Molecular Systems Biology (2005) doi:10.1038/msb4100010

www.molecularsystemsbiology.com
Article number: 2005.0006

molecular systems biology

A coherent feed-forward loop with a SUM input function prolongs flagella expression in *Escherichia coli*

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Received 17.1.05; accepted 9.3.05

Complex gene-regulation networks are made of simple recurring gene circuits called network motifs. The functions of several network motifs have recently been studied experimentally, including the coherent feed-forward loop (FFL) with an AND input function that acts as a sign-sensitive delay element. Here, we study the function of the coherent FFL with a sum input function (SUM-FFL). We analyze the dynamics of this motif by means of high-resolution expression measurements in the flagella gene-regulation network, the system that allows *Escherichia coli* to swim. In this system, the master regulator FlhDC activates a second regulator, FliA, and both activate in an additive fashion the operons that produce the flagella motor. We find that this motif prolongs flagella expression following deactivation of the master regulator, protecting flagella production from transient loss of input signal. Thus, in contrast to the AND-FFL that shows a delay following signal activation, the SUM-FFL shows delays after signal deactivation. The SUM-FFL in this system works as theoretically predicted despite being embedded in at least two additional feedback loops. The present function might be carried out by the SUM-FFL in systems found across organisms.
Molecular Systems Biology 29 March 2005; doi:10.1038/msb4100010
Subject Categories: metabolic and regulatory networks; microbiology
Keywords: transcription networks; expression dynamics; green-fluorescent protein; mathematical models; design principles

Introduction

One of the most significant network motifs (Milo *et al*, 2002; Shen-Orr *et al*, 2002) in transcription regulation networks is the feed-forward loop (FFL). This motif was first defined in *Escherichia coli* (Shen-Orr *et al*, 2002), and then found in diverse organisms including *Saccharomyces cerevisiae* (Lee *et al*, 2002; Milo *et al*, 2002), *Bacillus subtilis* (Eichenberger *et al*, 2004), *Caenorhabditis elegans* (Mangan *et al*, 2003) and humans (Mangan *et al*, 2003; Odom *et al*, 2004). In the FFL, transcription factor X activates a second transcription factor Y, and both activate the output gene Z (Figure 1A). There are eight types of FFLs, characterized by the signs of the transcription interactions (repression or activation) (Mangan and Alon, 2003). One of the most abundant FFL types, called the type-1 coherent FFL (Mangan and Alon, 2003; Ma *et al*, 2004), has three positive regulations.

In order to understand the function of the FFL, one needs to specify the input function that integrates the effects of X and Y on gene Z. Previous experimental work characterized the function of the FFL in the *ara* system of *E. coli*. This FFL has an AND input function, in which both X and Y are needed to activate Z (Mangan *et al*, 2003). The AND-FFL showed a delay in Z expression following step activation of X, and no delay following deactivation of X. Here, we focus on the case where *either* X or Y is sufficient to activate Z (similar to an OR gate). We choose an experimental system in which X and Y act additively to regulate Z. That is, the input function at the Z promoter sums over the two inputs. We term this motif the SUM-FFL. One of the simplest ways to implement an additive input function is to provide a gene with two different promoters, each responding to one of the inputs. Such multiple promoters are indeed found in many gene systems.

We previously modeled the FFL with OR-gate logic (Mangan and Alon, 2003), which can be considered as a Boolean approximation to a SUM input function. The mathematical models suggested that a coherent FFL with an OR gate can carry out an information-processing function termed 'sign-sensitive delay': The output Z responds rapidly when the level of X increases, whereas Z responds only at a delay once X levels decrease (Mangan and Alon, 2003). The delay is due to the presence of Y. After X is deactivated, it takes time for Y levels to decrease sufficiently to de-activate Z. Thus, this gene circuit can protect against transient deactivation, because Z production can proceed even if X activity is briefly lost. If the

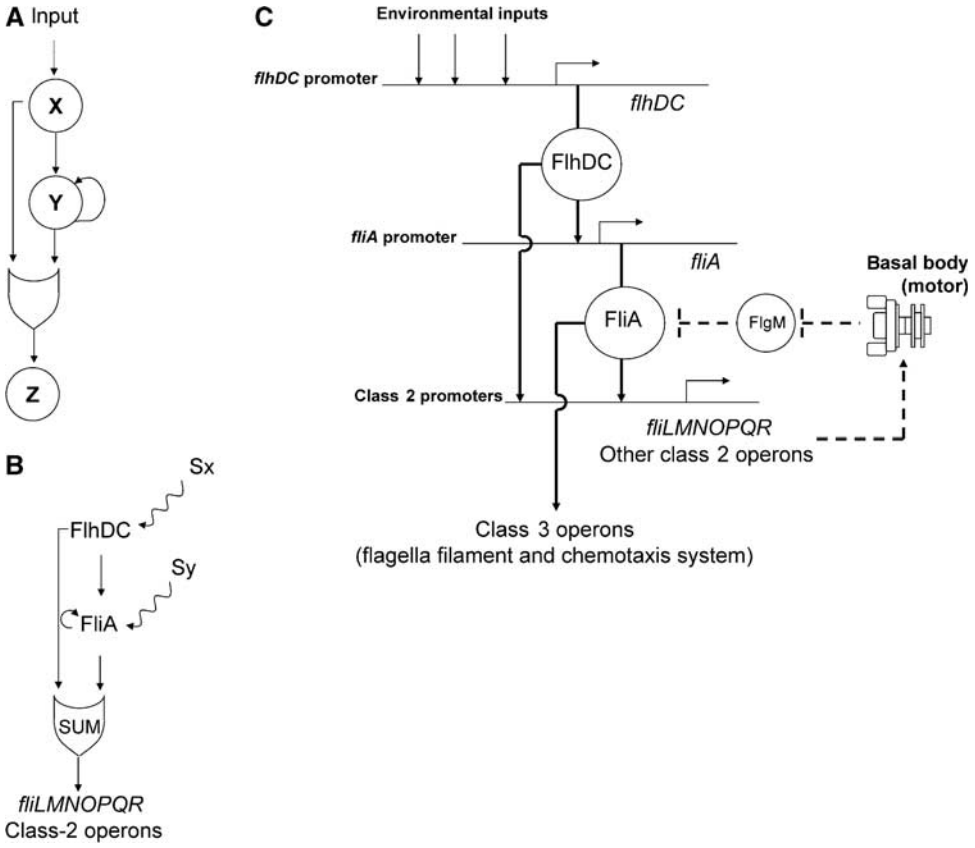

Figure 1 (**A**) The type-1 coherent FFL (Mangan and Alon, 2003). In many cases, Y regulates its own production as shown. (**B**) The SUM-FFL in the flagella class 2 regulation network. X is *flhDC*, Y is *fliA* and Z is the *fliLMNOPQR* operon (termed *fliL*) and other class 2 operons. In this circuit, the activator X regulates Y, X and Y act additively to activate the output gene *Z*. The input Sx is the production rate of X (or, more generally, a stimulus that activates X). The input Sy regulates the activity of Y. In the flagella system, Y positively regulates its own production. (**C**) A more detailed view of the flagella network and the basal-body checkpoint. The *flhDC* promoter is controlled by several transcription factors responsive to environmental stress and starvation. The class 2 genes encode the structural proteins that make up the basal bodies. FliA is involved in a positive feedback loop called the basal body checkpoint. In this loop, the activity of FliA as a transcription factor is inhibited by binding the protein FlgM (dashed –| sign indicating inhibition). FlgM is exported out of the cell once the first active basal bodies are formed, by a specific transport mechanism that exports FlgM through the basal bodies (dashed inhibition symbol between the basal body and FlgM). Thus, FliA helps activate genes that produce basal bodies, which export the inhibitor FlgM out of the cell, relieving the inhibition of FliA.

activator Y was removed from the circuit, Z would respond rapidly both to increases and decreases in X activity and the protection function would be lost.

As in the case of OR-FFL, a SUM-FFL can show a delay following OFF steps of X activity. This contrasts with the AND-gate FFL, which shows delay following ON but not OFF steps (Mangan and Alon, 2003; Mangan *et al*, 2003). Models of the SUM-FFL show a delay for a wide range of biochemical parameters, such as the production and degradation rates of the proteins, or the activation coefficients of the genes (Kalir and Alon, 2004). The length of the delay can be tuned by changing these parameters (Mangan and Alon, 2003). Positive feedback of Y on itself (Figure 1A) can further increase the delay time by slowing the reduction in Y levels following deactivation of X (see a simple mathematical analysis of positive auto-regulation in the appendix). In general, negative auto-regulation can speed responses, whereas positive auto-regulation slows response time (Savageau, 1974; Rosenfeld *et al*, 2002).

The above-mentioned theoretical treatment of the FFL deals with the interactions of three genes in isolation. In reality, this circuit is embedded in a network of interactions. It is therefore crucial to experimentally test the dynamical behavior of this motif in living cells. For this purpose, we consider a well-characterized gene-regulation system, the flagella biosynthesis network of the bacterium *E. coli* (Aldridge and Hughes, 2002).

When growth conditions become mildly unfavorable, *E. coli* produces several rotating flagella and swims away. The genes that make up the flagella motor are regulated by a SUM-FFL (Kalir and Alon, 2004) (Figure 1B). The master flagella activator X (FlhDC) activates a second activator Y (FliA). The activators X and Y function additively to activate the genes Z that build the flagella motor (Z represents the flagella class 2 genes arranged in operons such as *fliLMNOPQR*, here termed *fliL*).

The concentration and activity of the two regulators X and Y is affected by signals, termed Sx and Sy (Figure 1B). These inputs to this system are as follows: the rate of production of X is controlled by factors that respond to environmental signals such as glucose starvation (CRP) (Silverman and Simon, 1974), heat shock (dnaKJ and GrpE) (Shi *et al*, 1992), osmotic stress (ompR) (Shin and Park, 1995), low-PH (H-NS) (Soutourina *et al*, 1999) and cell density (QseBC) (Sperandio *et al*, 2002). Flagella in the best studied *E. coli* strains are

activated at late exponential growth phase, and deactivated in stationary phase (Amsler *et al*, 1993) as well as under high salt concentration and alcohol molecules (Shi *et al*, 1993).

The activity of Y is controlled by the signal Sy, a checkpoint that monitors the production of flagellar motors (basal bodies). The transcriptional activity of Y is inhibited by binding a protein inhibitor (FlgM, an anti-σ factor; Kutsukake and Iino, 1994). The inhibitor FlgM is exported out of the cells by completed basal bodies (Hughes *et al*, 1993; Karlinsey *et al*, 2000). Thus, when the first basal bodies are completed, FlgM is exported, relieving the inhibition of Y so that it begins to activate downstream genes Z (Figure 1C). Note that the presence of Sy (that is, the absence of FlgM) is required for the delay function of the SUM-FFL.

Here, we studied the effects of the SUM-FFL on the dynamics of the flagella gene expression using high-resolution measurements from living cells. We find that the SUM-FFL can generate a delay in the turn-OFF dynamics of the system, a delay that is dependent on the presence of Y. The delay is on a time-scale similar to that required for assembly of a flagellum.

Results

To study the dynamics of the SUM-FFL in the flagella system, we constructed *E. coli* cells in which X is under control of an inducible promoter. In these cells, the production of X (FlhDC) can be turned ON or OFF by means of a chemical inducer (L-arabinose) added externally to the cells (Kalir and Alon, 2004). The rate of Z (FliL) production from these cells was monitored in real time by means of a green-fluorescent protein (GFP) fused to a copy of the DNA regulatory region of gene Z (the *fliL* promoter) on a low-copy plasmid (Kalir *et al*, 2001). In order to measure GFP fluorescence, which corresponds to Z promoter activity, the cells were grown in an automated flourimeter during ON and OFF steps of X production (Kalir and Alon, 2004). As a control, we compared the dynamics to cells in which the gene for Y (*fliA*) was deleted.

To study turn-ON of gene expression, we added an inducer to the cells to initiate the production of X. We find that Z shows rapid production following an ON step of X production (Figure 2A). Cells deleted for Y showed about a 50% lower maximal Z expression. To study the response time, we normalized the fluorescence per cell signal to its maximal value. We find that cells deleted for Y show a rapid production of Z, similar to cells wild type for Y (Figure 2A). To study turn-OFF of gene expression, we shifted cells growing with inducer for 3 h to a medium without inducer (and with saturating anti-inducer D-fucose; Wilcox, 1974). We find that the deactivation of Z occurred at a delay of about 60–80 min compared to a cell in which Y is deleted (Figure 2B). Thus, the SUM-FFL displays a sign-sensitive delay, with a delay following OFF but not ON steps of X production.

The sign-sensitive delay also occurred in experiments in which X was deactivated following 4 or 5 h of induction (data not shown). During the activation phase of X, the basal body checkpoint appears to be activated so that Y can be active (as seen by the fact that genes regulated by Y and not X, such as the class 3 operon *fliC*, are activated). A much shorter delay occurred when X was induced for only 2 h before inactivation

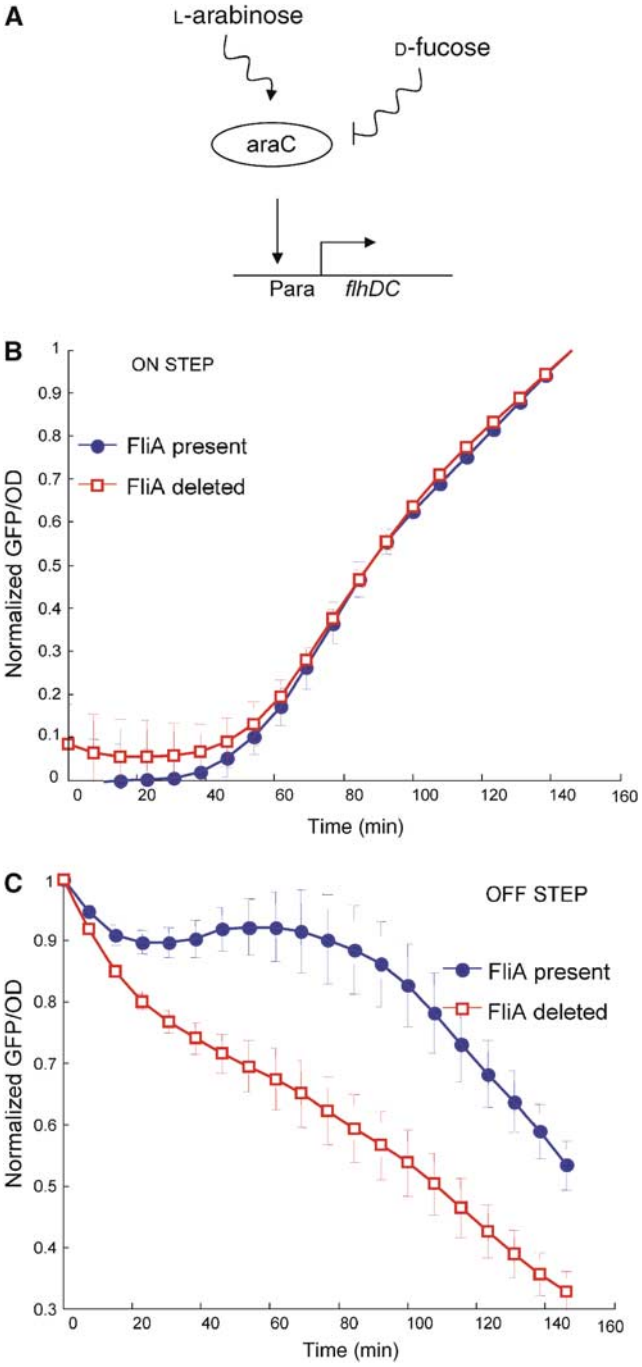

Figure 2 Experimental dynamics of *Z* (*fliL*) expression in a strain containing Y (*fliA*, strain U306 + pJM45 + pJM35, RP437Δ*flhD*, ●) and a strain deleted for Y (U307 + pJM45 + pJM35, RP437Δ*flhD*Δ*fliA*, □) (strains and plasmids were described in Kalir and Alon, 2004). (**A**) Production of X (FlhDC) regulated by the araBAD promoter on a low-copy plasmid was controlled by an inducer externally added to the cells (L-arabinose). The anti-inducer D-fucose allowed deactivation of X expression. (**B**) Dynamics of Z expression following induction of X. Cells were grown in defined glycerol medium as described (Kalir and Alon, 2004) with saturating inducer (2 mM arabinose), and Z production rate was monitored using GFP controlled by the Z promoter. GFP fluorescence divided by cell density (OD), normalized to a maximum of one, is shown. (**C**) Dynamics following turn-OFF of X production. Cells were grown with inducer for 3 h and then shifted to medium with no inducer and saturating anti-inducer (50 mM D-fucose).

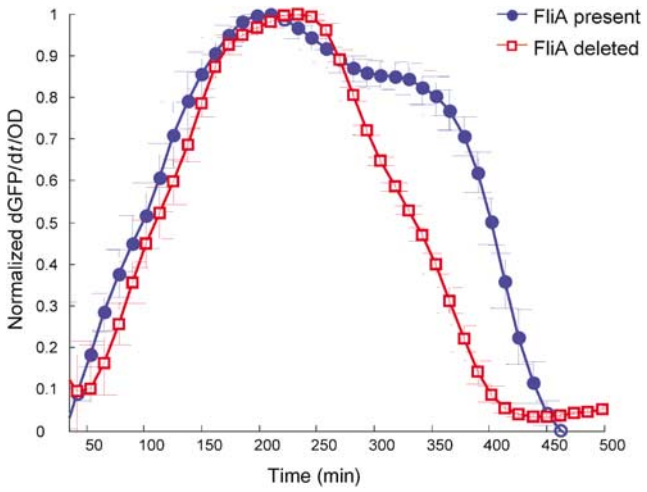

Figure 3 Experimental dynamics of Z (*fliL*) expression in wild-type cells (U16 + pJM35, RP437 ●), and in cells deleted for *fliA* (U309 + pJM35, RP437Δ*fliA* □). Cells were grown in defined glycerol medium (Kalir and Alon, 2004) and Z production rate was monitored using GFP controlled by the Z promoter. Promoter activity, defined (Kalir and Alon, 2004) as the rate of change of GFP flourescence divided by cell density (OD), normalized to a maximum of one, is shown.

(data not shown). A short period of FlhDC induction may not allow Y levels to accumulate, or might not be sufficient to form functional basal bodies, and therefore the action of Y would be blocked. This agrees with the theoretical prediction that the delay should depend on the presence of Sy.

We also studied the dynamics of *fliL* expression in wild-type RP437 cells in which *flhDC* is under control of its native promoter on the chromosome (that is, in which X is not produced by an inducible promoter as in Figure 2A). In these cells, flagella expression is turned OFF when cells approach the end of exponential growth (Amsler *et al*, 1993; Kalir *et al*, 2001; Kalir and Alon, 2004). We compared wild-type cells to cells in which the *fliA* gene is deleted (Δ*fliA*). The maximal promoter activity (Kalir and Alon, 2004) of the *fliL* promoter was lower by about 30% in the Δ*fliA* strain. To study the response time, we normalized the promoter-activity dynamics by the maximal promoter activity. We find that the wild-type and Δ*fliA* strains show similar normalized turn-ON dynamics (Figure 3). The turn-OFF of the wild-type cells is delayed with respect to the strains missing *fliA* (Figure 3). Thus, in the wild-type context, Y appears to prolong the production of Z in the turn-OFF phase of the dynamics.

Discussion

We find that the SUM-FFL in the flagella system displays sign-sensitive delay, with a significant delay following OFF steps of X production. This qualitatively agrees with theoretical predictions (Mangan and Alon, 2003) for the function of this FFL.

Why is a mechanism needed that prolongs flagella gene expression after the master regulator X is deactivated? The production of the flagella master activator X in wild-type cells is governed by multiple environmental inputs, such as carbon starvation, temperature, osmotic stress and cell density (Figure 1C) (Shi *et al*, 1993; Aldridge and Hughes, 2002). These factors fluctuate in the environment, especially if the cell swims from place to place. The present results suggest that the SUM-FFL makes the flagella system insensitive to brief periods in which X is deactivated. It allows the flagella system to turn-OFF only when the proper conditions are sensed for a lengthy period of time. The time-scale of the delay generated by the SUM-FFL, about 60–80 min under the present conditions, is comparable to the time needed to complete a flagellum, on the order of 1–2 h (Aizawa and Kubori, 1998; Kalir *et al*, 2001) (about 1–2 cell generations).

Network motifs appear to allow a qualitative understanding of the dynamics of gene expression in the simple systems studied so far. For example, they allow an understanding of the dynamics of the *B. subtilis* sporulation network, which is made of several cascaded FFLs, based on the features of each individual FFL (Eichenberger *et al*, 2004). In the flagella system, the SUM-FFL forms the backbone of the regulatory system, but it does not act in isolation. Rather, it is embedded within the network as part of at least two additional positive feedback loops: auto-regulation of FliA and the basal-body checkpoint (Figure 1C). Despite being embedded in a larger circuit, the flagella SUM-FFL performs sign-sensitive delay as predicted from theoretical analysis (Mangan and Alon, 2003) of the isolated motif. This raises the hope that motifs are, at least in some systems, wired into networks in such a way that allows understanding of the networks dynamics based on the behavior of each individual motif.

Network motifs can serve as qualitative models for the system dynamics, but they cannot be considered as fully detailed models. This is because a detailed model would require many additional subtle mechanisms and interactions, of which many are probably currently uncharacterized. For example, when comparing deletion mutants to wild-type cells, we may be changing not only the connections in the motif, but also other cell components. Thus, the present results should be tested with the quantitative blueprint models of mutant cells, similar to those established for the wild-type flagella system (Kalir and Alon, 2004).

The FFL network motif appears in the transcriptional wiring of organisms from bacteria to humans (Milo *et al*, 2002, 2004; Mangan *et al*, 2003; Odom *et al*, 2004). More generally, it is a basic building block of biological information-processing networks that range from the scale of molecules to the scale of connections between cells (Milo *et al*, 2002, 2004), such as the network of synaptic connections between neurons in *C. elegans*. In neuronal networks, the FFL motif seems not to result merely from spatial effects in which neighboring neurons tend to synapse to each other (White *et al*, 1986): such spatial effects would also produce three-neuron feedback loops, but such feedback loops are found to be rare (Itzkovitz and Alon, 2005). It is possible that the SUM-FFL can act as a delay element also in these networks, protecting the output from transient deactivation.

The present experimental study adds the SUM-FFL to previously studied motifs such as the AND-FFL that can act as a sign-sensitive delay element (Mangan *et al*, 2003), the incoherent FFL that can generate pulses and speed responses (Mangan and Alon, 2003; Basu *et al*, 2004), the single-input module that can generate 'just-when-needed' temporal gene

expression programs (Laub *et al*, 2000; Ronen *et al*, 2002; Shen-Orr *et al*, 2002; McAdams and Shapiro, 2003; Zaslaver *et al*, 2004), negative auto-regulation that can speed response times (Savageau, 1974; Rosenfeld *et al*, 2002) and decrease the variability of steady-state expression (Becskei and Serrano, 2000), and hybrid feedback loops that can generate oscillations (Goldbeter, 2002; Lahav *et al*, 2004; Nelson *et al*, 2004). It would be important to characterize and study additional motifs, in order to approach the goal of a complete dictionary of basic circuit elements and their functions (Elowitz and Leibler, 2000; Gardner *et al*, 2000; Batchelor and Goulian, 2003; Rosenfeld and Alon, 2003; Voigt *et al*, 2004; Wall *et al*, 2004).

Acknowledgements

We thank MG Surette, MB Elowitz and T Tlusty for discussions and help, all members of our lab for discussions, and HFSP, NIH, and Minerva for support.

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Appendix

Positive auto-regulation of FliA, a simple mathematical analysis

In the flagella system, FliA transcriptionally activates its own production. Previous experimental and theoretical work has shown that this system is well described by an additive linear input function (Kalir and Alon, 2004). That study indicated that the dynamics of FliA concentration, denoted *Y*, can be modeled by the following equation that includes FliA self-activated production and degradation:

$$dY/dt = \beta X + \beta' Y - \alpha Y$$

where βX is the production rate due to X and β' is the auto-activation rate. The parameter α is the protein degradation/dilution rate (Rosenfeld *et al*, 2002) of *Y*. After *X* has decayed, the dynamics of *Y* concentration obeys the same equation with $\beta X=0$:

$$dY/dt = \beta' Y - \alpha Y$$

The solution of this equation is an exponential decay:

$$Y(t) = Y_0 \exp(-(\alpha - \beta')t)$$

The time to decay to halfway of the initial concentration Yo is called the response time (Savageau, 1974; Rosenfeld *et al*, 2002), $T_{1/2}$. The response time can be found by solving for

$$Y(t = T_{1/2}) = \tfrac{1}{2} Y_0$$

yielding

$$T_{1/2} = \log(2)/(\alpha - \beta')$$

This response time is always longer than the response time in the case where there is no self-activation ($\beta'=0$), which is

$$T_{1/2} = \log(2)/\alpha$$

provided that the system is stable ($\beta' < \alpha$). The stronger the positive auto-regulation, the longer the response time. This contrasts with negative auto-regulation, which speeds response times (Savageau, 1974; Rosenfeld *et al*, 2002). Thus, positive auto-regulation of Y can help prolong the delay in the SUM-FFL following X deactivation.

Note that very strong auto-regulation, in which $\beta' > \alpha$, leads to instability and unchecked growth of Y in the model. In real systems, this instability will be limited by other factors (such as saturation of the input function), locking Y in an ON state of high expression even after its activating input βX vanishes.

Hence, strong positive auto-regulation can in principle lock genes ON even after their input signals have decayed. This is thought to occur in developmental transcription networks to act as a memory that determines a cell's fate. However, in the flagella system which requires reversible induction, it appears that the auto-regulation of FliA is not sufficient to act as a bistable switch and keep FliA expressed after FlhDC is deactivated (Kalir and Alon, 2004). Positive auto-regulation in this system can, as we have discussed, act to prolong the expression of FliA, and thus to prolong the delay generated by the SUM-FFL after X is deactivated.