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MONOCLONAL ANTIBODIES DIRECTED AGAINST THE CYCLIC AMP RECEPTOR PROTEIN OF *ESCHERICHIA COLI* AS PROBES OF STRUCTURE AND FUNCTION

by

Xiao-Miao Li

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

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ABSTRACT

MONOCLONAL ANTIBODIES DIRECTED AGAINST THE CYCLIC AMP RECEPTOR PROTEIN OF *ESCHERICHIA COLI* AS PROBES OF STRUCTURE AND FUNCTION

by

Xiao-Miao Li

Adviser: Joseph S. Krakow

Monoclonal antibodies (mAb) against the *E. coli* cAMP receptor protein (CRP) have been isolated and characterized. The anti CRP mAbs fall into two groups: Class I mAbs bind only to native but not urea denatured CRP. Class II mAbs bind equally well to both native and urea denatured CRP. The locations of the antigenic determinants of the mAbs have been studied. All Class I mAbs studied are located in the C-terminal domain of CRP and all Class II mAbs studied are located in the N-terminal domain. Class II mAb 64D1 strongly inhibits cAMP binding by CRP by altering the conformation of CRP and consequently also inhibits binding of CRP to the *lac* promoter and abortive initiation by RNA polymerase. CRP in the preformed open promoter complex is protected from attack by mAb 64D1 indicating that binding of RNA polymerase stabilizes the cAMP-CRP complex. The antigenic

determinants of mAb 66C3 and mAb 63B2 are located at the hinge region of CRP joining the large and small domains. Both mAbs are strong inhibitors of transcription initiation from the lac promoter; mAb 63B2 inhibits DNA binding by CRP while mAb 66C3 enhances this binding. DNase I footprinting indicates that cAMP-CRP-mAb 66C3 binds to CRP Site 1 (-50 to -70 bp) and also to CRP Site 2 (-10 to +10 bp) which overlaps part of the RNA polymerase binding site. 50% of the preformed open promoter complex resists attack by mAb 66C3. The results imply that two states of CRP in the open promoter complex might exist due to the asymmetry in the cAMP-CRP complex. Protein-protein interactions between CRP and RNA polymerase are also suggested by a comparison of the DNase I footprint patterns of lac P^{+} and lac L8UV5 complexes.

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TABLE OF CONTENTS

Abstract..iii

Acknowledgments...v

Table of contents...vi

List of Tables..viii

List of Figures...ix

Abbreviations...xi

Introduction..1

Materials and Methods.......................................11

1. Source of materials......................................11

2. Production of monoclonal antibodies......................11

3. Solid phase ELISA..13

4. Protein purification.....................................13

5. Preparation of labeled proteins..........................14

(1) [^{3}H]CRP labeling.....................................14

(2) [^{125}I]mAb labeling.................................14

6. DNA fragment purification................................15

7. Preparation of labeled lac DNA...........................15

(1) [^{3}H]lac P$^+$ labeling...............................15

(2) 3'-end [^{32}P]lac P$^+$ labeling.....................16

(3) 5'-end [^{32}P]lac P$^+$ labeling.....................17

8. Proteolytic cleavage of CRP or CRP-mAb complexes.17

(1) CRP core preparation....................................17

(2) 9.5 KDa and 13 KDa fragment formation and purification..18

(3) 10 KDa and 6 KDa fragment digestion.....................18

9. Western blotting...19

10. [^{3}H]cAMP binding assay..........................20

11. Gel electrophoresis for detecting
protein-DNA or protein complexes.................20

12. Immunoprecipitin assay for mAb
containing complexes.............................21

13. Sandwich immunoassay for mAb binding.............21

14. Abortive initiation assay........................22

15. DNase I footprinting.............................23

Results..25

1. Characterization of nine mAbs....................25

2. Characterization of mAb 64D1.....................31

3. Characterization of mAb 66C3.....................36

4. Comparison of mAb 66C3 and mAb 115D5.............40

5. Glycerol effect on protein-DNA interaction.......41

Discussion...43

Tables...60

Figures..67

Bibliography..113

LIST OF TABLES

1. Binding of mAbs to native and denatured CRP......64

2. Binding of Class I mAbs to CRP and Cores.........65

3. Effect of anti-CRP mAbs on lac P^+ DNA
directed abortive initiation.....................66

4. Effect of anti-CRP mAbs on cAMP binding..........67

5. Summary of functional properties of
anti-CRP mAbs....................................68

6. Immunoprecipitin assay for binding of
mAbs to CRP and CRP-DNA complexes................69

7. Lack of binding of [^{125}I]mAb 64D1
to mAb 64D1-CRP..................................70

LIST OF FIGURES

1. The amino acid sequence and DNA sequence of CRP....................................71
2. Drawing of the CRP dimer..........................73
3. Western blot analysis of binding of mAbs to CRP fragments............................75
4. Cleavage map of CRP and localization of mAb binding sites................................77
5. Effect of anti-CRP mAbs on binding of cAMP-CRP to the lac DNA promoter fragment........79
6. Gel electrophoresis analysis of binding of mAbs to CRP in solution...............81
7. Effect of time of preincubation with mAb 64D1 on stability of CRP complexes...........83
8. Effect of time of incubation with mAb 64D1 on CRP-DNA complexes.............................85
9. Effect of cAMP concentration on stability of CRP complexes..................................87
10. Effect of cAMP and cGMP on binding of [^{125}I]mAb 64D1 to CRP............................89
11. Effect of proteases on cAMP-CRP and mAb 64D1-CRP.91
12. Effect of subtilisin on CRP and mAb 64D1-CRP.....93
13. Effect of mAb 64D1 concentration on sensitization of CRP to attack by trypsin and subtilisin..................................95

14. Effect of cAMP and cGMP on binding of [125I]mAb 66C3 to CRP....97

15. Effect of mAb 66C3 on abortive initiation from the lac P+ promoter....99

16. Effect of mAb 66C3 on the stability of the initiation complex....101

17. Effect of mAb 66C3 concentration on abortive initiation from the lac P+ promoter....103

18. Effect of mAb 66C3 on binding of CRP and RNA polymerase to lac P+....105

19. Effect of mAb 66C3 on binding of CRP and RNA polymerase to lac L8UV5....107

20. Binding of mAb 66C3 and mAb 115D5 to Hpa II fragments of lac P+ and lac L8UV5....109

21. Comparison of the effects of mAb 115D5 and mAb 66C3 on binding of CRP and RNA polymerase to lac P+....111

22. Effect of glycerol on lac DNA binding by CRP and RNA polymerase....113

23. Effect of glycerol on abortive initiation from lac P+ and lac L8UV5....115

Abbreviations used:

CRP: cyclic AMP receptor protein;

mAb: monoclonal antibody;

cAMP: 3', 5'-cyclic AMP;

cGMP: 3', 5'-cyclic GMP;

ApA: adenylyl (3', 5,)adenosine;

lac: the lactose operon;

gal: the galactose operon;

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

RP_o: the open initiation complex;

Da: dalton;

KDa: kilo-dalton;

DMEM: Dulbecco's Modified Eagle's Medium;

ELISA: enzyme-linked immunosorbent assay;

PEG: polyethelene glycol;

BSA: bovine serum albumin;

SDS: sodium dodecyl sulfate;

EDTA: (ethylenedinitrilo) tetra-acetic acid;

DTT: dithiothreitol;

TCA: trichloroacetic acid;

Tris: Tris-(hydroxymethyl) aminomethane;

PBS: phosphate buffered saline;

WB: washing buffer;

BB: blocking buffer;

INTRODUCTION

The cAMP receptor protein (CRP also referred to as the catabolite gene activator protein, CAP) regulates transcription of at least 25 genes in E. coli (for general review see Ullman and Danchin, 1983 and de Crombrugghe et al.,1984). In the absence of cAMP, CRP retains only a nonspecific DNA binding activity. In the presence of cAMP, the cAMP-CRP complex binds to specific DNA sites located at or near a promoter and affects the rate of transcription initiation. Although the major role of CRP is to activate transcription from most catabolite repressible genes, in some systems such as the crp promoter (Aiba, 1983) it acts as a repressor. In the lac (Malan and McClure, 1984) and gal (Musso et al., 1977) operons, in which there are two overlapping promoters, the binding of cAMP-CRP complex stimulates transcription from one promoter while inhibiting the other. In araBAD (Lee et al., 1981), malK-malB and malE,F,G (Chapon,1982) operons, an additional protein, the Ara C protein or the Mal T protein, is also required for activating transcription.

CRP is a dimeric protein comprised of two identical subunits of 209 amino acids. The amino acid sequence and DNA sequence of CRP molecule is shown in Figure 1. The crystal structure of a CRP dimer complexed with cAMP has been determined at 2.9 A resolution (McKay and Steitz, 1981 and McKay et al., 1982) (Fig. 2). Each subunit has two

distinct structural domains. The large N-terminal domain from residues 1 to 135 has an approximate overall dimension of 25x30x35 A. It is classified as an anti-parallel beta structure. There is an eight-stranded antiparallel beta roll forming a pocket, which forms a major part of cAMP binding site. The large domain also contains a 40 A long C-alpha helix which connects the two domains and partially closes off one end of the cAMP binding pocket. The smaller C-terminal domain from residues 136 to 209 with about 20x20x30 A dimension contains three alpha helices; D,E and F. The three-helix motif is similar to that found in the lambda cro repressor, the CRP E and F helices have a conformation similar to the alpha 2 and 3 helices in the cro repressor (Steitz et al., 1982). The F-helix which clearly protrudes from the surface of the CRP dimer provides the major interaction sites with DNA targets, and the two F-helices run approximately parallel to each other at a distance of 34 A. Although there is a cleft between the two domains noncovalent contacts between the domains stabilize the relative orientation of the domains. The major contact region between the domains is located between beta roll 5 of the large domain and the E-helix of the small domain. However, in one of the subunits of the CRP dimer there is an additional contact between the two domains. It is the contact between Asn-65 of beta roll 5 and Gln-153 located between D-helix and beta roll 9. The extra contact in one of the two subunits results in an

asymmetric conformation of the CRP molecule. In one subunit, the two domains form an open cleft. The other subunit is in a closed conformation. Therefore there is no unique dyad axis relating them. If the two N-terminal domains of the two subunits are superimposed on each other, the C-terminal domains are oriented at an angle as large as 28° (McKay and Steitz, 1981). The subunit-subunit contacts are exclusively located between the N-terminal domains mainly provided by the C-helix and part of the beta rolls. The two C-terminal domains have no direct interaction.

The bound cAMP molecule is completely buried within the interior of the beta rolls of the large domain. In the crystallographic structure, cAMP is in an *anti* conformation, the only position of the adenine ring which is accessible to the solvent is the C8 position (McKay et al., 1982). Its phosphoribose moiety is adjacent to the beta rolls 6 and 7, and its adenine ring is oriented toward the pair of C-helices in the dimer interface. The phosphate group interacts with residues Ser-82 and Ala-83, the ribose moiety is bound by residues Trp-84 and Leu-72. The adenine ring interacts with residue Ser-127 from the same subunit and residue Glu-128 from the other subunit (McKay et al., 1982). By analyzing the effects of cAMP analogs on CRP structure and function, Ebright et al. (1985) proposed a cAMP-CRP binding model in solution, in which cAMP is bound in the *syn* conformation. They proposed that the tight contacts occur only at the phosphoribose

moiety. The N-6, C-2 face of cAMP is directed outward to the solvent, instead of the N-8 face as suggested by the cAMP *anti* conformation model based on the crystallographic structure of CRP (McKay et al., 1982). The *syn* model is also supported by a nuclear magnetic resonance study of the cAMP-CRP conformation (Gronenborn and Clore, 1982). In both models hydrogen bonds between adenine N-6 of cAMP and amino acid residues of CRP are important.

Binding of cAMP to CRP elicits conformational changes in the protein. Free CRP is resistant to cleavage by trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease and subtilisin. The cAMP-CRP complex is cleaved rapidly by all the four proteases (Krakow and Pastan, 1973 and Eilen et al., 1978). The intersubnit disulfide cross-linking of CRP by dithiobis-2-nitrobenzoic acid (DTNB) is markedly enhanced in the presence of cAMP (Eilen and Krakow, 1977). Apparently, binding of cAMP to CRP brings the two subunits closer. The cysteine 178 in each subunit come close enough to form the disulfide bond. In fact, small-angle X-ray scattering experiments detected a decrease of 4.0 A in the radius of gyration of CRP upon binding to cAMP (Kumar et al., 1980).

Another change in CRP elicited by binding of cAMP is an increased affinity for specific DNA sites. Without cAMP, CRP shows non-specific DNA binding mainly through formation of salt bridges between the DNA phosphate backbone groups and the basic amino acid side chains of

CRP. The non-specific complex formed has CRP and DNA separated by up to 12 A thus enabling CRP to move freely along DNA to search for specific binding sites (Weber and Steitz, 1984). For specific CRP-DNA binding, hydrogen bonds between DNA specific regions and CRP are involved which increase the affinity of CRP for DNA by six fold (Weber and Steitz, 1984). CRP can search for the proper hydrogen bonds as it is "sliding" along the DNA chain, (Weber et al. 1984 suggested that CRP can move in and out of the DNA grooves at a rate faster than "sliding"), until the correct base sequence is reached.

Comparative analysis of the sequences protected by cAMP-CRP against DNase I digestion and by methylation protection experiments with various promoters suggested the consensus sequence TGTGA is critical for CRP binding. Genetic data emphasize the importance of the consensus sequence. Point mutations in gal (Busby et al., 1982; Busby and Dreyfus, 1983) and lac (Hopkins, 1974; Majors, 1975) which prevent stable CRP binding are located in this region. Deletion in this region prevents CRP binding (Busby et al., 1983). In rare cases like tnaA and lac promoters, an inverted repeat sequence was found 6 base pairs downstream from the TGTGA motif (Deeley and Yanofsky, 1982; Dickson et al., 1977). In most cases, this symmetry is not observed. But the non-symmetric sequence following the TGTGA motif is not unimportant. In the gal promoter, deletion in this region blocked CRP action in vivo and in

vitro (Taniguchi et al., 1979; Kolb et al., 1983). However the affinity of CRP for DNA appears to be higher when the symmetry is present.

Based on the crystallographic structure of CRP, a CRP-DNA specific binding model was proposed (McKay and Steitz, 1981; Mckay et al., 1982; Weber and Steitz, 1984). They suggested that the two F-helices of CRP penetrate into two successive major grooves of a left-handed B-DNA. However, if the DNA undergoes a right-handed to left-handed transition elicited by cAMP-CRP binding, the binding of cAMP-CRP to a closed circular DNA would greatly change its linking number when the DNA is nicked then resealed. Later experiments showed that only small changes in linking number were found when cAMP-CRP complex bound to a relaxed DNA containing either the lac or gal CRP binding site followed by closing the circle and release of the complex (Kolb and Buc, 1982). Therefore, another model was proposed, in which the N-terminal part of the F-helices enter the two successive major grooves of DNA in a way that is similar to the interaction of helix "3" of the lambda repressor (Pabo and Lewis, 1982; Steitz et al, 1983). In this arrangement, eight to nine base pairs in a region of fourteen base pairs will allow interaction with the F-helices. It is shorter than the CRP binding site. However, a bend in DNA would provide additional contacts with the DNA backbone. In fact, experiments strongly suggest that binding of cAMP-CRP to DNA causes

conformational changes in DNA. Addition of cAMP-CRP to the <u>lac</u> P^+ promoter strongly decreases its rotation time, which is determined by the length of the DNA helix (Porschke et al., 1984). cAMP-CRP induced bending of <u>lac</u> P^+ fragment containing the promoter region was identified by gel electrophoresis (Wu and Crothers, 1984). Interestingly, it was found that the bending center is not the symmetric center of the CRP binding site. There is a six base pairs shift from the symmetric center to the upstream direction, right in the middle of the TGTGA consensus sequence between -67 and -68.

A CRP conformational change induced on binding DNA is also suggested. In the presence of calf thymus DNA or synthetic double stranded or several single stranded DNAs at low ionic strength, CRP becomes sensitive to trypsin digestion, and the protease cutting sites differ from those seen with the cAMP-CRP complex (Angulo and Krakow, 1984). Furthermore, in contrast to the sensitivity of the cAMP-CRP complex to protease digestion the cAMP-CRP-DNA complex becomes resistant to trypsin, <u>Staph</u>. <u>aureus</u> V8 protease and subtilisin digestion but not to chymotrypsin (Angulo and Krakow, 1986). The experiment suggests that cAMP binding induces a conformational change in the C-terminal domain to a more "open" structure. The protease cutting sites from C-terminus up to residue-116 are exposed to the solvent. When this complex binds to DNA most of the available sites are protected. The cAMP analog analysis (Ebright et al.,

1985) also suggests there is the possibility that the class D analogs, which can bind to CRP and induce similar conformational changes on CRP like cAMP but fail to stimulate DNA binding may block a DNA induced conformational change in CRP because of the additional bulky group in the C-2 or N-6 position of the adenine ring. Since both amino acid residue-116 and the cAMP binding site are located in the N-terminal domain, the DNA binding induced conformational changes must involve in both domains.

The most puzzling question is how the cAMP-CRP complex interacts with RNA polymerase, and how this complex activates transcription. Two models for the mechanism of the transcription regulation by CRP have been proposed. The first one suggests that a direct interaction between the cAMP-CRP complex and RNA polymerase occurs resulting in either increasing the affinity of RNA polymerase for the promoter or increasing RNA polymerase activity (Majors, 1975). The second model suggests that on binding to its specific DNA target site the cAMP-CRP complex destabilizes neighboring regions of DNA thereby facilitating the formation of an open RNA polymerase-promoter complex (Dickson et al., 1975). Unfortunately, thus far, there is no direct experimental data to support either model. There is no evidence indicating that CRP-DNA binding induces any significant changes in DNA thereby directly facilitating RNA polymerase binding. cAMP-CRP-DNA complex formation

does not unwind DNA. In contrast, it is found that CRP binding stabilizes the DNA duplex (Unger et al., 1983). The significance of the DNA bending induced by cAMP-CRP remains unexplained. On the other hand, the CRP binding site of different promoters can vary from -36 to -106 and the TGTGA motif can be found on either the template or the non-template strand (de Crombrugghe et al., 1984). The differences in the location and the orientation of CRP sites indicate that the distance between the CRP site and the start of transcription is not critical. It argues against an unique direct CRP-RNA polymerase interaction. The real mechanism is still unknown.

It is clear that CRP is an allosteric protein. Each function requires certain conformational changes. If the conformational change is blocked the function will be lost. The development of the hybridoma techniques by Kohler and Milstein (1975) has made it possible to raise monoclonal antibodies (mAb) directed against specific determinants in a protein. Such mAbs can provide a series of site specific probes for studying the role of structure and conformation in the function of CRP.

Generally speaking, the antigenic sites bound by an antibody can be divided into two structural categories: segmental and assembled topographical. A segmental site exists wholly within a continuous segment of the amino acid sequence. An assembled topographic site consists of amino acid residues far apart in the primary sequence but brought

together in the surface topography of the native protein by the way it folds in three dimensions, therefore a conformational change of the protein may destroy the antigenic sites. However even with segmental determinants antibody binding appears to have a higher affinity for a preferred conformation. The recognition involves more than just a linear sequence of side chains. It depends on three dimensional conformation complementary between the antibody binding site and the antigenic determinant.

Previous immunological efforts have been limited to using polyclonal anti-CRP antibody prepared in rabbits to investigate the amount of CRP in E.coli extracts (Guiso and Blazy, 1980) and the presence of CRP-like proteins in several bacterial species (Anderson and Pastan, 1973).

The research comprising this dissertation was undertaken to study nine monoclonal antibodies raised against CRP. The locations of the epitopes of nine anti CRP mAbs and their effects on CRP functions were studied and the mechanisms of some interesting effects were explored. The relation of conformation and function is discussed.

MATERIALS AND METHODS

Materials: Reagents were obtained as follows: adenosyl 3'-5' adenosine (ApA), trypsin, chymotrypsin, subtilisin, calf thymus DNA, heparin, bovine serum albumin (BSA), toluidine blue, p-nitrophenylphosphate and DNase I, Sigma; Tween 80, J. T. Baker Chemical Co.; Staph. aureus V8 protease, Miles Laboratories; ATP, CTP, GTP, UTP and cAMP, P-L Biochemicals; [^{3}H]cAMP. [^{3}H]UTP, [^{3}H]dTTP, [alpha-^{32}P]dATP and [gamma-^{32}P]ATP, ICN; Phosphatase-coupled avidin, Zymed Laboratories; [^{3}H]N-ethylmaleimide, New England Nuclear; fetal calf serum, Sterile Systems Inc.; Dulbecco's modified Eagle's medium (DMEM), GIBCO; BA 85 nitrocellulose sheets and Elutip-d minicolumn, Schleicher and Schuell; phosphatase-coupled and peroxidase-coupled goat anti-mouse immunoglobulin, Kirkegaard and Perry Laboratories; Freund's complete and incomplete adjuvants, PEG 4000, E. Merck Inc.; Protein A-Sepharose, Pharmacia; DNA polymerase I Klenow fragment, EcoRI and PvuII restriction endonucleases, Boehringer Mannheim Corp.; Immunoprecipitin, Bethesda Research Laboratories; TEMED and bisacrylamide, Bio-Rad; acrylamide, Serva; Scintisol, Isolab; mice were obtained from Jackson Laboratories.

Production of Monoclonal Antibodies: SJL/J female mice (except for mAb 115D5 where C57Bl/6 mice were used) were injected with 100 ug CRP emulsified in Freund's complete

adjuvant. This was followed by three booster shots of 50 ug 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 $2X10^4$ 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 $2X10^4$ 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 ug CRP or the indicated fragment in 50 ul 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 ul wash buffer (WB: PBS, 2 mg/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 ul of culture supernatant or 0.1 ug mAb in 50 ul PBS plus 2 mg/ml BSA and the plate was incubated for 60 minutes at 37°C. After washing three times with WB 50 ul 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 ul of a solution containing 1 mg/ml p-nitrophenylphosphate in 0.1 M diethanolamine (pH 9.0) + 2.5 uM $MgSO_4$ was added and incubated for 30 to 60 minutes at 37°C. After addition of 100 ul 1 M NaOH the absorbance at 410 nm in each well was determined using a Dynatech Microelisa Reader.

Protein purification: CRP was isolated by the method of

Eilen et al. (1978) from an E.coli strain containing the recombinant plasmid pHA7 (Aiba et al., 1982) donated by Dr. H. Aiba. RNA polymerase was isolated from E. coli K12 purchased from Grain Processing Corp. by a modification of the method of Burgess and Jendrisak (1975). Protein concentrations were determined using the extinction coefficients: CRP, $E^{1\%}_{280nm}$ = 8.8 (Aiba and Krakow, 1981); RNA polymerase holoenzyme: $E^{1\%}_{280nm}$ = 6.7 (Levine et al., 1980).

Preparation of labeled proteins:

[^{3}H]CRP labeling: CRP was selectively labeled with N-ethylmaleimide at Cys-178 (Marco et al., 1982) by the following procedure. The reaction mixture contained (final volume 60 ul): 0.28 M potassium phosphate (pH 7.5), 20 uCi [^{3}H]N-ethylmaleimide and 18 ug CRP. After incubation for 60 minutes at 37°C 20 ul of 10 mM mercaptoethanol was added to terminate the reaction. The specific activity of the [^{3}H]NEM-CRP was 120,000 cpm/ug as determined by TCA precipitation.

[^{125}I]mAb labeling: [^{125}I]mAb was prepared by Hunter's method (1967). The reaction mixture contained (final volume 57.5 ul): 0.2 M sodium phosphate (pH 7.5), 0.86 mCi ^{125}NaI, 20 ug chloramine-T and 25.6 ug (160 pmol) mAb. After 1 minute at room temperature 5 ul of freshly prepared $Na_2S_2O_5$ (20 mg/ml in 0.1 M sodium phosphate, pH 7.5) was

added followed by 50 ul of 0.1 M KI. After an additional 5 minutes at room temperature the reaction mixture was applied to a Sephadex G25 column (10 mL bed volume) pre-equilibrated with PBS + BSA 1 mg/ml. 0.5 ml fractions were collected and the specific activity of the [^{125}I]mAb was determined by TCA precipitation.

DNA fragments purification: *E.coli* containing the *lac* P$^+$ promoter or *lac* L8UV5 promoter cloned into pMB9 by Dr. S. Fuller were obtained from Dr. A. Revzin. Plasmid DNA was prepared by a modification of the method of Marko et al. (1982), The 203 bp fragment from -140 to +63 containing the *lac* promoter was excised by digestion with *Eco*R I 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 DNA:

[^{3}H]lac P$^+$ labeling: The reaction mixture contained (final volume 650 ul): 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 52 nmol dATP, 60 uCi [^{3}H]dTTP (70 mCi/mmol), 26 ug *lac* P$^+$ DNA fragment and 26 units of DNA polymerase I Klenow fragment. After 30 minutes incubation at 37°C the unreacted [^{3}HdTTP was removed by adsorption of the DNA on a Elutip-d minicolumn. The [^{3}H]*lac* P$^+$ DNA was recovered by elution with 0.4 ml

20 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA. The labeled DNA was concentrated by addition of two volumes of 95% ethanol. After centrifugation the [^{3}H]lac DNA was dissolved in 100 ul TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and stored at 0°C.

3'-end [^{32}P]lac labeling: The reaction mixture contained (final volume 50 ul): 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 4 ug 203-bp lac fragment, 50 uCi [alpha-^{32}P]dATP (600 Ci/mmol) and 5 units DNA polymerase I Klenow fragment. After 15 minutes incubation at room temperature 10 ul 0.65 uM cold dATP was added and incubated for an additional 15 minutes at room temperature. 200 ul stopping solution containing 3 M ammonium acetate and 30 mM EDTA was added followed by two volumes of 95% ethanol and set in a -70°C dry ice ethanol bath for 15 minutes to precipitate the labeled DNA. After centrifugation the DNA pellet was dissolved in 200 ul 0.3 M sodium acetate and reprecipitated with ethanol. The labeled DNA then was suspended in 50 ul buffer containing 10 mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 1 mM DTT, 50 mM NaCl. 25 units of Pvu II restriction enzyme (which cuts the lac fragment at -120) was added and the reaction mixture was incubated at 37°C for 1 hour. 35 ul of 5 M ammonium acetate was added to stop the reaction. After precipitating with ethanol twice as described above the Pvu II digestion was repeated to make sure that cleavage was complete. Finally, the

unique 3'-end labeled lac DNA was dissolved in 40 ul TE buffer and stored at 0°C.

5'-end [^{32}P]lac labeling: 4 ug of 203-bp lac fragment was mixed with 50 mM Tris-HCl (pH 8.0) and 5 units alkaline phosphatase in a final volume of 50 ul and incubated at 37°C for 30 minutes. An equal volume of Tris-HCl (pH 8.0) saturated phenol was added to extract the DNA and 50 ul 50 mM Tris-HCl (pH 8.0) was added to back extract once more. 200 ul of a mixture containing 0.3 M sodium acetate, 0.1 M magnesium acetate was added and the dephosphorylated DNA was ethanol precipitated twice. Kinase buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA was added to dissolve the DNA completely, then 120 uCi of [gamma-^{32}P]ATP (600 Ci/mmol) and 8 units of T4 polynucleotide kinase were added to a final volume of 50 ul and incubated at 37 °C for 30 minutes. 35 ul 5 M ammonium acetate was added to stop the reaction and ethanol precipitated twice. To obtain the unique 5'-end labeled lac DNA, the Pvu II digestion as described above was followed.

Proteolytic cleavage of CRP or CRP-mAb complexes:

CRP core preparation (Eilen, Pampeno and Krakow, 1978): The proteolysis reaction is carried out under the following conditions (in a volume of 20 ul): 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 1 mM cAMP,

5 ug CRP. Incubation for 20 minutes at 37°C with 0.5% (v/v) of subtilisin generated the 13.3 KDa CRP core, 30 minutes incubation at 37°C with 2.5% Steph. aureus V8 protease generated the 18.8 KDa CRP core, with 1% chymotrypsin generated the 15.5 KDa CRP core and with 1% trypsin generated the 14.9 K Da CRP core. Whenever mAb was present, 3 ug CRP and 11.5 ug mAb or the indicated amount of mAb were added.

9.5 KDa and 13 KDa fragment formation and purification (Aiba and Krakow, 1981): 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.5% SDS, 50% (v/v) ethylene glycol, 5 ug CRP and 1% (w/w) chymotrypsin in a final volume of 50 ul was incubated for 30 minutes at 37°C. To isolate and purify the fragments the protein bands were visualized in cold 0.25 M KCl (Hager and Burgess, 1980), cut out, homogenized and eluted from the gel in elution buffer containing 50 mM Tris-HCl (pH 8.0) and 0.25% SDS by incubation at 4 °C overnight. After centrifugation the supernatants were concentrated by negative pressure dialysis against 10 mM potassium phosphate (pH 7.0), 0.2 M NaCl, 1 mM EDTA and 0.1 mM DTT overnight at 4 °C.

10 KDa and 6 KDa fragments digestion (Angulo and Krakow, 1984): 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.5 mM mercaptoethanol, 0.4 ug calf thymus DNA, 5 ug CRP and 1% (w/w) trypsin in a final volume of 50 ul was incubated for

30 minutes at 37°C. The reactions were terminated by addition of phenylmethanesulfonyl fluoride to a concentration of 0.8 mM. Formation of the indicated fragments was determined by SDS polyacrylamide gel electrophoresis (Laemmli, 1970).

Western Blotting: CRP and CRP fragments resolved by SDS polyacrylamide gel electrophoresis were transferred electrophoretically to BA 85 nitrocellulose sheets (Towbin et al., 1979) using a unit purchased from Hoefer Scientific Instruments. The position of the transferred proteins was located by heparin-toluidine blue staining (Towbin et al., 1982; Vartio et al., 1982). After removing the stain the remaining protein-binding sites were blocked by incubating the nitrocellulose sheet for 60 minutes at 37°C or overnight at room temperature in blocking buffer (BB): 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.5% Tween 80. The nitrocellulose sheet was then incubated with the indicated mAb (2 ug/ml in BB) for 90 minutes at room temperature with occasional shaking. The nitrocellulose sheet was washed three times with BB with a distilled water rinse following each wash cycle. To visualize the mAb complexes the nitrocellulose sheet was incubated for 90 minutes at room temperature with peroxidase-coupled goat anti-mouse immunoglobulin (1/300 dilution in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mg/ml BSA). After washing three times with BB the nitrocellulose sheet was incubated in a solution

made by mixing 1.7 ml 4-chloro-1-naphthol (3 mg/ml in methanol) + 8.5 ml Tris-HCl (pH 7.4) + 4 ul H_2O_2 (30% solution). Color was allowed to develop for 15 to 60 minutes at room temperature.

[^{3}H]cAMP binding assay: Binding of [^{3}H]cAMP was assayed by the method of Anderson et al. (1971). CRP (2 ug) or CRP (2 ug) + mAb (35 ug) were preincubated in 50 ul potassium phosphate (pH 7.6) for 5 minutes at 0°C. Then the following were added (in 50 ul) to give a final concentration of: 10 mM potassium phosphate (pH 7.6), 10 mM 5'AMP, 1 uM [^{3}H]cAMP (3000 cpm/pmol) and 200 ug casein. After 3 minutes at 0°C 0.5 ml saturated ammonium sulfate (pH 8.0) was added. The precipitates were collected by centrifugation, dissolved in 0.7 ml PBS and counted in 5 ml Aquasol-2.

Gel electrophoresis for detecting protein-DNA or protein complexes: A modified gel electrophoresis method of Garner and Revzin (1981) was applied. The samples containing: 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA and the indicated amount of CRP, cAMP, lac P^+ and mAb in a volume of 15 ul were incubated for 10 minutes at 37°C. Prior to loading on the polyacrylamide gel 5 ul 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 2 hours electrophoresis at room temperature the DNA-containing bands were visualized by staining for 30 minutes with ethidium bromide (1 mg ethidium bromide per liter water), when ^{32}P-labelled DNA was used bands were visualized by autoradiography, protein bands were visualized by Coomassie brilliant blue staining.

Immunoprecipitin assay for mAb containing complexes: The reactions were carried out in 1.5 ml Eppendorf tubes in a mixture which contained (final volume 50 ul): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 133 nM [^3H]NEM-CRP and 320 nM mAb or 0.1 mM cAMP, 6 nM [^3H]*lac* P^+, 30 nM CRP, 320 nM mAb with or without 30 nM RNA polymerase holoenzyme as indicated. After 10 minutes at 37°C 20 ul of a 10% suspension of Immunoprecipitin (*Staphylococcus aureus*, Cowan I strain, with protein A formalin-fixed to the cell walls) was added. After an additional 10 minutes at room temperature the immune complexes were isolated by centrifugation for 1 minute at 13,000 g and washed twice by centrifugation with 100 ul of 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT. The pellet was solubilized by heating for 5 minutes at 100°C in 200 ul of 5% SDS, 50 mM mercaptoethanol and counted in 5 ml Scintisol.

Sandwich immunoassay for mAb binding: Costar 96-well EIA

polystyrene plates were coated with antibody by a 2 hour incubation at 37°C followed by incubation overnight at 4°C. Each well received 500 ng of the indicated mAb in 50 ul PBS. Remaining binding sites were blocked by incubation for 60 minutes at 37°C with 200 ul per well of washing buffer (WB see ELISA assay). The plates were then washed twice with WB. Binding of CRP to immobilized mAb was carried out by incubation for 2 hours at 37°C with a solution (50 ul) containing: 5.4 ug/ml CRP in PBS + 2 mg/ml BSA. After washing three times with WB 50 ul of a solution containing 10 nM [^{125}I]mAb in PBS + 2 mg/ml BSA plus the indicated concentration of cAMP or cGMP was added. After incubating for 2 hours at 37°C the plates were washed with WB. [^{125}I]mAb bound was assayed after addition of 200 ul of a solution containing 0.5 M NaOH + 5% SDS. After transfer to 10 x 75 mm tubes radioactivity was determined in a LKB Autogamma counter.

Abortive initiation assay: A modification of the abortive initiation assay of Malan et al. (1984) was used to determine the effect of mAb on CRP-dependent transcription from the lac promoter. The standard reaction mixture contained (final volume 50 ul): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1% glycerol. 0.1 mM cAMP, 2 nM lac P^+ DNA fragment, 40 nM RNA polymerase holoenzyme, 40 nM CRP and (where indicated) 240 nM mAb were added in the order indicated. After an additional preincubation at

37°C for 10 minutes or for the time stated 0.5 mM ApA and 50 nM [^{3}H]UTP (250 cpm/pmol) were added. The reaction was allowed to proceed for 15 minutes at 37°C when it was terminated by addition of 10 ul 0.5 M EDTA. Radioactive ApApUpU and UTP were resolved by paper chromatography in WASP solvent (McClure et al., 1978) . After cutting the chromatography strip into 1 cm segments the amount of oligonucleotide synthesized was determined by counting appropriate segments in Scintisol.

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 ul): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1% glycerol, 3 nM [^{32}P]*lac* fragment, 40 nM CRP, and where indicated 240 nM mAb, 120 nM RNA polymerase holoenzyme and the indicated concentration of cAMP. The components added, the order of addition and the times of incubation are given in the figure legends (if not specifically indicated 10 minutes is the normal incubation time). After formation of the complexes 1 ul of a solution containing 20 ng/ml DNase I in 20 mM potassium phosphate (pH 6.8), 1 mM EDTA and 50% glycerol was added and incubated for 30 seconds at 37°C. The reaction was terminated by addition of a solution containing 3.1 M ammonium acetate (pH 7.6), 25 mM EDTA and 63 ug/ml tRNA followed by phenol extraction, ethanol precipitation and

reprecipitation. After drying the pellets under vacuum 10 ul 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 (1977). After electrophoresis the gel was autoradiographed at -70°C using Kodak XAR-5 film and a Cronex II-Plus intensifying screen.

RESULTS

Characterization of nine mAbs:

The anti-CRP mAbs were assayed for their ability to bind to urea-denatured and/or native CRP adsorbed onto wells of Costar styrene plates. The binding of the mAbs was determined by ELISA. As shown in Table 1, five of the mAbs bound preferentially to native CRP, and these are termed Class I mAbs. The remaining four mAbs bound with a comparable affinity to native and denatured CRP and are termed Class II mAbs. All of the anti-CRP mAbs were of the IgG class and contained kappa light chains (data not shown).

Digestion of cAMP-CRP with chymotrypsin or the *Staph. aureus* V8 protease yields N-terminal cores which retain the dimeric structure of the native CRP. The subunit molecular weight of the chymotryptic and *Staph.aureus* V8 protease CRP cores formed after cleavage of cAMP-CRP are 15,000 and 18,800 Da, respectively. Following adsorption to assay plates the CRP cores were used to localize the Class I mAb binding sites (Table 2). The Class II mAb 65C3 bound equally well to native CRP and both N-terminal cores. Three of the Class I mAbs (62A2, 62D6 and 63A3) bound to native CRP but reacted only to a limited extent with either of the CRP cores. The low binding activity observed may be

due to residual intact CRP. The remaining two Class I mAbs (63B2 and 66C3) bound to the native CRP and the N-terminal 18,800 Da core but not to the 15,000 Da core. The lower extent of binding of mAb 63B2 and 66C3 to the 18,800 Da core may be a result of the loss of the C-terminal region which may be involved in maintaining the native conformation of CRP in this domain.

All four of the Class II mAbs bound equally well to CRP and the 15,000 Da chymotryptic core as assayed by solid-phase ELISA (Table 2). Since the Class II mAbs bind to denatured CRP, it was possible to utilize Western blotting to localize the mAb binding sites within CRP fragments resolved by SDS-polyacrylamide gel electrophoresis. CRP fragments were produced by three different procedures. Digestion of cAMP-CRP with subtilisin yields a N-terminal fragment of 13,300 Da (Eilen et al., 1978; Tsugita et al., 1982). CRP in the presence of SDS is cleaved by chymotrypsin to give a N-terminal fragment of 9,500 Da and a C-terminal fragment of 13,000 Da (Aiba and Krakow 1981). The DNA-CRP complex formed at low ionic strength in the absence of cAMP is cleaved by trypsin, yielding a 10,000 Da N-terminal fragment and a 6,000 Da C-proximal fragment with the N-terminal sequence: Val(131)-Gly-Asn.....(Angulo and Krakow 1986). After electrophoresis and transfer to nitrocellulose sheets the position of the fragments was visualized using heparin-toluidine blue staining (Towbin et al., 1982; Vartio et

al., 1982). As expected from the results obtained with the solid-phase ELISA, each of the Class II mAbs bound to the N-terminal fragments produced by cleavage of CRP with the S.aureus protease and chymotrypsin (Fig. 3). mAb 115D5 bound weakly to the 9,500 Da N-terminal fragment produced by cleavage of SDS-CRP by chymotrypsin while mAbs 64D1,65C3 and 64B4 (the fragment binding properties of the latter two mAbs are identical) bound to the C-terminal 13,000 Da fragment. mAb 65C3 and 64B4 did not bind to the 10,000 Da or 6,000 Da fragment; both mAbs bound to the 13,000 Da C-terminal fragment. This defines the location of the antigenic determinant(s) for mAbs 65C3 and 64B4 within the region spanning Phe 102...Lys 130. mAb 64D1 bound to the N-terminal 10,000 Da fragment but not to the N-terminal 9,500 Da fragment. Although the C-terminal sequence of the 10,000 Da fragment has not yet been determined, it is likely that the tryptic cut occurred at either Lys 89 or Lys 100. Should the 10,000 Da fragment terminate at Lys 89 it would appear that the determinant for mAb 64D1 is Val 86-Arg-Ala-Lys (Fig. 2). The location of the epitope for mAb 115D5 was determined by a competitive immunoassay, the data clearly showed that mAb 115D5 cross-reacted with both CRP and the 9,500 Da fragment but not with the 13,000 Da fragment (Li and Krakow, 1985). It would appear that the immobilization of the 9,500 Da fragment on nitrocellulose for Western blotting in some fashion occludes the determinant for mAb 115D5. Summarized in Fig.

4 are the conclusions derived from the immunoblotting and ELISA experiments.

Abortive synthesis of ApApUpU by RNA polymerase directed by the 203-base pair restriction fragment carrying the lac P^+ promoter is almost completely dependent on the presence of cAMP-CRP. The effect of the anti-CRP mAbs on this reaction was determined (Table 3). Three of the mAbs (Class I mAbs 66C3 and 63B2 and Class II mAb 64D1) almost completely blocked the action of CRP in stimulating abortive initiation. Of the three Class I mAbs which bind to determinants located near the C-terminus of CRP (Fig. 4), mAbs 62A2 and 62D6 inhibited abortive initiation by about 75% while mAb 63A3 effected a 33% inhibition. The mAb 115D5, whose antigenic determinant resides within the N-terminal domain of CRP, inhibited abortive initiation by about 50%. The remaining two Class II mAbs 64B4 and 65C3 did not inhibit this reaction.

Inhibition of CRP-dependent abortive initiation by the anti-CRP mAbs could be due to three possible effects: inhibition of cAMP and/or DNA binding and interference with possible contact between CRP and RNA polymerase. Of the three most effective inhibitors of the abortive initiation reaction (mAbs 63B2, 66C3, 64D1) only the Class II mAb 64D1 markedly inhibited cAMP binding (Table 4). The three Class I mAbs 62A2, 62D6 and 63A3 which partially inhibited abortive initiation also inhibited cAMP binding to a lesser extent. The four remaining mAbs 66C3, 65C3, 64B4 and 115D5

did not affect cAMP binding by CRP.

The gel electrophoresis method of Garner and Revzin (1981) was used to examine the effects of the anti-CRP mAbs on the binding of cAMP-CRP to the lac promoter fragment. Incubation of CRP with the DNA at a molar ratio of 4:1 resulted in almost complete binding of the DNA (Fig. 5, lane CRP). To determine the effect of added antibody the mAb was incubated with the preformed cAMP-CRP-DNA complex. Four of the mAbs which inhibited abortive initiation by 75% or better also resulted in a total (lane d, mAb 64D1; lane f, mAb 63B2) or nearly total inhibition (lane h, mAb 62A2; lane i, mAb 62D6) of lac DNA binding. The Class I mAb 63A3 (lane e) which partially inhibited abortive initiation also showed a partial inhibition of lac DNA binding. Two of the mAbs inhibited neither abortive initiation nor DNA binding (lane b, mAb 65C3; lane c, mAb 64B4). Two mAbs bound to the cAMP-CRP-DNA complex, these were mAb 115D5 which partially inhibited abortive initiation (lane j) and the strongly inhibitory mAb 66C3 (lane a).

mAbs 65C3 and 64B4 have no effect on formation of either CRP-cAMP or CRP-lac DNA complexes and do not inhibit abortive initiation but strongly bind to immobilized CRP on the EIA polystyrene plate. Friguet et al.(1984) have observed that binding of proteins to a EIA polystyrene plate may partially denature the protein. In order to check the affinity of the mAbs for native CRP in solution a gel electrophoresis assay was used, the results are shown

in Fig. 6. At a 2:1 ratio of mAb to CRP, four Class I mAbs 66C3, 63B2, 62A2 and 62D6 and two Class II mAbs 64D1 and 115D5 bound to CRP. The complexes migrated at a position different from mAb alone. Class I mAb 63A3 and two Class II mAbs 65C3 and 64B4 showed very poor binding. A large proportion of the mAb remains free in the presence of CRP. Apparently, the antigenic determinants for mAbs 65C3 and 64B4 are folded inside of the native CRP molecule in solution and probably also in the cAMP-CRP and cAMP-CRP-*lac* DNA complexes. This may explain the lack of an observable effect of these mAbs on CRP activity.

Table 5 summarizes the functional properties of the nine anti-CRP mAbs. ELISA assay classified five mAbs which bind to native CRP but not urea denatured CRP as Class I and four mAbs which bind equally well to both native and urea denatured CRP as Class II. Three mAbs 63A3, 65C3 and 64B4 bind poorly to CRP in solution. mAb 62A2 and mAb 62D6 located at the far C-terminal region show partial inhibition of formation of cAMP-CRP, cAMP-CRP-DNA complexes and abortive initiation. The two mAbs (65C3 and 64B4) which bind in the region including the B-alpha helix and part of the C-alpha helix showed poor binding of CRP in solution and had no observable effect on CRP activity. The two mAbs (63B2 and 66C3) located in the region including D-alpha helix and part of C-and E-alpha helices are strong inhibitors of abortive initiation but only mAb 63B2 inhibits cAMP-CRP-DNA binding, mAb 66C3 can

bind to this complex without its dissociation. Similarly, mAb 115D5 can also bind to this complex but its antigenic determinant is located in the N-terminal domain. mAb 64D1 which binds in the region from Val 86 to Lys 89 or Lys 100 strongly inhibits cAMP-CRP binding and consequently inhibits the formation of cAMP-CRP-DNA complex and abortive initiation.

The mechanism of mAbs 64D1, 66C3 and 115D5 effects have been analyzed.

Characterization of mAb 64D1:

The effects of mAb 64D1 on formation of cAMP-CRP, cAMP-CRP-lac P^+ and cAMP-CRP-lac P^+-RNA polymerase complexes were assayed by abortive synthesis (Fig. 7). While CRP is rapidly inactivated by mAb 64D1 the cAMP-CRP-lac P^+-RNA polymerase complex remains unaffected even after a 40 minute incubation with the antibody prior to assay for abortive initiation. Incubation of mAb 64D1 with cAMP-CRP or cAMP-CRP-lac P^+ results in a comparable and intermediate rate of inactivation. The results indicate that the presence of the lac P^+ DNA fragment does not stabilize the cAMP-CRP complex.

DNase I footprinting provides a direct visualization of the resistance of the open promoter complex, RP_o, to dissociation by mAb 64D1. Binding by cAMP-CRP to the lac P^+ fragment protects the region spanning -50 to -80 bp (Fig. 8, lanes b, c). Incubation of the preformed cAMP-CRP-

lac P^+ complex with mAb 64D1 results in a progressive decomposition of the complex so that after a 40 minute incubation (Fig. 8, lane f) binding of CRP to lac P^+ has been lost. In contrast to the relative instability of the cAMP-CRP-lac P^+ complex, the presence of RNA polymerase results in the formation of the RP_o (Fig 8, lane h) in which CRP remains unaffected even following a 40 minute incubation with mAb 64D1 (Fig. 8, lanes i, j).

The results presented in Figure 9 compare the effects of cAMP concentration on the stability of the cAMP-CRP, cAMP-CRP-lac P^+ and cAMP-lac P^+-RNA polymerase complexes to subsequent incubation with mAb 64D. CRP binds to its site in the lac P^+ promoter at 100 uM cAMP (Fig. 9, lane b) and 25 uM cAMP (Fig. 9, lane d). Preincubation of CRP and mAb 64D1 (Fig. 9, lane c) results in a complete loss of DNA binding activity. The effect of cAMP concentration on the sensitivity of CRP to inhibition by mAb 64D1 is shown in Figure 9, lanes e, f, g. Shown is the effect of incubation of the cAMP-CRP complex formed at different cAMP concentrations with mAb 64D1 followed by binding to the lac P^+. The cAMP-CRP complex formed with 100 uM cAMP is resistant to subsequent incubation with mAb 64D1. As the cAMP concentration is lowered the inhibitory effect of the antibody is seen; at 25 uM cAMP binding to lac P^+ is almost completely inhibited. The preformed cAMP-CRP-lac P^+ complex formed at different cAMP concentrations shows a similar response to incubation with mAb 64D1 (Fig. 9, lanes

i, j, k). The results indicated that the presence of the lac P^+ DNA does not increase the resistance of cAMP-CRP to attack by mAb 64D1. In contrast the presence of RNA polymerase effects an obvious increase in the affinity of CRP for its site on the lac P^+ promoter. This effect is seen at 6 uM cAMP (Fig. 9, lane o) where stable CRP binding is not observed in the absence of RNA polymerase. Incubation of the preformed cAMP-CRP-lac P^+-RNA polymerase complex with mAb 64D1 shows that while the RP_O formed at 25 uM cAMP (Fig. 9, lane q) is not affected, lower concentrations of cAMP (Fig. 9, lanes r, s) resulted in an increased dissociation of the complex in the presence of the antibody.

The lack of immediate inhibition of CRP activity in the preformed initiation complex could be due to the inability of mAb 64D1 to bind to CRP or that antibody can bind but CRP activity is retained. In order to show that mAb 64D1 binds to CRP, the protein was labeled at Cys-178 with [^{3}H]N-ethylmaleimide (Ebright et al., 1985). The data presented in Table 6 show that Immunoprecipitin binds to the immune complex formed between mAb 64D1 or mAb 66C3 and [^{3}H]NEM-CRP. Both monoclonal antibodies are of the IgG1 class for which Protein A has a variable affinity (Goding, 1978). The results presented in Figure 6 showed that mAb 66C3 binds to the cAMP-CRP-lac P^+ complex without its dissociation. The data presented in Table 6 show that the [^{3}H]lac P^+ DNA bound to cAMP-CRP sediments after incubation

with mAb 66C3 and Immunoprecipitin. In contrast, little or no label in the complex sediments after incubation with mAb 64D1. Incubation of the cAMP-CRP-lac P^+ complex with mAb 64D1 would ultimately result in its dissociation. Formation of the stable open promoter complex evidently precludes binding of CRP by mAb 64D1.

The CRP protomer consists of two identical subunits, accordingly there should potentially be two antigenic sites for mAb 64D1. The method of Pestka et al. (1983) provides a convenient assay for the availability of the mAb 64D1 determinants on the dimeric CRP. The procedure involves immobilization of unlabeled mAb 64D1 to the wells of a polystyrene RIA plate followed by incubation with CRP to allow formation of the immune complex. Binding of $[^{125}I]$mAb 64D1 to this complex would indicate that both of the antigenic sites on the CRP protomer are available. The data presented in Table 7 indicate that there was essentially no binding of the $[^{125}I]$mAb 64D1 to the immobilized mAb 64D1-CRP. When the assay was run using the immobilized mAb 64C4-CRP complex binding of $[^{125}I]$mAb 64D1 was observed. The data indicate that only one mAb 64D1 antigenic site is available on CRP or that both of the antibody combining sites are involved in binding to the antigenic determinant present on each of the CRP subunits.

It is clear that cAMP binding results in a conformational change in CRP. This has been shown to occur in the C-terminal DNA-binding domain (Eilen et al., 1978)

as well as the N-terminal cAMP-binding domain (Eilen and Krakow, 1977) where the determinant for mAb 64D1 resides (Fig. 5). This antibody binds to denatured or native CRP adsorbed to polystyrene or nitrocellulose. Under these conditions there is no observable effect of cAMP on mAb 64D1 binding. Adsorption to these matrices probably results in CRP structures with restricted conformational mobility. To determine the effects of ligands known to be allosteric effectors a variation on the sandwich radioimmunoassay was used. In this procedure CRP is bound to an immobilized monoclonal antibody having an epitope specificity different from that of mAb 64D1. The results presented in Figure 10 show that the binding of [^{125}I]mAb 64D1 is markedly inhibited by cAMP and to a lesser extent by cGMP. The data suggest that the conformation of the antigenic domain for mAb 64D1 in cAMP-CRP is different from that of either unliganded or denatured CRP. This could be a consequence of a cAMP-induced conformational change or a result of the contact of cAMP with an amino acid side chain comprising part of the antigenic determinant for mAb 64D1.

It would appear that cAMP binding establishes a conformation in CRP unfavorable for mAb 64D1 binding and conversely formation of the mAb 64D1-CRP complex alters the conformation of the cAMP binding domain in both CRP subunits. Limited proteolysis has been used to probe the effects of cAMP and DNA binding on CRP conformation (Angulo

and Krakow, 1985, 1986). Unliganded CRP is relatively resistant to proteolytic attack while cAMP-CRP is attacked to yield N-terminal cores which retain cAMP binding activity. The mAb 64D1-CRP complex is rapidly attacked by the S.aureus V8 protease, trypsin, chymotrypsin (Fig. 11) and subtilisin (Fig. 12 A). The size of the fragments generated by proteolytic attack on the mAb 64D1-CRP and cAMP-CRP complexes differs indicating that the CRP conformation in each complex is also different. Assay by Western blotting (Fig. 12 B) shows that mAb 64D1 can bind to the fragments demonstrating that cutting by subtilisin did not destroy the antigenic site for mAb 64D1.

Using proteolysis by trypsin and subtilisin to assay for mAb 64D1 binding the results presented in Figure 13 show that the presence of one mAb 64D1 per CRP protomer results in complete cutting of both CRP subunits. It is apparent that when the ratio of antibody to CRP subunit is less then one, residual intact CRP subunit is found. The results of this experiment indicate that the mAb 64D1-CRP complex does not dissociate during the course of the incubation with the proteases.

Characterization of mAb 66C3:

As shown in Figure 5 the antigenic determinant of mAb 66C3 is located within the region including the D-alpha helix and part of the C- and E-alpha helices. mAb 66C3 binds to native but not urea-denatured CRP indicating that

the determinant is an assembled topographic site formed by the native folding of CRP. The results presented in Figure 14 show that the determinant is in a region which is conformationally responsive to cAMP binding. The data demonstrate that the conformation elicited by binding of cAMP effects a five fold increase in binding of [^{125}I]mAb 66C3 over the binding observed in the absence of this allosteric ligand. In contrast only a small increase in the binding of [^{125}I]mAb 66C3 is seen in the presence of cGMP. These results also show that the antigenic determinants present on each of the CRP subunits are available for binding by mAb 66C3. The experimental approach used is based on the procedure of Pestka et al.(1983) similar to the one shown in Table 7 and Fig. 10 except that the immobilized mAb on the wells is mAb 66C3. Thus the binding seen is a consequence of the formation of a mAb 66C3-CRP-[^{125}I]mAb 66C3 complex.

The initial characterization of mAb 66C3 showed that it was a potent inhibitor of the abortive initiation reaction directed by the lac P^+ template although mAb 66C3 did not inhibit binding of cAMP (Table 4) or lac P^+ (Fig. 6, lane a). The data presented in Figure 15 show that abortive initiation from lac P^+ is almost completely inhibited when mAb 66C3 is incubated with cAMP-CRP prior to addition of RNA polymerase. In contrast addition of mAb 66C3 after formation of the open promoter complex results in a 50% inhibition of ApApUpU synthesis.

In the experiment shown in Figure 15 the preformed RP_o complex was incubated with mAb 66C3 for 10 minutes prior to assay for abortive initiation. To determine whether a more extended incubation with mAb 66C3 would result in a greater inhibition the experiment shown in Figure 16 was carried out. The data show that no further inhibition is effected after the initial 10 minute challenge. The results imply that about 50% of the CRP present in the RP_o complex is sensitive to attack by mAb 66C3. The results presented in Figure 16 also indicate that the preformed cAMP-CRP-mAb 66C3-lac P^+ complex cannot be activated by extended incubation with RNA polymerase. The antibody titration assay shown in Fig. 17 shows that mAb 66C3 concentration is saturated at a 2 to 1 mAb 66C3 to CRP ratio. At this point, similar to that shown in Fig. 15, preincubation of mAb 66C3 with cAMP-CRP resulted in a complete inhibition while adding mAb 66C3 after formation of the RP_o resulted in about 50% residual activity. Increasing the mAb 66C3 to CRP ratio to 15 to 1 did not result in a significant increase in inhibition.

DNase I footprinting was carried out to further define how binding of mAb 66C3 to cAMP-CRP affects lac P^+ DNA binding. Under the conditions used in the experiment shown in Figure 18 A and B (lane b) protection by cAMP-CRP of Site 1 on [^{32}P]lac P^+ is observed. In contrast one sees a more distinct protection by cAMP-CRP-mAb 66C3 within CRP Site 1 and also CRP Site 2 (Fig. 18 A and B, lane c). In

the presence of RNA polymerase binding of cAMP-CRP is stabilized and the characteristic CRP Site 1 and RNA polymerase footprint is obtained (Fig. 18 A and B, lane e). Formation of the cAMP-CRP-mAb 66C3-lac P^+ complex followed by incubation with RNA polymerase results in a footprint like that observed in the absence of added RNA polymerase (Fig. 18 A and B , lane f). The results explain the strong inhibition of abortive initiation by mAb 66C3 seen in Figure 14; prior formation of the cAMP-CRP-mAB 66C3-lac P^+ complex precludes binding by RNA polymerase. In contrast the preformed RP_o complex is relatively resistant to subsequent incubation with mAb 66C3 (Fig. 18 A and B, lane g).

The lac L8UV5 promoter contains a double mutation, the L8 mutation present in CRP Site 1 markedly lowers its affinity for cAMP-CRP, the UV5 mutation in the -10 consensus region enables RNA polymerase to form the RP_o in the absence of cAMP-CRP. The DNase I footprint shown in Figure 19 panel A shows that neither cAMP-CRP nor cAMP-CRP-mAb 66C3 are able to form a stable complex at CRP Site 1 or 2. As expected a strong protection extending downstream from -50 bp is seen in the presence of only RNA polymerase (Fig. 19 A, lane d). Binding of cAMP-CRP to Site 1 is stabilized in the presence of RNA polymerase (Fig. 19 A, lane e). In contrast to the inhibition of binding of RNA polymerase by cAMP-CRP-mAb 66C3 to the lac P^+ promoter one sees that in the lac L8UV5 promoter binding of RNA

polymerase inhibits binding by cAMP-CRP-mAb 66C3 (Fig. 19 A, lane f). In the present of 25% glycerol (Fig.19 panel B), Protection by cAMP-CRP alone cannot be seen; in the presence of mAb 66C3 protection at both CRP Site 1 and Site 2 is observed (Fig. 19 B, lanes b and c).

The lac P^+ and lac L8UV5 fragments are cut by Hpa 2 at -20 bp separating CRP Site 1 and Site 2 on each of the resultant restriction fragments. Figure 20 shows that cAMP-CRP binds to the wild type CRP Site 1 fragment but does not bind to either the L8 Site 1 or Site 2 fragment. cAMP-CRP-mAb 66C3 forms a complex with all of the wild type Site 1 fragment and much of the Site 2 fragment. In the case of the fragments generated from the lac L8UV5 a partial binding of cAMP-CRP-mAb 66C3 to both the L8 Site 1 and Site 2 fragments is seen. It appears that cAMP-CRP bound to mAb 66C3 shows an increase in affinity for DNA sites which do not have the optimal sequence for CRP binding.

Comparison of mAb 66C3 and mAb 115D5:

mAb 115D5, like mAb 66C3, is able to bind to the cAMP-CRP-DNA complex without its dissociation (Fig. 6, lane j) but its antigenic determinant is located in the N-terminal 9,500 Da fragment (Fig. 4) and it can bind to both native and denatured CRP (Table 1). mAb 115D5 shows a partial inhibition of abortive initiation and the effect is seen whether it is added before or after RNA polymerase

(Table 5 and data not shown). A DNase I footprint comparison of mAb 66C3 and mAb 115D5 is shown in Figure 21. As expected from the abortive initiation data mAb 115D5 shows partial inhibition of RP_o complex formation with or without preincubation. In the absence of RNA polymerase, mAb 115D5 induces CRP Site 1 and Site 2 binding similar to mAb 66C3 (Fig. 21, lanes c and d). In mAb-CRP-*Hpa* 2 fragment binding, mAb 115D5 shows similar effects to mAb 66C3 but with less intensity (Fig. 20, lane a and e). Both mAbs do not appear to induce any detectable conformational changes on CRP as assayed by protease digestion (data not shown). The significance of the similarities and the dissimilarities will be discussed in the Discussion section.

Glycerol effect on protein-DNA interaction:

Glycerol shows interesting effects on protein-DNA binding. As shown in Fig. 19, panel B, glycerol increases mAb 66C3-cAMP-CRP binding at CRP Site 1 and Site 2. Glycerol also enhances cAMP-CRP Site 1 binding. As shown in Figure 22, and Figure 18, the cAMP-CRP-*lac* P^+ binding pattern is different from mAb 66C3-cAMP-CRP-*lac* P^+ at CRP Site 1. mAb 66C3-cAMP-CRP protects CRP Site 1 better than cAMP-CRP (Fig, 22, lanes b, c. Fig. 18, lanes b,c). In the presence of 25% glycerol, cAMP-CRP protection of CRP Site 1 is comparable to mAb 66C3-cAMP-CRP but glycerol does not

induce CRP Site 2 binding by cAMP-CRP as mAb 66C3-cAMP-CRP does in the absence or presence of glycerol (Fig. 22, lanes c, d). RNA polymerase does not show specific binding to the *lac* P^+ promoter in the absence of CRP. In the presence of glycerol, RNA polymerase binding is obtained (Fig. 22, lanes e, g). This binding is different from that seen in presence of CRP (Fig. 22, lane f). The enhanced cutting in region -44 to -47 bp is not seen in the presence of CRP.

Figure 23 shows the glycerol titration effect on abortive transcription. In the absence of CRP transcription from *lac* P^+ is increased when the glycerol concentration is increased while transcription from *lac* L8UV5 and *lac* P^+ in the absence of CRP is decreased. This indicates that RNA polymerase binding in high glycerol in the absence of CRP retains transcription activity but at a lower efficiency. Glycerol increases the affinity of protein specific binding on the promoter but it also may have an effect on protein conformation and/or function.

DISCUSSION

As described in the Introduction, CRP is a protein with multiple functions. It interacts with cAMP, DNA and possibly with RNA polymerase to regulate transcription of several bacterial genes. Besides the crystal structure of the cAMP-CRP complex described by Steitz and co-workers (Figure 2) other potential conformations of CRP have been indicated by its sensitivity to attack by trypsin and other proteolytic enzymes (Angulo and Krakow, 1986; Eilen et al., 1978) and by analysis of cAMP analogue-CRP interaction (Ebright et al., 1985). Each of these conformations corresponds to the cAMP binding or DNA binding function. Site-specific monoclonal antibodies which bind to antigenic determinants in or able to attain a specific conformation can facilitate the study of the relation of CRP structure and function.

Nine anti-CRP mAbs have been characterized with regard to the region of CRP in which the antigenic determinants reside, and this has been correlated with the effect of each mAb on CRP activity. As summarized in Table 1, the nine mAbs fall in two groups as classified by ELISA assay. Five mAbs which bind to native CRP but not urea denatured CRP are referred to as Class I, and four mAbs which bind to both native and urea denatured CRP are referred to as Class

II. The antigenic determinants of all of the Class I mAbs are located within the C-terminal domain and all of the Class II mAbs are located within the N-terminal domain (Fig. 4). It is not known if this clear separation of the locations of Class I and II mAbs is only a coincidence. mAb 63A3, mAb 65C3 and mAb 64B4 show weak or no binding to CRP in solution. The antigenic determinants for mAb 65C3 and mAb 64B4 were located within the region including the B- and C-alpha helices and the determinant for mAb 63A3 was located within the C-terminal region. The determinants of these antibodies do not seem to be available in native CRP. The mAbs were initially selected for binding to CRP immobilized on the polystyrene surface of ELISA plates. Such binding may distort CRP, exposing regions of the protein not ordinarily available in native CRP. Such an effect has been described by Djavedi-Ohaniance et al. (1984). These mAbs may have been elicited by antigen which has been denatured or subject to partial proteolysis during the immunization process.

The Class II mAb 64D1 shows strong inhibition of cAMP-CRP binding. The antigenic determinant for mAb 64D1 is located within the region encompassing Val-86 to Lys-100 forming parts of the beta 7 and 8 rolls. As mentioned in the Introduction, cAMP binding occurs within a pocket formed by the eight-stranded antiparallel beta roll in the N-terminal domain (McKay et al., 1982). Contacts between cAMP and CRP amino acid residues Arg-87, Ser-83 and Ala-34

are close to the proposed determinant for mAb 64D1. Binding of as large a molecule as an immunoglobulin at a nearby region could result in steric hindrance. The data presented in Figures 11 and 12 showed that the mAb 64D1-induced conformational change is the reason for the inhibition of cAMP binding. In contrast to the insensitivity of the unliganded CRP to the proteases, both cAMP-CRP and mAb 64D1-CRP complexes are attacked by the *Staph. aureus* V8 protease, trypsin, chymotrypsin and subtilisin. The difference in the sites of cutting of cAMP-CRP and mAb 64D1-CRP by these proteases indicates that the conformation of these CRP complexes is different. In each case the fragments resulting from proteolytic attack of mAb 64D1-CRP are smaller than those resulting from attack on cAMP-CRP. Binding of mAb 64D1 to its antigenic site in the large N-terminal CRP domain apparently alters the conformation within this domain and as a consequence directly (sterically) or indirectly due to the altered conformation of the N-terminal domain affects the interaction between the N- and C-terminal domains of CRP. Whatever the actual mechanism it is apparent that binding of mAb 64D1 results in the exposure in CRP of otherwise inaccessible peptide regions thereby allowing for attack by the proteases used.

When the ratio of mAb 64D1 to CRP protomer is less than one, undigested CRP remains (Fig. 13) indicating that even after digestion the mAb 64D1 is remains bound to the

CRP core. mAb 64D1 did not dissociate from the CRP core to rebind to undigested CRP.

Although CRP consists of two subunits, binding of one mAb 64D1 per CRP protomer is sufficient to sensitize both subunits to proteolytic cutting (Fig. 13). The results of the double antibody experiment (Table 7) suggest that only one mAb 64D1 can bind to the CRP protomer. The apparent inaccessibility of the second antigenic determinant may be a consequence of steric hindrance resulting from the interaction of the antibody with one of the antigenic determinants. This would have to elicit the protease-sensitive conformation in both subunits. A second possibility is that each of the combining sites on the mAb 64D1 IgG are able to bind to each of the antigenic determinants in the CRP protomer. This would require that the conformation of CRP is such that each of the antigenic determinants is properly disposed to allow for such a bridged interaction with the antibody.

Binding of mAb 64D1 results in the inhibition of cAMP binding by CRP (Table 4). The preformed cAMP-CRP complex also inhibits mAb 64D1 binding by CRP. As shown in Figure 10, increasing concentration of cAMP results in a decreased binding of $[^{125}I]$mAb 64D1. CRP conformation is changed when bound to cAMP. This suggests that the antigenic determinant of mAb 64D1 is blocked in the cAMP-CRP complex, mAb 64D1 can only bind to unliganded CRP. Since the mAb 64D1-CRP complex is much more stable than the cAMP-CRP

complex, in the presence of 100 uM cAMP as in the abortive initiation and DNase I footprinting assays, mAb 64D1 appears to act by trapping unliganded CRP as it dissociates from the preformed complexes. The mAb 64D1-CRP complex formed will no longer be able to bind to cAMP and support formation of the RP_o. Therefore the experiments in Figures 7, 8, 9 actually show the dissociation of different complexes. Incubating free CRP with mAb 64D1 results in total loss of CRP activity (Fig. 7, curve a, Fig. 9, lane c). Preformed cAMP-CRP and cAMP-CRP-lac P^+ complexes seem to dissociate at a similar rate (Fig. 7, lanes b, c) and have similar sensitivities to changes in cAMP concentration (Fig. 9 lanes d, e, f, g, h, i, j, k). It seems that DNA binding does not appreciably enhance the stability of the cAMP-CRP complex. In contrast, when RNA polymerase is present, the cAMP-CRP complex in the RP_o is dramatically stabilized. In Figure 7, 40 minutes incubation in the presence of mAb 64D1 results in dissociation of about half of the cAMP-CRP and cAMP-CRP-lac P^+ complexes while almost all of the RP_o remains active. In Figure 9, in the presence of 25 uM cAMP, the preformed cAMP-CRP and cAMP-CRP-lac P^+ complexes are completely dissociated while the RP_o remains stable until the cAMP concentration drops to 6 uM. The resistance of the RP_o formed at low cAMP concentration relative to the sensitivity of the cAMP-CRP-lac P^+ complex may reflect a possible conformational change elicited in CRP on contact with RNA polymerase in the RP_o.

This may increase the affinity for cAMP by CRP present in the RP_o possibly by closing the pocket in which cAMP is bound. CRP in the complex is not completely resistant since at 6 uM cAMP the effect of mAb 64D1 on dissociation of the RP_o is evident. The results also indicate that RNA polymerase does not seen to dissociate as rapidly as CRP from its site on the lac P^+ promoter (Fig. 9, lanes r, s). However RNA polymerase which remains bound to the promoter under conditions where CRP has dissociated is not active as determined by the abortive initiation assay. It would appear that simultaneous binding of cAMP-CRP and RNA polymerase is required to form an active RP_o. The presence of RNA polymerase has been found to increase the affinity of cAMP-CRP for its binding sites on the lac and gal promoters (de Crombrugghe et al., 1984). From the data presented in this study one possible model to explain the inability of mAb 64D1 to rapidly attack CRP in the cAMP-CRP-lac P^+-RNA polymerase complex would involve reciprocal conformational changes in both CRP and RNA polymerase resulting from protein-protein contacts. This would increase the affinity of cAMP-CRP for its DNA site and lower the accessibility of the mAb 64D1 determinants on CRP.

Class I mAb 66C3 and mAb 63B2 are strong inhibitors. Their antigenic determinants are located in the region which contains part of the E- and C-alpha helices and all of the D-alpha helix. This region is the so called hinge

region. When cAMP is bound, the N- and C- terminal domains are brought closer by the bending in this region and the amino acid residues in this region could interact with the F-alpha helix to increase the DNA binding capacity of CRP (Garges and Adhya, 1985). Both mAb 66C3 and mAb 63B2 do not bind to denatured CRP and their binding is sensitive to conformational changes in CRP. The antigenic determinants of these mAbs apparently belong to the assembled topographic category. Even though both antibodies are inhibitors and their antigenic determinants are located within a similar region, the antibody effects on CRP-DNA binding are totally different. mAb 63B2 inhibits cAMP-CRP-<u>lac</u> P^+ binding completely while mAb 66C3 can bind to the cAMP-CRP-<u>lac</u> P^+ complex. In fact, the binding of mAb 66C3 to the cAMP-CRP complex increases its affinity to <u>lac</u> P^+ CRP Site 1 and induces CRP binding at the -10 to +10 region known as CRP Site 2. These differences indicate that the CRP hinge region is critical for its DNA binding activity. Changes in this region can result in a complete loss of DNA binding or a great increase of DNA binding capacity. Recently Liu-Johnson et al.(1986) proposed a model for CRP binding to DNA. According to this model the contacts between CRP and <u>lac</u> P^+ DNA are not only located within the consensus sequence but also the region 13bp away from the center of the dyad symmetry. DNA molecule bends by up to 90° around CRP to provide the extra contact. They suggested that CRP had a strong distal binding domain

responsible for this extra contact which is located somewhere above the major DNA binding F-alpha helix. The antigenic determinants for mAb 66C3 and mAb 63B2 as shown in Fig. 4 are away from the F-alpha helix. These determinants are probably within the proposed distal binding region thereby affecting binding of DNA by CRP.

The inhibition by mAb 66C3 of <u>lac</u> P^+ transcription initiation could be due to Site 2 binding by mAb 66C3-CRP. The cAMP-CRP-mAb 66C3 complex binds in the -10 to +10 region, and like a repressor, would block RNA polymerase binding. Interestingly, Class II mAb 115D5 which is located in N-terminal 9,500 Da fragment can also induce CRP Site 2 binding (Fig, 21, lane d) but it only causes a partial inhibition of abortive initiation. As described in the Introduction, besides the covalent linking of the N- and C-terminals there are noncovalent contacts between the two domains to stabilize their relative orientation. The major contacts occur at beta roll 5 of the N-terminal domain and the E-alpha helix of the C-terminal domain (McKay et al., 1982)). Binding of mAb 115D5 in the N-terminal domain could change the conformation of the C-terminal domain by altering the contact between the two domains resulting in a new conformation in the hinge region similar to the one induced by binding of mAb 66C3. Since mAb 66C3 binds to the C-terminal domain directly eliciting the conformational change within this domain while mAb 115D5 binds to the N-terminal domain indirectly

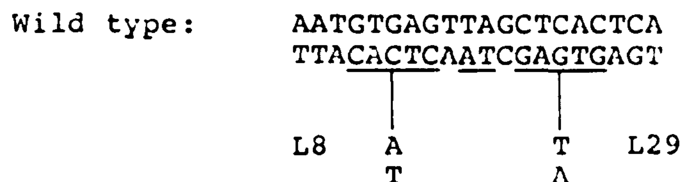
inducing the conformational change at the DNA binding domain, the binding to CRP Site 2 by cAMP-CRP-mAb 115D5 is not as strong as cAMP-CRP-mAb 66C3 (Fig. 20). RNA polymerase is able to replace mAb 115D5 in the cAMP-CRP-mAb 115D5 complex and form the RP_o (Fig. 21). Even though mAb 115D5 induces CRP Site 2 binding it is not a strong inhibitor of transcription initiation. In lac L8UV5 RNA polymerase binds to DNA in the absence of cAMP-CRP and the relative affinity of RNA polymerase to lac L8UV5 is higher than cAMP-CRP-mAb 66C3 or cAMP-CRP-mAb 115D5 to CRP Site 2. Thereby both antibodies do not inhibit initiation from lac L8UV5 (data not shown).

mAb 66C3 shows another interesting effect. The data presented in Figure 15 shows that abortive initiation from lac P^+ is almost completely inhibited when mAb 66C3 is incubated with cAMP-CRP prior to the addition of RNA polymerase. In contrast, addition of mAb 66C3 after formation of the RP_o results in only an approximately 50% inhibition. The antibody titration assay shown in Fig. 17 indicated that the mAb 66C3 concentration is not the limiting factor for the observed incomplete inhibition. The 50% residual activity remains at a mAb 66C3 to CRP ratio from 2:1 to 15:1. A 10 minute incubation of mAb 66C3 with the preformed RP_o results in a rapid decrease of transcription from lac P^+, but further incubation showed no additional inhibition. It seems that there may be two

types of initiation complexes. One is attacked by mAb 66C3 and dissociated within 10 minutes. The remaining RP_o seems resistant to mAb 66C3 attack. As mentioned in Introduction, CRP is an asymmetric dimeric protein. The relative orientation of the N- and C-terminal domains in the two subunits is not identical. The CRP dimer has the capacity to bind two cAMP molecules but binding of the second cAMP is negatively cooperative (Takahashi et al., 1980). Only one cAMP per promoter fragment was found both in the presence and absence of RNA polymerase and the RP_o is fully active with only one cAMP bound (Garner and Revzin, 1982). They suggested that CRP with one cAMP exhibited "half of the sites reactivity". On the other hand, cAMP-CRP lac P^+ binding appears symmetrical about the axis between -61 and -62 as determined by photochemical protection and exo III digestion protection (Simpson, 1980). Since the lac P^+ promoter has an inverse repeated consensus sequence in the CRP binding site, binding of CRP to lac P^+ could be asymmetrical. CRP-lac P^+ binding requires cAMP but only one cAMP bound per CRP dimer. Only the subunit of CRP which had bound cAMP would be in the right conformation to recognize the TGTGA motif but it will work equally well with either motif. The other subunit without cAMP probably has a weaker interaction with the second motif. The equal opportunity for tight binding in both motifs makes the protection by the protein appear symmetrical. In the review by de Crombrugghe et al.,

(1984) the possibility of two alternative asymmetrical arrangements of the CRP dimer at the lac P^+ CRP site with equal frequency has been considered. This model can explain the data obtained with mAb 66C3. It has been determined that two molecules of mAb 66C3 can bind per CRP dimer and mAb 66C3 preferentially binds to CRP in the presence of cAMP (Fig. 14). Since each subunit of CRP could be in different conformations mAb 66C3 could bind to the subunit with cAMP better than to the subunit without cAMP. In the preformed RP_o if the cAMP binding subunit of CRP is away from RNA polymerase, the strong binding of mAb 66C3 to this subunit will not interfere with RNA polymerase binding. The relatively weak mAb 66C3 binding to the subunit lacking cAMP might be prevented by the competing interaction with RNA polymerase. This RP_o form would be active and resistant to mAb 66C3 attack. If the cAMP bound subunit of CRP is facing RNA polymerase, the strong binding of mAb 66C3 could change the conformation of the subunit, block the protein-protein interaction between CRP and RNA polymerase or simply sterically compete with RNA polymerase for lac binding. In the lac P^+ promoter the -10 region for RNA polymerase diverges from the consensus sequence. The result of the competition of cAMP-CRP-mAb 66C3 with RNA polymerase is the loss of RNA polymerase binding (Fig. 18, lane f). In lac L8UV5, because of the mutations within the -10 RNA polymerase binding region and the -60 CRP binding region, RNA polymerase binding is

stronger than that by the cAMP-CRP complex. According to the model presented here, binding of mAb 66C3 to the subunit close to RNA polymerase results in release of CRP (Fig. 19 lane f). The results of Ebright et al. (1984) seem to support this model but also raise some questions. They selected CRP mutants, CRP', in which Glu 181 in the F-alpha helix was changed. CRP' mutants do not bind to lac P^+ but bind equally well to lac L8 and lac L29. The base change in lac L8 and lac L29 are symmetrically identical. Each change is a GC to AT substitution located 5 bp from the axis of symmetry in the CRP recognition site as shown below:



A single mutation in one of the two consensus sequences results in recovery of binding activity suggesting that both consensus sequences are functional. Tight binding in one of these sequences is enough to form a stable complex similar to that suggested in our model. But in lac L8 and lac L29 there is still an unchanged TGTGA sequence remaining, however wild type CRP could not form a stable complex with it. This suggests that the weak interaction between the unliganded CRP subunit and the TGTGA motif is important or the mutant TGTAA sequence pushes CRP away and

destabilizes the complex.

It is interesting to point out that in most of the CRP requiring promoters, CRP binding always directs RNA polymerase binding, but in <u>lac</u> L8UV5, it is RNA polymerase that directs CRP binding (Fig. 19, lanes b, e). The footprints show the similar protection pattern in cAMP-CRP-<u>lac</u> P^+-RNA polymerase and cAMP-CRP-<u>lac</u> L8UV5-RNA polymerase indicating that the contacts between the proteins and both promoters are similar. This suggests that proper interaction between protein and DNA can form a stable CRP-DNA or RNA polymerase-DNA complex. When this interaction is not strong enough to form a stable complex the interaction between CRP and RNA polymerase provides additional contacts to stabilize the weaker binding protein.

As discussed above RNA polymerase binding could induce conformational changes in CRP that stabilize the cAMP-CRP complex against attack by mAb 64D1. A conformational change in CRP induced by RNA polymerase is indicated by comparing the DNase I footprint patterns. In Figures 8, 9, 18 (the upper strand) and 19, cAMP-CRP complex protects the region from -48 to -75 (lane b). When RNA polymerase is added, bands in the -83 region which were not protected by CRP binding are covered and in the -85 region enhanced cutting is seen. The basic CRP protection pattern is unchanged. The small additional protection above the -80 region could not be due to another

CRP molecule binding. It could reflect the conformational changes in CRP induced by contact with RNA polymerase. Since the original CRP protection pattern is not changed, this conformational change of CRP should not affect the major DNA contacts with CRP. Data presented in Figure 19 also suggest that CRP binding could alter RNA polymerase conformation. In <u>lac</u> P^+ the effect can not be seen because polymerase binding occurs only in the presence of CRP. The <u>lac</u> L8UV5 mutant makes it possible to compare the binding pattern of RNA polymerase with and without the cAMP-CRP complex. In Figure 19, bands shown at the region from -44 to -47 with (lane e) or without (lane d) cAMP-CRP are the major differences seen in the RNA polymerase binding region. Without CRP the bands appear and with cAMP-CRP the bands disappear. When mAb 66C3 is added, CRP is released and those bands reappear (lane f). The presence of 25% glycerol enhances DNase I digestion in this region in the absence of cAMP-CRP. The contrast with and without cAMP-CRP is more impressive. Glycerol increases RNA polymerase affinity for <u>lac</u> P^+ so the comparison is possible in the presence of glycerol. Similar differences are seen in the <u>lac</u> P^+ system as in <u>lac</u> L8UV5 (Fig. 22 lands e, f, g). Region -44 to -47 is close to the CRP binding site. It is possible that the RNA polymerase domain located in this region is involved in the interaction with CRP. The observed change in the footprint pattern suggests that when interacting with the cAMP-CRP complex, this domain of RNA

polymerase changes its conformation as well as DNA binding activity. This conformational change could be independent of the catalytic domain, because in lac L8UV5 RNA polymerase is fully functional in either the absence or the presence of cAMP-CRP. The differences shown in the footprint do not affect RNA polymerase activity. Besides, in 25% glycerol where RNA polymerase shows binding to lac P^+, transcription initiation from lac P^+ is increased in the absence of cAMP-CRP (Fig. 23, b).

Summary and Suggestions:

As discussed above the nine mAbs were randomly selected. The classifications and the locations of the mAbs are interesting in their coincidence. All the Class I antibodies are located in the C-terminal domain and all the Class II antibodies are located in the N-terminal domain. This might give some information about the nature of the two domains. It is interesting to see if this rule is generally obeyed by making more monoclonal antibodies and checking their specificity by using similar methods.

The antigenic determinant of mAb 64D1 is near the cAMP binding site. mAb 64D1 binds to unliganded CRP only and this binding changes CRP conformation to one unable to bind cAMP. The C-terminal sequence of the 10,000 Da trypsin-DNA fragment to which mAb 64D1 can bind has not yet been accurately determined. The binding site of mAb 64D1 can be either from Val-85 to Lys-88 or from Val-85 to Lys-

100. More accurate antigenic determinant location can be done by assaying antibody binding with synthetic oligopeptide fragments spanning this region.

As shown in Table 7, only one mAb 64D1 molecule bound per CRP dimer and binding of one mAb 64D1 changes the conformation of both subunits of CRP (Fig. 13). It was proposed in this dissertation that the demonstrated binding of one antibody could be due to either interaction of both antibody combining sites of mAb 64D1 to the subunits of a CRP dimer simultaneously. This would therefore change the conformation on both subunits. It is also possible that steric hindrance imposed by the initially bound antibody blocks binding of the second antibody. This requires that the binding of one molecule of antibody should be able to change the conformation on both subunits. In order to distinguish between the two possibilities, similar experiments as those shown in Table 7 and Fig. 3 are suggested, using papain digested mAb. The Fab should contain only one combining site. This will indicate the Fab to CRP dimer ratio giving complete digestion and if a Fab-CRP-Fab complex forms.

The antigenic determinants of mAbs 66C3 and 63B2 are located in a similar region but mAb 66C3 enhances DNA binding by CRP while mAb 63B2 blocks this binding. More accurate location of the epitopes for these two mAbs will be very helpful in understanding CRP-DNA interaction. As Class I antibodies, both mAbs 66C3 and 63B2 do not bind to

denatured CRP. Methods used by Tsapakos et al. (1985) can help solve the problem. There are several protease cutting sites in the CRP C-terminal domain where the two mAbs bind. HPLC will be used to resolve and quantify the small peptide fragments. If the mAb binding site is within the protease cutting site, the HPLC pattern for protease digestion of CRP and mAb-CRP complex will be different.

The data presented strongly suggest direct protein-protein interaction between CRP and RNA polymerase and local conformational changes are observed by footprinting. However only the _lac_ system has been assayed in this study. Large differences in the distance between the CRP and RNA polymerase binding sites are found in other systems like _gal_ and _ara_. Modified _lac_ fragments with deletions or insertions in the region between the two proteins are suggested for footprint assay. Footprinting of CRP and RNA polymerase in _lac_ is well established. Changing the distance between CRP and RNA polymerase without damage to the binding sites of the proteins can be used to generate model systems to study the relation between the distance and the binding activities of the two molecules. This will be very helpful in understanding the role of CRP in transcription regulation of different CRP dependent promoters.

Table 1. Binding of mAbs to Native and Denatured CRP

		ELISA Assay	
mAb	Class	Native CRP	Denatured CRP
		(A 410nm)	
62A2	I	0.94	0.14
62D6	I	0.84	0.27
63A3	I	0.84	0.07
63B2	I	1.19	0.29
66C3	I	1.35	0.15
64B4	II	1.30	1.34
65C3	II	1.38	1.32
64D1	II	1.19	1.13
115D5	II	1.16	1.19

Native CRP (0.5 ug/50 ul PBS) or urea-denatured CRP (0.5 ug) were adsorbed onto wells of EIA plates. CRP was denatured by incubation of 50 ug CRP in 1 ml of 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 6 M urea for 30 minutes at 37°C. After diluting with 4 ml 50 mM Tris-HCl (pH 8.0) 50 ul was added to each well. Incubation with the indicated mAb (1.8 ug/50 ul PBS + 1 mg/ml BSA) was for 60 minutes at 37°C. Binding of mAbs was determined by ELISA as described in "Materials and Methods".

Table 2. Binding of Class I mAbs to CRP and Cores

		ELISA Assay		
mAb	Class	CRP	SAP-Core	CHY-Core
			(A 410 nm)	
62A2	I	1.26	0.15	0.06
62D6	I	1.44	0.08	0.05
63A3	I	1.17	0.10	0.06
63B2	I	1.53	0.49	0.11
66C3	I	1.46	0.59	0.09
64B4	II	1.57	1.53	1.46
65C3	II	1.63	1.62	1.75
64D1	II	1.66	1.64	1.63
115D5	II	1.53	1.47	1.25

Native CRP (0.5 ug/50 ul) or core derived from incubation of 0.5 ug CRP plus cAMP with Staph. aureus V8 protease (SAP) or chymotrypsin (CHY) (see Materials and Methods) was adsorbed onto wells of EIA plates. Incubation with mAb (0.5 ug in 50 ul PBS + 1 ml BSA) was for 60 minutes at 37°C. Binding of mAbs was determined by ELISA as described in "Materials and Methods".

Table 3. Effect of Anti-CRP mAbs on lac P^+ DNA Directed Abortive Initiation

mAb	Class	ApApUpU Synthesis
		(% activity)
62A2	I	25
62D6	I	27
63A3	I	67
63B2	I	2
66C3	I	0
64B4	II	94
65C3	II	89
64D1	II	0
115D5	II	43

CRP dependent abortive initiation was assayed as described in "Materials and Methods".

Table 4. Effect of Anti-CRP mAbs on cAMP Binding

mAb	Class	[^{3}H]cAMP Bound (% Activity)
62A2	I	66
62D6	I	65
63A3	I	81
63B2	I	117
66C3	I	95
64B4	II	104
65C3	II	95
64D1	II	14
115D5	II	109

Binding of [^{3}H]cAMP was assayed as described in "Materials and Methods".

Table 5. Summary of Functional Properties of Anti-CRP mAbs

mAb	Class	*CRP binding	Inhibition of cAMP binding	Inhibition of *lac* DNA binding	Inhibition of Abortive initiation
62A2	I	+++	+	++	++
62D6	I	+++	+	++	++
63A3	I	+	+	+	+
63B2	I	+++	-	+++	+++
66C3	I	+++	-	-	+++
64B4	II	-	-	-	-
65C3	II	-	-	-	-
64D1	II	+++	+++	+++	+++
115D5	II	+++	-	-	++

The symbols +++, ++, + and - refer to strong, moderate, weak and no effect on the assays listed above. mAb 66C3 and mAb 115D5 bind to the cAMP-CRP-*lac* DNA complex.

*: Binding of mAbs to CRP in solution (see Fig. 6).

Table 6. Immunoprecipitin Assay for Binding of mAbs to CRP and CRP-DNA Complexes

mAb	$[^3H]$NEM-CRP (cpm)	CRP-$[^3H]$DNA (cpm)	CRP-$[^3H]$DNA-RNP (cpm)
64D1	9087	0	97
66C3	26420	7748	9318

The Immunoprecipitin assay was carried out as described in "Materials and Methods". RNP, RNA polymerase holoenzyme; $[^3H]$ DNA, $[^3H]$lac P^+ DNA fragment.

Table 7. Lack of Binding of [^{125}I]mAb 64D1 to mAb 64D1-CRP

Immobilized mAb	[^{125}I]mAb 64D1 Bound
	(cpm)
64D1	2511
64C4	68306

Conditions used for the Immunoassay for [^{125}I]mAb 64D1 binding are described in "Materials and Methods".

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

1/1 | 31/11 | 61/21

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 ACG CTT ATT
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 thr leu ile

91/31 | 121/41 | 151/51

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 GGT AAA GAA ATG ATC
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 gly lys glu met ile

181/61 | 211/71 | 241/81

CTC TCC TAT CTG AAT CAG GGT GAT TTT ATT GGC GAA CTG GGC CTG TTT GAA GAG GGC CAG GAA CGT AGC GCA TGG GTA CGT GCG AAA ACC
leu ser tyr leu asn gln gly asp phe ile gly glu leu gly leu phe glu glu gly gln glu arg ser ala trp val arg ala lys thr

271/91 | 301/101 | 331/111

GCC TGT GAA GTG GCT GAA ATT TCG TAC AAA AAA TTT CGC CAA TTG ATT CAG GTA AAC CCG GAC ATT CTG ATG CGT TTG TCT GCA CAG ATG
ala cys glu val ala glu ile ser tyr lys lys phe arg gln leu ile gln val asn pro asp ile leu met arg leu ser ala gln met

361/121 | 391/131 | 421/141

GCG CGT CGT CTG CAA GTC ACT TCA GAG AAA GTG GGC AAC CTG GCG TTC CTC GAC GTG ACG GGC CGC ATT GCA CAG ACT CTG CTG AAT CTG
ala arg arg leu gln val thr ser glu lys val gly asn leu ala phe leu asp val thr gly arg ile ala gln thr leu leu asn leu

451/151 | 481/161 | 511/171

GCA AAA CAA CCA GAC GCT ATG ACT CAC CCG GAC GGT ATG CAA ATC AAA ATT ACC CGT CAG GAA ATT GGT CAG ATT GTC GGC TGT TCT CGT
ala lys gln pro asp ala met thr his pro asp gly met gln ile lys ile thr arg gln glu ile gly gln ile val gly cys ser arg

541/181 | 571/191 | 601/201

GAA ACC GTG GGA CGC ATT CTG AAG ATG CTG GAA GAT CAG AAC CTG ATC TCC GCA CAC GGT AAA ACC ATC GTC GTT TAC GGC ACT CGT TAA
glu thr val gly arg ile leu lys met leu glu asp gln asn leu ile ser ala his gly lys thr ile val val tyr gly thr arg OCH

Figure 1.

Figure 2. Drawing of the CRP dimer (from de Crombrugghe et al. 1984). The N-terminal domain consists of alpha-helix A, beta-sheets 1 to 8, and alpha-helices B and C. The DNA binding C-terminal domain consists of alpha-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.

Figure 2.

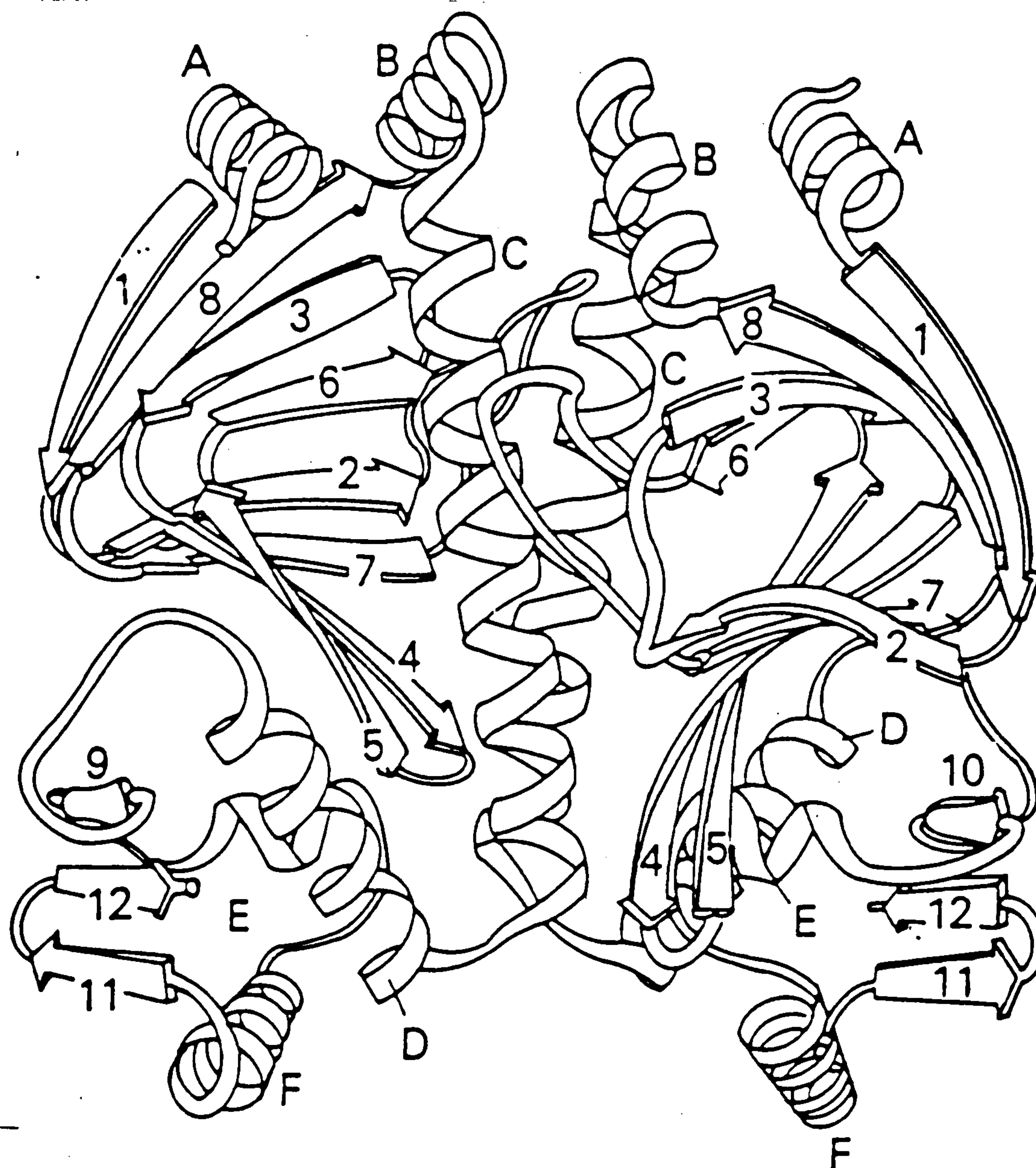


Figure 3. Western blot analysis of binding of mAbs to CRP fragments. CRP and fragments produced by partial proteolysis of CRP were resolved electrophoretically and transferred to nitrocellulose sheets. Binding of mAbs was determined as indicated in "Materials and Methods". CRP, intact CRP; SAP, core fragment produced after incubation of cAMP-CRP with *Staph.aureus* V8 protease; SUB, core fragment produced after incubation of cAMP-CRP with subtilisin; CHY+SDS, CRP fragments produced after incubation of CRP with chymotrypsin in the presence of SDS; TRY+DNA, CRP fragments produced after incubation of DNA-CRP with trypsin.

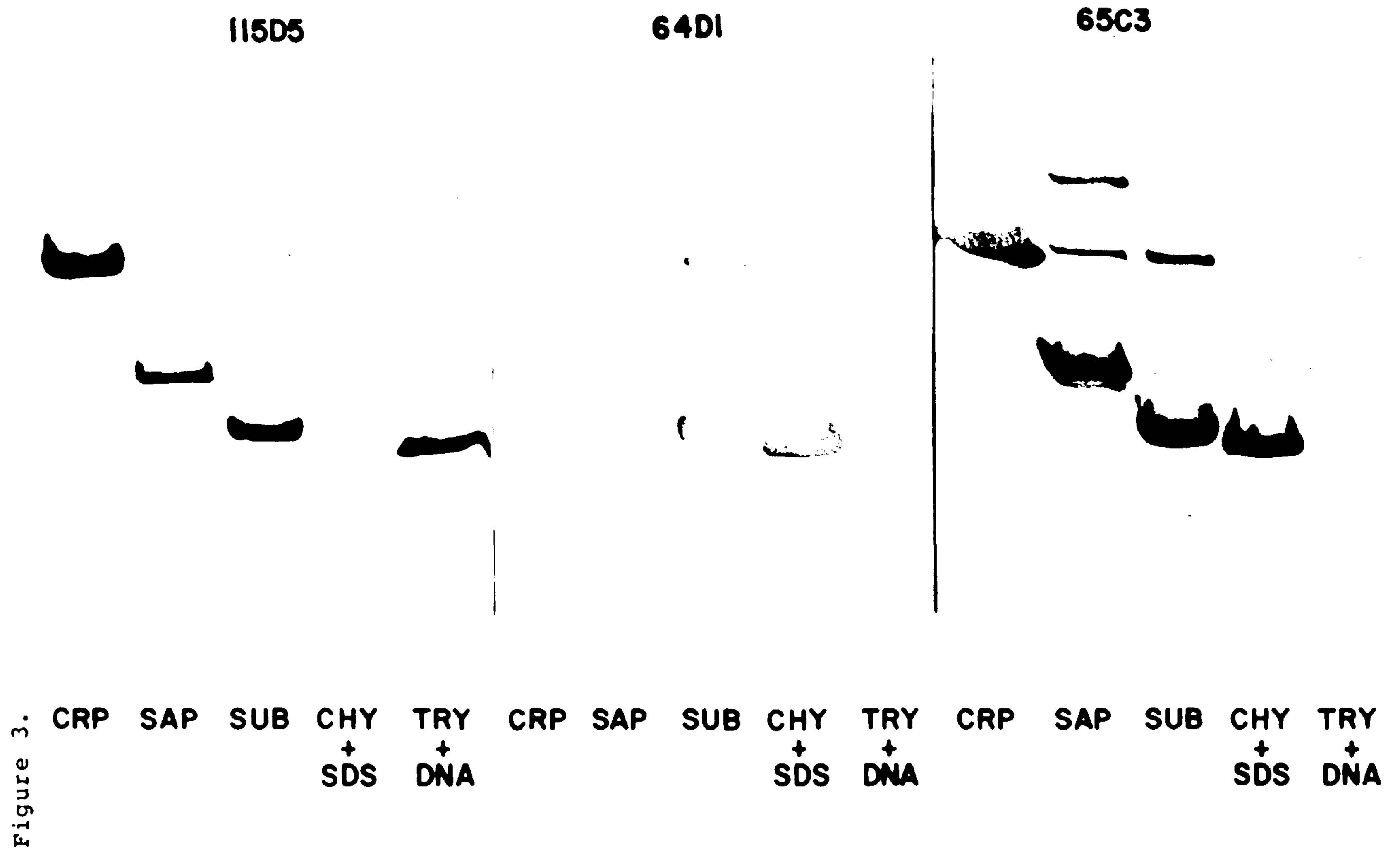


Figure 3.

Figure 4. 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 mAbs. Segments labelled A - F represent the alpha helical regions of CRP (McKay et al., 1982).

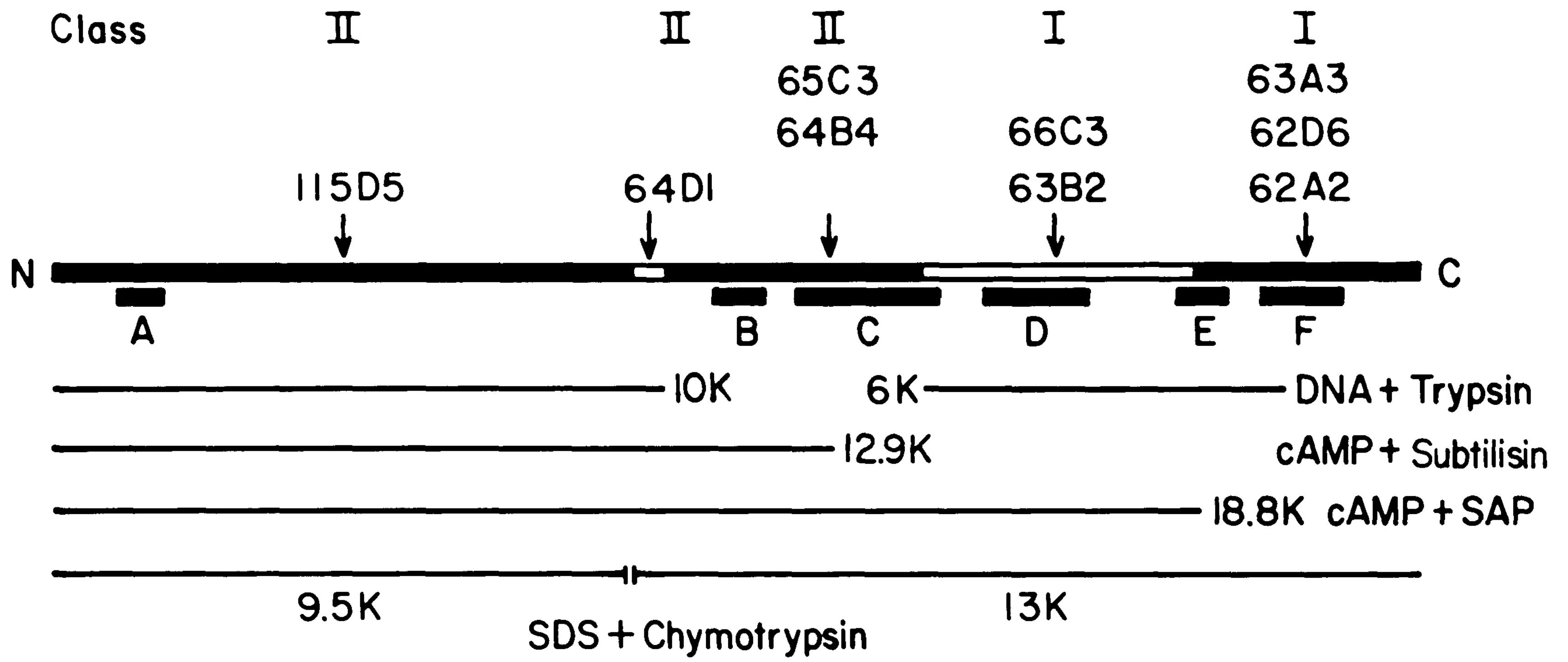


Figure 4.

Figure 5. Effect of anti-CRP mAbs on binding of cAMP-CRP to the lac DNA promoter fragment. Samples containing (final volume 15 ul) 0.2 uM lac DNA fragment, 24 uM cAMP, with or without 0.8 uM CRP and 4 uM mAb were resolved electrophoretically. lac P^+, no CRP added; CRP, lac DNA + cAMP-CRP; lanes a - j contained lac DNA, cAMP-CRP and the indicated mAb as follows: (a) mAb 66C3, (b) mAb 65C3, (c) mAb 64B4, (d) mAb 64D1, (e) mAb 63A3, (f) mAb 63B2, (g) mAb 63C2, (h) mAb 62A2, (i) mAb 62D6, (j) mAb 115D5.

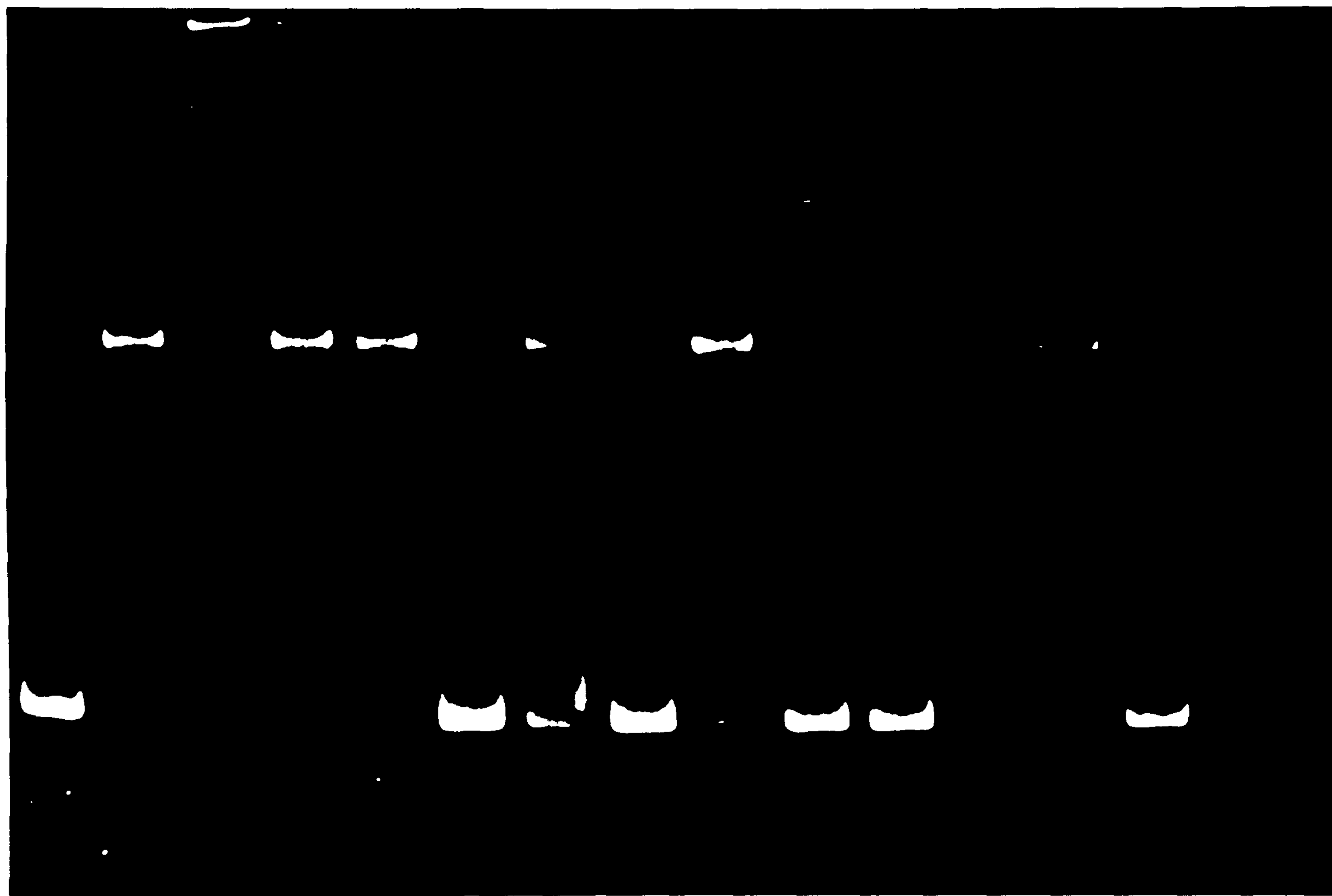


Figure 5.

Figure 6. Gel electrophoresis analysis of binding of mAbs to CRP in solution. Samples containing (final volume 15 ul) 2.4 uM of the indicated mAb with or without 4.4 uM CRP were resolved electrophoretically as described in "Materials and Methods". CRP is a positively charged molecule, during the electrophoresis it migrates in the opposite direction of mAbs and runs off the gel. The bands shown in the gel are the mAbs or mAb-CRP complexes only. a, mAb; b, mAb + CRP.

66C3 65C3 64B4 64D1 63A3 63B2 62A2 62D6 115D5

a b a b a b a b a b a b a b a b a b

Figure 6.

Figure 7. Effect of time of preincubation with mAb 64D1 on stability of CRP complexes. Conditions for the abortive initiation assay are described in "Materials and Methods". Preincubation (10 minutes at 37°C) components are: A, cAMP +CRP + RNA polymerase + *lac* P^+ fragment; B, cAMP + CRP + *lac* P^+ fragment; C, cAMP + CRP; D, CRP. After addition of mAb 64D1, the mixtures were incubated at 37°C for the times indicated. Residual activity was determined following addition of the missing components plus ApA and [^{3}H]UTP.

Figure 7.

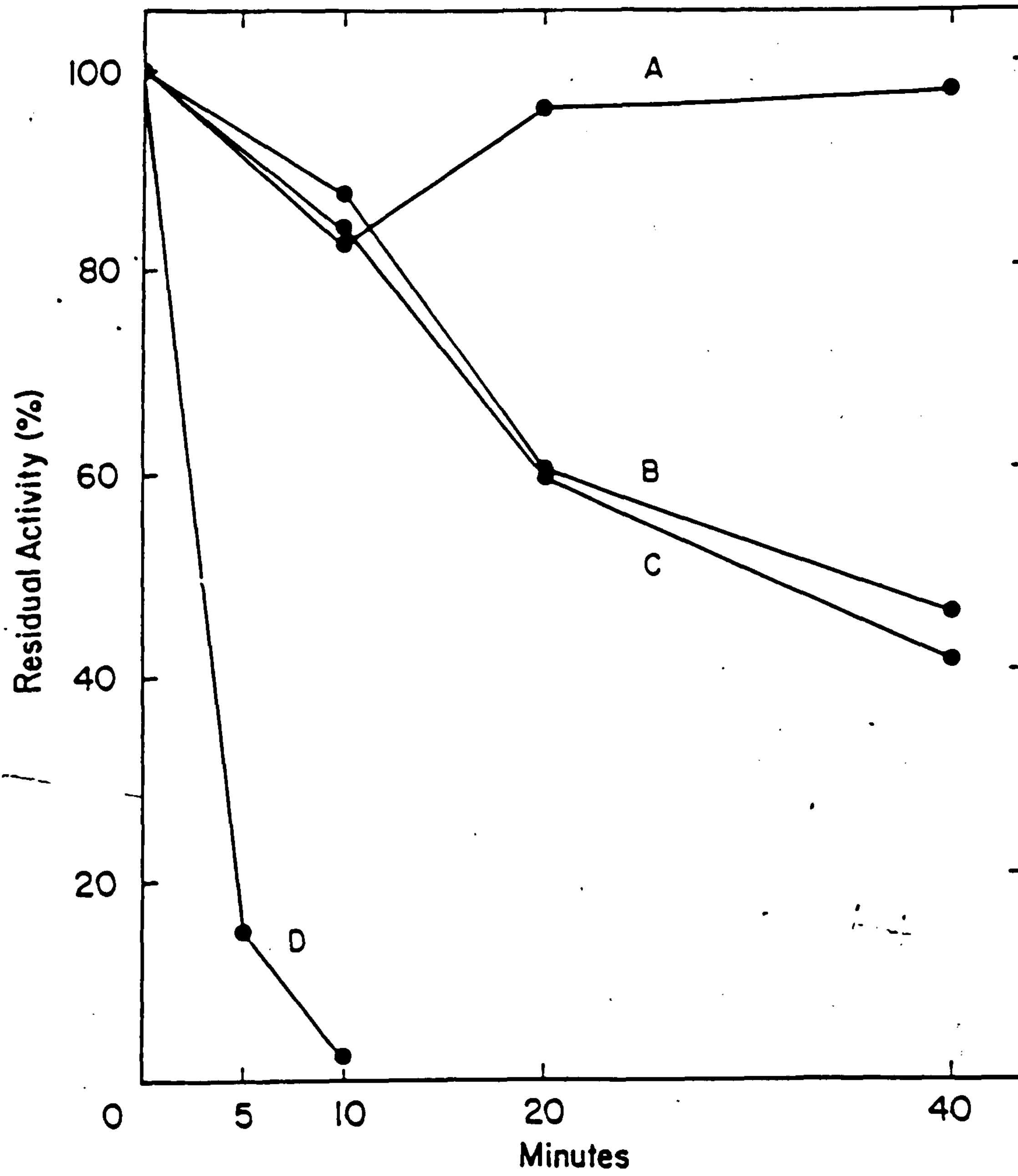


Figure 8. Effect of time of incubation with mAb 64D1 on CRP-DNA complexes. DNase I footprinting conditions are described under "Materials and Methods" using 3 uM [^{32}P]*lac* P^{+} fragment and where indicated 40 nM CRP, 100 nM cAMP, 120 nM RNA polymerase and 240 nM mAb 64D1. lane a, [^{32}P]*lac* P^{+} only; lanes b, c, cAMP + CRP + [^{32}P]*lac* P^{+} incubated for 20 and 40 minutes; lanes d, e, f, cAMP-CRP-[^{32}P]*lac* P^{+} complex formed after incubation for 10 minutes followed by incubation with mAb 64D1 for 10, 20 and 40 minutes; lane g, RNA polymerase + [^{32}P]*lac* P^{+} incubated for 10 minutes; lane h, cAMP-CRP-RNA polymerase-[^{32}P]*lac* P^{+} complex incubated for 40 minutes; lanes i, j, cAMP-CRP-RNA polymerase-[^{32}P]*lac* P^{+} complex formed after incubation for 10 minutes followed by incubation with mAb 64D1 for 20 and 40 minutes.

Figure 8.

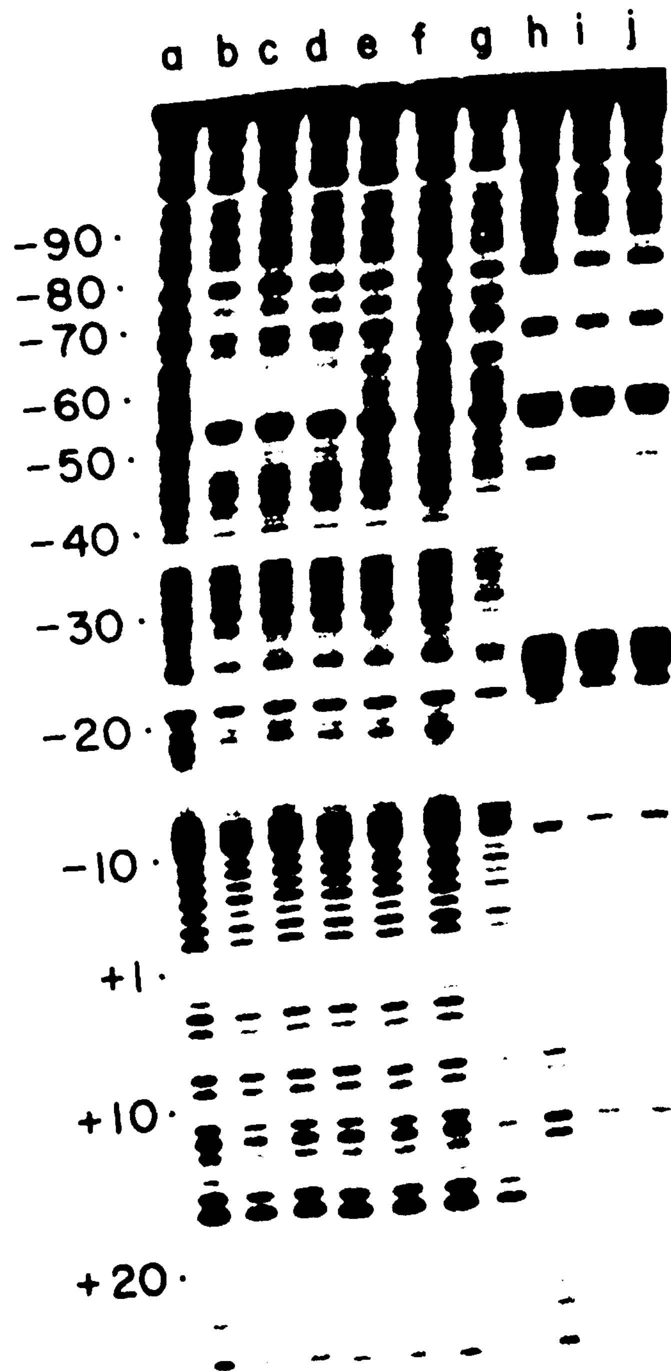


Figure 9. Effect of cAMP concentration on stability of CRP complexes. DNase I footprinting conditions are similar to those described in the legend for Figure 8 except that the cAMP concentration is varied. Lane a, [^{32}P]lac P^{+} only; lane b, [^{32}P]lac P^{+} + CRP and 100 uM cAMP; lane c, mAb 64D1 was incubated with CRP for 10 minutes then 100 uM cAMP and [^{32}P]lac P^{+} were added and incubated for 10 minutes; lane d, CRP + 25 uM cAMP incubated for 10 minutes followed by incubation with [^{32}P]lac P^{+} for 10 minutes; lanes e, f, g, CRP + 100 uM, 50 uM or 25 uM cAMP incubated for 10 minutes followed by incubation with [^{32}P]lac P^{+} and mAb 64D1 for 10 minutes; lane h, [^{32}P]lac P^{+} + CRP + 25 uM cAMP incubated for 10 minutes; lanes i, j, k, [^{32}P]lac P^{+} + CRP and 100 uM, 50 uM or 25 uM cAMP incubated for 10 minutes followed by incubation with mAb 64D1 for 10 minutes; lanes l, m, n, o, [^{32}P]lac P^{+} + CRP + RNA polymerase and 50 uM, 25 uM,12.5 uM or 6.25 uM cAMP incubated for 10 minutes; lanes p, q, r, s, [^{32}P]lac P^{+} + CRP + RNA polymerase and 50 uM, 25 uM, 12.5 uM or 6.25 uM cAMP incubated for 10 minutes followed by incubation with mAb 64D1 for 10 minutes.

Figure 9.

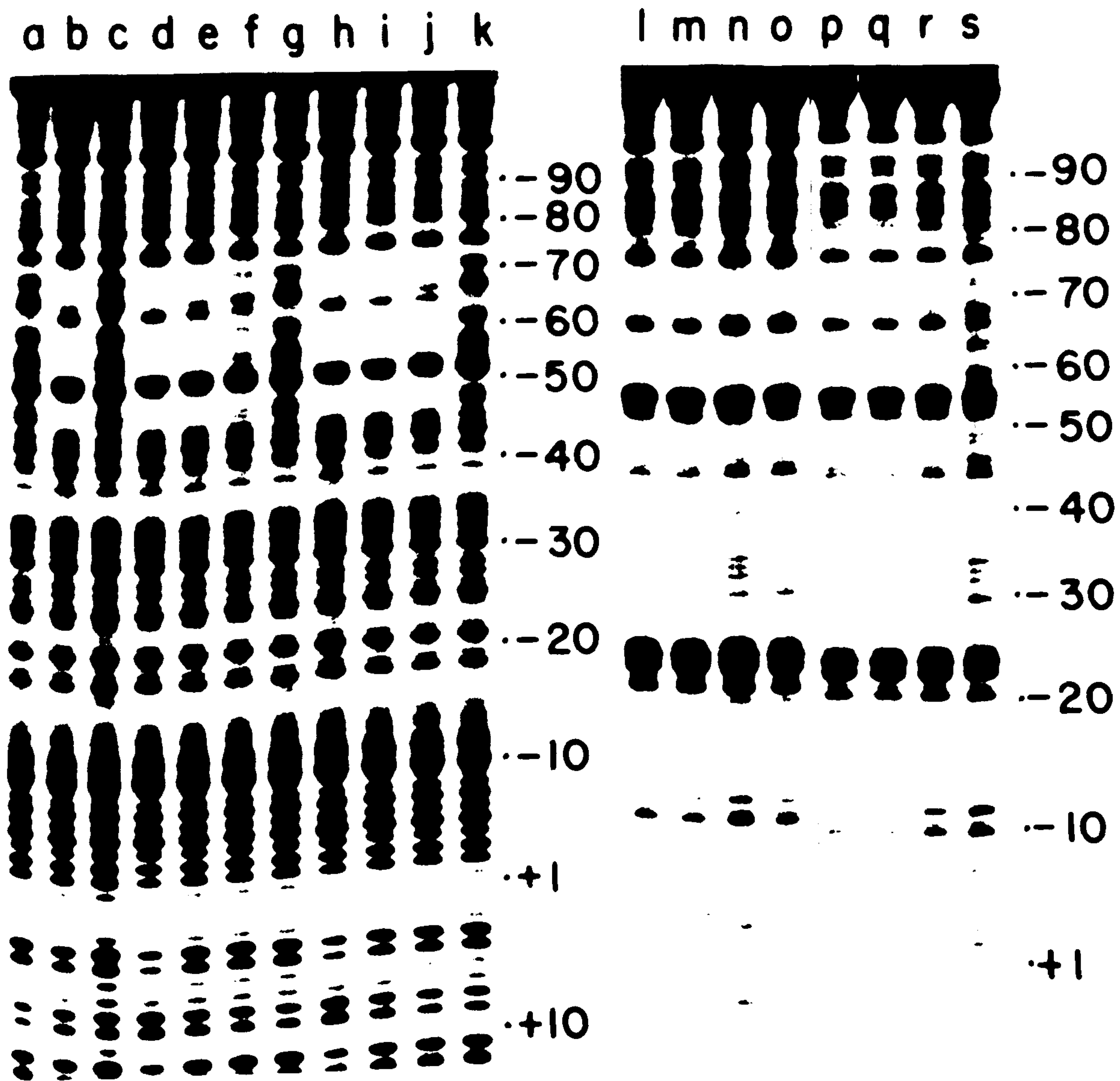


Figure 10. Effect of cAMP and cGMP on binding of [^{125}I]mAb 64D1 to CRP. Conditions are described under "Materials and Methods" with mAb 62A2 as the immobilized antibody. Binding of [^{125}I]mAb 64D1 was determined after incubation in the presence of the indicated concentrations of cAMP or cGMP.

Figure 10.

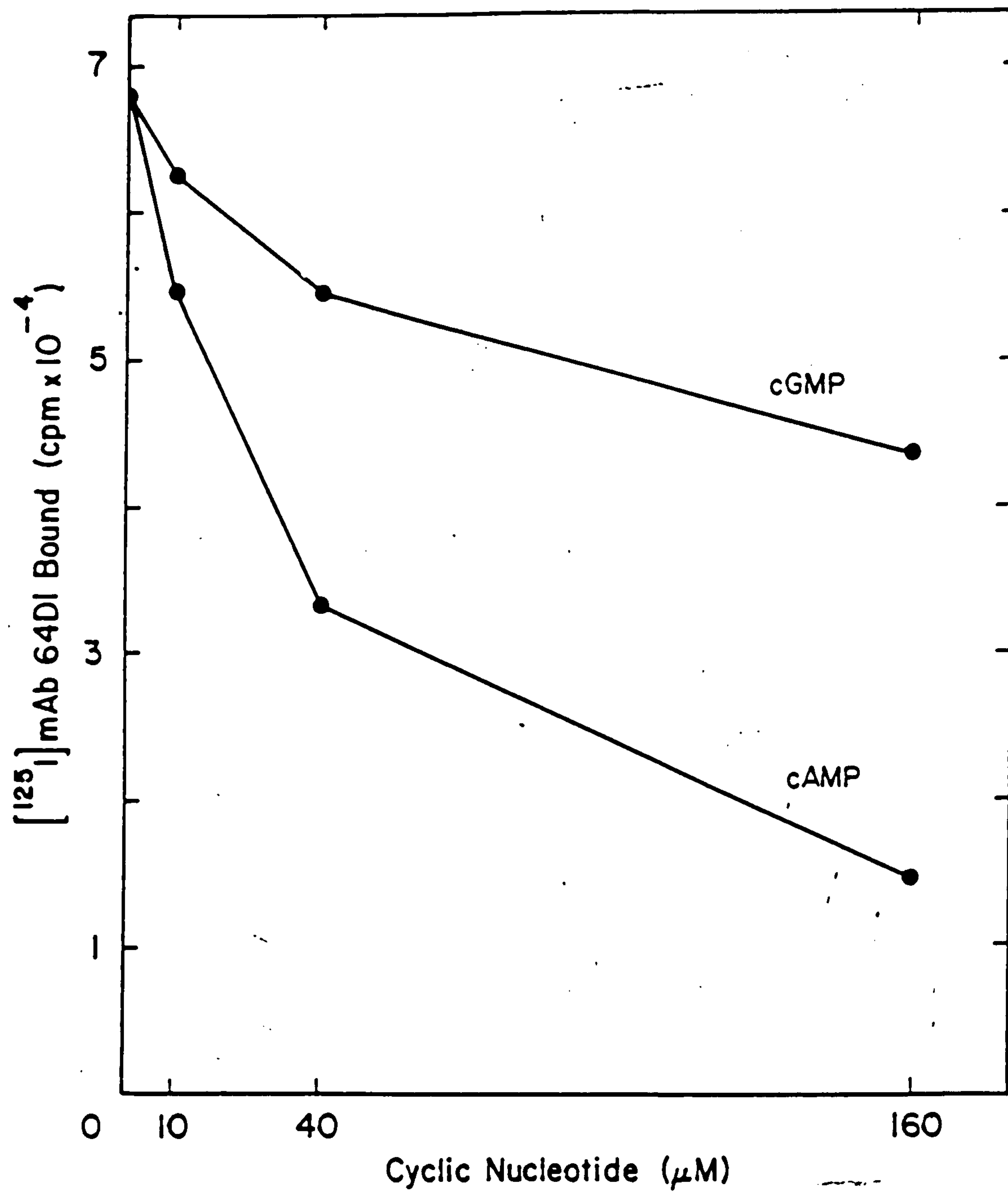


Figure 11. Effect of proteases on cAMP-CRP and mAb 64D1-CRP. Conditions are described under "Materials and Methods", the incubations with the indicated proteases were carried out for 40 minutes. *Staph. aureus* V8 protease (0.3 ug) plus: lane a, CRP; lane b, cAMP-CRP; lane c, mAb 64D1-CRP; lane d, mAb 64D1. Trypsin (0.15 ug) plus: lane e, CRP; lane f, cAMP-CRP; lane g, mAb 64D1-CRP; lane h, mAb 64D1. Chymotrypsin (0.07 ug) plus: lane i, CRP; lane j, cAMP-CRP; lane k, mAb 64D1-CRP; lane l, mAb 64D1; lane m, cyanogen bromide fragments of myoglobin.

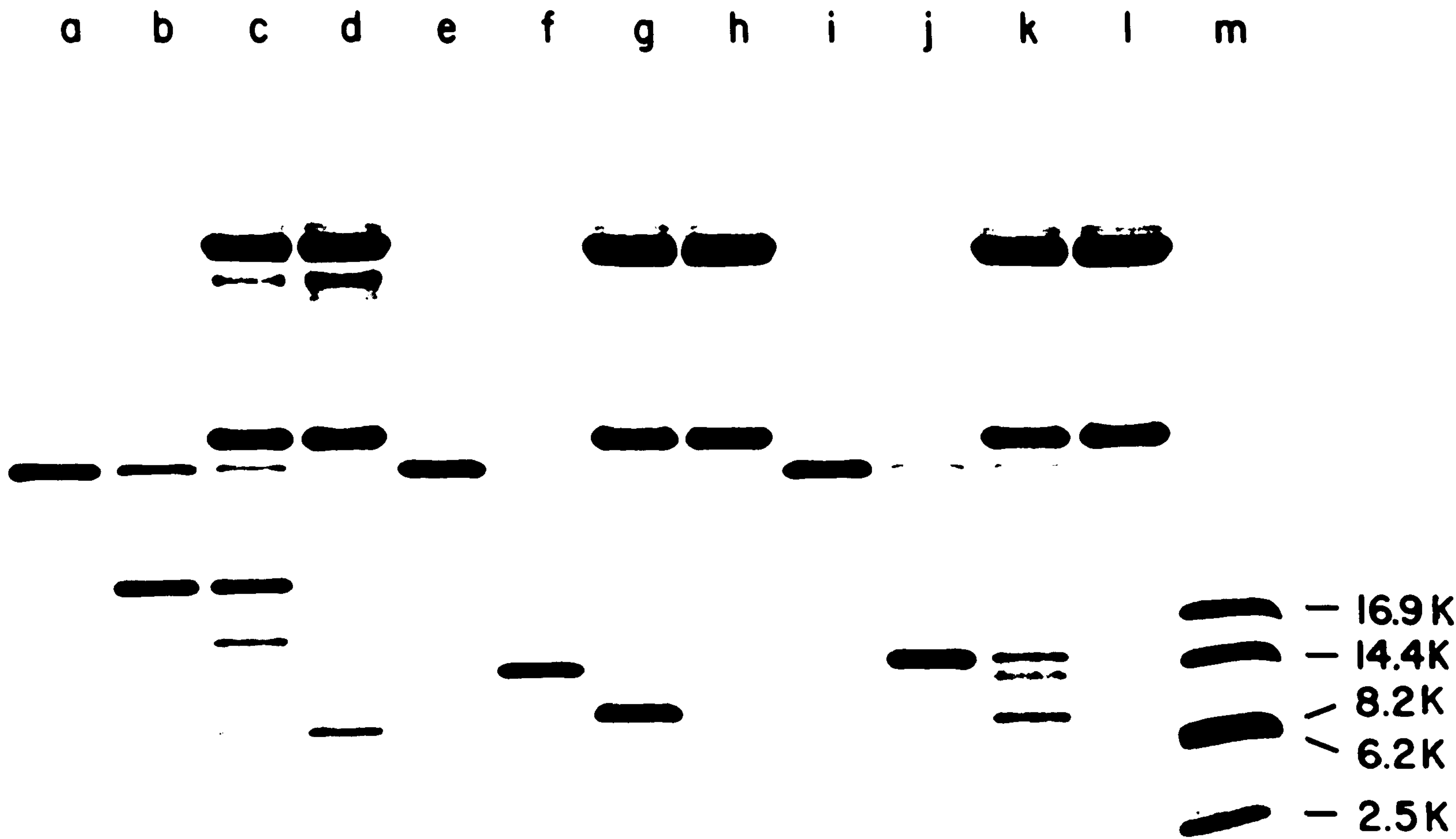


Figure 11.

Figure 12 A and B. Effect of subtilisin on CRP and mAb 64D1-CRP. Conditions are described in "Materials and Methods". Fig. 12 A (Coomassie blue staining): lane a, mAb 64D1; lane b, CRP; lanes c to f, CRP + 0.2 ug subtilisin incubated for 5, 10, 20, 40 minutes; lanes g to j, mAb 64D1-CRP + 0.2 ug subtilisin incubated for 5, 10, 20, 40 minutes; lane k, cAMP-CRP + 0.2 ug subtilisin incubated for 40 minutes. Fig. 12 B (Western blotting): lane a to e, mAb 64D1-CRP + 0.2 ug subtilisin incubated for 0, 5, 10, 20 and 40 minutes; lane f, cAMP-CRP + 0.2 ug subtilisin incubated for 40 minutes.

Figure 12.

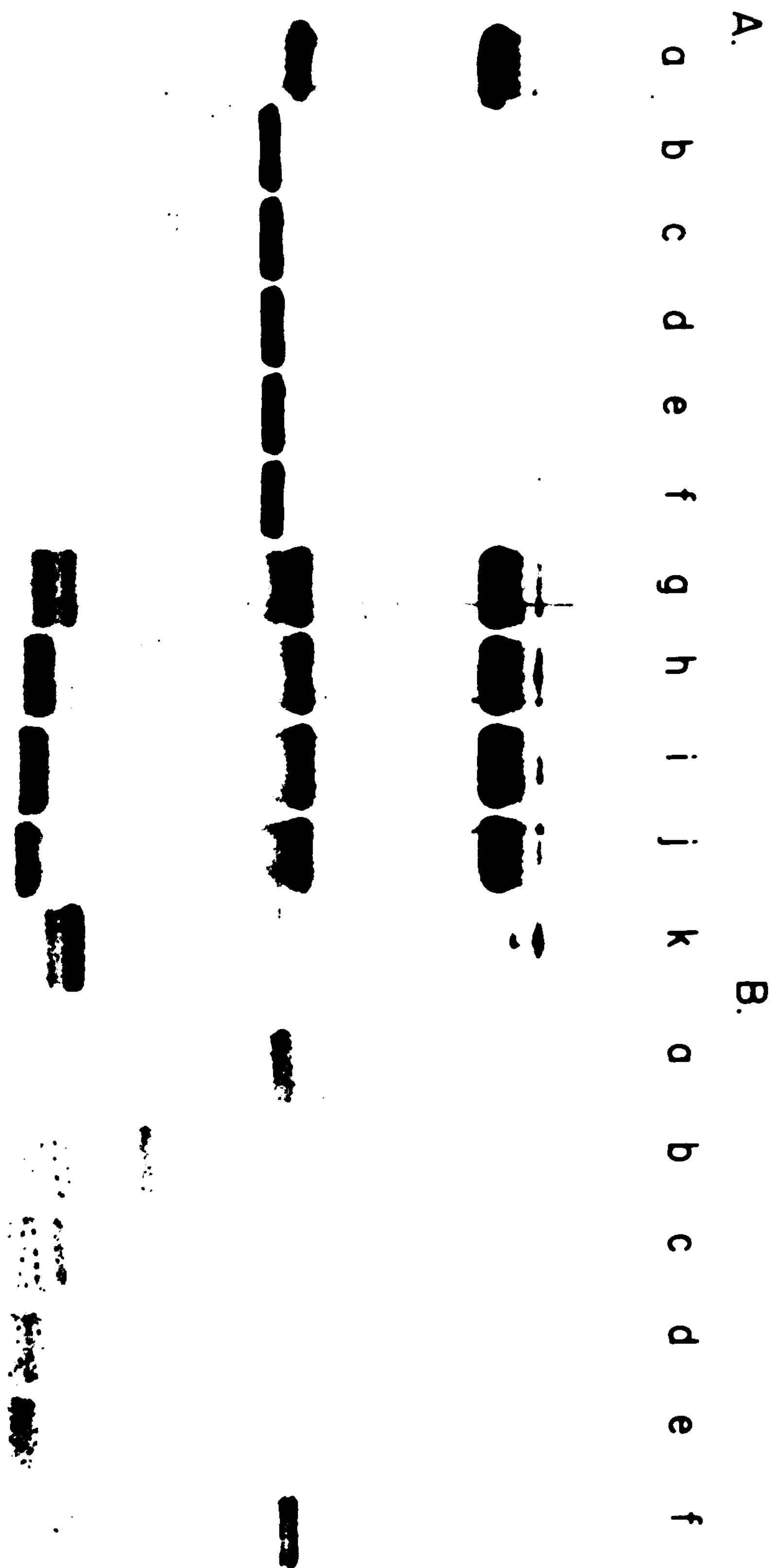


Figure 13. Effect of mAb 64D1 concentration on sensitization of CRP to attack by trypsin and subtilisin. Conditions are described under "Materials and Methods". 1.3 uM CRP was incubated with 0.15 ug trypsin (lanes a to d) or with 0.2 ug subtilisin (lanes e to i) for 40 minutes. Lanes a and e, CRP + 2 uM mAb 64D1; lanes b and f, CRP + 1.3 uM mAb 64D1; lanes c and g, CRP + 0.65 uM mAb 64D1; lanes d and h, CRP + 0.33 uM mAb 64D1; lane i, molecular weight markers: cytochrome C, 12,600 Da, trypsin inhibitor, 6,200 Da.

Figure 13.

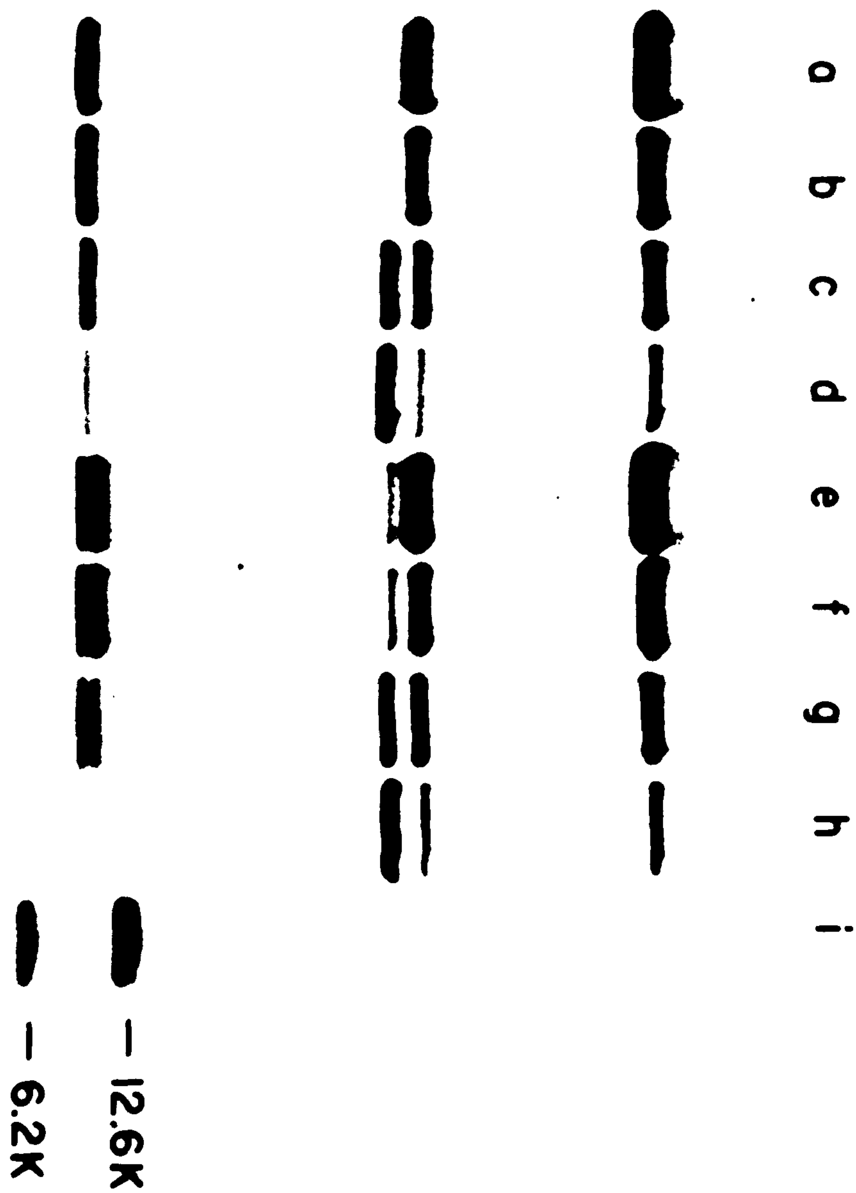


Figure 14. Effect of cAMP and cGMP on binding of [^{125}I]mAb 66C3 to CRP. Conditions are described under "Materials and Methods" with unlabeled mAb 66C3 as the immobilized antibody. Binding of [^{125}I]mAb 66C3 was determined after incubation in the presence of the indicated concentrations of cAMP or cGMP.

Figure 14.

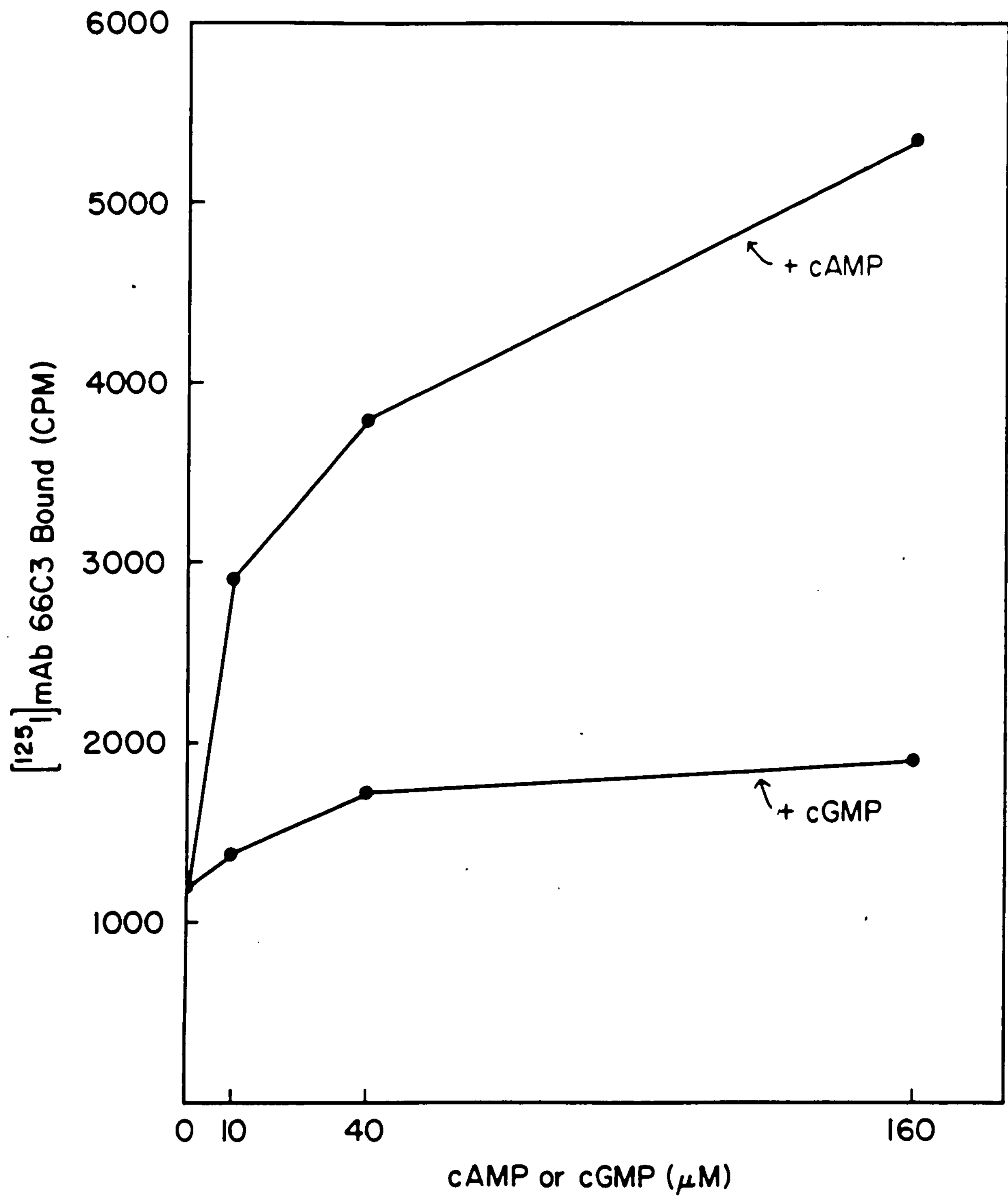


Figure 15. Effect of mAb 66C3 on abortive initiation from the *lac* P^+ promoter. Conditions for the abortive initiation assay are described in "Materials and Methods". RP_o (a,b) or mAb 66C3-cAMP-CRP-*lac* P^+ complex (c) are formed by incubation at 37°C for 10 minutes following by adding KPK buffer (a), mAb 66C3 (b) or RNA polymerase (c) for an additional 10 minutes incubation at 37°C. Abortive initiation activity was determined following addition of ApA and [^{3}H]UTP and incubations for the times indicated.

Figure 15.

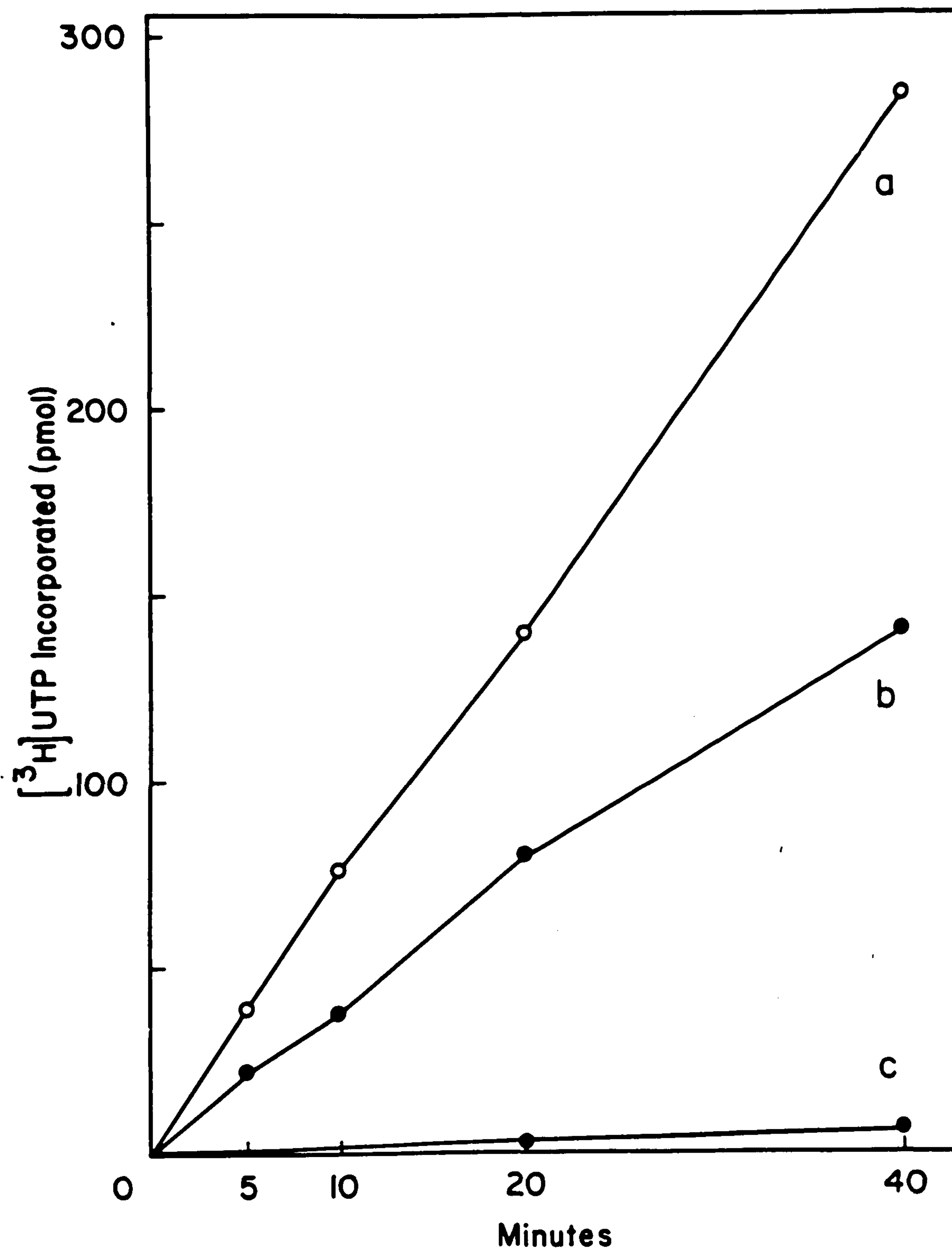


Figure 16. Effect of mAb 66C3 on the stability of the initiation complex. Abortive initiation conditions are described under "Materials and Methods". Preincubation components are: a, cAMP + CRP + *lac* P^+ + RNA polymerase; b, cAMP + CRP + *lac* P^+. After addition of mAb 66C3, the mixtures were incubated for the times indicated. Residual activity was determined following addition of the missing components plus ApA and [^{3}H]UTP.

Figure 16.

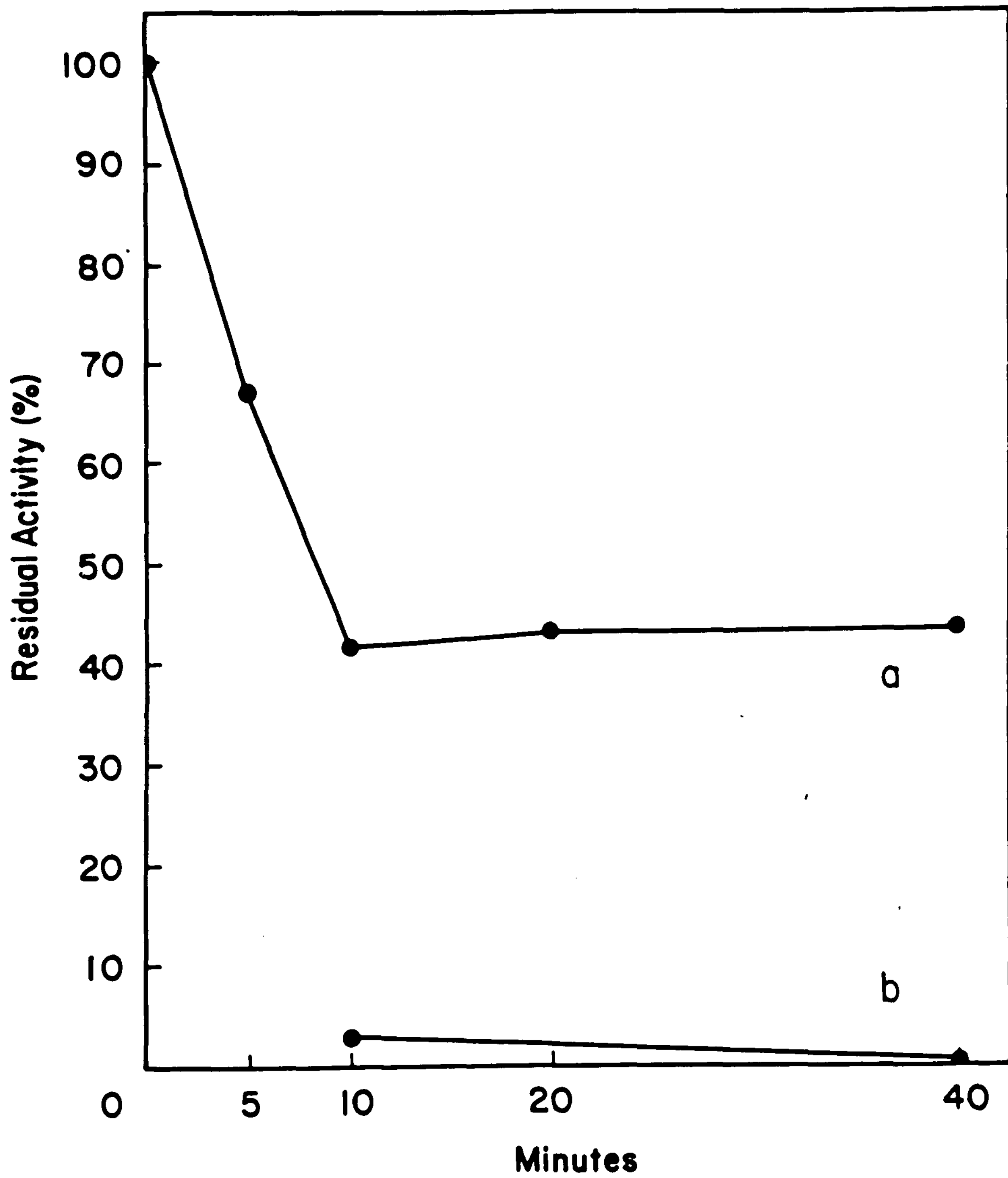


Figure 17. Effect of mAb 66C3 concentration on abortive initiation from the *lac* P^+ promoter. Abortive initiation conditions are described under "Materials and Methods" with a constant concentration of CRP and the indicated concentration of mAb 66C3. a, mAb 66C3 was added after the formation of RP_o; b, mAb 66C3 was added after the formation of the cAMP-CRP-*lac* P^+ complex, and after 10 minutes incubation RNA polymerase was added.

Figure 17.

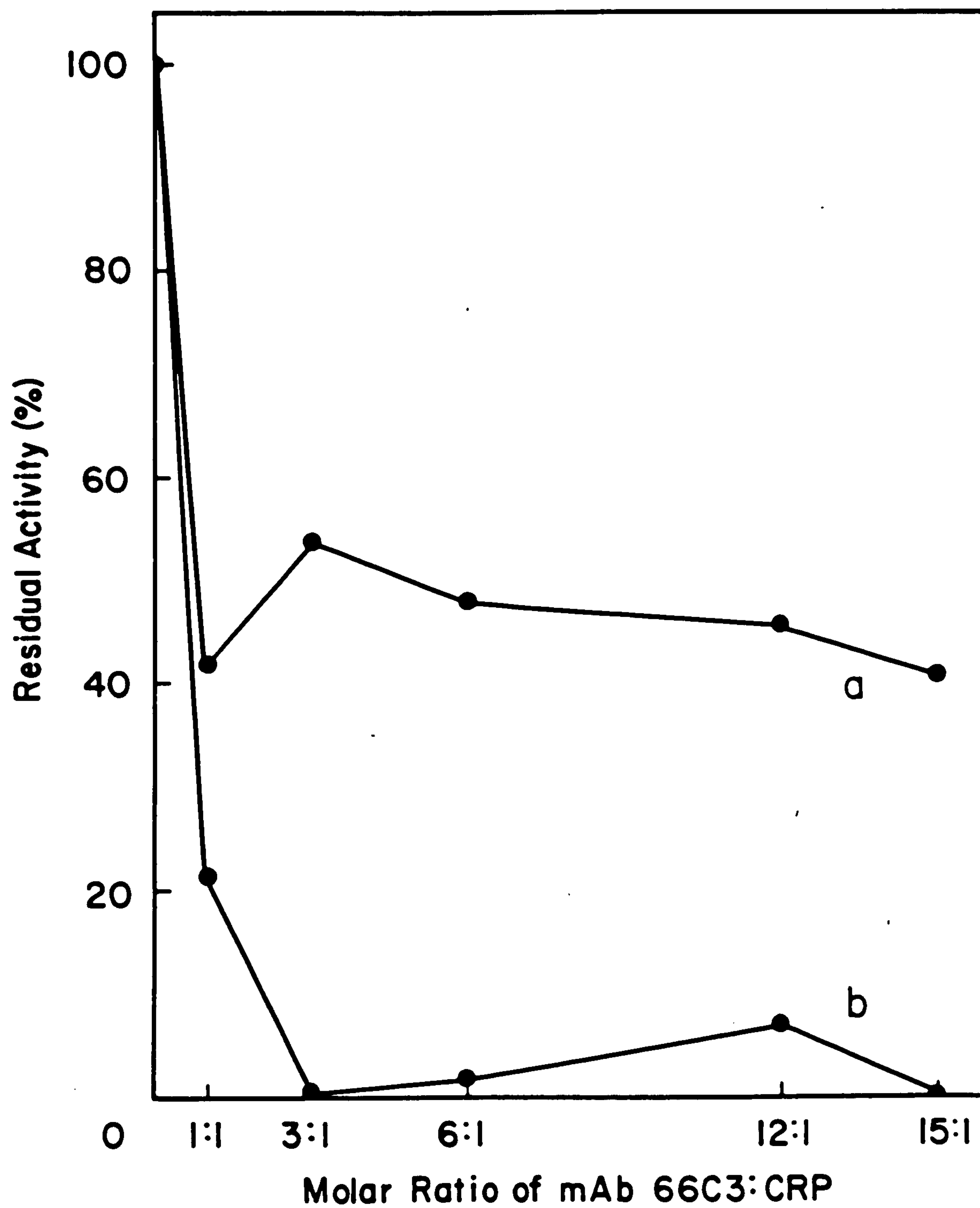


Figure 18. Effect of mAb 66C3 on binding of CRP and RNA polymerase to *lac* P^+. DNase I footprinting conditions are described under "Materials and Methods". Panel A, *lac* P^+ upper strand; panel B, *lac* P^+ lower strand. Lane a, [^{32}P]*lac* P^+ only; lane b, cAMP + CRP + [^{32}P]*lac* P^+; lane c, cAMP + CRP + [^{32}P]*lac* P^+ + mAb 66C3; lane d, [^{32}P]*lac* P^+ + RNA polymerase; lane e, cAMP + CRP + [^{32}P]*lac* P^+ + RNA polymerase; lane f, cAMP-CRP-[^{32}P]*lac* P^+-mAb 66C3 complex formed after incubation for 10 minutes followed by incubation with RNA polymerase; lane g, cAMP-CRP-[^{32}P]*lac* P^+-RNA polymerase complex formed after incubation for 10 minutes followed by incubation with mAb 66C3.

Figure 18.

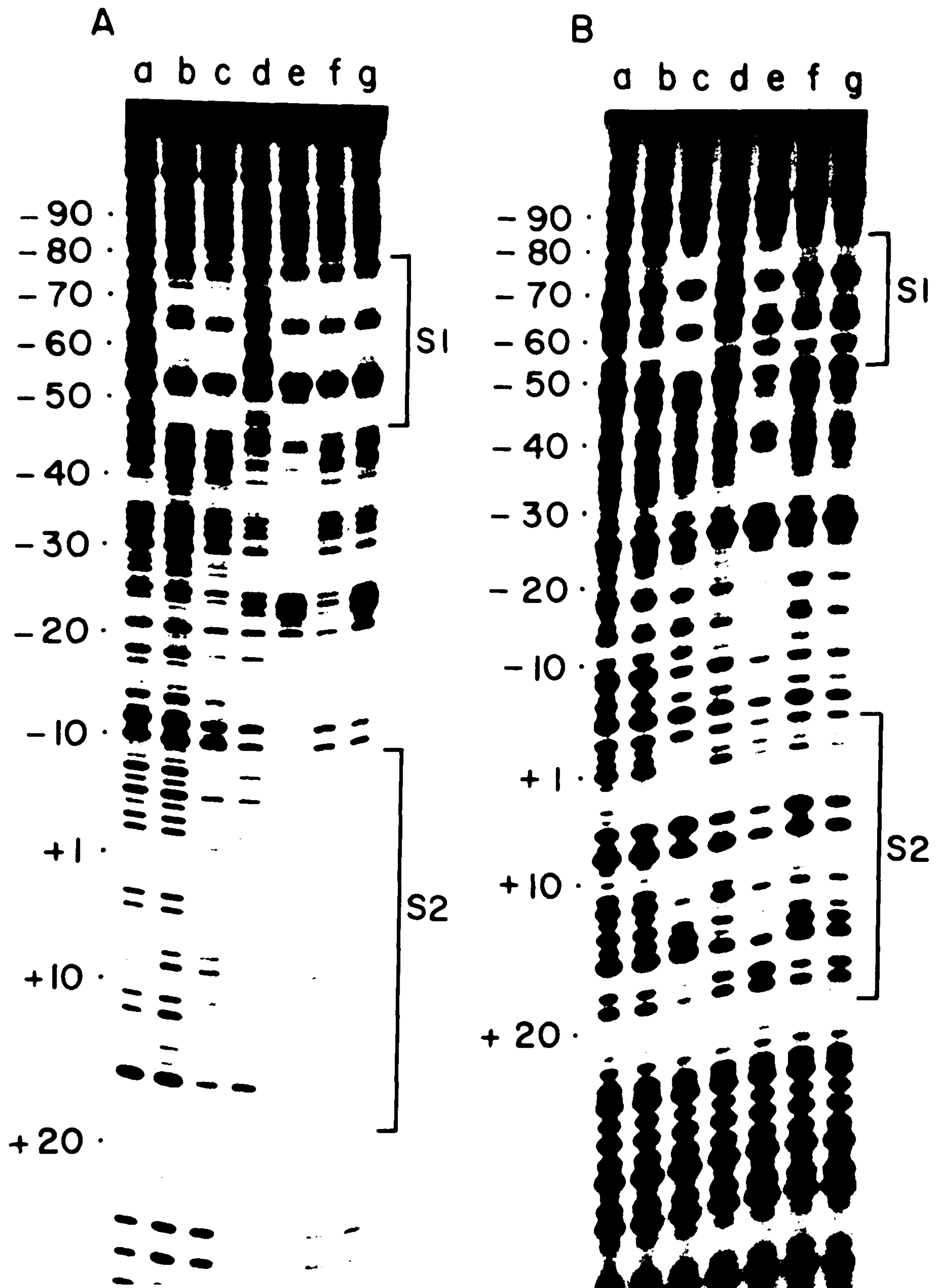


Figure 19. Effect of mAb 66C3 on binding of CRP and RNA polymerase to lac L8UV5. DNase I footprinting conditions are described under "Materials and Methods" with [^{32}P]lac UV5 upper strand. Panel A, with 1% glycerol (normal condition); panel B, with 25% glycerol. Lane a, [^{32}P] lac L8UV5 only; lane b, cAMP + CRP + [^{32}P] lac L8UV5; lane c, cAMP + CRP + [^{32}P] lac L8UV5 + mAb 66C3; lane d, [^{32}P] lac L8UV5 + RNA polymerase; lane e, cAMP + CRP + [^{32}P] lac L8UV5 + RNA polymerase; lane f, cAMP + CRP + [^{32}P] lac L8UV5 + RNA polymerase + mAb 66C3.

Figure 19.

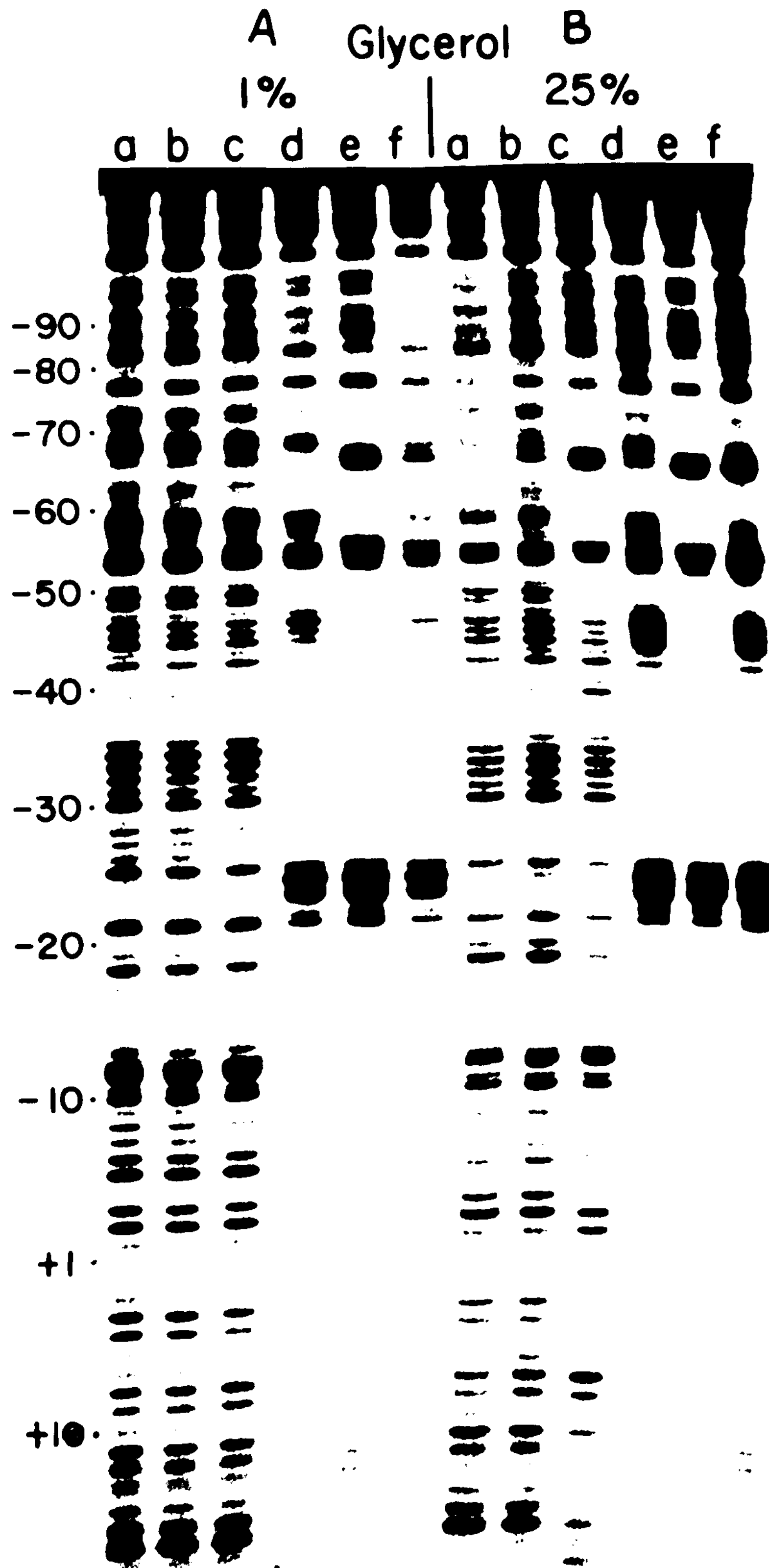
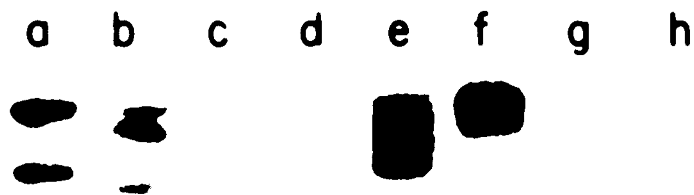


Figure 20. Binding of mAb 66C3 and mAb 115D5 to Hpa II fragments of lac P+ and lac L8UV5. Hpa II fragments of [^{32}P] lac P+ and [^{32}P]lac L8UV5 were prepared by incubation with 4 units of Hpa II restriction enzyme per pmol [^{32}P]lac 203 bp fragment (labelled at both ends) in Hpa II restriction 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 uM cAMP, 133 nM CRP and 0.4 uM mAb in a final volume of 15 uL. 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, mAb 115D5 + cAMP + CRP + [^{32}P]lac L8UV5; lane b, mAb 66C3 + cAMP + CRP + [^{32}P]lac L8UV5; lane c, cAMP + CRP + [^{32}P]lac L8UV5; lane d, [^{32}P]lac L8UV5 only; lane e, mAb 115D5 + cAMP + CRP + [^{32}P]lac P+; lane f, mAb 66C3 + cAMP + CRP + [^{32}P]lac P+; lane g, cAMP + CRP + [^{32}P]lac P+; lane h [^{32}P]lac P+ only.

Figure 20.



CRP-S1

Figure 21. Comparison of the effects of mAb 115D5 and mAb 66C3 on binding of CRP and RNA polymerase to _lac_ P^{+}. DNase I footprinting conditions are described under "Materials and Methods" with [^{32}P]_lac_ P^{+} upper strand. Lane a, [^{32}P]_lac_ P^{+} only; lane b, cAMP + CRP + [^{32}P]_lac_ P^{+}; lane c, cAMP + CRP + [^{32}P]_lac_ P^{+} + mAb 66C3; lane d, cAMP + CRP + [^{32}P]_lac_ P^{+} + mAb 115D5; lane e, RNA polymerase + [^{32}P]_lac_ P^{+}; lane f, cAMP + CRP + [^{32}P]_lac_ P^{+} + RNA polymerase; lane g, cAMP-cRP-[^{32}P]_lac_ P^{+}-mAb 66C3 complex formed after incubation for 10 minutes followed by incubation with RNA polymerase for 10 minutes; lane h, cAMP-CRP-[^{32}P]_lac_ P^{+}-RNA polymerase complex formed after incubation for 10 minutes followed by incubation with mAb 66C3 for 10 minutes; lane i, cAMP-CRP-[^{32}P]_lac_ P^{+}-mAb 115D5 complex formed after incubation for 10 minutes followed by incubation with RNA polymerase(for 10 minutes; lane j, cAMP-CRP-[^{32}P]_lac_ P^{+}-RNA polymerase complex formed after incubation for 10 minutes followed by incubation with mAb 115D5.

Figure 21.

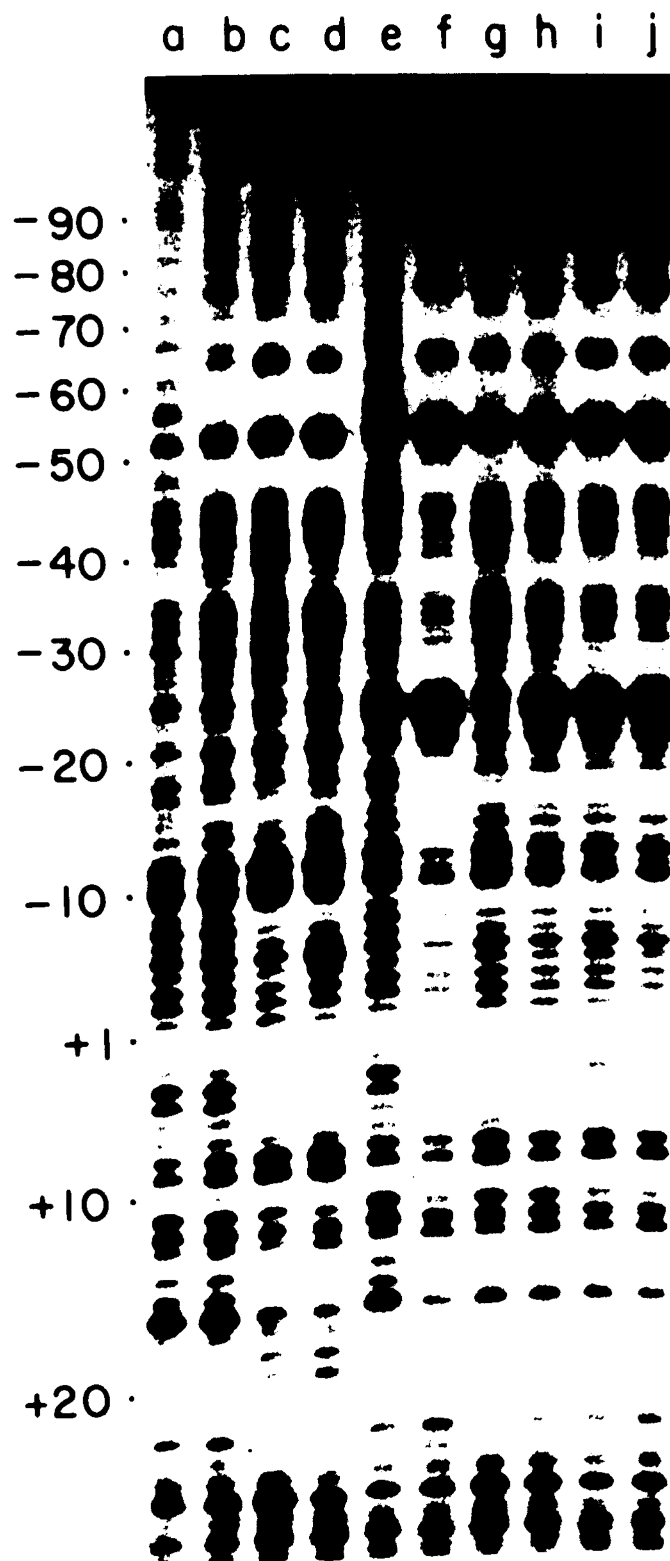


Figure 22. Effect of glycerol on *lac* DNA binding by CRP and RNA polymerase. DNase I footprinting conditions are described under "Materials and Methods" with [^{32}P]*lac* P^{+} upper strand. Lane a, [^{32}P]*lac* P^{+} only; lane b, cAMP + CRP + [^{32}P]*lac* P^{+} in 1% glycerol; lane c, cAMP + CRP + [^{32}P]*lac* P^{+} + mAb 66C3 in 1% glycerol; lane d, cAMP + CRP + [^{32}P]*lac* P^{+} in 25% glycerol; lane e, RNA polymerase + [^{32}P]*lac* P^{+} in 1% glycerol; lane f, cAMP + CRP + [^{32}P]*lac* P^{+} + RNA polymerase in 1% glycerol; lane g, RNA polymerase + [^{32}P]*lac* P^{+} in 25% glycerol.

Figure 22.

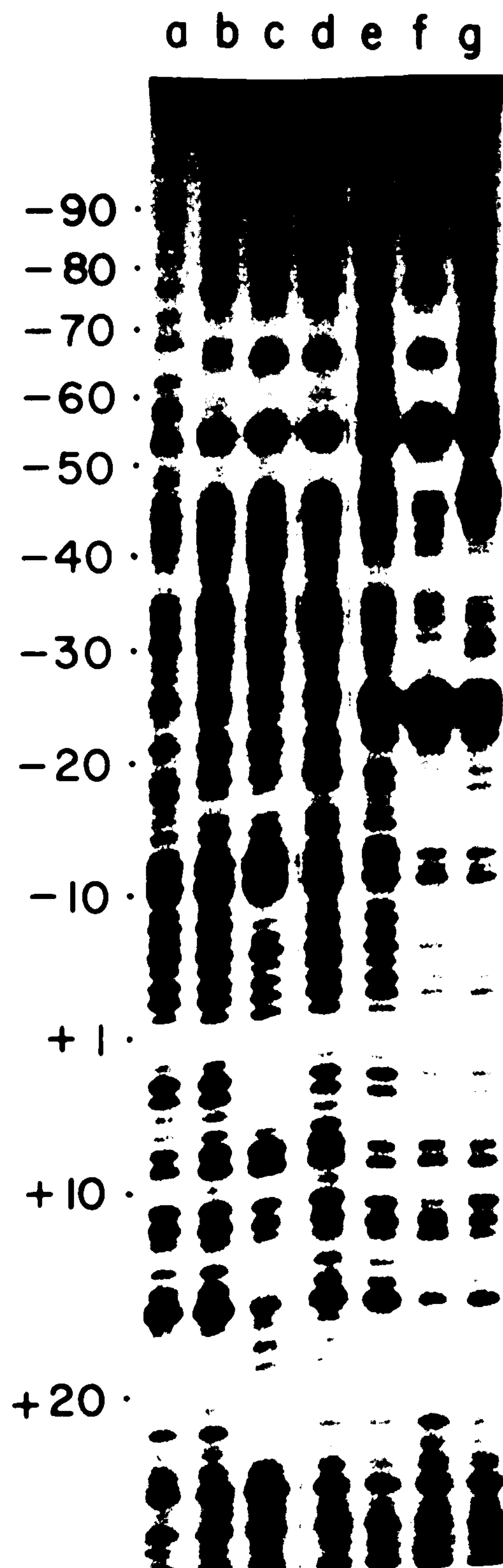
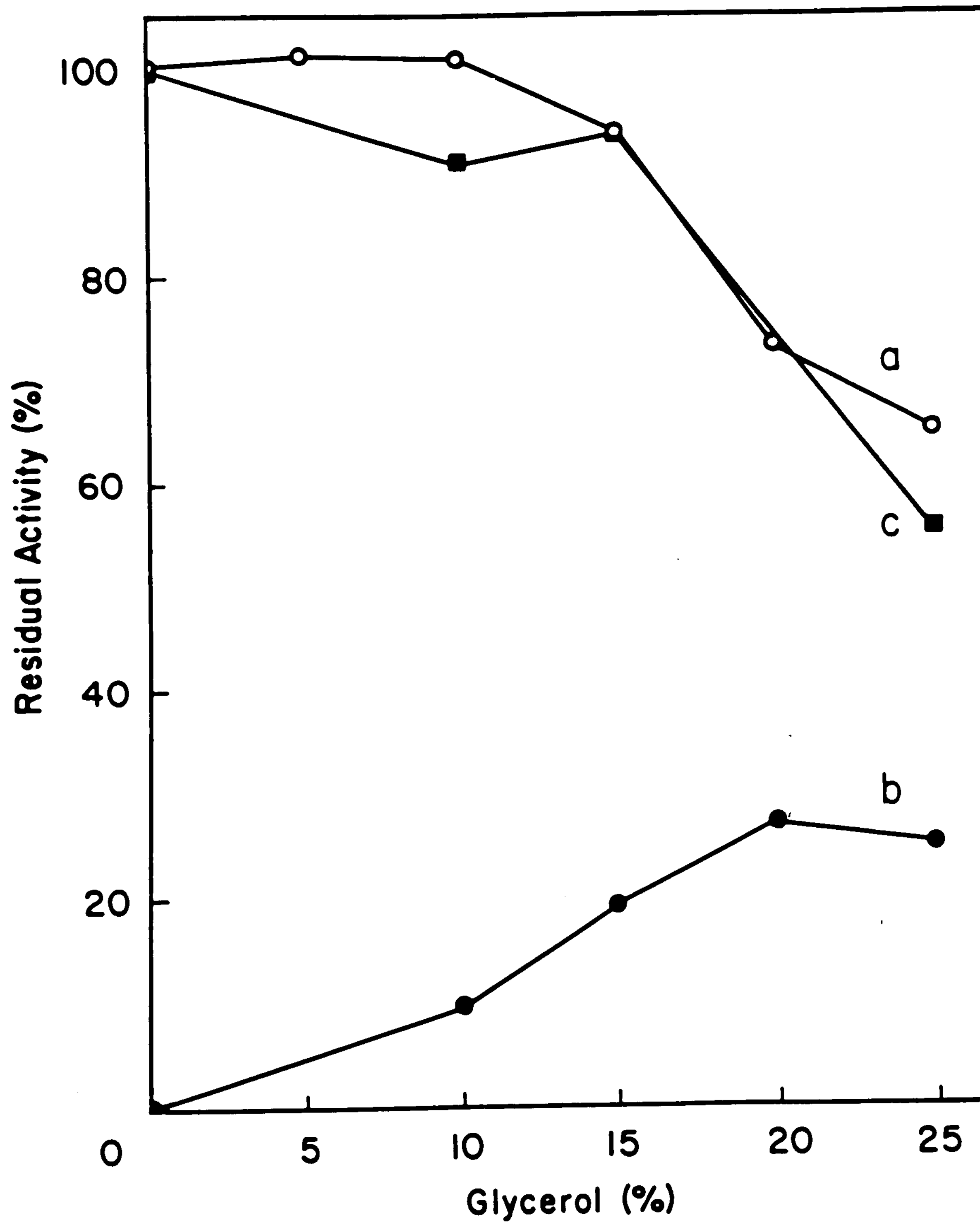


Figure 23. Effect of glycerol on the abortive initiation from lac P^+ and lac L8UV5. Conditions are described under "Materials and Methods" with glycerol concentration varied as indicated. (a), lac P^+ with cAMP-CRP; (b), lac P^+ without CRP; (c), lac L8UV5.

Figure 23.



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