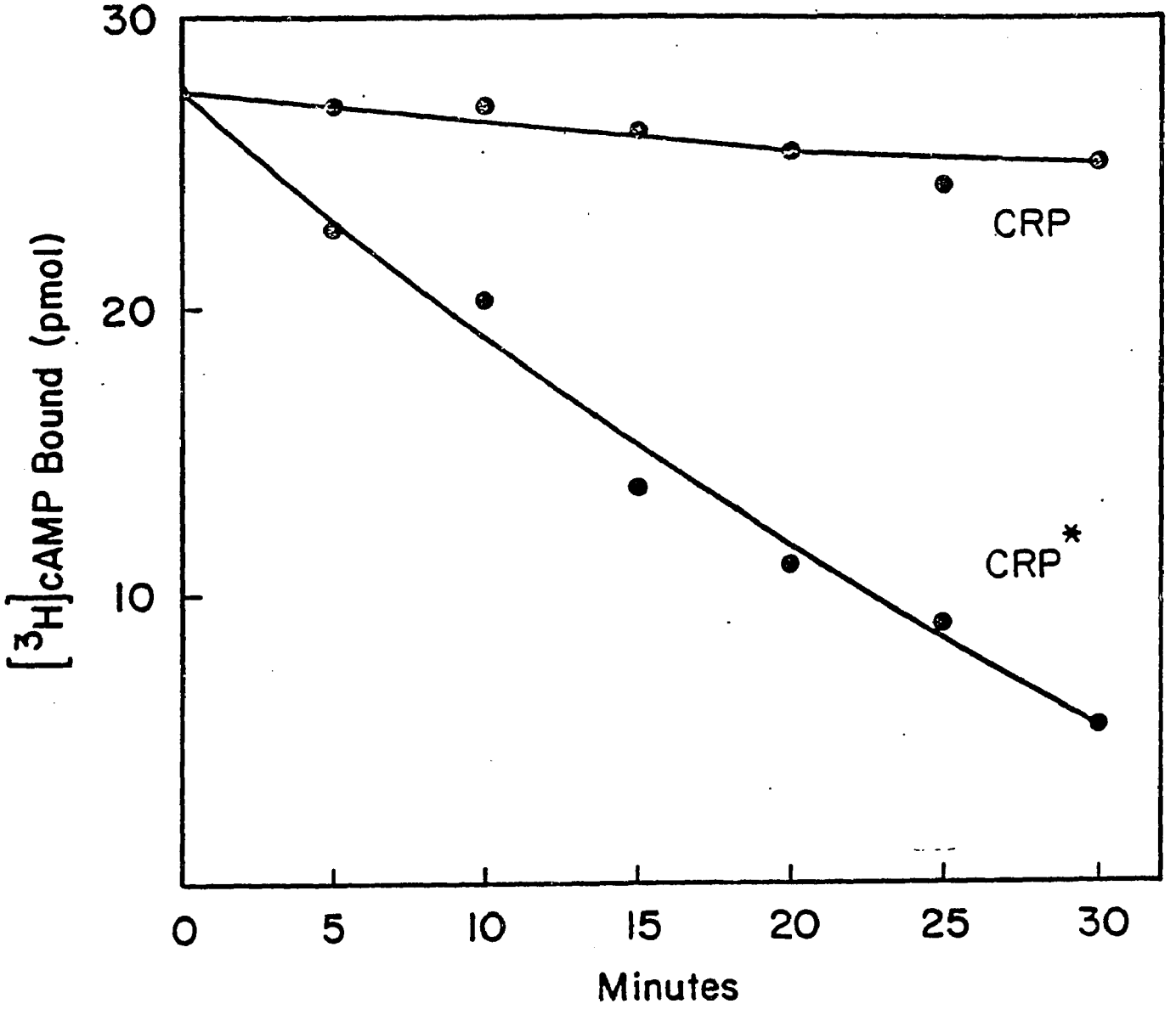

30
20
10
[3H]cAMP Bound (pmol)
CRP
CRP*
0
5
10
15
20
25
30
Minutes

Figure 8. Effect of dithionitrobenzoic acid on intersubunit crosslinking of CRP*598 and CRP. Conditions are described under "Materials and Methods". Lane a, CRP*598; lane b, cAMP-CRP*598 + dithionitrobenzoic acid; lane c, cGMP-CRP*598 + dithionitrobenzoic acid; lane d, CRP*598 + dithionitrobenzoic acid; lane f, cGMP-CRP + dithionitrobenzoic acid; lane g, CRP + dithionitrobenzoic acid; lane h, cAMP-CRP; lane i, CRP.

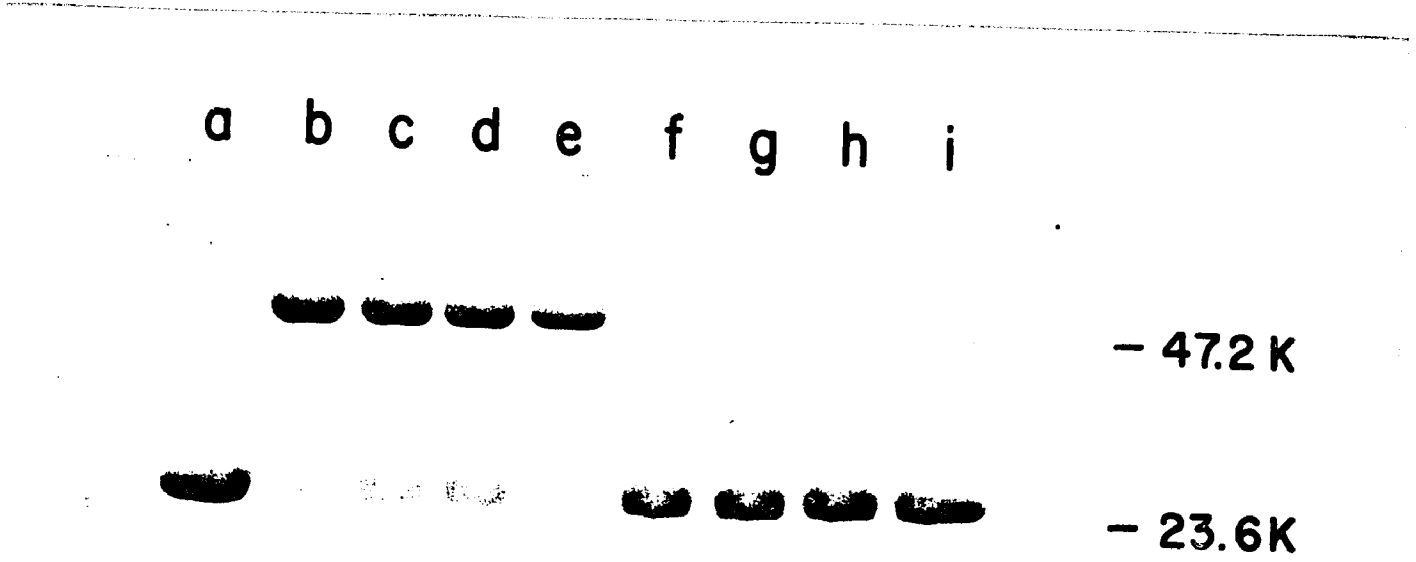

a b c d e f g h i
– 47.2 K
– 23.6K

Figure 9. Effect of cAMP concentration on the ability of CRP and CRP*598 to support *lac* P^+-directed abortive initiation. Conditions for the abortive initiation reaction are described in "Materials and Methods". Incorporation of [^{3}H]UMP in the absence of CRP or CRP*598 was 4 pmol. CRP, ●-●; CRP*598, o-o.

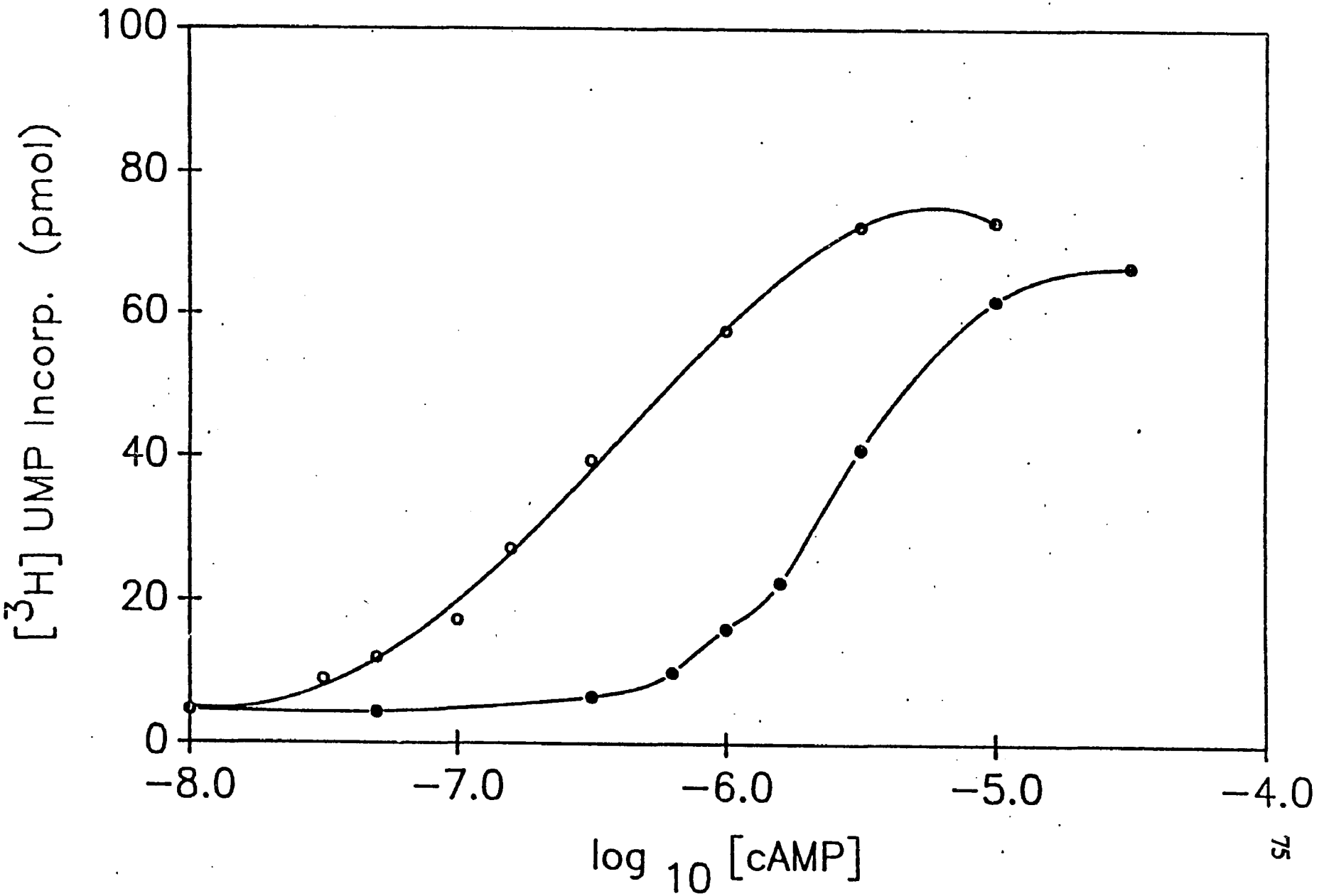

100
80
60
40
20
0
[3H] UMP Incorp. (pmol)
−8.0
−7.0
−6.0
−5.0
−4.0
log 10 [cAMP]

Figure 10. Scatchard representation of the binding of cAMP to CRP and CRP*598 determined by equilibrium dialysis and ammonium sulfate precipitation. The conditions used for equilibrium dialysis are presented in "Materials and Methods" with 4 μM CRP or CRP*598; the insert graph shows the results obtained using the ammonium sulfate precipitation method. The conditions are presented in Materials and Methods with 2 μM CRP or CRP*598. CRP, ●-●; CRP*598, o-o.

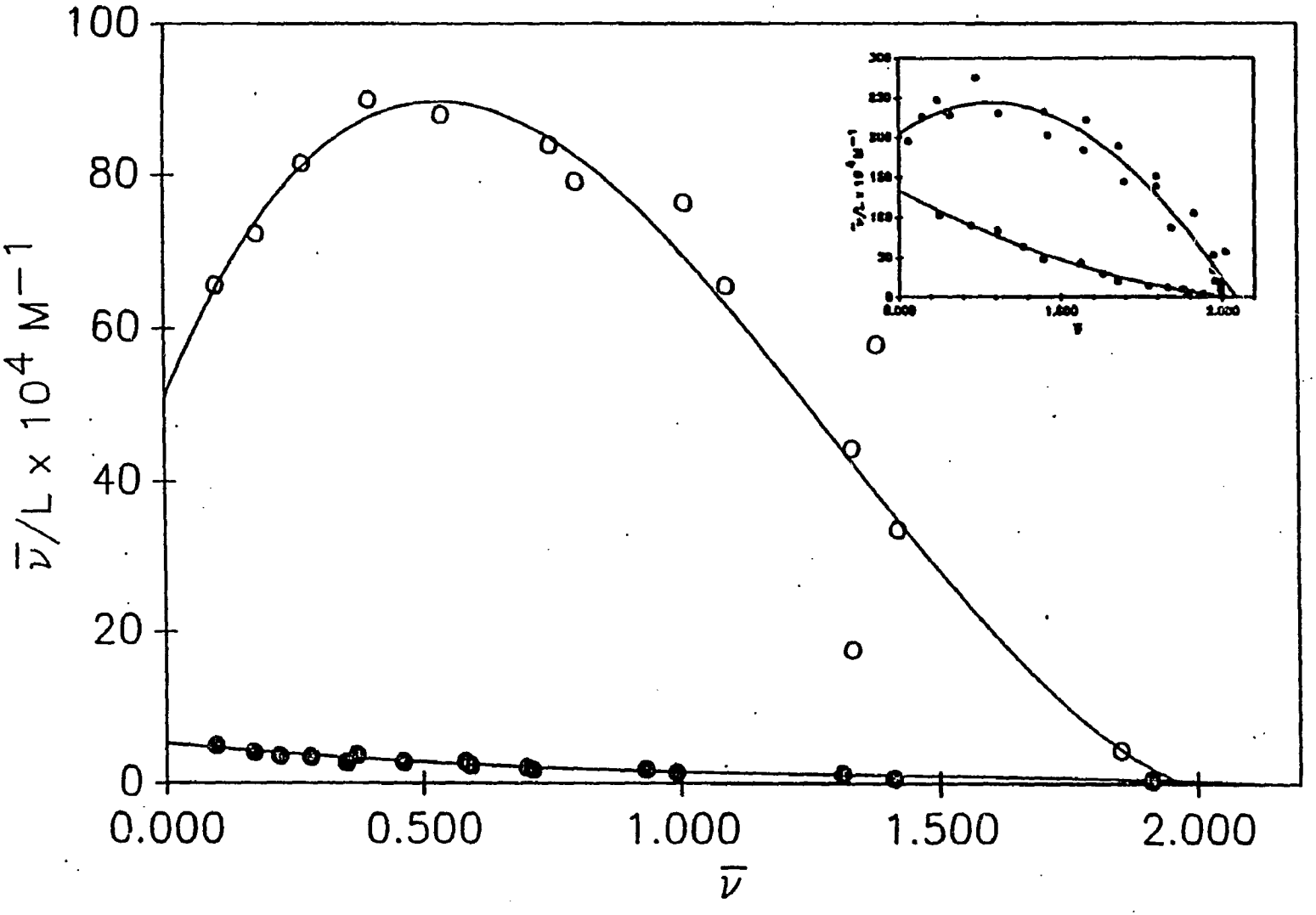

100
80
60
40
20
0
$\bar{\nu}/L \times 10^4\ M^{-1}$
0.000
0.500
1.000
1.500
2.000
$\bar{\nu}$

Figure 11. Scatchard representation of the binding of cGMP to CRP and CRP*598 as determined by equilibrium dialysis and ammonium sulfate precipitation. The conditions used for equilibrium dialysis are presented in "Materials and Methods" with 6 μM CRP or CRP*598; the insert graph shows the results obtained using the ammonium sulfate precipitation method. The conditions are presented in "Materials and Methods" with 2 μM CRP or CRP*598. CRP, ●-●; CRP*598, o-o.

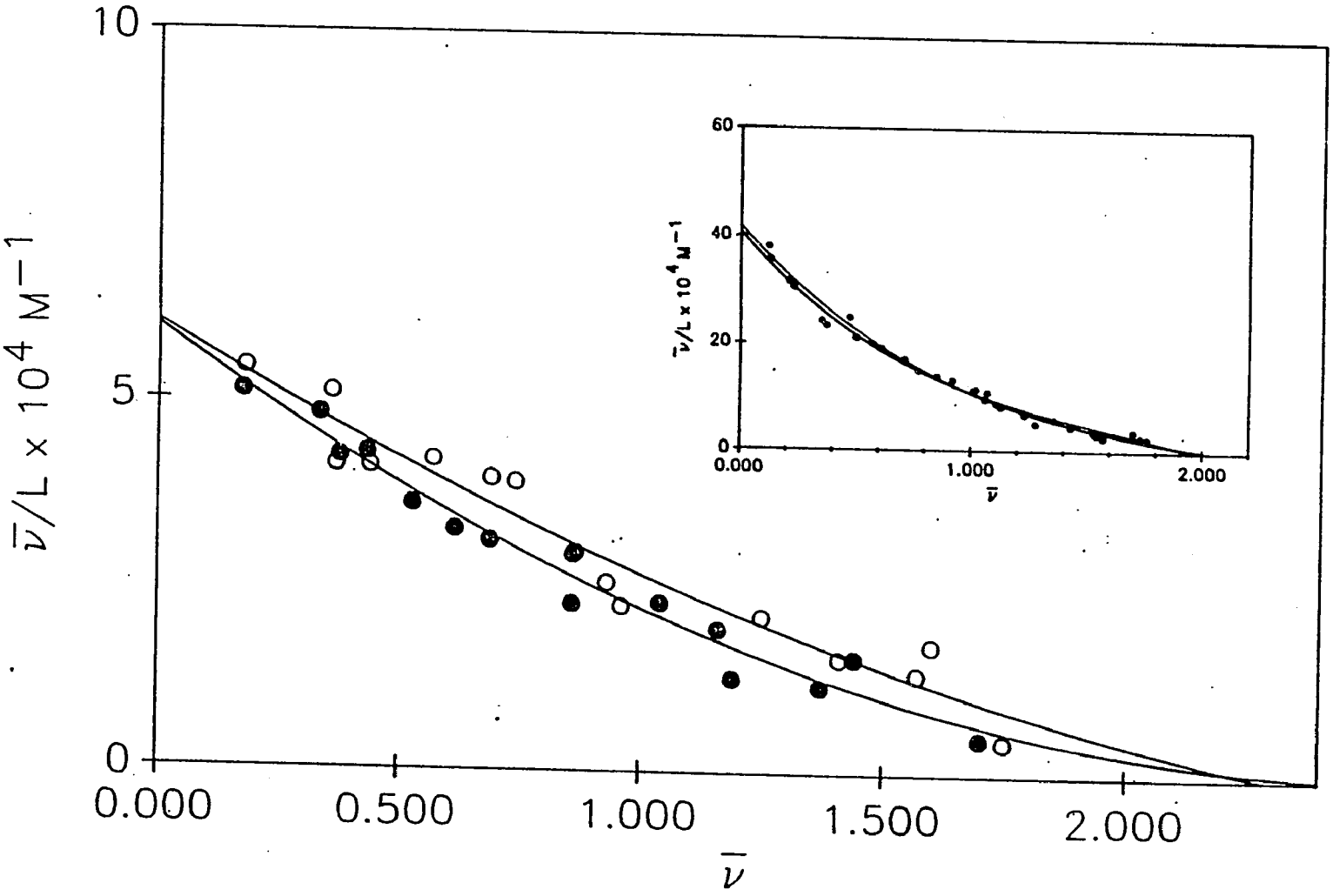

10
5
0
0.000
0.500
1.000
1.500
2.000
$\bar{\nu}$
$\bar{\nu}/L \times 10^4\ M^{-1}$
60
40
20
0
0.000
1.000
2.000
$\bar{\nu}$
$\bar{\nu}/L \times 10^4\ M^{-1}$

Figure 12. Displacement of [^{3}H]cGMP binding by cAMP with CRP or CRP*598. The incubation conditions are those presented in "Materials and Methods" using 2 x 10^{-6} M [^{3}H]cGMP and the cAMP concentrations indicated in the Figure. CRP, ●-●; CRP*598, o-o.

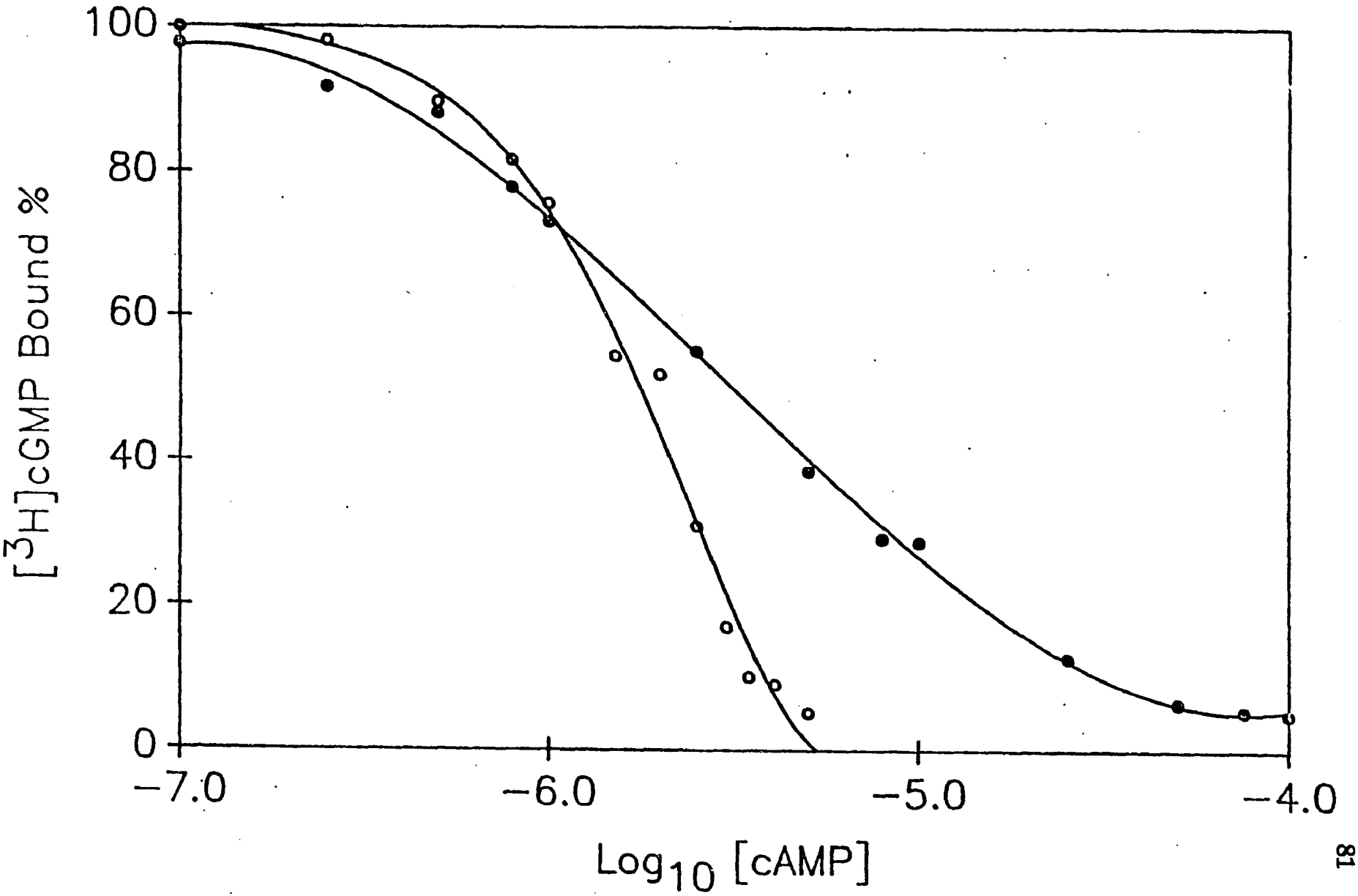

100
80
60
40
20
0
[3H]cGMP Bound %
−7.0
−6.0
−5.0
−4.0
Log10 [cAMP]

Figure 13. Displacement of [^{3}H]cGMP binding by adenosine with CRP or CRP*598. The incubation conditions are those presented in Materials and Methods using 2 x 10^{-6} M [^{3}H]cGMP and the adenosine concentrations indicated in the Figure. CRP, ●-●; CRP*598, o-o.

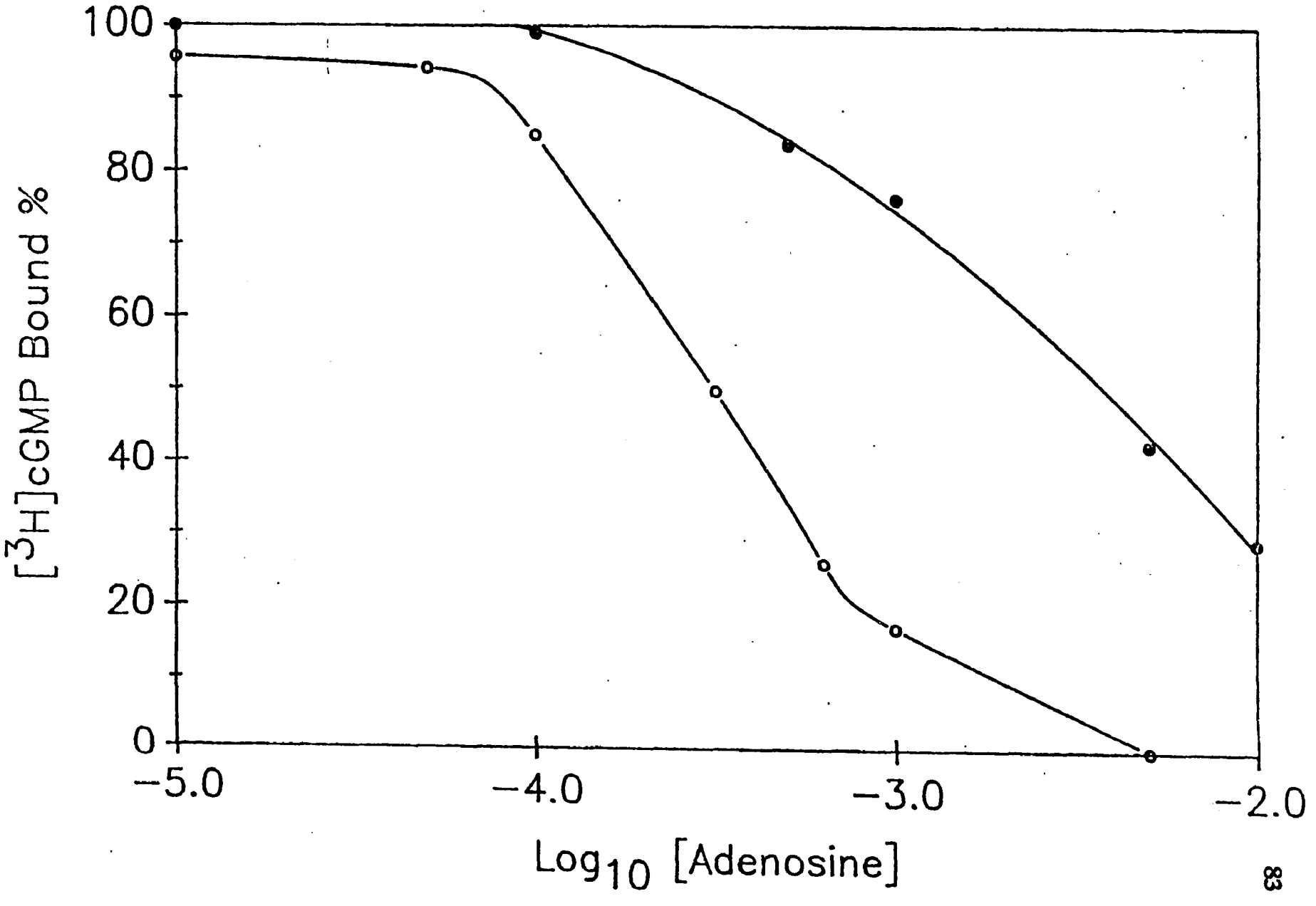

100
80
60
40
20
0
[3H]cGMP Bound %
−5.0
−4.0
−3.0
−2.0
Log10 [Adenosine]

Figure 14. Western blot analysis of the binding of anti-CRP*598 mAbs to CRP*598 fragments. CRP*598 fragments produced by partial proteolysis of CRP*598 were resolved electrophoretically and transferred to nitrocellulose sheets. Binding of mAbs was determined as indicated in "Materials and Methods". TRY, fragments produced after incubation of CRP*598 with trypsin; SAP, fragments produced after incubation of CRP*598 with *Staph. aureus* V8 protease; CY, fragments produced after incubation of CRP*598 with carboxypeptidase. A, mAb A517A1; B, mAb A521C6; C, mAb A54D3; E, mAb A521C6; F, mAb A54D3; G, control; H, mAb A54D3.

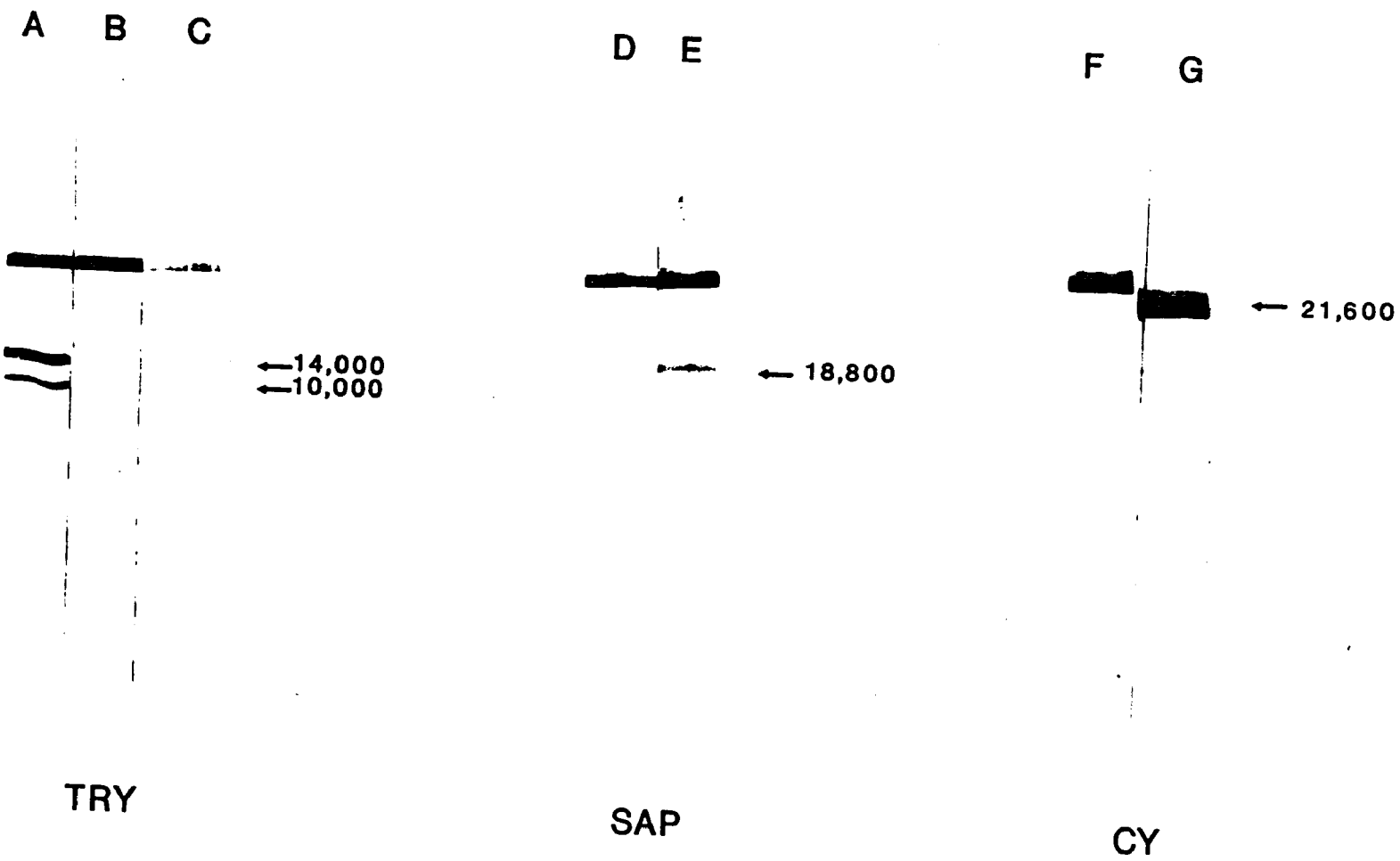

A B C
14,000
10,000
TRY
D E
18,800
SAP
F G
21,600
CY

Figure 15. Effect of anti-CRP*598 mAbs on binding of cAMP-CRP to the *lac* DNA promoter fragment. Samples containing (final volume 15 μl) 6.7 nM [^{32}P] *lac* DNA fragment, 100 μM cAMP, with of without 650 nM CRP/CRP*598 and 6.5 μM mAb were resolved electrophoretically. a, *lac* P$^+$; lanes b-i contained *lac* DNA and the indicated CRP and mAb as follows: b, cAMP-CRP*598; c, cAMP-CRP*598 + mAb A517A1; d, cAMP-CRP*598 + mAb A54D3; e, cAMP-CRP*598 + mAb A521C6; f, cAMP-CRP; g, cAMP-CRP + mAb A517A1; h, cAMP-CRP + mAb A54D3; i, cAMP-CRP + mAb A521C6.

a b c d e f g h i

Figure 16. Effect of mAb A517A1 and 66C3 on binding of cAMP-CRP209RD to the *lac* DNA promoter fragment. Samples containing (final volume 15 μl) 6.7 nM [^{32}P] *lac* DNA fragment, with or without 650 nM CRP and indicated mAbs were resolved electrophoretically. a, *lac* P$^+$; b, *lac* P$^+$ + cAMP-CRP ; lane c-g contained *lac* DNA, cAMP-CRP and indicated mAb as follows: c, 1.63 μM mAb A517A1; d, 3.25 μM mAb A517A1; e, 6.5 μM mAb A517A1; f, 1.63 μM mAb 66C3; g, 3.25 μM mAb 66C3.

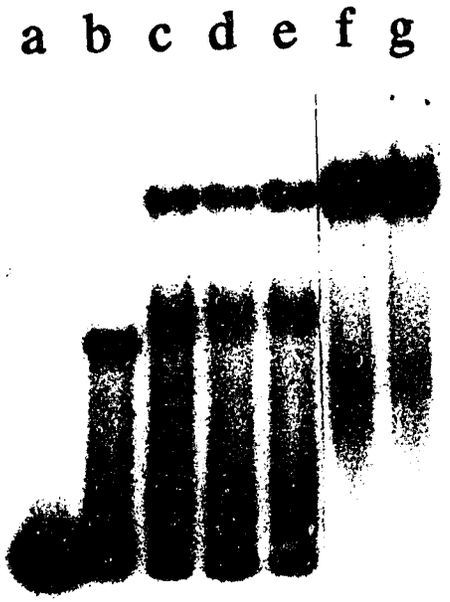

a b c d e f g

Figure 17. Binding of mAb A517A1-CRP and mAb 66C3-CRP to *Hpa*II fragments of *lac* P+. *Hpa*II fragments of [^{32}P] *lac* P+ were prepared by incubation with 4 units of *Hpa*II restriction enzyme per pmol [^{32}P] *lac* 203 bp fragment (labelled at both ends) in *Hpa*II restriction buffer for 10 minutes at 37° C. The binding assay conditions are described under "Materials and Methods" with 6.7 nM of each of the *Hpa*II fragments of [^{32}P] *lac* DNA, 100 μM cAMP, 133 nM CRP and indicated mAb in final volume of 15 μl. S1, position of *Hpa*II large fragment from -140 bp to -20 bp containing CRP Site 1; S2, position of *Hpa*II small fragment from -20 bp to + 65 bp containing CRP Site 2; CRP-S1, position of CRP-*Hpa*II small fragment complex. Lane a, [^{32}P] *lac* P+ only; Lane b-j contained *lac* DNA, cAMP-CRP and indicated as follows: b, no mAb; c, 0.16 μM mAb A517A1; d, 0.33 μM mAb A517A1; e, 0.65 μM mAb A517A1; f, 1.33 μM mAb A517A1; g, 0.16 μM mAb 66C3; h, 0.33 μM mAb 66C3; i, 0.65 μM mAb 66C3; j, 1.33 μM mAb 66C3.

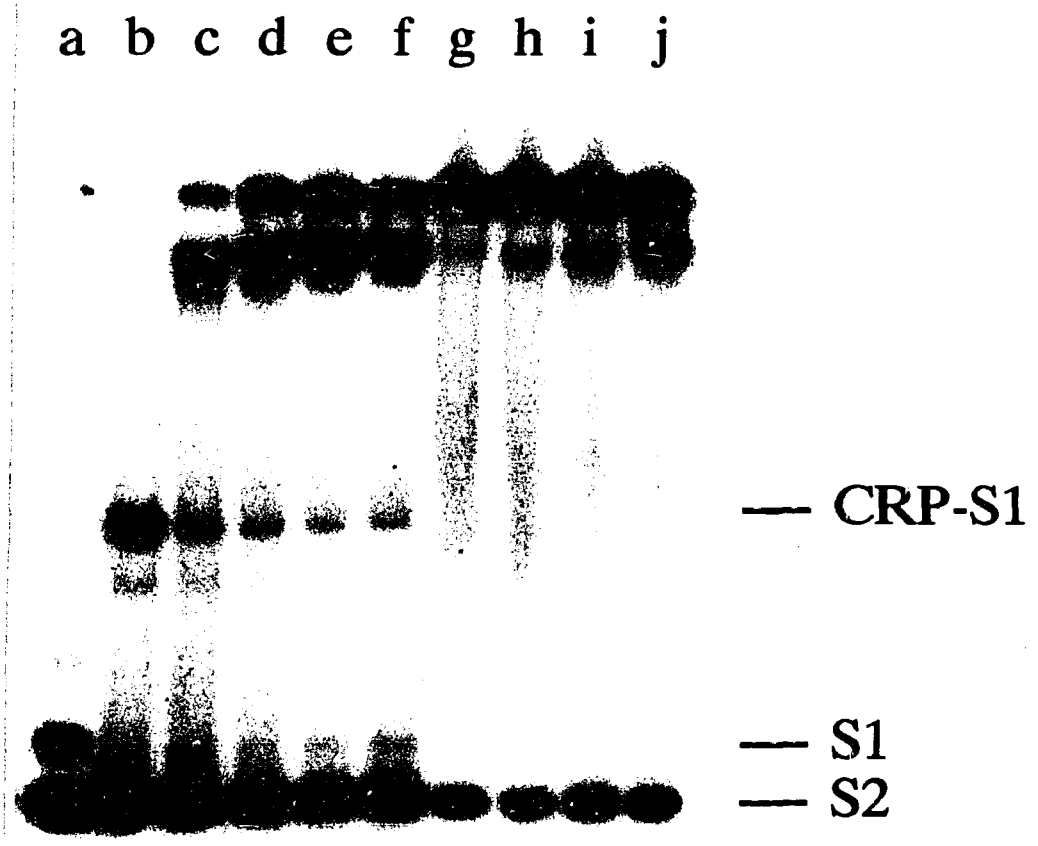

a b c d e f g h i j
— CRP-S1
— S1
— S2

Figure 18. Effect of mAb 64B4 on binding of cAMP by CRP and CRP*598. The incubation mixtures contained (final volume 100 μL): 40 mM Tris-HCl (pH 8.0), 400 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 100 μg casein, 2 μM [^{3}H]-cAMP (266 cpm/pmol), 0.5 μM CRP or CRP*598 and the indicated concentration of mAb 64B4. After 30 min at 0°C 600 μL of saturated ammonium sulfate was added. After an additional 10 min at 0°C the mixture was centrifuged at 10,000 rpm for 5 min. The pellet was dissolved in 500 μl H_2O and the radioactivity determined in Scintisol. CRP, ●-●; CRP*598, o-o.

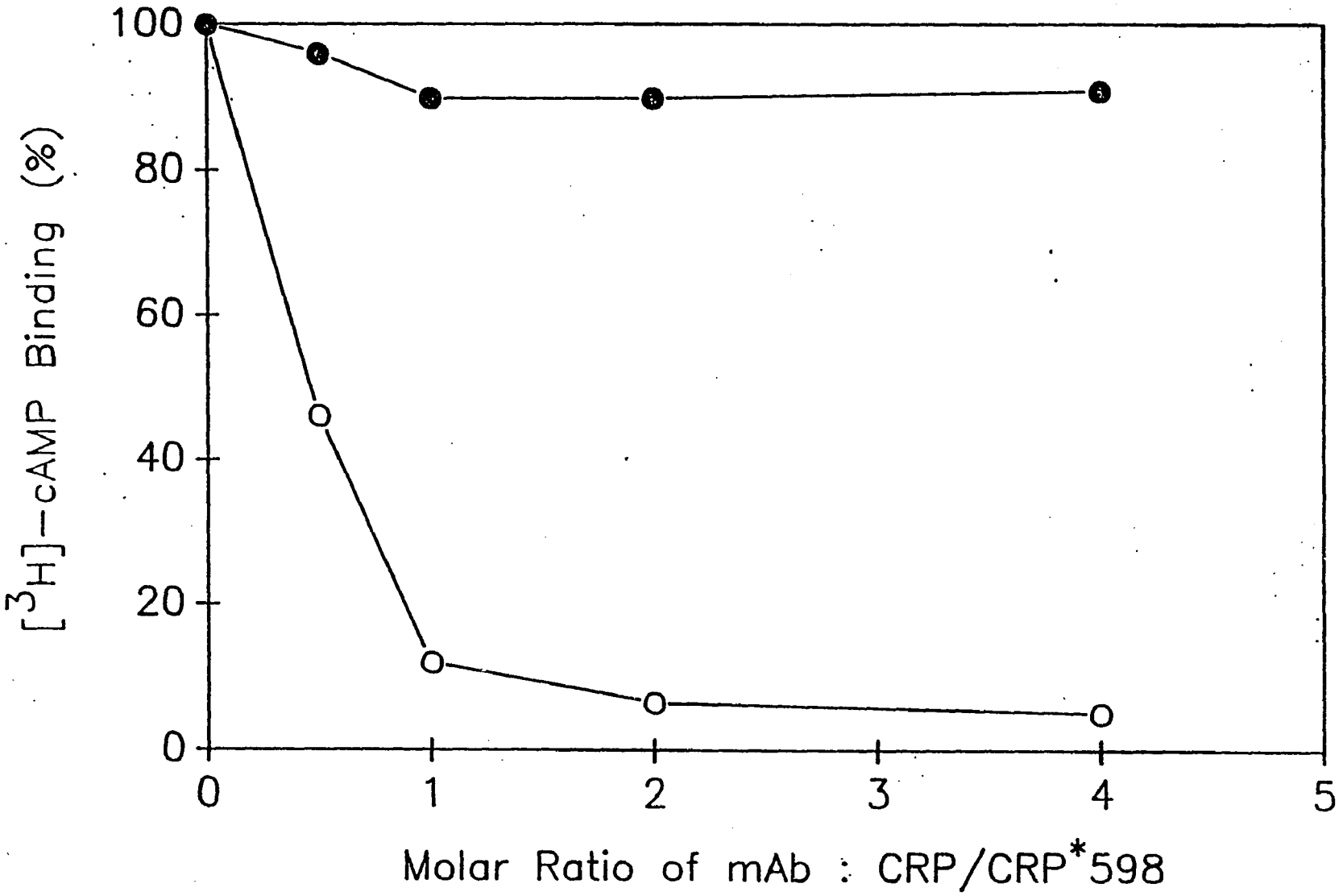

100
80
60
40
20
0
[3H]-cAMP Binding (%)
0
1
2
3
4
5
Molar Ratio of mAb : CRP/CRP*598

Figure 19. Effect of mAb 64B4 on binding of cGMP by CRP and CRP*598. The reaction conditions are described under Figure 18 legend with exception of 2 μM [^{3}H]-cAMP replaced by [^{3}H]-cGMP (200 cpm/pmol). CRP, ●-●; CRP*598, o-o.

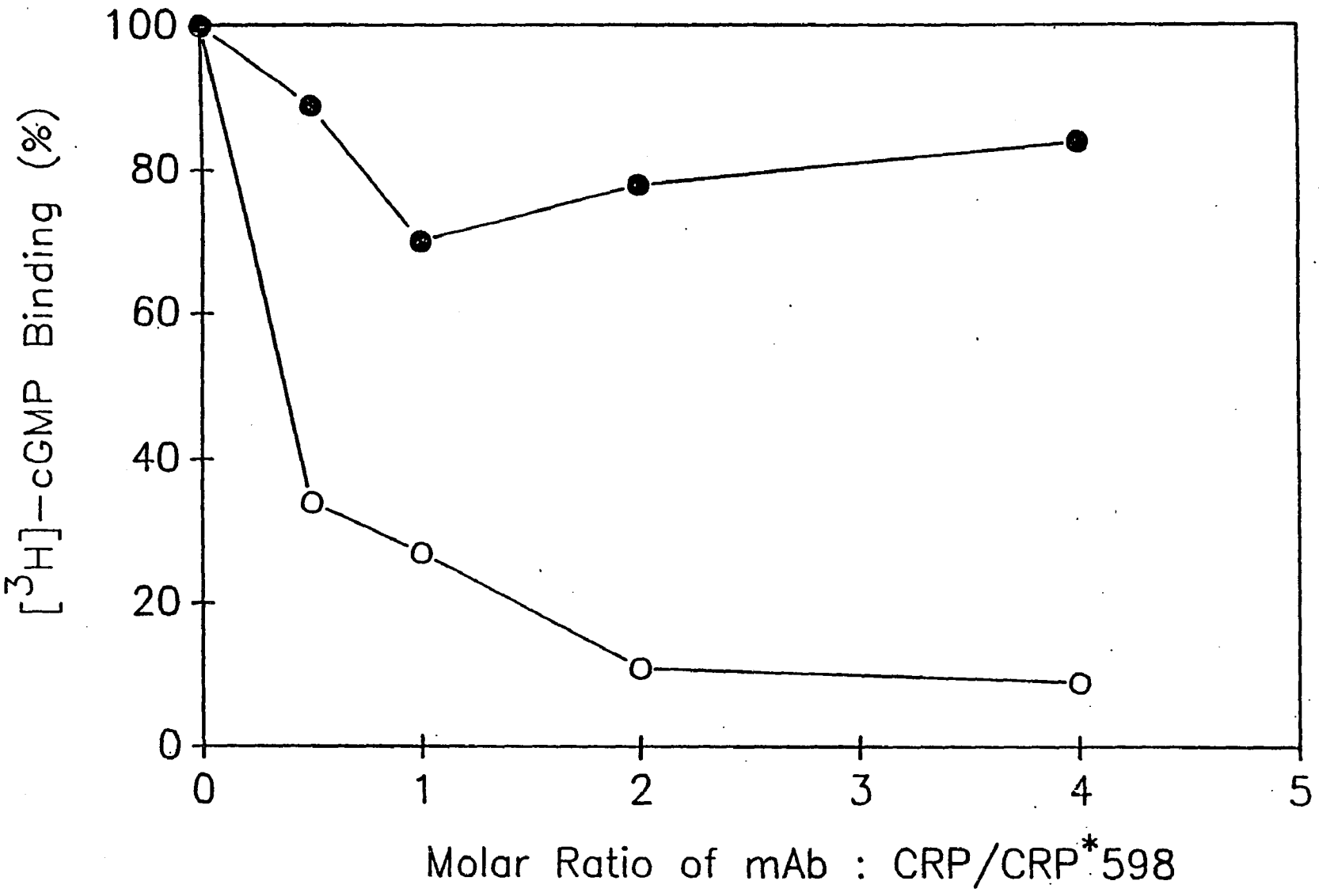
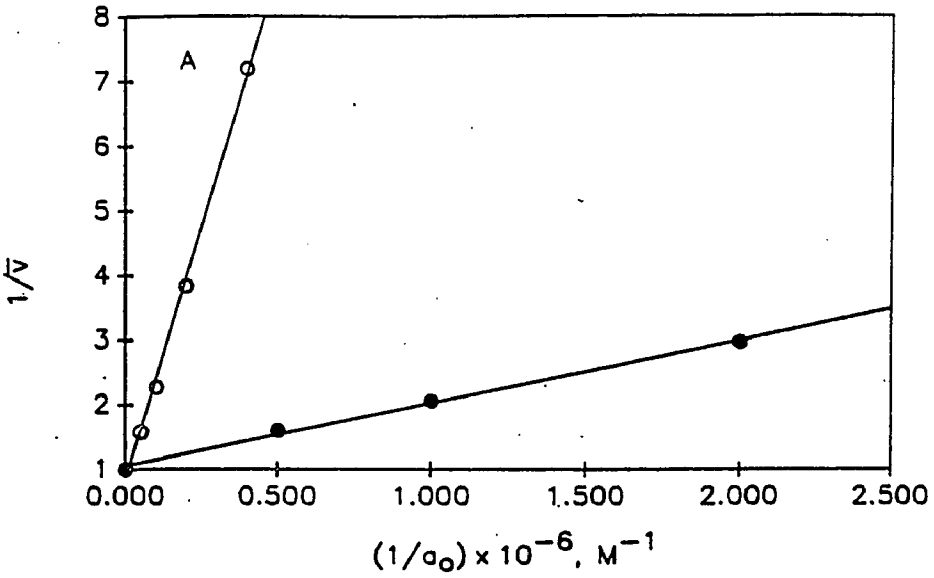
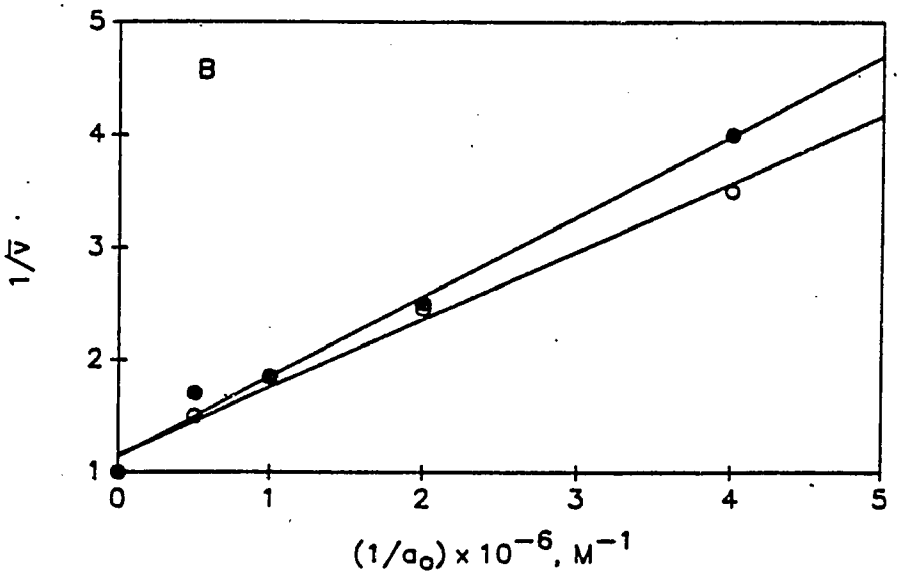

100
80
60
40
20
0
[3H]-cGMP Binding (%)
0
1
2
3
4
5
Molar Ratio of mAb : CRP/CRP*598

Figure 20. Klotz plots of the binding of CRP or CRP*598 to mAb 62D6. Decreasing concentrations of CRP (from 2 μM) and CRP*598 (from 20 μM) were incubated with a constant amount (2 nM) of mAb 62D6. The measurements were performed as described in Friguet *et al.* (1985). a_o is the total antigen concentration and ν is the fraction of the bound antibody. Panel A, no cyclic nucleotide; panel B, plus 25 μM cAMP; panel C, plus 25 μM N^6-butyryl-cAMP. CRP, ●-●; CRP*598, o-o.


A
8
7
6
5
4
3
2
1
1/v̄
0.000
0.500
1.000
1.500
2.000
2.500
(1/a_o) x 10^-6, M^-1


B
5
4
3
2
1
1/v̄
0
1
2
3
4
5
(1/a_o) x 10^-6, M^-1

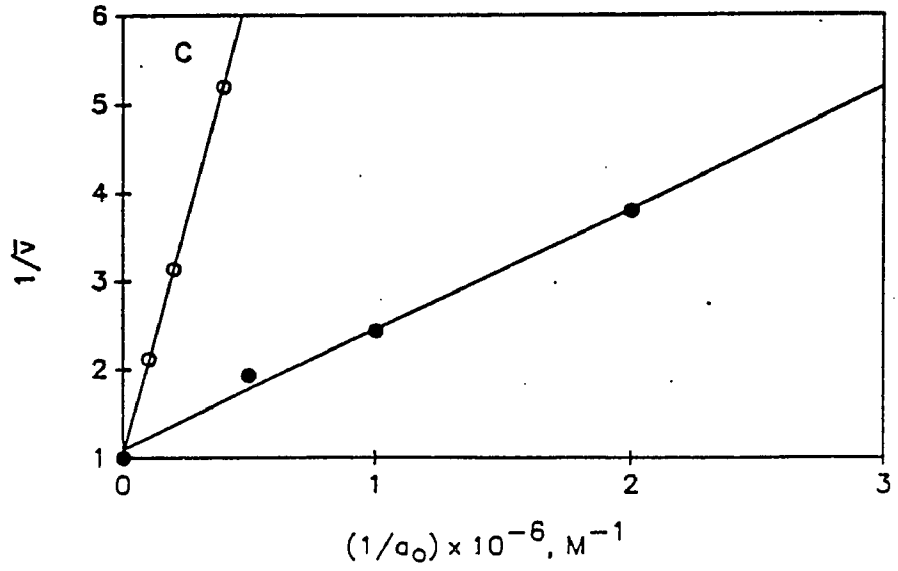
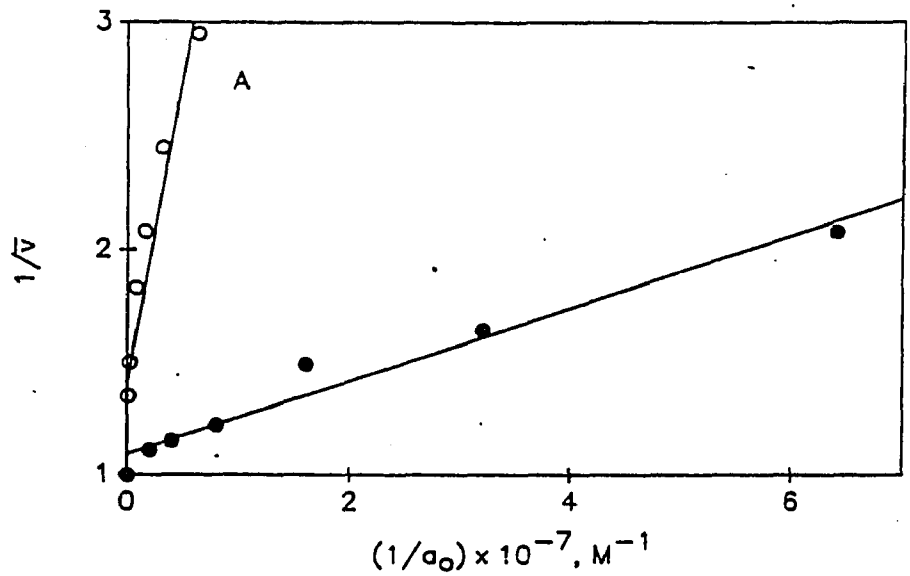
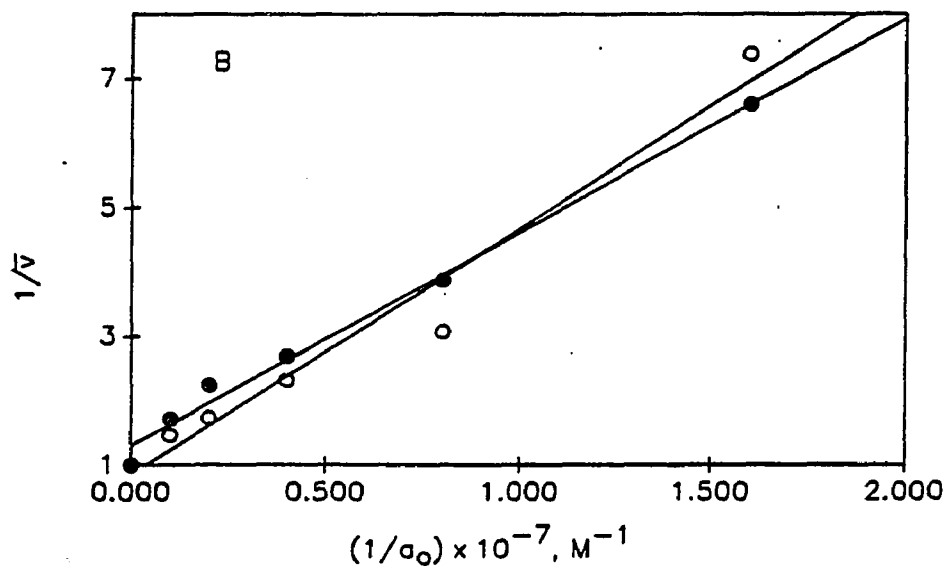

C
6
5
4
3
2
1
1/v̄
0
1
2
3
(1/a_o) x 10^-6, M^-1

Figure 21. Klotz plots of the binding of CRP or CRP*598 to mAb 66C3. Decreasing concentrations of CRP (from 1 μM) and CRP*598 (from 10 μM) were incubated with a constant amount (2 nM) of mAb 66C3. The measurements were performed as described in Friguet *et al.* (1985). a_o is the total antigen concentration and ν is the fraction of the bound antibody. Panel A, no cyclic nucleotide; panel B, plus 25 μM cAMP; panel C, plus 25 μM N^6-butyryl-cAMP. CRP, ●-●; CRP*598, o-o.


A
3
2
1
0
2
4
6
$1/\bar{v}$
$(1/a_o) \times 10^{-7}, M^{-1}$


B
7
5
3
1
0.000
0.500
1.000
1.500
2.000
$1/\bar{v}$
$(1/a_o) \times 10^{-7}, M^{-1}$

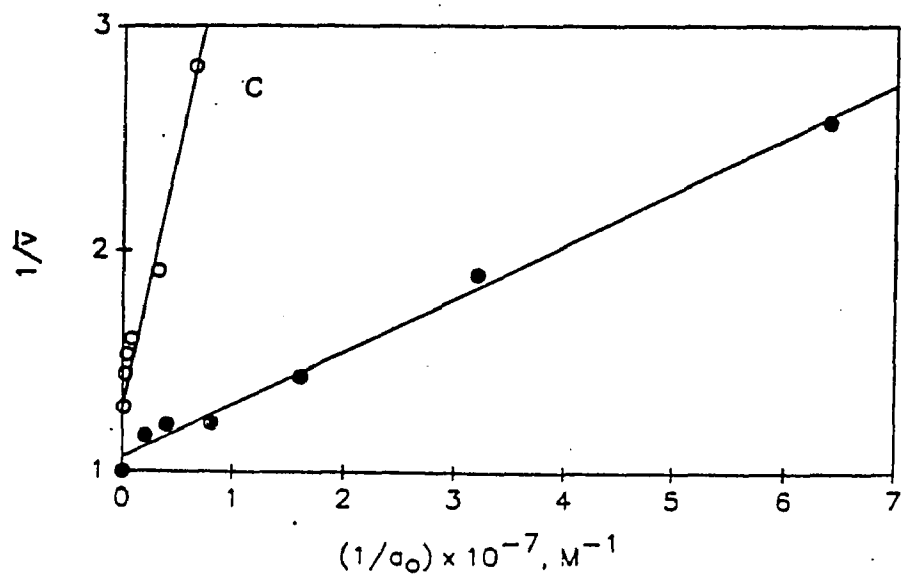
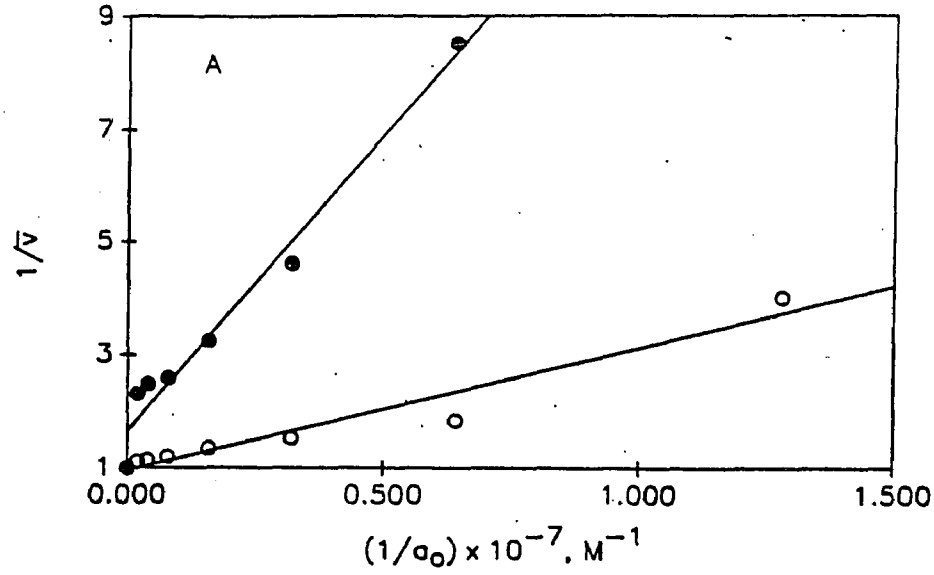
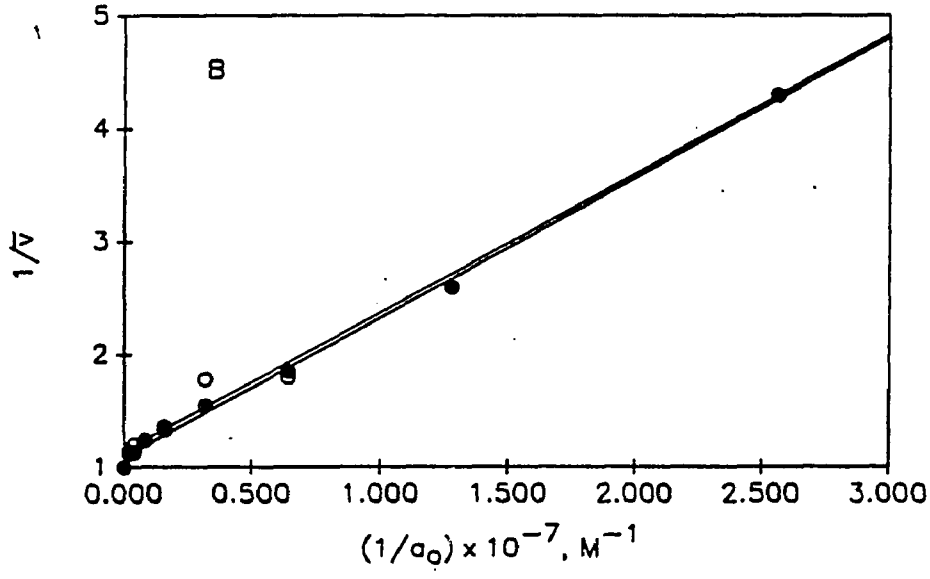

C
3
2
1
0
1
2
3
4
5
6
7
$1/\bar{v}$
$(1/a_o) \times 10^{-7}, M^{-1}$

Figure 22. Klotz plots of the binding of CRP or CRP*598 to mAb A54D3. Decreasing concentrations of CRP or CRP*598 (from 5 μM) were incubated with a constant amount of impure mAb A54D3. The measurements were performed as described in Friguet *et al.* (1985). a_o is the total antigen concentration and ν is the fraction of the bound antibody. Panel A, no cyclic nucleotide; panel B, plus 25 μM cAMP; panel C, plus 25 μM N^6-butyryl-cAMP. CRP, ●-●; CRP*598, o-o.


A
9
7
5
3
1
$1/\bar{\nu}$
0.000
0.500
1.000
1.500
$(1/a_o) \times 10^{-7}$, M^{-1}


B
5
4
3
2
1
$1/\bar{\nu}$
0.000
0.500
1.000
1.500
2.000
2.500
3.000
$(1/a_o) \times 10^{-7}$, M^{-1}

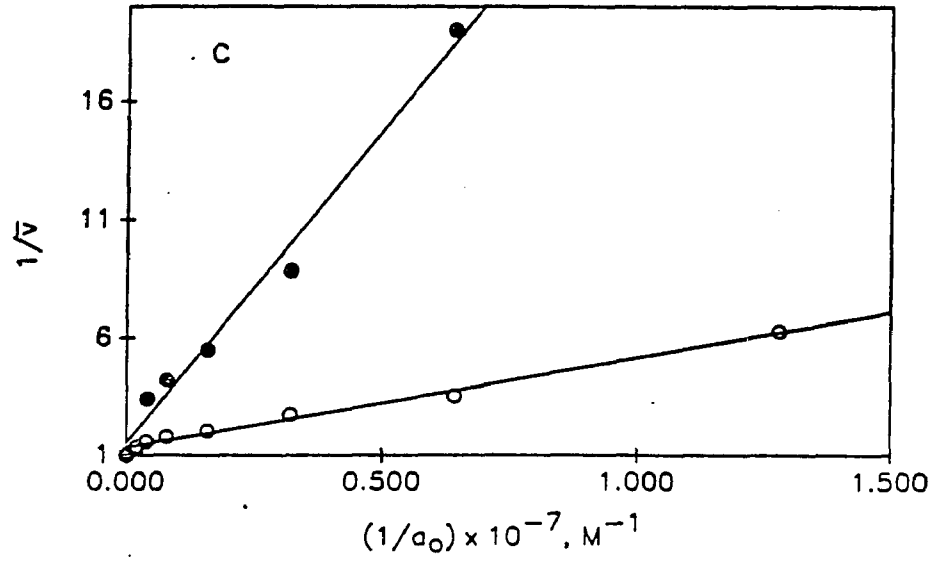
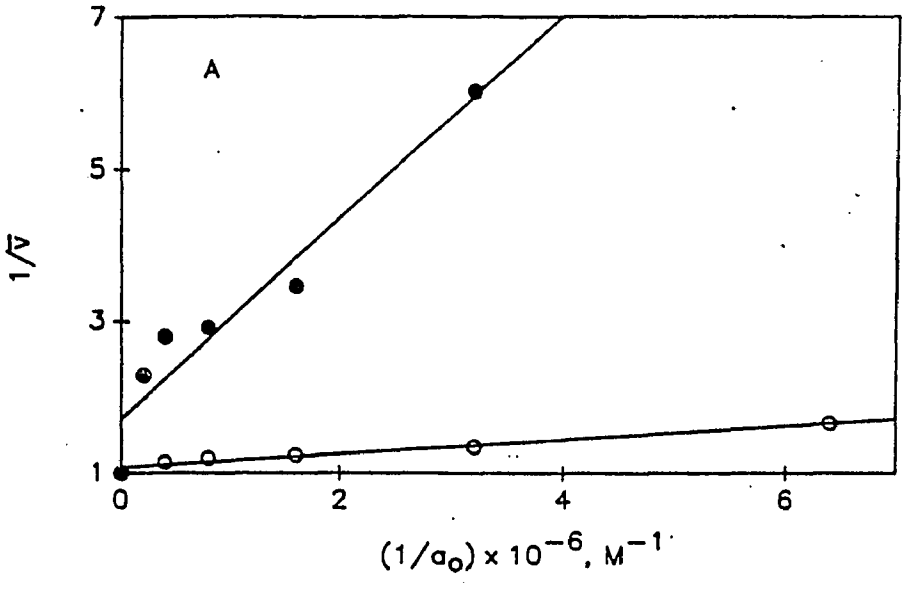
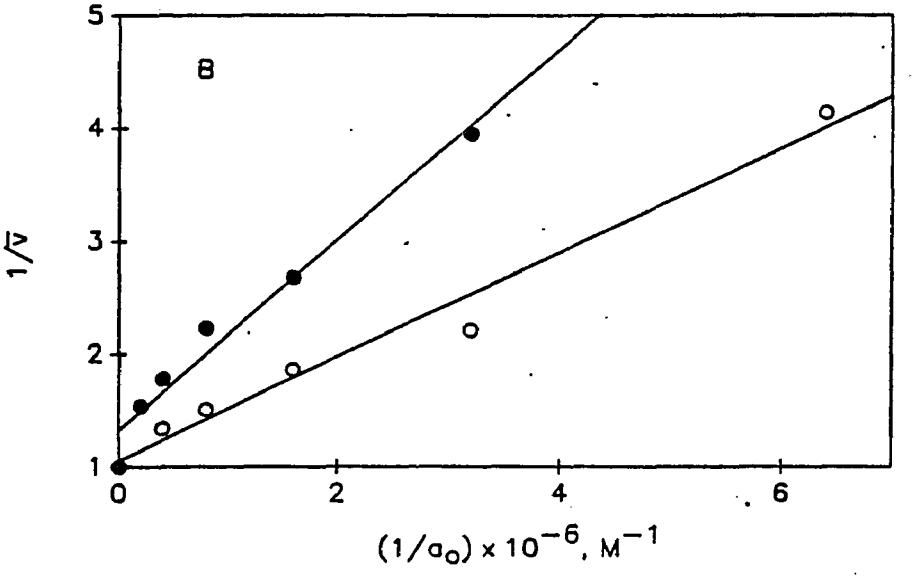

C
16
11
6
1
$1/\bar{\nu}$
0.000
0.500
1.000
1.500
$(1/a_o) \times 10^{-7}$, M^{-1}

Figure 23. Klotz plots of the binding of CRP or CRP*598 to mAb A521C6. Conditions used are described in the legend to Figure 22. a_o is total antigen concentration and ν is the fraction of the bound antibody. Panel A, no cyclic nucleotide; panel B, plus 25 μM cAMP; panel C, plus 25 μM N^6-butyryl-cAMP. CRP, ●-●; CRP*598, o-o


A
7
5
3
1
0
2
4
6
$1/\bar{v}$
$(1/a_o) \times 10^{-6}$, M^{-1}


B
5
4
3
2
1
0
2
4
6
$1/\bar{v}$
$(1/a_o) \times 10^{-6}$, M^{-1}

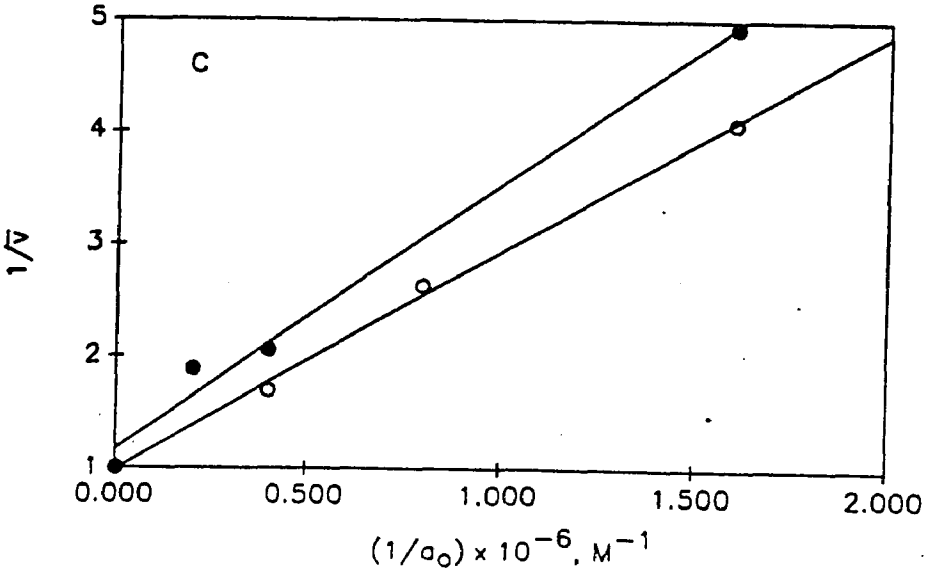

C
5
4
3
2
1
0.000
0.500
1.000
1.500
2.000
$1/\bar{v}$
$(1/a_o) \times 10^{-6}$, M^{-1}

Figure 24. Cleavage map of CRP and localization of mAb binding sites. Data obtained from Western blotting and ELISA were used to localize the binding sites of the anti-CRP and anti-CRP*598 mAbs. Segments labelled A-F represent the α helical regions of cAMP-CRP/CRP*598.

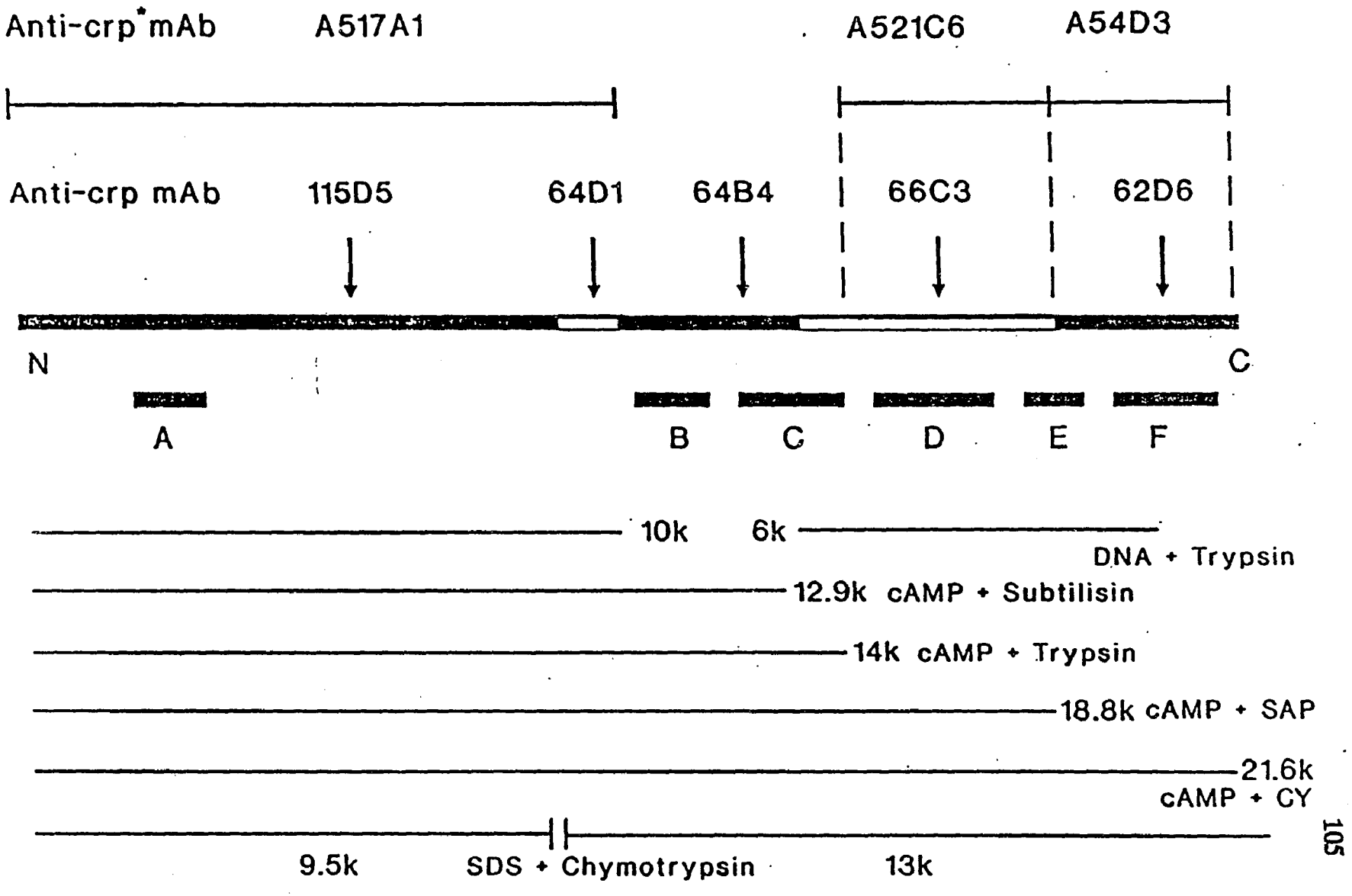
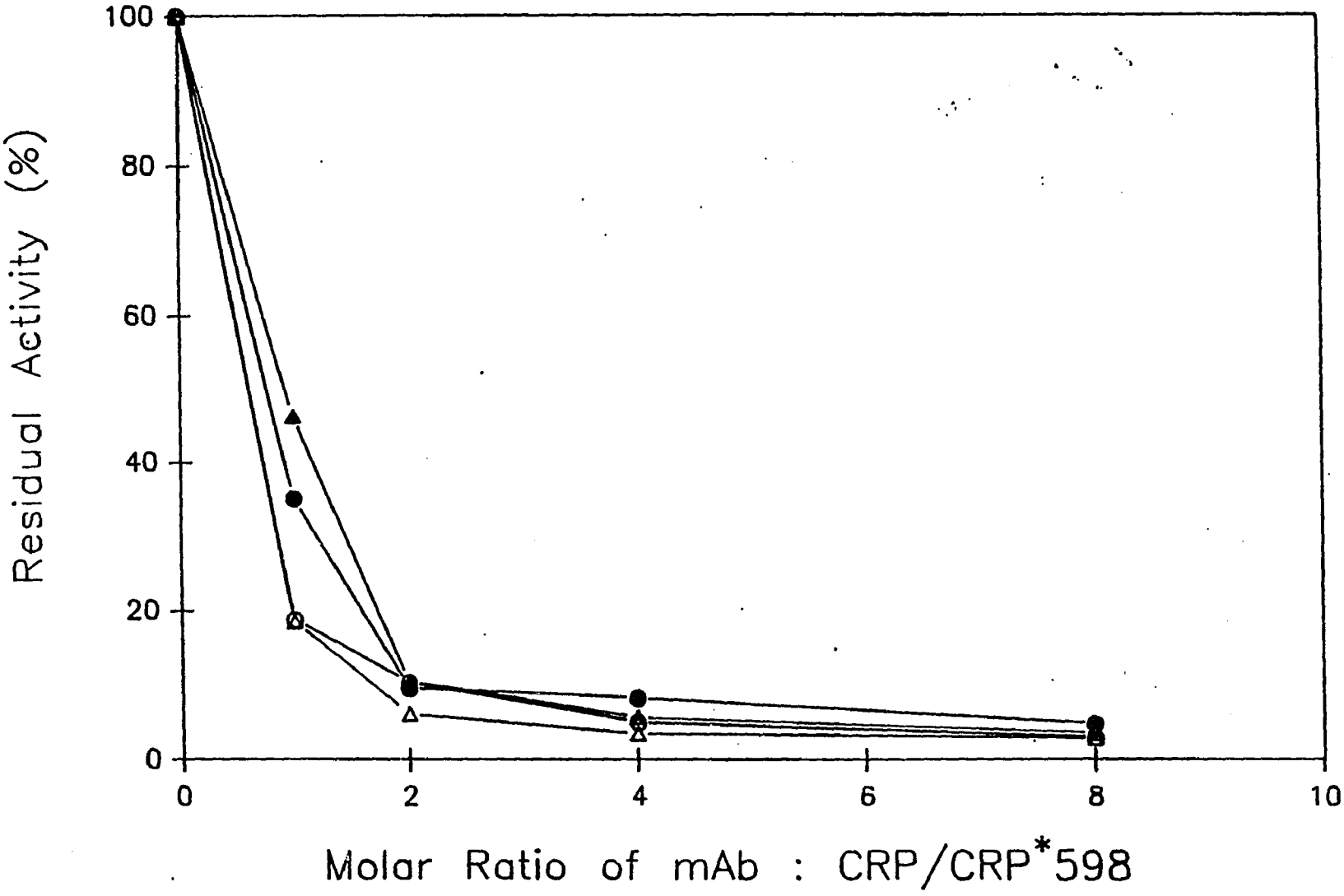

Anti-crp* mAb
A517A1
A521C6
A54D3
Anti-crp mAb
115D5
64D1
64B4
66C3
62D6
N
C
A
B
C
D
E
F
10k
6k
DNA + Trypsin
12.9k cAMP + Subtilisin
14k cAMP + Trypsin
18.8k cAMP + SAP
21.6k
cAMP + CY
9.5k
SDS + Chymotrypsin
13k

Figure 25. Effect of mAb A517A1 concentration on abortive initiation from the *lac* P^+ promoter. Abortive initiation conditions are described under "Materials and Methods" with a constant concentration of CRP/CRP*598 and the indicated concentration of mAb A517A1. ▲-▲, mAb A517A1 was added after the formation of the cAMP-CRP-*lac* P^+ complex, and after 10 minutes incubation RNA polymerase was added; ▵-▵, mAb A517A1 was added after the formation of RP_o with CRP; o-o, mAb A517A1 was added after the formation of cAMP-CRP*598-*lac* P^+ complex, and after 10 minutes incubation RNA polymerase was added; ●-●, mAb A517A1 was added after the formation of RP_o with CRP*598.


Residual Activity (%)
100
80
60
40
20
0
0
2
4
6
8
10
Molar Ratio of mAb : CRP/CRP*598

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