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# Stoichiometry of Catabolite Activator Protein/Adenosine Cyclic 3′,5′-Monophosphate Interactions at the *lac* Promoter of *Escherichia coli*†

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ABSTRACT: The stoichiometry of catabolite activator protein (CAP) and adenosine cyclic 3′,5′-monophosphate (cAMP) interactions at the *lac* promoter has been determined under ionic conditions in which CAP-stimulated initiation of transcription is observed. Polyacrylamide gel electrophoresis was used to separate long-lived CAP/cAMP–promoter complexes from unbound components. Quantitation of the DNA and cAMP in the "complex" band showed a 1:1 molar ratio between them, in both the presence and absence of RNA polymerase. Equilibrium dialysis of CAP/cAMP solutions demonstrated that there is only one cAMP bound per protein molecule under our conditions. Taken together, these results imply that a single CAP molecule bound to the *lac* promoter is sufficient for biological activity. This was confirmed by centrifugation experiments in which DNA–protein complexes were rapidly pelleted in a Beckman Airfuge and were then analyzed for DNA and CAP content. The bound CAP molecule was localized on the promoter by restriction endonuclease protection experiments. These results are consistent with one CAP molecule being bound from 55 to 70 base pairs upstream from the point of RNA chain initiation, as was indicated by previous studies [Simpson, R. B. (1980) *Nucleic Acids Res. 8*, 759]. Titrations of the *lac* promoter with CAP, monitored by quantifying the transcriptional activity of RNA polymerase, showed that it was necessary to add four CAP molecules per promoter to achieve maximal stimulation, under conditions where all active CAP molecules would be bound to the promoter. This result shows that only one of every four molecules in our preparation of CAP is active in specific binding and transcription stimulation, even though they are 100% active in nonspecific DNA binding. Since it is found that stoichiometric amounts of active CAP must be added to get full initiation of transcription, it appears that CAP cannot "cycle" out of a ternary CAP/cAMP–RNA polymerase–promoter complex but must remain bound to the promoter until RNA synthesis begins.

The catabolite activator protein (CAP) of *Escherichia coli* increases the rate of RNA synthesis at catabolite-sensitive operons in response to the absence of glucose in the growth medium (Pastan & Adhya, 1976). When *E. coli* is grown in media lacking glucose, the intracellular level of cAMP is elevated (Saier et al., 1975; Epstein et al., 1975). CAP binds adenosine cyclic 3′,5′-monophosphate (cAMP), causing a profound conformational change in the protein (Krakow & Pastan, 1973). The CAP/cAMP complex then binds to catabolite-sensitive promoters and somehow increases the rate at which RNA polymerase initiates transcription. This enhancement is thought to be due to a facilitation of the formation of an "open" ("melted-in") polymerase–promoter complex at the initiation site (Pastan & Adhya, 1976). Two models for the mechanism of CAP action have been postulated: (1) that CAP serves as a DNA melting protein and that the effects of opening the helix at the CAP site are transmitted to the polymerase binding sequence downstream ("action-at-a-distance" model) (Dickson et al., 1975; Wells et al., 1977); (2) that CAP interacts directly with RNA polymerase when both are bound to their respective sites on the promoter and that these protein–protein interactions are responsible for the stimulation of transcription (Gilbert, 1976). Of course, these two models are not mutually exclusive, and the actual mechanism may be a combination of the two.

The specific area of promoter to which CAP binds has been localized by determining which bases are protected from chemical modification, or from deoxyribonuclease or exonuclease III digestion, by bound CAP/cAMP (Simpson, 1980; Schmitz, 1981). In the *lac* promoter, the area of most intense protection appears to be 55–70 base pairs upstream from the point of initiation. The modification of bases adjacent to this site also appears to be modulated somewhat, which might suggest that more than one CAP molecule is binding. [It has been shown that a bound CAP molecule will cover about 13 base pairs of double helical B-DNA (Saxe & Revzin, 1979); this value is consistent with the probable dimensions of a 45 000-dalton protein (Kumar et al., 1980).] Although the CAP binding site may appear to be removed from the RNA polymerase binding site, −41 to +1, Siebenlist et al. (1980) have observed that the sites are not so far distant from each other to preclude protein–protein interactions.

We present here results relevant to the fundamental question of whether one or several CAP molecules are involved in controlling transcription at catabolite-sensitive operons. We note that the cooperative binding to DNA of regulatory proteins has been observed in other systems, including the repressor and *cro* proteins of bacteriophage λ (Ptashne et al., 1980) and the SV40 T-antigen (Myers et al., 1981). Comparison of base sequences and of protection experiments at the *lac* and *gal* promoters led O'Neill et al. (1981) to propose that two CAP molecules might interact at the *lac* operon. It is

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known that CAP can participate in DNA-mediated protein–protein interactions, since its nonspecific binding to DNA is highly cooperative (Saxe & Revzin, 1979; Takahashi et al., 1979; Garner & Revzin, 1981a). In addition, we found by using a gel electrophoresis technique that, under solution conditions which support transcription, it is necessary to add several CAP molecules per promoter to fully complex the DNA (Garner & Revzin, 1981b). This led us to consider whether every CAP molecule in a preparation is fully active in specific binding and whether more than one CAP molecule binds to the promoter. We show here that in fact only one CAP molecule binds to the *lac* promoter, indicating that not every molecule in a preparation of CAP is active in stimulation of transcription.

## Experimental Procedures

*Materials.* CAP was isolated and purified to greater than 95% homogeneity from *E. coli* K12 strain CR63 by a modification (Garner & Revzin, 1981b) of the method of Boone & Wilcox (1978). RNA polymerase holoenzyme was purified from *E. coli* K12 strain PR7 by the procedure of Burgess & Jendrisak (1975), as modified by Lowe et al. (1979). Purified $\sigma$ factor was added to assure 100% saturation as monitored by analytical ultracentrifugation (Revzin & Woychik, 1981). Our RNA polymerase preparations were typically about 20–30% active in the quantitative assay of Chamberlin et al. (1979).

Restriction fragments 211 base pairs long containing the *lac* promoter–operator region were isolated as previously described from *E. coli* strains containing this fragment cloned into the plasmid pMB9 (Garner & Revzin, 1981b). Fragments contained either the wild type *lac* or L8-UV5 mutant *lac* promoter region. The L8 mutation is in the CAP binding site and renders the promoter insensitive to catabolite repression (Ippen et al., 1968). The UV5 mutation, in the polymerase binding region, allows efficient initiation even in the absence of CAP/cAMP (Silverstone et al., 1970). An 800 base pair fragment containing the wild-type *lac* control region, which had been cloned by us into pBR322, was purified in the same manner.

Tritium-labeled UTP, CTP, and cAMP were obtained from ICN, and [$\alpha$-$^{32}$P]UTP was from New England Nuclear. The chemical and radiochemical purity of the [$^3$H]cAMP was checked by using cellulose thin-layer chromatography in the solvent system of Brooker (1971) and was shown to be greater than 95%. Restriction endonucleases were from Bethesda Research Laboratories or New England Biolabs. All other chemicals were ACS reagent grade and used without further purification.

*Buffers.* Unless indicated otherwise, all experiments were performed in a buffer containing 0.1 M KCl, 0.020 M Tris, pH 8.0 (at 22 °C), 0.003 M $MgCl_2$, 0.0001 M EDTA, and 0.0001 M dithiothreitol. When present, cAMP was at $2 \times 10^{-5}$ M, a level sufficient to give full stimulation of *lac* transcription.

*Binding Experiments.* Details of the gel electrophoresis method for examining protein–DNA complexes are described elsewhere (Garner & Revzin, 1981b). One applies, for example, a CAP–DNA solution to a polyacrylamide gel and separates the components by electrophoresis; both the DNA fragments and the CAP–DNA complexes form bands. To analyze solutions containing CAP, DNA, and radioactively labeled cAMP, we ran identical aliquots of a binding mixture on cylindrical tube gels. After electrophoresis, one gel was removed from its tube, soaked for 2 h or more in the electrophoresis buffer, and then scanned at 260 nm in a Gilford Model 250 spectrophotometer with an attached Model 2410S linear transport device to quantitate the DNA by comparison to known standards. It is necessary to soak the gel before scanning to allow small molecules that interfere with measuring the absorbance (e.g., unpolymerized acrylamide) to diffuse out of the gel. To determine the amount of [$^3$H]cAMP that migrates with the complex band, we cut one or more other gels immediately after electrophoresis into 0.25-cm slices, which were placed in scintillation vials along with 0.3 mL of Protosol (New England Nuclear) and 10 mL of toluene/PPO scintillation cocktail. The vials were stored overnight at 37 °C, cooled in the dark, and counted in a thin window counter. Quenching was monitored by the single channel ratio method and was essentially constant for the gel slices. The specific activity of cAMP was determined by treating an aliquot of [$^3$H]cAMP in the same manner with Protosol and scintillation cocktail, which gave the same degree of quenching seen in the gel slices.

In principle, one could simply radioactively label the protein to get a more direct measurement of the amount of CAP present in the DNA–protein complex band. However, difficulties associated with retaining biological activity in the labeled protein with protein of sufficient specific activity and in sufficient quantity precluded this possibility.

For studies of CAP–DNA binding, 7.5% polyacrylamide [46:1 acrylamide:bis(acrylamide)] gels were used; for experiments involving CAP–RNA polymerase–promoter complexes, the gels used were 5% (30:1) polyacrylamide.

*Nuclease Protection Experiments.* DNA–protein complexes were formed by incubating solutions containing promoter DNA and CAP, with or without RNA polymerase, at 37 °C for 10 min in the presence of $2 \times 10^{-5}$ M cAMP. If RNA polymerase was present, poly[d(A·T)] was then added, and the solutions were incubated 10 min more at 37 °C to dissociate and sequester any nonspecifically bound RNA polymerase (Cech & McClure, 1980). A restriction endonuclease was added, and the solution was again incubated at 37 °C for the appropriate time, after which the reactions were made 333 $\mu$g/mL in heparin and 0.017 M in EDTA and extracted with phenol. The solutions were then analyzed on 7.5% polyacrylamide (46:1) gels. In all cases, a control sample of DNA with no protein present was digested to monitor the restriction enzyme activity.

*Transcription Assays.* The appropriate DNA fragment and RNA polymerase were mixed, in the presence or absence of CAP/cAMP, in a final volume of 50 $\mu$L. A 2-fold ratio of polymerase molecules to promoters was sufficient to give full transcriptional activity. The solutions were then incubated for 10 min at 37 °C, after which time heparin was added to a concentration of 100 $\mu$g/mL. After 1 min an aliquot of nucleoside triphosphates was added to give the following final concentrations: ATP and GTP, 0.2 mM each; UTP, 10 $\mu$M; CTP, 2 $\mu$M; 2.5 $\mu$Ci of [$^3$H]CTP was present in each solution. Transcription was then allowed to proceed for 15 min at 37 °C, after which time the reactions were quenched with trichloroacetic acid, filtered, and counted in a toluene-based scintillation cocktail.

For analysis of the lengths of the transcripts, the above protocol was used, except that the concentrations of UTP and CTP were reversed and 0.4 $\mu$Ci of [$\alpha$-$^{32}$P]UTP was substituted for [$^3$H]CTP. Fifteen minutes after addition of the nucleotides the solutions were made 9 M in urea and electrophoresed on 25% polyacrylamide slab gels containing 7 M urea. These gels were then assayed by autoradiography.

*Equilibrium Dialysis.* All experiments were done in a CRC equilibrium dialysis apparatus. A solution (0.4 mL) containing CAP and [$^3$H]cAMP was placed on one side of the membrane, while the other compartment contained an equal volume of buffer only (no cAMP), and the solutions were allowed to dialyze for 8–16 h. This was sufficient time to ensure that equilibrium had been reached, as shown by a control experiment in which cAMP, initially placed in one chamber (no CAP), was found in equal concentrations on both sides of the membrane after 8 h. Following dialysis the concentration of protein in each chamber was checked by ultraviolet absorbance and the amount of CAP-bound cAMP determined by scintillation counting of aliquots of solutions from both compartments.

*"Airfuge" Experiments.* The binding of CAP to polymerase–promoter complexes was monitored by centrifuging DNA–protein mixtures at 150000*g* in a Beckman Airfuge at 22 °C. This technique is described in detail elsewhere (S. H. Shanblatt and A. Revzin, unpublished results). In our experiments an 800 base pair DNA fragment containing the wild-type *lac* promoter was mixed with CAP and RNA polymerase (four molecules of each protein per DNA fragment). The ionic conditions are such that most of the polymerase molecules are DNA bound, either to the promoter or to some other region. The nonspecifically bound polymerases cover less than 15% of the available base pairs, hence having little effect on CAP binding, but they do make the DNA–protein complexes heavy enough to be pelleted in 30 min in the Airfuge. Following a run, the pellet was resuspended in 200 μL of buffer, the absorbance spectrum was read, and an aliquot was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels (10% or 12% gels). The level of DNA was determined from the absorbance readings. Protein bands in the gel were visualized by the silver-staining procedure of Wray et al. (1981), and the amount of CAP in the pellet was quantitated by comparison with known amounts of CAP run in neighboring lanes.

## Results

The gel electrophoresis method used in these experiments separates protein–DNA complexes from unbound DNA on the basis of differences in electrophoretic mobility. As shown in Figure 1, the position in the gel of the CAP–DNA band corresponds with the peak of radioactivity which represents [$^3$H]cAMP in CAP/cAMP–*lac* promoter complexes. Quantitation of the DNA in the peak, by comparing the peak area with that from a DNA standard, and of the [$^3$H]cAMP level indicates that there is one cAMP per promoter in the complex peak (0.98 ± 0.07; average of four experiments, i.e., $n = 4$). Changing either the input ratio of CAP to DNA or the time of electrophoresis changes the absolute amounts of DNA and cAMP in the complex band, but the ratio of cAMP to DNA is always approximately 1 to 1. Addition of RNA polymerase leads to the formation of poly[d(A·T)]-insensitive "ternary" complexes of RNA polymerase, CAP/cAMP, and the *lac* promoter (Garner & Revzin, 1981b). Analyzing these complexes on polyacrylamide gels in a similar manner gives essentially the same result, one cAMP per promoter (1.06 ± 0.29; $n = 3$). Equilibrium dialysis of CAP/cAMP solutions showed that, at $2 \times 10^{-5}$ M cAMP and the ionic strength used in our assays, there is approximately one cAMP bound per CAP molecule (0.72 ± 0.11; $n = 3$). These results, taken together, would indicate that there is only one CAP molecule bound to the *lac* promoter.

This conclusion was verified by experiments using the Beckman Airfuge. Under our standard buffer conditions, one molecule of CAP (0.94 ± 0.11; $n = 3$) specifically bound per promoter fragment is found in the pelleted material. In deriving this result, we corrected for the small amount of CAP that is nonspecifically bound in our solutions. The correction involved an experiment in which an equivalent amount of T7 DNA was substituted for the *lac* promoter fragments. This control is appropriate since, although the *lac* DNA fragments have many more ends than does the long T7 DNA, CAP shows no tendency to interact with DNA ends (Garner & Revzin, 1981b).

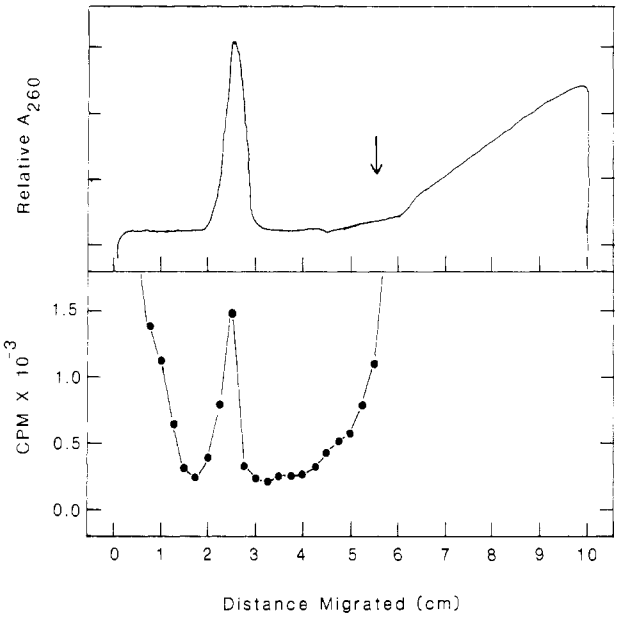


FIGURE 1: Absorbance and radioactivity profiles of CAP–promoter complex gels. Aliquots (30 μL) of a solution of $2.0 \times 10^{-7}$ M *lac* wild-type promoters, $7.7 \times 10^{-7}$ M CAP, and $2.0 \times 10^{-5}$ M [$^3$H]cAMP were incubated for 10 min at 37 °C. Then 5 μL of dye mix (33% glycerol, 0.03% bromophenol blue) was added to each, and the samples were applied to 0.5 × 10 cm 7.5% polyacrylamide tube gels. Electrophoresis was for 90 min at 2 mA per tube. The gels were then scanned or sliced and counted as described in the text. The left side of the diagram corresponds to the top of the gel. The large increase in radioactivity (and $A_{260}$) near the bottom of the gel is due to unbound cAMP. The arrow indicates the position where free DNA would migrate. (In the experiment shown, all DNA was bound to protein.) Lower percentage gels in which ternary CAP–RNA polymerase–promoter complexes are run show the same large separation of the complex and free DNA bands.

To further support these results and to localize the bound CAP molecule, we examined whether CAP can protect *lac* promoter DNA from various restriction endonucleases. The conditions of these experiments were such that only partial digestion was achieved. Figure 2a shows that, while digestion by *Alu*I is markedly affected, the presence of CAP causes little change in the restriction patterns for either *Hha*I or *Eco*RII. The apparently different degree of digestion in the *Hha*I lanes (4 and 5) did not appear in all experiments; our data certainly indicate that the *Hha*I site is accessible even if CAP is bound. We found that *Eco*RII was not very efficient at cleaving our DNA fragment. Longer incubation times or additional enzyme did not lead to more digestion than shown. Again, the presence of CAP did not affect the *Eco*RII patterns. We emphasize that identical results are obtained if RNA polymerase is present. It is of interest that the *Eco*RII site remains accessible even if both CAP and polymerase are bound. The locations of restriction sites in the *lac* promoter (Figure 2b) are such that, if more than one CAP molecule were contiguously bound to the promoter, either the *Hha*I or *Eco*RII site should be protected. Thus, the data in Figure 2a suggest that a single

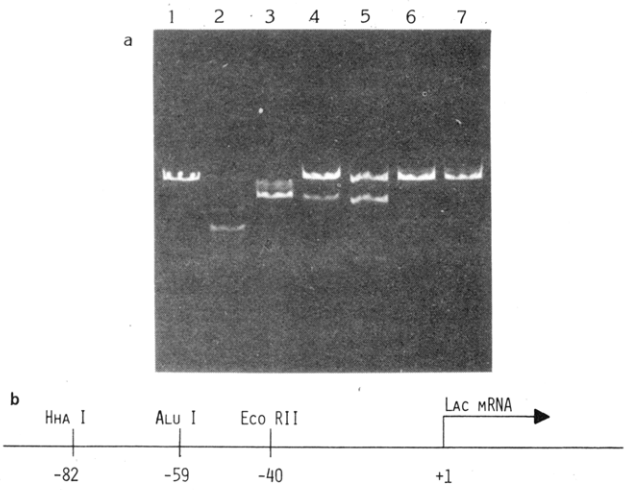


FIGURE 2: (a) Limited restriction endonuclease digestion of *lac* promoter DNA in the presence and absence of CAP. Solutions contained $2.0 \times 10^{-7}$ M promoters and $2.0 \times 10^{-5}$ M cAMP; CAP, where present, was $8.0 \times 10^{-7}$ M. Digestions were performed as described in the text with, in each case, about 0.24 "1 h" units of enzyme. The times of digestion were as follows: *Hha*I, 5 min; *Alu*I and *Eco*RII (*Bst*nI), 10 min. (Lane 1) No restriction enzyme added; (2) *Alu*I, no CAP; (3) *Alu*I, plus CAP; (4) *Hha*I, no CAP; (5) *Hha*I, plus CAP; (6) *Eco*RII, no CAP; (7) *Eco*RII, plus CAP. Digestions of RNA polymerase–*lac* promoter complexes (+CAP/cAMP) gave the same patterns (data not shown). (b) Location of relevant restriction sites in the *lac* promoter, relative to the start of initiation. Locations are based on the sequence data of Dickson et al. (1975).

CAP molecule binds to the promoter in the region −55 to −70, whether RNA polymerase is present or absent.

We also assayed the DNA–protein complexes for transcriptional activity under our solution conditions. The pattern of transcripts seen on urea gels is similar to that already reported (Munson & Reznikoff, 1981), i.e., a very intense, main transcript of about 65 bases corresponding to a single, full-length run-off of the *lac*-specific coding region present on this fragment and many small oligonucleotide fragments produced by the "stuttering" of polymerase at the promoter (data not shown). We have quantitated the number of long RNA molecules transcribed by means of a precipitation assay. Figure 3 shows a titration done with such an assay by increasing the concentration of CAP but keeping the DNA, cAMP, and RNA polymerase concentrations constant. The amount of RNA produced increases until at least four CAP molecules per wild-type *lac* promoter have been added, at which point it levels off at approximately a 5–6-fold increase over the transcription in the absence of cAMP or CAP and eventually begins to decline. This is consistent with the previous work of Majors (1975). The high initial level of transcription seen when the L8-UV5 mutant promoter is used also starts to diminish at higher concentrations of CAP, which suggests that this decline may be due to nonspecific binding by the excess CAP molecules.

## Discussion

The results of the Airfuge, gel electrophoresis, and transcription experiments described above demonstrate that a single CAP molecule binding to the *lac* promoter region is sufficient to stimulate initiation by RNA polymerase. This finding is supported by restriction nuclease protection experiments, which give no evidence for the binding of more than one CAP molecule. The restriction enzyme protection pattern seen for CAP bound to the *lac* promoter is consistent with the binding site determined for CAP by other methods (Simpson, 1980; Schmitz, 1981). Perhaps the changes in the patterns of chemical modifications seen outside this site are due to some

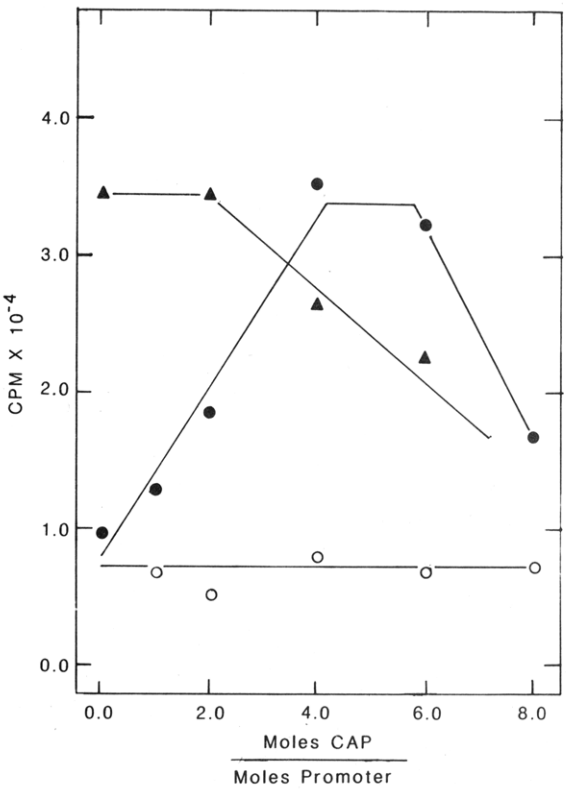


FIGURE 3: Titration of *lac* promoters with CAP, monitored by transcriptional activity. Transcription assays were performed as indicated in the text. In all samples the promoter concentration was $4.0 \times 10^{-8}$ M, RNA polymerase was $8.0 \times 10^{-8}$ M, and cAMP, where present, was $2.0 \times 10^{-5}$ M. Each point is an average of three replicates, which gave a standard deviation of approximately 10%. (○) Wild-type *lac* promoter, no cAMP; (●) wild-type *lac* promoter with cAMP; (▲) L8-UV5 *lac* promoter with cAMP.

nonspecific CAP binding, which should occur at the low ionic strengths used in those experiments.

The transcription results lead to several conclusions concerning our preparations of CAP. Since a 4:1 ratio of CAP to *lac* promoter fragments must be added to obtain maximum transcription, we infer that only one-fourth of the CAP molecules are active. This is consistent with previous results from our laboratory, which showed that four CAP molecules per promoter were just sufficient to complex essentially all the DNA [see Figure 2 of Garner & Revzin (1981b)]. The apparent inactivity of 75% of the CAP molecules appears to be maintained from one batch to another. This may be characteristic of our method of isolating the protein, or it could represent the in vivo situation. It is interesting to note, however, that our preparations are fully active in nonspecific binding (Saxe & Revzin, 1979; Garner & Revzin, 1981a).

It is also of interest that, since a full complement of active CAP molecules is necessary to obtain maximal transcription, the CAP molecule bound in a ternary CAP/cAMP–RNA polymerase–*lac* promoter "open" complex cannot "cycle". That is, it does not dissociate from this complex prior to initiation and bind to another promoter, leading to the formation of a second open complex, etc. If this were true, then a less than saturating concentration of active CAP molecules would give complete open-complex formation and transcription, in contrast to our results, which indicate that CAP must remain bound to the promoter at least until initiation has occurred.

Both the gel electrophoresis and nuclease protection results described above show that the number and position of CAP molecules binding to the promoter are not affected by the presence of RNA polymerase. This is in contrast to the *gal*

operon, where apparently the binding of RNA polymerase causes an additional CAP to bind to the promoter (S. H. Shanblatt and A. Revzin, unpublished results).

By quantitating the DNA and the CAP-bound cAMP in a gel band containing specific CAP/cAMP–*lac* promoter complexes, we find only one cAMP per promoter both in the presence and in the absence of RNA polymerase. Loss of a cAMP molecule from CAP during electrophoresis should not affect this result, since the CAP–promoter binding affinity is very low in the absence of cAMP (Garner & Revzin, 1981b), and cAMP-free protein molecules would dissociate rapidly from the promoter. The DNA would then migrate ahead of the band of DNA–protein complexes so that neither the lost [$^3$H]cAMP nor the corresponding DNA molecule would appear with the complex band.

Takahashi et al. (1980), using equilibrium dialysis, found that there are two binding sites for cAMP per CAP and that the binding of the two cyclic nucleotide molecules is negatively cooperative. Under our dialysis conditions, we find that only one cAMP molecule binds to each CAP molecule. Since we are working at a cAMP concentration sufficient to give full nonspecific binding (Saxe & Revzin, 1979; Takahashi et al., 1979), promoter-specific binding (Garner & Revzin, 1981b), and stimulation of transcription, this suggests that the binding of the second cAMP is gratuitous; the negative cooperativity found by Takahashi and co-workers may suggest that CAP exhibits "half of the sites reactivity" and is fully active with only one cAMP bound.

The control of transcription by RNA polymerase at catabolite-sensitive promoters is a complicated process involving several accessory factors. Complete understanding of the initiation process must be preceded by an understanding of the configuration of the components of the preinitiation complex. In this work, we have determined the number and location of CAP/cAMP molecules bound to the promoter in this complex. We are now applying other physical methods to the study of the *lac* operon in hopes of gaining an understanding of the precise mechanism of control.

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