Combinatorial transcriptional control of the lactose operon of *Escherichia coli*

Thomas Kuhlman*, Zhongge Zhang†, Milton H. Saier, Jr.†, and Terence Hwa*‡

*Center for Theoretical Biological Physics and †Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0374

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The goal of systems biology is to understand the behavior of the whole in terms of knowledge of the parts. This is hard to achieve in many cases due to the difficulty of characterizing the many constituents involved in a biological system and their complex web of interactions. The *lac* promoter of *Escherichia coli* offers the possibility of confronting "system-level" properties of transcriptional regulation with the known biochemistry of the molecular constituents and their mutual interactions. Such confrontations can reveal previously unknown constituents and interactions, as well as offer insight into how the components work together as a whole. Here we study the combinatorial control of the *lac* promoter by the regulators Lac repressor (LacR) and cAMP-receptor protein (CRP). A previous *in vivo* study [Setty Y, Mayo AE, Surette MG, Alon U (2003) *Proc Natl Acad Sci USA* 100:7702–7707] found gross disagreement between the observed promoter activities and the expected behavior based on the known molecular mechanisms. We repeated the study by identifying and removing several extraneous factors that significantly modulated the expression of the *lac* promoter. Through quantitative, systematic characterization of promoter activity for a number of key mutants and guided by the thermodynamic model of transcriptional regulation, we were able to account for the combinatorial control of the *lac* promoter quantitatively, in terms of a cooperative interaction between CRP and LacR-mediated DNA looping. Specifically, our analysis indicates that the sensitivity of the inducer response results from LacR-mediated DNA looping, which is significantly enhanced by CRP.

DNA looping | gene regulation | *lac* promoter | systems biology

The *lac* promoter (P*lac*) of *Escherichia coli* is one of the most extensively studied systems of molecular biology (1–6). The knowledge and insight gained from these studies have shaped much of how we now think about gene regulation. It is well known that *E. coli* cells repress the expression of the *lac* operon when glucose is abundant in the growth medium. Only when the glucose level is low and the lactose level is high is the operon fully expressed. Thus, the regulation of this operon represents an example of "combinatorial control" widely seen in prokaryotes and eukaryotes (7, 8). In this case, the combinatorial control is implemented molecularly by two transcription factors, the Lac repressor (LacR), which represses transcription and the cAMP receptor protein (CRP) which activates transcription. Activation by CRP requires the inducer cAMP, which is used by *E. coli* cells as a signal of glucose shortage (9–15). Repression by LacR is activated in a nearly all-or-none manner upon varying the amount of lactose or one of the several synthetic inducers in growth medium with poor carbon sources (16, 17). The fold-change in repression is very large (>1,000-fold) and has been shown to involve LacR-mediated DNA looping (5, 6, 18–21).

Here we quantitatively investigate the competing effects of activation and repression on the P*lac in vivo*. We focus on two perplexing issues. (*i*) According to biochemical studies (22–25), LacR–inducer interaction is only weakly cooperative. By what mechanism(s) does the observed induction response become so abrupt (26, 27)? (*ii*) Despite the well known role CRP plays in activating transcription (28), structural studies (29, 30) suggest that CRP enhances repression by facilitating the LacR-mediated DNA looping. Moreover, *in vitro* biochemical studies indicate that CRP stabilizes LacR–DNA binding (31–33). What functional role(s) does CRP actually play in the control of this operon? We approached these issues by first identifying mutants of *E. coli* MG1655 that allowed us to directly control the activities of the activators and repressors by varying the levels of two inducers in the growth medium. We then characterized the promoter activity systematically for numerous combinations of the inducers. The gene expression data obtained clearly reveal the effect of CRP in enhancing the steepness of the inducer response. We developed a thermodynamic model of gene regulation (8, 34–36), incorporating the known molecular mechanisms of LacR-induced DNA looping and its coupling to CRP through DNA bending (32, 33). The success of the model is manifested in its ability to describe the complex codependence of gene expression on the two inducer levels quantitatively by invoking a single parameter, the cooperativity between CRP and LacR-mediated DNA looping, with the fitted value of the cooperativity agreeing well with that determined from *in vitro* biochemical measurements (32, 33). Our study presents a proof of concept that the complicated web of interactions that couple repressors, activators, promoters, and DNA loops *in vivo* can be quantitatively dissected, provided that the right modeling together with a precise sequence of experiments on a systematically picked set of mutants are carried out.

Results

Repression by LacR. We first quantitatively characterized the activity of the P*lac* subject to various degrees of repression by the LacR for *E. coli* cells in the exponential growth phase. In our experiments, the activity of LacR was modulated by the synthetic gratuitous inducer isopropyl β-D-thiogalactopyranoside (IPTG) (37). We performed the β-galactosidase assays for wild-type *E. coli* MG1655 cells in M9 minimal medium with 0.5% glucose as the carbon source and up to 1 mM IPTG. P*lac* activity, defined here as the product of the β-galactosidase activity and the cell-doubling rate [see supporting information (SI) *Methods*], is plotted against the corresponding IPTG concentrations as the black crosses in Fig. 1*a*.

The data points are fitted to the Hill function,

$$\alpha_{\mathrm{IPTG}} = b_{\mathrm{IPTG}} \cdot \frac{1 + f_{\mathrm{IPTG}} \cdot ([\mathrm{IPTG}]/C_{\mathrm{IPTG}})^{m_{\mathrm{IPTG}}}}{1 + ([\mathrm{IPTG}]/C_{\mathrm{IPTG}})^{m_{\mathrm{IPTG}}}}, \qquad [1]$$

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Abbreviations: LacR, Lac repressor; CRP, cAMP-receptor protein; IPTG, isopropyl β-D-thiogalactopyranoside; AC, adenylate cyclase; PDE, cAMP-phosphodiesterase.

‡To whom correspondence should be addressed at: Center for Theoretical Biological Physics, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0374. E-mail: hwa@ucsd.edu.

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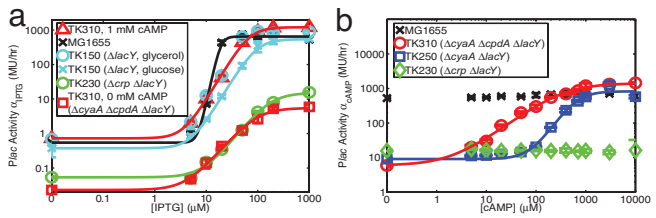

Fig. 1. Dependence of P*lac* activity on the inducers. (*a*) The IPTG response for *E. coli* MG1655 and various Δ*lacY* mutants grown in minimal M9 medium with various amounts of IPTG and 0.5% glucose except for the cyan circles (0.5% glycerol). No cAMP was added to the medium except for the red triangles, which indicate that 1 mM cAMP was added. The lines are best fits to the Hill function (Eq. **1**). (*b*) The cAMP response for *E. coli* MG1655 and various Δ*cyaA* mutants, grown in minimal M9 medium plus 0.5% glucose, 1 mM IPTG, and various amount of cAMP. The lines are best fits to the Hill function (Eq. **2**).

shown as the black line in Fig. 1*a*. The Hill function is used here (and elsewhere in *Results*) merely to extract the qualitative features of the promoter activity; the appropriate quantitative description of the data presented will be provided below in *Analysis*. The features of the IPTG-dependent promoter activity (the "IPTG response") are conveyed by the best-fit Hill parameters listed in Table 1 (row 1): The overall fold change, quantified by $f_{IPTG} \approx 1{,}200$, is in good agreement with previous studies (5, 6, 18–20, 38, 39). However, the "sensitivity" of the response, quantified by the slope of the transition region in a log–log plot and given approximately by the Hill coefficient ($m_{IPTG} \approx 4.5$), is much larger than the expected behavior based on the known biochemistry (see below).

One factor contributing to the hypersensitivity observed is a positive-feedback effect due to the expression of *lacY*, which encodes the Lac permease, as previously reported (16, 39). We therefore deleted *lacY* from *E. coli* MG1655 to form strain TK150 (see SI Table 4), and repeated the β-galactosidase assays for this strain. P*lac* activities obtained are plotted as the cyan crosses in Fig. 1*a*. Fitting again to the Hill form yields the cyan line with the best-fit parameters shown in Table 1 (row 2). The IPTG response of the Δ*lacY* mutant exhibits a broader transition, with $m_{IPTG} \approx 2.6$.

Another possible cause of this hypersensitivity is a suggested cooperative interaction between the LacR and the activator CRP, which also binds in the promoter region (31–33). To investigate this possibility, we deleted the *crp* gene from *E. coli* TK150 to form strain TK230 (SI Table 4) and repeated the β-galactosidase assays for this strain. P*lac* activities obtained are plotted as the green circles in Fig. 1*a*. Fitting again to the Hill form yields the green line with the best-fit parameters shown in Table 1 (row 3). The IPTG response of the Δ*crp* Δ*lacY* double mutant is broader still, with $m_{IPTG} \approx 2$.

Activation by CRP. We next characterized the dependence of P*lac* activity to different degrees of activation by cAMP–CRP (the "cAMP response"). To avoid possible complications due to interaction between LacR and CRP, all experiments were performed under saturating IPTG concentration (1 mM) to disable LacR–operator interaction. This was complemented by direct deletion of LacR in some cases (see below). We also deleted *lacY* in all subsequent experiments to avoid possible feedback. In all cases discussed below where we directly compared the P*lac* activity of *lacY*$^-$ and *lacY*$^+$ cells, differences of no more than 2-fold were obtained (data not shown).

Table 1. Hill parameters for the IPTG response

Strain	b_{IPTG}, MU/hr	f_{IPTG}	C_{IPTG}, μM	m_{IPTG}
MG1655	0.5	1,200 ± 80	20 ± 4	4.5 ± 0.7
TK150 (Δ*lacY*)	0.4	1,445 ± 185	100 ± 18	2.6 ± 0.2
TK230 (Δ*crp* Δ*lacY*)	0.05	255 ± 19	150 ± 15	2.0 ± 0.1
TK310 (Δ*cyaA* Δ*cpdA* Δ*lacY*), no cAMP	0.02	238 ± 26	90 ± 5	2.0 ± 0.2
TK310, 1 mM cAMP	0.7	1,600 ± 180	70 ± 8	2.8 ± 0.1

Parameters derived from fit of the IPTG dependence of the P*lac* activity to the Hill form (Eq. **1**) for various strains of *E. coli* derived from MG1655, grown in medium with 0.5% glucose and various amounts of IPTG. For the last row, 1 mM cAMP was also added to the growth medium. MU, Miller unit.

Control by cAMP. One way to manipulate the cellular level of cAMP–CRP is to subject cells to different levels of cAMP in the medium and rely on the diffusion of cAMP into cells. This approach requires shutting off the endogenous synthesis of cAMP by the enzyme adenylate cyclase (AC), encoded by *cyaA* (41–44). Setty *et al.* (28) attempted this approach by growing cells in medium with 0.2% glucose and various levels of cAMP, expecting that AC activity would be repressed via catabolite repression (10, 14, 15, 45–49). However, they observed only a few-fold change in P*lac* activity despite large variations in the extracellular cAMP levels (0–20 mM) (27). The observed change was surprisingly small given that >50-fold difference in P*lac* activity was obtained between the wild-type and Δ*crp* strains (3). In fact, a nearly 10-fold difference in P*lac* activity can be seen by simply growing wild-type cells on various sugars [see SI Fig. 3 (blue bars)].

Effect of AC deletion. We reasoned that the small change in P*lac* activity obtained by Setty *et al.* (28) might have resulted from the incomplete repression of AC activity by glucose uptake; hence, we repeated the experiment with the deletion of *cyaA*. *E. coli* TK250 strain (Δ*cyaA* Δ*lacY*; see SI Table 4) was grown in M9 minimal medium with 0.5% glucose, 1 mM IPTG, and up to 10 mM cAMP. β-Galactosidase activity was assayed as described above. The resulting P*lac* activity displays a smooth sigmoidal dependence as shown in Fig. 1*b* (blue squares). [Almost identical results (data not shown) were obtained for the *LacR*-null mutant (strain TK320), indicating that LacR is indeed not a factor with saturating IPTG (1 mM) in the growth medium.] A ≈100-fold difference is seen between the low and high cAMP concentrations, comparable to the difference in P*lac* activity reported between the *crp*-null mutant and wild-type strains of *E. coli* (3). In contrast, P*lac* activity of the wild type (Fig. 1*b*, black symbols) as well as the Δ*lacY* mutant (data not shown) grown in glucose displayed only an ≈3-fold change over the same range of cAMP levels, similar to the before-mentioned finding by Setty *et al.* (28). As a negative control, we show in Fig. 1*b* (green circles) the promoter activity obtained for the *crp*$^-$ strain (TK230); its lack of cAMP dependence indicates that the observed cAMP dependence for the *crp*$^+$ strain was mediated primarily by cAMP–CRP. Additional negative controls on possible indirect effects of cAMP variations on P*lac* activity are shown in SI Fig. 4*b*. We found the variation of CRP expression and the nonspecific effects of CRP on P*lac* activity to be small (≈2-fold), compared with the 100-fold difference observed for the Δ*cyaA* mutants over the same range of cAMP concentrations.

The cAMP response exhibited by strain TK250 was analyzed by fitting to the Hill function,

$$\alpha_{cAMP} = b_0 \cdot \frac{1 + f_{cAMP} \cdot ([cAMP]/C_{cAMP})^{m_{cAMP}}}{1 + ([cAMP]/C_{cAMP})^{m_{cAMP}}}, \qquad [2]$$

and plotted as the blue line in Fig. 1*b*. The best-fit parameters are shown in Table 2 (row 1). The sensitivity of the cAMP response, characterized by the Hill coefficient $m_{cAMP} \approx 2$, is in disagreement with the noncooperative nature of cAMP–CRP interaction (50–53). This suggests a nonlinear relationship between the extracellular and intracellular cAMP concentrations in Δ*cyaA* strain and prompted us to look for additional factors regulating intracellular cAMP levels.

Effect of phosphodiesterase deletion. One such factor is cAMP degradation catalyzed by the enzyme cAMP-phosphodiesterase (PDE) (54–58), encoded by *cpdA* (59). We deleted the *cpdA* gene to obtain

Table 2. Hill parameters for the cAMP response

Strain	b_{cAMP}, MU/hr	f_{cAMP}	C_{cAMP}, μM	m_{cAMP}
TK250 (Δ*cyaA* Δ*lacY*)	9.1	91 ± 5	645 ± 43	2.1 ± 0.1
TK310(Δ*cyaA* Δ*cpdA* Δ*lacY*)	5.9	240 ± 13	320 ± 32	1.0 ± 0.1
Prediction based on thermodynamics	—	>20	10–1,000	1

The first two rows give the parameters derived from fit of cAMP response of *cyaA* mutants to the Hill function (Eq. **2**). The last row gives the best estimates of these parameters according to the thermodynamic model and the known biochemical parameters (see *SI Methods* for details).

strain TK310 (Δ*cyaA* Δ*lacY* Δ*cpdA*) (see SI Table 4) and repeated the β-galactosidase assay and analysis. The cAMP response obtained (Fig. 1*b*, red circles) is more gradual than that of *cpdA*$^+$ cells (blue squares). Fitting the data to the Hill form (Eq. **2**) yielded the red line with the Hill parameters listed in Table 2 (row 2). Specifically, the Hill coefficient $m_{cAMP} \approx 1$ is consistent with the naïve expectation based on the noncooperative nature of cAMP–CRP interaction (50–53).

Combinatorial Control. We next investigated the codependence of P*lac* activity on the two regulators, LacR and cAMP–CRP. We showed above that the Δ*crp* Δ*lacY* strain (TK230) could be used to characterize the bare IPTG response, whereas the Δ*lacY* Δ*cyaA* Δ*cpdA* strain (TK310) could be used to characterize the bare cAMP response. To characterize the codependence of the promoter on IPTG and cAMP, we first verified that strain TK310 exhibited nearly the same IPTG dependence as TK230 in growth medium with no cAMP added; compare[§] the red squares and green circles in Fig. 1*a*. Fitting the IPTG response of TK310 (red squares) to the Hill form (Eq. **1**) yields the solid red line; the corresponding parameters are provided in Table 1 (row 4).

We repeated the β-galactosidase assay and analysis for TK310 cells grown in media with various combinations of IPTG and cAMP concentrations. As evidence of an interaction between IPTG-mediated and cAMP-mediated regulations, we show in Fig. 1*a* (red triangles) the IPTG response for TK310 strain in growth medium containing 1 mM cAMP. This response is nearly indistinguishable from the physiological P*lac* activity exhibited by *cyaA*$^+$ *cpdA*$^+$ cells in glycerol medium (cyan circles). The dashed red line is the best fit to the Hill function (Eq. **1**), with parameters listed in Table 1 (row 5). Note that the overall fold-change (f_{IPTG}) is increased from <250-fold when no cAMP was added in the medium to >1,500-fold with 1 mM cAMP in the medium. The latter fold-change is comparable to those obtained for wild-type *E. coli* cells grown in the absence or saturating concentration of IPTG (Fig. 1*a*, black crosses) (5, 6, 18–20, 38, 39). Additionally, the sensitivity of the IPTG response increased from $m_{IPTG} \approx 2.0$ (Table 1, row 4) for TK310 cells grown in the absence of cAMP to $m_{IPTG} \approx 2.8$ (Table 1, row 5) for the same cells grown in 1 mM cAMP. Fitting the IPTG responses of these cells obtained at a variety of cAMP concentrations, we found a trend of increasing Hill coefficient (from 2 to 3) and fold-change (from 250- to 1,800-fold) for cAMP levels from 1 μM to 1 mM (see SI Fig. 5*a*). The complete codependence of P*lac* activity on IPTG and cAMP is shown as the 3D plot in SI Fig. 6*a*.

Analysis

We have seen that the IPTG and cAMP responses of various mutant strains of *E. coli* MG1655 fitted well to Hill functions, with the Hill parameters summarized in Tables 1 and 2. However, the Hill function itself has been invoked so far without justification; it was merely a familiar form used to quantify key features of the response, e.g., the overall fold-change and sensitivity. Below we will analyze and interpret the results obtained in light of the rich knowledge on the molecular biology of the P*lac* and the biochemistry of the associated components using a thermodynamic model of transcriptional regulation (see refs. 8 and 36; see also the brief review in *SI Methods*).

Activation by cAMP–CRP Is Noncooperative. The cAMP response found for strain TK310 (Δ*cyaA* Δ*cpdA* Δ*lacY*) exhibited a broad transition (Fig. 1*b*, red circles), well fitted by the Hill form (red line). This is in agreement with the thermodynamic model, which predicts the Hill form for response to simple activation by cAMP–CRP (*SI Methods*) (35). The Hill coefficient $m_{cAMP} \approx 1$ obtained is in good agreement with the biochemistry finding that it takes one cAMP molecule to activate the CRP dimer (51–53).

The thermodynamic model further relates the other Hill parameters f_{cAMP} and C_{cAMP} to the biochemical parameters that describe CRP-mediated transcriptional activation (see *SI Methods*). The best estimates of f_{cAMP} and C_{cAMP} based on knowledge of the biochemical parameters are given in Table 2 (row 3). The available information is not sufficient for a quantitative comparison of the parameter C_{cAMP}, whose value depends on the CRP–operator binding affinity *in vivo* as well as the relationship between the intra- and extracellular cAMP concentrations, both of which can be estimated only very crudely (*SI Methods*) (9, 60). The parameter f_{cAMP}, which describes the maximal fold-change in the cAMP response, is given by the cooperativity of CRP and RNA polymerase interaction according to the thermodynamic model. Our result $f_{cAMP} = 240 \pm 13$ is significantly larger than the cooperativity factor of ≈20 obtained from *in vitro* biochemical measurements (61, 62). This discrepancy is analogous to one noted earlier by Beckwith *et al.* (3). It may have resulted from the accumulation of a number of small factors. For example, in addition to recruiting RNA polymerase, CRP was shown to stimulate the transition of promoter DNA from the closed to open conformation, thereby enhancing the transcription rate by ≈50% (61). In addition, the autoregulation of CRP expression may account for ≈2-fold difference (see SI Fig. 3), and another ≈2- to 3-fold difference may be attributed to the deletion of *cpdA* (Fig. 1*b*, compare the vertical ranges of the blue and red lines).

PDE Provides Insulation to Variations in cAMP. We are not aware of any significant phenotype reported for cells with a PDE deletion. Only small differences of 2- to 3-fold in P*lac* activity were seen between the wild-type and Δ*cpdA* strains (SI Fig. 3, blue and red bars), and no systematic trend can be seen in the growth rates of the two strains (SI Fig. 3, numbers on top of the bars). However, the effect of *cpdA* expression on the *cyaA* mutant is striking. Comparison of the blue and red lines in Fig. 1*b* suggests that PDE expression insulates the cell from extracellular cAMP variations of up to 100 μM. This may be important for cells in environments where AC activity is significantly repressed.

LacR-Mediated DNA Looping Increases the Sensitivity of the IPTG Response. The IPTG response of the Δ*crp* Δ*lacY* double mutant (TK230) exhibited a reduced sensitivity (Fig. 1*a*, green circles) compared with the *lacY* mutant (TK150; Fig. 1*a*, cyan crosses). The difference is due to the activated CRP in the latter strain (which has *cyaA* intact and, hence, synthesizes cAMP endogenously). We will discuss the effect of CRP shortly; for now, we first discuss the IPTG response in the absence of CRP, i.e., that of strain TK230. This response is well fitted by the Hill form (Fig. 1*a*, green line), with the Hill coefficient $m_{IPTG} \approx 2.0$ (Table 1, row 3). Although a cooperative IPTG response with Hill coefficient ≈ 2 is widely quoted in the molecular biology literature (26) and, moreover, a cooperative IPTG–LacR interaction was suggested based on a structural study of LacR (29), *in vitro* biochemical studies of IPTG–LacR binding

[§]However, *cpdA* mutants show a 2-fold overall reduction in gene expression for unknown reasons.

Table 3. Parameters for model with DNA Looping.

Strain	K_{IPTG}, μM	L_0
TK230 (Δ*crp* Δ*lacY*)	15.7 ± 1.5	4.1
TK310 (Δ*cyaA* Δ*cpdA* Δ*lacY*)	12.3 ± 1.4	3.7
TK250 (Δ*cyaA* Δ*lacY*)	17.8 ± 1.7	5.0

Parameters derived from the fit of IPTG response of given strains to the thermodynamic model with DNA looping (Eq. **3**).

in fact found a noncooperative IPTG–LacR interaction, which became only weakly cooperative (Hill coefficient = 1.4~1.6) in the presence of operator DNA fragments (22–25). We reasoned that the apparent cooperativity ($m_{IPTG} \approx 2$) observed *in vivo* might have resulted from LacR-mediated DNA looping[¶] known to occur in wild-type *E. coli* cells (6, 18–20). The corresponding thermodynamic model is developed in *SI Methods*, with the following form of the IPTG response:

$$\alpha_{IPTG} = \alpha_0 \Big/ \left[1 + \frac{R}{(1 + [IPTG]/K_{IPTG})^2} + \frac{R \cdot L_0}{(1 + [IPTG]/K_{IPTG})^4} \right]. \quad [3]$$

Although Eq. **3** is not of the Hill form, it resembles a spectrum of different Hill functions, with the maximal fold-change

$$f_{IPTG} = 1 + R \cdot (L_0 + 1), \quad [4]$$

and with the sensitivity also increasing with the parameters R and L_0 (see *SI Methods*). The parameter R depends on the LacR concentration and LacR–operator affinity; it gives the fold-repression between zero and saturating IPTG levels in the absence of DNA looping ($L_0 = 0$). The values of $R \approx 20$–50 can be inferred from two previous studies by Oehler *et al.* (6, 21), who characterized the activities of P*lac*s bearing various Lac operator mutations (see *SI Methods*). In the analysis below, we use $R \approx 50$; results of similar quality (data not shown) can be obtained for $R \approx 20$. The parameter L_0 describes the enhancement in the "local concentration" of LacR molecules due to DNA looping (6, 35). There is currently no direct measurement of L_0 for the P*lac* in the absence of CRP binding.

Before applying Eq. **3** to our results, we first discuss its form in the absence of DNA looping ($L_0 = 0$). In a very recent report, Oehler *et al.* (62) used various mutants to characterize the IPTG responses of the P*lac* incapable of DNA looping. Their data are reproduced in SI Fig. 7*a* (red triangles and blue squares) and shown to fit well to Eq. **3** with $L_0 = 0$ (solid line). The data are equally well fitted in SI Fig. 7*b* by the Hill form (Eq. **1**) with an effective Hill coefficient of 1.5, in agreement with results of previous *in vitro* studies (24).

We next confront DNA looping by fitting the IPTG response of strain TK230 (Fig. 1*a*, green circles) to Eq. **3**, with two fitting parameters K_{IPTG} and L_0. (The saturation value α_0 is fixed by the promoter activity at 1 mM IPTG.) The best-fit curve (data not shown) is nearly indistinguishable from the green line in Fig. 1*a*, although Eq. **3** contains one fewer fitting parameter than Eq. **1**. In particular, the observed sensitivity of the IPTG dependence is well reproduced. The best-fit parameters obtained are shown in Table 3 (row 1). The value of the loop parameter simply reflects the maximal fold-change of the IPTG response as given by Eq. **4**. The value of the IPTG–LacR dissociation constant K_{IPTG} obtained is comparable to the results of *in vitro* biochemical studies (*SI Methods*) (4, 64, 65). Similarly, we found that the DNA looping model described the IPTG response of strain TK310 (Δ*cyaA* Δ*cpdA* Δ*lacY*) very well in medium with no cAMP. The corresponding best-fit parameters are given in Table 3 (row 2); the best-fit curve (data not shown) is nearly indistinguishable from the lower red line in Fig. 1*a*.

Cooperativity Between CRP and LacR-Mediated DNA Looping Enhances the Sensitivity of the IPTG Response. Strain TK310, which exhibited the expected cAMP response in medium with 1 mM IPTG and the expected IPTG response in medium with no cAMP added, was used to investigate the combinatorial control of P*lac* activity by LacR and cAMP–CRP. According to the thermodynamic model, if there is no interaction between cAMP–CRP-mediated activation and LacR-mediated repression, then the codependence of P*lac* activity on IPTG and cAMP would simply be the algebraic product of the IPTG response and the cAMP response, i.e., the product of the red lines in Fig. 1. A manifestation of this product form is a simple vertical shift of the IPTG responses of the same strain at different cAMP concentrations in the log–log plots. However, this clearly cannot be the case for strain TK310, because both the sensitivity and the maximal fold-change of its IPTG response increase with increasing cAMP levels (Fig. 1*a* and SI Fig. 5; see also SI Fig. 8*a* for a direct comparison).

Various forms of interactions between CRP and LacR in the P*lac* region have been previously proposed or reported (29, 31–33, 66, 67). Because CRP causes a 90–130° bend in DNA (20, 68, 69), the binding of CRP to its site in the P*lac* could potentially bring the Lac operators O1 and O3 into closer proximity, promoting the formation of DNA loop between them (SI Fig. 9) (29). The existence of this interaction is supported by a computational study based on the available LacR–operator and CRP–operator cocrystal structures (30). Moreover, Hudson and Fried (33) and Vossen *et al.* (34) found cooperativity in the formation of a ternary complex of CRP, LacR, and P*lac* DNA *in vitro*, with the simultaneous presence of LacR and cAMP–CRP increasing the affinity of both proteins for their respective binding sites by a cooperative factor (Ω) of 4- to 12-fold.

To see whether CRP-assisted DNA looping may quantitatively explain the observed codependency of P*lac* activity, we extended the thermodynamic model with DNA looping to include the CRP-dependent looping effect (*SI Methods*). The extended model predicts the IPTG response to have the same form as Eq. **3** but with the prefactor α_0 replaced by the Hill function α_{cAMP} for strain TK310 (Eq. **2** with parameters given by Table 2, row 2) and with the loop parameter L_0 replaced by

$$L = L_0 \cdot \frac{1 + \Omega \cdot [cAMP]/C_{cAMP}}{1 + [cAMP]/C_{cAMP}}, \quad [5]$$

where Ω is the cooperativity factor of the CRP–LacR–loop interaction.

To test the predictions of the extended thermodynamic model, we used Eq. **4** to infer the value of the loop parameter L from the maximal fold-change of the IPTG response at each cAMP value; the result is plotted as the circles in Fig. 2*a*. We then used Eq. **3** to compute the expected IPTG responses using these values of L. The results are shown as the dashed lines in Fig. 2*b* for several representative cAMP values, together with the experimental data for TK310 cells grown in media with the corresponding cAMP concentrations. Note that the dashed lines are not fits of the data to the model. The striking congruence between the dashed lines and the data points (for [cAMP] ≤ 1 mM) shows that the cAMP-dependent sensitivity, which requires a series of different Hill coefficients within the Hill function description (SI Fig. 5*a*), can be naturally described by the DNA looping model in terms of the cAMP-dependent loop parameter L with no other adjustable parameters.

[¶]We note that the increased in cooperativity by DNA looping was discussed recently by Vilar and Saiz (75) in the context of the lysis-lysogeny control of Phage λ. However, the mechanism of cooperativity there is the oligomerization of regulatory proteins made possible through DNA looping. In contrast here, the tetramerization of LacR is independent of DNA looping. What DNA looping enhances here is the sensitivity to IPTG rather than LacR.

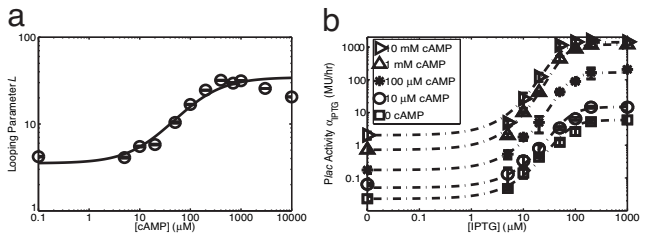

Fig. 2. Combinatorial control of P*lac* activity for strain TK310. (*a*) The cAMP-dependent loop parameter L is inferred from Eq. **4** by fixing the maximal fold-change f_{IPTG} to be the ratio of α_0 and the observed promoter activity in medium with no IPTG. α_0 is generated by the bare cAMP response α_{cAMP} by using the parameters in Table 2 (row 2). The result obtained (circles) is fitted to Eq. **5** by using a single fitting parameter, $\Omega = 10.3 \pm 0.1$, with $C_{cAMP} = 320$ μM (Table 2, row 2) and $L_0 = 3.7$ (Table 3, row 4). (*b*) The IPTG responses obtained at different cAMP levels (symbols) are plotted together with predictions of the CRP-assisted DNA-looping model (Eq. **3**) with no adjustable parameters. We used $R = 50$ and $K_{IPTG} = 12.3$ μM (Table 3, row 3), with values of L taken from *a* and α_{cAMP} for α_0 (Eq. **2** with parameters values given in Table 2, row 2).

To further examine whether the cAMP-dependence of L conforms to the known energetics of CRP-assisted DNA looping, we fitted the inferred values of L (Fig. 2*a*, circles) to Eq. **5** by allowing only the parameter Ω to vary. The result of the fit (Fig. 2*a*, solid line) described the data well, except again at the highest cAMP level (10 mM), where cell growth significantly slowed down and other effects (e.g., overexpression of nucleoid proteins) might significantly affect DNA bending and looping (70). The value of the best-fit parameter obtained, $\Omega = 10.3 \pm 0.1$, is in agreement with the 4- to 12-fold cooperativity found *in vitro* (*SI Methods*) (31, 33). SI Fig. 6*b* shows the 3D plot of the expected P*lac* activity according to the extended thermodynamic model. Collectively, these results provide strong quantitative support for the hypothesis of CRP-assisted repression by DNA looping (31–33).

Finally, we repeated the analysis for the combinatorial control of TK250 cells which are *cpdA*$^+$. Despite the unexpected cooperative nature of the cAMP response of this strain (Fig. 1*b*, blue symbols), with $m_{cAMP} \approx 2$, the IPTG responses at various cAMP levels (SI Fig. 10*b*) are still well described by Eq. **3** (with the best-fit values of K_{IPTG} and L_0 given in Table 3, row 3). Moreover, except for the highest cAMP concentrations again, the cAMP-dependence of the loop parameter L agrees well with the corresponding Hill function, with similar value of the cooperativity factor, $\Omega \approx 9.7$ (see SI Fig. 10*a*). These results indicate that *cpdA* expression affects only the activation of CRP by extracellular cAMP but not the interaction of cAMP–CRP with DNA looping itself.

Discussion

Systematic, quantitative characterization of gene expression may yield unique insights on the mechanisms of gene regulation. A major obstacle in this effort is to quantify the amount of active transcription factors in cells in a given environment. As attempted earlier by Setty *et al.* (28), we tackled this problem for the P*lac* by controlling the amount of inducers IPTG and cAMP extracellularly but with the *cyaA* mutant of *E. coli*, which cannot synthesize cAMP endogenously. One danger of this approach is the array of mechanisms the cell may employ to control the intracellular effector levels. We encountered two such mechanisms in our study. Through the positive feedback of the Lac permease, *E. coli* cells sensitize themselves to the presence of inducers of the LacR, whereas through the activity of PDE, *E. coli* cells appear to insulate themselves from low levels of cAMP in the environment. The LacY effect is well known for induction with lactose in the growth medium (16, 17). However, the PDE effect observed here is previously unknown. It may yield important clues toward a comprehensive understanding of cAMP transport and control central to carbon metabolism in *E. coli*.

DNA Looping Is Assisted by CRP. The innate regulation of P*lac* activity is revealed in *E. coli* cells bearing *cyaA lacY cpdA* triple deletion. We found that with a saturating amount of IPTG in the medium, the cAMP–CRP-mediated activation behaved in accordance with the thermodynamic model, given the noncooperative nature of the cAMP–CRP interaction. In addition, in the absence of cAMP, the LacR-mediated repression behaved in accordance with the thermodynamic model with DNA looping. Moreover, the codependence on IPTG and cAMP can be explained by the extended thermodynamic model with a single cooperativity parameter, Ω, whose value is of the order found by *in vitro* experiments. Both the quality of the fit and the simplicity of the model (i.e., one parameter for codependence) are superior compared with other simple but ad hoc models, as we show explicitly in SI Fig. 8). These results strongly support the hypothesis of CRP-assisted DNA looping (29, 31) and shed light on the seemingly conflicting roles CRP plays in the regulation of the P*lac*: CRP is an activator in that it increases gene expression at each IPTG level. However, it increases gene expression in a nonlinear way such that the maximal fold-change and the sensitivity of the IPTG response are both enhanced.

Hypersensitivity of IPTG Response Resulted from Multiple Factors. Even in the absence of *lacY*-mediated feedback, P*lac* activity depended sensitively on the IPTG level with an apparent Hill coefficient of 2.5~3 at physiological intracellular cAMP concentrations. This is rather remarkable given that the basic interaction of IPTG with the Lac tetramer is not cooperative at all in the absence of operator DNA (24). Whereas the involvement of DNA looping in the IPTG hypersensitivity is shown qualitatively by the experiment of Oehler *et al.* (62), the results and analysis of this study reveal the complex way in which this hypersensitivity is attained. First, the noncooperative IPTG–LacR binding becomes weakly cooperative (effective Hill coefficient of ≈1.5) in the presence of operator DNA (SI Fig. 7) (22–25), due presumably to the allosteric coupling between inducer and operator binding (see *SI Methods*). The involvement of DNA looping then increases the cooperativity (effective Hill coefficient of ≈2), because both dimeric units of the Lac tetramer are required to bind specifically to operators to form DNA loops. Finally, in the physiological range of intracellular cAMP concentrations where CRP is activated, the cooperative interplay between DNA looping and CRP-mediated DNA bending further enhances the looping-induced cooperativity to the observed effective Hill coefficient of 2.5~3.

As shown by Novick and Weiner (16) and by Ozbudak *et al.* (17), the hypersensitivity of P*lac* activity to the inducer of LacR is crucial for the bistable (i.e., the all-or-none) nature of *E. coli*'s lactose utilization strategy (16, 17). The latter is the molecular basis of important physiological effects, such as the diauxie shift (71). It is interesting that hypersensitivity resulted in this case from an agglomeration of weak interactions. This distributed way of implementing an important molecular function (instead of, e.g., a highly cooperative LacR–inducer interaction) may reflect the serendipity of the evolutionary dynamics or, alternatively, a robust evolutionary strategy to preserve important system-level functions.

The Importance of Being Quantitative. Critical to this study was the recognition of various "artifacts" produced by processes not directly related to the regulation of the P*lac*. The key discriminating feature we used was the quantitative comparison of the observed responses to the expectations of the thermodynamic model, in light of the known biochemical processes. Although fitting the experimental data to a Hill form is in itself not a very discriminating task, we find that the "reasonableness" of the numerical values of the fitting parameters, especially the apparent Hill coefficients of the re-

sponses, can be quite revealing. Nevertheless, the power of the quantitative analysis is by itself limited, because it can only suggest the existence of problems but does not identify the sources. It is through the quantitative comparison of the characteristics of a series of key mutants that the major conclusions of this study are established. It is important to note that some of our most discriminating mutants did not display much difference in the high/low states of expression but exhibited clear differences in the abruptness of the transition between states.

The P*lac* is one of the prototypical model systems of gene regulation. Classic studies on the regulation of this promoter have established numerous fundamental concepts as well as laying down the appropriate methodology for studying the molecular biology of gene regulation. Quantitative studies of the P*lac* can again play important roles in laying down the foundation of quantitative systems biology, whose goal is to understand the behavior of a "system" in terms of the relevant properties of its components. The P*lac* is admittedly a rather simple system. Nevertheless, we see from this study that system-level properties, such as the sensitivity of the IPTG response, resulted from a closely intertwined set of interactions among the molecular constituents. We demonstrated how this system can be dissected by careful quantitative characterization and targeted genetic manipulations, along with guidance from quantitative modeling and the knowledge of the biochemistry of the molecular constituents. The experience gained here may be of value to the study of other more complex biological systems.

Methods

Plasmids and Strains. All strains used in this study were derived from *E. coli* K-12 MG1655 as listed in SI Table 4 and detailed in *SI Methods*. Chromosomal gene deletion was performed by using the method of Datsenko and Wanner (71), and transferred from one strain to another by using P1 transduction. All mutations were verified with PCR.

Cell Growth and β-Galactosidase Assay. Overnight cultures were grown in M9 minimal medium containing the standard concentrations of necessary antibiotics in a 37°C water bath until stationary phase. The carbon source was 0.5% glucose unless otherwise indicated. These cultures were diluted 100- to 1,000-fold into 24-well plates (Costar) containing the same growth medium plus various concentrations of IPTG and cAMP. The plates were grown with vigorous shaking in a humidity-controlled incubator maintained at 37°C, with OD_{600} measurements taken every 2 h in a Tecan Genios Pro plate reader. When OD_{600} of a sample reached 0.2–0.4, it was assayed for β-galactosidase activity. These assays were performed in triplicate or more according to Miller (73) and Griffith (74), with minor modifications detailed in *SI Methods*. The β-galactosidase activity obtained (A) was expressed in Miller units (73), and the promoter activity (α) reported was taken to be the product of A and the cell-doubling rate $\lambda_{1/2}$. Serial dilution experiments were used to verify that the entire range of promoter activity reported in the figures lay within the linear responsive regime of the measurements (see *SI Methods*).

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