

# Negative Autoregulation Speeds the Response Times of Transcription Networks

Nitzan Rosenfeld<sup>1</sup>, Michael B. Elowitz<sup>2</sup> and Uri Alon<sup>1\*</sup>

<sup>1</sup>Department of Molecular Cell Biology, Department of Physics of Complex Systems  
Weizmann Institute of Science  
Rehovot 76100, Israel

<sup>2</sup>Rockefeller University, 1230  
York Avenue, New York, NY  
10021, USA

Cells regulate gene expression using networks of transcription interactions; it is of interest to discover the principles that govern the dynamical behavior of such networks. An important characteristic of these systems is the rise-time: the delay from the initiation of production until half maximal product concentration is reached. Here we employ synthetic gene circuits in *Escherichia coli* to measure the rise-times of non-self-regulated and of negatively autoregulated transcription units. Non-self-regulated units have a rise-time of one cell-cycle. We demonstrate experimentally that negative autoregulation feedback (also termed autogenous control) reduces the rise-time to about one fifth of a cell-cycle. This agrees with an analytical solution of a mathematical model for negative autoregulation. This may help in understanding the function of negative autoregulation, which appears in over 40% of known transcription factors in *E. coli*.

© 2002 Elsevier Science Ltd. All rights reserved

**Keywords:** autogenous control; biophysics; design principles; feedback kinetics; systems-biology

\*Corresponding author

## Introduction

A major current challenge is to understand the design principles of gene regulation networks. It is therefore of interest to study the properties of regulatory structures, or “motifs”, that occur frequently in the networks. Understanding the function of a recurring motif is likely to shed light on the design of numerous systems in which it appears. One such common motif is negative autoregulation, in which transcription factors negatively regulate their own transcription.<sup>1</sup> This occurs, for example, in over 40% of known *Escherichia coli* transcription factors (Table 1).<sup>2,3</sup> Examples of transcription factors that negatively regulate their own transcription include Crp, a global transcription factor that mediates the catabolite response of several hundred genes, LexA, the master regulator of the SOS DNA repair system, and numerous other well-characterized transcription factors involved in metabolism and stress responses. Negative autoregulation has been shown to reduce cell-to-cell fluctuations in the steady-state level of the transcription factor.<sup>4</sup> Here, we explore the effect of negative autoregulation on the kinetics of transcription.

The kinetics of simple transcription units are slow.<sup>5</sup> If the protein product is long lived, the typical time-scale for changes in the concentration is the cell-cycle. One strategy to achieve faster kinetics is to shorten the lifetime of the product by degrading it.<sup>6</sup> This incurs the metabolic cost of constant protein production and turnover. Here, we consider an alternative strategy, where a transcription factor represses its own transcription. Negative autoregulation has been theoretically suggested to decrease rise-time without need for high protein turnover rates.<sup>1,7,8</sup> To study this experimentally, we use a synthetic circuit in *E. coli*<sup>4,9,10</sup> to demonstrate that negative autoregulation can speed up transcription responses, in agreement with mathematical models.

## Results

### Theory

Assume that the design goal is a circuit which produces a given steady-state protein concentration when induced. We compare two strategies of gene regulation: a simple transcription unit and a negative autoregulatory circuit (Figure 1). The two circuits can be designed to reach the same steady-state level of protein concentration, by having differing values for the maximal production rate of their promoters. We will show that the

Abbreviations used: aTc, anhydrotetracycline; GFP, green fluorescence protein.

E-mail address of the corresponding author: [urialon@weizmann.ac.il](mailto:urialon@weizmann.ac.il)

**Table 1.** Transcription factors in *E. coli* that repress their own transcription (negative autogulation, also termed autogenous control)<sup>2</sup>

Negatively autoregulating transcription factors	Additional transcription regulation	Function
AraC	Crp	Arabinose utilization
ArgR		Arginine biosynthesis
Crp		Catabolite repression, global regulator
CysB	DsdC	Cysteine biosynthesis
DsdC		Regulator of D-serine dehydratase
EmrRAB		Multidrug resistance pump
ExuR	Fis	Carbon utilization
Fis		rRNA and tRNA operons and DNA replication
Fnr		Aerobic, anaerobic respiration, osmotic balance
Fur	Crp	Iron transport
GalS		Galactose utilization
GcvA	Crp, RpoN	Cleavage of glycine
GlnA		Glutamine synthesis
Hns		Global regulator
Ihf		Integration host factor, global regulator
IlvY		Isoleucine and valine synthesis
LexA		SOS DNA repair
Lrp		Leucine response, amino acid limitation, global regulator
LysR		Lysine biosynthesis
MarA	Rob	Multiple antibiotic resistance
ModE		Molybdate transport
MtlADR		Mannitol utilization
Nac	RpoN	Histidine utilization/nitrogen assimilation
NadR		NAD biosynthesis, other roles
NagC		Repressor of genes for catabolic enzymes
OxyR	Fis	Oxidative stress
PhdR		Activator of hca cluster, other roles
PurR		Purine biosynthesis
PutAP	Nac	Proline synthesis, other roles
RpiR		Ribose catabolism
SoxS		Superoxide stress
SrlA-D	SoxR	Glucitol/sorbitol utilization
TrpR		Tryptophan biosynthesis
UxuABR		Mannonate utilization

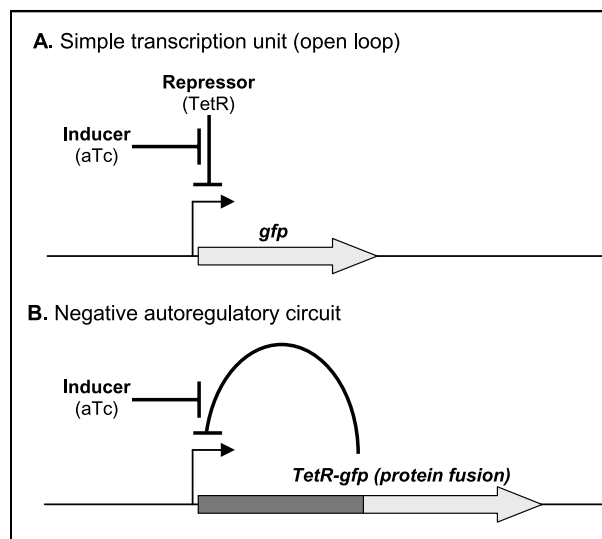
Several of these operons have additional transcription factor inputs.

negative autoregulatory circuit approaches its steady-state value much faster than the non-autoregulatory circuit.

For both models, the rate of change of the concentration of the protein product  $x$  has been described<sup>5,11</sup> (see Table 2, and for more details see also math primer†):

$$\frac{dx(t)}{dt} = A(t) - \alpha x \quad (1)$$

with a production term  $A(t)$  and a dilution/degradation term  $-\alpha x$ . For a long lived gene product,

**Figure 1.** Synthetic transcription circuits. (a) Simple transcription unit (open loop, Dh5α + pZS12-tetR + pZE21-gfp). Cells expressing TetR can be induced, by adding aTc to the medium, to produce GFP. (b) Negative autoregulation (Dh5α + pZS<sup>21</sup>tetR-egfp<sup>4</sup>): the *tet* promoter controls the production of its repressor, TetR fused to GFP. The TetR–GFP fusion protein represses its own promoter.<sup>4</sup>

the latter accounts for the dilution of the protein when the cells grow and divide, with a cell-cycle time of  $\tau$ , where  $\alpha = \ln(2)/\tau$ . In order to study the kinetics of induction we will examine cases where the initial protein concentration of zero.

We define the rise-time  $t_r$  as the time required for a gene product to reach half of its steady-state concentration,  $x(t_r) = x^{st}/2$ .

### Kinetics of a simple transcription unit

For a simple transcription unit with a constant rate of production  $A_1 = \beta_1$ , the steady-state concentration is:

$$x_1^{st} = \beta_1/\alpha \quad (2)$$

**Table 2.** Variables and parameters used in the models

	Description
$x(t)$	Protein concentration in cells
$A(t)$	Protein production rate
$\tau$	Cell-cycle time
$\alpha$	Growth rate $\alpha = \ln(2)/\tau$
$\beta$	Protein production rate from the fully induced promoter
$\beta_1, x_1(t)$	Subscript 1 indicates the simple transcription unit
$\beta_2, x_2(t)$	Subscript 2 indicates the negatively autoregulated circuit
$\beta/\alpha$	Steady-state protein concentration from fully induced promoter
$k$	Dissociation constant of the repressor to its own promoter

† <http://www.weizmann.ac.il/mcb/UriAlon/>

and the kinetics of step induction are:<sup>5,11</sup>

$$\frac{x_1(t)}{x_1^{\text{st}}} = 1 - e^{-\alpha t} \quad (3)$$

so that the deviation of  $x$  from its steady-state value drops by half each cell-cycle. Thus, a simple transcription unit has a rise-time of one cell-cycle:

$$t_r = \tau$$

### Negative autoregulation speeds response times

A genetic autoregulatory circuit is one in which the rate of production of the gene product depends on its intracellular concentration. For simplicity we assume a Michaelis–Menten-like form for the activity of the promoter:

$$A_2(t) = \frac{\beta_2}{1 + \frac{x_2(t)}{k}} \quad (4)$$

where  $\beta_2/\alpha$  is the steady-state product concentration from an unrepressed promoter and  $k$  is the dissociation constant ( $=1/\text{affinity}$ ) of the repressor to its own promoter. The steady-state is:

$$x_2^{\text{st}} = \frac{\sqrt{k^2 + 4k\beta_2/\alpha} - k}{2} \quad (5)$$

For strong autorepression ( $\beta_2/\alpha \gg k$ ), the steady-state approaches:

$$x_2^{\text{st}} = \sqrt{k\beta_2/\alpha} \quad (6)$$

and the kinetics approach a simple limiting form:

$$\frac{x_2(t)}{x_2^{\text{st}}} = \sqrt{1 - e^{-2\alpha t}}, \quad \beta_2/\alpha \gg k \quad (7)$$

The rise-time is:

$$t_r = (\log_2(4/3)/2)\tau = 0.21\tau$$

compared to  $t_r = \tau$  for the simple transcription unit. The parameters of the two designs can be set to achieve an equal steady-state ( $x_1^{\text{st}} = x_2^{\text{st}}$ ) by assigning a relatively weak promoter to the unrepressed circuit and a strong promoter to the autorepressed circuit. The rise-time of the negatively autoregulated circuit is about one fifth of the equivalent circuit (with the same steady-state) without negative autoregulation.

The rise-time approaches the limiting value of about one-fifth of a cell-cycle for strong autorepression,  $\beta_2/\alpha \gg k$ . When autorepression is weak,  $\beta_2/\alpha \ll k$ , there is effectively no autoregulation and  $t_r = \tau$ . For intermediate value of  $\beta_2/\alpha$ , the rise-time changes continuously between these limiting values (equations (9) and (10) in Materials and Methods, and Figure 2, marked with  $T = 0$ ). Interestingly, there is a broad region where the rise-time is about  $0.2\tau$ , independent of the biochemical parameters  $\beta_2/\alpha$  and  $k$ . For example, using reasonable parameters for bacterial repressors,<sup>7</sup> at an unrepressed level of 4000 pro-

teins per cell (equivalent to a concentration of roughly  $\beta_2/\alpha = 4 \mu\text{M}$ ) and a binding constant of  $k = 10 \text{ nM}$ , the expected steady state level is 200 molecules per cell and the expected rise-time is  $t_r = 0.24\tau$ .

### Effects of cooperativity

Analysis of cooperativity in autorepression  $A_3 = \beta_3/(1 + x^H/k^H)$  (equation (11) in Materials and Methods), suggests that cooperativity in the repression of the transcription factor's own promoter can further decrease the rise-time. (The lower limit of  $t_r/\tau$  decreases as the cooperativity increases ( $t_r^{H=1} \approx 0.21\tau$ ,  $t_r^{H=2} \approx 0.06\tau$ ,  $t_r^{H=3} \approx 0.02\tau$ , and  $t_r^{H=4} \approx 0.01\tau$ ).

### Effects of protein degradation

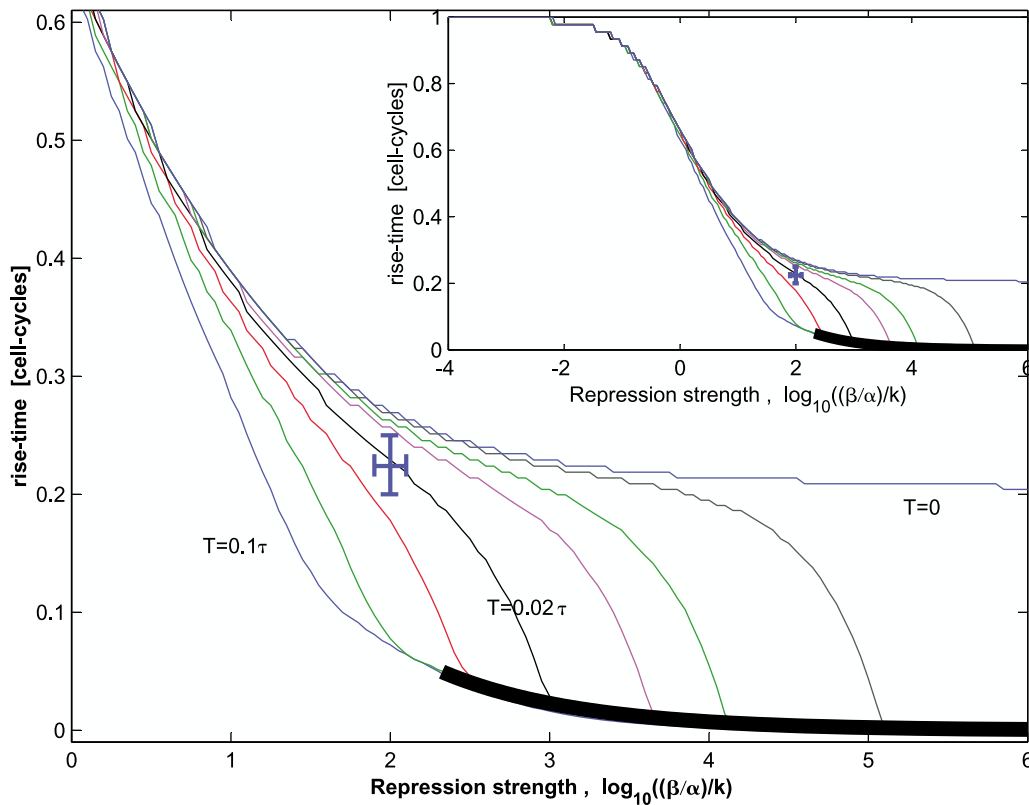
For a gene product with a finite half-life time  $\tau_{\text{deg}}$ , we substitute everywhere the dilution rate  $\alpha = \ln(2)/\tau$  with  $\alpha' = \ln(2) \times (\tau^{-1} + \tau_{\text{deg}}^{-1})$ , so that for short lived proteins  $\alpha' = \ln(2)/\tau_{\text{deg}}$ . All the above analysis holds for degradable gene products, replacing  $\alpha$  by  $\alpha'$ . The rise-time is faster by the same factors, replacing  $\tau$  with  $\tau' = (\tau^{-1} + \tau_{\text{deg}}^{-1})^{-1}$ . Thus, for rapidly degradable proteins with lifetime  $\tau_{\text{deg}}$ , much smaller than  $\tau$ , a simple transcription unit has a rise-time of  $t_r = \tau_{\text{deg}}$ , while a strong negatively autoregulated circuit has a rise-time of  $t_r \approx 0.2\tau_{\text{deg}}$ .

### Effects of explicitly treating mRNA levels

Note that for simplicity we did not explicitly include equations for the production and degradation of mRNA in the equations above. mRNA concentration is assumed to be at a quasi-steady-state proportional to  $A(t)$ , due to the short mRNA lifetime compared to that of the proteins. For example, mRNA lifetime in prokaryotes is usually on the order of a few minutes, while protein dilution and degradation rates are generally on the order of tens of minutes to many hours.<sup>12,13</sup> Indeed, numerical solutions of the system of equations obtained by explicitly calculating the mRNA levels (equations (12a) and (12b) in Materials and Methods) yield kinetics that nearly coincide with the solutions of the equations above.

### Effects of delays in the formation of active proteins

Delays on the order of a few minutes are expected between transcription initiation and the formation of the active repressor able to negatively regulate the activity of its own promoter.<sup>12,14</sup> Such delays are due to the cumulative effect of steps such as elongation, termination, ribosome binding and peptide elongation, protein folding, formation of complexes such as dimers, and their diffusion to the DNA-binding site. A simple way to



**Figure 2.** Numerical calculation of the rise-time (in cell-cycles) of a non-cooperative negative autoregulatory circuit. The rise-time is plotted as a function of the repression ratio  $(\beta/\alpha)/k$ .  $\beta/\alpha$  is the steady-state concentration produced from an unregulated promoter, and  $k$  is the repressor dissociation constant to its own promoter. The rise-times for various values of the delay  $T$  between transcript initiation and active protein formation are shown, for  $T = [0.1 \ 0.06 \ 0.035 \ 0.02 \ 0.01 \ 0.006 \ 0.002 \ 0]$  in units of the cell-cycle. The cross marks the measured parameters (with errors) of the negative autoregulatory circuit used in this study. From the measured position of the cross, the effective time delay may be estimated, and is found to be  $0.02(\pm 0.01)$  cell-cycles, or roughly three minutes. The bold black line marks the regime where large overshoots in protein concentration occur. Note that without production delay ( $T = 0$ ), when  $\beta_2/\alpha \gg k$  the rise-time approaches a constant fraction of the cell-cycle,  $t_r^{\min} = 0.21\tau$ .

represent the effects of the cumulative delay  $T$  is:

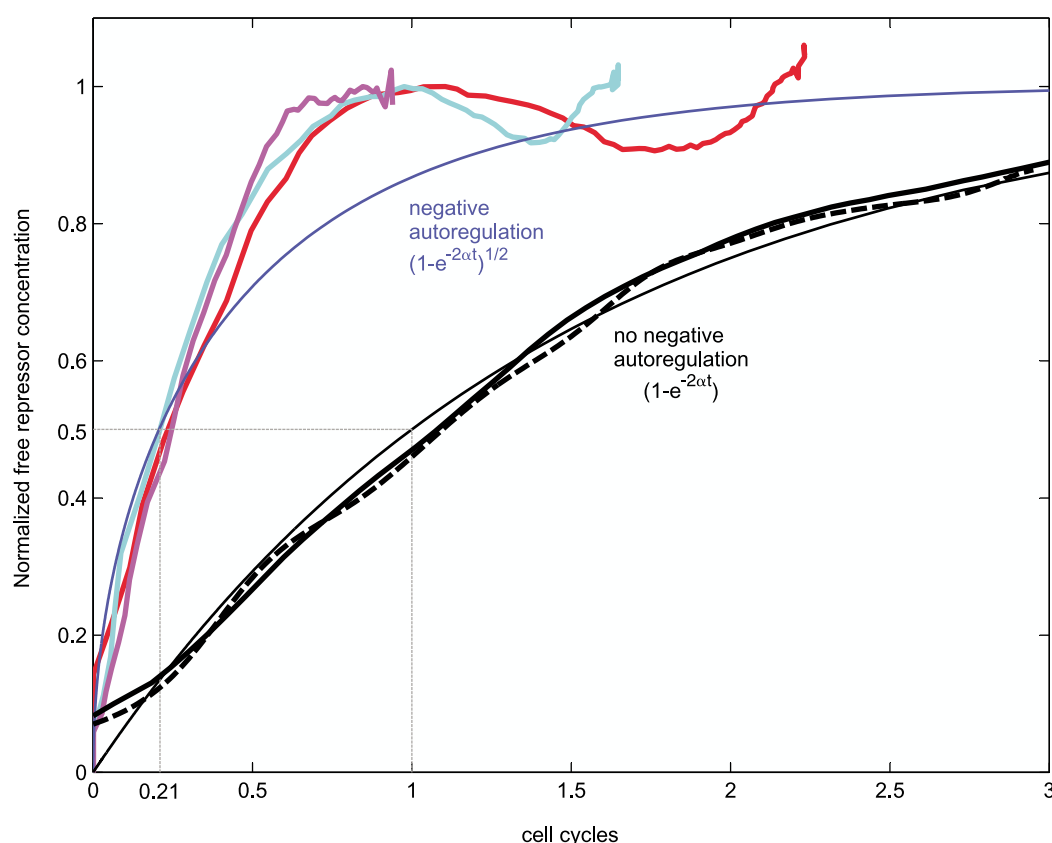
$$\frac{dx}{dt}(t) = \frac{\beta}{1 + \frac{x(t-T)}{k}} - \alpha x(t) \quad (8)$$

We find that the delay has a significant effect only for promoters so strong that the production during the delay time  $T$  is of the order of the steady-state level of the repressor (equation (6)), that is  $\beta T \sim \sqrt{k\beta/\alpha}$ . In this case, by the time the first repressors become active, many repressors are already in production. Therefore, the feedback is unable to stop production and a large overshoot in protein concentration can occur. Figure 2 shows the rise-times obtained for various values of the delay time  $T$  obtained by numerical solutions of equation (8). The delay leads to a decrease in the rise time in comparison to the model with no delay (equations (9) and (10) in Materials and Methods), marked by  $T = 0$  in Figure 2. The bold line shows cases where an overshoot is obtained. In many cases a large overshoot is probably undesirable, due to possible toxic

effects<sup>15,16</sup> and increased production cost. In addition, this excess protein takes a long time to dilute out when the system is turned off. Therefore, an optimal design may favor intermediate values of the repression strength  $(\beta/\alpha)/k$ , which have a rapid rise-time but are far from the overshoot region.

### Experimental kinetics of simple transcription units and negative autoregulatory circuits

The simple transcription unit (Figure 1(a)) was represented by cells bearing a plasmid carrying a green fluorescent protein (*gfp*) gene controlled by the *tet* promoter, which is repressed by a constitutively produced repressor TetR. The bacteria were grown in a multi-well fluorimeter that allows automated measurements of fluorescence and cell density at a temporal resolution of minutes.<sup>23</sup> Cells from overnight cultures were diluted into fresh medium containing the inducer anhydrotetracycline (aTc), which binds and inactivates the repressor TetR. After a short lag the fluorescence



**Figure 3.** Comparison of the experimentally observed kinetics of a negative autoregulatory circuit and a simple transcription unit. Fluorescence per cell, normalized by its maximal value, is plotted *versus* time in cell-cycles. The rise-time is the time to reach half of the maximal product concentration (thin dashed lines). Bold black line, induction of a simple transcription unit (open loop). Fine black line, theory (equation (3)). Red, cyan, and purple lines, kinetics of negative autoregulatory circuit. Blue, analytical solution of the mathematical model of negative autoregulation in the limit of strong autorepression,  $\beta_2/\alpha \gg k$  (equation (7)). Broken black line, kinetics of the autoregulatory circuit prior to aTc depletion, where *tetR* is fully inactivated and the feedback is cut. The simple transcription unit has a rise-time of one cell-cycle, while the negative autoregulatory circuit has a rise-time of 0.2 cell-cycles.

per cell kinetics agrees with equation (3) (Figure 3) and shows a rise-time of one cell-cycle.

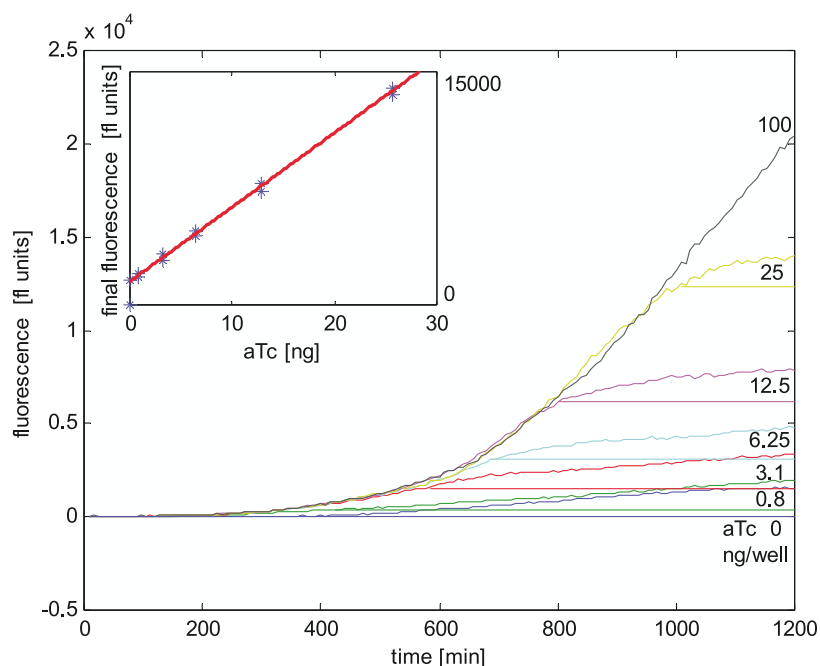
To measure the effect of negative autoregulation on the rise kinetics, we employed the synthetic circuit of Becskei & Serrano,<sup>4</sup> in which a transcription factor (TetR–GFP fusion) represses its own production (Figure 1(b)). To observe the effects of negative autoregulation on the induction kinetics, one needs to turn on the production of repressor from a low initial concentration of active repressor. To do this, we made use of an extraordinary property of the *tet* system: TetR binds to its inducer molecule aTc with an extremely high affinity ( $10^{12} \text{ M}^{-1}$ ).<sup>17</sup> Because of the high affinity, it is well established that aTc can be titrated out of the medium by TetR.<sup>18</sup> During growth of the cells, the amount of TetR–GFP fusion protein increases until the concentration of TetR–GFP equals that of aTc. At this point (Figure 4, horizontal lines), virtually all TetR are bound and inactivated by aTc. The inducer aTc is titrated out of the medium, and the new TetR produced are free to repress their own promoter. From this point on, the

kinetics qualitatively changes due to negative autoregulation.

We find that the rise-time of the negative autoregulatory circuit is much smaller than the rise-time of an unregulated unit ( $t_r \approx 0.2\tau$ , Figure 3). We compare the observed kinetics to the model of a negative autoregulatory transcription unit (equation (7)). It is striking that this limiting solution, which has no free parameters, displays a timescale similar to the experimentally observed kinetics (Figure 3). At late times (after 0.2 cell-cycles) the experimental rise is even faster than the model (possible reasons are discussed in Materials and Methods). Under conditions where aTc is not depleted, the *tet* repressor is fully inactivated, the feedback loop is cut, and the behavior of the autoregulated circuit is identical with that of the non-autoregulated transcription unit, showing a rise time of one cell-cycle (Figure 3, broken black line).

To compare the experimental results to the more detailed theoretical model including delays between transcription initiation and active





**Figure 4.** Fluorescence (continuous lines) of the negative autoregulatory circuit (Figure 1(b)) in response to different concentrations of aTc shows two distinct regimes, an exponential increase in fluorescence followed by a transition to a slower rate of increase. The fluorescence at the transition (horizontal lines) is proportional to the concentration of aTc, and the final fluorescence equals the fluorescence at the transition plus a constant. Inset: blue stars, final fluorescence data from two repeated experiments. Red, fit to a straight line.

protein formation, we estimated the repression ratio  $(\beta/\alpha)/k$  from the measured ratio of the steady-states at saturating aTc and under negative autoregulation. We find that  $(\beta/\alpha)/k = 100(\pm 20)$ . Using this value in equation (8), we find that the kinetics are well described with a delay time of  $3(\pm 1)$  minutes ( $0.02$  cell-cycles). The kinetics virtually coincides with the analytical solution without delay (equation (7)). We note that a three minute delay time agrees with typical delays between induction and formation of the first active proteins in *E. coli*.<sup>12,14</sup>

## Discussion

We demonstrate experimentally that negative autoregulation can significantly speed up the rise-times of transcription units. It has long been known that simple transcription cascades composed of long-lived transcription factors with no autoregulation are typically very slow. For example, the classic experiments on beta-galactosidase production showed that though the first enzymes are produced within minutes the rise-time is on the order of a cell-cycle.<sup>5</sup> Our findings suggest that negative autoregulation can reduce these delays to a fraction of the cell-cycle time. Consider evolution as an engineer, whose goal is to design a transcription unit that gives rise to a given steady-state product concentration. Two designs are compared: (A) a relatively weak promoter with no negative autoregulation, and (B) a strong promoter with negative autoregulation. Design B will show a faster rise-time to the same steady-state. The rise-time is expected to decrease with increased cooperativity in the binding of the repressor to its own promoter.

The fundamental reason that the negative autoregulatory circuit has a shorter rise-time is that the unrepressed promoter creates a fast initial rise, while at later times the newly produced repressor shuts off its own production to reach the required steady-state concentration. Note that a strong non-autoregulated promoter will reach any given concentration faster, but will stabilize at a much higher steady-state. Steady-state concentrations of the product that are much higher than its functional range are undesirable due to the metabolic cost of unneeded production, possible toxic effects and the long time required for its subsequent dilution when production is ceased.<sup>15,16</sup>

An alternative way of speeding the responses is to introduce degradation of the gene product. The response time is then determined by the degradation rate, and so to achieve fast responses at a given steady-state level requires increased production. This has the drawback of increased metabolic cost ("futile cycle"). There are qualitative differences in the kinetics of systems employing degradation compared with those using negative autoregulation: degradation speeds up both the rise-time and the turn-off time of protein levels (the time to reach half steady-state levels after transcription is turned off), while negative autoregulation speeds up the rise-time, but does not generally affect the turn-off time. It is interesting that eukaryotic cells seem to use the degradation strategy for transcription factors more often than prokaryotes.<sup>6</sup> Even in a system that has degradation, adding negative autoregulation still speeds up the response time to a fraction of the degradation time.

The present results should apply to a broad variety of gene regulation systems in *E. coli* and other organisms (Table 1). For example, the SOS

DNA repair system genes are transcriptionally repressed by a negatively autoregulating repressor LexA.<sup>19</sup> Upon DNA damage, the LexA cleavage rate is increased, its level drops and the SOS repair genes are expressed.<sup>20</sup> After damage is repaired, the cleavage process stops, and LexA levels build up to repress the system. Negative autoregulation can help speed up the recovery of LexA levels.<sup>8</sup> Additional examples include transcription factors that are transcriptionally regulated by other transcription factors, and negatively regulate their own production (Table 1). Such is the case of the negatively autoregulating transcription factor AraC, which controls the arabinose utilization genes.<sup>21</sup> The transcription of the *araC* gene is activated by Crp, a global regulator responsive to glucose starvation. Upon removal of glucose from the medium, Crp becomes activated to induce AraC transcription. The negative autoregulation of AraC should speed up its production, leading to a faster utilization of the sugar arabinose in place of glucose.

This study contributes to the emerging understanding of genetic regulatory networks. It would be interesting to characterize the kinetic behavior of additional regulatory circuit elements. Positive autoregulation,<sup>22</sup> for example, is expected to slow down the response times of transcription units.<sup>1</sup> Artificial gene circuits could be very useful tools for isolating and analyzing such circuits in detail. This will be important in approaching a systems-level understanding of networks composed of such recurring regulatory motifs.<sup>2</sup>

## Materials and Methods

### Bacterial strains and plasmids

*E. coli* strain Dh5 $\alpha$ , which expresses *lacI*, was used in all experiments. Plasmid pair pZS12-tetR + pZE21-gfp was used to make the non-autoregulatory network. The plasmids were based on the modular system of Lutz & Bujard.<sup>16</sup> For pZS12-tetR, *tetR* was cloned into a vector containing the low-copy SC101 origin of replication, ampicillin resistance cassette, and P<sub>lac</sub>O1 promoter.<sup>16</sup> pZE21-gfp contained a ColE1 origin, kanamycin resistance cassette, P<sub>tet</sub>O1 promoter, and gfpmut3.<sup>23</sup> pZS\*21tetR-egfp (kindly obtained from Becskei & Serrano<sup>4</sup>) was the negative autoregulatory circuit.

### Growth conditions and measurements

Cultures (2 ml) inoculated from single colonies were grown overnight in defined medium (M9 salts (Bio 101 Inc.) + 0.05% (w/v) Casamino acids + 0.1% (v/v) glycerol + 2 mM MgSO<sub>4</sub> + 0.1 mM CaCl<sub>2</sub> + 1.5  $\mu$ M thiamine + antibiotics: either kanamycin (50  $\mu$ g/ml) + ampicillin (100  $\mu$ g/ml) or kanamycin (25  $\mu$ g/ml)) at 37 °C with shaking at 300 rpm. For the simple transcription unit, 0.08 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to all media to induce the *lac* promoter to produce TetR, the repressor of the *tet* promoter. The cultures were diluted 1:100 into the same medium

plus anhydrotetracycline (aTc) (Acros Organics), which inactivates TetR, as inducer: 500 ng/ml for the simple transcription unit, and varying amounts of aTc (0–500 ng/ml, see Figure 4) for the negative autoregulatory circuit. Growth rate was not affected at the aTc concentrations used. The dilution was done into flat-bottom 96 well plates at a final volume of 200  $\mu$ l per well. Cultures were grown in a Wallac Victor2 multi-well fluorimeter with injectors set at 37 °C and assayed with an automatically repeating protocol of absorbance (*A*) measurements (600 nm filter, 1.0 second, absorbance through approximately 0.5 cm of fluid), fluorescence readings (485 nm and 535 nm filters, 0.5 second, CW lamp energy 2482 units), and shaking (2 mm double-orbital, normal speed, 203 seconds).<sup>24</sup> Once every three repeats the shaking was replaced by automated injection of 7  $\mu$ l double-distilled water into each well to counteract evaporation. Time between repeated measurements was eight minutes. Background fluorescence of cells bearing a promoterless gfp vector was subtracted. Relative error between repeated experiments using the ptet-tetR-gfp construct was less than 5%.

For the open-loop kinetics (Figure 3, continuous black line), bacteria were diluted from overnight culture into medium with inducer (500 ng/ml aTc). After a short lag phase the bacteria enter exponential growth, with a cell-cycle time of  $\tau = 135$  minutes, and reach a maximum fluorescence per *A* of  $\sim 50,000$  fl units per *A* unit. The strain carrying the negative autoregulatory circuit grew at a maximum rate of one doubling every  $\tau = 72$  minutes. For the negative autoregulation kinetics (Figure 3, colored lines), we calculated the amount of repressor needed to titrate the inducer by a straight line fit (Figure 4 inset). We then calculated the amount of new repressor molecules produced after the inducer aTc was titrated out, by the difference between the fluorescence (continuous lines in Figure 4) and the fluorescence at the point of titration (horizontal lines in Figure 4). The fluorescence difference was divided by the absorbance to get relative fluorescence per *A*, which was normalized by its maximum value of  $\sim 5600$  fl units per *A* unit. This was plotted versus the growth of the cells in cell-cycles, calculated by  $\log_2(A(t)/A(t_0))$ . The lines correspond to the continuous lines in Figure 4 with 3.1, 6.25 and 12.5 ng/well aTc. With saturating aTc (when no qualitative change in the expression was observed due to depletion of aTc by TetR and the effects of negative autoregulation) the negatively autoregulatory circuit reached a fluorescence level of  $55,000(\pm 5000)$  fl units per *A* unit. Roughly 15 hours from the beginning of the experiment (time  $\sim 900$  minutes in Figure 4) the cells start to enter stationary phase and their growth rate drops substantially, so only a few cell-cycles are measured in Figure 3. A slight increase in *tet* promoter activity is observed under the present conditions, as the cells leave exponential growth and enter stationary phase, in both open loop and closed loop circuits.

The unusually high affinity of aTc to TetR allowed us to calibrate fluorescence in terms of number of GFP molecules (using the fluorescence at the point where all TetR–GFP molecules are bound by aTc, Figure 4 and inset). Assuming that all the aTc is active and binds TetR molecules, we estimate that one fl unit equals  $4.4(\pm 1)$  fmol GFP molecules per well. Note that if only a fraction  $0 < \theta < 1$  of the aTc molecules are active,<sup>18</sup> then correspondingly one fl unit equals  $\theta \times (4.4 \pm 1)$  fmol GFP molecules per well. One *A* unit corresponds to about  $10^8$  cells per well.

### Simple model of the kinetics of a negative autoregulatory circuit

Equations (1) and (4) can be integrated, using equation (5):

$$t(x) - t(x_0) = -\frac{1}{2\alpha} \left[ \log((x - x_{st})(x + k + x_{st})) + \frac{k}{k + 2x_{st}} \log\left(\frac{x - x_{st}}{x + k + x_{st}}\right) \right]_{x_0}^x \quad (9)$$

When  $(\beta_2/\alpha) \gg k$  this equation can be approximated by equation (7), and when  $k \gg (\beta_2/\alpha)$  by equation (3). From equations (5) and (9), we can calculate the rise-time:  $t_r$ :

$$t_r = t\left(\frac{x_{st}}{2}\right) - t(0) = f\left(\frac{\beta_2/\alpha}{k}\right) \quad (10)$$

which turns out to be a function only of the dimensionless ratio,  $\rho = (\beta_2/\alpha)/k$ .

For cooperative autorepression  $A_3 = \beta_3/(1 + x^H/k^H)$ , we obtain at the limit  $\beta_3/\alpha \gg k_3$ :

$$\frac{x_3(t)}{x_3^{st}} = (1 - e^{-(H+1)\alpha t})^{\frac{1}{H+1}}, \quad x_3(t_0 = 0) = 0, \quad (11)$$

$$\beta_3/\alpha \gg k$$

### System of equations obtained by explicitly calculating mRNA levels

$$\frac{dm}{dt} = \frac{\tilde{\beta}}{1 + \frac{x(t)}{k}} - \alpha_m m(t) \quad (12a)$$

$$\frac{dx}{dt} = \gamma m(t) - \alpha x(t) \quad (12b)$$

### Biochemical parameters of the tetracycline system

For completeness, we include the values of some of the known biochemical parameters of the *tet* system. TetR occurs as homodimers, with  $K_d$  smaller than  $10^{-7}$  M.<sup>25</sup> In the absence of inducers (such as tetracycline or aTc) TetR dimers bind tightly ( $K_d = 10^{-11}$  M)<sup>26,27</sup> to the specific DNA operator sequence  $O_2$ .  $P_{LtetO-1}$  (used in the construction of the circuits used here) contains the  $O_2$  operator twice,<sup>16</sup> yet they are not known to have cooperative effects. Two *lac* operators in corresponding positions show that the repression effect of the operator in one position is 50–70 times stronger than in the other position,<sup>28</sup> indicating that the promoter may be adequately modeled by a non-cooperative repression model. aTc inactivates the DNA-binding abilities of TetR by tightly binding to it ( $K_d \sim 10^{-12}$  M).<sup>17</sup> The binding of the first aTc reduces the affinity of the TetR dimer to DNA by two to three orders of magnitude and the second bound aTc reduces the affinity by a further four to seven orders.<sup>26</sup>

One may speculate that the positive deviation of the negative autoregulation kinetics from the model after about half a cell-cycle (Figure 3) may be due to system details that were not included in the model, such as TetR dimerization. For example, consider the following hypothetical scenario: upon aTc depletion, TetR dimers are all doubly bound, both TetR monomers being bound to aTc. As fresh TetR is produced, it can no longer bind

aTc since all aTc is already bound. However, aTc or dimer dissociation and re-association on a slow timescale lead to formation of bound/unbound TetR heterodimers, which are inactive as repressors. Thus, repression would be less strong than expected in the model, leading to an overshoot (effectively, depletion is acting at a delay). Eventually virtually all inactive dimers would be singly bound, and the newly produced TetR dimers would be active repressors.

The presently observed agreement between the induction kinetics of the TetR-GFP fusion prior to aTc depletion, the kinetics of GFP from a fully induced promoter, and the model for long-lived proteins (broken, bold, and light black lines in Figure 3) suggests that the lifetimes of both GFP and TetR-GFP are much longer than the cell-cycle.

### Acknowledgements

We thank A. Becskei, L. Serrano, R. Lutz and H. Bujard for plasmids. We thank P. Bashkin for assistance. We thank N. Barkai for illuminating discussions and suggestions. We thank M. Surette, S. Leibler, O. Stock, A. Levine and all members of our laboratory for discussions. This work was supported by the Israel Science Foundation, the Human Frontiers Science Project and the Minerva foundation. M.E. is supported by the Seaver institute and the Burroughs-Wellcome Fund. N.R. dedicates this work to the memory of his father, Yasha Rosenfeld.

### References

1. Savageau, M. A. (1974). Comparison of classical and autogenous systems of regulation in inducible operons. *Nature*, **252**, 546–549.
2. Shen-Orr, S. S., Milo, R., Mangan, S. & Alon, U. (2002). Network motifs in the transcriptional regulation network of *Escherichia coli*. *Nature Genet.*, **31**, 64–68.
3. Thieffry, D., Huerta, A. M., Perez-Rueda, E. & Collado-Vides, J. (1998). From specific gene regulation to genomic networks: a global analysis of transcriptional regulation in *Escherichia coli*. *Bioessays*, **20**, 433–440.
4. Becskei, A. & Serrano, L. (2000). Engineering stability in gene networks by autoregulation. *Nature*, **405**, 590–593.
5. Monod, J., Pappenheimer, A. M. & Cohen-Bazire, G. (1952). La cinétique de la biosynthèse de la  $\beta$ -galactosidase chez *E. coli* considérée comme fonction de la croissance. *Biochim. Biophys. Acta*, **9**, 648–660.
6. Schimke, R. T. (1969). On the roles of synthesis and degradation in regulation of enzyme levels in mammalian tissues. *Curr. Top. Cell. Regul.*, **1**, 77–124.
7. McAdams, H. H. & Arkin, A. (1997). Stochastic mechanisms in gene expression. *Proc. Natl Acad. Sci. USA*, **94**, 814–819.
8. Little, J. W. (1996). The SOS regulation system. In *Regulation of Gene Expression in Escherichia coli* (Lin, E. C. C. & Simon Lynch, A., eds), pp. 453–479, R.G. Landes Company, Austin, TX, USA.



9. Elowitz, M. B. & Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature*, **403**, 335–338.
10. Gardner, T. S., Cantor, C. R. & Collins, J. J. (2000). Construction of a genetic toggle switch in *Escherichia coli*. *Nature*, **403**, 339–342.
11. Gorini, L. & Maas, W. (1957). The potential for the formation of a biosynthetic enzyme in *Escherichia coli*. *Biochim. Biophys. Acta*, **25**, 208–209.
12. Lin, E. C. C.; Simon Lynch, A. (eds) (1996). *Regulation of Gene Expression in Escherichia coli*, R.G. Landes Company, Austin, TX.
13. Bernstein, J. A., Khodursky, A. B., Lin, P. H., Lin-Chao, S. & Cohen, S. N. (2002). Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc. Natl Acad. Sci. USA*, **99**, 9697–9702.
14. Wagner, R. (2000). *Transcription Regulation in Prokaryotes*, Oxford University Press, Oxford.
15. Oehmichen, R., Klock, G., Altschmied, L. & Hillen, W. (1984). Construction of an *E. coli* strain overproducing the Tn10-encoded TET repressor and its use for large scale purification. *EMBO J.* **3**, 539–543.
16. Lutz, R. & Bujard, H. (1997). Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucl. Acids Res.* **25**, 1203–1210.
17. Scholz, O., Schubert, P., Kintrup, M. & Hillen, W. (2000). Tet repressor induction without Mg<sup>2+</sup>. *Biochemistry*, **39**, 10914–10920.
18. Hillen, W., Klock, G., Kaffenberger, I., Wray, L. V. & Reznikoff, W. S. (1982). Purification of the TET repressor and TET operator from the transposon Tn10 and characterization of their interaction. *J. Biol. Chem.* **257**, 6605–6613.
19. Walker, G. (1984). Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. *Microbiol. Rev.* **48**, 60–93.
20. Ronea, M., Rosenberg, R., Shraiman, B. & Alon, U. (2002). Assigning numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics. *Proc. Natl Acad. Sci. USA*, **99**, 10555–10560.
21. Schleif, R. (2000). Regulation of the L-arabinose operon of *Escherichia coli*. *Trends Genet.* **16**, 559–565.
22. Becskei, A., Seraphin, B. & Serrano, L. (2001). Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J.* **20**, 2528–2535.
23. Cormack, B. P., Valdivia, R. H. & Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.
24. Kalir, S., McClure, J., Pabbaraju, K., Southward, C., Ronen, M., Leibler, S. *et al.* (2001). Ordering genes in a flagella pathway by analysis of expression kinetics from living bacteria. *Science*, **292**, 2080–2083.
25. Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K. & Meier, I. (1983). Control of expression of the Tn10-encoded tetracycline resistance genes. Equilibrium and kinetic investigation of the regulatory reactions. *J. Mol. Biol.* **169**, 707–721.
26. Lederer, T., Takahashi, M. & Hillen, W. (1995). Thermodynamic analysis of tetracycline-mediated induction of Tet repressor by a quantitative methylation protection assay. *Anal. Biochem.* **232**, 190–196.
27. Saenger, W., Orth, P., Kisker, C., Hillen, W., Hinrichs, W., Tovar, K. *et al.* (2000). The tetracycline repressor—a paradigm for a biological switch. *Angew. Chem. Int. Ed. Engl.* **39**, 2042–2052.
28. Lanzer, M. & Bujard, H. (1988). Promoters largely determine the efficiency of repressor action. *Proc. Natl Acad. Sci. USA*, **85**, 8973–8977.

Edited by M. Yaniv

(Received 9 May 2002; received in revised form 2 September 2002; accepted 7 September 2002)