Multiple Specific CytR Binding Sites at the Escherichia coli deoP2 Promoter Mediate Both Cooperative and Competitive Interactions between CytR and cAMP Receptor Protein*

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Binding of cAMP receptor protein (CRP) and CytR mediates both positive and negative control of transcription from Escherichia coli deoP2. Transcription is activated by CRP and repressed by a multi-protein CRP-CytR-CRP complex. The latter is stabilized by cooperative interactions between CRP and CytR. Similar interactions at the other transcriptional units of the CytR regulon coordinate expression of the transport proteins and enzymes required for nucleoside catabolism. A fundamental question in both prokaryotic and eukaryotic gene regulation is how combinatorial mechanisms of this sort regulate differential expression. To understand the combinatorial control mechanism at deoP2, we have used quantitative footprint and gel shift analysis of CRP and CytR binding to evaluate the distribution of ligation states. By comparison to distributions for other CytR-regulated promoters, we hope to understand the roles of individual states in differential gene expression. The results indicate that CytR binds specifically to multiple sites at deoP2, including both the well recognized CytR site flanked by CRP1 and CRP2 and also sites coincident with CRP1 and CRP2. Binding to these multiple sites yields both cooperative and competitive interactions between CytR and CRP. Based on these findings we propose that CytR functions as a differential modulator of CRP1 versus CRP2-mediated activation. Additional high affinity specific sites are located at deoP1 and near the middle of the 600-base pair sequence separating P1 and P2. Evaluation of the DNA sequence requirement for specific CytR binding suggests that a limited array of contiguous and overlapping CytR sites exists at deoP2. Similar extended arrays, but with different arrangements of overlapping CytR and CRP sites, are found at the other CytR-regulated promoters. We propose that competition and cooperativity in CytR and CRP binding are important to differential regulation of these promoters.

In Escherichia coli, the enzymes and transport proteins required for nucleoside catabolism and recycling are encoded by genes belonging to the CytR regulon. This gene family consists of at least nine unlinked transcriptional units (for review, see Ref. 1). Expression of these transcriptional units is coordinately regulated by the interplay of two transcriptional regulatory proteins, CRP1 (also referred to as CAP) and the CytR repressor. Transcription is activated in response to intracellular cAMP levels by CRP, repressed by CytR, and induced by cytidine. A few of the transcriptional units are also separately regulated by a second repressor, DeoR (2–4), via an independent mechanism. A key feature of the CytR regulon is that the individual cistrons are differentially expressed. Extents of activation, repression, and induction all vary among the different transcription units (cf. Ref. 5). This is achieved by nesting levels of local repression, mediated by DeoR and CytR, on a more global regulation mediated by CRP. This illustrates a process, common to both E. coli and higher order eukaryotes, in which complex patterns of expression are controlled using a small number of regulatory proteins. For example regulation of cell growth and differentiation often combines tissue-specific or developmental stage-specific factors with more global control elements. The mechanism of such broad regulatory programs is a fundamental issue in gene regulation. Presumably combinatorial mechanisms that rely on different local features of different genes are involved.

Several of the CytR-regulated operons have been investigated at the molecular level. The promoter deoP2 of the tetracistrionic operon that directs the synthesis of purine and pyrimidine phosphorylases and enzymes required for sugar utilization has generated the greatest interest (6). Others that have been investigated include tsx (7), encoding an outer membrane protein, cdd (8), encoding cytidine deaminase, udp (9) encoding uridine phosphorylase, cytr (10) encoding CytR, and most recently nupG (11) encoding a membrane nucleoside transport protein. In all cases, these studies have implicated interactions of CRP and CytR with sequences located in the 80–100 bp immediately upstream of the various transcription start sites and interactions between the proteins as the basis for positive and negative gene regulation. Thus, differential and coordinate gene regulation must depend on different dispositions of CRP and CytR binding sites, different protein binding affinities, and/or different levels of site-site interaction or cooperativity.

Most CytR-regulated promoters contain tandem CRP sites. CRP1 (at about −41.5 bp from transcription start sites) and CRP2 (at about −92.5 bp) appear to be class II and class I sites, respectively (12, 13). The significance of these classifications is the suggestion that they direct different kinetic mechanisms of activation. At class I sites, CRP is proposed to increase the apparent affinity of RNAS for the promoter, whereas at class II

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The abbreviations used are: CRP, E. coli cAMP receptor protein; bp, base pairs; bis-tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxyethyl)propane-1,3-diol; RNAP, E. coli RNA polymerase; CytR, E. coli cytidine repressor protein; CRP1 and CRP2, CRP operator sites 1 and 2, respectively; P1 and P2, deo operon promoters 1 and 2, respectively.

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sites, CRP is proposed to increase the rate of formation of the open transcription complex. At deoP2, CRP1 alone, but not CRP2 alone, substantially activates transcription (5). CRP1 has been proposed to substitute for the lack of a ~35 consensus promoter sequence (14), whereas CRP2 is proposed to be necessary only for CytR to bind. However, in the absence of kinetic studies on any of these promoters there is little direct evidence to suggest which kinetic mechanism of activation is involved under any condition. The highest levels of expression are achieved with both sites functional, suggesting some synergy in activation (15).

The tandem CRP sites in different promoters are separated by DNA of variable length and sequence. Based on footprinting analysis of purified CytR binding to deoP2 and other promoters, this intervening sequence is now thought to contain the CytR binding site (6, 8, 16). A putative recognition motif has been identified (6, 17), and pairs of such motifs, arranged as either direct or inverted repeats, have been implicated as the CytR operator (9, 17). It is ironic, given the results we report here, that until relatively recently CRP and CytR were believed to compete to bind to the same sites (cf. Ref. 4).

The mechanism of CytR-mediated repression is indirect. CytR has no effect on basal level transcription but instead requires CRP binding to mediate repression (16, 18). Of course, this necessarily means that CytR functions only under conditions of CRP-dependent activation. Under these circumstances CytR typically does not completely reduce the activated level of expression to the basal level (5). Despite this role of CytR as a functional antagonist of CRP, the two proteins interact cooperatively, resulting in substantially increased CytR binding affinity when CRP is present (6). The role of cooperativity is widely thought to be to recruit CytR at otherwise sub-saturating concentrations. The crucial role that cooperativity plays in repression is highlighted by the fact that when CytR binds cytidine, induction occurs as a result of the loss of cooperativity and despite no effect of cytidine binding to CytR on intrinsic binding of CytR to DNA (6).2

The complexity of these regulatory properties has generated confusion about the nature of key molecular interactions. The most perplexing questions involve the CRP-CytR cooperativity. For example, heterologous cooperativity has been reported to require both CRP sites, from which a lack of pairwise interactions is inferred (20). Yet, regulation of cytRP, in which there is only one CRP site, also depends on cooperative CytR and CRP binding (10). In addition, it was recently observed (11, 21) that the apparent cooperativity, when assessed by the effect of saturating CRP concentration on CytR affinity, is substantially greater than when cooperativity is assessed by the effect of saturating CytR concentration on CRP binding. This “one-way stimulation” (11) represents an apparent conflict with the laws of thermodynamics that still awaits a molecular explanation.

Several lines of evidence implicate protein-protein interactions as providing the driving force for cooperativity. First, mutations have been located on the surface of CRP in a putative protein interacting domain that interfere with cooperativity with CytR and with CytR-mediated repression (22). Second, CytR and CRP are reported to mutually antagonize each other’s protein binding induced bends in the cytRP sequence (10). Since such coupled DNA structural transitions necessarily contribute unfavorably to cooperativity, the driving force for cooperativity in cytRP must be derived from favorable protein-protein contacts. Third, a truncated CytR which is lacking the DNA-binding helix-turn-helix motif was reported to bind to deoP2 in the presence of CRP and further to do so with only moderate reduction in overall affinity (23) compared with the full-length protein. This would suggest that CytR is primarily a protein-protein bridge, dependent on its interactions with CRP bound to the flanking CRP sites for association with its operator. However, the question remains open how even these favorable protein-protein contacts could possibly compensate for the loss of a direct DNA binding interaction with a \( K_d \) in the 10 nM range.

Previous approaches used to investigate CytR-regulated promoters have not been fully successful in developing an understanding of the macromolecular interactions that regulate transcription. The confusion stems in large part from the fact that only qualitative reasoning has been used to address quantitative questions. For example, most of the in vitro studies have been conducted as combinations of plus/minus the various components (CRP, CytR, cAMP, cytidine, and promoter elements) with insufficient understanding of the mutual effects of interactions between these molecules to know what concentrations are necessary to achieve particular effects. Our immediate goal was to understand how the protein-DNA and protein-protein interactions control the distribution of operator configurations at deoP2. Our approach has been to use DNase footprinting to obtain complete individual site isotherms for binding of CytR and CRP to deoP2, at different configurations of empty and filled sites for the other regulatory protein. From such data, the complete population distribution of promoter configurations can be determined as a function of both CRP and CytR concentration. We anticipated being able to deduce connections between the individual promoter configurations in the distribution and biologically functional states.

The results indicate a much more complex promoter structure than was previously supposed. Multiple, specific CytR sites are arranged over an extended region of DNA that includes both CRP1 and CRP2 as well as the sequence they flank. CytR binding to these sites mediates both cooperative and competitive interactions with CRP. Together these explain quantitatively the apparent one-way stimulation. Similar specific interactions of CytR with extended DNA sequences have been identified at deoP1 and also near the middle of the approximately 600-bp sequence separating P1 and P2. Comparison of the DNA sequences of these sites further clarifies the CytR binding motif. The distribution of such motifs, both at deoP2 and at other CytR-regulated promoters, define arrays of contiguous and overlapping CytR binding sites. This entirely new phenomenon is consistent with the interpretation that CytR functions as a modulator of CRP-mediated activation. We envision such a function operating at two levels: first as a differential modulator of class I versus class II CRP activation at individual promoters, and second as a differential modulator of activation of the various CytR cistrons.

**MATERIALS AND METHODS**

*Reagents and Enzymes—*Crystalline adenosine 3'-5' cyclic monophosphate (cAMP) and crystalline cytidine (both >99%) were purchased as free base from Sigma and as free acid from ICN, respectively. Stock concentrations (in 50 mM bis-tris base, pH 7.0, 1 mM EDTA) were determined, and purity was assessed spectrophotometrically by comparing observed spectra to published molar extinction coefficients and absorbance ratios (61). Bovine pancreas deoxyribonuclease I (DNase I, code D) from Worthington was treated as described (24). [\( \alpha^{32} \)]-dATP (3000 Ci/mmol) was purchased from Amerham Corp. or ICN; unlabeled dNTPs were from Life Technologies, Inc. Buffer components and reagents were electrophoresis grade if available and reagent grade otherwise.

**CRP and CytR Purification**—CRP was expressed from *E. coli* strain K12 ΔHtrp transformed with the expression plasmid pPLcCRP (25) and purified as described (26). No contamination is detectable by Coomassie staining of overloaded SDS-polyacrylamide gels from which we

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estimate at least 98% purity. CRP concentration was estimated based on ε(%) = 9.2 at λmax = 278 nm (27).

CytR was expressed and purified as described. On SDS-polyacrylamide gels, the purified material used in these studies was at least 90% full-length CytR (M, = 37,800). The remaining material was contained in two bands, with apparent molecular weights of 31,000 and 27,000. This has comprised from 5 to 20% of the total material in different CytR preparations. Under native conditions, purified CytR elutes from a Pharmacia Superose 6 column in a single, sharp peak with apparent molecular weight 72,500 ± 2,500. This is consistent with sedimentation equilibrium analysis that indicates CytR to be homogeneous dimer in solution. This peak accounts for all observable UV absorbing material.

Thus, the lower molecular weight bands appear to be products of endogenous proteases, as has been observed with other members of the LacI repressor family (29, 30). We conclude on this basis that the CytR preparation is at least 95% pure. Concentration was estimated using an extinction coefficient, ε = 0.30 ± 0.02 mg⁻¹ cm⁻¹ at 280 nm.

**Operator DNA Preparation—** Fig. 1 shows the deo DNA fragments used. Plasmid pSS1332 contains the deo P1/P2 sequence cloned from –801 to +151 relative to the P2 start site for transcription, cloned into the BamHI site of pUC13. Insertion of an 8-bp NotI linker into the Smal site of the vector generated an 879-bp NotI/HinII fragment in which the +32P-labeled NotI end is 192 bp downstream from CRP1. A 285-bp NotI/Smal fragment containing only the P2 regulatory region was generated by inserting a NotI linker into a BamHI site at –117 in the deo sequence. All DNA fragments were agarose gel purified after banding the plasmid preparations twice in CsCl gradients. DNA was protein free, as determined from A<sub>260</sub>/A<sub>280</sub> (31). Fragments were labeled at their NotI sites using the Klenow fill-in reaction as described (32).

Mutant promoters were generated in which site-specific CRP binding to CRP1 (CRP1 – pLP01) or to CRP2 (CRP2 – pLP02) was eliminated. The BamHI fragment from pSS1332 was subcloned into pM13mp8, and single-stranded DNA was isolated as described (33). Site-directed mutagenesis was conducted using the kit from Amersham Corp. Mutagenic oligonucleotides, 30 nucleotides in length, were designed to produce symmetric G to A transitions in both TGTGA, CRP recognition motifs of the mutated site. Sequences of the mutants (Fig. 1) were confirmed by dideoxy DNA sequencing. CRP1 and CRP2 operator fragments (879 bp) were isolated as described.

**Individual Site Binding Experiments—** Quantitative DNase I footprint titrations were conducted as described (32, 34) in 10 mM bis-tris (pH 7.00 ± 0.1), 100 mM NaCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 50 μg/ml bovine serum albumin, and 1 μg/ml calf thymus-DNA. Binding reaction mixtures (200 μl) were equilibrated in a water bath at 20 °C (±0.2 °C) for between 40 min and 2 h prior to DNase I exposure. Measurements are independent of the incubation time over this range. These were exposed to 2–6 ng of DNase I, added in a 5.0-μl volume, for 12.0 min, and quenched by addition of ½ volume of 50 mM Na₂EDTA before addition of stop solution (34). Two-dimensional optical scanning of footprint autoradiograms and analysis of the digitized images was as described (34).

**Mobility Shift Titrations—** Mobility-shift titrations were conducted as described (35, 36) using 5% acrylamide gels (29:1, acrylamide:bis) and 0.5 × TBE electrophoresis buffer (31, 33). CRP and deoP2 DNA (10 μl; 285-bp fragment) were incubated (40–60 min) at 20 °C (±0.1 °C) in the DNase I footprint binding buffer but with 2 μg/ml CT-DNA and with 1.5% Ficoll added to facilitate gel loading. Aliquots (20 μl) of equilibrated binding reaction mixtures containing 1400 dpm of 32P were loaded onto 1.5-mm minigels in a Bio-Rad Mini Protean II device that had been pre-electrophoresed for 5 min. Gels were loaded with current on and electrophoresed at a constant 200 V for 35 min.

Dried gels were imaged using a Molecular Dynamics PhosphorImager 435SI. Phosphor plates were exposed for 8–10 h and scanned at 176-μm spatial resolution. Analysis of the digital images was conducted using the program IPLabGel (Signal Analytics Corp.) or ImageQuant (Molecular Dynamics, Inc.) essentially as described (35, 36). The combination of long exposure and high specific radioactivity yielded ratios of average pixel intensity to background of about 100, minimizing concerns about local background variation (24, 32).

**Numerical Analysis—** Binding data were analyzed by using the nonlinear least squares parameter estimation program, NONLIN (37). NONLN estimates parameter values corresponding to a minimum in the variance, and worst case joint confidence limits for each parameter corresponding to approximately one standard deviation. Simple, non-cooperative binding of a single protein to an individual DNA site is described by

\[
Y = \frac{k_l \cdot L}{I + k_i \cdot L}
\]

where L is the concentration of free protein ligand, and \( k_i \) is the intrinsic association constant for binding of the protein to the individual site. Binding of either CRP or CytR alone was analyzed to obtain the Gibbs free energy change corresponding to \( k_i \) in Equation 1 (ΔG° = −RT ln \( k_i \)).
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In the analysis of footprint titration data, where fractional protection rather than total saturation is the quantity experimentally determined, it was also necessary to fit the fractional protection endpoints as adjustable parameters for each separate titration (24).

Equations that describe the cooperative binding of CRP and CytR were derived by considering the relative probability of each of the deoP2 configurations (Table III) as specified by the Specific, and Nonspecific interactions. For noncooperative binding, \( \Delta G_s \) equals \( \Delta G^\prime \) (Equation 1). Equation 1 can also provide an accurate estimate of \( \Delta G_{s,app} \) for binding experiments in which both CytR and CRP were present. \( \Delta G_{s,app} \) is related to the integral of an individual site binding curve and reflects the sum of free energy changes for both intrinsic binding and the effect of all cooperative interactions. For noncooperative binding, \( \Delta G_{s,app} \) equals \( \Delta G^\prime \) (Equation 1). Equation 1 can also provide an accurate estimate of \( \Delta G^\prime \), and of its confidence limits when two different proteins interact cooperatively in binding to different DNA sites, and when binding of one is titrated while the concentration of the second is held constant (39). It is necessary that the concentration held constant be saturating. In this limit, the probability that the protein being titrated will bind to DNA that is already liganded by the first protein is made arbitrarily close to unity, and the shape of the binding curve is described by Equation 1.

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Analysis of CRP Binding to deoP2—Footprint titrations of CRP binding show the expected protected regions correspond-

![Figure 2](image2.png)  
**FIG. 2.** CRP binding to CRP1 and CRP2. Background, DNase I footprint titration showing both protection and hypersensitivity in CRP1 and CRP2. Titrations of 879-bp deoP1/P2 DNA under standard conditions plus 150 \( \mu \mathrm{M} \) cAMP. Foreground, individual site binding curves for CRP1 (squares) and CRP2 (diamonds). Data plotted versus total CRP dimer concentration. Solid curves fit Equation 1. This yields \( \Delta G_{s,app} = -11.9 \pm 0.1 \text{ kcal/mol} \) and \( \Delta G_{s,app} = -13.3 \pm 0.1 \text{ kcal/mol} \).

![Figure 3](image3.png)  
**FIG. 3.** CRP binding versus [cAMP]. Points are \( \Delta G_{s,app} \) for binding to CRP1 (squares) and CRP2 (diamonds) relative to a 1 \( \mu \text{M} \) total CRP dimer standard state. Individual site binding curves from separate DNase I footprint titration experiment at each [cAMP] were analyzed using Equation 1. Error bars are confidence limits to fitted \( \Delta G_{s,app} \). The solid curves drawn through the points represent analysis of these data as described in the text.

Fraction of CRP as Functionally Active CRP(cAMP)1—The role of cAMP as an allosteric effector of CRP functional states is well known (26, 41). Free CRP dimers have three quaternary conformational states corresponding to the three cAMP ligation states. cAMP binding is negatively cooperative, thus favoring the singly liganded species, CRP(cAMP)1, which is the functionally active, site-specific DNA binding form (42). However, as a consequence of overlapping transitions for binding of the first and second cAMPs, only a fraction of CRP is in this functionally active form even at optimal cAMP concentration. Therefore it was necessary to analyze the cAMP concentration dependence of CRP binding to deoP2 to determine concentrations of active CRP(cAMP)1 dimer in our experiments. Results are shown in Fig. 3.

Consistent with previous reports, two overlapping transitions yield a maximum in apparent affinity between 100 and 200 \( \mu \text{M} \) cAMP. Affinities for CRP1 and CRP2 parallel each other over the entire range of cAMP concentrations, consistent with the conclusion that cAMP is an effector of pre-existing conformational states of free CRP dimers. These data were analyzed quantitatively using Equation 4 of Heyduk and Lee (41) to estimate the free energy changes corresponding to macroscopic, step-wise association constants for binding of one \( (K_1) \) and two \( (K_2) \) cAMPs to free CRP dimers and for binding of CRP(cAMP)1 and CRP(cAMP)2 to CRP1 and CRP2. The operator DNA binding affinity of unliganded CRP is assumed equal to 0 in this model. This analysis yielded \( K_1 \) equal to \( (2.6 \pm 2.4) \times 10^4 \text{ M}^{-1} \) and \( K_2 \) equal to \( (1.8 \pm 1.8) \times 10^4 \text{ M}^{-1} \). These estimates at pH 7.0 are 6 times less and 2 times greater than estimates at pH 7.8 (41) indicating both lower intrinsic cAMP binding affinity and lower (negative) cooperativity, i.e. weaker...
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Free energy changes for saturation of deoP2 operator sites CRP1 and CRP2 with CRP(cAMP)1 or the intervening CytR site with CytR, in the presence or absence of effector ligands as indicated. Values of $\Delta G_i$ (kcal/mol) determined by analysis of individual site binding curves as described in the text.

| deoP2 valency | Titrant | Effector(s) | No. expl.a | Operator site | CRP2 | CytR | CRP1 |
|---------------|---------|-------------|------------|---------------|------|------|------|
| Wild-type CRP | cAMP    | 11          | $-13.0 \pm 0.2$ | $-11.7 \pm 0.2$ |
| CRP1 CRP     | cAMP    | 5           | $-13.1 \pm 0.3$ |
| CRP2 CRP     | cAMP    | 5           | $-11.6 \pm 0.1$ |
| Wild-type CytR | CytR, cAMP | 7         | $-13.5 \pm 0.3$ |
| CRP1 CytR    | CytR, cAMP | 2         | $-13.8 \pm 0.3$ |
| CRP2 CytR    | CytR, cAMP | 4         | $-12.2 \pm 0.5$ |
| Wild-type CytR | None or cAMP | 14       | $-10.4 \pm 0.4$ |
| CRP1 CytR    | None or cAMP | 3         | $-10.4 \pm 0.2$ |
| CRP2 CytR    | None or cAMP | 2         | $-10.5 \pm 0.5$ |
| Wild-type CytR | CytR, cAMP | 9          | $-13.1 \pm 0.2$ |
| CRP1 CytR    | CytR, cAMP | 3          | $-12.4 \pm 0.3$ |
| CRP2 CytR    | CytR, cAMP | 2          | $-12.4 \pm 0.5$ |

a Effector concentrations: CRP, 0.1 $\mu$M (total dimer); cAMP, 150 $\mu$M; CytR, 0.4 $\mu$M (dimer).

b $\Delta G_i$ values shown are means of multiple determinations ($\pm$ S.D. of mean). Number of experiments represented in the means are indicated. Mean values for binding to wild type deoP2 reflect approximately equal numbers of titration experiments conducted using 285 bp of P2 containing and 879 bp of Pu/P2 containing DNA fragments. There was no difference in results obtained using these two fragments.

coupling between cAMP binding and equilibria between quaternary conformational states.

Based on these values of $K_i$ and $K_d$, the fraction of CRP-(cAMP)$_1$ is calculated to reach a maximum of 0.635 ($\pm$0.021 by propagation of errors) of total CRP at 150 $\mu$M cAMP, the concentration used in all subsequent experiments. Although no unique estimate for the affinity of CRP(cAMP)$_2$ for binding to either CRP1 or CRP2 was obtainable, the analysis did yield as an upper limit to the operator binding affinity of CRP(cAMP)$_2$ a value 100-fold lower than that of CRP(cAMP)$_1$. On this basis, the simplifying assumption was made that only CRP(cAMP)$_1$ is functionally active in all subsequent analyses. Free energy changes reported for CRP binding use a standard state of 1 $\mu$M dimeric CRP(cAMP)$_1$. Estimates of the free energy changes for binding of CRP(cAMP)$_1$ to CRP1 and to CRP2 are in Table 1. Affinity for CRP2 is 10-fold higher than for CRP1, consistent with results reported at other experimental conditions (6).

**Footprint Titration Analysis of CytR Binding**—CytR binding to deoP2 was also investigated by footprint titration. In contrast to the CRP footprints, which are localized to the 22-bp CRP binding sites, CytR binding protects an extended region from about bp −25 to −110 bp relative to the start site for transcription (Fig. 4). This extended footprint includes not only the previously identified CytR binding site (6) but also both flanking CRP binding sites. Based on literature reports that CytR binds to a single specific site flanked by CRP1 and CRP2, routine analysis of fractional protection included only this sequence, i.e. approximately bp −55 to −80 (see Fig. 1). Analysis of these data using the simple binding model (Equation 1) yielded an apparent free energy change for CytR binding to this site, equal to $-10.4 \pm 0.4$ kcal/mol (Table 1).

Two additional CytR footprints were observed, one centered at about −235 bp and another at −592 bp from the P2 transcription start site. The latter is in the P1 regulatory region and overlaps DeoR operator site, O1 (43). Hence, we denote this as the “P1” CytR site. We denote the former as the “upstream” CytR site. The CytR concentration dependences for protection at these sites differ from that for the P2 site. Therefore, these represent distinct, specific CytR binding sites that have not been previously described. Quantitative analysis of the upstream site yieldede the binding free energy change of $-11.6 \pm 0.2$ kcal/mol (mean of four experiments). Thus, CytR’s affinity for this site is nearly 10-fold greater than its affinity for the P2 site. At 750 bp from the $^{32}$P-labeled end of the DNA fragment, the electrophoretic resolution of bands in the P1 site is inadequate for quantitative analysis.

To evaluate the possibility of cooperative interactions between CytR bound to deoP2 and CytR bound to the upstream
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Fig. 5. Linkage schematic showing relationships between molecular configurations of deoP2 and free energy states. In this model CRP(cAMP)₁ binds to CRP₁ and CRP₂ and CytR binds to a single intervening site. We denote CRP₁ and CRP₂ as sites 1 and 2, and therefore, the CytR site as site 3. Shading indicates liganded sites. The standard free energy of each state relative to the unliganded reference state is given as a sum of contributions due to the intrinsic Gibbs free energy changes for binding to each of the operator sites (Δ₆,ᵢ = 1, 2 or 3) and the Gibbs free energy changes for pairwise or three-way cooperative interaction between liganded sites (ΔGᵢⱼₖ). Cooperativity is defined thermodynamically as the difference between the total free energy change to saturate two or more sites simultaneously and the sum of intrinsic free energy changes to fill them separately.

and/or P₁ site(s), the P₂ regulatory region and the upstream and P₁ regions were isolated on separate DNA fragments as described above. Footprint titration experiments conducted using these DNA fragments yielded apparent binding free energies for the deoP₂ (−10.5 ± 0.1 kcal/mol; mean of five experiments) and upstream (−11.2 ± 0.3 kcal/mol; mean of nine experiments) CytR binding sites that are indistinguishable from those obtained using the larger P₁/P₂ containing DNA fragment. This result indicates no cooperative interaction between CytR bound to P₂ and CytR bound to the upstream site. We infer also no interaction between P₁ and P₂ sites. Therefore, the apparent free energy changes obtained in these analyses are intrinsic free energy changes for binding of CytR to the local sites. Quantitative protection data for the P₁ site were obtained by labeling the P₁-containing fragment at the end near P₁. Analysis of these data yielded an intrinsic free energy change for CytR binding equal to −10.5 ± 0.6 kcal/mol (mean of eight experiments). Thus, affinities of CytR for the P₁ and P₂ sites are approximately equal.

Analysis of Heterologous Cooperative Interaction between deoP₂-bound CRP and CytR—To evaluate cooperative interactions between different proteins binding to DNA, we considered the thermodynamic cycle for their simultaneous binding (cf. Ref. 44). Fig. 5 illustrates the approach based on the deoP₂ structure diagrammed in Fig. 1. The total free energy change to fill an individual site with ligand, including the effects of interactions with other ligands, is the individual site loading free energy change, ΔGᵢ, (38) a model independent quantity. ΔGᵢ(CytR), the loading free energy change for CytR binding alone (no CRP) is equal to the intrinsic free energy change, ΔGᵢ, (Fig. 5). The loading free energy change for CytR binding to deoP₂ that is saturated by CRP(cAMP)₁ (ΔGᵢ(CRP₁)) includes contributions from both intrinsic binding and cooperativity, i.e. ΔGᵢ + ΔGᵢ₂₃. Thus ΔGᵢ₂₃ = (ΔGᵢ(CRP₁) − ΔGᵢ(CytR)). ΔGᵢ₂₃ can also be evaluated by comparing CRP(cAMP)₁ binding in the presence versus the absence of saturating CytR. Cooperativity contributes unequally to CRP₁ and CRP₂, dependent on their relative intrinsic affinities for CRP(cAMP)₁ binding (ΔG₁ and ΔG₂, Fig. 5). Therefore, the CytR-mediated differences in loading free energy changes for CRP(cAMP)₁ binding to CRP₁ and CRP₂ are summed to yield ΔGᵢ₂₃ = (ΔGᵢ(CRP₁) − ΔGᵢ(CRP₂)) + (ΔGᵢ₁(CRP₂) − ΔGᵢ₂(CRP₂)). These two independent methods for evaluation of ΔGᵢ₂₃ provide a critical control: if the molecular model properly accounts for all molecular configurations and free energy states, the same value for ΔGᵢ₂₃ must be obtained either way.

To evaluate the cooperative free energy change in this manner, loading free energy changes were determined for binding of CytR and CRP(cAMP)₁ to deoP₂, each in the presence and absence of a fixed concentration of the other (Table I). As a practical approximation to the limit of saturating CRP(cAMP)₁, 0.1 μM CRP (total dimer) and 150 μM cAMP were used. Saturation of CRP₁ and CRP₂ are 0.97 and >0.99 at the resulting CRP(cAMP)₁ concentration (64 nM). The fixed CytR concentration used was 0.5 μM, which yields 0.97 saturation. Table I reflects many repetitions of titration experiments on wild type deoP₂. This is because titration experiments were conducted using both the longer, P₁/P₂ containing DNA fragment and the shorter P₂ containing fragment (Fig. 1). Identical results were obtained for the two operator fragments, as described earlier. As an additional control, CytR titrations were conducted in the presence and absence of cAMP. At 150 μM, cAMP had no effect on intrinsic CytR binding. ΔGᵢ₂₃ and ΔGᵢ₃, which pertain to pairwise cooperative interactions between CytR binding and CRP bound to either CRP₁ or CRP₂, were similarly evaluated using reduced valency mutants in which specific binding to either CRP₁ (CRP₁⁻) or CRP₂ (CRP₂⁻) was eliminated. The mutants were produced by introducing a G to A transition into each of the TGTGA motifs for either site (45–47). We mutated both TGTGA motifs for each site because this is reportedly necessary to completely abolish CRP activation of deoP₂ (20).

No specific binding of CRP to either mutated site was observed. Intrinsic binding to the remaining site (CRP₁ or CRP₂) was identical to binding to CRP₁ and CRP₂ in the wild type operator (Table I). This is consistent with the conclusion that CRP binding is noncooperative. CytR titrations of CRP₁⁻ and CRP₂⁻ were conducted to evaluate whether there was any effect of mutating either CRP₁ or CRP₂. CytR binding to CRP₁⁻, CRP₂⁻, and wild type deoP₂ were identical (Table I) indicating no such effect. We infer that there is also no effect of mutation of either CRP site on the remaining CRP site. Table II lists ΔG_i values obtained by taking differences between values in Table I, as described above. Whereas the apparent cooperativity as evaluated from CytR titrations is substantial, consistent with previous reports, the apparent cooperativity as evaluated from CRP titrations is much weaker.
In particular, pairwise binding of CytR and CRP to either site appears to be essentially noncooperative when evaluated from CRP titrations. This phenomenology is consistent with recent reports of “unidirectional stimulation” in CRP and CytR interaction with *nupG* (11). However, when couched in quantitative terms as in Table II, it is evident that this phenomenology reflects the failure of Fig. 5 to account for all configurations of bound CytR and/or cAMP-CRP. Additional sites of interaction for one or both proteins are necessary to explain these results. Control experiments cited above demonstrate that these effects are not the result of interactions with the upstream or P1 CytR sites. Thus, we conclude that as yet uncharacterized sites near *deoP2* must be responsible.

**Analysis of CytR Binding Reveals Multiple Interactions with *deoP2*—**Considering it unlikely that high affinity CRP sites would be overlooked since the sequence specificity for CRP binding is well known (45–48), we decided to analyze the extended DNase I protection pattern conferred by CytR binding (Fig. 4). The CytR concentration dependence of separate regions of the extended CytR footprint was evaluated systematically to determine whether the entire footprint represents a single binding event or multiple binding of CytR to separate sites. To obtain adequate electrophoretic resolution of the CytR footprint for this purpose, CytR titrations were conducted using the shorter, P2 DNA fragment (Fig. 1). Titration curves were constructed from the fractional protection in a series of blocks of DNA bands covering the sequence from bp −31 to −100 relative to the P2 start site. In defining blocks of bands for analysis, it is necessary to choose well resolved DNA bands (24). As a consequence the blocks analyzed represent a mixture of contiguous and overlapping blocks. Results of this analysis are shown in Fig. 6.

The apparent free energy change for the usual CytR block extending from approximately bp −55 to −80 is consistent with the value listed in Table I. Sub-partitions of this block yield values indistinguishable from this, albeit with lower precision in some cases. This indicates that the CytR concentration dependence of the protection over this region is the same, consistent with a single molecular event, binding of CytR to a single site. By contrast, the fractional protection that extends over CRP1 and CRP2 shows a different CytR concentration dependence as reflected in smaller apparent free energy changes for CytR binding. Therefore, this protection reflects different molecular events; it cannot be due to CytR binding to the −55 to −80 region. It suggests additional CytR binding sites with somewhat lower affinity than the previously identified CytR site (site 3; Fig. 5) and which partially or wholly overlap CRP1 and CRP2. This extended protection reflects specific binding of CytR to DNA. The more or less uniform protection of the entire DNA fragment that results from non-specific binding is observed, but only at higher CytR concentrations than were used in these experiments.

**Figure 6. Analysis of extended CytR protection pattern.** A, footprint titration of the 285-bp *deoP2* fragment showing increasing protection as a function of CytR concentration in lanes 1–12. A Maxam-Gilbert A + G sequencing reaction (lane 13) was used to determine absolute base pair positions relative to the CRP operators, CRP1 and CRP2 (marked). The CytR concentration dependence of protection was analyzed in a series of separate blocks of bands as delineated. Results, shown as apparent individual site loading free energy changes, indicate a 10-fold range of apparent binding affinities. B, individual site curves for CytR binding to its high affinity site flanked by CRP1 and CRP2 and for binding to CRP1. *Solid curves* are the fits of these data to Equation 1; this yields apparent free energy changes for intrinsic binding equal to −10.8 ± 0.2 (included in average shown in Table I) and −9.7 ± 0.3 kcal/mol.

I and apparent free energy changes in Fig. 6, two models that incorporate two additional CytR binding sites and which constitute opposing possibilities were evaluated. The possibilities are 1) that the additional CytR sites that overlap CRP1 and CRP2 are specific binding sites to which CytR binds noncooperatively and with defined affinity; and 2) that the additional binding is nonspecific but cooperative. Competitive binding was formulated as rules (constraints) that (i) CRP(cAMP)ᵢ₁ binding to CRP1 and CytR binding to the site overlapping CRP1 (denoted site 4) are mutually exclusive; and (ii) similarly, that CRP(cAMP)ᵢ₂ binding to CRP1 and CytR binding to the site overlapping CRP2 (denoted site 5) are mutually exclusive. In the second model, CytR bound to the high affinity site 3 nucleates nonspecific binding on either side. Since our data do not precisely define which base pairs constitute the additional
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Table III
Operator configurations and free energy states for CRP and CytR binding to deoP2: specific and nonspecific models for higher order CytR binding

deoP2 configurations with sites denoted as filled (CRP or CytR) or empty (○). The total Gibbs free energy of each configuration relative to the unliganded reference state is given as a sum of contributions from eight free energy changes for intrinsic binding of CRP and CytR and for cooperative interaction between liganded sites. Intrinsic binding and cooperative interactions among CRP1, CRP2, and the intervening CytR site are defined in Fig. 5. Two phenomenological models for competition between CytR and CRP define additional CytR binding sites that occlude CRP1 and CRP2 in terms of thermodynamic properties. These are 1) Specific Binding, CytR binds specifically, but non-cooperatively to two additional operators that occlude CRP1 and CRP2, with intrinsic free energy changes $\Delta G_s$ and $\Delta G_n$, respectively; and 2) Non-specific (n.s.), Cooperative (C), Binding, CytR binds non-cooperatively with intrinsic free energy change, $\Delta G_{n.s.}$, and binding to directly adjacent sequences is weakly cooperative, with free energy change, $\Delta G_c$.

| Operator configurations | Free energy contributions if inhibition is due to |
|-------------------------|--------------------------------------------------|
| CRP2 (CytR) | CRP | CytR | CRP1 |
| 1 | ○ | ○ | ○ | 0 |
| 2 | ○ | ○ | CRP | $\Delta G_s$ |
| 3 | CRP | CytR | ○ | $\Delta G_n$ |
| 4 | CytR | ○ | CytR | $\Delta G_{n.s.}$ |
| 5 | CytR | CytR | ○ | $\Delta G_c$ |
| 6 | CytR | ○ | CytR | $\Delta G_{n.s.}$ |
| 7 | CRP |○ | CRP | $\Delta G_s + \Delta G_{n.s.}$ | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 8 | CRP | CytR | CRP | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 9 | CRP | CytR | CRP | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 10 | CRP | CytR | CytR | $\Delta G_s$ |
| 11 | CytR | CytR | CRP | $\Delta G_s$ |
| 12 | CytR | CytR | CytR | $\Delta G_s$ |
| 13 | CytR | CytR | CytR | $\Delta G_s$ |
| 14 | CytR |○ | CytR | $\Delta G_s$ |
| 15 | CRP | CytR | CRP | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 16 | CRP | CytR | CytR | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 17 | CytR | CytR | CRP | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |
| 18 | CytR | CytR | CytR | $\Delta G_s + \Delta G_{n.s.} + \Delta G_c$ |

CytR sites, the models define these sites only in terms of thermodynamic properties. Table III lists the operator configurations and free energy states that result.

The titration data represented by the $\Delta G_1$ values in Table I were analyzed according to these models, which we denote Specific, and Nonspecific, Additional Sites. The concentrations of both CytR and CRP(cAMP)$_1$, whether used as tiritant or as held constant, were the independent variables. The fractional protection of individual sites was the dependent variable. No data for putative CytR sites 4 and 5 were included in the analyses. Instead we used the data for the known sites, 1–3, to determine (i) whether such additional CytR binding sites could account quantitatively for the cooperative free energy changes in Table II, (ii) whether in so doing, well behaved parameter values, $\Delta G_s$ and $\Delta G_n$, (or $\Delta G_{n.s.}$ and $\Delta G_c$) would be obtained, and (iii) whether the parameters so obtained are consistent with the analysis of extended protection in Fig. 6.

Including both the eight free energy contributions listed in Table III and the individual site, fractional protection end points (24), 176 adjustable parameters are required for a global analysis of all 84 binding curves represented in Table I. Since this exceeds limitations of our software and hardware, we instead analyzed two representative titrations from each line in the table, 24 in all. In three cases these were the only data. In all other cases, the criteria for selection were (i) that the $\Delta G_1$ values for the pair of experiments chosen reflect the mean and standard deviation of the entire set as far as possible and (ii) that subject to criterion i, the data be of the highest precision and best distribution of independent variable available. Criterion i ensures the greatest possible sensitivity to systematic differences between experiments with different titrants and different operators, thus imposing the most critical possible standard on evaluation of goodness of fit.

To minimize effects of subjective bias in the selection of representative curves, we repeated the analysis using a second data subset in which different titrations were selected whenever possible. A third analysis was conducted in which the concentrations of titrants that yield half-saturation were calculated separately for each binding curve from the $\Delta G_1$ values. This yielded 84 calculated points in which the independent variables are the protein concentrations (CytR and CRP(cAMP)$_1$) and the dependent variable is a constant 0.5 (cf. Ref. 49). Analysis of these half-titration point data requires only the eight free energy changes defined in Table III as adjustable parameters.

Results obtained from the first of these analyses are summarized in Table IV. Results obtained in the subsequent two analyses were indistinguishable from these except that the half-titration point data yielded slightly less precise parameter estimation. The Specific and Nonspecific Additional Sites models both describe the data well. Indistinguishable variances are obtained and these reflect the inherent precision of the individual data as defined by variances to separate fits of individual binding curves. Fig. 7 demonstrates how the Specific Additional Sites model rationalizes both the apparent high cooperativity of CytR binding in the presence of CRP(cAMP)$_1$ at fixed, saturating concentration and apparent weak cooperativity of CRP(cAMP)$_1$ binding in the presence of CytR at fixed, saturating concentration. The net effect of cooperativity and competition results in the leftward shift of the solid curves with respect to their noncooperative broken curve counterparts. The net effect on CytR binding is substantial while the net effect on CRP binding to CRP2 is slight.

The parameter values obtained from the two models for intrinsic binding and cooperative interactions between CytR, cAMP-CRP, and operator sites 1–3 are nearly identical. In addition, both models yield well bounded values for intrinsic affinities of CytR sites 4 and 5 even though no data for binding to these sites was analyzed. These parameters are determined solely on the basis of their effect on apparent CytR-CRP cooperativity. Nevertheless, the values obtained are completely consistent with the analysis of the extended CytR protection data in Fig. 6.

It is possible to distinguish between the two models as follows.
Table IV

| Parameter | Specific | Non-specific |
|-----------|----------|--------------|
| $\Delta G_{i}$ | -11.7 ± 0.1 | -11.7 ± 0.1 |
| $\Delta G_{2}$ | -13.0 ± 0.2 | -13.0 ± 0.2 |
| $\Delta G_{3}$ | -10.6 ± 0.2 | -10.5 ± 0.1 |
| $\Delta G_{4}$ | -1.4 ± 0.3 | -1.8 ± 0.2 |
| $\Delta G_{23}$ | -1.5 ± 0.3 | -1.2 ± 0.2 |
| $\Delta G_{13}$ | -3.1 ± 0.4 | -2.9 ± 0.2 |
| $\Delta G_{12}$ | -8.7 ± 0.5 | |
| $\Delta G_{23}$ | -9.3 ± 0.4 | |
| $\Delta G_{123}$ | -2.2 ± 0.2 | -2.5 ± 0.2 |
| $s$ | 0.057 | 0.058 |

The Nonspecific Additional Sites model results in a free energy change for pairwise cooperative interaction between CytR dimers bound to adjacent sites that is positive, thus indicating negative cooperativity. This requires nonspecific binding of CytR adjacent to the specific CytR site to be less probable at a given CytR concentration than elsewhere on the DNA. Direct observation contradicts this; protection by CytR of DNA sequences overlapping CRP1 and CRP2 occurs at lower CytR concentration than elsewhere on the DNA. Accordingly, we reject the Nonspecific Additional Sites Model and conclude that CytR binding to sites overlapping CRP1 and CRP2 is specific.

**Gel Mobility Shift Analysis of CytR Binding to deoP2**—The parameter values in Table IV predict significant populations of deoP2 species with 0, 1, 2, and 3 CytR dimers bound at intermediate CytR concentration, since the binding affinities of CytR for its three thermodynamically defined deoP2 binding sites differ by only 20-fold from highest to lowest. Gel mobility shift titrations of CytR binding were conducted to evaluate the existence of the predicted higher order ligation states. The 285-bp deoP2 DNA fragment was used in these experiments to avoid complications due to CytR binding to high affinity upstream and P1 sites.

In addition to the unliganded deoP2 band, at least four distinct shifted bands are observed as a function of increasing CytR concentration (Fig. 8, lanes 6 to 14). Similar observations have recently been reported independently (50). At the highest CytR concentrations, deoP2 migrates as a single band whose mobility continuously decreases with increasing CytR concentration (lanes 18-20). To investigate whether nonspecific CytR binding is responsible for these observations, the concentration of carrier, CT-DNA, was systematically varied from 0 to 3.2 μg/ml. The latter is a 2500-fold excess over deoP2 DNA and provides approximately a 1:1 mol ratio of nonspecific binding sites to the highest CytR dimer concentration used. Even at this concentration, CT-DNA still had no affect on the CytR concentration dependence of first three of the lower mobility bands. Therefore, these represent specific, CytR decoP2 complexes. Decreasing mobility of the final band was shifted toward higher CytR, suggesting that it represents nonspecific DNA binding.

The fraction of deoP2 in each band was determined as described under “Materials and Methods.” For purposes of this analysis, the unliganded deoP2 band and the next three highest mobility bands were individually quantitated to yield $\Theta_{3}$; lower mobility bands apparent in lanes 15 and above are not well resolved on the gel and so were lumped together to yield $\Theta_{4}$. To make a quantitative comparison to our model in Table III, the data were analyzed using the expression

$$\Theta_{i} = e^{-\Delta G_{i}/RT} \cdot [\text{CytR}] \cdot Z$$

(Eq. 3)

where $\Delta G_{i,M}$ is the free energy change for binding of $i$ CytR dimers to unliganded deoP2 (i.e., related to the macroscopic product association equilibrium constant by $K_{i,M} = e^{-\Delta G_{i,M}/RT}$) and $Z$ is the binding polynomial (51) equal to

$$\sum_{i=0}^{4} e^{-\Delta G_{i,M}/RT} \cdot [\text{CytR}]^i.$$  

(Eq. 4)

This formulation interprets each successive retarded band in the gel shift as representing one additional CytR dimer bound. This is the simplest, albeit not the only, possible interpretation. The lowest mobility bands that were lumped together must represent a distribution of binding stoichiometries. But for simplicity, the fraction $\Theta_{4}$ was treated as representing exactly 4 CytR dimers bound.

Estimates of $\Delta G_{i,M}$ obtained in this manner are compared in Table V to values predicted using the Specific, Additional Sites model in Table IV (e.g. $\Delta G_{1,M} = -RT \ln(e^{-\Delta G_{1,M}/RT} + e^{-\Delta G_{23,M}/RT} + e^{-\Delta G_{12,M}/RT} + e^{-\Delta G_{13,M}/RT})\cdot \Delta G_{i}$ defined in Table III). By this comparison, the gel mobility shift titration assesses slightly weaker overall binding (~3-fold in affinity) than does footprint analysis. Such differences are common, presumably due to well understood kinetic artifacts in the gel mobility shift experiment (55, 56, 52–55). A more salient observation is that this difference is constant for $\Delta G_{1,3}$, $\Delta G_{2,3}$, and $\Delta G_{1,2,3}$. Thus, the relative distributions of deoP2 species with 1, 2, or 3 CytR dimers bound as assessed by gel mobility shift titration is identical to what is predicted when the footprint titration data are analyzed according to the Specific Additional Sites model.

**Inhibition of CRP Binding by Cytidine-ligated CytR**—A second testable prediction of our model is based on the unusual mechanism of induction in which cytidine is an effector of heterologous cooperative interaction between CytR and CRP (62) rather than of intrinsic binding of CytR to DNA. By eliminating cooperativity between CytR and CRP bound to flanking sites, cytidine should make CytR into a simple competitor for CRP binding to CRP1 and CRP2. To test this prediction quantitatively, we conducted parallel titrations of CRP binding in the presence of saturating cytidine, with and without fixed but now subsaturating CytR (Fig. 9).

Free energy changes obtained for CRP binding alone in this experiment were $\Delta G_{1} = -11.8 \pm 0.2$ kcal/mol and $\Delta G_{2} = -13.3 \pm 0.1$ kcal/mol. These are indistinguishable from the intrinsic free energy changes, $\Delta G_{1}$ and $\Delta G_{2}$ (Tables I and IV), thus confirming no effect of cytidine on intrinsic CRP binding. When 0.2 μM cytidine-ligated CytR was subsequently added, CRP affinity for CRP1 and CRP2 dropped by over 2-fold, giving $\Delta G_{\text{CRP1}} = -11.3 \pm 0.1$ kcal/mol and $\Delta G_{\text{CRP2}} = -12.9 \pm 0.2$ kcal/mol. These are within 0.2 kcal/mol of what is predicted, when calculated using parameter values listed in Table IV for CytR binding. $\Delta G_{13}$, $\Delta G_{12}$, and $\Delta G_{123}$ were set equal to 0 to mimic the loss of cooperativity that results from cytidine binding by CytR. Predicted and observed curves are compared in Fig. 9.

Experimental resolution of CRP binding is limited at higher CytR concentrations; substantial protection from DNase I cleavage by CytR binding alone obscures any changes when CytR is displaced by CRP. However, we do observe an additional few-fold decrease in apparent CRP affinity when the CytR concentration is increased to 0.4 μM, consistent with a simple competitive model.

**DISCUSSION**

The most significant aspect of the CytR regulon is that a complex pattern of coordinate and differential regulation of a large number of unlinked transcriptional units is provided by interactions between CRP, CytR, and the DNA sequences of the various promoters. This illustrates a common strategy by which
a small number of transcription factors directs broad gene regulatory programs. Our goal was to provide a quantitative description of these interactions at *deoP2*, both in order to understand how individual macromolecular interactions and promoter states combine to regulate transcription from *deoP2* and to serve as a model for investigation of other CytR-regulated promoters. This has led to the discovery of a feature of *deoP2* which has not been described previously for this or other gene regulatory systems and which might lie at the heart of the regulatory mechanism of all CytR-regulated promoters. Specifically, CytR binds to multiple closely spaced or even overlapping sites on DNA with differing affinities and not to just a single operator as previously presumed. Interaction of CytR with these sites provides a complex pattern of both competitive and cooperative interactions between CytR and CRP bound to CRP1 and CRP2.

**Cooperative and Competitive Interactions of CytR**—The initial indication and one functional consequence of these interactions is that while saturation of CRP1 and CRP2 greatly increases the apparent affinity of CytR for its putative operator located between these, saturation by CytR has a much lesser effect on the apparent affinity of CRP for CRP1 and CRP2 (Table I) (21). A similar effect has been noted also for the *nupG* promoter (11). Because the Gibbs free energy is a thermodynamic function of state, the total free energy change to saturate either of these promoters with both proteins, whether by CRP binding followed by cooperative CytR binding or by the reverse, is necessarily the same. It is evident that the experimental observations can only be rationalized by additional macromolecular interactions, ones that have not been properly accounted.

Neither contamination of one or another of the protein preparations by another interacting molecule nor some similar experimental artifact provides a plausible explanation; similar observations were made on different regulatory regions by different laboratories using different experimental protocols. On the other hand, CytR binding to additional sites that compete with CRP(cAMP)1 for binding to CRP1 and CRP2 presents a plausible hypothesis based on just a qualitative evaluation of the effects. At high CytR concentration, favorable interactions with CytR would increase apparent affinity of CRP(cAMP)1 for CRP1 and CRP2. But this would also be partly offset by unfavorable competitive interactions.

As our analysis indicates, such competition also accounts...
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Free energy changes corresponding to macroscopic product equilibrium constants for CytR binding to deoP2

Table V
Comparison of distributions of CytR-ligated deoP2 as obtained from analysis of gel mobility shift titrations and predicted by the Additional Specific CytR Binding model (Table III). Gel mobility shift data were analyzed assuming four CytR sites to yield free energy changes corresponding to the macroscopic product equilibrium constants for binding of one \( \Delta G_{1,M} \) to four \( \Delta G_{4,M} \) CytR dimers. Predicted values were calculated from the parameter values listed in Table IV as described in the text.

| Observed: \( \Delta G_{1,M}^a \) | \( \Delta G_{2,M} \) | \( \Delta G_{3,M} \) | \( \Delta G_{4,M} \) | \( \Delta G_{1,M}^a \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| \( -9.8 \pm 0.1^a \) | \( -19.2 \pm 0.1 \) | \( -27.9 \pm 0.1 \) | \( -38.2 \pm 0.2 \) | \( 0.033 \) |

\( ^a \) kcal/mol at 20°C.

Quantitatively for the binding. Excellent fits to all of the titration data for both CRP and CytR, whether alone or in combination, and for both wild type and reduced valency deoP2 were obtained by global analysis using simple models for the competing sites. The mathematical formulations considered only thermodynamic properties of the additional CytR sites. These yielded well bounded estimates of CytR affinity for the competing CytR sites even though the data for these sites were not analyzed. These are specific sites for CytR binding. CytR binding to a single operator does not simply nucleate nonspecific binding to adjacent DNA sequences.

The model's quantitative predictions were tested against three independent criteria and found consistent with the experimental facts in each instance. First, estimated affinities for the hypothesized additional CytR sites match the CytR concentration dependence of the DNase I protection in CRP1 and CRP2. Second, gel mobility shift analysis demonstrated multiple, specific deoP2-CytR complexes, whose populations are consistent with quantitative predictions of the model. Third, under inducing conditions in which binding of cytidine to CytR eliminates positive cooperativity between CRP and CytR (6), CytR was found to be a competitive inhibitor of CRP binding.

CytR Binding Sites Upstream from deoP2 Define a Recognition Motif and Demonstrate an Array of Overlapping deoP2 Sites—In addition to these specific CytR binding sites in the P2 regulatory region, we have identified two other CytR binding loci. These are located about 240 bp upstream from P2 and in the −10 region of deoP1, respectively. These sites are also specific for CytR binding as judged both by the occurrence of localized DNase I protection and by competition with non-regulatory DNA in both footprint and gel shift experiments. In fact, the P2 upstream site has the highest affinity CytR binding that has been reported (cf. Refs. 6, 8, 10). Therefore, these DNA sequences constitute specific CytR recognition sequences.

We have compared these sequences to the regulatory region of deoP2 and other CytR-regulated promoters (Fig. 10) to further refine the consensus CytR recognition motif. Guiding this comparison is a 5-bp motif, TGCAA, previously proposed based on comparative sequence analysis (6) and supported by an analysis of mutations that affect CytR binding and regulation of deoP2 (17). Pairs of inverted and/or direct repeats of this motif with variable spacing (2–5 bp) are thought to constitute CytR operators in the different CytR-regulated promoters (cf. Refs. 9, 17 and Fig. 10). However, if based on the criterion of most probable nucleotide at each position in these putative CytR operators, then the 6-bp sequence, TTGCAA, defines the consensus. This palindromic motif obviates any distinction between inverted and direct repeats, as previously noted for deoP2 (17).

Fig. 10 shows the results when the deoP2 regulatory sequences are searched for both individual matches to the 6-bp motif (with the criterion that at least 4 of 6 bp match) and pairs of such motifs with the requisite spacing (2–5 bp). By either criterion, what emerges is a continuous array of adjacent and overlapping sites, ranging from the promoter distal edge of CRP2 to at least the promoter distal edge of CRP1. The consensus is best matched by the site previously identified as the CytR operator, centered at −70.5 bp. This site coincides with the highest affinity binding we observe by footprint titration. The putative flanking sites in both directions match the consensus less well, consistent with the footprints which indicate decreasing affinity for CytR binding to these sequences.

The sequences protected from DNase I cleavage by CytR binding to the upstream and P1 sites are also coincident with appropriately spaced pairs of motifs (Fig. 10). These sites both appear to comprise pairs of overlapping CytR binding sites. The P1 site contains a single perfect match to the motif flanked by less perfect matches, only 3 of 6, yet the affinity for CytR binding here is the same as for the −70.5 P2 site. This is significant in relation to the CytR binding observed at CRP1. While there is no pair of motifs in CRP1, individual motifs are arrayed there and even downstream to the −10 promoter element. At least in CRP1, these apparently suffice for weak yet still specific CytR binding, consistent with the protection observed.
not unique to deoP2 but instead are also found in other CytR-regulated promoters (Fig. 10). Evidence of their functional significance in those promoters is abundant. For example, while extended protection patterns in nupG (both DNase I and chemical modification reagents) have been interpreted as resulting from CytR binding to two perfect TGCAA motifs separated by an 11-bp spacer (11), this overlooks several additional CytR recognition motifs, including one located in the middle of the proposed 11-bp spacer. Together these define four separate, adjacent, and overlapping binding sites. Because titration studies were not conducted it isn’t possible to assess either individual affinities for CytR or the mutual effects of CRP binding and of CytR binding to different sites. However, the array of CytR sites overlaps CRP1, the lower affinity of the two CRP sites in nupG (11) and consequently the most easily affected by competition from CytR. A single CytR recognition motif within CRP2 might also constitute a weak binding site, analogous to the situation in deoP2. The structure of nupG is an inversion of deoP2, and similar interactions between CytR and CRP, no doubt, explain the apparent one-way stimulation (11).

Extended binding has been observed also for both cytRP (10) and udp (9). In both cases, CytR concentrations in the 10 nM range protect a single site. But additional protection is observed at only a few-fold higher CytR concentration, exactly in line with our deoP2 data. The particular protection patterns conform to the distribution of CytR recognition motifs shown in Fig. 10: individual loci mapped by hydroxyl radical footprinting in the −50 to −70 region of cytRP (10) and DNase I protection of the entire CRP1/CRP2 region of udp (9). This additional protection has been interpreted as aggregation of CytR on the DNA (10). However, since CytR does not aggregate in solution at sub-μM concentration3 this should be interpreted as protein binding to DNA rather than protein aggregation per se. It is also noteworthy that gel mobility shift experiments on these systems have not provided evidence for multiple liganded complexes, whether in the presence or absence of CRP(cAMP)3 binding. However, none of these experiments (cf. Refs. 6, 9–11) was conducted at saturating CytR concentration.

TsxP2 also offers evidence of similar interactions with CytR. CytR is reported to protect only a 20-bp sequence (7) corresponding to the putative CytR operator that overlaps CRP1 (Fig. 10). Because these experiments used extracts of unmutated CytR concentration, they don’t address whether additional lower affinity sites also exist. But when CRP is added to this same CytR concentration, cooperative binding of CytR and CRP produces a footprint that extends beyond CRP1 to the −10 region of the promoter. The region protected is much larger than by either protein alone, and the protection pattern in CRP1 is distinctly different from that produced by CRP binding alone. Supporting the interpretation that this represents additional CytR binding is a promoter mutation that greatly reduces CytR-mediated repression (7). The mutation, located in the protected region downstream from CRP1 (Fig. 10), is in a CytR recognition motif.

Energetics of Competition and Cooperativity—The model we developed to analyze our binding data was developed as the simplest model to rationalize the observed energetics. It clearly oversimplifies the actual situation. As there appear to be more than three CytR binding sites, the saturating CytR stoichiometry, whether in the presence or absence of CRP(cAMP)3, isn’t obvious. It shouldn’t be a surprise that the gel mobility shift assay indicates more than three ligation states (Fig. 8). Also since adjacent sites overlap, CytR binding to these should be self-competitive, a feature we did not incorporate. Perhaps this accounts for the small difference between predicted and observed competitive binding curves in Fig. 9. In recognition of these facts, our formulation of competition should be considered as a phenomenological description only.

On the other hand, the free energy changes for cooperativity between CytR and CRP are independent of the particular model and so appear to provide accurate descriptions of these interactions. It seems significant that ΔG13 and ΔG23 are equal. If direct protein-protein interactions provide the driving force for cooperativity, as has been presumed (23 58), then CytR bound to the intervening site must make substantially identical interactions with CRP, whether bound to CRP1 or to CRP2. This situation is reminiscent of the bacteriophage λ CI repressor which similarly makes identical pairwise cooperative interactions between adjacent sites with spacing ranging from 3 to 7 bp (56). Second, it may also be significant that the three-way cooperativity, i.e. with both CRP sites filled and CytR bound in between, equals the additive sum of free energy changes for these pairwise interactions. This suggests that CytR bound to the intervening site simultaneously contacts CRP bound to both CRP1 and CRP2 and with no influence of either pairwise interaction on the other. We might refer to these as ‘complementary pairwise’ interactions by comparison to the CI repres-
sor pairwise interactions that are not independent of one another (39, 56) and are referred to as “alternate pairwise.”

How can CytR can make the same interaction with CRP bound to CRP1 and CRP2 given spacers of 15 and 8 bps? There are several possibilities. First, CytR might have substantial structural flexibility as might result if its DNA binding head and inducer binding domain are only loosely tethered to one another. Such flexibility could also be invoked to explain CytR’s ability to bind pairs recognition motifs with variable spacing of 2–5 bp in different operators. The structures of two homologues, PurR and LacR, are known from crystallographic studies (57–60). In these cases, the DNA binding helix-turn-helix and core domains are connected by hinge helices which form stable structures only when the repressors are bound to DNA (58, 60). Perhaps CytR differs from those by not forming stable structures even when bound. Second, there might be a specific three protein, CRP-CytR-CRP complex that is asymmetric with respect to its interactions with DNA. Third, interaction with CRP might involve a repositioning of CytR on the DNA as has been suggested independently (62). Our data do not address this question; further investigation is necessary.

Role of Competition and Cooperativity in deoP2 Regulation—The important question remaining is how these multiple competitive and cooperative interactions between CytR and CRP regulate transcription. The energetics alone cannot provide a definitive answer but do suggest possibilities (and eliminate others). The common perception is that heterologous cooperativity serves to recruit CytR as CRP fills the CRP sites (6, 11). In this view, CytR is presumed to be an ineffective inhibitor of transcription in the absence of CRP binding only because its free concentration is insufficient to fill the site. The total effect of cooperativity with both CRP sites on CytR affinity is 100–1000-fold in different promoters and conditions (cf. Table I and Refs. 6, 8) just about sufficient to provide such a switch. But simple recruitment of such sort makes little functional sense, as it would yield only two significantly populated states, i.e., CRP and CytR sites empty or CRP and CytR sites filled. The result would be two-state, on or off, regulation. Since the combination of appropriate promoter (strong or weak) and either protein alone is sufficient to provide such regulation, it isn’t clear why a more complex mechanism would have evolved.

On the other hand, considering both cooperativity and competition together suggests a substantially more complex role for CytR. We start with the premise that CytR is a modulator of CRP activation rather than a repressor per se, a view consistent with the fact that CytR is ineffective in the absence of CRP binding. In this view the state of CRP activation is as important as occupancy by CytR. The tandem CRP sites and pairwise heterologous cooperativity are crucial to such a role as is the suggestion that the molecular mechanism of CRP activation differs at class I versus class II sites (63). CRP is proposed to activate transcription by making direct protein-protein contacts with the C-terminal domain of the α-subunit of RNAP, thereby directing interactions between RNA and otherwise nonspecific DNA sequences (64), substituting for the UP promoter element found in some particularly active genes (cf. Ref. 65 and references therein). A surface loop of CRP that is involved in the protein-protein contact has been identified (66). The subunit of CRP that contacts the α-RNAP subunit depends on the promoter architecture; it is the proximal subunit at class I sites (CRP2) but the distal subunit at class II sites (63). In either case, the new RNAP-DNA interaction would be in the sequence between CRP2 and CRP1, precisely where CytR binds. In fact, RNAP has been reported to make minor groove contacts in this region, dependent on CRP binding (62). CytR should function as an RNAP antagonist by competing with this upstream RNAP-DNA interaction.

At low CytR concentration, increasing cAMP concentration directs a two-stage activation: first, of class I as CRP2 fills and second, of class II as CRP1 subsequently fills. Because heterologous cooperative interactions are complementary pairwise in nature, recruitment of CytR via heterologous cooperativity is also a two-stage process. First, pairwise cooperativity between CytR and CRP (cAMP)1 bound to CRP2 yields about a 10-fold increase in effective CytR affinity. Although a significant effect, this alone is not sufficient to shut down CRP2-mediated activation. However as CRP(cAMP)1 subsequently binds CRP1, the additional pairwise cooperative interaction should now cause CytR to compete effectively for CRP2-RNAP interactions and so tend to shut down CRP2-mediated activation.

We suggest that cooperative CytR binding has very different effects on class I CRP activation mediated by CRP2 versus class II activation mediated by CRP1. Under these conditions of low CytR concentration, CytR might be an ineffective repressor of the latter. As such, CytR would act initially as a switch from one kinetic mode of CRP activation to another. There are two reasons for thinking this should be so. First, activation at class II sites has been reported which appears to bypass these critical interactions between α-RNAP and its interactions with DNA (63, 67) but which instead involves interactions with other components of RNAP. CytR binding should not compete with such a mechanism. Second, while mutant deoP2 with only CRP1 functional (i.e., CRP2) displays nearly the same extent of CRP activation (presumably class II-mediated) as wild type, it is not effectively repressed by CytR (20). Occupancy of the DNA sites by CytR site cannot account for this. At the high CRP(cAMP)1 concentration required to significantly load CRP1, the net effect of cooperativity and competition produces only a slight difference in CytR occupancy whether deoP2 is wild type or CRP2 (Table I).

Additional CytR sites and competition with CRP might play several roles in such a scheme. The primary role, regardless of specific mechanism, is probably to provide exactly the observed one-way stimulation. This is accomplished by partially decoupling CRP binding from CytR binding. Simple cooperativity is of course a two-way street, and as such, CytR would be a net recruiter of CRP as well as the reverse. This would proportionally diminish the effectiveness of CytR as a negative regulator. On the other hand, the combined effect of competition and cooperativity on CRP binding to both CRP1 and CRP2 is small (Fig. 7). Consequently, CytR is not a significant net recruiter of CRP. Thus, while repression, which we view as modulation of activation, depends on a complex interplay between cAMP, CytR, and cytidine concentrations, the initial activation is controlled only by cAMP concentration and is largely independent of CytR and cytidine. The second, simpler role of competition comes into play at very high CytR concentrations where CytR simply becomes a net inhibitor of CRP binding. This effect occurs first at CRP2 due to the higher affinity of CytR for its competing site(s), thus helping to facilitate the switch from class I to class II CRP activation. However, it also provides a final, cutoff of CRP-mediated activation at sufficient CytR concentration.

Finally, one can speculate on the role for overlapping multiple CytR sites (Fig. 10). Presumably these cannot be occupied simultaneously. Repositioning of CytR between these sites would be possible, particularly in configurations with only one CRP site (CRP1 or CRP2) occupied. Repositioning might provide a configuration in which RNAP makes favorable contacts with upstream DNA sequences even while CytR is still bound. These RNAP-DNA interactions would now compete solely with the difference in free energy changes (intrinsically cooperative) to bind CytR at one location versus another. The free energy
penalty is substantially less than if CytR must be displaced entirely in order for RNP to interact with upstream DNA sequences. Thus, repositioning of CytR could have a profound effect on RNP-CRP-DNA interactions and therefore on the effectiveness of CytR-mediated repression.

**Role of Competition and Cooperativity in Differential Regulation**—Bacterial promoters are often described as having a small number of functional states, e.g. basal, activiting, repressing, and inducing. The accuracy of this view is questionable, even when considering simple catabolic operons which need only be responsive to availability and requirement for an individual metabolite. Coordinate regulation of unlinked operons poses an even more complex question. CytR regulates expression from different cistrons of enzymes and transport proteins involved in both catabolism and recycling of nucleosides. To balance the flux of nucleoside metabolites, regulation of both absolute and relative enzyme levels must be responsive to widely varying metabolic conditions. Regardless of the particular details, the defining feature of deoP2 regulation is a complex and highly interdependent array of ligation states. This in turn controls the expression of a large number of promoter units. The reason for this would appear to be to provide for continuous modulation of each of the genes as a multi-variant function of the metabolic state of the cell.

Based on the structures of the promoters and on experimental observations made on other promoters, it seems evident that cooperative and competitive interactions such as we detail here are not unique to deoP2. Instead this appears to be a general feature of CytR-mediated control of gene expression. In comparing the structures of the different CytR-regulated promoters, most have similar tandem CRP sites. What appears to distinguish them are their very different arrays of CytR binding sites. These presumably result in very different patterns of cooperative and competitive interactions. While one necessarily looks to unique features of the different promoters to provide for their differential regulation, the existence of a common theme or organizing principle to those differences is inherently attractive. Therefore, while we cannot predict the specific effects of these different arrays of CytR sites on regulation of other promoters, we can reasonably speculate that the different balance of similar effects is critically involved in the mechanism of their differential regulation.

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