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The Incoherent Feed-forward Loop Accelerates the Response-time of the *gal* System of *Escherichia coli*

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Complex gene regulation networks are made of simple recurring gene circuits called network motifs. One of the most common network motifs is the incoherent type-1 feed-forward loop (I1-FFL), in which a transcription activator activates a gene directly, and also activates a repressor of the gene. Mathematical modeling suggested that the I1-FFL can show two dynamical features: a transient pulse of gene expression, and acceleration of the dynamics of the target gene. It is important to experimentally study the dynamics of this motif in living cells, to test whether it carries out these functions even when embedded within additional interactions in the cell. Here, we address this using a system with incoherent feed-forward loop connectivity, the galactose (*gal*) system of *Escherichia coli*. We measured the dynamics of this system in response to inducing signals at high temporal resolution and accuracy by means of green fluorescent protein reporters. We show that the galactose system displays accelerated turn-on dynamics. The acceleration is abolished in strains and conditions that disrupt the I1-FFL. The I1-FFL motif in the *gal* system works as theoretically predicted despite being embedded in several additional feedback loops. Response acceleration may be performed by the incoherent feed-forward loop modules that are found in diverse systems from bacteria to humans.

Keywords: systems biology; design principles; computational biology; network motifs; fluorescent reporter

Introduction

Cells respond to external signals by means of transcription regulation networks.[1–9] These networks are made of recurring regulatory circuits called network motifs.[10,11] Network motifs were first defined in the transcription network of *Escherichia coli*.[10] The same set of motifs were then found to appear in the transcription networks of diverse organisms, including yeast,[11–13] *Bacillus subtilis*,[14,15] *Drosophila*[14] and humans.[16,17] Each network motif has been suggested to perform a key information processing function.[10,18,19] It is important to experimentally study the function of each network motif in living cells, because this can shed light on the dynamical behavior of many systems across organisms that have the motif.

One of the most significant network motifs is the feed-forward loop (FFL).[10,11,18,20–24,53,54] The FFL is made of two cascaded transcription factors that jointly regulate a gene. In the FFL, transcription factor X regulates transcription factor Y, and both jointly regulate gene Z. Since each of the three interactions of the FFL can be either activation or repression, there are eight types of FFLs (Figure 1), corresponding to the different combinations of positive and negative regulation for each interaction. The frequency of each FFL type was counted in transcription regulation databases for both *E. coli* and *Saccharomyces cerevisiae*.[18] Two FFL types are more common than others. These are termed the coherent type-1 FFL (C1-FFL), which contains three positive interactions (that is, X and Y are both transcriptional activators), and the incoherent type-1 FFL (I1-FFL) in which X activates Y and Z while Y represses Z. Coherent and incoherent type 1 FFLs can appear in interesting combinations, for example cascaded I1-FFLs and C1-FFls govern the gene regulation system for sporulation in *B. subtilis*.[15] Often, the same X and Y regulate several genes, Z1,…Zn, forming a multi-output FFL.[25,26]

Theoretical analysis suggested that the C1-FFL motif robustly performs a dynamical function

Abbreviations used: GFP, green fluorescent protein; FFL, feed-forward loop; I1-FFL, incoherent type 1 FFL.

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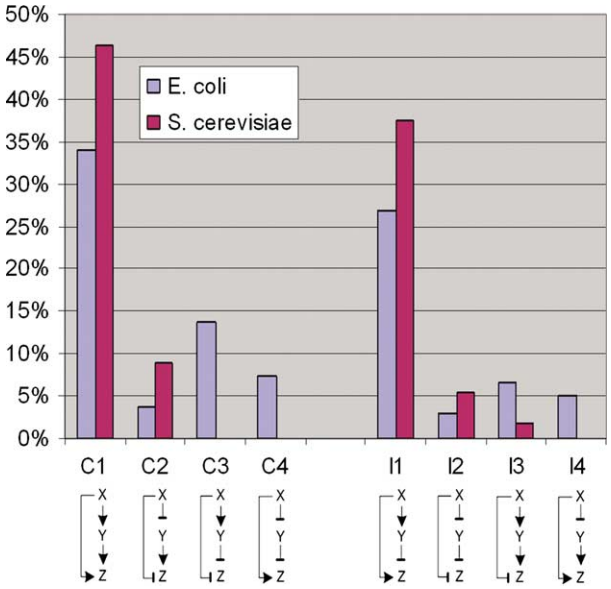

Figure 1. The eight FFL types and their relative abundance in the transcription networks of *E. coli* and *S. cerevisiae*. Relative abundance is the fraction of each type relative to the total number of FFLs in the network (138 in *E. coli* and 56 in *S. cerevisiae* in the networks presently studied). The coherent FFL types are denoted C1 through C4, and the incoherent types I1 through I4.

termed sign-sensitive delay.[18] This delay depends on the input function at the Z promoter. For example, when X and Y are both needed to activate Z, forming a C1-FFL with an AND input-function at the Z promoter, the C1-FFL shows a delay of Z expression following induction of X activity, but no delay following deactivation of X. Thus the C1-FFL can filter out brief fluctuations that activate X. This function was experimentally demonstrated in living cells in the arabinose (*ara*) utilization system of *E. coli*.[20] In another example, when either X or Y are sufficient to activate Z, forming a C1-FFL with an OR or SUM input-function at the Z promoter, a delay occurs after deactivation of X but not after activation. This function, which allows continuous expression even if the input signal is briefly lost, was experimentally demonstrated in living cells using the flagella class 2 system of *E. coli*.[24]

Here, we experimentally study the second common FFL type, the type-1 incoherent FFL (I1-FFL) (Figure 1). In this motif, X positively regulates Y and Z, whereas Y represses Z expression. Thus, the two regulators X and Y act in opposite signs to control Z, hence the name "incoherent FFL". I1-FFLs regulate over 100 different genes in *E. coli*[21] and yeast,[18] comprising about one-third of the total number of FFLs in these organisms (Figure 1). It is much more common than any of the other three types of incoherent FFL (types 2, 3 and 4 incoherent FFL). Recently, an I1-FFL involving a micro-RNA has been identified in mammalian cells.[27]

Theoretical analysis suggested that the I1-FFL can perform response acceleration:[18] expression of the output gene Z has a shorter response time, defined as the time to reach halfway to the steady-state level, than a corresponding "simple regulation" system that reaches the same steady state. To see this, note that when X is induced, Z begins to be expressed. In parallel, the level of the repressor Y also increases. When Y activity exceeds the repression threshold for the Z promoter, Z expression is repressed, and drops to a low steady-state level. The result is an overshoot dynamics, which shortens the response-time. The response-time of Z in the I1-FFL is smaller than the response time of a corresponding simply regulated system in which X regulates Z without an I1-FFL. In the simply regulated system, the dynamics[28] are exponential convergence to a steady state x_{st}, such that $x/x_{st}=1-e^{-\alpha t}$. The response time for stable proteins (not actively degraded in the cell) has been shown to be about one cell-generation time.[28,29] In contrast, the I1-FFL shows accelerated responses, by using a strong promoter to achieve rapid initial induction and a repressor to reduce production at a delay and reach the desired steady state. When the biochemical parameters are such that Y fully represses Z, the resulting dynamics resembles a pulse of Z expression.[18]

The I1-FFL was recently constructed as a synthetic gene circuit in *E. coli* by Weiss and colleagues.[30] Well-studied transcription factors were connected in an I1-FFL pattern with a strong repression of Z by Y, whose readout was green-fluorescent protein. These experiments demonstrated that the I1-FFL can generate a pulse of expression following induction of X.

Both synthetic gene circuit experiments and the theoretical models deal with the I1-FFL as a three-gene circuit in isolation. In the real cell, this motif is embedded inside a network of additional interactions. It is therefore important to experimentally study its function in a natural system in living cells.

Here, we examine the dynamics of the I1-FFL in living cells using the well-studied *crp-galS-galE* system of *E. coli*,[31–36] which has an I1-FFL structure (Figure 2(a)). The *gal* system allows *E. coli* to grow on the sugar galactose. Expression of the *gal* genes is inhibited in the presence of glucose, a superior energy source. In the *gal* system, the galactose utilization operon *galETK*, called *galE* throughout this study, is transcriptionally regulated by *CRP*, an activator induced by glucose starvation. The *galE* promoter is also repressed by *GalS*. *GalS* unbinds from the *galE* promoter in the presence of the inducer β-D-galactose or its non-metabolizable analog D-fucose (Figure 2(a)), relieving the repression. The gene that encodes the repressor *GalS* is itself positively regulated by *CRP*, so that an I1-FFL is formed. Furthermore, *GalS* negatively regulates its own expression, forming a negative autoregulation loop. This loop can act to speed[28,37] and stabilize[38] the expression of the *GalS* gene.

The *GalE* I1-FFL is embedded in the cell within a number of additional interactions (Figure 2(b)), including regulation by the repressor *GalR* (which

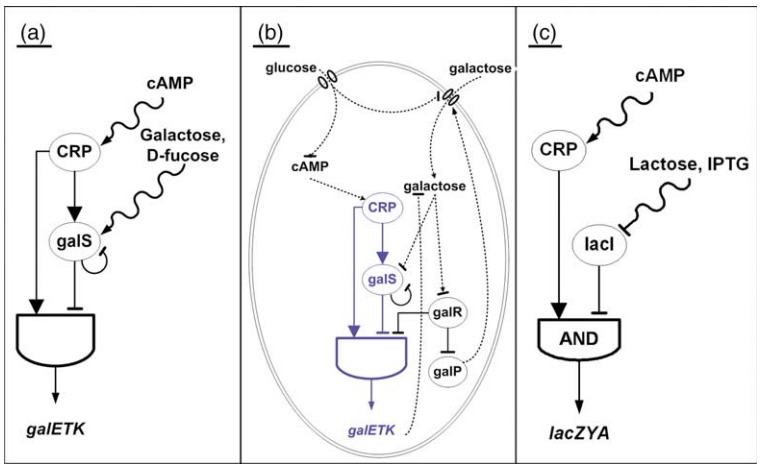

Figure 2. The galactose and lactose systems of *E. coli*. (a) The I1-FFL in the galactose system. CRP activates galS and *galETK* (denoted *galE* throughout). *galS* represses *galE* and its own promoter. The inducers are cAMP for *CRP* and galactose/D-fucose for *GalS*. D-Fucose is a non-metabolizable inducer. (b) A partial map of the interactions in the galactose system. Continuous lines represent transcription interactions, and broken lines represent non-transcriptional interactions (see the text). (c) The lactose system, in which *lacZYA* (denoted *lacZ* throughout) is regulated by CRP and *lacI*. The inducer IPTG is used to deactivate *LacI* and therefore to allow full expression of the *lacZ* promoter. Here, AND-gates mean binding of the activator AND NOT the repressor to the promoter region of the target gene.

does not appear to be itself regulated[35]); the degradation of the inducer galactose by the *galETK* genes; inhibition of galactose pumps by glucose (known as inducer exclusion) and induction of galactose pumps by galactose, which act to increase inducer levels in the cell.

Here, we focus on the dynamic behavior of the *galE* promoter, as a representative I1-FFL system. We used a green fluorescent protein (GFP) reporter system to obtain high-resolution dynamical expression measurements of the *gal* system in living *E. coli* cells, and compared the dynamics to various control systems, such as the *lac* system (Figure 2(c)) that does not display the FFL connectivity and mutants deleted for the *gal* repressor genes. We find that the *galE* I1-FFL circuit exhibits response-acceleration. This acceleration is abolished in mutants and conditions that disrupt the I1-FFL motif. These findings support the theoretical predictions about the function of this motif and provide a view of its design and dynamics in a natural system.

Results

Incoherent type-1 FFLs in the transcription networks of both *E. coli* and yeast

Transcription regulation databases of *E. coli* have increased in scope since the original study of the FFL types.[18] Recently, Ma *et al.*[21] have extended the FFL counts by using updated databases including EcoCyc and RegulonDB, as well as the data described by Shen-Orr *et al.*[10] They counted the cases of FFL using genes as nodes, and reported several hundred new FFLs, including many I1-FFLs.

We repeated this counting, but defined each node in the network as an operon (a set of genes transcribed on the same mRNA) as Shen-Orr *et al.*,[10] rather than using one node per gene as Ma *et al*. This avoids counting self-regulated operons, which contain both a transcription factor and another gene, as FFLs (see Materials and Methods). We find, in agreement with Ma *et al*., that I1-FFLs together with the C1-FFL make up most of the FFLs in the network (Figure 1). The other six types are much more rare. The distribution of FFL types is similar between the transcription networks of *E. coli* and yeast: in both organisms, I1-FFL and C1-FFL are the most abundant types.

galE is regulated in an I1-FFL by *CRP* and *GalS*

To quantify the regulatory effects in this system, we used reporter strains, in which GFP is fused to the promoter region of the gene of interest on a low copy plasmid, in *E. coli* K12 strains. In each case, the entire inter-genic region was used to control GFP. This method allows accurate measurements of promoter activity dynamics, as was previously demonstrated.[20,24,39–41] GFP fluorescence and $A_{600\mathrm{nm}}$ were measured by growing strains in a multi-well fluorimeter. Gene expression was calculated by the fluorescence per $A_{600\mathrm{nm}}$ unit, $P = \mathrm{GFP}/A$, where GFP is the fluorescence signal and A is the absorbance (see Materials and Methods for details).

We measured the activity of promoters in *E. coli* MG1655 strains (Table 1A). We find that *galS* promoter has undetectable activity when bacteria are grown on glucose. Its promoter activity increases to easily detectable values, once cells are grown on a non-glucose medium, such as on mannose as a carbon source (Table 1A). Similar results are found for other carbon sources (maltose, glycerol, data not shown). This is consistent with regulation of the *galS* promoter by *CRP*.[35,36] Adding the inducer D-fucose to the medium further increases *galS* expression by 30($\pm$10)% (Table 1A).

We find that the *galE* promoter has a significant basal activity level in glucose (Table 1A). Saturating

Table 1. Activity of *gal* promoters under various conditions

A. *Steady state expression of WT reporters in MG1655*

	Reporter [AU]	
Condition	*galS*	*galE*
Glucose	0.0	0.4
Glucose+mannose	0.0	0.4
Mannose	0.4	0.5
Mannose+D-fucose	0.5	4.7

B. *Effect of galS, galR deletion on galE reporter in MC4100*

	Growth on mannose without inducer			
	WT	*ΔgalS*	*ΔgalR*	*ΔgalR galS*
galE	0.6	1.6	3.9	5.2

C. *Effect of galS+galR deletion on galE reporter in MC4100*

	Growth under specific inducer			
	With D-fucose		With galactose	
	WT	*ΔgalR galS*	WT	*Δgal RgalS*
galE	4.6	5.5	2.5	3.5

The steady state expression was obtained by calculating GFP/*A* (see Materials and Methods). Measurements were taken at mid-exponential phase. All values have the same arbitrary units scale. Measurement errors are $\sim \pm 0.1$.

level of 20 mM D-fucose causes a twofold increase in glucose medium above the basal level (Figure 3), indicating that this inducer enters the cell in the presence of glucose in sufficient amounts to cause

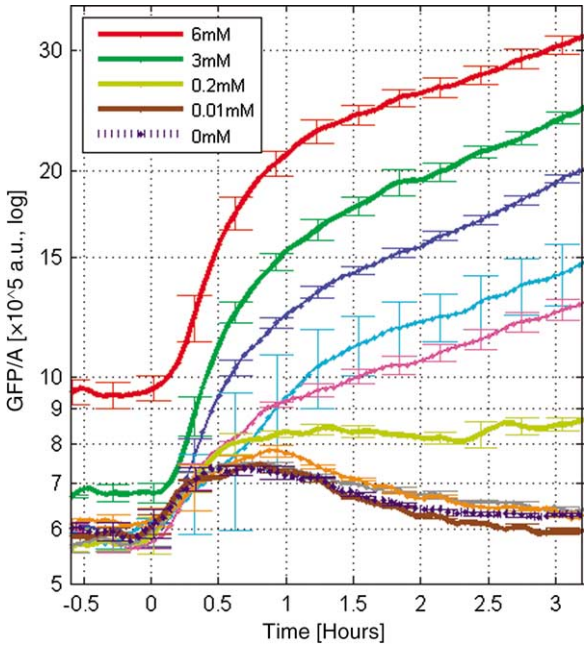

Figure 3. Dynamics of the *galE* promoter with different levels of the inducer D-fucose. Shown is GFP per *A* during exponential growth on minimal medium with 0.02% glucose and 0.8% mannose. The *galE* promoter is induced upon depletion of glucose at time $t=0$ (about 5 h from start of experiment). D-Fucose concentration was 6 mM (red), 3 mM (green), 1.5 mM (blue), 0.75 mM (cyan), 0.4 mM (magenta), 0.2 mM (yellow), 0.05 mM(gray), 0.02 mM (orange), 0.01 mM (brown), 0 mM (purple).

partial induction of the system. In the absence of glucose, the *galE* promoter activity is ninefold induced by adding 20 mM D-fucose (Table 1A).

We also measured the activity of the *galE* promoter in *E. coli* MC4100 strains deleted for *galR*, *galS* and both *galS* and *galR*, compared to their parental strain (Table 1B). We find that both repressors have a measurable effect on the *galE* promoter, and that *galR* has a stronger effect. Removal of *galS* causes a 2.6-fold increase in expression levels. *galS* appears to have an effect even in the absence of *galR*, as seen by the 1.5-fold increase in the Δ*galR*Δ*galS* strain compared to the Δ*galR* strain. This suggests that the *galE* promoter is regulated by *GalS in vivo*. Note that deletion of both repressors leads to a higher activity level than addition of saturating inducer to the wild-type strain (Table 1C). This suggests that the repressors have a residual repressing effect even when fully induced.

These measurements are consistent with the I1-FFL connectivity of the *CRP-GalS-GalE* system suggested by previous studies.[35] The measurements suggest that the input function at the *galE* promoter is similar to an AND gate, requiring both the presence of D-fucose/galactose and the lack of glucose for high activity. Unlike a pure AND input-function, however, the *galE* promoter appears to have a basal activity in the presence of glucose.[35]

The *GalE* promoter is activated upon depletion of glucose

We measured the dynamics of the *GalE* promoter activity during growth on a minimal medium with limiting glucose (0.005%) and saturating mannose (0.8%). In this medium, the bacteria utilize all the glucose when reaching a certain A_{600} level at mid-exponential growth. At this point the cells continue to grow on mannose but do not stop growth (that is, they do not show the slow-down of growth that occurs in diauxic transitions between other sugars such as glucose and lactose). We tracked GFP fluorescence and A_{600} at a temporal resolution of 3 min over several hours of growth in a temperature-controlled multi-well fluorimeter.

We find that the *galE* promoter is activated when glucose is depleted from the medium. The point in time (as well as the A_{600} level), in which *galE* begins to be activated, is delayed upon increasing the glucose concentration in the medium (data not shown). A *lacZ* reporter strain, used as a control because its promoter is also inhibited by glucose through the effect of CRP (Figure 2(c)), becomes activated at the same time as the *galE* reporter (see below). The same applies for the other reporter strains used in this study.

We used these conditions to study the effect of different levels of the inducer D-fucose on the production levels of *galE*. We find that *galE* promoter activity increases with increasing D-fucose levels (Figure 3). In saturating D-fucose the dynamics is a monotonic increase from a basal level to a new, higher steady-state level. At low levels of inducer, the dynamics shows an overshoot and stabilization at a

lower steady-state level. The dynamics transit between these two extremes in a continuous fashion at intermediate levels of inducer.

The response of *galE* promoter is accelerated

The *galE* promoter activity is enhanced upon depletion of glucose from the medium, resulting in an increase of expression. To measure the response time, the time to reach 50% of steady state, we normalized the dynamics. This was done by subtracting the basal expression obtained in growth on a medium with saturating glucose+mannose, and dividing by the steady-state activity where growth is on mannose alone (see Materials and Methods). This allows comparison of the shape of the dynamic curves of different promoters and in different conditions (Figure 4). We find that the normalized dynamics of the *galE* promoter without inducer shows an overshoot, and is accelerated. It reaches 50% of its steady-state level after 0.34($\pm$0.04) cell generations. In contrast, the dynamics with saturating D-fucose induction is a monotonic increase that resembles the theoretical solution of constant production and dilution, resulting in a monotonic convergence to steady state. The normalized expression levels with D-fucose reach 50% of the steady-state level after more than one cell generation time (Figure 4).

The *lac* operon shows no acceleration

We also measured the dynamics of the *lacZ* promoter under the same conditions. The *lacZ* promoter is regulated by CRP in a simple regulation configuration with no FFL because CRP does not regulate *LacI* (Figure 2(c)). We supplied the inducer IPTG at saturating levels (0.25 mM) to inhibit *LacI* that represses the *lacZ* promoter and hence to induce high levels of expression. When glucose is depleted, *lacZ* expression increases. We find that the *lacZ* promoter does not show accelerated dynamics. It reaches 50% of its steady-state level after more than one cell generation time (Figure 4).

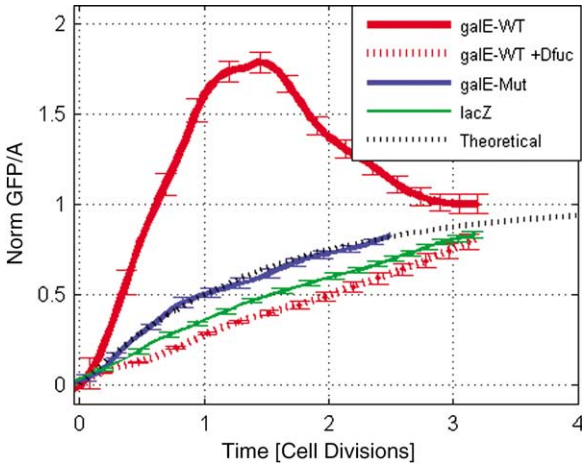

Figure 4. The expression of the *galE* promoter normalized relative to steady state. Time is in cell divisions, and $t=0$ is the point at which glucose is depleted. Continuous red curve, wild-type promoter with no inducer D-fucose. Broken red curve, wild-type promoter with 20 mM D-fucose. Blue line, mutant *galE* promoter in which the *galS* binding site was mutated. Green line, *lacZ* promoter. Broken black line, theoretical curve for simple gene regulation, where $x/x_{st}=1-e^{-\alpha t}$ with $\alpha=\log(2)/\tau$ and τ is the cell-generation time. Response time is the time to reach 50% of the steady-state level, namely 0.5 on the *y*-axis.

Accelerated response is dependent on the repressor binding site in the *galE* promoter

We measured the dynamics of a mutant *galE* reporter plasmid in which the main binding site of *galS*/*galR* was deleted. This mutated promoter loses its responsiveness to D-fucose, but not to glucose (data not shown). We find that the normalized dynamics of this promoter shows no acceleration following the depletion of glucose (Figure 4). Its dynamics has a response time of about one cell generation.

Discussion

This study examined the dynamic function of the incoherent type-1 FFL network motif, using the well-studied *gal* system of *E. coli*. Using high-resolution dynamic measurements, we found that this network motif exhibits overshoot dynamics that accelerates the response time of *galE* gene expression upon removal of glucose. This acceleration is lost when the inducer D-fucose is added, or when the FFL is disrupted by removing the binding site of the repressor *galS* in the *galE* promoter. These findings support the theoretical predictions for the dynamical function of this motif.

Response acceleration by the I1-FFL is due to the fact that at early times, CRP strongly activates the *galE* promoter, resulting in rapid production. In parallel, CRP activates GalS production. Thus, at a delay, GalS builds up to repress the promoter, locking the system at the desired steady-state promoter activity. An alternative design, in which GalS was not under CRP control but rather constitutive (a non-FFL design similar to the design of the lac system Figure 2(c)), could only reach the desired steady state by using a weaker promoter activity, resulting in a slower rise of *galE* gene products. A strong promoter would result in an undesirably high level of expression. Thus, the acceleration in the I1-FFL is due to the decoupling between initial promoter strength and final steady-state level. A similar acceleration effect is provided by negative autoregulation,[28] another common network motif.

The present experimental results provide an estimate for the response time in this system: the response time, defined as the time to reach 50% of the steady-state level, is about one-third of the cell generation time. This is ~threefold faster than

systems with no acceleration, which have a typical response time of about one cell generation time.[28] Such systems include the *lacZ* simply regulated promoter. In models, the magnitude of the acceleration in the I1-FFL depends on the molecular parameters of the system such as the expression level of the repressor and its repression threshold for the target gene. For example, the stronger the repression of Z (*GalE*) by Y (*GalS*), the faster the response time.[18] These parameters can be tuned over evolutionary timescales to optimize the response time and other features of the system.[23]

The response acceleration is seen upon glucose starvation, which leads to an activation of the *galE* promoter. If glucose is added back to the medium, *CRP* becomes rapidly inactive. Thus, the *galE* promoter activity is expected to drop to basal levels, resulting in an exponential decay of the *galE* gene products. Such a rapid drop in production and exponential decay following glucose addition was experimentally demonstrated in the *lac* and *ara* systems.[20] Hence, response is accelerated compared to simple-regulation when the I1-FFL is activated, but response is similar to simple-regulation when the I1-FFL is deactivated.[18]

What could be the function of response acceleration that occurs in the absence of the inducer of the *gal* system? One possibility is that when glucose runs out, the cell prepares to utilize other sugars such as galactose. It needs to express low levels of the galactose utilization and transport system in order to detect and import this sugar once it appears in the environment. The I1-FFL helps to accelerate this process, and potentially to allow the *gal* system to reach functional protein levels faster, and thus to be ready to use galactose earlier if it appears.

We note that the principle function of GalS and GalR is to allow regulation of the *gal* genes by the inducer galactose.[31–35] The present study focused on a possible functional reason why *galS* is regulated by *CRP* rather than constitutively expressed.

This study tested the function of the I1-FFL network motif in living cells, indicating that the I1-FFL in the *gal* system exhibits response acceleration as predicted by models. This motif functions in the predicted way despite the fact that it is embedded within many additional interactions not included in the theoretical model. This supports the suggestion that network motifs are often linked to the rest of the network in a way that preserves their independent dynamical functions.[42] Such a design might allow building complex networks out of circuit elements that can be reliably wired to each other, keeping the proper internal workings of each circuit somewhat insulated from the rest of the network. The same idea is used by engineers to build intricate devices by repeated use of a small set of elementary electronic circuits. Based on this suggestion, it would be important to see whether the full network dynamics can be understood at least partially by considering separately the dynamics of each network motif. The present experimental approach, supplemented by comprehensive libraries of reporters,[40,43,44] could readily be extended to address such questions in the living cell.

Materials and Methods

Plasmids and strains

Promoter regions were PCR amplified from MG1655 genomic DNA with the following start and end genomic coordinates:[45] *lacZYA* (365438-365669), *galETK* (791278-790262), *galS* (2239688-2238648). This included the entire region between open reading frames (ORFs) with an additional 50–150 bp into each of the flanking ORFs. Each promoter region was sub-cloned into XhoI and BamHI sites upstream of a promoterless *gfpmut2*[46] gene in a low copy pSC101-origin plasmid as described,[41] to create a separate low-copy reporter plasmid for each genomic promoter. These plasmids have an estimated intracellular copy number of about ten plasmids per cell.[47] The plasmids were transformed into *E. coli* strain MG1655[45] (wild-type for *gal* and *lac*). Note that all endogenous genes including transcription factors are expressed from their normal genomic locations, and their effect on the promoter of interest is monitored by the fluorescence level of the *gfp* protein. The *gfp* reporter becomes fluorescent within minutes after transcription.[41]

In some cases, reporter plasmids have been known to titrate out transcription factors and hence to affect the behavior of the system of interest. For example, a *lac* promoter, even on a low copy plasmid, can affect the intracellular level of *lac* repressor *LacI*, present at about ten tetramers per cell, to an extent that measurably affects the degree of repression.[48] In the present system, *GalS* is estimated to function at levels exceeding 100 dimers per cell, based on its *in vitro* affinity to the *galE* promoter (30–70 nM)[33] and on *in vitro* transcription regulation experiments in which it begins to repress effectively at concentrations of several hundred nM.[33] Hence, the present *galE* reporter strains, which introduce of the order of ten plasmids with a *galE* promoter region, should not significantly titrate out the repressor and affect the behavior of the system. We also note that reporter plasmids similar to the present plasmids were found not to affect system behavior: for example, in the flagella system, low-copy reporter plasmids for flagella promoters did not affect the swimming behavior of cells.[41]

Mutated reporter plasmid for the *galETK* promoter, denoted *galE-Mut1*, in which *GalR/GalS* binding site (also known as galE-O_E) was deleted, was prepared by mutagenesis (BaseClear Labservices). The deleted sequence of the galE-O_E operator near −60 bp from the transcription start site was TGTGTAAACGATTCCAC,[36] located at genomic coordinate 791357-791373. Mutant *E. coli* strains[36] for *gal* repressors (kindly provided by S. Adhya), were based on MC4100 (WT): MW130 (MC4100 Δ*galR*) denoted Δ*galR*; MW131 (MC4100 *galS*::ΔTn*10*) denoted Δ*galS*; and MW132 (MC4100 Δ*galR galS*::ΔTn*10*) denoted Δ*galRgalS*.

Growth medium

The media used are denoted as follows: M0 is minimal medium (M9 salts supplemented with 1 mM $MgSO_4$,

0.1 mM $MgCl_2$, 25 mg/l kanamycin). Overnight medium MON is M0 supplemented with 0.4% (w/v) glucose, 0.5% (v/v) glycerol and 0.1% (w/v) Casamino acids; medium M1C is M0+0.8% of a specific carbon source, such as mannose, or galactose as specified; M1G is M1+0.8% glucose; M1GC is M1+0.8% glucose+0.8% of specified carbon source; M1Cg (M1C+0.005 to 0.02%glucose) is a medium with a small amount of glucose used for testing the response to glucose depletion. In addition, in some cases D-fucose was added at 20 mM (or otherwise as specified).

Culture and measurements

Single colonies grown on agar plates were used to inoculate 5 ml of MON, and grown overnight at 37 °C with shaking at 250 rpm. The cultures were diluted 1:2000 into 250 μl of medium in a 96-well plate (Nunc 167008), with different combinations of reporter strains and media: M1C, M1Cg, M1G; with/without D-fucose. The *lacZ* strain was supplemented by 0.25 mM IPTG for full induction. Each well was covered with 60 μl of mineral oil (Sigma M-3516) to prevent evaporation. We previously found that this covering did not significantly affect growth rate or lead to up-regulation of anaerobic promoters. The plate was placed in a Wallac Victor2 multi-well fluorimeter at 37 °C with a repeating protocol that included shaking (2 mm orbital, normal speed, 30 s), absorbance (A) measurements (600 nm, 0.5 s), and fluorescence (GFP) readings (excitation 485 nm, emission 535 nm, 1 s, CW lamp energy 15,000).[28,39,41] The protocol repeated the set of shake, GFP, A, GFP. Time between repeated GFP measurements was about 3 min. Each plate contained 6–18 replicates of each combination of strain-medium-supplement, where the strains were: *galE*, *galS*, *lacZYA*, promoterless vector, and a reference well with medium only. The measurement outputs were used to determine the promoter dynamics of each strain in each medium (M1C, M1G, etc.). Cell generation time in the various media, calculated as the time it takes the A_{600} to double in mid-exponential phase, varied between different conditions and strains, and ranged between 0.75 h and1.5 h at 37 °C.

Data analysis

Background absorbance from wells containing medium only was subtracted from all absorbance readings. Background GFP fluorescence, that was determined by the GFP reading from a strain bearing the promoterless vector U66[40] at the same condition and absorbance in the same experiment, was subtracted from the GFP readings. GFP fluorescence per cell was $GFP(t)/A(t)$. To normalize the step response of the tested strain, growing on M1Cg, into dimensionless values between zero and 1, we subtracted the baseline of growth on high glucose+carbon medium (M1GC), and divided by the top level of growth on sole carbon medium (M1C). The response time is a measure of the time it takes a gene product to reach its physiologically determined steady-state level. We use the traditional definition of response time: the time to reach 50% of the steady state-level.[2,28]

Transcription network databases

We used the EcoCyc databases[49–52] (Version 8.6) of direct transcription interactions for *E. coli*[10] and a previously described transcription network for *S. cerevisiae*.[11] The networks are available†. We enumerated the FFLs using methods described earlier.[10,11] In *E. coli*, several FFLs have a "dual-regulation" transcription factor, which behaves as an activator in the presence of an inducer and as a repressor in the absence of an inducer, which were counted as positive regulators. Note that each node in our *E. coli* network is an operon, and not an individual gene. Using genes as nodes can lead to mis-counting of FFLs, in cases where both Y and Z components are on the same operon. For example, in the *betIBA* operon, *ArcA* regulates the operon which is also auto-regulated by one of the products, the transcriptional activator *BetI*, resulting in the mis-recognition of *ArcA-BetI-BetBA* as an FFL.

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