radicals, respectively. This mechanism of drug and reduced oxygen free radical generation could explain the tumoricidal mechanism of the drug. The absence of an obligatory requirement for metal or enzyme activation could result in a unique spectrum of antitumor activity.

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Role of a Second Catabolite Activator Protein Molecule in Controlling Initiation of Transcription at the Galactose Operon of *Escherichia coli*†

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ABSTRACT: The molecular mechanisms whereby RNA polymerase, catabolite activator protein (CAP), and cyclic AMP (cAMP) participate in transcriptional regulation at the galactose operon have been probed by a variety of in vitro techniques. Interactions between purified proteins and promoter-containing DNA fragments were assayed by gel electrophoresis, by resistance to restriction endonuclease digestion, and by monitoring runoff transcripts. The data bear on the multiple functions that CAP performs in *gal* control. A CAP–cAMP complex can exclude RNA polymerase from one of the two overlapping promoter regions (P2), thereby targeting the enzyme to the other (P1); this process is markedly influenced by the cAMP level. In addition, a second CAP molecule is involved in a cooperative process, which, at low cAMP, is required for efficient formation of transcriptionally competent complexes at P1. This second CAP may serve to stabilize the 1:1:1 CAP–polymerase–*gal* DNA intermediate under physiological conditions, thus enhancing initiation from P1 relative to P2. Kinetic analysis reveals that the modest effect of CAP on the rate of P1 open complex formation can be resolved into about a 4-fold increase in the binding of RNA polymerase to the P1 region, plus a 1.5-fold elevation in the rate of isomerization of enzyme–promoter complexes to the open state.

Regulation of transcription at the *Escherichia coli* galactose operon is a complex process involving RNA polymerase, the catabolite activator protein (CAP) and its effector cAMP,[1] and the *gal* repressor (Adhya & Miller, 1979). In addition, transcription can occur from either of two overlapping promoter sites (Musso et al., 1977). In the absence of CAP–cAMP, RNA polymerase binds to the P2 promoter and initiates at nucleotide −5. When the intracellular concentration of cAMP rises, CAP activates P1 and enhanced levels of transcription occur from +1 start site. The binding of CAP at its primary site (−35 to −50) (Taniguchi et al., 1979; Busby et al., 1982) can, in principle, play two roles in stimulating

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[1] Abbreviations: CAP, catabolite activator protein; cAMP, adenosine cyclic 3′,5′-phosphate; bp, base pair; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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initiation from P1. It may competitively inhibit RNA polymerase from binding at P2, thus channeling more enzyme molecules to P1, and it may directly increase the ability of polymerase to begin synthesis at P1. These two roles can be functionally separated. The *gal* P-11 mutant cannot initiate at P1 due to a transversion at −11 (Musso et al., 1977); however, under certain conditions CAP is able to bind at its primary site and can inhibit P2 transcription. CAP also appears to function in a positive manner by increasing the actual number of P1 transcripts relative to the basal P2 level (Sklar et al., 1977).

Both P1 and P2 are used in vivo where they respond to CAP–cAMP just as they do in vitro (Aiba et al., 1981). It has also been shown that *two* CAP molecules are involved in controlling transcription at the *gal* operon (Shanblatt & Revzin, 1983). In the presence of RNA polymerase these CAP molecules are adjacent on the DNA, one at the −35 to −50 region, the other covering base pairs −50 to −66. The sequence of events at the *gal* control region, in the presence of cAMP (and the absence of repressor), is that a single CAP–cAMP complex binds at the primary site and then RNA polymerase binds to the P1 promoter followed by the second CAP molecule upstream of the first. In this paper we report in vitro studies of the interactions of CAP, cAMP, and RNA polymerase with DNA fragments containing the wild-type or P-11 mutant *gal* promoter region, in order to elucidate the molecular mechanisms whereby two CAP molecules may increase the number of productive initiation events from the P1 promoter over the amount synthesized from P2 in their absence.

Experimental Procedures

gal Promoter Fragments. All fragments are numbered relative to the P1 mRNA start site designated +1. DNA fragments 1100 bp long (−650 to +450) containing the wild type or P-11 mutant *gal* promoter were purified from plasmids pBdCl and pBdC5 (DiLauro et al., 1979), respectively, which were generously provided by Sankar Adhya. The "−90" fragment is 135 bp long and extends from a *Cfo*I site at −90 to a *Hpa*II site at +45. The 105-bp "−60" fragment covers a region from the *Hin*fI site at −60 to +45. In addition, 105-bp fragments in which the *Hin*f site at −60 was either made blunt or changed to an *Eco*R1 site were also used in binding and transcription experiments; results were quantitatively the same with all three fragments.

Restriction fragments were purified by separation in polyacrylamide gels followed by electroelution and chromatography through DEAE-Sephadex (Maniatis et al., 1982). DNA fragments used in binding gels and *Hinf* protection experiments were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase following treatment with calf intestinal phosphatase. Concentrations of DNA were determined spectrophotometrically with ϵ_{260} = 13 000 M^{-1} cm^{-1} (Felsenfeld & Hirschman, 1965) and are expressed as molar in promoter fragments.

Proteins. RNA polymerase (Burgess & Jendrisak, 1975; Lowe et al., 1979) and CAP (Boone & Wilcox, 1978) were prepared as previously described (Garner & Revzin, 1981). The polymerase was 45% active in specific binding to the *lac* L8-UV5 promoter when assayed by gel binding and about 30% active when assayed by the method of Chamberlin et al. (1979). On the basis of the amount required to fully stimulate *lac* P1 transcription in vitro, CAP was estimated to be 25% active. Protein concentrations were determined spectrophotometrically [see Garner and Revzin (1981)]. *Active* CAP and RNA polymerase concentrations are reported in the text.

Binding Reactions. All complexes were formed in a buffer that contained 5 nM DNA fragment in 40 mM Tris-HCl (pH 7.9 at 22 °C), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1% glycerol, and the indicated cAMP concentration. Where indicated, CAP was added to 10–50 nM and the reactions were incubated for 10 min at 37 °C, followed by RNA polymerase addition to 25 nM and an additional 30-min incubation at 37 °C. These times are sufficient for equilibrium to be reached. Reaction volumes were 25 μL.

CAP–DNA Binding Gels. CAP–DNA complexes were formed as described above by using radiolabeled "−90" or "−60" fragments and 12.5, 25, or 50 nM CAP and 5 μM cAMP. Following a 10-min incubation at 37°C, $^1/_{10}$ volume of 25% Ficoll was added and the reactions were immediately loaded onto a 0.1 × 10 cm vertical 5% polyacrylamide gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA). The gel and reservoir buffer contained 5 μM cAMP. Gels were electrophoresed at 15 V/cm for 45 min, dried onto Whatman 3MM paper and autoradiographed with Kodak XAR-5 film.

Hinf Protection Experiments. Binding reactions were performed with 5 nM end-labeled 1100-bp promoter fragment, 5 μM cAMP, and various amounts of CAP. Following the 30-min incubation with 25 nM RNA polymerase, heparin was added to 80 μg/mL. (The heparin removes nonspecifically bound enzyme and prevents reinitiation from occurring in the transcription assays. Heparin was not added to reactions containing CAP only.) One minute later 25 units of *Hin*fI was added and the digests were incubated for 30 min. They were then extracted with phenol–chloroform (1:1) and then ether and loaded onto a 5% polyacrylamide gel in TBE. Gels were dried and autoradiographed. The extent of protection was quantified by cutting out and counting the (A + B) and A bands (see Figure 5) from each lane.

Runoff Transcription. Restriction fragments (5 nM) extending from −90 to +45 or from −60 to +45 were used as templates, with 25 nM RNA polymerase, 25 nM CAP, and the indicated cAMP concentration. Excess glycerol was not added to stimulate P2 transcription in the absence of cAMP–CAP (Musso et al., 1977). Following formation of CAP–RNA polymerase–DNA complexes, $^1/_{10}$ reaction volume of a mix containing heparin and nucleoside triphosphates in binding buffer was added. Final concentrations were 80 μg/mL heparin, 200 μM each ATP, GTP, and CTP, and 50 μM [α-^{32}P]UTP (10 μCi; 3000 Ci/mmol). After a 5-min incubation, reactions were stopped and the RNA was extracted with phenol–chloroform (1:1), followed by ethanol precipitation. Products were suspended in 90% formamide in 1× TBE, heated at 90 °C for 3 min, and quick chilled on ice. They were loaded onto a 0.04 × 20 cm 8% polyacrylamide, 7 M urea gel in TBE (Maxam & Gilbert, 1980) and electrophoresed until the bromphenol blue reached the bottom of the gel. Size markers are ^{32}P-end-labeled *Hpa*II fragments of pBR322. Gels were dried and autoradiographed by using Kodak XAR-5 film.

Kinetic Studies. The rate of open complex formation was assayed by measuring the number of transcripts synthesized from a single round of initiation. Complexes were formed in binding buffer with 20 nM DNA fragment (235 bp; −90 to +145) and 20 μM cAMP in the presence or absence of 125 nM CAP at 30 °C. At time zero, prewarmed RNA polymerase was added. At various subsequent times aliquots were removed and mixed with heparin and the four nucleoside triphosphates as described above. Following a 20-min incubation, reactions were stopped with an equal volume of 10 M urea and, after heating to 90 °C, were loaded onto an 8% polyacrylamide gel containing 7 M urea as described above.

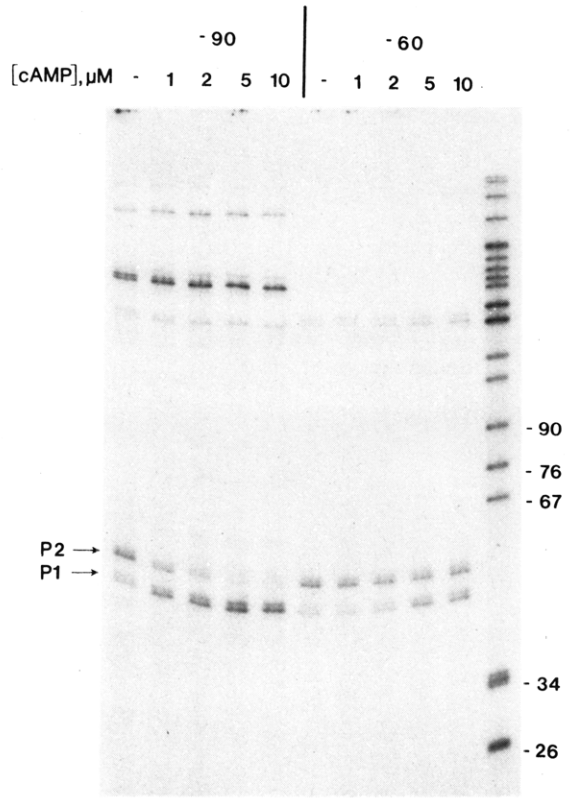

FIGURE 1: Runoff transcripts synthesized from wild-type −90 to +45 or −60 to +45 *gal* promoter fragments at varying cAMP concentrations. Complexes were formed by the sequential addition of CAP and polymerase at the indicated cAMP levels. RNA synthesis was then performed in the presence of heparin. Reaction products were extracted, precipitated, and fractionated on an 8% polyacrylamide, 7 M urea gel in TBE. Size markers are *Hpa*II fragments of pBR322; numbers at right refer to length, in bases, of some of the standards. The P2 runoff transcript is 5 nucleotides longer than that from P1.

Gels were dried and autoradiographed, and the band corresponding to the P1 runoff transcript was excised and counted. The longer fragment and higher DNA concentration were needed to ensure that the number of counts per transcript was adequate for accurate analysis. It is not possible to achieve this by lowering the UTP below 25 μM (hence increasing its specific activity), since low concentrations of UTP result in the synthesis of short RNAs (9 bp) rather than the full-length transcript (Musso et al., 1977). Reactions were performed at 30 °C instead of 37 °C in order to slow down the rate and make the sampling times more manageable. The kinetics of heparin-resistant complex formation were also measured on binding gels by mixing DNA and protein, quenching the reaction at various times by adding heparin, and then electrophoresing the DNA–protein complexes (no nucleotides added) through a 5% polyacrylamide gel in TBE buffer followed by quantification of the amount of labeled DNA in the free and complex bands.

RESULTS

Transcription from gal DNA Promoter Fragments. Whether CAP can actually stimulate initiation at P1 as well as occlude RNA polymerase from P2 is best studied by doing transcription experiments. Analysis of runoff transcripts synthesized from either −90 or −60 to +45 *gal* promoter DNA fragments shows markedly different responses to changes in cAMP concentrations for the two fragments (Figure 1). At 2 μM cAMP, in the presence of both CAP and RNA polymerase, most of the transcripts from the −90 fragment initiate at P1, while the transcripts from the −60 fragment remain

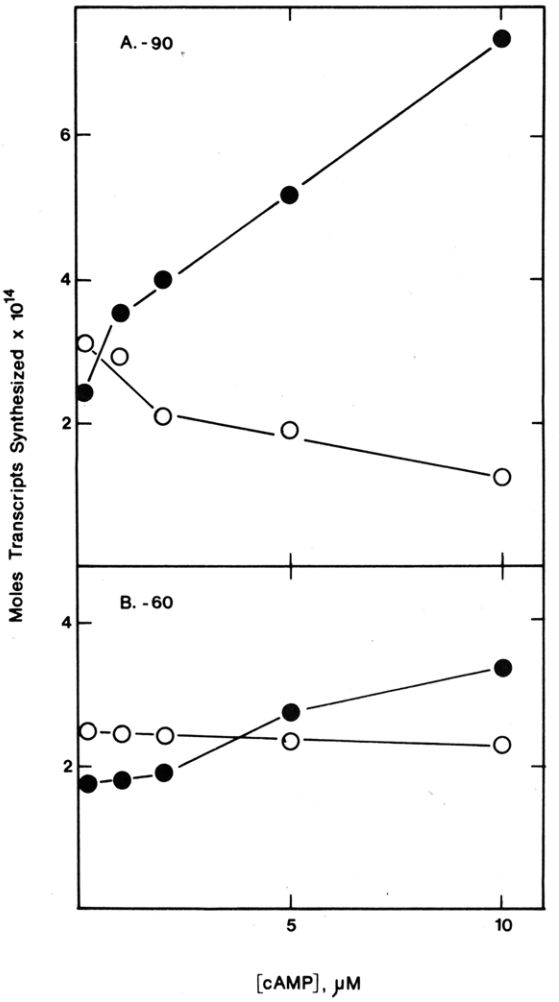

FIGURE 2: Quantitation of transcripts visualized on the gel in Figure 1. The regions of the gel corresponding to the P1 and P2 transcripts were excised and counted. Data are expressed as the number of moles of either P1 (closed circles) or P2 (open circles) transcripts synthesized in each reaction, taking into account the differing number of uridine residues in each transcript. (A) cAMP titration of the −90 to +45 fragment corresponding to the left 5 lanes of the gel. (B) Same titration of the −60 to +45 fragment, corresponding to the right 5 lanes of the gel in Figure 1.

equally distributed between the two promoters. This is clearly illustrated in Figure 2, in which the amounts of P2 and P1 transcript have been quantified. It is possible to inhibit P2 transcription somewhat and still obtain transcripts from P1 on the −60 fragment at elevated cAMP (above 20 μM), though little CAP–cAMP stimulation of P1 transcription over the P2 level occurs from the −60 template (data not shown).

The observation that more cAMP is required to inhibit P2 on the −60 fragment than on the −90 fragment is substantiated by CAP titrations at a given cAMP concentration of 20 μM. The data in Figure 3 were obtained by quantifying the P1 and P2 transcripts following resolution by gel electrophoresis, similarly to Figures 1 and 2. More than twice as much CAP is required to see any effect on the relative amounts of P1 and P2 transcripts from the −60 fragment compared to the −90 fragment. Thus high cAMP or CAP concentrations can mimic the effect of the second CAP, allowing transcription from P1 on a −60 fragment, which can accommodate the binding of only one CAP molecule.

Binding of CAP to −90 and −60 Fragments. One possible explanation for the previous results is that a CAP molecule binds more tightly to the −90 fragment than to the −60 so that the CAP–cAMP complex is better able to compete with RNA

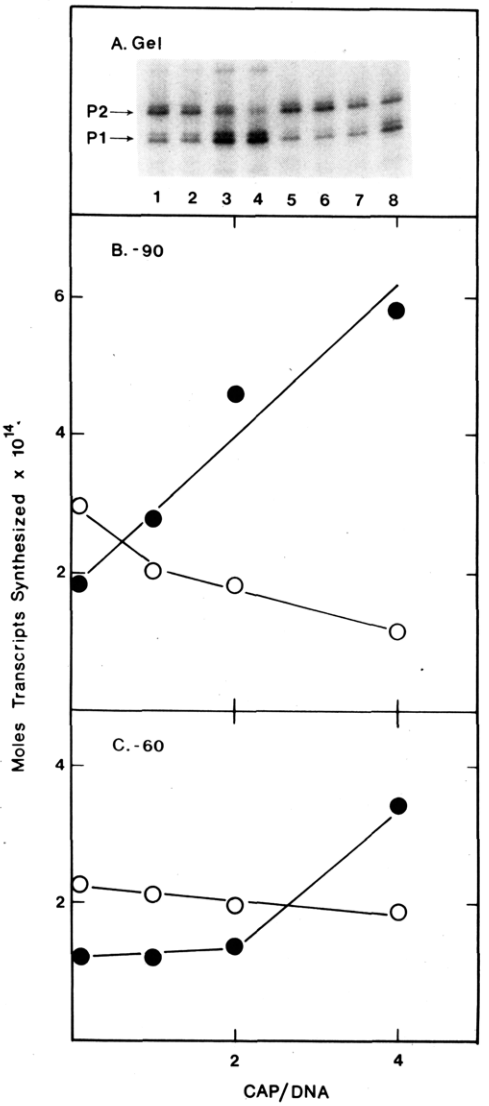

FIGURE 3: Effect of increasing CAP concentration on relative amounts of P1 and P2 transcripts synthesized. Reactions were done as described in Figure 1, except that the cAMP concentration was maintained at 20 μM while the amount of CAP added was varied. (A) Runoff transcripts synthesized from the wild-type −90 to +45 (lanes 1–4) or −60 to +45 (lanes 5–8) *gal* promoter DNA: lanes 1 and 5, no CAP; 2 and 6, 5 nM CAP; 3 and 7, 10 nM CAP; 4 and 8, 20 nM CAP. P1 (closed circles) and P2 (open circles) transcripts were quantified as described in the legend to Figure 2. (B) CAP titration on the −90 to +45 restriction fragment. (C) Same titration on the −60 to +45 fragment.

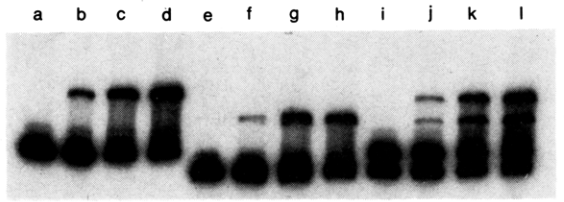

FIGURE 4: CAP binding to *gal* DNA fragments. The 135-bp "−90" fragment (lanes a–d and i–l) or the 105-bp "−60" DNA (lanes e–l) was used as substrate. Lanes a–h contained the indicated fragment at 5 nM, while in lanes i–l each fragment was present at 2.5 nM. [^{32}P]DNA was incubated without (a, e, and i) or with 12.5 (b, f, and j), 25 (c, g, and k), or 50 nM CAP (d, h, and l) and 5 μM cAMP. Binding mixtures were electrophoresed through a 5% polyacrylamide gel in TBE containing 5 μM cAMP. DNA and CAP–DNA complexes were visualized by autoradiography.

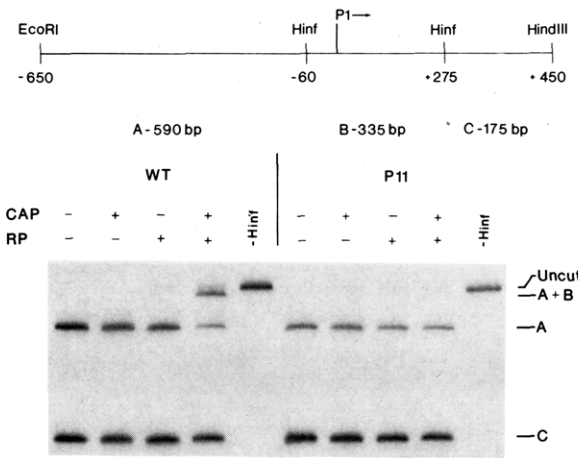

FIGURE 5: Protection of the −60 *Hinf* site by CAP and RNA polymerase. (Upper) Schematic diagram of the 1100-bp *gal* promoter DNA fragment showing the two restriction sites and sizes of the three fragments resulting from *Hinf* digestion. The CAP-dependent P1 initiation site is at +1. (Lower) Autoradiogram of polyacrylamide gel showing the 1100-bp *gal* DNA *Hinf* digestion products produced in the presence of CAP–cAMP, RNA polymerase, or both. The wild-type and P-11 fragments, 5′-end-labeled, were used as substrates for binding and subsequent digestion. DNA was present at 5 nM, CAP and RNA polymerase were present at 25 nM, and cAMP was present at 5 μM. Uncut DNA ("*–Hinf*" lanes) is the 1100-bp starting material and the (A + B) band is the fragment resulting from protection of the −60 site. The internal, unlabeled B fragment is not visualized on the autoradiogram.

polymerase binding to P2 at lower CAP and cAMP concentrations on the longer fragment. The gel binding assay is a convenient way to measure the relative affinities for CAP of two DNAs of different lengths (Garner & Revzin, 1981; Fried & Crothers, 1981; Kolb et al., 1983a). Although CAP binds much less tightly to *gal* promoter DNA than to *lac*, binding at relatively low cAMP concentrations and CAP/*gal* promoter molar ratios can be demonstrated by this technique (Kolb et al., 1983b). At 5 μM cAMP some CAP–*gal* DNA complexes dissociate during the electrophoresis run so that the complex band may not be a true measure of the number of complexes present at the beginning of the run.

Figure 4 represents a gel in which preformed CAP–*gal* DNA complexes were electrophoresed to separate the complexes from unbound DNA. ^{32}P-End-labeled DNA restriction fragments extending from either −90 or −60 to +45 were used in these experiments; the primary CAP binding site covers the region from −35 to −50. Figure 4 shows that both DNAs form stable complexes with CAP. Furthermore, they bind CAP with equivalent affinities as judged by the fact that the same percentage of input DNA is found in CAP–DNA complexes with the two fragments. In addition the −60 fragment containing the mutant P-11 promoter binds to CAP as well as the wild-type DNAs (data not shown). This is not unexpected since the mutation is at −11, well separated from the CAP binding site. While the wild-type −90 and −60 fragments show marked differences in their ability to switch from P2- to P1-promoted transcription at a given cAMP or CAP concentration, this is not due a difference in the binding affinity of the first CAP molecule to its primary site at −35 to −50.

Protection of Wild Type but Not P-11 Hinf Site. Since DNAse I and exonuclease III protection data imply that the second CAP spans the −50 to −66 region (Shanblatt & Revzin, 1983), protection from *Hinf* digestion at −60 is a convenient assay for the presence of this CAP molecule. The 1100-bp *gal* promoter fragment (−650 to +450) contains two *Hinf* sites, one at −60 and the other at +275. Complete digestion of the fragment, therefore, yields three smaller pieces of lengths 590 (A), 335 (B), and 175 bp (C) as indicated in Figure 5. Selective protection at −60 (the A–B junction) yields a new,

larger fragment of 925 bp with concomitant loss of the individual A and B fragments. The distal C fragment, completely within the *gal*E gene, provides a positive control for digestion since its appearance should be unaffected by the presence of CAP and RNA polymerase. The *Hinf* B fragment is not visualized on an autoradiogram since it is an internal fragment of the end-labeled 1100-bp DNA and is therefore not radioactive.

Protection from *Hinf* digestion at −60 at the wild-type *gal* promoter occurs only when both CAP and RNA polymerase are bound, as shown in Figure 5. Neither protein alone affords protection from digestion; even at high cAMP and protein concentrations, CAP by itself does not protect the −60 *Hinf* site (data not shown). However, when CAP and then RNA polymerase are added to the DNA, subsequent treatment with *Hinf* yields the (A + B) partial fragment, indicative of a second CAP molecule bound upstream of the first. This complex of *gal* DNA with RNA polymerase and two CAPs in the presence of heparin has a much longer half-life than the 30-min *Hinf* incubation time so that very little dissociation occurs during the digestion period.

The necessity for RNA polymerase to be bound stably at P1 in order for the second CAP to bind is illustrated by studies using the P-11 mutant promoter. The mutation at −11 results in RNA polymerase being unable to initiate transcription from P1; we find no heparin-insensitive complexes at this promoter on the mutant DNA. One CAP molecule can bind to the P-11 promoter region, and this binding can be stable enough to prevent open complex formation at P2 (Spassky et al., 1984; S. Shanblatt, data not shown). Nevertheless, from Figure 5 it is evident that no protection at −60 occurs on the mutant DNA fragment, even if both CAP–cAMP and RNA polymerase are present. This result has been obtained at both low and high cAMP concentrations.

CAP "Redistribution" Experiments. Strong interaction of the second CAP molecule at the wild-type *gal* promoter was further confirmed by an experiment designed to emphasize that this binding is cooperative and therefore involves a stabilization of RNA polymerase bound at P1. This consisted of incubating various amounts of CAP with the 1100-bp DNA fragment, then adding RNA polymerase, and determining the presence of a second CAP molecule by the degree of protection from *Hinf* digestion at −60. Results of a series of such experiments are shown in Figure 6. Protection of end-labeled DNA was quantified from the radioactivity in the (A + B) band relative to the A band. The solid line in Figure 6 (lower) represents the actual data obtained from the autoradiogram shown there. Protection is observed even at input CAP/promoter ratios of less than unity, indicating that a redistribution of CAP molecules occurs when RNA polymerase is added. (This conclusion derives from the fact that in the absence of polymerase, any specific CAP–DNA complex will have a single CAP molecule bound at the primary CAP site. If there is less than one CAP per promoter fragment in the solution, every active CAP molecule will be in complex with DNA at the moment of RNA polymerase addition.) The alternative possibility, that none of the second CAP sites are filled until all of the primary sites are occupied, is depicted by the dashed line in Figure 6. It is clear that the data are inconsistent with this latter notion, which serves to stress the cooperative nature of the binding of the second CAP to the 1:1:1 CAP–RNA polymerase–*gal* promoter DNA intermediate.

Effect of CAP on the Rate of P1 Open Complex Formation. One way to assess the function of a transcriptional activator is to examine its effect on the kinetics of RNA polymerase

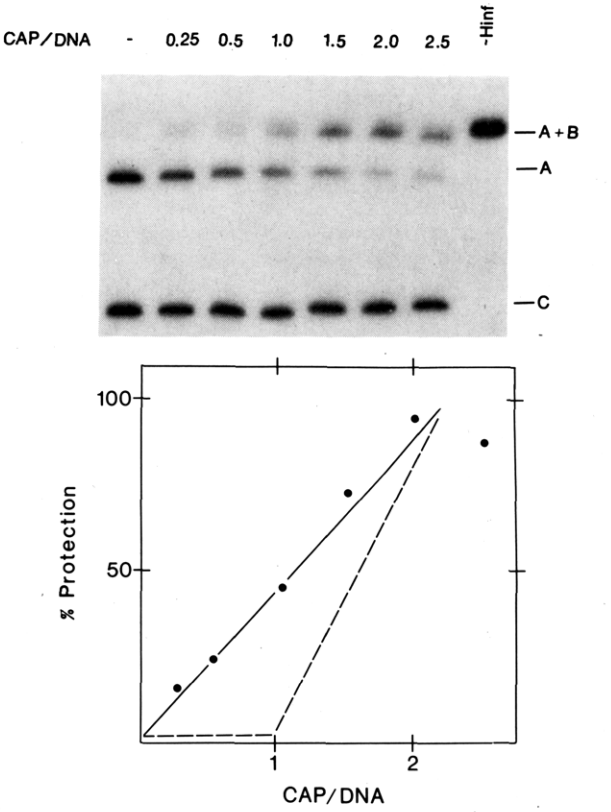

FIGURE 6: Binding of the second CAP quantified by protection from *Hinf* digestion. (Upper) Autoradiogram showing the *Hinf* digestion products produced in the presence of RNA polymerase and varying amounts of CAP. End-labeled 1100-bp wild-type *gal* DNA was incubated with the indicated amount of CAP at 5 μM cAMP. Subsequently RNA polymerase, then heparin, and the *Hinf* enzyme were added. Following protein removal the DNA products were separated on a 5% polyacrylamide gel. (Lower) Plot of percent protection at the −60 site vs. the input CAP/DNA molar ratio. The percent protection was quantified from the radioactivity in the (A + B) and A bands; maximal protection was normalized to 100%. The solid line shows the actual data. The dashed line depicts the result that would have been obtained if the binding of the second CAP molecule were not cooperative (see text).

open complex formation. The TAU analysis of McClure (McClure, 1980) permits one to dissect the overall process into two steps, namely, the initial binding of the enzyme to form a closed complex (K_B) followed by the irreversible isomerization of a closed to an open complex (k_2). This species is characterized by resistance to heparin and is transcriptionally competent. We have used the amount of RNA synthesized from one round of transcription (i.e., in the presence of heparin) as a measure of the number of open complexes present at a given time. It is convenient to follow open complex formation by assaying transcripts, since this permits separation of any contribution of P1 from that of P2. In this regard it is noteworthy that a significant number of P1 transcripts are synthesized even when no CAP–cAMP is present (Figure 1, lane 1). The rates of open complex formation on the −90 fragment, determined at various excess RNA polymerase concentrations in the absence or presence of CAP, were then used to construct the TAU plot shown in Figure 7. At a given polymerase concentration, the kinetics results were first plotted as ln $(1 - F)$ vs. time (where F = fraction of transcripts at time t, relative to the level at 30 min, at which point the reaction is virtually complete). The slope of this line is $-1/\tau_{obsd}$, as described by McClure (1980), where $\tau_{obsd} = 1/K_B[R]k_2 + 1/k_2$. From a plot of τ_{obsd} vs. $1/[R]$, K_B is then calculated as intercept/slope and k_2 is 1/intercept, where [R] is the total

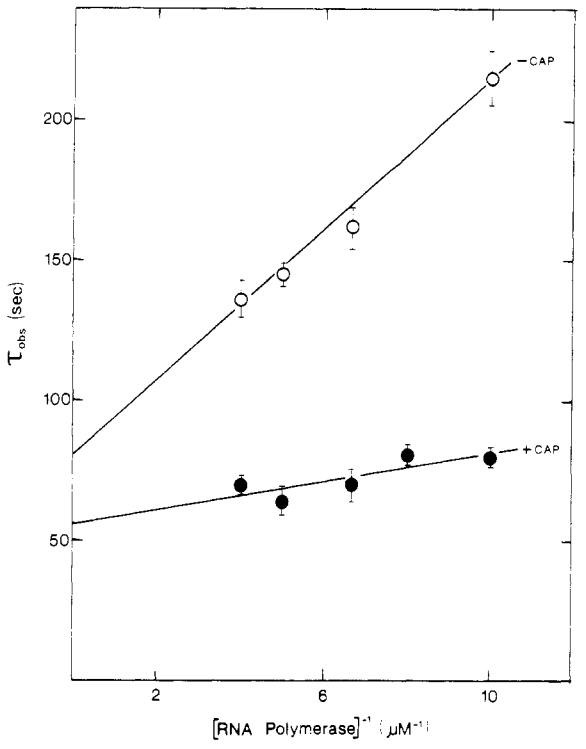

FIGURE 7: TAU plot analysis of kinetics of *gal* P1 promoter open complex formation. The rate of complex formation was assayed in the absence (open circles) or presence (closed circles) of CAP–cAMP at the indicated RNA polymerase concentrations, by measuring one round of runoff transcription, as described under Experimental Procedures, from the −90 to +145 template. The data are from two separate experiments; the lines are linear least-squares fits of the data.

Table I: Kinetic Parameters Describing *gal* P1 Open Complex Formation

	K_B (M^{-1})	k_2 (s^{-1})
no CAP–cAMP	5.9×10^6	0.012
plus CAP–cAMP	22.0×10^6	0.018

RNA polymerase concentration.[2] The parameters K_B and k_2 extracted from this plot are presented in Table I. CAP has only a modest effect on each of these quantities, K_B increasing about 4-fold and k_2 about 1.5-fold in the presence of the activator. Similar results were obtained by using binding gels to measure the rates of formation of heparin-resistant complexes. Thus the level of transcription is indeed proportional to the degree of promoter occupancy.

Discussion

The results presented above enable us to draw a number of conclusions about the molecular mechanisms involved in stimulation of transcription at the *gal* operon. A central question has been whether the action of CAP requires primarily protein–protein interactions or is mediated mainly through conformational changes in the DNA that may help

[2] This expression for τ invokes perhaps the simplest sequence of events in open complex formation. Similar equations can be derived for more complicated mechanisms involving one or two CAP molecules, which would be relevant to the −60 or −90 fragments, respectively. In these cases, τ_{obsd} again splits into $K_{B,app}$ and $k_{2,app}$ terms, which are functions of the CAP and cAMP concentrations. Thus TAU analysis at various CAP and cAMP levels can in principle be used to probe the details of initiation at the *gal* promoter. Preliminary results, however, indicate that uncertainties in the experimental data points coupled with the relatively small differences observed with and without CAP may render such an analysis impractical at this time.

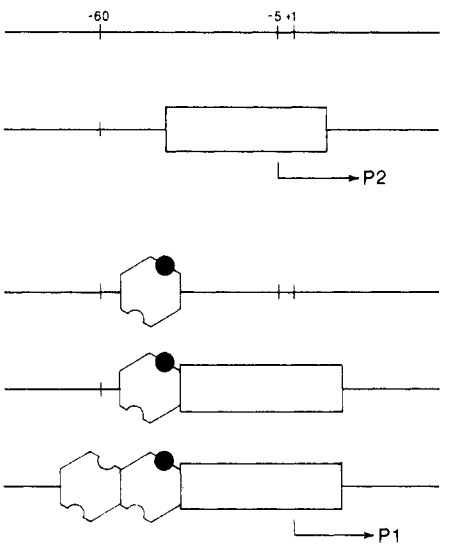

FIGURE 8: Model for CAP and RNA polymerase interactions at the *gal* promoter region. In the absence of CAP, an RNA polymerase molecule (rectangle) binds at P2. In the absence of polymerase, a single CAP (hexagon) to which one cAMP is bound will bind at its primary site. If an RNA polymerase molecule then binds at the P1 site, a second CAP molecule will also bind; no additional cAMP is needed for this step. Binding of the first CAP is competitive with RNA polymerase interactions at P2. Binding of the second CAP protects the *Hinf* site at −60 from digestion.

RNA polymerase to "melt in" and form open complexes (Gilbert, 1976). The latter possibility seems less likely due to evidence that CAP does not unwind double-helical DNA (Kolb & Buc, 1982; DeGrazia et al., 1985). While unambiguous proof for direct CAP–RNA polymerase interactions is not yet at hand, it is difficult to explain regulation at the *gal* promoter in their absence. It has been suggested, from analysis of *gal* mutants deficient in CAP binding, that polymerase bound at P1 can stabilize weakly bound CAP (Spassky et al., 1984). Thermodynamically the converse must also be true. Our data encompass a role for a second CAP molecule in the stabilization of RNA polymerase at P1, at cAMP concentrations likely to be encountered in vivo.

A model for the molecular events that occur at the *gal* operon control region is shown in Figure 8 (Shanblatt & Revzin, 1983). If RNA polymerase is incubated with DNA fragments containing the *gal* promoter, open complexes are formed at P2. [These are displaced only very slowly to P1 if CAP is then added (Spassky et al., 1984).] On the other hand, mixing CAP with *gal* promoter DNA leads to binding at the primary site (Taniguchi et al., 1979); when RNA polymerase is then added, the enzyme is directed to P1 and the resulting CAP–polymerase–promoter complexes are made stable by the binding of a second CAP molecule. It is hard to imagine how structural changes in the DNA alone could be responsible for this complicated series of events. In addition, we report here that the second CAP molecule binds *only* when RNA polymerase can melt in at P1, i.e., at the wild type but not at the P-11 mutant promoter. From this we infer that the second CAP molecule is necessary for open complex formation to occur efficiently at P1. We note also that no specific *gal* DNA sequences upstream of −60 are required for binding of the second CAP (Taniguchi & deCrombrugghe, 1983). We suggest that conformational changes in the first CAP molecule that occur as RNA polymerase binds at P1 are critical in providing an environment attractive to the second CAP. Thus direct and specific interactions between CAP and RNA polymerase are likely crucial factors in regulation at the *gal* promoter. It will be of interest to learn whether CAP faci-

litates structural changes in RNA polymerase that may be a rate-limiting step in the formation of open complexes (Roe & Record, 1985).

The presence of overlapping promoter sequences at both the *lac* (Malan & McClure, 1984; Spassky et al., 1984; Peterson & Reznikoff, 1985) and *gal* (Musso et al., 1977) operons has led to the idea that CAP could stimulate transcription from these catabolite-sensitive operons by inhibiting polymerase access to a less productive promoter (e.g., P2), thus guiding enzyme molecules to P1. Our results suggest that a critical function of CAP is indeed to exclude RNA polymerase from P2 at the *gal* operon in a process closely tied to the cAMP level. We find that exclusion from P2 occurs at high concentrations of cAMP, irrespective of whether the second CAP molecule can bind (−90 fragment) or not (−60 fragment). At 1–2 μM cAMP, open complex formation at P2 is inhibited only when two CAP molecules are able to bind (i.e., on the −90 fragment but not on the −60). Note that the redistribution experiments shown in Figure 6 indicate that P1 binding and therefore exclusion from P2 is very efficient, even at low cAMP, if both CAP molecules can bind. We have also observed that the P-11 P2 promoter is not efficiently inhibited by CAP at 2 μM cAMP, presumably because only one CAP binds. At higher cAMP concentrations this singly bound CAP is able to prevent P2 transcription by effectively competing with RNA polymerase for the overlapping binding site.

The data also support the hypothesis that the second CAP molecule functions to stabilize the 1:1:1 CAP–RNA polymerase–promoter intermediate (Figure 8). We find by using the −60 fragment (which can accommodate only one CAP) that exclusion of polymerase from P2 may occur at high cAMP but not at the 1–2 μM levels at which the bulk of the complexes are at P1 on the −90 fragment (Figure 1). Because the first CAP binds with equal affinity to the two fragments (Figure 4), the observed differences in transcriptional response to cAMP must be attributable to the binding of a second CAP molecule to the −90 fragment. Since we also find that complexes of CAP with its primary site are more stable at elevated cAMP (data not shown; Kolb et al., 1983b), we conclude the following. At high cAMP, one CAP bound to the −60 fragment can eliminate access of RNA polymerase to P2; melting in to transcriptionally competent complexes seems eventually to occur at P1, even though a second CAP molecule cannot bind. At low cAMP, however, CAP does not have a high enough affinity to exclude RNA polymerase from P2. The 1:1:1 intermediate that forms at low cAMP readily dissociates on the −60 fragment (so that P2 becomes available) but is firmly stabilized as the second CAP binds to the −90 fragment.

Does CAP have any effect on transcription other than serving to inhibit access to P2? It has been proposed that in the *lac* system CAP functions both in this manner and as an activator (Malan & McClure, 1984). These authors infer from their data that CAP increases K_B at the *lac* P1 promoter by at least an order of magnitude but that it has no effect on k_2 (Malan et al., 1984). The effect of CAP on the *gal* P1 promoter is to enhance K_B about 4-fold and k_2 about 1.5-fold. The isomerization rate of *gal* P1 in the absence of CAP is equivalent to that of the supercoiled *lac* promoter in the presence of the activator. Conversely, the fully activated *gal* P1 exhibits a K_B equivalent to that of the *lac* promoter in the absence of CAP. The overall strengths of the *lac* and *gal* P1 promoters in the absence and presence of CAP are quite similar despite the fact that the individual kinetic parameters are disparate. CAP does exert direct effects on *gal* P1 open complex formation, although they are not as pronounced as with *lac*.

The results of our studies of the P-11 mutant (Figure 5) reveal that the second CAP molecule enhances melting in of RNA polymerase at the wild-type P1 promoter. In addition, the level of P1 transcription on the −90 fragment, at either low or high cAMP, is more than twice that observed from the P2 promoter, while no CAP stimulation of transcription occurs on the −60 fragment even at high cAMP. However, this result is tempered by the following observation. Using the gel binding technique to analyze solutions used in transcription experiments, we find that all DNA fragments are in complex with RNA polymerase (at P2 in the absence of CAP or cAMP, at P1 in their presence). So we have the dilemma that there are differences in the numbers of transcripts made, from apparently fully occupied promoters, in assays in which heparin was added to ensure but a single round of initiation. This paradox has yet to be resolved. Experiments in which we visualized all transcripts, including the oligonucleotides synthesized during the initiation event at *gal* (at high nucleoside triphosphate and cAMP concentrations), did not reveal any startling differences between transcription from P1 on the −90 fragment or the −60 fragment. It is nevertheless tempting to speculate that CAP may play an additional role in enhancing the transcription process at some step beyond open complex formation. One could, for example, envision CAP aiding in the escape of RNA polymerase from the promoter site perhaps by altering somehow the conformation of the enzyme.

The studies described here have been done in the absence of the *gal* repressor and thus reveal elements of transcriptional control in the induced state. Recent findings by Adhya and co-workers indicate that repression is a more complicated process than one might have expected (Irani et al., 1983; Majumdar & Adhya, 1984). Two operator regions have been discovered, one at about −53 to −67, the other within the *gal*E gene itself (+45 to +60). A mutation in either of these DNA sequences can lead to constitutive transcription. One hypothesis put forth to explain these results [and similar behavior in the *ara* system (Dunn et al., 1984)] is that a loop is formed when repressor(s) interacts (interact) with both operator DNA regions simultaneously. Whether CAP molecules have any special role in repression is an open question; it is of particular interest that the second CAP site and the extragenic operator region are virtually coincident. This issue can be resolved by additional in vivo and in vitro studies.

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Registry No. cAMP, 60-92-4; RNA polymerase, 9014-24-8; galactose, 59-23-4.

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Structural Aspects of the Copper Sites in Cytochrome *c* Oxidase. An X-ray Absorption Spectroscopic Investigation of the Resting-State Enzyme†

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ABSTRACT: Copper K-edge X-ray absorption spectroscopy (XAS) has been used to investigate the structural details of the coordination environment of the copper sites in eight resting-state samples of beef heart cytochrome *c* oxidase prepared by different methods. The unusual position and structure of the resting-state copper edge spectrum can be adequately explained by the presence of sulfur-containing ligands, with a significant amount of S → Cu(II) charge transfer (i.e., a covalent site). Quantitative curve-fitting analysis of the copper extended X-ray absorption fine structure (EXAFS) data indicates similar average first coordination spheres for all resting-state samples, regardless of preparation method. The average coordination sphere (per 2 coppers) mainly consists of 6 ± 1 nitrogens or oxygens at an average Cu-(N,O) distance of 1.99 ± 0.03 Å and 2 ∓ 1 sulfurs at an average Cu-S distance of 2.28 ± 0.02 Å. Quantitative curve-fitting analysis of the outer shell of the copper EXAFS indicates the presence of a Cu···Fe interaction at a distance of 3.00 ± 0.03 Å. Proposed structures of the two copper sites based on these and other spectroscopic results are presented, and differences between our results and those of other published copper XAS studies [Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465-498] are discussed.

Cytochrome *c* oxidase (ferrocytochrome c/O_2 oxidoreductase, EC 1.9.3.1) catalyzes the final step of mitochondrial respiration, the four-electron reduction of O_2 using reducing equivalents from the electron transport chain.[1] The enzyme utilizes four spectroscopically distinct redox-active metal sites to accomplish this task. Two iron atoms are present in the form of a heme *a* prosthetic group and are labeled heme *a* and heme a_3, forming the basis for the alternative name of this enzyme, cytochrome aa_3. Two copper atoms are also asso-

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[1] For an excellent review of cytochrome *c* oxidase, see Wikström et al. (1981).

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