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Mode of Regulation and the Insulation of Bacterial Gene Expression

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SUMMARY

A gene can be said to be insulated from environmental variations if its expression level depends only on its cognate inducers, and not on variations in conditions. We tested the insulation of the *lac* promoter of *E. coli* and of synthetic constructs in which the transcription factor CRP acts as either an activator or a repressor, by measuring their input function—their expression as a function of inducers—in different growth conditions. We find that the promoter activities show sizable variation across conditions of 10%–100% (SD/mean). When the promoter is bound to its cognate regulator(s), variation across conditions is smaller than when it is unbound. Thus, mode of regulation affects insulation: activators seem to show better insulation at high expression levels, and repressors at low expression levels. This may explain the Savageau demand rule, in which *E. coli* genes needed often in the natural environment tend to be regulated by activators, and rarely needed genes by repressors. The present approach can be used to study insulation in other genes and organisms.

INTRODUCTION

Genes are regulated by transcription factors according to biological signals, such as chemical inducers. The expression of a gene as a function of its inducers—induction curves or input functions—has been extensively studied. Input functions have been measured by varying one or more inducers, usually resulting in sigmoidal functions (Yagil and Yagil, 1971; Setty et al., 2003; Bintu et al., 2005; Kaplan et al., 2008). Recent studies have characterized the stochastic variation in induction curves between genetically identical cells, also known as expression noise (Rosenfeld et al., 2005; Eldar and Elowitz, 2010; So et al., 2011).

Here we consider the question of how gene input functions vary across conditions. If we assume that promoters are designed to control expression in response to specific, cognate signals, it might be useful for induction curves to be insensitive to variations in conditions that do not affect the cognate signals. One may say that a promoter is perfectly "insulated" if its input function does not vary when conditions change. The first aim of the present study is to measure the degree to which simple bacterial promoters are insulated from noncognate variations in the environment (for other types of insulation, see Geyer [1997] and Del Vecchio et al. [2008]).

A second aim is to study the effect of the mode of gene regulation on insulation. Recently, Shinar et al. (2006) proposed, on theoretical grounds, that bacterial promoters would be more insulated when the cognate regulators bind their sites on the promoter than when they do not bind their sites. This is because unbound sites are more prone to binding by nonspecific factors. The activity of such potential nonspecific factors is expected to vary with conditions—and thus cannot be canceled out by adjusting the DNA sequence of the promoter (Itzkovitz et al., 2006).

Similar predictions relate to insulation from variations in the activity of the cognate regulators themselves caused by different environments. Such variation can be due to unwanted interactions with condition-specific factors. When sites are strongly bound by their cognate regulator, the binding curve is near saturation and thus changes in regulator activity have small relative effects. When the site is weakly bound by its cognate regulator, changes in regulator activity can have large relative effects because the binding curve is in a high-slope regime. For both sources of variation—variation in cognate regulator activity or in non-specific binding—a promoter bound to its cognate regulator is predicted to be more insulated than a promoter weakly bound to its cognate regulator.

This assumption explains a long-standing correlation noted by Michael Savageau, that bacterial genes expressed often in the environment (high-demand genes), tend to be regulated by activators, whereas genes rarely expressed (low-demand genes) tend to be regulated by repressors (Savageau, 1974, 1998a, 1998b; Gerland and Hwa, 2009). In both cases, the unifying principle is that the regulator binds its site most of the time, reducing variation across conditions, and maintaining the desired input function (Shinar et al., 2006).

To experimentally address the question of variability across conditions, we measured the activity of *E. coli* promoters under a range of conditions at high resolution and accuracy. In each of the conditions, we obtained the input function by measuring promoter activity at varying levels of inducer(s). We find that the input functions show sizable variation across conditions. We further find that this variation is markedly reduced when

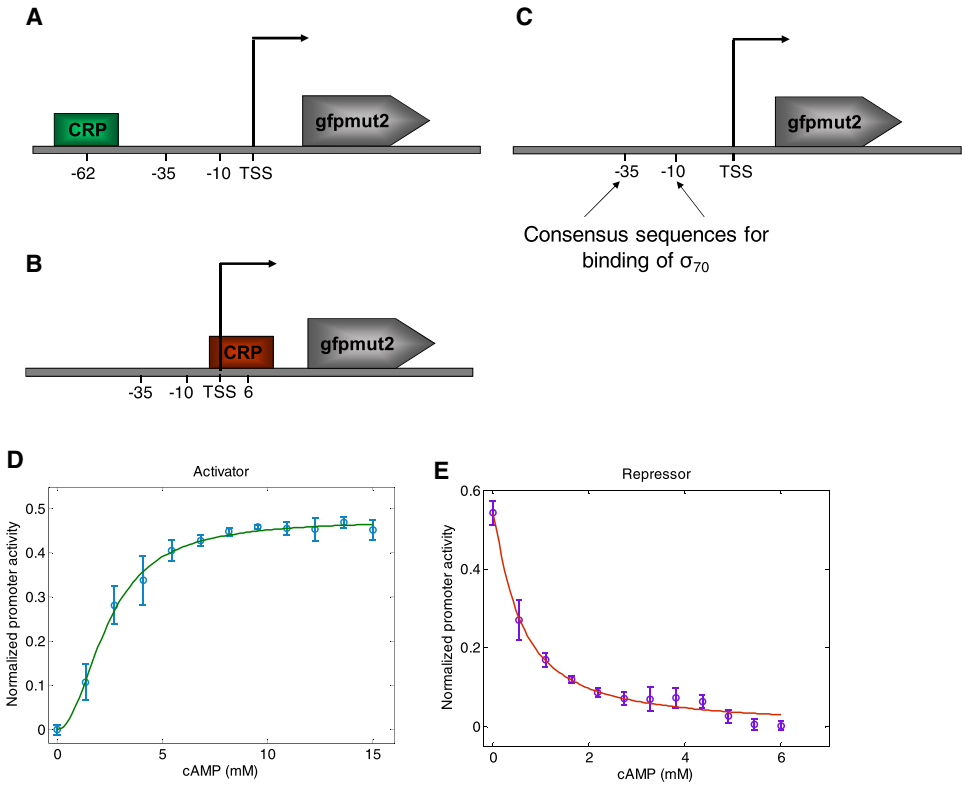

Figure 1. Constructs in which CRP Is Either an Activator or a Repressor

(A) Construct (U435) in which CRP is an activator of a GFP reporter gene.

(B) Construct (U436) in which CRP is a repressor of a GFP reporter gene.

(C) Construct (U449/U371) in which the RNA polymerase binding site is unregulated (σ_{70} reporter construct). This construct was used to normalize the promoter activities to control for variations in growth rate and protein production. All constructs use the same low-copy plasmid vector.

(D) Activator construct shows increased promoter activity as a function of the inducer cAMP.

(E) Repressor constructs shows decreasing promoter activity as a function of the inducer cAMP. Promoter activity is rate of GFP fluorescence accumulation divided by cell density (OD) at mid exponential growth, normalized to the promoter activity of the σ_{70} reporter (U449). All measurements are on *E. coli* strain U451 in which cyaA is deleted so that no endogenous cAMP is produced, grown in M9 minimal medium with 0.2% glucose. Error bars represent the standard error of four repeats.

See also Figure S1.

regulators bind their sites as compared to the same expression level reached when regulators are unbound. This provides a link between insulation of gene expression and the mode of gene regulation.

RESULTS

Reporter Constructs in which CRP Is a Repressor or an Activator

To test the relation between mode of regulation and the variability of expression across conditions, we constructed two synthetic reporter plasmids based on the same parental low-copy plasmid (pSC101 origin). In both plasmids, CRP controls the expression of a rapidly folding green fluorescent protein reporter gene. In one construct, U435 (Figure 1A), CRP acts as an activator, binding at −62 upstream of the RNA polymerase binding site (at −10 and −35). In this configuration, CRP binding enhances promoter activity (Collado-Vides et al., 1991).

In the second construct, U436 (Figure 1B), CRP acts as a repressor. Its binding site is located at +6, downstream of the −10 and −35 RNA polymerase sites. When bound to this site, CRP sterically blocks RNA polymerase binding (Ingraham and Neidhardt, 1987; Collado-Vides et al., 1991; Cox et al., 2007).

To obtain a similar expression range for the two constructs, the sequence of the RNA polymerase binding site (−10 and −35 sites) was made closer to consensus in the repressor construct than in the activator construct (see the Experimental Procedures). This leads to high expression levels for the repressor construct when CRP is unbound, which are comparable to the high expression in the activator construct which occurs when CRP binds the promoter. The plasmid constructs are otherwise identical (same ribosomal binding site in the gfp gene, etc.).

We transformed the plasmids into *E. coli* MG1655 deleted for the *cyaA* gene (U451) (Kuhlman et al., 2007). This strain does not

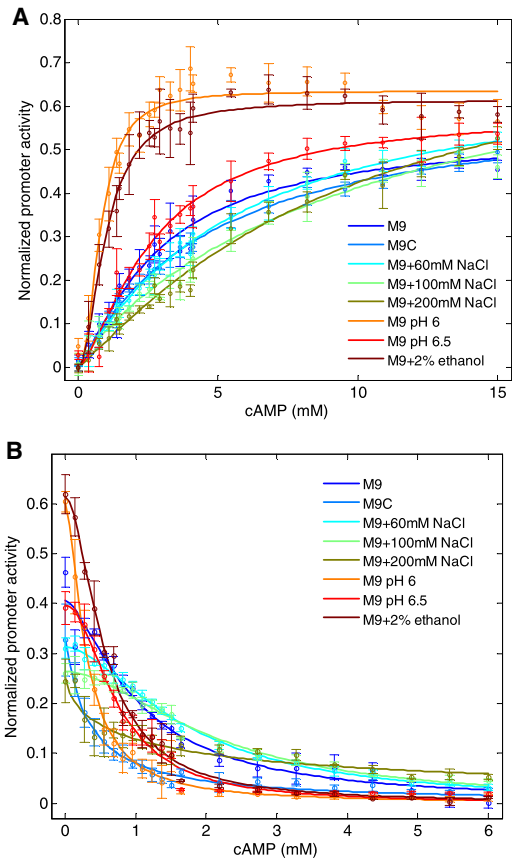

Figure 2. Repressor and Activator Construct Show Variability across Growth Conditions

(A) Activator promoter activity as a function of cAMP shows significant variation across eight different growth conditions.

(B) Repressor promoter activity as a function of cAMP shows significant variation across eight different growth conditions. Full lines are fits of the data to Hill functions $\beta \cdot K^n/(K^n + x^n)$, where x is cAMP level, n is the Hill coefficient, β is the maximal expression, and K is the halfway induction point. Error bars represent the standard error of eight independent repeats.

See also Figure S1.

endogenously produce cAMP, the inducer of CRP, allowing a wide range of induction using externally added cAMP. We measured the cell density and GFP fluorescence in 12 external cAMP levels in 96-well plate cultures, by means of a robotic incubator-fluorimeter system as previously described (Kaplan et al., 2008). Measurements were at a temporal resolution of 8 min. We tracked promoter activity, defined as the rate of GFP production per OD unit, dGFP/dt/OD, throughout the growth curve. All promoter activities reported here are averages of the promoter activity in mid-exponential growth. Promoter activity was normalized to the promoter activity of a σ_{70} reporter construct based on the same plasmid vector (Figure 1C) grown under identical conditions. This normalizes for global effects due to growth rate differences between conditions (Zaslaver et al., 2009; Klumpp et al., 2009; Scott et al., 2010).

We measured the induction curves of both strains in M9-glucose minimal medium. Promoter activity in the activator construct (U435) increased with inducer cAMP, reaching 50% induction at cAMP = 2.3 ± 0.2 mM (Figure 1D). The repressor construct showed decreasing promoter activity with cAMP, with a 50% repression at cAMP = 0.6 ± 0.2 mM (Figure 1E). The expression range of the two constructs is similar—the maximal promoter activity normalized to that of the σ_{70} reporter is about 0.4 and 0.5 for the repressor and activator respectively. These constructs are thus examples of a repressor and an activator that generate comparable control of gene expression. We next asked how this control varies across noncognate conditions.

Reporters Show Sizable Variability across Conditions

We measured the induction curve of the repressor and activator constructs as a function of cAMP levels in eight conditions, including high salt, ethanol, low pH, and amino acids (see the Experimental Procedures for a list of conditions). Each induction curve is averaged over four replicate experiments. The experiments were repeated on two to four different days. The relative error between repeats is on the order of 5%. We find that the input functions (induction curves) vary across conditions, with a standard deviation between curves that is about 10%–35% of the mean (Figures 2A and 2B).

Mode of Regulation Affects Variability across Conditions: Activator Shows Higher Variability at Low Expression Level, and Lower Variability at High Expression Level, than the Repressor

We compared the variability across conditions between the activator and repressor constructs. We find that for the repressor, the standard deviation between conditions increases with promoter activity (Figure 3B). In contrast, standard deviation first rises and then decreases for the activator construct (Figure 3A). Thus, the maximal expression level of the repressor was more variable than that of the activator (0.13 ± 0.02 versus 0.05 ± 0.01; Figure 3C). In contrast, at intermediate expression levels (e.g., comparing the data at promoter activity of 0.23, which is at about half maximal induction), the repressor shows less variation that the activator (SD = 0.06 ± 0.01 versus 0.15 ± 0.04, coefficient of variation [SD/mean] of 0.26 ± 0.04 versus 0.65 ± 0.15; Figure 3D). These effects were not due to variation in the control vector (σ_{70}), whose promoter activity was nearly independent on cAMP (Figure S1 available online).

In sum, the activator seems to be more insulated (less variable across conditions) than the repressor at the high end of their expression range; at the mid and low regions of the expression range, the repressor seems to be more insulated. These findings are in line with the hypothesis that there is more variability across conditions when the regulator is unbound to its cognate regulator than when it is bound.

The *lac* Input Function Shows Large Variability across Conditions

We further tested variability across conditions in a promoter with two transcription factor inputs, the promoter of the *lac* operon. The two transcription factors are the activator CRP and the repressor LacI. We used a low-copy reporter plasmid in which the full intergenic region upstream of *lacZ* regulates gfp (U410)

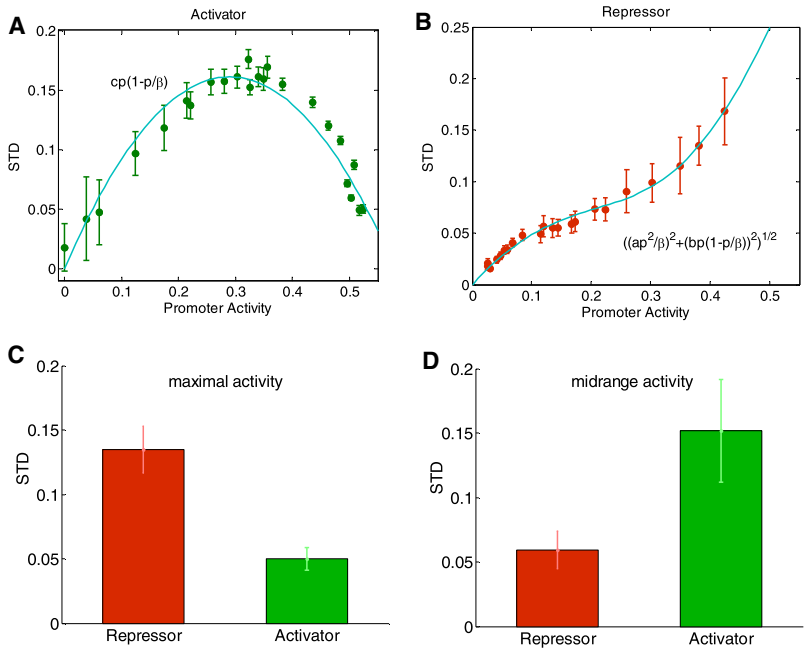

Figure 3. Variation across Conditions Depends on Mode of Regulation: Activator Is More Variable at Low Expression, and Repressor Is More Variable at High Expression

(A) Standard deviation of activator promoter activity (U435) first rises and then decreases as a function of the mean expression. Full line, fit to model with $\beta = 0.58$, $c = 1.11 \pm 0.02$.

(B) Standard deviation of repressor promoter activity (U436) across conditions rises as a function of the mean expression. Full line, fit to model with $\beta = 0.39$, $a = 0.36 \pm 0.01$, $b = 0.64 \pm 0.02$.

(C) Standard deviation across conditions of maximal expression is higher for repressor than activator.

(D) Standard deviation across conditions of expression at the middle of the induction range. Variation of normalized promoter activity is compared at cAMP concentrations (1 mM and 1.6 mM for repressor and activator, respectively) that yield mean promoter activity of 0.23 for repressor and activator. Error bars were calculated by bootstrapping, using 95% confidence.

See also Figures S3 and S5.

(Figure 4A). We included on the plasmid a copy of the *lacI*^{q} gene, which produces high levels of the repressor, sufficient to achieve strong repression without inducer (Lee and Bailey, 1984; Setty et al., 2003).

We transformed *E. coli* MG1655 with the plasmid and used 96 combinations of the two inducer levels, cAMP and IPTG, to regulate CRP and LacI, respectively. The *lac* promoter activity increases with IPTG and cAMP as has been previously described (Setty et al., 2003; Mayo et al., 2006; Kuhlman et al., 2007) and can be represented as a two-dimensional input function (Kaplan et al., 2008) (Figure 4B).

We measured this input function in 15 different conditions, using the same 96 levels of inducers. These conditions included high salt, ethanol, low pH, and low temperature (see the Experimental Procedures). We normalized the promoter activity in each condition to the σ_{70} reporter strain grown in the same conditions, to control for differences in growth rate. This control strain showed little variation in promoter activity (less than 30%) as a function of ITPG and cAMP (Figure S2).

The variability of the input function was measured by the standard deviation across conditions of the promoter activity at each of the 96 IPTG and cAMP levels. We find that the *lac* promoter input function varies across conditions, with the standard deviation over mean ranging from about 10% to 100%.

Equi-Expression Lines Show Higher Variability across Conditions when *lac* Promoter Is Bound then when It Is Unbound

We analyzed the variation across conditions of the *lac* input function on lines of equal promoter activity. These are contour lines on the mean input function (the input function averaged over all conditions). On each equi-expression line, concentrations of the inducers vary in a way that keeps the mean *lac* promoter activity constant. At one end of each line, cAMP levels are high and IPTG levels are low, so that both CRP and LacI tend to bind their sites. At the other end of the line, cAMP levels are low and IPTG levels are high, so that both regulators tend to be unbound (Figure 4B). Thus, an equi-expression line allows comparison of states of relatively high and low promoter occupancy, at the same mean expression level.

To follow each equi-expression line, we define an angle θ (Figure 4B, inset at top) that is equal to zero on the side where the regulators are expected to be relatively unbound to the promoter (low cAMP and high IPTG), and increases along the line until reaching its maximal value of $\theta = 90^\circ$ at the other end where the regulators are expected to be relatively bound to the promoter (high cAMP high and low IPTG). This angle is a coordinate whose use is to go along the contour from high to low occupancy. Plotting variability across conditions as a function of the angle θ allows one to examine the effect of promoter occupancy on insulation. We find that the variability between conditions decreases with promoter occupancy (Figure 5A). This conclusion applies to all expression levels (that is to all equi-expression lines) tested, represented by different colors in Figure 5.

We also computed the promoter occupancy at each inducer combination, using a mathematical model for the *lac* promoter (see the Experimental Procedures). Again, we find that the variability between conditions decreases with the promoter occupancy (Figure 5B).

Simple Mathematical Model Agrees with Present Results

To further understand the present results, we analyzed the effects of various sources of variability on a gene's input

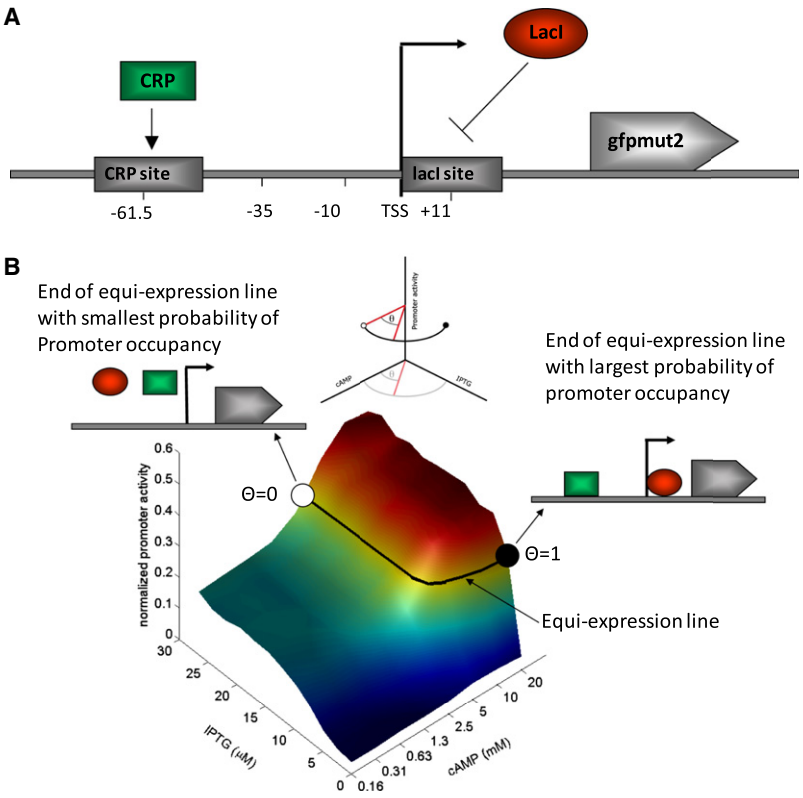

Figure 4. The *lac* Promoter Input Function Increases with Its Inducers IPTG and cAMP; Equi-Expression Lines Allow One to Study the Transition between a Bound and an Unbound Promoter Configuration while Keeping Constant Promoter Activity

(A) The *lac* promoter is regulated by the activator CRP and the repressor LacI. CRP binds its sites tightly when bound to the inducer cAMP, whereas LacI binds its site tightly when it is not bound to its inducer IPTG. Only one of the two lacI sites on the plasmid is shown; the other is at –82.

(B) Promoter activity of the *lac* promoter, normalized to promoter activity of a σ_{70} promoter (U371), in M9 + 0.2% glucose, for 96 combinations of cAMP and IPTG. Equi-expression lines connect inducer levels at which expression is constant. At one end of an equi-expression line is a configuration in which both CRP and LacI are relatively bound often (high cAMP and low IPTG); at the other end of the line is a configuration in which the two regulators are bound rarely. The angle θ (inset on top) is a coordinate along each line. When θ moves from 0° to 90°, a point moves along the equi-expression line from the open to the closed configuration sides.

See also Figure S2.

function. For this purpose, we consider the simplest mechanism for regulation, the well-studied mass-action model of binding of regulators to the promoter, which yields Michaelis-Menten expressions for the induction curve (Buchler et al., 2003; Setty et al., 2003; Bintu et al., 2005). Consider a regulator, whose concentration in its active, DNA binding form is X, which binds its site with dissociation constant K. The Michaelis-Menten expression for an activator is $\beta \cdot X/(K+X)$, and for a repressor $\beta \cdot K/(K+X)$, where β is the maximal activity.

We consider two sources of nonspecific binding. The first is nonspecific binding that interferes with RNA polymerase, modeled by a factor with concentration N. The second is nonspecific binding that interferes with the binding of the transcription factor. We model this by a factor of concentration N'. Both N and N' vary between conditions. Mass-action analysis results in promoter activity for an activator of the form $P_A = \beta \cdot X/(K+X+N+N')$, and for a repressor $P_R = \beta(K+N')/(K+X+N+N')$. The factor N' appears in the numerator of the repressor equation because N' binding precludes repressor binding leading to full promoter activity. It does not appear in the numerator of the activator equation, because N' binding precludes activator binding and thus leads to no expression.

The nonspecific factors N and N' vary between conditions. Thus $N = \langle N \rangle + dN$, where dN is a random variable with standard deviation σ_N. Similarly, $N' = \langle N' \rangle + dN'$, where dN' has standard deviation $\sigma_{N'}$. Assuming nonspecific binding that is much weaker than the binding of the cognate factor X, namely $dN, dN' << K$, one finds that, to a first approximation, the variation across conditions for the activator is

$$std(P_A) = cP_A \cdot \left(1 - \frac{P_A}{\beta}\right),$$

where $c = (\sigma_N^2 + \sigma_{N'}^2)^{1/2}/(K + \langle N \rangle + \langle N' \rangle)$. This function describes a parabola: variation across conditions first increases with expression and then decreases. The function fits well the experimental findings for the activator construct (full line in Figure 3A).

For a repressor, the variation in nonspecific binding yields the following standard deviation for the promoter activity:

$$std(P_R) = \left[\left(\frac{a \cdot P_R^2}{\beta}\right)^2 + \left(b \cdot \left(1 - \frac{P_R}{\beta}\right) \cdot P_R\right)^2\right]^{1/2},$$

where $a = \sigma_N/(K + \langle N' \rangle)$ and $b = \sigma_{N'}/(K + \langle N' \rangle)$. Thus, for a repressor, variation increases with promoter activity. It rises linearly with promoter activity, then briefly saturates, and then rises quadratically with promoter activity. This gives a good fit with the experimental data (full line in Figure 3B; detailed derivation of equations is given in Figure S5).

Note that good fits are obtained with very few free parameters. The parameter β for each construct is determined by its measured maximal promoter activity. The nonmonotonic form of the activator variation is captured by a single free parameter c = 1.11 ± 0.02. The kinked rising curve for the repressor is fit very well with two free parameters, a = 0.36 ± 0.01 and b = 0.64 ± 0.02. We note that using Hill functions instead of Michaelis-Menten functions yields precisely the same equations and results for the variability.

In summary, modeling of the effect of nonspecific binding suggests that data is well explained by nonspecific factors that vary between conditions and block the binding of RNA polymerase and the cognate transcription factor.

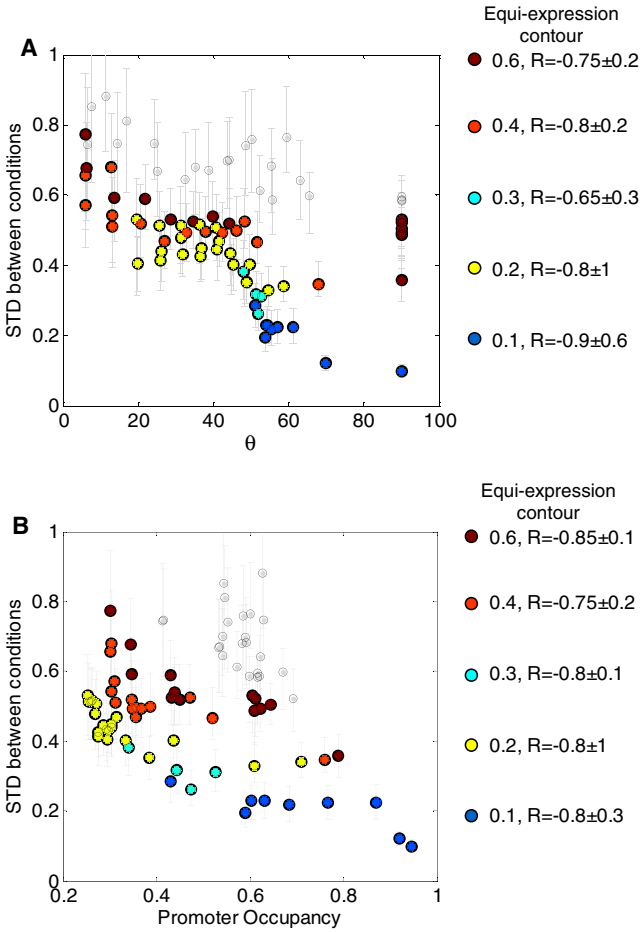

Figure 5. Lac Promoter Shows Higher Variability across Conditions when Unbound to Its Regulators than when It Is Bound at Equal Mean Expression Level

(A) Variation across conditions plotted for different equi-expression lines as a function of the angle θ, defined in Figure 4.

(B) Variation across conditions decreases with computed promoter occupancy. Occupancy is calculated from a model in which the two transcription factors bind independently, and expression results in states where only the activator is bound (see the Experimental Procedures).

In both (A) and (B), variation is from growth in 15 different conditions (see the Experimental Procedures for conditions). Promoter activity is normalized to a σ_{70} reporter grown in the same conditions. Contour levels of normalized promoter activity are at 0.1, 0.2, 0.3, 0.4, and 0.6; the correlation coefficient R between variation and θ or occupancy at each contour are indicated on the right. White dots indicate data from regions of the input function where slope is steepest (more than 75% of the maximal slope in the input function; Figure S4). Due to the slope, variation in these points is expected to be more sensitive to experimental errors, and they were not included in the regression computation. The points that line up on the right side of (A) are at the flat, maximum region of the input function. They line up because of the way we define θ—approximately the same angle (90°) corresponds to different wells in the plate with about the same promoter activity. Error bars were calculated by bootstrapping, using 95% confidence. See also Figure S4.

We also modeled the effects of variation in the cognate regulator activity. Here, the promoter activity depends on $X+dX$, where dX is a random variable. Repeating the analysis, we find the same qualitative conclusions: activators have variability that rises and then falls with promoter activity; repressors show monotonically increasing variability. Thus, activators are better insulated at high expression and repressors at low expression. However, the quantitative shape of the curves does not fit the measured variability as well as the models for nonspecific binding described above. Details are provided in the Figure S3.

DISCUSSION

We explored the variability of *E. coli* promoter activity across conditions, and its dependence on the mode of regulation. We tested constructs in which CRP acts as an activator or a repressor. We find that both modes of regulation can show sizeable variation across conditions. Activator and repressor differ in when this variation is most strong: the activator showed large variation at low and intermediate expression levels, whereas the repressor showed large variation at high expression levels. In other words, the activator input function is better insulated from variations in conditions at high expression levels; the repressor is better insulated at low expression levels. Equivalently, insulation seems to be highest when the promoter is occupied by its cognate regulator and lowest when the promoter is unbound by its regulator.

The same trend was found in the *lac* promoter, which has two cognate regulators—CRP and LacI. By following equi-expression lines, we could measure variability across conditions, comparing different degrees of promoter occupancy, while keeping mean expression constant. We find that the bound *lac* promoter has lower variation across conditions than the unbound promoter.

These findings can be rationalized in terms of a simple model of nonspecific binding to a promoter that interferes with the binding of RNA polymerase and with the transcription factors. The model predicts that activator variability should be nonmonotonic with promoter activity, first rising and then decreasing, whereas repressor variability should increase with promoter activity. This model gives excellent fit to the data.

Intuitively, when cognate regulators bind they are close to saturating their binding curves; small perturbations such as nonspecific binding or changes in regulator activity thus have small relative effects, so that insulation is good. When the regulators are weakly bound, they can be in a high-slope region of their binding curve; small perturbations can have large relative effects, and insulation is compromised.

Given that insulation of a gene is highest when its promoter is bound, a good strategy to provide insulation is to choose a mode of regulation that keeps the regulator(s) bound most of the time. Figure 6 shows a comparison between the activator and repressor measurements of Figure 3: At low-intermediate expression levels, the activator shows more variability across conditions than the repressor. At high levels, the reverse is true.

Thus a gene which, in its natural environment, is expressed most of the time at low-intermediate region of its expression range (a low-demand gene) would benefit from a repressor mode of control; genes needed most of the time at the high end of their expression range (a high-demand gene) would benefit from an activator mode of control.

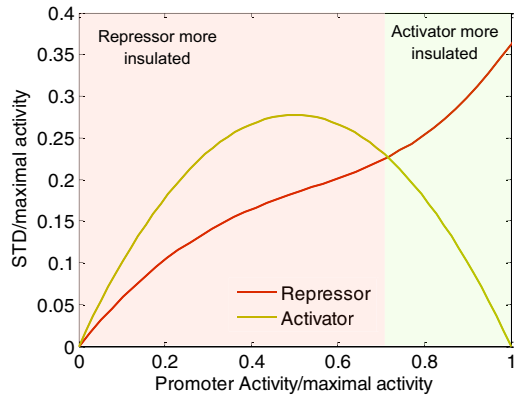

Figure 6. Variability as a Function of Promoter Activity Suggests that the Repressor Is Better Insulated at Low Expression and Activator at High Expression

Data are from Figure 3, normalized to maximal promoter activity. This suggests that genes needed most often at the low end of their expression range would benefit from a repressor mode of control, to minimize variability. An activator is better when the gene is needed often near the high end of its expression range. See also Figures S3 and S5.

This provides support for the theoretical explanation by Shinar et al. (2006) for the Savageau demand rule. According to the Savageau rule, high-demand genes have activator control and low-demand genes show repressor control (Savageau, 1974, 1998a, 1998b). Genes with multiple regulators seem to follow the same principle (Shinar et al., 2006): in the natural environment of *E. coli*, starvation is common and lactose is rare, and thus cAMP is usually high and lactose usually absent, so that both CRP and LacI bind the *lac* promoter. Thus, the *lac* promoter with its regulatory design of an activator and a repressor insures that the most common environmental state is the one with highest promoter occupancy and hence with the highest insulation. This state has a low-intermediate expression level, which can be reduced by adding glucose, and increased by adding lactose. Had the *lac* promoter been designed with, say two activators or two repressors, insulation would have been compromised (Shinar et al., 2006).

Variation of an input function across conditions may be adaptive or nonadaptive in terms of fitness. If a condition leads to altered expression of a gene, it could be a deviation that reduces fitness and requires better insulation. It could also be a highly evolved response using a mechanism we don't yet understand that increases fitness. One may argue that it is not possible to evolve dedicated fine-tuned responses to all of the conditions that can be potentially encountered by the cell because their number is combinatorially huge—consider all possible combinations of nutrients and stresses. Thus insulation is likely to be important, even if it does not relate to all observed variations. The assumption that variation between conditions is deleterious can be tested in each particular case by means of accurate fitness measurements in different conditions (Elena and Lenski, 2003; Dekel and Alon, 2005; Oxman et al., 2008).

The present approach can be used to study insulation in other genes and organisms. This adds to our understanding of insulation of gene circuits, complementing work on other aspect of insulation of modules (Babiskin and Smolke, 2011), such as minimizing the retroactive effects of downstream components on the function of a gene circuit (Del Vecchio et al., 2008). Insulation of genes from effects of their genomic context (rather than insulation from varying conditions) has also been studied (Geyer, 1997).

It is likely that additional mechanisms exist to increase or decrease insulation from environmental variations. For example, in eukaryotes, nucleosome occupancy is known to correlate with the degree in which gene expression varies across conditions (Landry et al., 2007; Field et al., 2008; Tirosh et al., 2009). The potential connection of mode of control and insulation addressed in this study could provide a link between the ecological demand for a gene and its regulatory design.

EXPERIMENTAL PROCEDURES

Strains

The following strains were used in this study:

- U66: MG1655 + promoterless vector (Zaslaver et al., 2006)
- U371: MG1655 wt + σ_{70} reporter (Kaplan et al., 2008)
- U410: MG1655 wt + lacZ reporter containing $lacI^q$ gene (this study)
- U435: MG1655 ΔcyaA + CRP as an activator construct (Kaplan et al., 2008; this study)
- U436: MG1655 ΔcyaA + CRP as a repressor construct (Kaplan et al., 2008; this study)
- U449: MG1655 ΔcyaA + σ_{70} reporter (this study)
- U450: MG1655 ΔcyaA + promoterless vector (this study)
- U451: MG1655 ΔcyaA (this study)

cyaA deletion in MG1655 was achieved by P1 transduction from the Keio knockout collection (Baba et al., 2006). The genomic deletion was verified by PCR. Kanamycin resistance was eliminated from the deleted strain with FLP recombinase, as described (Datsenko and Wanner, 2000).

Vector Preparation

Synthetic activator, repressor, and σ_{70} reporter preparation was previously described (Kaplan et al., 2008). The vectors are based on a low-copy plasmid (pSC101 origin [Lutz and Bujard, 1997; Kalir et al., 2001]) with Kanamycin resistance. In the plasmid, a promoter of interest controls green fluorescent protein gene (gfpmu2) (Zaslaver et al., 2006). This plasmid is not measurably lost from cells, and has very low cell-cell variation in promoter activity of promoters with medium to high expression as those used here (Silander et al., 2012). Its copy number was found not to vary as a function of cAMP under conditions similar to the present experiments (Kaplan et al., 2008). The parental sequence for the promoter region in the three reporters was the MG1655 *lac* promoter region, genomic coordinates 365637–365530 (complementary strand) (Karp et al., 2002). LacI binding sites were eliminated from the activator and repressor constructs by reshuffling of their sequence. For the σ_{70} reporter, both the LacI and CRP binding sites were reshuffled and replaced the σ_{70} site with the consensus site. The constructs were used to transform MG1655 ΔcyaA strain (Zaslaver et al., 2006).

The repressor vector was further point mutated with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA) and the strength of its −10 binding site was reduced in order to obtain expression levels similar to the activator vector (TATAAT was replaced with GATAAT).

The promoter region sequence of the activator was as follows: GAGA GGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCA CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGCATGGATA AGTAGCTAGGAATTTCACACTGCAAACAGCT.

The promoter region sequence of the repressor was as follows: GAGAGG CATGGGGACGGGAACACTACCAGATCAAATGTGTCGTTTCCAGACAGGTC

AGCCCCTTGACACTGCTGTCGTGTCCTTCAGATAATGGTAAATGTGAGTTAGCTCACACTTTTTCACACTGCAAACAGCT.

The promoter region sequence of the σ_{70} reporter was as follows: CGTCAGGAGGAGAGGGGCAGTGAGCGCAACGCAATCAGATCAAATGTGTCGTTTCCATAGGCACCCCAGGCTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGCATGGATAAGTAGCTAGGAATTTCACACTGCAAACAGCT.

The *lacI*^{q} gene was amplified from pTRC99A plasmid by PCR using primers harboring XhoI and BglII restriction sites. The PCR fragment was then purified, digested with the appropriate enzymes and ligated to a lacZ reporter plasmid containing a 250 bp region that includes 36 bp into the *lacI* gene, the entire intergenic region and 92 bp into the *lacZ* gene (Zaslaver et al., 2006). MG1655 was transformed with the ligated plasmid and the sequence was verified by sequencing.

Growth Conditions

Experiments were carried out in M9 minimal media consisting of M9 salts (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.01% NH_4Cl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$). For amino acid media (M9C), M9 media were supplemented with 0.05% casamino acids and 0.2% glycerol. All media contained 0.2% glucose, 50 μg/ml kanamycin unless otherwise indicated, and growth was at 37°C unless otherwise indicated.

The conditions used for the activator and repressor construct experiments were as follows: (1) M9, (2) M9C, (3) M9 + 60 mM NaCl, (4) M9 + 100 mM NaCl, (5) M9 + 200 mM NaCl, (6) M9 (pH = 6), (7) M9 (pH = 6.5), and (8) M9 + 2% ethanol.

The conditions used for the *lac* experiments were as follows: (1) M9, (2) M9C, (3) M9 + 30 mM NaCl, (4) M9 + 60 mM NaCl, (5) M9 + 100 mM NaCl, (6) M9 + 350 mM NaCl, (7) M9 + 3.5% ethanol, (8) M9 + 0.03% H_2O_2, (9) M9 (pH 4.5), (10) M9 (pH 5), (11) M9 (pH 5.5), (12) M9 (pH 6), (13) M9 (pH 6.5), (14) M9 + 0.2% galactose instead of glucose, and (15) M9 at 27°C instead of 37°C.

Robotic Experiments

Bacteria were inoculated from frozen stocks and grown overnight at 37°C with shaking at 250 rpm either in M9 or in M9C media containing 0.2% glucose and 50 μg/ml kanamycin (inoculate for cultures grown in M9 based media were grown overnight in M9, inoculates for cultures in M9C were grown overnight in M9C). A robotic liquid handler (FreedomEvo, Tecan) was used for preparation of experiments: cultures were diluted 1:500 into the specific experimental medium at a final volume of 150 μl per well in 96-well plates (Nunc).

For the activator and repressor experiments, a cAMP (Sigma) gradient with 12 different concentrations ranging from 0 to 6, 8, or 15 mM was created along one axis of the plate and four replicates were done for each cAMP concentration. The same experimental set up was used for each growth condition. Relative error between replicates is about 5%, much smaller than the variation observed between conditions.

For the lac experiments, a cAMP gradient with eight different concentrations ranging from 0 to 20 mM was created along one axis of the plate and an IPTG (Fermentas) gradient with 12 different concentrations ranging from 0 to 30 μM was created along the second axis.

The plates were covered with 100 μl mineral oil (Sigma) per well to prevent evaporation as described (Zaslaver et al., 2006) and were then transferred into an automated incubator and were grown with shaking at 37°C. Every 8 min each plate was transferred by the robotic arm into a multiwell fluorimeter (Infinite F200, Tecan) that read OD (600 nm) and GFP fluorescence (535 nm).

Data Analysis

Data was processed with Matlab (MathWorks). Promoter activity for each well was calculated from the OD and GFP measurements after subtraction of the OD and GFP background. GFP background was obtained for each well from promoterless control strains U66 and U450 (the latter for the repressor and activator experiments) grown under the same conditions. Promoter activity was calculated by computation of the rate of accumulation of GFP fluorescence per unit time divided by the OD ($dGFP/dt/OD$) (Ronen et al., 2002). At all conditions, promoter activity reached an approximately constant value over a window at mid-exponential growth and was averaged over this window. Window size was determined for each condition such that the variation in growth rate across the window was less than 10%—the window included 15–20 time points (about 2–3 hr) around the point of maximal growth rate. Results are insensitive to varying the window size.

To normalize for changes in growth rate, we divided each promoter activity at each time point by the activity of a σ_{70} reporter grown under the same conditions. This control reporter (Kaplan et al., 2008) is based on the same pSC101-orign vector (U66). It has consensus σ_{70} −10 and −35 sites driving the gfpmut2 reporter gene. Normalizing by this vector controls for condition-specific variations in global transcription rates, posttranscriptional processes, gfp protein properties or changes in plasmid copy number.

Model for Promoter Occupancy

To compute occupancy for the lac promoter construct employed in this study, we use a mass-action model based on previous studies (Setty et al., 2003; Bintu et al., 2005). The inputs are the two inducers S_x = cAMP and S_y = IPTG that bind X = CRP and Y = LacI, respectively. The promoter activity is proportional to the fraction of time the promoter is bound by the activator and not by the repressor: $P = \beta X^*/(K_x + X^*)K_y/(K_y + Y^*)$, where the DNA-binding forms of the transcription factors are CRP-cAMP, namely $X^* = X_t S_x/(K_{sx} + S_x)$, and LacI unbound to ITPG, namely $Y^* = Y_t K_{sy}^n/(K_{sy}^n + S_y^n)$, and n is a Hill coefficient. Here X_t and Y_t are total transcription factor concentrations, and the K parameters are the dissociation constants for the sites and inducers. The promoter occupancy of the CRP site is $O_x = X^*/(K_x + X^*)$, and the promoter occupancy of the LacI site is $O_y = Y^*/(K_y + Y^*)$. Importantly, the promoter activity in this model is simply related to occupancy as follows: $P/\beta = O_x(1 - O_y)$, where β is the maximal promoter activity. This model predicts, therefore, that the normalized input function is separable to a product of two functions, one that depends only on cAMP (namely, O_x), and the other that depends only on IPTG [namely, $(1 - O_y)$]. Indeed, we find that the two-dimensional input function, normalized to its maximum value, is given to a good approximation by the product of two one-dimensional functions (Kaplan et al., 2008): g(cAMP), the normalized activity as a function of cAMP at maximal ITPG induction, and f(ITPG), the normalized activity as a function of IPTG at maximal cAMP induction. In other words, P(cAMP, IPTG) / β = g(cAMP) · f(IPTG), with mean relative error of about 10%. To compute the occupancy, we equated these functions with the occupancy as follows: $O_x = f$(cAMP), and $O_y = 1 - g$(IPTG). Figure 5B shows average occupancy $O_a = (O_x + O_y) / 2$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.molcel.2012.04.032.

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