Design and characterization of a dual-mode promoter with activation and repression capability for tuning gene expression in yeast

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ABSTRACT

Modularity in controlling gene expression artificially is becoming an essential aspect of synthetic biology. Artificial transcriptional control of gene expression is one of the most well-developed methods for the design of novel synthetic regulatory networks. Such networks are intended to help understand natural cellular phenomena and to enable new biotechnological applications. Promoter sequence manipulation with cis-regulatory elements is a key approach to control gene expression transcriptionally. Here, we have designed a promoter that can be both activated and repressed, as a contribution to the library of synthetic biological ‘parts’. Starting with the minimal cytochrome C (minCYC) promoter in yeast, we incorporated five steroid hormone responsive elements (SHREs) and one lac operator site, respectively, upstream and downstream of the TATA box. This allows activation through the testosterone-responsive androgen receptor, and repression through the LacI repressor. Exposure to varying concentrations of testosterone (to vary activation) and IPTG (to vary repression) demonstrated the ability to tune the promoter’s output curve over a wide range. By integrating activating and repressing signals, the promoter permits a useful form of signal integration, and we are optimistic that it will serve as a component in future regulatory networks, including feedback controllers.

INTRODUCTION

The living cell is an extraordinarily complex system and one of the aims of synthetic biology is to predictively and quantitatively understand the level of complexity by integrating minimal networks into host cells (1). Creating circuits in electronics is facilitated by the availability of a wide range of relatively simple, well-characterized components. The genetic ‘circuits’ often employed in synthetic biology applications suffer from the scarcity of robust and modular biological ‘parts’ (promoters and transcription factors, for example) available (2,3). Increasing the number of available parts and characterizing the behavior of the new components is thus an essential aspect of the current effort to create more complex synthetic networks. Given the limited number of synthetic components that can be incorporated into designs without placing an unacceptable burden on the host metabolism, parts that can serve multiple functions are an attractive prospect for synthetic biology. Here, we describe the design and characterization of one such part, a single promoter capable of both activation and repression in yeast.

Most eukaryotic and prokaryotic promoters contain regulatory elements for multiple transcription factors, and are responsible for regulating biological processes. Transcription factors bind cis-regulatory DNA sequences upstream of cognate genes to repress or activate their expression by hindering or enhancing the binding of RNA polymerase (RNAP) to the promoter. There are many transcription factors that have been employed artificially to regulate gene expression (4). There are two general, non-exclusive, ways of using transcription factors to regulate gene expression: one is the use of programmable DNA-binding proteins to recognize native promoters and the other is the manipulation of promoter sequences such that they are recognized by known DNA-binding proteins (5). Examples include the use of zinc finger DNA-binding proteins and transcription activator-like effectors (TALEs) (6–9), uncovering regulatory proteins by high-throughput ‘mining’ in E. coli (10,11), and using the classic inducible lac, ara and tet (Tn10) operon elements as elements in DNA sequences targeted by natural regulatory proteins (12–15). Some of the most promising current approaches to combining programmable repression and activation in a single element are based on the CRISPR-Cas system (11,16–19). In bacteria, a single-element system combining programmable repression and activation has been reported (11). The approach em-

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employs the RNA-guided DNA-binding protein dCas9 to up- and down-regulate gene expression in *E. coli* and *S. pneumoniae*. A mutant dCas9 protein (lacking endonuclease activity) was used as a repressor, preventing RNAP binding through base pair complementarity between an RNA guide and the cognate gene sequence without requiring changes to the promoter sequence. Activation of the cognate gene was accomplished by fusing dCas9 to the omega subunit of RNAP (17). Another CRISPR-Cas-based system (16) has demonstrated the ability to use distinct guide RNAs to direct the same CRISPR-transcription factor to different positions in a yeast promoter region. The design allows for activation, repression, or combined activation and repression from a single element, and was successfully implemented in both yeast (*S. cerevisiae*), and human (HEK293T) cells (16).

Here, we pursue an approach based on the construction of a synthetic promoter sequence; control of gene expression with synthetic promoters has been a central method in synthetic biology to date (2,10,20,21). The discovery of *cis*-regulatory sequences from naturally occurring promotor elements has led to the construction of these synthetic promoters using a building block approach, which can either be carried out either systematically or in a randomized fashion (22). A promoter derived from the lipoprotein gene in *E. coli* was one of the first examples of a synthetic promoter that was used to express a number of tRNA genes (23). Subsequently, techniques were developed in bacteria that enabled mutation of promoter sequences while simultaneously placing known regulatory protein-binding sequences into positions flanking the essential -10 and -35 promoter elements; this enabled the efficient construction of synthetic promoters for use in bacteria and other prokaryotes (16). Considerable effort in synthetic biology has focused on using lac, ara and tet (Tn10) operon elements to design controllable synthetic promoters (12,13), and the approach has been successfully used in prokaryotic systems to produce promoters with predictable behavior (15,20,21,24). Here, we present a conceptually similar approach to synthetic promoter design, but in the context of the eukaryotic *Saccharomyces cerevisiae*. The structural differences between prokaryotic and eukaryotic promoters require somewhat different methods in creating synthetic promoters in this cellular context.

Combinatorial synthetic promoter design in yeast has been done to measure the noise in gene expression, where multiple tet operator sites were placed in different positions after the TATA box of a GAL1 promoter to control the expression of yeast-enhanced green fluorescent protein (yEGFP) (2). The yeast GAL1 promoter with tetO and Olac operator sites has also been used to make libraries of promoters with predictable behavior (25). The cytochrome C promoter has been augmented with activating responsive elements (REs) upstream, to control expression of lacZ and EGFP (26,27). Modification of the activation and repression properties of yeast promoters have each been investigated separately, but combined activation and repression from a single promoter has yet to be explored. Here, we report the creation of such a promoter, displaying both activation and repression capability. This kind of promoter would be a useful tool for designing artificial networks that require the ability to integrate multiple signals with opposite effects, for example as an approach for maintaining homeostatic behavior (28,29). The ability to create response curves with different profiles also suggests that the promoter will be helpful in tuning gene expression levels and responses, an issue of increasing interest in synthetic biology (15,30,31).

The paper will begin by detailing the design of our synthetic promoter: it contains five human androgen receptor REs upstream of the TATA box of the minimal cytochrome C (minCyc) promoter, and one lac operator site downstream of the TATA box. Repression occurs when the LacI repressor protein binds to its operator site and disrupts the binding of the preinitiation complex (PIC) of the transcriptional machinery to the core promoter region (32) of *S. cerevisiae*. Androgen receptor driven activation is accomplished by recruitment of transcriptional machinery to the core promoter region upon binding of the androgen receptor Res (32). Both repression and activation are chemically controllable: the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) reduces the binding affinity of the LacI protein for its target sequence, while adding testosterone facilitates the binding of the androgen receptor protein. After establishing the promoter’s design, the remainder of the paper presents an experimental characterization of its steady-state response to exposure to our two chemical inducers: using yEGFP as a reporter of gene expression from the promoter, we have characterized the activation and repression of the promoter by sweeping both IPTG and testosterone levels.

**MATERIALS AND METHODS**

**Strains, media and reagents**

The *Saccharomyces cerevisiae* strain YPH500 (α, ura3–52, lys2–801, ade2–101, trplΔ63, his3Δ200, leu2Δ1) (ATCC Cedarlane Corporation, Burlington, Canada) served as the host strain for plasmid chromosomal integrations. Genomic integrations were specifically targeted to the redundant ura3–52 locus (25). A transformation kit (Fast yeast transformation kit, G-Biosciences, St. Louis, USA) was used to transform all plasmids in yeast. The LEU2 and TRP1 selectable marker genes within the plasmids pLAREG and pRR-AR (this study) were used for selection. Synthetic drop-out media and agar plates without tryptophan and leucine (SD-TRP-LEU) were prepared according to standard protocols (33) using components (amino acids mixture, ammonium sulfate, nitrogen base, galactose and adenine hemisulfate) purchased from Sigma-Aldrich Canada (Oakville, Canada). Testosterone was purchased from Toronto Research Chemicals (Toronto, Canada). Restriction enzymes, Antarctic phosphatase, and T4 DNA ligase were purchased from New England Biolabs Canada (Whitby, Canada). PfuTurbo hotstart polymerase chain reaction (PCR) master mix was purchased from Stratagene (La Jolla, USA). Dimethyl sulfoxide (sterile biological grade), agar and IPTG were purchased from Bioshop Canada Inc. (Burlington, Canada).

**Plasmid construction and genomic integration**

The yeast integrative plasmid pRS4D1 (2) was used as a backbone to insert the modified dual-mode promoter and
enhanced green fluorescent protein, with the GAL1 promoter driving LacI expression cloned into the same plasmid. Synthetic codon optimized LacI (34) and the GAL1 promoter were amplified from plasmid pTXLX (25) (kindly provided by Tom Ellis, Imperial College London) by PCR. The resulting plasmid is referred to hereafter as pLAREG (Figure 1A). The pRR-AR plasmid (Figure 1B), a non-integrative plasmid that maintains approximately a single copy per cell (35,36), was used to express the glyceraldehyde phosphate dehydrogenase (GPD) driven androgen receptor gene. This plasmid was purchased from Addgene and modified by excising the 5RE-cyc and lacZ portions using the BpiI and AatII restriction enzymes. The minCYC with five androgen receptor sites was PCR-amplified from the pRR-AR-5Z plasmid (27) and inserted upstream of the yEGFP gene (37) using overlapping PCR (38). The yEGFP gene was PCR-amplified from the pLXGTX plasmid (25) (again kindly provided by Tom Ellis, Imperial College London). The resulting fragment was then used to insert the Olac site downstream of the minicyc promoter’s TATA box using overlapping PCR. This new construct was PCR-amplified with 5′-phosphate-added primers. The vector with GAL1 promoter expressing LacI was cut with the EagI and AleI restriction enzymes and dephosphorylated using Antarctic phosphatase. After ligation of the phosphorylated insert and the dephosphorylated vector, the plasmid was transformed into E. coli strain DH5α. All constructs were confirmed with restriction digestion as well as by sequencing. All plasmids were constructed and used to transform E. coli strain DH5α to harvest DNA for yeast transformations. The pLAREG plasmid was linearized with the StuI restriction enzyme before yeast transformation. Yeast YPH500 strain competent cells were prepared before transformation by following the protocol provided by the fast yeast transformation kit. A multiple plasmid transformation protocol was followed to transform both plasmids pLAREG and pRR-AR. Transformation was done by adding a total of 0.2–1.0 μg plasmid DNA in 10 μl of competent cells along with appropriate amount of reagents (see the protocol) provided by the fast yeast transformation kit. Cells were spread on SD-TRP-LEU agar plates after incubating at 30°C for 90 min. Growth of transformants was seen on agar plates after five days of incubation at 30°C.

**yEGFP activation, repression and dose response experiments**

Yeast cells were plated on SD-TRP-LEU plates containing 2% galactose, and single colonies were used to inoculate 10 mL SD-TRP-LEU medium, also supplemented with 2% galactose. The selected colonies were grown at 30°C with 260 RPM orbital shaking until reaching an OD600 of between 1.5 and 2.0. The inoculated cultures were spun down at 600 g for 3 min and diluted into fresh SD-TRP-LEU media for activation, repression and dose response assays. The yeast cells were diluted to an OD600 of 0.4 (26) before adding any testosterone (0 to 50 μM) or IPTG (0 to 20 mM). Stock solution of 50 mM testosterone and 1 M IPTG solution were prepared in DMSO and water, respectively. All the cultures of yeast for activation, repression and dose response contained 1% DMSO to enhance the uptake of the inducers (27). Culturing of the yeast was carried out in 24 well plates (Costar 3526, Corning, Mississauga, Canada) in a total volume of 1 mL. Cells were grown at 30°C with 330 RPM orbital shaking using the a microplate shaker (LSE digital microplate shaker, Corning, Mississauga, Canada) for 35 to 40 h and diluted in Phosphate buffered saline before fluorescent measurements were taken in 96 well optical bottom black plates (Thermo Scientific, Ottawa, Canada). A plate reader (Infinite M1000 Pro, Tecan, San Jose, CA) was used to measure both bulk yEGFP fluorescence and OD600 for each well. All fluorescence values were normalized by OD600 to provide approximate compensation for differing rates of cell growth between wells.

**RESULTS AND DISCUSSION**

Figure 1 shows the components used to implement and test the dual-mode promoter. An integrative plasmid, pLAREG (Figure 1A; LAREG standing for ‘(L)acI, androgen receptor (AR), (EG)FP’), served to insert the promoter into the yeast genome, constitutively expressing the LacI repressor under the control of the GAL1 promoter and reporting under the dual-mode promoter’s activity with yEGFP. The non-integrative plasmid pRR-AR (Figure 1B; a modified pRR plasmid, with AR standing for ‘androgen receptor’) constitutively expresses the androgen receptor (generally known as a steroid hormone receptor, SHR) required for the promoter’s activation. Plasmid pRR-AR incorporates an autonomous replicating sequence (ARS) and a centromeric DNA sequence (CEN) that ensure efficient episomal replication and segregation, respectively; the CEN element also helps to maintain a single copy of the plasmid (36), as accumulation of multiple CEN-containing plasmids may be toxic to yeast (35). The androgen receptor was expressed under the control of the GPD promoter since it is active in both glucose- and galactose-containing media. Galactose-containing media were used for all tests of combined activation and repression in the dual-mode promoter, since the repressor LacI was expressed under the control of the glucose-repressed promoter Gal1. Glucose-containing media were used for initial tests of the activation-only mode of the promoter (data not shown). The pRR-AR and pLAREG plasmids can be transformed into any trp1 strain of S. cerevisiae along with the reporter plasmid (pLAREG) to confer the ability to sense and respond to hormonal activation.

Figure 1A and C shows the schematic design of the dual-mode promoter: five response element (RE) sequences with binding affinity for the androgen receptor (AR) protein are placed upstream of the TATA box of the minimal S. cerevisiae cytochrome C promoter (minCYC), and a LacI binding sequence (Olac) is placed downstream of the TATA box. Androgen receptor binding to the response elements serves to activate the promoter, while LacI binding to its operator site serves to repress promoter activity (Figure 1C), as measured by yEGFP expression levels (below). Figure 1D presents the complete sequence of the dual-mode promoter, indicating the five androgen receptor response elements (bold, underlined), the TATA box (bold, uppercase), the Lac operator site (boxed) and the start codon for the yEGFP gene (bold, italics, uppercase).

The range of possible expression levels from the dual-mode promoter is shown in Figure 2 as a bar plot. The ad-
Figure 1. Dual-mode promoter system for *S. cerevisiae*. (A) pLAREG plasmid is an integrative plasmid which was integrated into *S. cerevisiae* genome at URA3 locus. This plasmid expresses lacl repressor from the gal1 promoter and the reporter protein EGFP from the hybrid dual-mode promoter (PR). The dual-mode promoter consists of five androgen receptor binding elements (underlined bold in panel D) upstream and one lac operator site (boxed in panel D) downstream of the TATA box (uppercase bold in panel D) of the minimal *S. cerevisiae* cytochrome C promoter. (B) pRR-AR is a yeast non-integrative plasmid that maintains stable copy number (single copy/cell), and was used to express human androgen receptor from the glyceraldehyde phosphate dehydrogenase (GPD) promoter. (C) Yeast-enhanced green fluorescent protein (yEGFP) was used as a reporter to test the dual-mode promoter’s activation in the presence of testosterone and repression in the presence of LacI. The level of LacI repression can be tuned with isopropyl-β-D-1-thiogalactopyranoside (IPTG). (D) The sequence of the dual-mode promoter (PR). The following features are highlighted: five androgen responsive elements (RE) (underlined bold), one lac operator site (boxed), TATA box (uppercase bold) and the yEGFP start site (uppercase bold italic).
Figure 2. Activation and repression of yEGFP from the dual-mode promoter. Fluorescence of yEGFP (normalized to the highest value obtained) shows that full activation (both inducers present: \( T = 10 \mu M, I = 10 \) mM; the \( +T+I \) case) was 7.6-fold higher compared to complete repression (neither inducer present, \( T = I = 0 \); the \( -T-I \) case). The presence of only one of the inducers in the \( +T-I \) (\( T = 10 \mu M, I = 0 \)) and \( -T+I \) (\( T = 0, I = 10 \) mM) cases led to much lower increases of approximately 1.6- and 1.4-fold above the \( -T-I \) level, respectively. Yeast cultures were grown overnight, then diluted to \( \text{OD}_{600} \) of 0.4, 10 \( \mu M \) testosterone and 10 mM IPTG with 1\% DMSO were then added, followed by 40 h growth after addition. The error bars indicate one standard deviation, based on three replicates.
since LacI’s binding affinity has been reduced as far as possible), with yEGFP fluorescence measurements normalized to the highest value observed. The same data are presented on semilog (Figure 3A) and linear (Figure 3B) plots, and illustrate that the dual-mode promoter’s response to testosterone saturates by a concentration of 10 μM.

Tuning the response curves of synthetic biological components is a key step in designing systems: components need to operate at matching levels if they are to be interfaced successfully, and the overall dynamics of the system are set by the shapes of the response curves of its individual elements (30,31). By varying the two chemical inducer concentrations, the dual-mode promoter offers a variety of steady-state response curves, as functions of either testosterone (Figure 4A) or IPTG (Figure 4B). For each curve, one inducer concentration was kept fixed at a series of values, while sweeping the other inducer’s concentration; the values shown are normalized to the highest fluorescence observed within each full set of curves. The continuous curves shown on Figure 4A and B are the results of non-linear least-squares fitting with a simple Michaelis–Menten function (or equivalently, a Hill function with n = 1); in almost all cases, a good fit is obtained with this simple function \((y/(K_m+x))+y\), where \(x\) represents the inducer concentration. In Figure 4A, the Michaelis–Menten constants \(K_m\) or equivalently, half maximal values) for testosterone activation with different fixed IPTG concentrations (0 mM, 0.1 mM, 1 mM, 5 mM and 10 mM) are 0.00746 μM, 0.81 μM, 0.48 μM, 0.616 μM and 0.76 μM, respectively. In Figure 4B, the \(K_m\) (half maximal) values for IPTG activation with different fixed testosterone concentrations (0 μM, 0.01 μM, 0.1 μM, 1 μM, 5 μM, 10 μM and 20 μM) are 0.0385 mM, 0.0529 mM, 0.029 mM, 0.2 mM, 0.183 mM, 0.223 mM and 0.188 mM, respectively. The shape of the curves indicates a lack of cooperativity in the promoter’s response: the responses increase smoothly to saturation, with no switch-like behavior. The half-maximal value for testosterone with fixed saturated concentration of IPTG (10 mM) was 0.76 μM, while the half-maximal value for IPTG with fixed saturated concentration of testosterone (20 μM) was 0.188 mM. The testosterone response for the minCYC promoter containing just the five hormone REs in the absence of a lac operator site has been measured (27), reporting a half-maximal value of 0.045 μM testosterone; our significantly higher value of 0.76 μM suggests that the presence of the lac operator has a significant influence on the genetic context of the promoter.

Figure 5 combines the data in Figure 4 into a three-dimensional (3D) plot showing the effect of both inducers. The 3D log plot shows that the correct combination of testosterone and IPTG concentration can lead to a wide range of promoter output levels.

The number of REs for androgen receptor binding and the proximity of the lac operator site to the TATA box of the minCYC promoter has a substantial impact on the promoter’s ability to activate or repress gene expression. Initially, the promoter was constructed with five RE sites and one lac operator site, with the lac site placed 115 bp downstream of the TATA box. This version of the promoter activated gene expression, but no repression was observed (data not shown). The lack of repression might be due to either to disruption of binding of the PIC to the core promoter region or to the open loop of the lac operator site inhibiting the binding of LacI. Another version of the promoter incorporated two REs rather than the five REs presented above, with the single lac operator site in the same position as in the 5-RE version. This version, too, displayed only activation by the androgen receptor, but no repression by LacI (data not shown). Productive future investigations could involve systematically varying the number and position of the REs and lac operator sites, to produce a family of related promoters with varying strengths of activation and repression.
Figure 4. Dose-response curve for the dual-mode promoter, sweeping concentrations of testosterone and IPTG. Each curve represents yEGFP fluorescence, normalized by the highest value obtained. The saturating value of the induction curve of each inducer can be tuned by varying the value of the other inducer. Semilog plots have been used to help distinguish the lower inducer concentrations; for convenience, the data for inducer concentrations of zero have been artificially plotted two orders of magnitude below the lowest non-zero concentration. The error bars indicate one standard deviation, based on three replicates.

Figure 5. 3D plot showing dual-mode promoter output (measured by yEGFP fluorescence, normalized by the highest value obtained) as a function of both testosterone and IPTG; this plot combines the individual curves of Figure 4 into a surface. A wide range of desired promoter output levels can be obtained by manipulating one or both of the testosterone and IPTG concentrations.
We have described an approach to modifying existing promoters to add an extra level of regulation by introducing binding sequences for a repressor protein to a promoter activated by a different transcription factor. This dual-mode behavior is a useful property for designs in synthetic biology for applications in which multiple signals must be integrated into a single output: for example, arranging for the production of a protein or other molecular species to respond positively to one stimulus and negatively to another would allow a cell-based biosensor to produce outputs more elaborate than those limited to strict activation or repression by a single input. As an element in a control network, the dual-mode promoter can play a central role in the construction of a network implementing an integral feedback control scheme; previous theoretical work (28, 29) indicates that such a controller, with the property of adapting near-perfectly to sustained perturbations, could be used as a sensory or control element to regulate the level of a target output. The design depends critically on the ability to have a promoter that can accept one activating input signal while being repressed by a second control signal. As mentioned above, the ability to tune a system’s response curve of synthetic systems is increasingly required in synthetic biology (30, 31), both to match signal levels across multiple components and to create desired dynamical behaviors. Having two ‘knobs’ to adjust allows our system to provide a range of response curves, and thus may facilitate the process of tuning its responses when integrating it into a larger network.

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