The *Escherichia coli* Cyclic AMP Receptor Protein Forms A 2:2 Complex With RNA Polymerase Holoenzyme, *In Vitro* †

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Abstract

Sedimentation equilibrium studies show that the *Escherichia coli* cyclic AMP receptor protein (CAP) and RNA polymerase holoenzyme associate to form a 2:2 complex *in vitro*. No complexes of lower stoichiometry (1:1, 2:1, 1:2) were detected over a wide range of CAP and RNAP concentrations, suggesting that the interaction is highly cooperative. The absence of higher stoichiometry complexes, even in the limit of high [protein], suggests that the 2:2 species represents binding saturation for this system. The 2:2 pattern of complex formation is robust. A lower-limit estimate of the formation constant in our standard buffer (40 mM Tris (pH 7.9), 10 mM MgCl₂, 0.1 mM DTT, 5% glycerol, 100 mM KCl) is $2 \times 10^{20}$ M⁻³. The qualitative pattern of association is unchanged over the temperature range $4^\circ C \leq T \leq 20^\circ C$, by substitution of glutamate for chloride as the dominant anion, or on addition of 20µM cAMP to the reaction mix. These results limit the possible mechanisms of CAP-polymerase association. In addition, they support the idea that CAP-binding may influence the availability of the monomeric form of RNA polymerase that mediates transcription at many promoters.
Introduction

The *Escherichia coli* cyclic AMP receptor protein (CAP\(^1\)) regulates the transcriptional activity of at least 100 promoters (reviewed in (1-3)). Under conditions of high intracellular [cAMP] it binds target sequences within or near promoters and interacts with RNA polymerase holoenzyme to modulate transcription. CAP is a homodimer with a subunit molecular weight of 23,619 (4). High-resolution crystal structures of CAP•cAMP and CAP•cAMP•DNA complexes have been obtained (5-8). The CAP-DNA complex is 2-fold symmetric, with each monomer providing one high-affinity binding site for cAMP, 1/2 of the DNA-contacts and two potential RNA polymerase interaction surfaces\(^2\), designated activating regions (AR) 1 and 2 (3). This polyvalency implies that it is theoretically possible for CAP to interact with >1 RNA polymerase molecule at a time.

There is ample precedent for this notion in evidence for regulatory models in which CAP simultaneously binds two other transcription factors (10), two additional molecules of CAP (11,12), one additional transcription factor and RNA polymerase (12,13) or two RNA polymerase alpha subunits (2,3). However, these interactions take place when CAP and its protein partners are DNA-bound and although CAP has been shown to interact with polymerase

\(^1\) Known also as CRP.

\(^2\) A third non-native activating region (AR3, residues 52-58) is created by substitution of lys52 by a neutral or negatively charged residue (3,9).
holoenzyme in free solution (14-16), lack of information about the stoichiometry of these complexes has impeded their characterization.

The *E. coli* RNA polymerase occurs in two forms, a core enzyme with subunit composition \((\alpha)_2\beta\beta'\) (\(M_r \sim 385,000\)) and a holoenzyme with subunit composition \((\alpha)_2\beta\beta'\sigma\) (\(M_r \sim 455,000\); reviewed in (17-19)). The sigma subunit is required for sequence-specific promoter binding and thus for high-fidelity transcription initiation (20). It also contains a surface motif that can interact with CAP when the proteins are bound at Class II promoters (3,21). Two additional motifs that can interact with CAP are located within the \(\alpha\) subunit. The best characterized of these is the “287 determinant” within the C-terminal domain of \(\alpha\) (3). Mutations of the residues that comprise this structure interfere with CAP-dependent transcription activation at Class I promoters (22,23) and reduce cooperative binding in the formation of CAP-\(\alpha\)-DNA ternary complexes (24). A second motif, located within the N-terminal domain of the \(\alpha\) subunit has been implicated in CAP interactions at Class II and some Class III promoters (3,25). The multiplicity of CAP-binding motifs indicates single RNA polymerase molecules have the potential to interact with two or more CAP dimers; evidence for such interactions at some Class III promoters has been reviewed by Busby and Ebright (3). At present it is not known whether similar interactions take place in the absence of DNA.

In addition to directing promoter binding and providing a CAP-interaction motif, the \(\sigma\)-subunit exerts a significant influence on the self-association pattern of RNA polymerase. The
holoenzyme exists as an equilibrium mixture of monomers and dimers at low salt ([KCl] ≤ 300 mM) while at higher [salt] the monomer form prevails ((26,27); this study). In contrast, the core enzyme associates to form complexes at least as large as tetramer, although the exact mechanism of core enzyme self-association remains controversial (27,28). While the biological role of RNA polymerase self-association is unknown, the observations that the holoenzyme binds to the lacUV5 promoter as a monomer and to the tyrT promoter as a dimer have prompted the suggestion that dimerization might influence the distribution of polymerase between available promoters (29). In addition, it is possible that dimerization controls the availability of the holoenzyme form of RNA polymerase, because dimers form preferentially when the σ-subunit is present (see above). Since CAP interacts with the RNA polymerase holoenzyme, it seems plausible that it might modulate the self-association and functions of RNA polymerase that take place when it is not DNA-bound. The data presented below represent a first test of this notion.
CAP-RNA POLYMERASE INTERACTION

Materials and methods

Proteins.  E. coli RNA polymerase holoenzyme was the kind gift of Dr. T. Heyduk. The holoenzyme was prepared by incubating core enzyme (30) with a 2-fold molar excess of $\sigma_{70}$ (31) and purifying the reconstituted holoenzyme by MonoQ chromatography (32). By SDS-PAGE and sedimentation equilibrium criteria, these holoenzyme preparations were fully saturated with $\sigma_{70}$ (results not shown). The E. coli cyclic AMP receptor protein was isolated from strain pp47 containing plasmid pH5 (kindly provided by Dr. H. Aiba). The purification followed the protocol of Fried (33) and gave protein of greater than 95% purity as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis. The preparation used in this study bound 10 nmol of cAMP per mg of protein in the cAMP binding assay of Anderson et al. (34), and was ~80% active in cAMP-dependent binding to the lac promoter, according to the method of Fried & Crothers (35). Protein concentrations were determined spectrophotometrically using $\epsilon_{280} = 2.82 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$ per RNAP holoenzyme monomer (18) and $\epsilon_{280} = 3.5 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ per CAP dimer (34).

Sedimentation equilibrium assays. Samples were dialyzed extensively against 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol containing either 100 mM KCl or 300 mM KCl, as indicated. Aliquots were then centrifuged to equilibrium in a Beckman XL-A analytical ultracentrifuge equipped with an AN-60 rotor. Absorbance values were measured at 280 nm as functions of radial position. Five scans were averaged for each sample at each rotor
speed. The approach to equilibrium was considered to be complete when replicate scans separated by $\geq 8$ h were indistinguishable.

At sedimentation equilibrium, the absorbance at a specified wavelength and position in the solution column is given by equation 1.

$$A(r) = \sum_n \alpha_{n,0} \exp[\sigma_n(r^2 - r_0^2)] + \zeta$$

Here $A(r)$ is the absorbance at radial position $r$, the summation is over all species, $n$; $\alpha_{n,0}$ is the absorbance of the $n$th species at the reference position $r_0$, $\sigma_n = M_n(1 - \bar{v}_n \rho)\omega^2/2R$ with $M_n$ the molecular weight of the $n$th species, $\bar{v}_n$ its partial specific volume (0.737 for CAP (36), 0.742 for RNAP (27,37)), $\rho$ the solution density, $\omega$ the rotor angular velocity, $R$ the gas constant and $T$ the absolute temperature. Buffer densities were measured with a Mettler density meter. The baseline offset term $\zeta$ compensates for slight position-independent differences in the optical properties of different cell assemblies.

For a system in which monomers (M) are in equilibrium with dimers (D), Eq. 1 becomes

$$A(r) = \alpha_{M,0} \exp[\sigma_M(r^2 - r_0^2)] + \alpha_{D,0} \exp[\sigma_D(r^2 - r_0^2)] + \zeta$$
Here $\sigma_D = 2M_M (1 - \overline{v_M} \rho) \omega^2 / 2 R \gamma$ and the absorbance term for dimer depends on the absorbance scale association constant, $\alpha_{D,0} = K (\alpha_{M,0})^2$. Association constants were estimated by simultaneous least squares fitting of Eq. 2 to multiple data sets (“global analysis”) using the program NONLIN3, running on a Macintosh computer (38). Typical analyses used nine data sets corresponding to three samples differing in nominal protein concentration, centrifuged at three rotor speeds.

**$\chi^2$ analysis of molecular weights.** Our current implementation of the global analysis program NONLIN allows the modeling of self-association reactions but not hetero-associations like that of CAP with RNA polymerase. To circumvent this limitation we fit single data sets to hetero-association models, testing several fixed values of the molecular weights of the CAP-polymerase complex. The molecular weight value that minimizes the $\chi^2$ parameter (Eq 3; (39)) is the “best” estimate, by the least-squares criterion.

$$\chi^2 = \sum_i \left( \frac{1}{\sigma_i^2} [A_i - A(r)]^2 \right)$$  \hspace{1cm} (3)

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3 NONLIN for the Macintosh was obtained from the software archive located in the Reversible Associations in Structural and Molecular Biology (RASMB) website [http://www.bbri.org/RASMB/rasmb.html](http://www.bbri.org/RASMB/rasmb.html).
Here the summation is over radial positions \( r \), the observed absorbance is \( A_r \), the standard deviation in \( A \) is \( \sigma_r \), and the absorbance predicted by the fit is \( A(r) \). A valuable feature of the \( \chi^2 \) parameter is that it is additive. Thus, the sum of \( \chi^2 \) values obtained from fitting different individual data sets (with the same binding equation) is itself a \( \chi^2 \) parameter (40). The molecular weight that minimizes this sum of \( \chi^2 \) parameters is the value most consistent with the data by this “global” criterion.
Results

*The salt-dependent monomer-dimer equilibrium of RNA polymerase holoenzyme.* Both holoenzyme and core RNA polymerases are known to undergo [salt]-dependent self-association reactions (26-28,41,42). Thus, a characterization of the assembly states of our samples of RNA polymerase is a prerequisite to the studies of CAP-polymerase interaction described below. Representative sedimentation profiles for the RNA polymerase holoenzyme, at 4°C, are shown in Fig. 1. Curve A is a profile obtained at 300 mM KCl; the solid line through the data is a global least squares fit of the expression for a single species (Eq. 1 with $\sigma_n$ corresponding to RNA polymerase monomer) to 9 data sets (3 concentrations, 3 rotor speeds). The small, symmetrically distributed curve-fitting residuals demonstrate the compatibility of the single-species model with the data. The value of $M_r$ (± 67% confidence interval) returned by this analysis was 454,000 ± 6,000 in good agreement with the value of the monomer molecular weight ($4.55 \times 10^5$) predicted on the basis of the subunit composition ($\alpha_2\beta\beta'\sigma$) of the holoenzyme (18,27). This molecular weight and the small confidence interval demonstrate that the enzyme is $\sigma$-saturated. In addition, these values are incompatible with significant self-association or degradation in our samples under these experimental conditions.

Curve B is a profile obtained at 100 mM KCl; the solid line through the data is a global least squares fit of the expression for a monomer-dimer equilibrium (Eq. 2) to 9 data sets as described above. The molecular weight of the monomer was set at 454,000; this analysis
returned an estimate of the association constant $K_a = 7.37 \pm 3.55 \times 10^5 \text{M}^{-1}$. The small values of the residuals and lack obvious systematic dependence on radial position indicate that the monomer-dimer model is consistent with the data from these samples and argues strongly against the presence of higher molecular weight assemblies under these solution conditions. These results are in excellent agreement with those of Record and co-workers, who found a monomer-dimer association with an apparent association constant of $\approx 10^6 \text{M}^{-1}$ under similar ionic conditions (27).

**CAP sediments as a single, ideal species in the absence of RNA polymerase.** Representative sedimentation profiles of CAP, obtained at 4°C and 19,000 rpm, are shown in Fig. 2. The solid curves through the data are global least squares fits of the expression for a single species (Eq. 1 with $\sigma_n$ corresponding CAP) to 6 data sets (2 concentrations, 3 rotor speeds). The small, symmetrically distributed residuals demonstrate the compatibility of the single-species model with the data. The molecular weights returned by these analyses were 48,400 ± 1,400 for CAP in buffer containing 300 mM KCl (curve A) and 47,800 ± 1,400 for CAP in buffer containing 100 mM KCl (curve B). The agreement with the molecular weight derived from sequence ($M_r$ (CAP dimer) = 47,238) indicates that this protein is neither degraded nor aggregated under our experimental conditions.

**CAP and RNAP interact in the absence of DNA.** Solutions in which CAP and RNA polymerase were combined contained an additional species with a weight-average molecular
weight ($\sim 1.1 \times 10^6$) significantly greater than that observed for the RNA polymerase dimer, in
the experiments described above (908,000 $\pm$ 12,000). Shown in Fig. 3 are sedimentation
equilibrium profiles acquired from mixtures containing RNA polymerase in slight molar excess
(curve A) and CAP in slight molar excess (curve B). The data were fit by sedimentation models
with 2-species (Eq. 1 with two terms). When RNA polymerase was in excess (curve A), the
model used contained terms for polymerase monomer ($M_r$ set at 454,000) plus an additional
species (for which $M_r$ was a parameter of the fit). The molecular weight returned by this analysis
was 1,090,000 $\pm$ 43,000, consistent with the presence of two equivalents of RNA polymerase
monomer in the assembly. The small, uniformly-distributed residuals (upper panel) attest to the
compatibility of this model to the data. The 3-species model with terms for free CAP, RNA
polymerase monomer and a complex, fit the data as well as the 2-species (polymerase +
complex) model, but returned values for the concentration of free CAP that were within error
equal to zero (result not shown)$^4$. Other models tested, including that corresponding to the RNA
polymerase monomer-dimer equilibrium, RNA polymerase dimer plus a complex, and one
including terms for free CAP plus a polymerase complex fit the data less well, as judged by
significantly larger $\chi^2$ values and evidence of systematic dependence of residuals on radial
position (results not shown).

$^4$ As discussed below, this result suggests that the formation constant for the CAP-RNA
polymerase complex may be quite large.
In contrast, when CAP was in slight molar excess over RNA polymerase (Fig. 3 curve B), the model most consistent with the data was one that included a term for free CAP and one for an additional species. The molecular weight of the additional component obtained by model fitting to this data set was $1,020,000 \pm 57,400$, suggesting the presence of two equivalents of RNA polymerase monomer in the assembly. As before, the small, symmetrical residuals demonstrate the compatibility of this model with the data. In this case, three species models, with terms for CAP, RNA polymerase (either monomer or dimer) plus a complex fit the data as well as the two-term (CAP + complex) model, but returned values for the concentration of free RNA polymerase that were within error equal to zero. Other models tested (one with terms for RNA polymerase monomer and dimer only, one with terms for free polymerase monomer plus a complex and one with terms for free polymerase dimer plus a complex), fit the data significantly less well, as judged by large $\chi^2$ values and evidence of systematic dependence of residuals on radial position (results not shown).

The CAP:RNA polymerase stoichiometry is 2:2. The molecular weights of the CAP-RNA polymerase complex that are returned by fitting individual data sets suggest that the complex contains two monomer-equivalents of RNA polymerase, but the values lack the precision needed to specify a unique CAP:RNA polymerase stoichiometry. Global analysis with NONLIN could not be done, because the current implementation of that program does not allow modeling of hetero-associations like that of CAP with RNA polymerase. Analysis of the dependence of the sum of $\chi^2$ values on molecular weight of the complex for ensembles of data
sets (9 in which CAP was in molar excess and 9 with RNA polymerase in excess) revealed broad minima centered at $M_w(\text{complex}) = 1 \times 10^6$ (Figure 4). Although this value is most consistent with a 2:2 stoichiometry, the broad distributions do not rule out 2:1 and 2:3 molar ratios.

To narrow the range of possible stoichiometries, the continuous variation (Job) method (43) was used to measure the optimal combining ratio of CAP and RNA polymerase. Seven samples prepared with total protein fixed ([CAP] + [RNA polymerase] = $7.2 \times 10^{-7}$M), but ranging in mole fraction of CAP from 0 to 1, gave values of $M_c(\text{complex})$ compatible with a 2:2 molar ratio (mean ± SD = 1,060,000 ± 52,000; Fig. 5). In addition, the amount of complex observed depended on the mole-fraction of CAP, giving a maximum when the mole fraction of CAP = 0.5 (equivalent to a molar ratio of 1 CAP/RNA polymerase). Together, these results support the notion that the complex has a 2:2 stoichiometry. The preferential formation of 2:2 complex and the absence of complexes of other stoichiometries (e.g., CAP:polymerase = 1:2 under conditions of polymerase excess, or CAP:polymerase = 2:1 under conditions of CAP excess) strongly suggests that the binding of CAP to RNA polymerase is cooperative. Further, the absence of detectable amounts of complex of higher molecular weight indicates that association does not proceed beyond the 2:2 ratio.

The 2:2 pattern of interaction is robust. Our data suggest that an overall association mechanism of the type
operates at 4°C under our solution conditions (40 mM Tris (pH 7.9), 100 mM KCl, 10 mM MgCl₂, 0.1 mM DTT, 5% glycerol). This pattern is valid over a wide range of protein concentrations. For a typical experiment in which RNA polymerase binds half of the available CAP, we estimate⁵ that the free CAP concentrations range from the detection limit (A₂₈₀ ~ 0.002 = 5 × 10⁻⁸ M) near the meniscus to approximately 6 × 10⁻⁶ M near the bottom of the centrifuge cell. Similarly, in an RNA polymerase-excess experiment in which CAP is half-saturating, we estimate that 6 × 10⁻⁹ M ≤ [RNA polymerase] ≤ 1.5 × 10⁻⁶ M. Since no free CAP can be detected in a polymerase-excess experiment, [CAP]₉₉ must be <5 x 10⁻⁹ M under these conditions. Taken together, the lower limit of [CAP]₉₉ and the upper limit of [RNA polymerase]₉₉ provide us with a lower-limit estimate for the formation constant, Kₐ ≥ 2 × 10²⁰ M⁻³. With several caveats (discussed below), this formation constant is compatible with previous in vitro estimates of the apparent association constant for CAP with RNA polymerase holoenzyme, in the absence of DNA.

To ensure that the 2:2 pattern of interaction described above was not an artifact of our choice of solution conditions, we performed a series of experiments in which buffer composition, salt concentration, temperature and [cAMP] were varied. Shown in Figure 6 are sedimentation

⁵ Based on data obtained at 7,000 rpm and 4°C, in our standard buffer, 40 mM Tris (pH 7.9), 100 mM KCl, 10 mM MgCl₂, 0.1 mM DTT, 5% glycerol.
profiles of CAP-RNA polymerase mixtures brought to equilibrium in buffers containing 300 mM KCl (curve A) and 300 mM potassium glutamate (curve B). The data shown in curve A were obtained under conditions of CAP-excess; the difference in molecular weights of CAP dimer (47,238) and 2:2 CAP-polymerase complex ($1.0 \times 10^6$) accounts for the biphasic character of the curve. The formation of a complex containing two equivalents of RNA polymerase under conditions in which the free holoenzyme is monomeric (c.f., Fig. 1), raises the possibility that CAP binding might stabilize the dimeric form of polymerase. The data shown in curve B were obtained under conditions of slight RNA polymerase excess, and the model fit to the data is that of RNA polymerase monomer ($M_r = 455,000$) plus a CAP-polymerase complex. The molecular weight of the complex returned by this analysis is 1,030,000 ± 26,000; this value is most consistent with a 2:2 CAP:polymerase stoichiometry. The formation of similar 2:2 complexes in chloride- and glutamate-containing buffers suggests that the CAP-polymerase association pattern is not highly sensitive to the identity of the dominant solution anion.

CAP is a cAMP-dependent transcription activator (1,44,45) so it was of interest to determine whether cAMP binding affects its interaction with the polymerase holoenzyme. Shown in Figure 7, curve A, are data obtained at 4°C in buffer supplemented with 20 µM cAMP. This concentration of cAMP is sufficient to form the complex containing 1cAMP per CAP dimer that is active in sequence-specific DNA binding, under conditions of pH and salt concentration comparable to those used here (35,46). As shown by the high quality of the fit, the data are compatible with a 2-species model (RNA polymerase monomer plus a CAP-polymerase
complex). The quality of the fit is not significantly improved by the inclusion of terms for additional species (result not shown), indicating that the binding is not weakened by cAMP to the point that free CAP has become detectable. Analysis using the 2-species model returns a molecular weight estimate for the complex of 1,070,000 ± 48,000. This is in good agreement with values obtained in the absence of cAMP and is consistent with the notion that the association pattern is independent of [cAMP] over the range 0 < [cAMP] < 20µM.

Kinetic and enzymatic protection studies show that RNA polymerase-promoter complexes undergo a substantial temperature-dependent isomerization, corresponding to the formation of one or more ‘open’ complexes (c.f., (47-49)). To determine whether the CAP-RNA polymerase complex undergoes a parallel temperature-dependent change in association, samples were brought to sedimentation equilibrium at 4°C (Fig. 3), 10°C, 20°C and 37°C. Curve B of Figure 7 shows representative data obtained at 20°C. The sample contained a molar excess of RNA polymerase, so the data were initially analyzed according to the 2-species model that includes RNA polymerase monomer plus a CAP-polymerase complex. The high quality of the fit indicates that this model is consistent with the data. The inclusion of terms for additional species did not significantly improve the fit (result not shown) supporting the notion that additional species are not present in substantial concentration at 20°C. This analysis gave a molecular weight for the CAP-polymerase complex of 1,100,000 ± 37,000, compatible with that

While this prevents any conclusion about whether cAMP alters the stability of the CAP-polymerase interaction, it is also incompatible with a large destabilization of the complex.
predicted for a 2:2 complex. Closely similar results were obtained at 10°C. At 37°C a qualitatively similar species distribution is found, but ~30% of the optical density of samples is lost early in the centrifuge run, most likely as a consequence of precipitation \(^7\) (results not shown). Taken together, these results indicate that the 2:2 CAP:polymerase association pattern is maintained over the range \(4^\circ C \leq T \leq 20^\circ C\) and that it may extend to somewhat higher temperatures.

\(^7\) SDS polyacrylamide gel electrophoresis analyses of recovered samples confirm that this apparent precipitation was not accompanied by proteolytic degradation.
Discussion

The results presented above indicate that CAP and RNA polymerase holoenzyme form a 2:2 complex under quasi-physiological conditions of [salt], [cAMP] and temperature. The interaction is robust in that modest changes in these variables do not alter the qualitative pattern of association. The 2:2 complex is the only CAP-polymerase complex detected over a wide range (~1000 fold) of protein concentrations and does not depend on the input CAP:RNA polymerase ratio. The absence of detectable concentrations of species of lower stoichiometry (e.g., 1:1, 1:2 or 2:1) implies that complex formation is a cooperative reaction. This notion is reinforced by the observation that only 2:2 (and not 1:1) complexes are found under solution conditions (300 mM KCl) in which RNA polymerase is monomeric when sedimented alone. In addition, the absence of higher-order complexes, even under conditions of high [protein], suggests that the 2:2 species represents binding saturation.

Together, these results place stringent limits on possible mechanisms of association. Both CAP and RNA polymerase have pairs of binding determinants that interact during the formation of transcription-activation complexes at class I and II promoters (see above). These determinants are logical candidates for the sites of protein-protein interaction in the absence of DNA. However, ‘open’ polymerization reactions that are theoretically possible on the basis of this polyvalency (Fig. 8A), are not compatible with the absence of 1:1 and 2:1 complexes or the limiting 2:2 stoichiometry that is observed. Alternative models that use pairs of binding surfaces...
and are compatible with a 2:2 limiting stoichiometry are shown schematically in Fig. 8B. In these models, surfaces on each CAP monomer (possibly activating region 1 or 2 (25,50)) interact with complementary surfaces on the alpha subunit of RNA polymerase. We envision two arrangements, one in which each CAP dimer binds the pair of alpha subunits on a single polymerase monomer (a ‘cis’ configuration) and one in which each CAP dimer bridges between alpha subunits on two polymerase monomers (a ‘trans’ configuration). We currently favor the trans configuration because cross-bridging between polymerase monomers has the potential to stabilize a polymerase dimer, and dimer stabilization by the first CAP molecule has the potential to account for the cooperative binding of a second CAP molecule. These effects are not available in the cis configuration.

Our data allow us to obtain a lower limit estimate of the formation constant for the CAP-polymerase complex, $K_a \geq 2 \times 10^{20}$ M$^{-3}$. Several attempts to measure the stability of CAP-polymerase complexes predate this report, but all have depended on the assumption of a 1:1 stoichiometry and thus yield estimates of a “monomer-equivalent” association constant ($K_{\text{apparent,1:1}}$). In 1980, Blazy and co-workers used non-equilibrium sucrose-gradient centrifugation to obtain $K_{\text{apparent,1:1}} \sim 3.3 \times 10^5$ M$^{-1}$ in the presence of cAMP (14). Parallel experiments carried out in the absence of cAMP failed to detect binding, although this may be ascribable to the non-
equilibrium nature of their assay. Later, Pinkney and Hoggett used fluorescence polarization measurements with fluorescein-labeled CAP to obtain a monomer-equivalent estimate $K_{\text{apparent,}1:1}$ \( \sim 3.3 \times 10^5 \text{ M}^{-1} \) in the presence of cAMP (15). Most recently, Heyduk and colleagues used the fluorescence polarization of a fluorescein-labeled 42 bp CAP binding site DNA to monitor the formation of a DNA•CAP•RNA polymerase ternary complex, from which they obtained the estimate $K_{\text{apparent,}1:1:1} \sim 3.5 \times 10^6 \text{ M}^{-1}$ in the presence of cAMP (16). Due to the presence of DNA, it is not clear that the latter interaction follows the same mechanism as the one described here. However, assuming that all studies characterized the same interaction, and assuming equal CAP and RNA polymerase concentrations, the previously-reported $K_{\text{apparent,}1:1}$ values predict formation constants for the 2:2 complex in the range $3.6 \times 10^{16} \text{ M}^{-3} \leq K_a \leq 4.3 \times 10^{19} \text{ M}^{-3}$. Given the differences in solution conditions and possible differences in the activities of protein preparations, these values are in reasonable agreement with our lower-limit estimate of the formation constant.

The in vitro self-association of RNA polymerase has been studied intermittently since 1966 (26-28,41,42,51), with later studies gaining impetus from the suggestion (29) that dimerization might play a transcription-regulatory role by influencing the distribution of RNA polymerase between competing promoters. Here we have shown that CAP binds preferentially to the dimeric form of polymerase holoenzyme. This implies that CAP stabilizes the polymerase dimer, although this conclusion comes with a caveat, since the sedimentation equilibrium data provide no information about the arrangement of molecules within the CAP-polymerase
complex. Thus we cannot yet say whether the polymerase-interactions that form the dimer in free solution are identical to those present when CAP is bound. Similarly, we are not yet in a position to say whether CAP-polymerase complexes of the kind investigated here form \textit{in vivo}. None-the-less, these results open to future investigation the intriguing possibility that CAP might regulate functions of the RNA polymerase holoenzyme that take place prior to promoter binding.
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Figure Legends

Figure 1. *Sedimentation equilibrium analyses of RNA polymerase holoenzyme.* Representative data obtained at 4°C in buffers consisting of 40 mM Tris (pH 7.9), 10 mM MgCl₂, 0.1 mM DTT, 5% glycerol and 300 mM KCl (curve A) or 100 mM KCl (curve B). Sample A was centrifuged at 9,000 rpm; the data are offset by 0.1 absorbance unit for clarity. The smooth curve is the global fit of the ideal single-species model (Eq. 1 with a single term) as described in *Experimental Procedures.* The value of $M_r$ returned by this analysis was 454,000 ± 6,000. Sample B was centrifuged at 7,000 rpm. The smooth curve is the global fit of the monomer-dimer model (Eq. 2), with monomer molecular weight set at 454,000 as described in *Experimental Procedures.* In both cases the curve-fitting residuals (upper panels) are small and lack obvious systematic dependence on radial position, demonstrating that the corresponding models are consistent with the mass distributions present in these samples.

Figure 2. *Sedimentation equilibrium analyses of CAP protein.* Representative data obtained at 4°C in buffers consisting of 40 mM Tris (pH 7.9), 10 mM MgCl₂, 0.1 mM DTT, 5% glycerol and 300 mM KCl (curve A) or 100 mM KCl (curve B). Both samples were centrifuged at 19,000 rpm; the data of curve A are offset by 0.1 absorbance unit for clarity. The smooth curves are global fits of the ideal single-species model (Eq. 1 with a single term) as described in *Experimental Procedures.* The values of $M_r$ returned by this analysis were: 48,400 ± 1,400 for
CAP in 300 mM KCl (curve A) and 47,800 ± 1,400 for CAP in 100 mM KCl (curve B). The curve-fitting residuals are shown in the upper panels.

Figure 3. CAP and RNAP interact in the absence of DNA. Representative data obtained under conditions of RNA polymerase excess (curve A) and CAP excess (curve B). In both cases, the binding buffer contained 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol and 100 mM KCl; both data sets were obtained at 4°C and 5,000 rpm. Bottom panel: sample A contained 2.7 × 10$^{-7}$ M CAP and 4.5 × 10$^{-7}$ M RNA polymerase. The smooth curve is the fit to the two-species model (free RNA polymerase monomer plus a CAP-polymerase complex) to the data set. The molecular weight of the complex returned by this analysis was 1,090,000 ± 43,000.

Sample B contained 3.6 × 10$^{-7}$ M CAP and 3.2 × 10$^{-7}$ M RNA polymerase. The smooth curve is the fit to the two-species model (free CAP plus CAP-polymerase complex) to the data set. The molecular weight of the complex returned by this analysis was 1,020,000 ± 57,400. The fitting residuals are shown in the upper panels.

Figure 4. Global dependence of $\chi^2$ on the molecular weight of the CAP-polymerase complex. Each curve represents the analysis of 9 data sets, corresponding to 3 protein concentrations and 3 rotor speeds. (○) data obtained under conditions of RNA polymerase molar excess; (●) data obtained under conditions of CAP molar excess, offset by +0.01 absorbance unit for clarity. Each data set was fit using the version of Eq. 1 corresponding to the appropriate model (free CAP + complex in samples with excess CAP, free RNA polymerase monomer + complex in
samples with excess polymerase), but with the molecular weight of the complex held constant. The sum of $\chi^2$ values from these fits is a measure of the deviation of the model from the data ensemble (39,40). Varying the input value of the molecular weight of the complex yields a distribution of summed $\chi^2$ values; the minimum value is the global least-squares “best” estimate of the molecular weight. Both $\chi^2$ distributions are minimized near $M_w(\text{complex}) = 1 \times 10^6$.

Figure 5. Continuous variation (Job) plot showing that the optimal combining ratio is 1:1. Lower panel: dependence of normalized [complex] ($\pm$ 95% confidence limits) on mole fraction of CAP. The total protein concentration was fixed ([CAP] + [RNA polymerase] = $7.2 \times 10^{-7}$M), but the mole fraction was allowed to vary as indicated. The binding buffer was 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol, 100 mM KCl. Individual data sets were fit using 2-species models; under conditions of CAP molar excess, the model corresponded to free CAP plus a complex, under conditions of RNA polymerase excess, the model corresponded to free polymerase monomer plus a complex. Upper panel: the molecular weights ($\pm$ 95% confidence limits) returned by these analyses. The horizontal line indicates the molecular weight expected for a 2:2 CAP:polymerase complex ($M_c = 1.00 \times 10^6$).

Figure 6. CAP and RNA polymerase holoenzyme form 2:2 complexes in solutions containing 300 mM potassium chloride and 300 mM potassium glutamate. Representative data obtained at 7,000 rpm and 4°C in buffers containing 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol and 300 mM KCl (curve A) or 300 mM K-glutamate (curve B). Bottom panel: sample
A contained $4.9 \times 10^{-7}$ M CAP and $3.3 \times 10^{-7}$ M RNA polymerase. The smooth curve is the fit to the two-species model (free CAP plus a CAP-polymerase complex) to the data set. The molecular weight of the complex returned by this analysis was $1,010,000 \pm 47,000$. Sample B contained $3.2 \times 10^{-7}$ M CAP and $3.8 \times 10^{-7}$ M RNA polymerase. The smooth curve is the fit to the two-species model (free RNA polymerase monomer plus CAP-polymerase complex) to the data set. The molecular weight of the complex returned by this analysis was $1,030,000 \pm 26,000$. The small, symmetrical residuals (upper panels) attest to the compatibility of these models with the data.

Figure 7. Effects of cAMP and elevated temperature on binding. Curve A: binding in the presence of 20µM cAMP. Representative data obtained at 4°C in 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol, 100 mM KCl, 20µM cAMP. The sample contained $3.7 \times 10^{-7}$ M CAP and $3.8 \times 10^{-7}$ M RNA polymerase; the data were fit by the 2-species model that includes RNA polymerase monomer plus a CAP-polymerase complex. The molecular weight returned for the complex was $1,070,000 \pm 48,000$. Curve B: data obtained at 20°C in 40 mM Tris (pH 7.9), 10 mM MgCl$_2$, 0.1 mM DTT, 5% glycerol, 100 mM KCl buffer. The sample contained $3.7 \times 10^{-7}$ M CAP and $4.5 \times 10^{-7}$ M RNA polymerase; the data were fit by the 2-species model that includes RNA polymerase monomer plus a CAP-polymerase complex. The molecular weight returned for the complex was $1,100,000 \pm 37,000$. 

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Figure 8. Schematic models of CAP-RNA polymerase association.  A. A hypothetical ‘open’ polymerization mechanism that is compatible with the polyvalent characters of CAP and RNA polymerase. RNA polymerase (magenta) and CAP (blue) assemble in alternation. The carboxy-terminal domains of RNA polymerase alpha subunits are labeled ($\alpha$). This pattern allows 1:1, 1:2 and 2:1 stoichiometries as well as stoichiometries greater than 2:2. The data presented in this paper argue against mechanisms of this kind. B. Hypothetical 2:2 complexes in which CAP dimers (blue) bridge between adjacent alpha subunits ($\alpha$). Left and center drawings represent the \textit{trans} configuration, in which CAP bridges between alpha subunits of different RNA polymerase monomers. The right drawing represents a \textit{cis} configuration in which CAP bridges between alpha subunits of the same polymerase monomer.
Figure 2

Residuals

A

B

A

B

[Graph showing residuals and A_280 values against (r - r_0)]
Figure 4
Figure 8
The Escherichia coli cyclic AMP receptor protein forms a 2:2 complex with RNA polymerase holoenzyme, in vitro

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