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The Cooperative Binding Energetics of CytR and cAMP Receptor Protein Support a Quantitative Model of Differential Activation and Repression of CytR-Regulated Class III *Escherichia coli* Promoters

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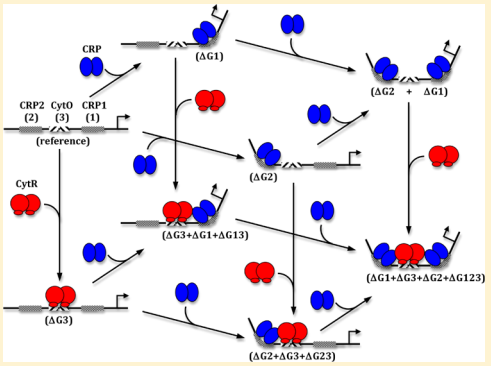
Supporting Information

ABSTRACT: cAMP receptor protein (CRP) and CytR mediate positive and negative control of nine genes in *Escherichia coli*, most of which are involved in nucleoside catabolism and recycling. Five promoters share a common architecture in which tandem CRP sites flank an intervening CytR operator (CytO). CytR and CRP bind cooperatively to these promoters to form a three-protein, DNA-bound complex that controls activation and repression, the levels of which vary markedly among the promoters. To understand the specific combinatorial control mechanisms that are responsible for this outcome, we have used quantitative DNase I footprinting to generate individual site isotherms for each site of protein−DNA interaction. The intrinsic affinities of each transcription factor for its respective site and the specific patterns of cooperativity and competition underlying the molecular interactions at each promoter were determined by a global analysis of these titration data. Here we present results obtained for *nupGP* and *tsxP2*, adding to results published previously for *deoP2*, *udpP*, and *cddP*. These data allowed us to correlate the reported levels of activation, repression, and induction with the ligation states of these five promoters under physiologically relevant conditions. A general pattern of transcriptional regulation emerges that allows for complex patterns of regulation in this seemingly simple system.



The *Escherichia coli* CytR regulon comprises nine unlinked transcriptional units[1] that encode enzymes and transport proteins involved in nucleoside catabolism and recycling. Twelve promoters are regulated both positively and negatively by the combined interactions of two proteins: the cAMP receptor protein (CRP), a global regulator of carbon metabolism,[1,2] and the cytidine regulator (CytR), a member of the LacR family of homologous bacterial repressors.[3] CRP activates transcription of hundreds of genes via direct contacts with components of RNA polymerase.[4] CytR is distinguished from all other LacR family members by two unique features that underlie its regulatory mechanism. First, CytO-bound CytR does not by itself repress transcription; instead, CytR functions only in concert with the CRP homodimer−cAMP complex (CRP_2−cAMP)[5] with which it binds cooperatively to DNA.[6] Second, CytR is a highly adaptive DNA binder. Its operators (CytO) contain a pair of recognition motifs, usually but not always inverted, that are separated by a central spacer whose length varies from 0 to 9 bp in different promoters.[7]

Promoters for five of the nine CytR-regulated genes share a common architecture in which tandem CRP operators flank an intervening CytO. CytR interacts directly with both of the flanking CRP_2−cAMP complexes to bind cooperatively,[8] yielding a three-protein complex in which CytR interferes with CRP-mediated activation.[9] Binding of cytidine to CytR attenuates the CRP−CytR interactions to restore CRP-mediated activation. This three-protein, DNA-bound complex constitutes a seemingly simple regulatory machine, but it is capable of surprisingly sophisticated control that is characterized by widely variable regulation of expression of these five genes. This raises the fundamental question of how a system featuring a similar arrangement of so few components is able to fine-tune the regulatory response to the differential requirements for expression of multiple genes.

One critical underlying factor in this process is that the two CRP operators direct distinct activation mechanisms. The distal site (CRP2) is centered ∼92 bp upstream from the transcription start in these CytR-regulated promoters (Figure 1). CRP2 directs a class I activation mechanism in which an activating region (AR1) of the proximal subunit of CRP_2−cAMP interacts directly with the C-terminal domain of the α-subunit (αCTD) of RNA polymerase (RNAP). This interaction directs the αCTD to bind DNA, thereby recruiting RNAP to the promoter.[10−14]

The proximal site (CRP1) is located ∼41 bp upstream from the start site where it occludes the −35 promoter element (Figure 1). CRP1 directs a class II mechanism in which a second activating region on CRP (AR2) of the proximal CRP_2−cAMP subunit interacts with the N-terminal domain of the RNAP α-subunit (αNTD). This interaction enhances the

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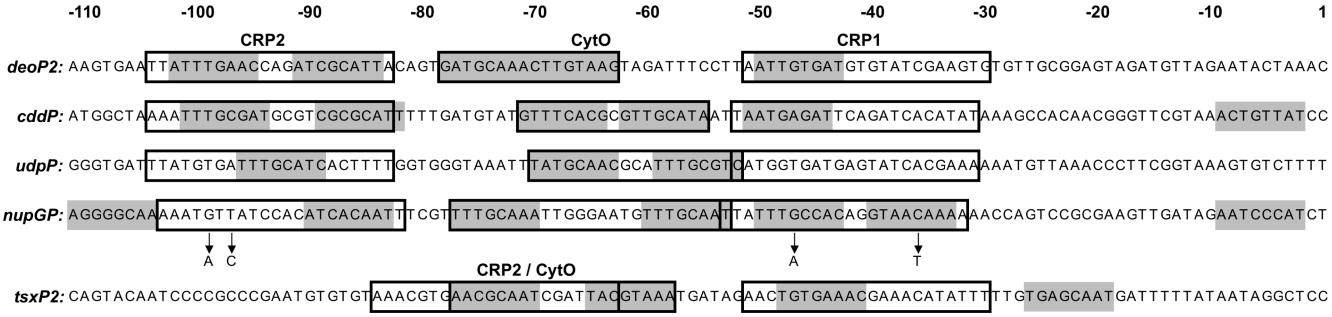

Figure 1. Type III CytR-regulated promoters. Sequences are numbered from +1 at the transcription start site. CRP sites (CRP1 and CRP2) and the CytR operator (CytO) are delineated by boxes with a white background; CytR recognition motifs are highlighted in gray. Transitions of 2 bp each in *nupGP* CRP1 and CRP2 as indicated eliminate sequence-specific binding by CRP to yield reduced valence promoters that we designate CRP1$^-$ and CRP2$^-$, respectively.

rate of open complex formation,[4,15,16] which is typically the rate-limiting step in initiation. In addition, AR1 of the distal CRP_2−cAMP subunit can interact with the CTD of the second RNAP α-subunit, thereby contributing to class I activation. Notice that while binding to the CytO places CytR in position to block both the AR1−αCTD and αCTD−DNA interactions that underlie the class I mechanism, it does not provide an explanation for the role of CytR in repressing the class II activation mechanism.

Promoters that feature multiple activators are termed class III. This is used somewhat imprecisely in the literature. However, in the context of the CytR-regulated promoters, it denotes both the fact that multiple CRP dimers are involved and a combination of class I and class II activation mechanisms. Several examples of class III activation are known in which the multiple activators operate synergistically to yield different outcomes depending on the particular promoter architecture.[17−20] The CytR-regulated promoters are distinct among these in two respects. (1) CRP recruits CytR as a corepressor, and (2) different outcomes are generated in different genes that share the same, rather than different, promoter architectures.

Differences in the energetics of the various macromolecular interactions contribute to these outcomes. To delineate these effects, we investigated and reported previously the detailed energetics of cooperative CytR and CRP interactions for three of the five class III CytR-regulated promoters, *deoP2*,[21] *udpP*,[22] and *cddP*.[23] These studies have documented substantial differences in intrinsic CRP binding affinity whose functional significance is the different distribution of class I, class II, and class III activation complexes. For example, the unusually high affinity of CRP for CRP1 of *cddP* correlates with unusually strong CRP-mediated activation of *cddP*.[23]

We also found considerable differences in the cooperative energetics and in the effect of binding of the inducer to CytR thereon.[21−23] The manner in which the separate pairwise cooperative interactions in the two protein CRP2−CytO and CytO−CRP1 complexes combine to generate the cooperative free energy in the three-protein, CRP2−CytO−CRP1 complex defines a range of effects from an additive combination of the pairwise interactions for *deoP2*[21] to mutually exclusive pairwise interactions in *cddP*.[23] The response to cytidine is similarly variable as a result of effects on the individual pairwise interactions that differ both between promoters and between CRP2−CytO and CytO−CRP1 within the same promoter.

These findings suggest that repression and induction are governed by interactions between the DNA-bound proteins and not by CytR binding per se. Indeed, we find that the fractional occupancy of CytO at *cddP* is approximately the same under both repressing and inducing conditions,[22] yet these differ in transcriptional activity by 20-fold.[24] The effect on *udpP* is even more stunning, yielding similar transcriptional activity with the CytR−cytidine species bound to CytO as compared to CytO empty (see below).

These observations point to significant interplay between CytR conformational states and CytR−CytO interactions. Swint-Kruse and colleagues have analyzed the critical role of the peptide linking the DNA-binding and ligand-binding domains of LacR family proteins in coupling conformational transitions in the ligand binding domains to control interactions with the DNA by the DNA-binding domains.[25−28] However, compared to the other family members, the CytR DBD is also highly dynamic. As we have shown, the CytR DBD is largely unfolded when free in solution, remains only loosely folded when bound nonspecifically to DNA, and coalesces to its fully folded structure only upon binding to specific DNA recognition sequences.[29] Further, we have shown that the variable spacing between operator half-sites controls the conformation and flexibility of operator-bound CytR.[30,31] The functional significance of this conformational adaptation is confirmed by a recent genomic analysis that found a high degree of conservation of orthologous CytO sites in 24 bacterial genomes, largely but not exclusively enterobacterial, as opposed to the variability of sites in different genes within the same genome.[32] These several observations suggest a conserved mechanism of differential gene regulation, one in which CytR is an effector of CRP-mediated activation, RNAP activity, or both, and not a more typical, purely passive bacterial repressor.

To assess these possibilities, we have now investigated binding of CytR and CRP to *nupGP* and *tsxP* to obtain the complete interaction energetics for all five class III promoters. The results extend the range of variations found previously. More significantly, the ΔG values allow us to compute the individual probability of every promoter configuration as a function of transcription factor and effector concentration. We have used this to analyze a large body of published data derived from reporter gene studies of these promoters conducted *in vivo*. The results of this analysis point to a very clear delineation of regulatory mechanisms. We find that occupancy of CRP1 and CRP2 alone accounts for differential activation due to the additive contributions of the class I and class II mechanisms. In contrast, CytO occupancy alone explains neither differential repression nor differential induction. Instead, these require additional control mechanisms that rely on the detailed architecture of the cytidine operators.

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Table 1. General Model for Binding of CRP and CytR to CytR-Regulated Promoters Showing Configurations and Free Energy States

	binding sites and configurations[a]						
	CRP2 (site 2)	CytR at CRP2 (site 5)	CytR (site 3)	CRP1 (site 1)	CytR at CRP1 (site 4)	free energy contribution[b]	free energy state[c]
1	O	O	O	O	O	reference state	ΔG_{s_1}
2	O	O	O	CRP	O	ΔG_1	ΔG_{s_2}
3	CRP	O	O	O	O	ΔG_2 ($\Delta G_2'$)	ΔG_{s_3}
4	O	O	CytR	O	O	ΔG_3	ΔG_{s_4}
5	O	O	O	O	CytR	ΔG_4	ΔG_{s_5}
6	O	CytR	O	O	O	ΔG_5	ΔG_{s_6}
7	CRP	O	O	CRP	O	$\Delta G_1 + \Delta G_2$	ΔG_{s_7}
8	O	O	CytR	CRP	O	$\Delta G_1 + \Delta G_3 + \Delta G_{13}$	ΔG_{s_8}
9	O	CytR	O	CRP	O	$\Delta G_1 + \Delta G_5$	ΔG_{s_9}
10	CRP	O	CytR	O	O	$\Delta G_2 + \Delta G_3 + \Delta G_{23}$	$\Delta G_{s_{10}}$
11	CRP	O	O	O	CytR	$\Delta G_2 + \Delta G_4$	$\Delta G_{s_{11}}$
12	O	O	CytR	O	CytR	$\Delta G_3 + \Delta G_4$	$\Delta G_{s_{12}}$
13	O	CytR	CytR	O	O	$\Delta G_3 + \Delta G_5$	$\Delta G_{s_{13}}$
14	O	CytR	O	O	CytR	$\Delta G_4 + \Delta G_5$	$\Delta G_{s_{14}}$
15	CRP	O	CytR	CRP	O	$\Delta G_1 + \Delta G_2 + \Delta G_3 + \Delta G_{123}$	$\Delta G_{s_{15}}$
16	CRP	O	CytR	O	CytR	$\Delta G_2 + \Delta G_3 + \Delta G_4 + \Delta G_{23}$	$\Delta G_{s_{16}}$
17	O	CytR	CytR	O	CytR	$\Delta G_3 + \Delta G_4 + \Delta G_5$	$\Delta G_{s_{17}}$
18	O	CytR	CytR	CRP	O	$\Delta G_1 + \Delta G_3 + \Delta G_5 + \Delta G_{13}$	$\Delta G_{s_{18}}$

[a]Binding sites arranged in order from promoter distal to promoter proximal. Each table row describes a particular configuration of protein-bound (CRP or CytR) and empty (O) sites. [b]Sum of free energy contributions from intrinsic binding of CRP and CytR (ΔG_i) and cooperative interaction between liganded sites [$\Delta G_{ij(k)}$] for each configuration of empty and filled sites. [c]ΔG_{s_N} denotes the total Gibbs free energy of configuration N relative to the unliganded reference state. This is equal to the sum of contributions shown in the previous column. The configuration number, N, indexes the s configurations specified in eqs 3 and 4.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes. Cytidine and cAMP stock solutions were prepared as described previously.[21] Bovine pancreas DNase I (code DPRF from Worthington) was stored in a buffer containing 50 mM Tris (pH 7.20), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, and 50% (w/v) glycerol. [α-^{32}P]-Deoxyribonucleoside 5′-triphosphates (3000 Ci/mmol) and unlabeled deoxyribonucleoside triphosphates were purchased from PerkinElmer and Invitrogen, respectively. Buffer components were electrophoresis grade or equivalent if available, and reagent grade otherwise.

CRP and CytR Purification. Expression of CRP from plasmid pPLcCRP1[33] in *E. coli* strain K12 and purification to at least 98% homogeneity[21] and expression CytR in *E. coli* strain BL21(DE3) and purification to at least 95% homogeneity have been described previously.[24] Total protein subunit concentrations were estimated on the basis of extinction coefficients (ε) of 18400 M^{-1} cm^{-1} at 280 nm for CRP[21] and 9460 ± 250 M^{-1} cm^{-1} at 276 nm for CytR.[31]

Promoter DNA Preparation. DNA fragments containing the *E. coli nupGP* and *tsxP2* promoters were obtained from *E. coli* strain K12 genomic DNA by polymerase chain reaction (PCR). The 295 bp region from position −151 to 144 relative to the *nupG* transcription start, and the 298 bp region from position −152 to 146 relative to the *tsx* transcription start were amplified. The purified PCR products were cloned into the Srf1 site of the Stratagene PCR-Script Amp SK(+) plasmid to generate plasmids pMRnupG and pMRtsx. Promoter-containing DNA fragments (~300 bp) generated by restriction digestion with *Sma*I (at a site created by ligation of the PCR product into the vector Srf I site) and *Bam*HI were purified by agarose gel electrophoresis. The *Bam*HI site was used for ^{32}P labeling using the Klenow fill-in reaction.[34]

nupGP mutants were designed to eliminate sequence-specific binding of CRP to either CRP1 (CRP1$^-$) or CRP2 (CRP2$^-$) based on systematic studies of the relative contributions from individual base pairs to binding[35] and transcriptional regulation.[36] Transitions of 2 bp in CRP1 to generate CRP1$^-$ and separately of 2 bp in CRP2 to generate CRP2$^-$ are indicated in Figure 1. These were generated using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. Sequences were confirmed by dideoxy sequencing.

Individual Site Binding Experiments. Quantitative DNase footprint titrations were conducted as described previously[21−23] in binding buffer consisting of 10 mM bistris, 100 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.0 mM NaN_3, 50 mg/L ovalbumin, and 1 mg/L CT-DNA. Mixtures containing CRP included 150 μM cAMP, a concentration that maximizes the fraction of CRP in its active form (0.64 ± 0.2) under these buffer conditions.[21] Binding titrations of either CytR or CRP in the presence of a fixed, near-saturating concentration of the other used 0.1 μM dimer for both CRP and CytR. At this concentration of CytR, CytO is ~85% saturated in both *nupGP* and *tsxP2*, though the weakest *nupGP* site that overlaps CRP1 is only ~50% saturated. At 0.1 μM CRP, the CRP operators are between 95 and >99.9% saturated.

DNase I exposure and electrophoresis were conducted exactly as described previously.[23] Dried gels were imaged by exposing a GE Healthcare storage phosphor screen for approximately 3 days. These were scanned at 176 μM

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resolution using either a Molecular Dynamics PhosphorImager 435 SI or GE Healthcare Typhoon 9410 instrument.

SAFA (semiautomated analysis of footprinting, National Center for Biomedical Computation at Stanford[37]) was used to define contiguous groups (or blocks) of bands protected from DNase I cleavage when a transcription factor binds to an individual operator site. A ^{32}P-labeled 100 bp DNA ladder was used to locate the promoter sites corresponding to these blocks. Blocks so identified were analyzed using ImageQuant version 5.2 (GE Healthcare) to obtain the individual site fractional protection, as described previously.[34,38] These data were first analyzed separately according to

$$P_{\mathrm{obs},i} = P_{\mathrm{o},i} + (P_{\mathrm{max},i} - P_{\mathrm{o},i}) \times \frac{e^{(-\Delta G_{\mathrm{app},i}/RT)+\ln[\mathrm{L}]}}{1 + e^{(-\Delta G_{\mathrm{app},i}/RT)+\ln[\mathrm{L}]}} \quad (1)$$

where P_{obs} is the fractional protection observed for binding site i at the free protein ligand concentration, [L], $\Delta G_{\mathrm{app},i}$ is the standard Gibbs free energy change that corresponds to the apparent association equilibrium constant for binding to site i ($\Delta G = -RT \ln K_{\mathrm{a}}$), and P_{o} and P_{max} are the baseline and maximal fractional protection attained at site i, respectively.[39] $\Delta G_{\mathrm{app},i}$ in this simple 1:1 binding equation provides a reasonable estimate of the individual site loading free energy change, $\Delta G_{\mathrm{Load},i}$,[40] and its confidence limits.[41]

Subsequent global analysis of the individual site CRP and CytR binding data was conducted according to the model defined by the promoter configurations described in Table 1. By accounting for all of the binding data in this manner, we are able to determine accurately the contributions to the free energy changes from cooperativity, competition, and induction in addition to the intrinsic affinity of each transcription factor for its respective site. The relative probability that a promoter will adopt any single configuration of occupied sites is

$$f_{s_N} = \frac{e^{(-\Delta G_{s_N}/RT)+i\ln[\mathrm{CRP(cAMP)_1}]+j\ln[\mathrm{CytR}]}}{\sum_N e^{(-\Delta G_{s_N}/RT)+i\ln[\mathrm{CRP(cAMP)_1}]+j\ln[\mathrm{CytR}]}} \quad (2)$$

ΔG_{s} is the sum of all free energy contributions for configuration s (Table 1) and i and j are the stoichiometries of bound CRP(cAMP)$_1$ complexes and CytR dimers in configuration s, respectively. The equation for saturation of a particular site by either transcription factor is obtained by summing the relative probabilities for all configurations in which the protein is bound to the site. For the reduced valence operators, CRP1$^-$ and CRP2$^-$, binding of CRP to the mutated site was treated as described in the text.

Global nonlinear least-squares analysis was performed using IgorPro version 5.05 (Wavemetrics Inc.) as described previously.[23] Variances from individual analyses of each binding curve using eq 1 were used to calculate normalized weights. The model described in Table 1 is nonlinear, featuring strongly correlated parameters. Parameter confidence limits for each of the ΔG values in the model were estimated by varying each parameter systematically while repeating the global analysis to estimate the remaining parameters (both ΔG values and $P_{\mathrm{o},i}/P_{\mathrm{max},i}$). The ratio of the variance obtained for each such fit to the variance at the global minimum was calculated. Ratios of variances were also separately calculated considering the data obtained for each binding site and set of effectors, e.g., for binding of CytR to CytO of the wild-type promoter in the presence of CRP and cytidine. This was done to eliminate combinations of parameters that yield an acceptable global fit at the expense of a poor fit to an individual component of the data set. The confidence limits reported correspond to the more restrictive of two criteria: (i) a ratio of variances for the global analysis corresponding to Fstat at the 65% confidence interval or (ii) a ratio of variances for any binding site and set of effectors corresponding to Fstat at the 95% confidence interval.

RESULTS AND DISCUSSION

Energetics of the Molecular Interactions for CytR-Regulated Promoters. The regulatory regions of the five class III, CytR-regulated promoters, aligned with the transcription start locations, are compared in Figure 1. With the exception of CRP2 in *tsxP2*, there is very little variation in the location of the CRP sites. However, the precise location of CytO does vary, and perhaps more significantly, so does the length of central spacers that separate the inverted sequences recognized by the repressor.

Our previous reports on *deoP2*, *udpP*, and *cddP* have highlighted differences in both the pattern of CytR–CRP cooperativity and the competition between CytR and CRP, the latter due to binding of CytR to variable arrays of satellite sites in addition to CytO.[21−23] Because the goal is to assess how these variations underlie differential regulation of CytR-regulated genes, the necessary first step is to analyze the cooperative energetics for the two remaining promoters, *nupGP* and *tsxP2*. We have described previously the required experimental strategy in our reports on *deoP2*, *udpP*, and *cddP*.[21−23] Consequently, we only summarize that strategy here and report the major findings. Details of the analysis are provided in the Supporting Information.

The experimental approach to delineate the effects of cooperativity and competition on the occupancy of the operator sites is to analyze the thermodynamic cycle for binding of each repressor alone to a given promoter, and also for the binding of both simultaneously (cf. the abstract graphic). Because each repressor binds to multiple sites, DNase I footprint titrations are employed to generate separate isotherms for each of the individual binding sites.

Figure 2 illustrates this approach with representative experimental data for the simplest example, that of binding of

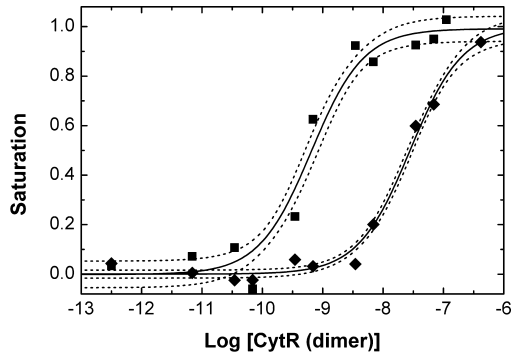

Figure 2. Binding of CytR to CytO of *nupGP*. Fractional saturation of CytO by CytR alone (squares) or by CytR in the presence (diamonds) of CRP at a concentration (0.1 μM) sufficient to yield 95% saturation of the CRP operators (see Experimental Procedures) is plotted as a function of log CytR dimer concentration. Solid curves represent analysis according to eq 1; dashed curves are the 65% confidence limits of the fitted curves. This analysis yields ΔG_{Load} values of −10.1 ± 0.08 and −12.3 ± 0.19 kcal/mol in the absence and presence of CRP, respectively. The relationship of the difference, $\Delta(\Delta G_{\mathrm{Load}})$, to total three-way cooperativity, ΔG_{123}, is discussed in the text.

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Table 2. Interaction Free Energy Changes for Binding of CRP and CytR to CytR-Regulated Promoters

	cddP[a]	*deoP2*[a]	*udpP*[a]	*nupGP*	*tsxP2*
ΔG_1	−13.6 ± 0.2	−11.7 ± 0.1	−12.0 ± 0.2	−11.2 ± 0.1	−12.0 ± 0.4
ΔG_2	−12.2 ± 0.3	−13.0 ± 0.2	−13.7 ± 0.2	−14.1 ± 0.1	not available
ΔG_3	−11.6 ± 0.2	−10.5 ± 0.2	−10.9 ± 0.2	−10.4 ± 0.3	−10.7 (+0.2, −0.3)
ΔG_{13}	−1.0 ± 0.4	−1.4 ± 0.3	−1.4 ± 0.2	−1.8 ± 0.1	−0.9 (+0.5, −0.2)
ΔG_{23}	−1.7 ± 0.4	−1.5 ± 0.3	−1.3 ± 0.3	−1.5 ± 0.1	
ΔG_{123}	−1.5 (+0.4, −0.3)	−3.1 ± 0.4	−2.3 ± 0.1	−2.3 ± 0.1	
$\Delta\Delta G_{13}$	−0.2 (+0.2, −0.3)	1.4	1.0 ± 0.3	0.7 ± 0.1	
$\Delta\Delta G_{23}$	1.1 ± 0.4	1.5	0.3 ± 0.3	0.5 ± 0.1	
$\Delta\Delta G_{123}$	0.2 ± 0.4	2.7	0.8 ± 0.4	1.0 ± 0.1	

[a] ΔG values in kilocalories per mole ± the 65% confidence interval. Asymmetric confidence limits are noted separately in parentheses where applicable. Sources are ref 23 for *cddP*, ref 21 for *deoP2*, and ref 22 for *udpP*.

CytR to CytO both alone and in the presence of a fixed and near-saturating concentration of CRP. The former represents the leftmost edge of the abstract graphic, and the latter approximates the rightmost edge. Analyzing each titration can be done in a model-independent manner to estimate the loading free energy change ($\Delta G_{\mathrm{Load},i}$), a model-independent quantity that reflects all interactions, including not only intrinsic binding to the local site but also contributions due to cooperative and competitive interactions resulting from interactions at other sites.[40,41] As illustrated in the abstract graphic, $\Delta(\Delta G_{\mathrm{Load},i})$ for the pair of titrations in Figure 2 would represent the cooperativity between CytR binding to CytO and CRP binding to the flanking sites, CRP1 and CRP2. Similar titrations of reduced valence promoters, in which specific binding of CRP to either CRP1 (CRP1$^-$) or CRP2 (CRP2$^-$) is eliminated via appropriate mutation of those sites, provide the means of assessing strictly pairwise cooperative interactions.

In actuality, $\Delta(\Delta G_{\mathrm{Load,CytO}})$ approximates only the cooperative free energy change for two reasons. (1) The CRP concentration is not sufficient for full saturation. (2) Binding of CytR to additional satellite sites competes for CRP binding and prevents complete filling of CRP1 and CRP2. The effect of the latter in particular can be strong. Delineation of the effects to obtain the Gibbs free energy changes for intrinsic binding and cooperativity requires model-dependent global analysis of fractional protection data for all combinations of titration experiments, conducted on both the wild-type and reduced valence mutant promoters. The complete data set for *nupG*, consisting of 77 individual site binding isotherms obtained from 42 separate footprint experiments, is summarized in Table S1 of the Supporting Information. Similarly, the *tsxP2* data set is summarized in Table S2 of the Supporting Information. The general molecular model is defined by the promoter configurations listed in Table 1. This model is well-established from our previous analyses of *deoP2*,[21] *udpP*,[22] and *cddP*.[23] Its major features are supported independently by the results obtained here for *nupG* and *tsxP2*. Specific features, such as the particular arrays of satellite binding sites for CytR and overlapping regions of DNase I protection, differ between promoters. These results are reported, and the application of the model to *nupG* and *tsxP2* is discussed in the Supporting Information.

Interaction free energy changes for all five *E. coli* class III CytR-regulated promoters, including those for *deoP2*, *udpP*, and *cddP* reported previously as well as for *nupG* and *tsxP2*, are listed in Table 2. These highlight several features that would be expected to contribute to differential regulation based on the distributions and functional activities of individual complexes. First, there is considerable variation in intrinsic CRP binding affinity. The values of ΔG_1 and ΔG_2 vary by ∼3 kcal/mol among the promoters, accounting for a 150-fold range of association constants from 2.3×10^8 to 3.3×10^{10} M^{-1} under these conditions. In consideration of our results for CRP2 of *tsxP2*, the range may extend even below 10^7 M^{-1}. Clearly, this confers very different sensitivities of the individual promoters to the cAMP concentration and, hence, to growth conditions. In addition, the relative affinity for CRP1 versus that for CRP2 is highly variable, ranging from approximately 10-fold for *cddP*, *deoP2*, and *udpP* to 25-fold for *tsxP2* and 200-fold for *nupGP*. CRP2 has the higher affinity in three cases and CRP1 in two. Considering the different modes of activation mediated by the two CRP sites, this leads to the expectation of highly variable stepwise responses to cAMP.

In contrast to CRP, CytR shows little variation in intrinsic affinity for CytO binding: affinities for four of five sites are indistinguishable from the mean value. Only *cddP* CytO, which features the optimal spacing between operator half-recognition sites,[7] has affinity outside this range, i.e., 8-fold higher. These results suggest relatively low intrinsic sequence specificity, a conclusion that is also supported by the existence of satellite binding sites in all five promoters. Consequently, the recruitment and positioning of CytR are dependent on cooperative interaction with CRP, as discussed previously.[8,42]

Pairwise cooperativity, for both CRP2−CytR and CytR−CRP1, is also relatively constant, with no ΔG_{ij} value being distinguishable from the mean value ($\Delta\bar{G}_{ij} = -1.4 \pm 0.3$ kcal/mol). However, the manner in which pairwise interactions combine in the three-protein, CRP−CytR−CRP regulatory complex is highly variable. Only in *deoP2* are pairwise interactions essentially additive; conversely, they appear to be mutually exclusive in *cddP*. This suggests that the particular arrangement of regulatory sequence, e.g., spacing in CytO, constrains the ability of CytO to interact freely with both flanking CRP dimers.

The effect of binding of cytidine to CytR on cooperativity, both pairwise and three-way, is similarly variable. *deoP2* exhibits a complete loss of cooperativity. The effects are similar but more modest at *nupGP*. More intriguing are *cddP*, for which the effect on CRP2−CytO cooperativity is substantial and the effect on CytO−CRP1 cooperativity is minimal, and *udpP*, for which the effect on CRP2−CytO cooperativity is minimal and the effect on CytO−CRP1 cooperativity is substantial. Evidently, there is not a simple linkage between binding of cytidine to CytR and CytR−CRP association; rather, there is interplay between cytidine-induced CytR conformational states and scaffolding constraints imposed by the promoters.

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Most intriguingly, the resulting effect on three-way cooperativity is minimal to none for both *cddP* and *udpP*, yet despite the fact that CytO remains occupied by CytR, both *cddP* and *udpP* are induced strongly.[24] Recall that because of the location of CytO, CytR would be expected to compete for both RNAP−CRP interaction and RNAP α-subunit binding to DNA. This mechanism of repression is necessarily disproved by the observation of strong induction when CytO remains occupied. However, if it is not simple occlusion, then what is the mechanism of repression and its relief under inducing conditions?

Modeling Activation and Repression of CytR-Regulated Promoters. To begin to address this question, we have developed a simple quantitative model of regulation. It derives from three simple principles. First, the distribution of ligation states for each promoter is governed by the energetics of the molecular interactions. Second, the individual ligation states are grouped to comprise a smaller number of functional states that are distinguished by differing transcriptional activity. Third, control of the distribution of functional states is the basis for gene regulation. The model is developed by iterating the mapping of individual ligation states into these distinct functional states while fitting for the transcriptional activities of the functional states that match experimental observations.

The feasibility of this approach derives from a fairly rich, albeit incomplete, historical set of functional data derived from reporter gene studies of these promoters in which genetic knockouts were used to assess the roles of CytR (*cytr*$^-$) and CRP (*crp*$^-$ and *cya*$^-$). Different combinations of mutations yield basal (*cytr*$^-$ plus *crp*$^-$ or *cya*$^-$), activated (*cytr*$^-$), repressed (wild-type), and induced (wild-type supplemented with cytidine in the growth medium) levels of transcription. Data from 19 separate published investigations, typically conducted in AB minimal medium supplemented with 0.5% glycerol, are listed in Table 3. For comparison using a common scale, the data were converted into relative transcription activity using the basal rate measured locally in the same study as an internal reference. A few of these studies determined the fold repression relative to the CRP-dependent activated rate rather than fold activation relative to the basal rate. In these cases, the average activated relative transcription rate was used to reference the values to the basal rate. Despite different studies using different reporter genes, strains, and genotypes, e.g., whether *crp*$^-$, *cya*$^-$, or *crp*$^-$ and *cya*$^-$, and whether *deor*$^+$ or *deor*$^-$, and despite slight variation in growth media, the experimental data are remarkably consistent with one another. This is evidenced by the high level of precision in 13 of the 25 values listed in Table 3 that were determined independently in more than one study.

To develop the model, we start by testing the hypothesis that CRP-mediated activation is governed solely by occupancy of CRP1 and CRP2 by CRP. Recall that CRP activates via distinct mechanisms depending on the site to which it is bound, thus generating classes of activation depending on whether it is bound to only CRP1 (class II) or CRP2 (class I) or to both CRP1 and CRP2 (class III). Accounting for these as four distinct functional states (basal plus three activated) yields

$$A_{[\mathrm{CRP}]} = f_1 + A_{\mathrm{II}} f_2 + A_{\mathrm{I}} f_3 + A_{\mathrm{III}} f_7 \qquad (3)$$

where $A_{[\mathrm{CRP}]}$ is the CRP concentration-dependent, relative transcription of a given promoter. The f_i terms are the fractional probabilities (given by eq 2) of a particular configuration of empty and filled CRP sites. These configurations are defined and indexed in Table 1. Note that because

Table 3. Transcription of CytR-Regulated Promoters Relative to the Basal Level[a]

	activating (*cytr*$^-$)			repressing (wild-type)			inducing (wild-type, cytidine)		
promoter	class III (CRP1/CRP2)	class I (CRP1-)	class II (CRP2-)	class III (CRP1/CRP2)	class I (CRP1-)	class II (CRP2-)	class III (CRP1/CRP2)	class I (CRP1-)	class II (CRP2-)
cddP	525 ± 601 (2)[47,48]	not determined	220 (1)[24]	16.5 ± 5.2 (5)[47−50]	not determined	22 (1)[49]	340 (1)[24]	not determined	not determined
deoP2	29.5 ± 2.1 (5)[5,47,51−53]	12.0 (1)[5]	19.3 ± 5.8 (2)[5,47]	3.5 ± 0.8 (9)[5,47,50−52,54,55]	3.7 (1)[5]	14.5 ± 3.6 (4)[5,47,56]	34 ± 6 (3)[46,57]	not determined	not determined
nupGP	45.1 ± 7.6 (2)[58]	not determined	3.8 (1)[58]	13.7 ± 1.1 (1)[58]	not determined	4.2 (1)[58]	not determined	not determined	not determined
tsxP2	not applicable	not applicable	16 ± 7.1 (2)[59]	not applicable	not applicable	3.6 ± 1.3 (3)[50,59]	not determined	not applicable	not determined
udpP	52.6 ± 17.6 (3)[60−62]	5.3 ± 1.8 (2)[60,62]	22.8 ± 13.8 (2)[60,62]	5.2 ± 1.0 (3)[60−62]	5.3 ± 1.8 (2)[60,62]	24.9 ± 3.9 (2)[60,62]	380 (1)[24]	not determined	not determined

[a]Relative enzyme activities in reporter gene studies using basal levels for internal reference standards as described in the text. Basal transcription was assessed in strains lacking both CytR (*cytr*$^-$) and CRP (*crp*$^-$ or *cya*$^-$). Values reported means ± the standard deviation, with the number of multiple independent measurements given in parentheses.

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activation is evaluated using *cytr*$^-$ strains, none of the CytR binding sites can be occupied; hence f_i for each of the other 14 species defined in Table 1 is zero. A_I represents the functional state defined by the ratio of activated (CRP site occupied) to basal (CRP site empty) transcription. This simple model attributes the differences in fold activation between the promoters to different site occupancies under the same conditions as governed by different binding affinities.

Data for reduced valence CRP1$^-$ and CRP2$^-$ promoters (Table 3) allow for initial separate analysis of class I and II mechanisms. We start with class II (CRP2$^-$), for which experimental observations are reported for all five promoters (Table 3, column 4). The solid curve in Figure 3 shows the

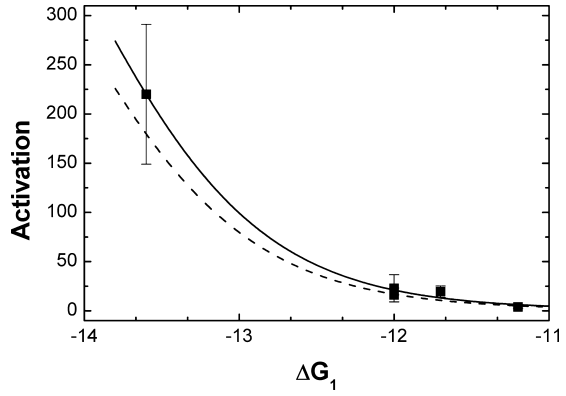

Figure 3. Class II activation of CytR-regulated promoters due to binding of CRP to CRP1 in *cytr*$^-$, *crp1*$^-$ strains. Solid points are relative transcription of values from column 4 of Table 3. The solid and dashed lines show the local fit of these data (eq 3) and the global fit of the data in Table 3 (eq 4), respectively. Parameter values are given in the text.

results of analysis according to eq 3 and based on the ΔG_i values that are listed in Table 2. A_{II} and $\ln[\mathrm{CRP(cAMP)}_1]$ (eq 2) are fitting parameters. This yields an A_{II} of 690, indicating a nearly 3 order of magnitude effect on transcription, and $\ln[\mathrm{CRP(cAMP)}_1] = -24.11$ (or 34 pM). This is several orders of magnitude lower than estimates for total cellular CRP based on Western blot analysis,[43] but Western blots measure total CRP, for both DNA-bound and free forms, and both the active and inactive ligation states of free CRP. Given the large number of CRP regulatory sites on the *E. coli* genome, the concentration of free $\mathrm{CRP(cAMP)}_1$ is necessarily buffered by the affinity of these sites to be in the 10−100 pM range, in good agreement with our model. The agreement between the observed values and this simple model is excellent, suggesting that features of the individual promoters, such as sequence context, promoter strength, etc., confer only secondary effects.

Class I-mediated activation (CRP1$^-$) has been investigated for only two promoters, *deoP2* and *udpP*, which precludes an unconstrained fit to eq 3. However, with $\ln[\mathrm{CRP(cAMP)}_1]$ as determined above, these two data points yield as an average value an A_I of 22 . Evidently, class I activation, which relies on the recruitment of RNAP, is ∼30-fold less effective than class II activation, which employs allosteric activation of RNAP.

Extending this analysis to the wild-type promoters, we conducted a global analysis of the activation data for the wild type and reduced valence mutants (Table 3, columns 2−4) using standard deviations of the mean values as weights. This yields parameter values that are indistinguishable from those from a separate analysis of the class I and II mechanisms [i.e., $A_{II} = 550 \pm 308$; $A_I = 16 \pm 6.5$; $A_{III} = 6000 \pm 5650$; $\ln[\mathrm{CRP(cAMP)}_1 = -24.06 \pm 0.51]$ (±standard errors)] and a reduced χ^2 of 2.1. This reflects a remarkably good fit considering the nature of the underlying functional data.

In considering activation of the wild-type promoters, a salient question is how class I and class II mechanisms combine in class III activation. We note the similarity between A_{III} and the product $A_I A_{II}$, suggesting that the class I and class II mechanisms are independent. To evaluate this hypothesis, we also analyzed the data subject to the constraint $A_{III} = A_I A_{II}$. This resulted in insignificant changes to parameter values ($A_I = 15 \pm 6.0$; $A_{II} = 610 \pm 331$; $\ln[\mathrm{CRP(cAMP)}_1] = -24.25 \pm 0.48$) and a reduced χ^2 of 1.9. The very slight decrease in χ^2 compared to that of the unconstrained fit indicates that A_{III} is not justified as an additional adjustable parameter.

Given the considerable success of this simple model to account for differential, CRP-mediated activation of the promoters, we have also attempted to extend the model to account for CytR-mediated repression. Equation 4 accounts for repression by including terms for the CytR-liganded species in Table 1 and adding the parameter R to represent repression, i.e.

$$A_{[\mathrm{CRP}]} = f_1 + A_{II}f_2 + A_I f_3 + f_4 + A_{III}f_7 + (1/R)(A_{II}f_8 + A_I f_{10} + A_{III}f_{15}) \quad (4)$$

Note that this defines R as constant fold repression when CytR is bound in any configuration. Other formulations that were evaluated, e.g., a constant transcription rate when CytR is bound in any configuration, do not fit the data. More complex formulations, e.g., a different fold repression for each configuration in which CytR is bound, are underdetermined by the data available.

Analysis of repression of the wild-type promoters yields a $\ln[\mathrm{CytR}]$ of -19.92 ± 0.35 ($\chi^2 = 2.5$). This corresponds to 2.2 (+1.1, −0.5) nM free CytR, in excellent agreement with an independent assessment of 4 nM reported previously.[44] This yields only a lower bound to R, the fold repression, as greater than several thousand. Such high repression is sufficient to

Table 4. Predicted Transcription of CytR-Regulated Promoters Relative to the Basal Level[a]

	activating (*cytr*$^-$)			repressing (wild-type)		
	class III (CRP1/CRP2)	class I (CRP1-)	class II (CRP2-)	class III (CRP1/CRP2)	class I (CRP1-)	class II (CRP2-)
cddP	260	1.5	174	19.5	0.94	53.8
deoP2	28.1	2.8	10.2	4.9	2.1	8.8
nupGP	38.5	7.8	4.9	11.4	4.2	4.4
tsxP2	not applicable	not applicable	16.2	not applicable	not applicable	13.4
udpP	89.6	5.5	16.2	7.8	2.7	12.1

[a]Relative enzyme activities calculated according to eq 4 and using parameter values as discussed in the text.

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largely counteract CRP-mediated activation when CytR is bound. Most of the transcription activity that remains is a reflection of the relatively low CytR concentration, which is subsaturating, though variably so, for all five promoters.

Table 4 presents the results of this analysis as the relative transcriptional activity for all five promoters. These fitted values correspond quite well to the experimental observations in Table 3 for activation of all five promoters, both the wild-type and reduced valence mutants, and for repression of the wild-type promoters. In particular, this simple model predicts accurately the rank order of effects of 14 of 15 experimental observations. The model does less well, however, for the repression of the reduced valence mutants. Comparing the values for repression to activation of the same promoters indicates that this model expects little or no repression by any two-protein, CytR−CRP complex. While this is consistent with experimental findings for many of the two-protein complexes (i.e., *udpP* class I and *deoP2*, *nupGP*, and *udpP* class II), it contradicts observation of very strong repression (75−90%) by the rest (*deoP2* class I and *cddP* and *tsxP* class II).

For *tsxP2*, the failure to account for the effect of the weak CRP2 site may contribute to the model's performance. Recent studies are somewhat equivocal as to whether CRP2 contributes substantially to the regulation of tsxP2.[35,36] However, effects of point mutations in CRP2 reported much earlier[45] provide fairly conclusive evidence that CRP2 does contribute significantly to CytR-mediated repression. We note that CytR−CRP cooperativity should enhance the specificity of CRP for CRP2, such that even quite weak and relatively nonspecific intrinsic binding might contribute to the formation of the three-protein repression complex. However, while this might account for the discrepancy between the predictions of the model and the experimental observations in the particular case of *tsxP2*, it cannot account for strong repression mediated by two-protein complexes in the other promoters.

Thus, it is evident that differences in the distribution of species alone cannot explain the observed pattern of repressive effects. It is also evident that this model does not explain the limited data for induction, in particular the relative transcription activity of *udpP* that is substantially greater in the presence of cytidine than even the level of fully activated expression in the absence of CyR. Promoter-specific effects are necessary to explain these patterns. On the basis of studies of *udpP*[22,24] and *deoP2*,[46] both we and others have proposed CytR to be an allosteric effector of CRP bound to CRP1 that switches its activity from activator of RNAP to repressor. This remains a plausible explanation in the face of the more comprehensive analysis we now present.

CONCLUSION

The utility of this simple transcription model is that it delineates effects that are governed entirely by the protein−DNA assembly energetics from those that are not. We find that differential activation of the CytR-regulated promoters is achieved simply by the variable affinity for CRP binding. Effects due to variations in promoter strength, the exact location of CRP operators, and flanking sequences are secondary. Class II activation mediated by CRP1 is the dominant mechanism of activation, with class I activation mediated by CRP2 being secondary. The comparative analysis supports our previous discussions of two-step activation resulting from the higher affinity of CRP2 than of CRP1 and subsequent recruitment of CytR to form the three-protein repression complex. The active CRP concentration is sufficient to saturate only the higher-affinity operators to an appreciable extent; the lower-affinity operators rely on CytR cooperativity to fill, so that activation mediated by these sites and repression are coincident. In this manner, remarkable differences in activation of these promoters are achieved simply by varying the CRP1 and CRP2 binding affinity. For example, the unusually strong activation of *cddP*, i.e., an order of magnitude greater than those of the other promoters, is due primarily to the reversal of CRP1 versus CRP2 affinity.

The model also delineates where it is necessary to invoke higher-order regulatory interactions to explain the effects. Although repression of the wild-type class III promoters appears to be described well by our model, repression mediated by two protein species, CRP−CytR and CytR−CRP, is not. Repression and induction both appear to require models that incorporate allosteric effects mediated by CytR−CRP interactions to explain the findings fully. The limited extent of experimental data in the last five columns of Table 3 precludes in-depth analysis of these effects. We intend to correct this deficiency by generating a comprehensive and internally consistent set of experimental observations of relative transcriptional activity and hope to extend this analysis based thereon.

ASSOCIATED CONTENT

Supporting Information

Detailed report of the analysis of the cooperative and competitive binding of CRP and CytR to *nupG* and *tsxP2* that includes results from individual experiments, the establishment of the interaction model, and energetic parameters from global analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; cAMP, adenosine 3′,5′-cyclic monophosphate; CRP, *E. coli* cyclic AMP receptor protein; CytR, *E. coli* cytidine repressor protein; DNase I, deoxyribonuclease I; RNAP, bacterial RNA polymerase; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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