Designing a genetic toggle switch for \textit{E. coli} that uses sequestration of a eukaryotic repressor as a mechanism for ultrasensitivity

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Abstract

Bistable gene expression—when a gene’s output can achieve and alternate between two distinct, stable states—plays a critical role in the regulation of various cell and developmental processes including cell-cycle progression, differentiation, and signaling. To study and harness this regulatory process in bacteria, synthetic biologists have created gene circuits in *E. coli* that use sequestration of bacterial activators driving their own expression by inducible inhibitors to generate ultrasensitive positive feedback that leads to bistability in their expression. While capable of bistability, these circuits can be affected by cross-interference with native *E. coli* regulatory processes and cause toxic squelching that make studying these circuits difficult. As such, gene circuits that can produce bistable gene expression in *E. coli* via sequestration-based ultrasensitivity while avoiding cross-interference and toxic squelching would be valuable tools for synthetic biology. Based on a premise that using eukaryotic repressors should avoid both toxic squelching and cross-interference in bacterial hosts, I here present efforts to create a circuit in *E. coli* that uses sequestration of the eukaryotic repressor C/EBPα by a synthetic inhibitor called 3HF to generate bistable gene expression. While I did not obtain a working circuit, I made progress toward selecting promoters and replication origins that balance the expressions of C/EBPα and 3HF, and toward selecting a fluorescent protein tag that is compatible with 3HF in *E. coli*. 
Introduction

Bistable gene expression is a phenomenon wherein the expression of a gene can alternate in a switch-like manner between two distinct states that are stable and can be maintained across cell divisions. In natural systems, this alternation, known as toggling, often occurs between a stable state of no expression (canonically called the “off” state) and a stable state of expression (canonically called the “on” state). For prokaryotic and eukaryotic organisms, such “on/off” bistable gene expression plays an essential role in the regulation of cellular and developmental processes that involve sharp and stable activation or deactivation of gene expression (Dubnau & Losick 2006; Veening et al. 2008). Prominent examples of such processes include: lactose utilization and antibiotic resistance in *E. coli* (Ozbudak et al. 2004; Deris et al. 2013); genetic competence, sporulation, and biofilming in *B. subtilis* (Maamar & Dubnau 2005; Veening et al. 2005; Chai et al. 2008); maturation of Xenopus oocytes (Ferrell 2002; Xiong & Ferrell 2003); mating response, cell cycle progression, and GAL network regulation in *S. cerevisiae* (Paliwal et al. 2007; Zhang et al. 2011; Stockwell et al. 2015); cell differentiation, cell cycle progression, and apoptosis in *H. sapien* (Vermeulen et al. 2003); and dorsal-ventral differentiation and eye formation in *D. melanogaster* (Lai et al. 2004).

To study and harness bistable gene expression as a tool for synthetic biology in bacteria, biologists have constructed synthetic gene circuits in *E. coli* that use a variety of schemes to generate bistable expression of different proteins (Gardner et al. 2000; Ferrell 2002; Atkinson et al. 2003; Wang et al. 2007; Chen & Arkin 2012; Shopera et al. 2015). One such scheme involves using an inducible inhibitor to sequester a transcriptional activator that drives its own expression (Figure 1) (Chen & Arkin 2012). The positive feedback generated by the activator’s self-regulation provides a foundation for bistable expression of the activator by stabilizing an “on” state of
expression. Sequestration of the activator by an inhibitor builds on this foundation to generate bistable expression of the activator by causing expression of the activator to drop rapidly in a sharp, “all-or-nothing” ultrasensitive response after (and only after) a threshold concentration of inhibitor sufficient to sequester all activator is induced (Figure 2). As demonstrated by the steady state rate-balance plot in Figure 3 (using the top-left scheme), such ultrasensitive responsiveness allows the expression rate of the activator to equal its degradation rate at three different concentrations of activator such that (within a certain range of inducible inhibitor concentrations) three unchanging concentrations, or steady states, of the activator are possible (Ferrell & Ha, 2014). The lower and higher steady states are stable, since deviation from either unbalances the expression and degradation rates in a manner that pushes the system back toward those steady states. The intermediate steady state, in contrast, is unstable because deviation from it unbalances the expression and degradation rates in a manner that pushes the system away from the intermediate steady state. As such, for any induced concentration of inhibitor within a certain range, the activator can be expressed at a stable high steady state (“on”) or a stable low steady state (“off”). Moreover, toggling between those two stable steady states requires sufficient toggling stimulus to push the system across the unstable intermediate steady state. Circuits that employ this scheme therefore generate bistable expression of the activator.

While effective at generating bistable gene expression, circuits that employ sequestration of bacterial activators to achieve ultrasensitivity can cause problems that can make using those circuits in E. coli difficult. High expression of activators from synthetic circuits can be toxic to host cells by binding and depleting transcription machinery needed for expression of essential genes—an effect known as squelching (Cahill et al. 1994). Moreover, natural regulatory pathways in E. coli from which circuit components are derived can interfere with the activity of those circuit
components in a process called cross-interference (Cardinale & Arkin 2012). As such, synthetic biologists might benefit from circuits that can generate bistable gene expression via sequestration-based ultrasensitivity without using activators or components derived from bacteria, since such circuits could be used bacteria without fear of toxic squelching or cross-interference.

My objective for this thesis project was to create such a gene circuit in *E. coli*. Because DNA-binding repressors prevent transcription by blocking procession of RNA polymerases without binding them, I reason that they should not cause toxic squelching (though toxic off-target repression could still occur) (Hanna-Rose & Hansen 1996). Moreover, I reason that eukaryotic repressors that do not occur naturally in bacteria should experience less cross-interference with native *E. coli* regulatory pathways (Rosano & Ceccarelli 2014). I therefore took the premise that using sequestration of a eukaryotic repressor to couple positive feedback in gene expression with ultrasensitivity might lead to bistable gene expression while avoiding toxic squelching and cross-interference in *E. coli*. Based on that premise, I endeavored to create a synthetic gene circuit in which the DNA-binding domain of the eukaryotic repressor C/EBPα (Landschulz *et al.* 1988; Buchler & Cross 2009) expressed under control of an IPTG-inducible promoter inhibits expression of the synthetic sequestering inhibitor 3HF (Krylov *et al.* 1995) expressed under control of a synthetic promoter containing binding sites for C/EBPα (Buchler & Cross 2009) (Figure 4).

Because sequestration of C/EBPα by 3HF should reinforce expression of 3HF in a form of indirect positive feedback and lead to ultrasensitive deactivation of 3HF at a threshold concentration of C/EBPα, this circuit should exhibit bistable expression of 3HF that toggles depending on the concentration of IPTG present in the host. The steady state rate-balance plot in Figure 3 demonstrates this logic if the response curves represent ultrasensitive expression of 3HF at different concentrations of C/EBPα (i.e. using the top-right scheme). Were this circuit to produce
bistable expression of 3HF without toxicity or cross-interference, it would serve as a proof-of-principle that synthetic gene circuits that use sequestration of eukaryotic repressors to couple ultrasensitivity with positive feedback in gene expression can be effective tools for implementing bistable gene expression in *E. coli*. Moreover, insights gained through its creation could be applied toward development of other sequestration-based circuits that reproduce eukaryotic bistable expression processes in bacteria for the sake of investigating those processes in less-interfering environments or investigating the effect of eukaryotic bistable expression processes in bacterial hosts.

Ultimately, I did not obtain a working version of the circuit. But I did make progress toward three objectives necessary for its completion: 1) selection of a promoter to control expression of C/EBPα that has a wide inducible range that allows for fine assessment of the circuit’s response to IPTG; 2) selection of a suitable fluorescent protein tag for fusion to 3HF; and 3) selection of replication origins for the plasmids encoding the circuit that balance the relative expression levels of C/EBPα and 3HF as driven by their respective promoters. I accomplished the first objective by testing two IPTG-inducible promoters for wide inducible ranges of C/EBPα expression. I accomplished the second objective by testing the expression and brightness of two potential fluorescent protein tags for 3HF and their compatibility when fused to 3HF. I accomplished the third task by testing replication origins on the plasmids encoding the components of the circuit until I found a pair that cause the maximum expression of C/EBPα to greatly exceed the maximum expression of 3HF at full induction. To determine the expression of the circuit’s components, I used flow cytometry that measured the intensity of fluorescence produced by mCherry fused to C/EBPα-mCherry and the fluorescent tags that I tested for 3HF.
Materials and Methods

Strains and Plasmids

I used the DH5αZ1 strain of *E. coli* (Lutz & Bujard 1997) as the host for all constructs that I created during this project. I selected this strain because it should tightly repress IPTG-inducible promoters in the absence of IPTG as a result of overexpressing the lac repressor (lacI) from two extra copies of the *lacI* gene encoding lacI that were integrated into its genome (Lutz & Bujard 1997). Moreover, this strain also has a high chemical transformation efficiency that makes it ideal for a project that requires frequent transformation of plasmids (Lutz & Bujard 1997).

Growth Conditions

I grew all cultures at 37°C with constant shaking in LB media. To ensure maintenance of plasmids, I added Ampicillin (100 µg/mL) to the media for strains carrying plasmids encoding C/EBPα components of the circuit and Chloramphenicol (25 µg/mL) for strains carrying plasmids encoding 3HF components of the circuit. For strains carrying plasmids encoding the C/EBPα and the 3HF components (each on their own plasmids), I added both Ampicillin (100 µg/mL) and Chloramphenicol (25 µg/mL) to the culture media. Although I did not initially do so, I later added Spectinomycin (50 µg/mL) to all cultures (see results and discussion sections for an explanation).

DNA Extraction and Purification

To extract and purify plasmids, I inoculated 5 mL cultures containing antibiotics (as described above) with single colonies of the strain(s) carrying the desired plasmid(s). I then incubated those cultures overnight (~14 hours), centrifuged them, and extracted plasmid DNA from the resulting pellets using Qiagen’s QIAprep Miniprep Kit and Protocol (Qiagen 2015). I
assessed the purity and concentration of extracted plasmid DNA using a ND-1000 Spectrophotometer running the ND-1000 operating software, version 3.3 (Thermo Scientific 2011).

**Cloning and Transformation**

I performed all cloning using restriction digestion and ligation protocols provided in Springer’s *Methods in Molecular Biology* (Walker 2016) and restriction endonucleases produced by New England Biolabs® Inc (www.neb.com/products/restriction-endonucleases). I created inserts containing the C/EBPα-mCherry or 3HF-yEVenus genes via PCR amplification from yeast plasmids used in previous studies (Buchler & Louis 2008). I created an insert containing the GFP gene via PCR amplification of the GFP gene from a stock plasmid kept in the Buchler lab (using primers 5’-GGTACCATGAGCGAACTGATC-3’ and 5’-CGATCGGCTACTAGTATTATTAATTCAG-3’). I confirmed the DNA sequences of all plasmids that I cloned with Sanger sequencing performed by the Duke Center for Genomic and Computational Biology through the Duke University Genome Sequencing Shared Resource (https://genome.duke.edu/cores-and-services/sequencing-and-genomic-technologies).

I made DH5αZ1 chemically competent using a calcium chloride-based chemical competency protocol provided in Springer’s *Methods in Molecular Biology* (Walker 2016) and transformed plasmids into resulting chemically competent strains using a heat shock transformation protocol also provided in Springer’s *Methods in Molecular Biology* (Walker 2016).

**Optical Density Measurements**

I measured the optical densities of all *E. coli* cultures with a Genesys 20 Spectrophotometer at a wavelength of 660 nm. To compensate for a disparity between the measured and actual OD$_{660}$
that occurs consistently with this particular spectrophotometer, I corrected the measured OD_{660} to the actual OD_{660} using the following equation: 

\[ \text{OD}_{\text{corrected}} = 2.055 - \sqrt{4.224 - 3.580 \times \text{OD}_{\text{measured}}} . \]

This disparity was identified and modeled by a lab technician who compared results from this photometer with those of other photometers (Unpublished data).

**Flow Cytometry**

To assess the expression of circuit components, I grew 5 mL cultures overnight (~14 hours) and then diluted them 1000-fold into fresh 3 mL cultures containing a range of IPTG concentrations. After growing the diluted cultures to mid-log phase (~3 hours; OD_{660} = 0.6), I measured the average fluorescence per cell generated by expressed circuit components using the yellow laser (561 nm at 516 V with a 615/20 nm filter) and the blue laser (488 nm at 332V with a 525/50 nm filter) of a MACS Quant VYB Flow Cytometer running MACSQuantify™ software version 2.8 (Miltenyi Biotech GmbH 2013; Miltenyi Biotech GmbH 2015; Miltenyi Biotech GmbH 2016).

**Data Analysis**

I analyzed all flow cytometry data using the FlowJo software, version 9.9.5 (TreeStar Inc. 2013). I excluded fluorescence measurements taken from cellular debris or dividing cells by gating the data to exclude measurements from entities with especially large or small side- or forward-scatters. I determined the mean, standard deviation, and margin of error of the fluorescence measurements taken for each sample using functions built into the FlowJo software (TreeStar Inc., 2013). I made graphs and figures of fluorescence expression data in Microsoft Excel.
Results

Objective 1: Testing IPTG-inducible promoters to control expression of C/EBPα-mCherry

My first objective toward creating the circuit was to identify an inducible promoter to control expression of C/EBPα-mCherry that has a wide inducible range that can be fine-tuned (changed in small increments) to allow for good resolution of the IPTG concentration at which the circuit toggles. I selected two IPTG-inducible promoters created by Lutz & Bujard (1997) that can be induced ~1700-fold and ~600-fold, respectively: $P_{lac/ara-1}$ and $P_{LlacO-1}$. I cloned these promoters in front of the C/EBPα-mCherry gene on separate plasmids bearing the low-copy-number 15A replication origin (15A ori) (constructs A and B in Figure 5) and then measured the average fluorescence driven by those promoters in DH5αZ1 cells across a range of IPTG concentrations (note that I did not include Spectinomycin in the culture media for this experiment). As shown in Figure 6, the mean fluorescence driven by $P_{LlacO-1}$ ranged from 1.79 units/cell at 0 µM IPTG to 1.95 units/cell at 500 µM IPTG. The mean fluorescence driven by $P_{lac/ara-1}$, in contrast, ranged from 0.22 units/cell at 0 µM IPTG to 0.33 units/cell at 1000 µM. 95% confidence intervals (CIs) calculated for the mean fluorescences driven by the two promoters and for the mean fluorescence of untransformed DH5αZ1 cells indicate that both promoters drove fluorescence significantly above the background fluorescence of DH5αZ1 (0.135-0.17 units/per cell) and that the mean fluorescences generated by $P_{LlacO-1}$ greatly exceeded that driven by $P_{lac/ara-1}$. However, they also indicate that the fluorescence generated by the promoters did not vary significantly with increasing concentrations of IPTG. As such, both promoters drove significant expression of C/EBPα-mCherry in DH5αZ1 at all IPTG concentrations, and $P_{LlacO-1}$ drove a higher magnitude of fluorescence than $P_{lac/ara-1}$. But neither promoter exhibited a discernible inducible range for lack of suppression at low IPTG concentrations.
I speculated that my DH5αZ1 strain poorly repressed P_{lac/ara-1} and P_{LlacO-1} because it was losing from its genome (perhaps through recombination) the extra copies of the lac\textsuperscript{\textregistered} gene that cause overexpression of the lac repressor that should tightly repress IPTG-inducible promoters in the absence of IPTG (Lutz & Bujard 1997). Close examination of how the DH5αZ1 strain was created (Lutz & Bujard 1997) revealed that the two extra copies of the lac\textsuperscript{\textregistered} gene were integrated into its genome with a nearby Spectinomycin-resistance gene that was used to select for cells that successfully integrated the extra copies into their genomes. I therefore conjectured that adding Spectinomycin to the culture media might restore proper repression of P_{lac/ara-1} and P_{LlacO-1} in DH5αZ1 by allowing growth only of cells that maintain the segments of their genomes containing the extra copies of lac\textsuperscript{\textregistered} and the Spectinomycin-resistance gene. I tested that conjecture by measuring the fluorescence generated by P_{LlacO-1} driving expression of C/EBPα-mCherry from plasmids carrying either the 15A or ColE1 ori (constructs B and C in Figure 5) in DH5αZ1 cells grown in media also containing Spectinomycin. I did not test P_{lac/ara-1} because it showed poor expression in the previous experiment. Moreover, I retested P_{LlacO-1} paired with both the 15A and ColE1 oris because the ColE1 ori has a much higher copy number than does the 15A ori (causes more plasmids to exist per cell) and I wanted to determine whether increasing the number of plasmids encoding P_{LlacO-1} driving expression of C/EBPα-mCherry altered the magnitude of the inducible range of C/EBPα-mCherry expression.

As shown in Figure 7, the mean fluorescence driven by P_{LlacO-1} when paired with the ColE1 orI increased approximately 13.26-fold over the range of IPTG concentrations tested, from 2.64 units/cell at 0 µM IPTG to 35 units/cell at 2000 µM IPTG. In contrast, the fluorescence driven by P_{LlacO-1} when paired with the 15A ori increased only 5.49-fold, from 0.37 units/cell at 0 µM IPTG to 2.03 units/cell at 2000 µM IPTG. 95% CIs calculated for the reported fluorescences
indicate that \( P_{\text{LlacO-1}} \) drove fluorescence significantly above background levels (~0.247 units/cell) when paired with either ori. They also indicate that \( P_{\text{LlacO-1}} \) drove significantly different levels of fluorescence across all but the highest concentrations of IPTG tested when paired with the ColE1 ori, but not when paired with the 15A ori. As such, \( P_{\text{LlacO-1}} \) exhibited an inducible range when Spectinomycin was added to the media. Moreover, \( P_{\text{LlacO-1}} \) had a stronger and wider inducible range when paired with the ColE1 ori than when paired with the 15A ori.

**Objective 2: Testing yEVenus and GFP as fluorescent tags for 3HF**

My second objective when creating the circuit was to identify a fluorescent tag that is compatible with 3HF in DH5\( \alpha \)Z1 so that I could measure its expression. I began by separately cloning the yeast-enhanced yellow fluorescent protein yEVenus (Buchler & Cross 2009) and a 3HF-yEVenus fusion under control of a C/EBP\( \alpha \)-repressible promoter called \( P_{\text{GCAAT}} \) (constructs D and F in Figure 5) that I created by replacing the tetO sequences of the tetO2 promoter system (Gari et al. 1997) with binding sites for C/EBP\( \alpha \) (Landschluz et al. 1988; Pei & Shih 1991). I elected to try yEVenus because it is an effective fluorescent tag for 3HF in yeast (Buchler & Cross 2009). However, I anticipated that DH5\( \alpha \)Z1 might not express yEVenus efficiently due to its gene’s sequence being optimized for efficient translation in yeast (Sheff & Thorn 2004). I therefore also cloned a green fluorescent protein (GFP) (Cha et al. 2000) under control of the \( P_{\text{GCAAT}} \) promoter on a different plasmid (construct E in Figure 5). I then measured the fluorescence of cells carrying the plasmid encoding yEVenus, cells carrying the plasmid encoding 3HF-yEVenus, and cells carrying the plasmid encoding GFP. I had also planned to test the fluorescence of cells carrying a plasmid encoding a 3HF-GFP fusion, but was unable to acquire the data in time.
As shown in Figure 8, cells carrying the plasmid encoding yEVenus exhibited a mean fluorescence of 6.5 units/cell, while those carrying the plasmid encoding GFP exhibited a mean fluorescence of 8.493 units/cell. Cells carrying the plasmid encoding 3HF-yEVenus, in contrast, exhibited a mean fluorescence of only 0.22 units/cell. 95% CIs calculated for all reported mean fluorescentences indicate that yEVenus and GFP both generated fluorescence significantly greater than the background fluorescence of untransformed DH5αZ1 (~0.25 units/cell) and that GFP generated significantly more fluorescence than yEVenus. They also indicate, in contrast, that the fluorescence of cells carrying the plasmid encoding 3HF-yEVenus plasmid was not significantly greater than the background fluorescence of untransformed DH5αZ1 cells. As such, both yEVenus and GFP express and fluoresce significantly in DH5αZ1, though GFP more-so than yEVenus. But 3HF-yEVenus does not express and/or fluoresce significantly in DH5αZ1.

**Objective 3: Testing repression of P<sub>GCAAT</sub> by C/EBPα-mCherry and balancing the maximum expressions of C/EBPα and 3HF**

My third objective toward creating this circuit was to verify that C/EBPα-mCherry represses P<sub>GCAAT</sub> in DH5αZ1 and to determine whether pairing P<sub>LlacO-1</sub> with the ColE1 ori (construct C in Figure 5) and P<sub>GCAAT</sub> with the 15A ori (construct D, E, and F in Figure 5) balances the maximum expressions of C/EBPα-mCherry and tagged 3HF such that C/EBPα-mCherry can overcome 3HF and toggle the circuit when fully induced. To do so, I measured the mean red and yellow fluorescentces of DH5αZ1 cells carrying a plasmid encoding P<sub>LlacO-1</sub> driving C/EBPα-mCherry paired with the ColE1 ori (construct C in Figure 5) and another plasmid encoding P<sub>GCAAT</sub> driving yEVenus paired with the 15A ori (construct D in Figure 5) across a range of IPTG concentrations. As shown in Figure 9, the mean red fluorescence of those cells gradually increased.
16.358-fold over the IPTG concentrations tested, from 1.58 units/cell at 0µM IPTG to 25.847 units/cell at 2000µM IPTG. In contrast, the mean yellow fluorescence of those cells gradually decreased 5.133-fold, from 8.403 units/cell at 0µM IPTG to 1.637 units/cell at 2000µM IPTG. 95% CIs calculated for the mean red and yellow fluorences of those cells and for untransformed DH5αZ1 cells indicate that both circuit components produced fluorences significantly above the background fluorescence of DH5αZ1 (~0.14 units/cell) at all IPTG concentrations. They also indicate that the fluorescence produced by the C/EBPα-mCherry component differed significantly across all IPTG concentrations, while the fluorescence generated by yEVenus differed significantly only across lower concentrations of IPTG. As such, expression of yEVenus decreased significantly with significant increases in expression of C/EBPα-mCherry at lower concentrations of IPTG. Moreover, the maximum fluorescence generated at full induction by C/EBPα-mCherry driven by P_LlacO-1 paired with the CoIE1 ori greatly exceeded the maximum fluorescence generated by yEVenus driven by P_GCAAT paired with the 15A ori.

**Objective 4: Testing the complete circuit**

Although 3HF-yEVenus does not appear to express or fluoresce well in DH5αZ1, I wanted to attempt my final objective in the creation of this circuit: to test a complete version for bistable expression of 3HF. I therefore measured the mean red and yellow fluorences of DH5αZ1 cells carrying plasmids separately encoding P_LlacO-1 driving C/EBPα-mCherry paired with the CoIE1 ori (construct C in Figure 5) and P_GCAAT driving 3HF-yEVenus paired with the 15A ori (construct F in Figure 5) across a range of IPTG concentrations. As shown in Figure 10, the mean red fluorescence of those cells gradually increased ~11-fold over the range of IPTG concentrations tested, from 1.67 units/cell at 0 µM IPTG to 18.357 units/cell at 500µM IPTG. In contrast, the
mean yellow fluorescence of the cells decreased only ~1.45-fold, from 0.711 units/cell at 0 µM IPTG to 0.488 units/cell at 500µM IPTG. 95% CIs calculated for the mean red and yellow fluorescences of those cells and of untransformed DH5αZ1 cells indicate that the circuit generated red and yellow fluorescences significantly above the background fluorescence of DH5αZ1 (~0.31 units/cell). They also indicate that the red fluorescence generated by the circuit differed significantly across all IPTG concentrations while the yellow fluorescence did not, particularly at higher concentrations of IPTG. As such, a complete version of the circuit generated a significant inducible range of C/EBPα-mCherry expression, but did not produce a substantial range of 3HF-yEVenus expression. Moreover, the circuit did not exhibit any readily apparent ultrasensitive deactivation of 3HF-yEVenus expression with increasing expression of C/EBPα-mCherry.

Discussion

Objective 1: Selecting an IPTG-inducible promoter to control expression of C/EBPα-mCherry

My first objective for this project was to determine whether P_{lac/ara}-1 and P_{LlacO}-1 drive wide inducible ranges of C/EBPα-mCherry expression in response to IPTG in DH5αZ1. As Figure 6 shows, P_{LlacO}-1 drove significantly higher expression of C/EBPα-mCherry than did P_{lac/ara}-1 when induced with IPTG. Neither, however, appeared to be repressed at low concentrations of IPTG, and thus did not exhibit inducible ranges. Because Lutz & Bujard (1997) found that IPTG induces P_{lac/ara}-1 to a stronger and wider range (~1700-fold) than P_{LlacO}-1 (~600-fold) in DH5αZ1, I sought to explain why P_{LlacO}-1 unexpectedly outperformed P_{lac/ara}-1 and why neither promoter seemed to be repressed by my strain of DH5αZ1 at low concentrations of IPTG. I suspected that experimental error accounted for why P_{LlacO}-1 unexpectedly outperformed P_{lac/ara}-1. However, sequencing results rule out inaccurate cloning or mutation as a potential cause, and replication of the experiment ruled
out procedural error. One possible explanation is that the discrepancy between my results and those reported by Lutz & Bujard (1997) might occur because I used \( P_{\text{LlacO-1}} \) and \( P_{\text{lac/ara-1}} \) to drive expression of a eukaryotic protein in DH5\( \alpha \)Z1 while Lutz & Bujard (1997) used them to drive expression of a bacterial protein. In any case, being motivated by time constraints to continue, I decided simply to work with \( P_{\text{LlacO-1}} \).

As explained in the results section, I speculated that my DH5\( \alpha \)Z1 strain poorly repressed \( P_{\text{lac/ara-1}} \) and \( P_{\text{LlacO-1}} \) at low concentrations of IPTG because it was losing the segment of its genome where extra copies of the \( \text{lacI}^\Theta \) encoding the lac repressor were integrated along with a gene for Spectinomycin resistance. I therefore speculated that adding Spectinomycin to the culture media might restore repression of \( P_{\text{LlacO-1}} \) by allowing growth of only DH5\( \alpha \)Z1 cells that retained that segment of their genomes. The results of my experiment to retest \( P_{\text{LlacO-1}} \) after adding Spectinomycin to the culture media (Figure 7) confirm that speculation. I therefore included Spectinomycin in the media for all cultures. Moreover, those results also demonstrate that pairing \( P_{\text{LlacO-1}} \) with the higher-copy-number ColE1 ori leads to a stronger and wider inducible range of C/EBP\( \alpha \)-mCherry expression than does pairing it with the lower-copy-number 15A ori. I therefore also decided to keep \( P_{\text{LlacO-1}} \) paired with the ColE1 ori in the circuit.

**Objective 2: Testing yEVenus and GFP as fluorescent tags for 3HF**

My second objective for this project was to determine whether yEVenus and GFP are effective fluorescent tags for 3HF. I reasoned that yEVenus might work because it has been successfully used as a fluorescent tag for 3HF in yeast (Buchler & Cross 2009) and appears to express and fluoresce well in DH5\( \alpha \)Z1 (Figure 8). Similarly, I reasoned that GFP might work because it has effectively tagged other proteins in *E. coli* (Drew *et al.* 2006) and also appears to
express and fluoresce well in DH5αZ1 (Figure 8). Unexpectedly, the low fluorescence of DH5αZ1 cells carrying a plasmid encoding 3HF-yEVenus (Figure 8) suggests that 3HF-yEVenus is either unstable or poorly expressed in DH5αZ1 and thus is not a suitable fluorescent tag for 3HF. Unfortunately, because I was not able to obtain an input-output curve for GFP or synthesize and test a 3HF-GFP fusion, I cannot conclude whether GFP is a compatible fluorescent tag for 3HF.

There are several possible explanations for the apparent instability and/or poor expression of 3HF-yEVenus in DH5αZ1. 3HF might be unstable in E. coli (a host in which 3HF has never been used) such that 3HF-yEVenus is also unstable and degraded quickly. Moreover, yEVenus might interfere with folding of 3HF such that it is unstable and quickly degraded in E. coli. Or, conversely, 3HF might interfere with proper folding of yEVenus such that it does not fluoresce properly or is quickly degraded in E. coli. It is also possible that both mutually interfere with one another’s folding such that 3HF-yEVenus is both quickly degraded and non-fluorescent in DH5αZ1. Additionally, DH5αZ1 might poorly express 3HF-yEVenus as a result of the sequence for yEVenus being optimized for efficient translation in yeast. However, because yEVenus appears to be expressed and fluoresce well in DH5αZ1 (Figure 8), this explanation seems less likely.

**Objective 3: Confirming repression of the P_{GCAAT} by C/EBPα-mCherry and balancing the maximum expressions of 3HF and C/EBPα-mCherry**

My third objective for this project was confirm that C/EBPα-mCherry suppresses the P_{GCAAT} promoter in DH5αZ1 and to pair P_{GCAAT} with a replication origin that balances the maximum expression of 3HF such that C/EBPα-mCherry can overcome sequestration by 3HF and toggle the system when fully induced. The input-output curve that I obtained for yEVenus when expressed under control of the P_{GCAAT} promoter from a plasmid with the 15A ori (Figure 9)
suggests that C/EBPα-mCherry suppresses the P_{GCAAT} promoter. Moreover, because yEVenus is ~3X brighter per molecule than mCherry (Nagai et al. 2002; Shaner et al. 2004), that the maximum fluorescence produced by C/EBPα-mCherry in the system is ~3X brighter than that produced by yEVenus strongly suggests that pairing P_{GCAAT} with the 15A ori drives a maximum expression of 3HF that is well below that of C/EBPα driven by P_{LlacO-1} paired with the ColE1 ori. I therefore decided to keep P_{LlacO-1} driving C/EBPα-mCherry paired with the ColE1 ori and P_{GCAAT} driving 3HF paired with the 15A ori.

**Objective 4: Behavior of the complete circuit**

The ultimate goal of my project was to create and test a complete version of the circuit to determine whether it generates bistable gene expression. To that end, while I recognized that 3HF-yEVenus does not appear to express and/or fluoresce well in DH5αZ1, I decided none-the-less to test a complete version of the circuit that uses 3HF-yEVenus. Not unexpectedly, the results of the experiment (Figure 10) provide no indications of bistable expression of 3HF. This finding very likely results from the instability and/or poor expression of 3HF-yEVenus already demonstrated in Figure 8. As such, my experiment to test a complete version of the circuit for bistability was unsuccessful in terms of providing insight as to whether the circuit has potential to achieve bistable gene expression.

**Conclusions**

Ultimately, I was not able to complete a working version of the circuit and thus could not formally determine whether it generates bistable gene expression while avoiding toxic squelching and cross-inference in *E. coli*. I did, however, make significant progress toward its development.
Primarily, I determined that the P_{LacO-1} promoter paired with the ColE1 ori should control expression of C/EBPα-mCherry while the P_{GCAAT} promoter paired with the 15A ori should control expression of 3HF. This arrangement should allow for C/EBPα-mCherry to overcome 3HF when (or before) the system is fully induced.

**Future Work**

Because I was unable to determine whether GFP is a suitable fluorescent tag for 3HF in DH5αZ1, future work should first involve obtaining an input-output curve for GFP (like the one obtained for yEVenus) and synthesizing a 3HF-GFP fusion that can be tested for expression and fluorescence in DH5αZ1. If that 3HF-GFP fusion appears to express and fluoresce in DH5αZ1, a complete version of the circuit using 3HF-GFP should be created and tested. If the 3HF-GFP fusion does not work, then an investigation should be conducted to determine whether 3HF is itself expressed stably in DH5αZ, since instability of 3HF in DH5αZ1 would cause poor expression, stability, and fluorescence of 3HF fusions in DH5αZ1. Such investigation could include western blotting or other protein expression assays. If 3HF is expressed stably on its own in DH5αZ1, then testing more fusions of 3HF with other fluorescent proteins or employing a different means of fluorescently tagging 3HF would be appropriate. If 3HF is not expressed stably in DH5αZ1, then the circuit should be redesigned to use a different eukaryotic repressor and corresponding inhibitor.

If a working version of the circuit is created, future work should then include efforts to evaluate the premise of this project: that the circuit can produce bistable gene expression while avoiding cross-interference and toxic squelching in *E. coli*. Whether the circuit generates toxic squelching could be determined by looking for signs of toxicity like high cell death count, poor growth, and poor gene expression, especially at the extremes of the circuit’s inducible range.
Moreover, squelching could be probed for by measuring changes in the output of constitutively expressed genes across different states of the circuit. Determining whether the circuit is free of cross-interference would be more difficult given the wide range of regulatory processes and networks that exist in *E. coli*. But most importantly, the circuit should produce bistability regardless of culture age, phase of cell cycle, and growth conditions. Whether or not that is the case could be investigated by determining whether the circuit retains an independent ability to toggle and remain stable regardless of those central regulatory processes in *E. coli*. If the circuit proves to generate bistable expression of 3HF without toxic squelching or cross-interference, the insights gained through its creation could be applied toward the creation of other bistable gene circuits in *E. coli* that use sequestration of eukaryotic repressors to couple ultrasensitivity with positive feedback in gene expression and thereby generate bistable gene expression.

**Author Contributions**

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References


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Figure 1: Diagram depicting a generic circuit scheme in which an inducible inhibitor (shaded squares) sequesters an activator (empty circles) that drives its own expression. When not sequestered by the inhibitor, the activator can induce its own expression. But when sequestered by the inhibitor into a complex (empty circle touching shaded square), the activator can no longer induce its own expression.
No induction – activator is “on”

Activator remains “on”

Induction below toggling threshold

Induction above toggling threshold

Activator turns “off”

Figure 2: Diagrams depicting (A) how sequestration of a transcriptional activator driving its own expression by an inducible inhibitor generates a toggling threshold for the expression of that activator; and (B) the ultrasensitive deactivation of activator expression that occurs across that toggling threshold. (A) The system is “on” before inducer is introduced (left) and a reservoir of activator accumulates. If inducer is introduced at a concentration below the toggling threshold (top-right), the system remains “on” because insufficient inhibitor is expressed to sequester the entire reservoir of free activator. If inducer is introduced at a concentration above the toggling threshold (bottom-right), sufficient inhibitor is expressed to sequester all activator such that none is available to drive its own expression and the system toggles “off”. (B) When a toggling threshold concentration of inhibitor is induced, sequestration of the activator causes ultrasensitive deactivation of activator expression (solid line). This ultrasensitive response contrasts with a typical Michaelian (dashed lines) deactivation response wherein the concentration of a gene product decreases gradually as the concentration of repressor increases. Adapted from Buchler & Cross 2009.
Figure 3: A steady state rate-balance plot showing how ultrasensitivity achieved through sequestration can lead to bistable gene expression in two circuit schemes: (top-left) sequestration of an activator driving its own expression by an inducible inhibitor; and (top-right) sequestration of an inducible repressor by an inhibitor that preserves its own expression. The top-left scheme corresponds to the previously made circuits described in this paper’s introduction. The top-right scheme corresponds to the proposed circuit. (Bottom-left) Ultrasensitive expression rate curves (solid lines) of X at different induced concentrations of Y (decimal values above curves) are plotted against a degradation rate curve of X (dashed line). Expression of X increases in an ultrasensitive manner as the concentration of X present in the system (x-axis) exceeds that of Y. Where the expression rate and degradation rate curves intersect, the concentration of X does not change and is therefore in a steady state. Thus, for every induced concentration of Y (excluding when not or fully induced) the system can achieve two stable steady states of X (shaded circles) and one unstable steady state (unshaded circles). When Y is not or fully expressed, the response curve for X is flat and intersects the sequestration rate line only once such that only one steady state is possible. (Bottom-right) As a result of having one unstable and two stable steady states for every degree of repressor induction (except 0 and 1), the system is bistable at those degrees of repressor induction (darker gray region). At no or full induction, the system is monostable. The range of bistability in this figure was arbitrarily selected for the sake of general demonstration.
Figure 4: Schematic of the gene circuit that I aimed to create for this project. In the circuit, C/EBPα is expressed under control of an IPTG-inducible promoter while 3HF, which sequesters free C/EBPα, is expressed under control of the C/EBPα-repressible P\textsubscript{GCAAT} promoter. Before IPTG is introduced, the circuit exists in an “on” state in which 3HF is expressed and accumulates into a titrating barrier that prevents lesser concentrations of C/EBPα from repressing expression of 3HF. But if enough IPTG is introduced to the system to drive expression of C/EBPα to a concentration greater than that of the titrating barrier of 3HF, free C/EBPα becomes available that begins to repress expression of 3HF. At that point, the system should toggle to an “off” state and IPTG can be removed to a degree without reversion to an “on” state (because less C/EBPα-mCherry is needed to repress 3HF expression when no 3HF is present to sequester C/EBPα).
Figure 5: Diagrams of the six plasmids created for this project (to give a quick visual summary of their components). Promoters are represented by triangles and genes encoding circuit components or fluorescent tags are represented by solidly colored rectangles. Origins of replication on the plasmids are represented by patterned rectangles. Labels for the components are included beneath or within the shapes representing them. Each plasmid diagram is labeled with a letter (A-F) for easy reference elsewhere in this paper.
Figure 6: Mean fluorescences of DH5αZ1 cells carrying a plasmid encoding C/EBPα-mCherry under control of the P_{lac/ara-1} or P_{LacO-1} promoter and cultured in the absence of Spectinomycin. The values reported for the DH5αZ1 control are the mean background fluorescences of untransformed DH5αZ1 cells. 95% confidence intervals are included for each mean fluorescence reported. The units of fluorescence are arbitrary.
Figure 7: Mean fluorescences of DH5αZ1 cells carrying a plasmid encoding C/EBPα-mCherry under control of the P_{LlacO-1} promoter paired with either the ColE1 ori or the 15A ori when cultured in media containing Spectinomycin. The value reported for the DH5αZ1 control is the mean background fluorescence of untransformed DH5αZ1 cells. 95% confidence intervals are included for all means except those for the DH5αZ1 control. For those means, the average confidence interval was ±0.0178. The units of fluorescence are arbitrary.
Figure 8: Bar plot showing the mean fluorescence of DH5αZ1 cells carrying plasmids encoding either yEVenus, 3HF-yEVenus, or GFP under control of the P_{GCAAT} promoter paired with the 15A ori. The value reported for the DH5αZ1 control is the mean background fluorescence of untransformed DH5αZ1 cells. 95% confidence intervals are included for all means. The units of fluorescence are arbitrary.
Figure 9: An input-output plot showing how the yellow fluorescence of DH5αZ1 cells carrying a plasmid encoding yEVenus under control of the P_{GCAAT} promoter paired with the 15A ori and another plasmid encoding C/EBPα-mCherry under control of the P_{LlacO-1} promoter paired with the ColE1 ori changes in response to increasing induction of C/EBPα-mCherry expression. The value reported for the DH5αZ1 control is the mean background fluorescence of untransformed DH5αZ1 cells. 95% confidence intervals are included for all means except that of the DH5αZ1 control. The 95% confidence interval for that mean is $\pm 0.0146$. The units of fluorescence are arbitrary.
Figure 10: A plot showing how the yellow fluorescence of DH5αZ1 cells carrying a plasmid encoding 3HF-yEVenus under control of the $P_{GCAAT}$ promoter paired with the 15A ori and another plasmid encoding C/EBPα-mCherry under control of the $P_{LlacO-1}$ promoter paired with the ColE1 ori changes in response to increasing induction of C/EBPα-mCherry expression. The value reported for the DH5αZ1 control is the mean background fluorescence of untransformed DH5αZ1 cells. 95% confidence intervals are included for all means except that of the DH5αZ1 control. The confidence interval for that mean is ± 0.0287. The units of fluorescence are arbitrary.