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CAP and RNA polymerase interactions with the *lac* promoter: binding stoichiometry and long range effects

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ABSTRACT

The binding stoichiometries of the complexes formed when the E. coli cyclic AMP receptor protein (CAP) binds to 203 bp lac promoter-operator restriction fragments have been determined. Under quantitative binding conditions, a single dimer of CAP occupies each of two sites in the promoter. Different electrophoretic mobilities are observed for 1:1 complexes formed with L8-UV5 mutant, L305 mutant, and wild type promoter fragments, indicating sequence-specific structural differences between the complexes. The differences in gel mobility between L8-UV5 and wild type complexes disappear when the promoter fragments are cleaved with Hpa II restriction endonuclease. Models in which CAP alters DNA conformation or in which CAP forms a transient intramolecular bridge between two domains of a DNA molecule could account for these observations. The selective binding of RNA polymerase to CAP-promoter complexes is demonstrated: the binding of a single CAP dimer to the promoter is sufficient to stimulate subsequent polymerase binding. Functional CAP molecules are not released from the promoter on polymerase binding.

INTRODUCTION

The cyclic AMP receptor protein (CAP) is responsible for the coordinate regulation of the transcription of a large number of genes in Escherichera coli. In the presence of cAMP, CAP binds to one or more sites within catabolite sensitive promoters, modulating the initiation of transcription (1-6). Two general models have been proposed for the role of CAP in transcriptional control (7). One requires protein-protein contacts between CAP and RNA polymerase, and the other a CAP-induced conformational change in the promoter. Either of these processes could stabilize a promoter-RNA polymerase complex, thereby increasing the probability of a transcription initiation event.

In this paper we report results characterizing the binding of CAP to the lac promoter. The stoichiometric ratio of protein to DNA in the observed complexes is determined, and evidence is presented implying the existence of long range interactions between a CAP-promoter complex and

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regions of DNA well outside of the binding site. Such interactions could result in a promoter conformation change. In addition, we demonstrate that RNA polymerase binds to CAP-DNA complexes in preference to free DNA, and that certain CAP-promoter complexes are bound more tightly than others. This allows assessment of the possible importance of each CAP-DNA complex to the control of lac transcription.

MATERIALS AND METHODS

Reagents. Acrylamide and N,N-methylene bisacrylamide were from BioRad. [^{3}H] sodium borohydride (5 Ci/mmole) and [γ^{32}P]ATP were from New England Nuclear. cAMP and bovine serum albumium were from Sigma, and formaldehyde from Malinkrodt. Unless noted, all other reagents were of the highest grade commercially available.

Proteins. Lac repressor was the gift of M. Leahy, and Sigma saturated E. coli RNA polymerase was the gift of W. McClure. CAP was isolated from E. coli K-12 as described (8). A sample of CAP crystals suspended in mother liquor was the gift of T. Steitz and D. McKay.

Isolation and 5' end-labeling of restriction fragments. Isolates of E. coli strain MM 294 harboring the lac promoter containing plasmids pOP 203 (Wt)-1 and pOP 203 (L8-UV5)-1 were the gift of F. Fuller. These plasmids contain 203 bp lac promoter-operator sequences cloned in the EcoR1 site of vector pMB9 (9). L8-UV5 is a double mutation. UV5 is a strong "up" mutation which stimulates transcription from the lac promoter even in the absence of CAP (10). L8 is a point mutation in the primary CAP site which renders the lac promoter insensitive to CAP in vivo (11). Plasmid DNA was prepared by a standard cleared lysate procedure (12), and purified by banding in CsCl gradients. Restriction enzymes EcoR1 and Hpa II were purchased from New England Biolabs, and were used according to manufacturers instructions. The 203 bp Eco R1 fragment containing the entire lac promoter-operator region was isolated from plasmid digests by preparative polyacrylamide gel electrophoresis (13). Linear form pMB9 was similarly isolated. A 202 bp promoter-operator fragment containing the L305 point deletion was the gift of J. Gralla. DNA molecules were labeled with ^{32}P at 5' termini according to Maxam and Gilbert (14). Hpa II digestion of 203 bp wild type and L8-UV5 molecules gave two fragments, of 121 bp and 82 bp, in equimolar amounts (Figure 1). The 121 bp fragment contains the genetically defined CAP sequence (site 1), while the 82 bp molecule contains the entire lac operator (4). A secondary CAP bin-

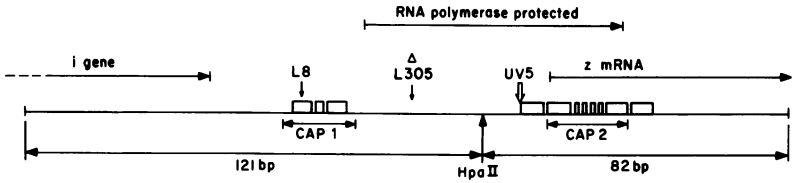


FIGURE 1. Partial restriction and binding-site map of the lac promoter-operator region. Binding site data from references 1 and 27.

ding site (site 2) has been found within the operator sequence (15).

Tritium labeling of CAP by reductive methylation. Reductive methylation of CAP was carried out according to the method of Rice and Means (16). The labeling reactions and purification of [^{3}H]CAP were performed in a special facility, under the supervision of the department of Health Physics at Yale University. CAP (1.6 mg/ml) was incubated for 10 min on ice in 100 µl of reaction buffer (45 mM potassium phosphate (pH 8.0 at 4° C), 4.5 mM EDTA, 10 µM cAMP, 10% glycerol). Formaldehyde was added with mixing to give a final concentration of 6 mM. Following a 5 min incubation at 4° C, several grains (ca. 20 µg) of solid [^{3}H] sodium borohydride were added, and the mixture incubated for 10 min further. A second addition of [^{3}H]-sodium borohydride was made, and the mixture allowed to react until no further evolution of gas was apparent. Linear form pMB9 DNA was added to give a final concentration of 0.1 mg/ml, and the solution incubated 20 min at room temperature. Protein-DNA complexes were isolated by chromatography on Sephadex G-100. Under the conditions employed, free protein (inactive in DNA binding) and ^{3}H-labeled by-products were retained, while CAP-DNA complexes eluted in the void volume. This step ensures that the [^{3}H]CAP used in subsequent experiments is fully active in DNA binding. Any residual ^{3}H-labeled contaminants were removed by the centrifuge column technique of Penefsky (17). The protein concentration was determined, after suitable dilution, by the method of Sedmak and Grossberg (18), and scintillation counting was performed in Aquasol.

Formation of protein-DNA complexes. DNA concentrations were determined spectrophotometrically (ϵ_{260} = 1.3 x 10^4 M^{-1} cm^{-1} (per mole bp)), or by titration with a standardized lac repressor preparation, under quantitative binding conditions (21). Protein concentrations were determined by the method of Sedmak and Grossberg (18) or spectrophotometrically, using ϵ_{280} = 3.9 x 10^4 M^{-1} cm^{-1} for CAP (dimer) (19), and ϵ_{280} = 3.0 x

10^5 M^{-1} cm^{-1} for RNA polymerase (20). Protein stock solutions were gently diluted in 10 mM Tris (pH 7.4 at 21° C), 1 mM EDTA, 3mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml BSA, 10% glycerol. Binding reactions were carried out in 10 mM Tris (pH 7.4 at 21° C), 1 mM EDTA, 50 mM KCl, 10 μM cAMP, 0.1 mg/ml BSA, containing 1-5 x 10^{-8} M restriction fragments, and variable amounts of CAP and RNA polymerase. After equilibration for 30 min at 21° C, solutions were diluted 10-fold with 10 mM Tris (pH 7.4 at 21° C), 1 mM EDTA, 10 μM cAMP, 0.1 mg/ml BSA, 10 μg/ml xylene cyanol FF, 5% glycerol; aliquots were loaded on a 5% polyacrylamide gel, and electrophoresis begun immediately.

Protein distribution analysis. Polyacrylamide gels were prepared and run as described (21). Autoradiograms were obtained with Dupont Cronex X-ray film, exposed at -20°C. Care was taken to ensure that film exposure was within the linear range of dose-response. Developed film was scanned with a Joyce-Loebl microdensitometer. Peak areas were determined by planimetry.

We have previously reported that lac repressor-DNA complexes with lifetimes of a few min can be detected by the gel assay, even though the run time of the analytical gel is on the order of hours (21,22). Recently this feature of the technique has also been observed for short-lived CAP-DNA complexes (23). Apparently, the duration of the analytical gel run has little effect on the distribution of protein-DNA complexes. A model for the stabilization of short-lived complexes by the gel matrix has been proposed (21).

RESULTS

Determination of binding stoichiometry. Titration of 203 bp promoter-operator fragments with CAP produces complexes that are resolvable by polyacrylamide gel electrophoresis (figure 2). Three gel bands, designated h, m, and l, are observed when the fragment contains the wild type promoter sequence, while only two (h and l) are found when the sequence contains the UV5 and L8 mutations. No other complexes are visible at higher protein to DNA ratios: at very high ratios, high molecular weight complexes form which barely enter the gel.

The ratio of CAP to DNA in each band was determined by forming complexes between [^{3}H]CAP and [^{32}P] labeled lac 203 bp promoter fragments, each of known specific activity. Protein-DNA complexes were formed as described, except that [^{3}H]CAP was added as its non-specific complex with carrier (pMB9) DNA. The much greater affinity of CAP for its specific

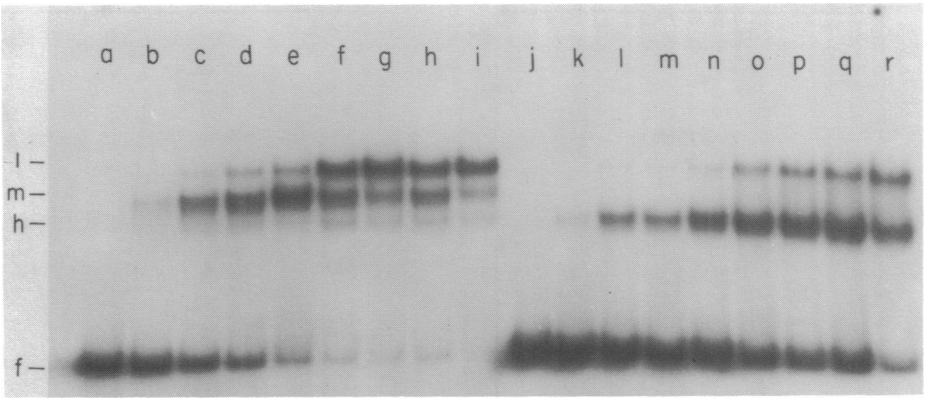


FIGURE 2. Titration of the 203 bp wild type and L8-UV5 fragments with CAP (lanes a-i and j-r respectively). Binding buffer consisted of 10 mM Tris (pH 7.4 at 21° C), 50 mM KCl, 1 mM EDTA, 0.1 mg/ml BSA, 10 μM cAMP. CAP:DNA ratios for lanes a-i respectively are: 0, 0.69, 1.38, 2.19, 2.92, 3.65, 4.38, 5.11, 6.57. CAP:DNA ratios for lanes j-r respectively are: 0, 0.58, 1.15, 1.73, 2.31, 2.88, 3.46, 4.04, 5.19. Equilibration was for 30 min at 21° C. Polyacrylamide gel run in TE + 10 μM cAMP at room temperature.

sequence caused protein transfer from the carrier DNA to take place once samples were mixed. Figure 3 shows the radioactivity profiles obtained when [^{3}H]CAP-203 bp wild type and [^{3}H]CAP-203 bp L8-UV5 promoter complexes are resolved by gel electrophoresis. Band mobilities and experimentally determined stoichiometries are presented in table I. The variation of molar ratios found on repetition of this experiment was approximately ±

TABLE I: Band mobilities and stoichiometries

DNA	Band	R_D[a]	Molar ratio $\frac{CAP}{DNA}$ (dimers)[b]
203 wt	h	0.62	0.78
	m	0.56	0.95
	l	0.49	1.76
203 L8-UV5	h	0.63	1.17
	l	0.50	2.24

[a]Fractional mobility of band with respect to free DNA
[b]Determined from data of Figure 3

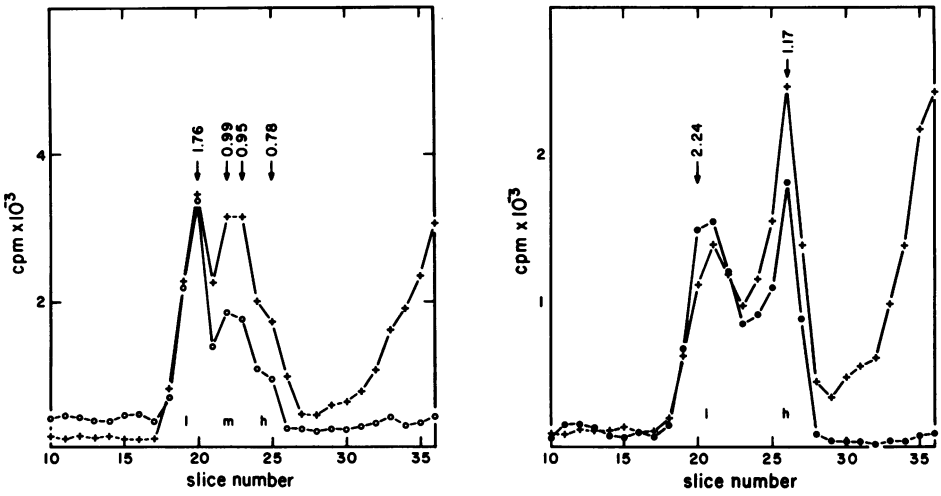


FIGURE 3A. Electrophoretic profile of [^{3}H] CAP binding to [^{32}P] 203 (Wt) DNA. [^{3}H]CAP (4.4 x 10^8 cpm mole^{-1}) was mixed with [^{32}P] 203 (Wt) (7.7 x 10^8 cpm mole^{-1}). Binding conditions are described in the text. Cylindrical 5% polyacrylamide gels were run at 21° C in 10 mM Tris (pH 8.0 at 21° C). 1 mM EDTA, 10 µM cAMP. Gel slices 1.2 mm thick were digested overnight in 100 µl of 30% H_2O_2 at 55° C; the resulting solution was counted in 5 ml aquasol. Profiles are corrected for ^{32}P carryover into the ^{3}H channel, and background subtracted. Peaks h, m, and l are designated. Symbols: (○), ^{3}H; (+), ^{32}P.

FIGURE 3B. Electrophoretic profile of [^{3}H]CAP binding to [^{32}P] 203 (L8-UV5) DNA. [^{3}H]CAP (4.4 x 10^8 cpm mole^{-1}) was mixed with [^{32}P] 203 (L8-UV5) (5.1 x 10^8 cpm mole^{-1}). Binding conditions and analysis are as described in Figure 3A. Symbols: (●), ^{3}H; (+), ^{32}P.

0.25. Taken together, these gel mobilities and molar ratios are compatible with 1:1 and 2:1 protein:DNA stoichiometries for bands h and l respectively, while the most probable value for wild type band m is 1:1. Nuclease protection studies have shown that there are two binding sites for CAP in the lac promoter-operator region (15). To explain the protection results, models requiring the binding of one (24) and two (25) dimers of CAP per binding site have been advanced. The low binding ratios reported here clearly favor the interpretation of one CAP dimer per binding site.

CAP binding is quantitative, and sequence-specific. To determine the fractional DNA binding activity of CAP, binding reactions were carried out keeping the DNA and cAMP concentrations constant, but varying CAP. Figure 4 shows the results of such titrations performed with and without a 5-fold excess (in terms of bp) of non-specific plasmid over lac promoter

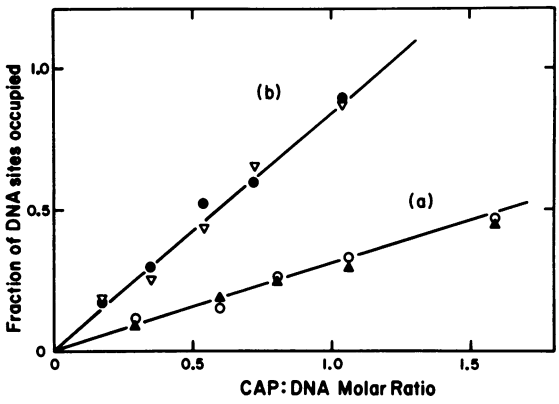


FIGURE 4. Effects of crystallization and non-specific DNA on lac promoter binding. Curve a: titration of the 203 bp wild type promoter fragment (1.3×10^{-8} M) with CAP, in the presence (▲) or absence (○) of linear form pMB9 plasmid (1.3×10^{-5} M base pairs). Curve b: titrations as in curve a, in the presence (▽) or absence (●) of non-specific DNA, except that CAP was from a preparation that had been crystallized (24), and redisolved just prior to use.

DNA. For the preparation of CAP used in this study, a DNA binding activity of 30% was obtained (curve a). This was a typical level of DNA binding activity in several other preparations tested. Increasing the concentration of lac promoter DNA in the binding assay over a 10-fold range did not alter the extent of DNA binding at a given CAP:DNA ratio (results not shown). Taken with the linearity of the binding curve, this indicates that DNA binding is quantitative under these assay conditions. The finding that a 5-fold excess of non-specific DNA does not significantly reduce the fractional binding indicates that essentially all of the active CAP is bound specifically, i.e. to sites of greater than average affinity. Specific binding is also supported by the observation of increasing relative affinity of the promoter fragment for CAP as cAMP concentration increases, compared to bulk DNA (22,23).

Curiously, a sample of CAP that had been crystallized (26) and then redissolved, showed significantly greater DNA binding activity than untreated samples (curve b). A rigorous comparison of binding activities is not justified because the samples were derived from different preparations of CAP. Nonetheless, it is tempting to speculate that crystallization is a selective process in which only active forms of CAP participate, or that CAP is activated in some manner by crystallization. A

more detailed investigation of this phenomenon may yield some valuable correlations between CAP structure and function.

DNA sequence and occupancy effects. In a previous study of lac repressor-DNA interactions, we found that the electrophoretic mobility of a protein-DNA complex was proportional to its protein:DNA ratio, but insensitive to the distribution of protein among the available binding sites (21). Similar behavior is observed for complexes of CAP with 203 bp L8-UV5 fragment. The formation of two electrophoretically distinct 1:1 complexes between CAP and the 203 bp wild type fragment is the first exception to this rule that we have observed. For this reason we believe that the altered gel mobility of the band m complex reflects structural differences between it and the other 1:1 complex band h. Because the L8-UV5 mutations prevent formation of the band m structure in vitro, and abolish CAP dependent expression of the lac operon in vivo, such differences may have functional significance.

The identical electrophoretic mobility of 2:1 complexes formed with wild type and L8-UV5 DNA fragments suggests that the second binding event either abolishes or compensates for the structural differences between 1:1 complexes. The nuclease protection data of Schmitz (15), show that 73 bp separate the center of site 1 from the center of site 2 in the wild type promoter. Hence the structural effect of the second binding event must extend along the DNA for a considerable distance. Other experiments presented below support this view.

The L305 mutation. As a means of exploring the effect of sequence context on site-specific CAP binding, we examined the interaction of CAP with a 202 bp promoter-operator fragment carrying the L305 mutation. This mutation is a deletion of a single G-C base pair located at position -37 in the wild type lac promoter (27). L305 decreases the transcriptional activity of the promoter, and its ability to be stimulated by CAP + cAMP (28). Nuclease protection studies with the wild type promoter indicate that the L305 deletion is located about 10 bp "downstream" from the edge of the primary CAP binding site (15,29). The results of a CAP titration of the L305 promoter fragment are shown in Figure 5. Three new bands (a b and c) are observed, while two (m and 1) comigrate with wild type complex bands m and 1. The high mobility of band a indicates that it is most probably a 1:1 complex, while band b may contain a 2:1 complex. The new low mobility band c complex probably contains more than two dimers of CAP.

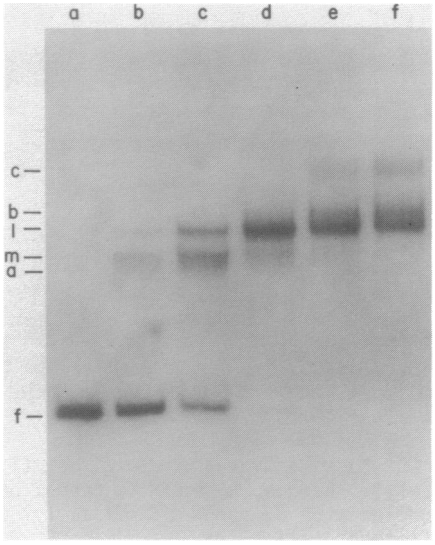


FIGURE 5. Titration of the 202 bp L305 promoter-operator fragment with CAP. CAP:DNA ratios for lanes a-f are: 0, 0.925, 2.77, 4.62, 6.47, 8.32. Binding conditions and gel electrophoresis as described in Figure 2.

Because the L305 and wild type sequences are identical except for the deletion at -37, these new complexes reflect perturbations in the CAP-promoter interaction due to this deletion alone. Historically, the effects of L305 and other mutations of the -35 region have been interpreted as the results of altered interactions of RNA polymerase with the modified sequences (30). The present results indicate that the sequence of the -35 region has an effect on CAP binding, and may help to explain the altered cAMP sensitivity of the L305 promoter *in vivo*. Some structural implications of the L305 mutation are considered in the discussion.

Hpa II cleavage abolishes band m. Hpa II cleavage of the 203 bp DNA produces fragments of 121 and 82 bp. The "upstream" half of the promoter, including the genetically defined CAP site (site 1) is located on the 121 bp fragment, while the *lac* operator sequence and second CAP site (site 2) are found on the 82 bp fragment. The Hpa II cut is at least 27 bp "downstream" from the edge of the primary CAP site, as defined by nuclease protection (15,29). As shown in Figure 6, only one 1:1 complex is formed with the 121 bp fragment, regardless of whether it contains the wild type or the L8 sequence. Thus, the Hpa II cleavage alters some aspect of

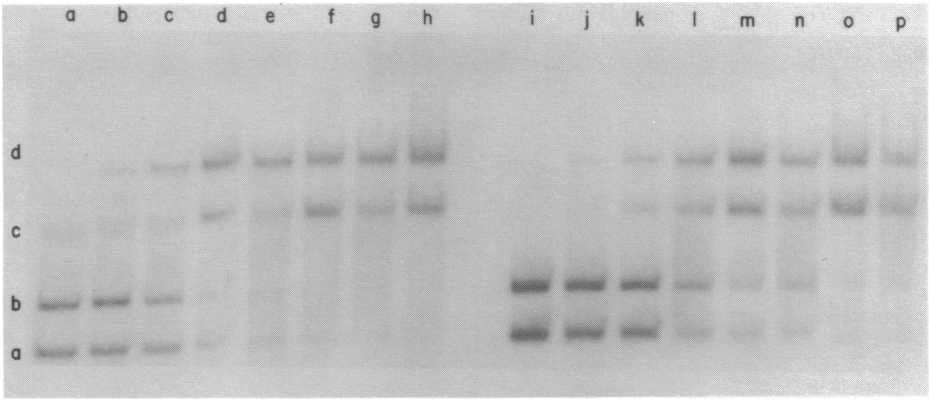


FIGURE 6. Binding of CAP to wild type and L8-UV5 Hpa II fragments (lanes a-h and i-p, respectively). Band a, 82 bp DNA (free); band b, 121 bp DNA (free); band c, 1:1 complex with 82 bp DNA; band d, 1:1 complex with 121 bp DNA. CAP: 121 bp fragment ratios for lanes a-h: 0, 0.51, 1.02, 2.04, 3.06, 4.09, 5.11, 7.15. CAP: 121 bp fragment ratios for lanes i-p: 0, 0.45, 0.98, 1.96, 2.94, 3.92, 4.90, 6.86. Binding conditions are described in Figure 2.

promoter structure required for formation of the 1:1 band m complex. Because the 121 bp fragment contains an intact -35 region, this observation suggests that additional sequences lying far outside of the primary CAP site are important for formation of the band m complex.

RNA polymerase selectively binds to CAP-promoter complexes. When E. coli RNA polymerase is equilibrated with CAP-promoter complexes, new structures of decreased gel mobility are formed (Figure 7A, bands a and b). Densitometric analysis indicates that CAP complexes are initially bound in preference to free DNA (Figures 7B and 7C), although free DNA is bound by polymerase when CAP is absent (data not shown). Since free CAP is expected to bind quantitatively to DNA under these conditions, the small initial changes in the free DNA concentration indicate that active CAP is not released from the promoter when RNA polymerase binds.

The relative strength of RNA polymerase binding to CAP-promoter complexes and to free DNA is reflected by the differential removal of each from solution. Only small differences of affinity for polymerase are observed among wild type promoter complexes: the order of preference appears to be m > h > l (Figure 7B). Thus the 1:1 complexes are bound with slightly higher affinity than the 2:1. The difference is more

striking for L8-UV5 promoter complexes, where a strong initial preference for 1:1 over 2:1 complexes is evident (Figure 7C).

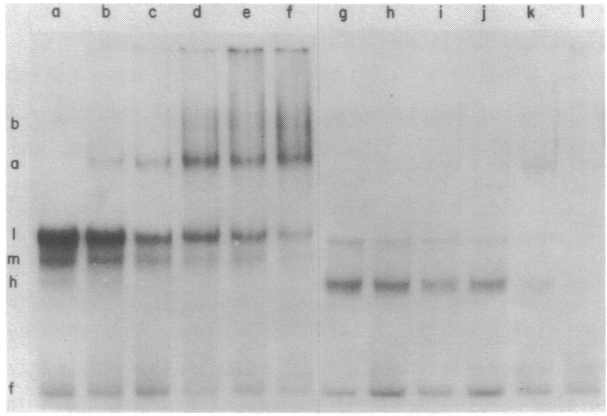
RNA polymerase binding to 1:1 CAP-promoter complexes is substantially complete at a polymerase:CAP ratio of 1. From this we infer that polymerase binds quantitatively to the CAP-promoter complexes and that the most probable stoichiometry for the CAP-RNA polymerase - lac promoter complex is 1:1:1.

DISCUSSION

CAP:promoter stoichiometry. Although nuclease and chemical protection studies provide detailed maps of specific protein-DNA contacts, they can not in general reveal the stoichiometry of binding. Such information is essential for correct interpretation of the protection patterns, and for understanding the functional interactions they reflect. The double label experiment described here represents a general solution to this problem. The data indicate that one dimer of CAP is bound at each specific site in the lac promoter. Due to the cooperative nature of some CAP-DNA interactions (19, 31) this finding may not extend to other promoters: comparative studies with other CAP binding sites are called for.

Long range effects on CAP-promoter interactions. The effect of the L8 mutation on CAP binding in vitro supports the suggestion that the wild type 1:1 complex (band m) contains a dimer of CAP bound at site 1 in the promoter. Our interpretation of the second 1:1 complex (band h), is that it contains CAP bound at site 2, plus any 1:1 complexes with CAP at site 1 which have not undergone a structural transition to the band m form. CAP binding to site 1 in the L8-UV5 promoter does not induce this conformational change, and so both site 1 and site 2 L8-UV5 complexes comigrate as band h on the gel.

Any model for the structural differences between complexes h and m must be compatible with the observations that that the electrophoretic differences disappear upon formation of a 2:1 complex with CAP, and that electrophoretically identical 1:1 complexes are formed with the Hpa II fragments. Possible models fall into two broad categories. In the first, site-specific binding results in a conformational change in CAP sufficient to alter the electrophoretic mobility of the complex. Such changes in CAP structure could conceivably modulate RNA polymerase activity through protein-protein contacts. Although there is no direct evidence against this possibility, we believe it is unlikely in view of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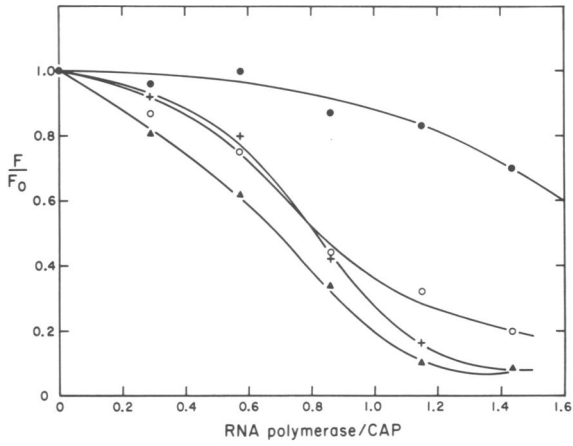
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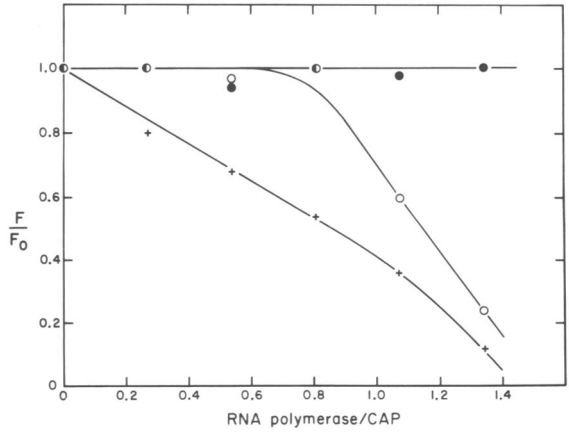
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the unexpectedly long-range effects of site 2 binding and Hpa II cleavage.

In the second category are models that require a conformational change in the DNA. A specific proposal of this sort has recently been advanced, involving a CAP-induced conformational transition in the promoter, from the right-handed B-form to a left-handed form (24). Other possible transitions include bending or kinking (33), and formation of a bridged hairpin complex (depicted schematically in Figure 8). The bridged structure is exceptional in that it does not require the long range transmission of structural changes by the DNA, although a growing body of evidence suggests that such transmission is possible (34).

We estimate that a 10% increase in DNA length would account for the difference in electrophoretic mobility of bands h and m. Length increases of this magnitude have been observed on formation of certain drug-DNA complexes (34), and for the cooperative B-Z transition of poly (dG-dC) (35). However, the effects of Hpa II cleavage are not well explained by a simple model in which CAP binding to the functional site increases the length of the DNA molecule. Such length increases should also occur in smaller molecules containing the specific sequences. Thus the mobility change due to CAP binding to the 121 bp fragment (site 1) should be significantly larger than that due to CAP binding to the 82 bp fragment. Inspection of Figure 6 indicates that this is not the case. CAP binding decreases the gel mobilities of these two fragments by very nearly the same extent.

Bending or kinking the DNA should also change the rate at which it runs on a gel. However, if CAP binding to the wild type site 1 induces a

FIGURE 7A. Binding of RNA polymerase to CAP-DNA complexes. CAP-203 bp DNA complexes were formed as described. Wild type and L8-UV5 fragment concentrations were 5 and 5.2 x 10^{-8} M, respectively. CAP:DNA ratios were 4.04 (wild type) and 3.15 (L8-UV5). Following equilibration RNA polymerase was added to give the following polymerase-CAP ratios: Wild type DNA, lanes a-f: 0, 0.29, 0.57, 0.86, 1.15, 1.17. L8-UV5 DNA, lanes g-l: 0, 0.27, 0.54, 0.81, 1.08, 1.34. Samples were equilibrated for 30 min, and gel lectrophoresis performed as described.

FIGURE 7B. RNA polymerase binding to CAP complexes formed with the 203 bp wild type promoter fragment. Data of Figure 6 plotted as the normalized fraction of free CAP-complex (F/F_0) vs. molar ratio of polymerase to CAP. Free DNA, (●); band h, (+); band m, (▲); band l, (○).

FIGURE 7C. RNA polymerase binding to CAP complexes formed with the 203 bp L8-UV5 promoter fragment. Data of Figure 6 plotted as the normalized fraction of free CAP-complex (F/F_0) vs. molar ratio of polymerase to CAP. Free DNA, (●); band h, (+); band l, (○).

FIGURE 8. Equilibrium between a linear CAP-DNA complex and the postulated bridged-hairpin form.

kink, but binding to the L8-UV5 site 1 does not, the mobilities of wild type 2:1 complexes should be different from L8-UV5 2:1 complexes. Because this is not the case, we conclude that the binding of a second CAP at site 2 must change the structure of the CAP-site 1 complex. Unless the two sites are interacting with the same CAP molecule, the effect must be transmitted along the DNA.

The bridged hairpin model is a variation on the bending theme which can account for many of our observations without requiring long range transmission of structural changes by the DNA. A bent structure could be maintained, on average, by transient binding of site 1 complexes to DNA in other regions (Figure 8). Interaction with the specific CAP sequence at site 2 could stabilize such a bridged structure, or the second binding site might be non-specific. Since no CAP functional mutants at site 2 have been reported, bridging to a non-specific site seems more likely. Recently Salemme has shown that the topology of the CAP dimer could allow it to bridge between two double helices (36). A single, sharp gel band will result if equilibration between bridged and non-bridged forms is rapid (as it should be for a first order process) (37).

The affinity of CAP for the L8 mutant site 1 is less than that of CAP for the wild type site (38). Thus a bridged structure formed with the 203 bp L8-UV5 fragment might be less stable than a comparable structure formed with the wild type fragment. Under conditions of rapid equilibration, the effect of an unstable bridge should be small, leading to electrophoretic mobilities very little different from the linear form. According to this model, band h contains the linear form of 1:1 complex, and band m the bent form. Less bending is seen in L8-UV5 complexes.

According to the bridge model, 2:1 wild type and L8-UV5 complexes with identical gel mobilities could be formed in two possible ways. A highly stable hairpin could result if CAP dimers at both site 1 and site 2 formed bridges. This added stabilization could compensate for the effect of the L8 mutation, enabling the L8 fragment to form a hairpin as well as the wild type. Alternatively, the second CAP dimer, bound at site 2, could prevent bridge formation with site 1 complexes by neighbor

exclusion or for steric reasons. In this case both wild type and L8 2:1 complexes would be linear.

Hpa II cleavage of the 203 bp promoter produces DNA fragments of 82 bp and 121 bp. Such fragments are likely to be too short to accommodate the hairpin bend needed for unimolecular bridge formation (39). Furthermore, bridging from site 1 to site 2 is transformed into a second-order reaction by this cleavage, greatly decreasing its probability of occurrence. These consequences of the bridge model are compatible with the experimental observation that only one 1:1 complex is formed between CAP and each of the Hpa II promoter fragments.

Formation of a bridged structure is one mechanism that has been proposed for the rapid transfer of gene regulatory proteins from site to site (40). Recently, evidence of bridged complexes between lac repressor and small operator fragments has been reported (41, 42). Although similar observations have not been reported for interactions between CAP and DNA, we have found that the dissociation kinetics of CAP from site 1 are second order in DNA at sufficiently high DNA concentrations (to be published). One candidate for the structure of the transition state in this reaction is a ternary complex with a CAP bridge between two DNA molecules. Thus the bridge model appears to be compatible with much of the available data.

The L305 deletion. CAP binding to L305-mutant 202 bp promoter fragments produces two complexes with gel mobilities identical to the wild type 1:1 and 2:1 complexes m and 1, as well as three new complexes. The gel mobilities of the new bands a and b are compatible with the idea that they represent structural variations of complexes m and 1. The low gel mobility of band c on the other hand, suggests that it represents a new complex with a CAP:DNA ratio greater than 2. The L305 deletion decreases the center-to-center distance between sites 1 and 2 from 73 to 72 bp, and changes the relative orientation of these sites by 36° about the helix axis. Either of these changes, or both, could stabilize the binding of additional dimers of CAP between the strong sites 1 and 2, utilizing the cooperative character of CAP-DNA binding (19). The 72 bp interval is evenly divisible by either 3 or 4, allowing either 2 or 3 CAP dimers to bind at DNA sites containing 24 bp dimer or 18 bp/dimer, respectively. Both of these binding site sizes lie within the range of published values (15, 19).

RNA polymerase binding. The binding of RNA polymerase to CAP-

promoter complexes is probably the first step in CAP-stimulated transcriptional initiation. The finding that 1:1 CAP-promoter complexes are preferentially bound by polymerase suggests that initiation complexes contain a single dimer of CAP. From the observed saturation of polymerase binding, we conclude that such complexes contain a single molecule of RNA polymerase as well. CAP is apparently not displaced from the promoter when RNA polymerase binds, at least not in functional form, since it would bind to free DNA and reduce the amount of that component in contradiction to the experimental result. This is particularly clear for the CAP:L8-UV5 promoter complex (Figure 7C). Surprisingly, even the 2:1 CAP complex appears not to lose CAP when polymerase binds. Since CAP site 2 overlaps with the region protected by RNA polymerase (43), it is possible that both CAP and polymerase can bind to the same region of DNA, perhaps on opposite sides of the helix. Alternatively, CAP may be displaced to another site on the DNA molecule without dissociation. If this is the case, then contacts with RNA polymerase must stabilize the new CAP:DNA complex. Nuclease footprinting experiments should be able to differentiate between these possibilities.

The observed binding of RNA polymerase to both CAP:L8-UV5 and CAP:wild type promoter complexes in preference to free DNA raises a significant question for further research: does the role of CAP extend beyond stabilization of polymerase-promoter contacts? If such stabilization is the only role of CAP, then mutations such as L8 which weaken CAP-promoter interactions should not affect stages of the initiation process subsequent to polymerase binding. The results presented here show that conditions (high CAP concentration) allowing CAP to bind to the L8 promoter result in selective binding of CAP-L8 promoter complexes by RNA polymerase. Under these conditions, CAP should stimulate transcription. If, on the other hand, the L8 mutation prevents CAP stimulation of transcription from these complexes, perhaps because of the lack of the DNA conformational change which produces band m, then the role of CAP must extend beyond the binding of RNA polymerase, into the initiation process itself.

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