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RESEARCH ARTICLE

A combinatorial approach to synthetic transcription factor-promoter combinations for yeast strain engineering

Zain Y. Dossani^{1,2*} | Amanda Reider Apel^{1,2*} | Heather Szmidt-Middleton^{1,2} |
Nathan J. Hillson^{1,2,3,4} | Samuel Deutsch^{2,3,4} | Jay D. Keasling^{1,2,5,6} |
Aindrila Mukhopadhyay^{1,2} 

¹DOE Joint Bioenergy Institute, Emeryville, California, USA

²Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA

³DOE Joint Genome Institute, Walnut Creek, California, USA

⁴DOE Agile BioFoundry, Emeryville, California, USA

⁵Department of Chemical and Biomolecular Engineering and Department of Bioengineering, University of California, Berkeley, CA, USA

⁶Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Building 220 Kemitorvet, 2800Kgs, Lyngby, Denmark

Correspondence

Aindrila Mukhopadhyay, DOE Joint Bioenergy Institute, Emeryville, California, USA.
Email: amukhopadhyay@lbl.gov

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Abstract

Despite the need for inducible promoters in strain development efforts, the majority of engineering in *Saccharomyces cerevisiae* continues to rely on a few constitutively active or inducible promoters. Building on advances that use the modular nature of both transcription factors and promoter regions, we have built a library of hybrid promoters that are regulated by a synthetic transcription factor. The hybrid promoters consist of native *S. cerevisiae* promoters, in which the operator regions have been replaced with sequences that are recognized by the bacterial LexA DNA binding protein. Correspondingly, the synthetic transcription factor (TF) consists of the DNA binding domain of the LexA protein, fused with the human estrogen binding domain and the viral activator domain, VP16. The resulting system with a bacterial DNA binding domain avoids the transcription of native *S. cerevisiae* genes, and the hybrid promoters can be induced using estradiol, a compound with no detectable impact on *S. cerevisiae* physiology. Using combinations of one, two or three operator sequence repeats and a set of native *S. cerevisiae* promoters, we obtained a series of hybrid promoters that can be induced to different levels, using the same synthetic TF and a given estradiol. This set of promoters, in combination with our synthetic TF, has the potential to regulate numerous genes or pathways simultaneously, to multiple desired levels, in a single strain.

KEYWORDS

hybrid promoter, *Saccharomyces*, strain engineering, synthetic biology

1 | INTRODUCTION

The yeast *Saccharomyces cerevisiae* is one of the most well-understood and widely used organisms in biological research as well as in bio-production processes. In addition to natively produced ethanol, *S. cerevisiae* has been engineered to produce a wide variety of products including pharmaceuticals, fuels and industrial chemicals (Peralta-

Yahya, Zhang, del Cardayre, & Keasling, 2012). For optimizing the production of bulk chemicals, multiple traits, in addition to an optimal biosynthetic pathway, must be developed (Lechner, Brunk, & Keasling, 2016). These necessitate the use of multigenic pathways and genes that modulate other phenotypes, such as for tolerance to the final product (Mukhopadhyay, 2015), pretreatment reagents (Frederix et al., 2014) and optimal carbon uptake (Reider Apel, Ouellet, Szmidt-Middleton, Keasling, & Mukhopadhyay, 2016). Yet the number of promoters used for genetic engineering in *S. cerevisiae* has remained

*These authors contributed equally.

limited to a few dozen native promoters, either constitutive promoters or galactose-inducible promoters that are the staple of the yeast genetic engineer's toolbox (Alper, Fischer, Nevoigt, & Stephanopoulos, 2005; Lee, DeLoache, Cervantes, & Dueber, 2015; Reider Apel et al., 2017). In cases where promoter inducibility is desired, galactose induction is particularly problematic because of the limitations it places on the types of carbon sources that can be used for cultivation.

Both promoter sequences and transcription factors (TF) are modular in nature. The first effort to use this aspect of regulatory regions and DNA binding proteins was a synthetic TF for *S. cerevisiae* reported in 1993 (Louvion, Havaux-Copf, & Picard, 1993) that used the Gal4 protein as the scaffold. By simply replacing the ligand binding domain of Gal4 with a human estrogen binding domain and using the VP16 viral activators protein that recruits the RNA Pol II complex, the authors could obtain gratuitous regulation of galactose responsive genes in response to estradiol. Although promising, this system was not used in any reported metabolic engineering efforts, possibly owing to the fact that the TF binds to native Gal responsive promoters, leading to crosstalk and unwanted changes in metabolism. More recently, Mclsaac and coauthors developed a similar system, where they replaced the Gal4 DNA binding domain (DBD) with zinc-finger DBDs (Mclsaac et al., 2013). Further, using a variable number of operator sequences in the corresponding promoters, they obtained superior dynamic range for the synthetic TF and promoter combinations. Yet the system suffers from low controllability at low- to mid-range expression levels, owing to high basal activity of the zinc fingers (Mclsaac, Gibney, Chandran, Benjamin, & Botstein, 2014). Similarly, Ottoz et al. have developed a variation that uses the DBD from the bacterial TF, LexA. The authors tested several variations of the LexA-based TF where they varied the activating domain to obtain a range of highly regulated TF-promoter combinations, adjusting the output

by increasing the number of LexA-binding sites, without changing basal promoter activity (Ottoz, Rudolf, & Stelling, 2014). Another recent study achieved a similar range of expression levels without the need for externally added inducer compounds (Rantasalo et al., 2016).

Alternative strategies to enable similar regulatory control have focused primarily on modifying the promoter regions for a gene of interest, changing the inducing molecule, or using tunable CRISPR-based transcription factors (crisprTFs). Examples include Blazeck, Garg, Reed, and Alper (2012), where a large number of native yeast promoters were used as the basis for a hybrid promoter that split the promoter into intact core regions and altered upstream activation sequences, and a similar, but broader, approach that altered both the upstream regions of native promoters as well as the corresponding synthetic DBDs using zinc finger motifs (Khalil et al., 2012). In addition to galactose and estradiol regulated promoters, tetracycline-inducible and camphor-repressible versions have been created to regulate gene expression (Cuperus, Lo, Shumaker, Proctor, & Fields, 2015; Gari, Piedrafita, Aldea, & Herrero, 1997; Ikushima, Zhao, & Boeke, 2015). dCas9 has also been used as a RNA-guided scaffold to recruit different protein effectors, thereby resulting in gene modulation (Gilbert et al., 2013). Targeting crisprTFs to sequences upstream of TATA boxes resulted in gene activation that was further enhanced through addition of multiple operator sites (Farzadfard, Perli, & Lu, 2013).

In this study, we used the LexA DBD, fused with the human estrogen binding domain and the viral activator domain, VP16, as the synthetic-chimeric TF. Then, by using a combination of operator sequences in promoter scaffolds of native *S. cerevisiae* promoters, we aimed to generate a series of promoter combinations that, when coupled to the same TF, can be used to modulate gene expression to different extents using the same concentration of the inducer molecule (Figure 1). The potential of such a promoter library would be to

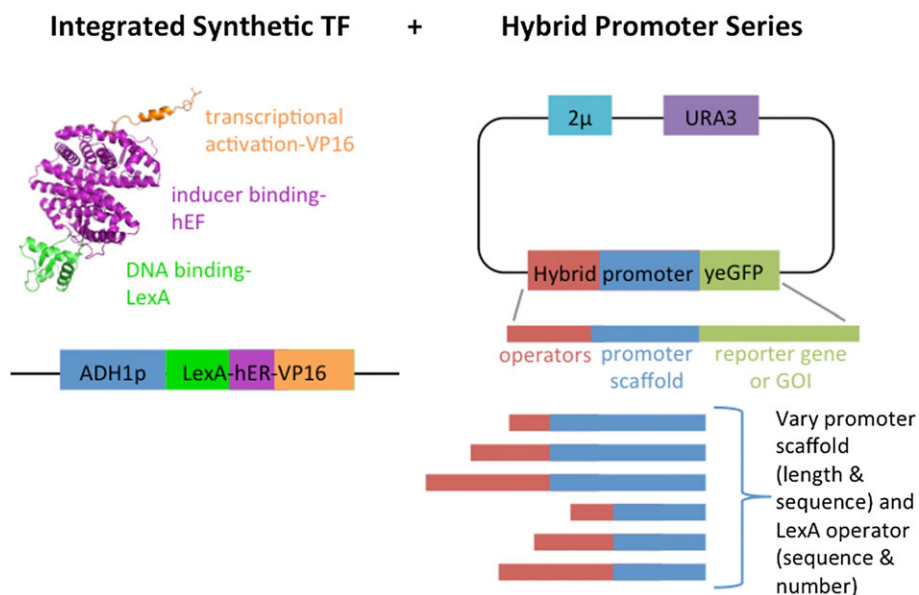


FIGURE 1 Design of modular transcription factor (TF) and hybrid promoter strains. Schematic of our two-part control system where we (left) constructed a strain (ZyD1) in which our hybrid transcription factor (*ADH1p*-LexA-hER-VP16) was integrated into locus *YPRCΔ15* for stable expression. (right) a series of hybrid promoters were constructed with promoter scaffolds of varying lengths (100 or 250 bp) and identities (*GAL1p*, *LEU2p*, *SPO13p*, *TEF1p*, *HHF2p*, *GCN4p*, *CUP1p*, *HEM13p*, *ZRT1p*, and *SSL1p*) that were paired with one to three copies of each of four different operator sequences (consensus, *uvrA*, *umuDC* and *colE1*). These 154 promoter combinations were expressed from a pRS426 plasmid and transformed into ZyD1 for analysis [Colour figure can be viewed at wileyonlinelibrary.com]

induce multiple genes to discrete desired levels, with the same synthetic TF and a small inducer molecule added to the culture medium. Using a combination of the j5 DNA assembly design software (Hillson, Rosengarten, & Keasling, 2012) and the PR-PR laboratory automation platform (Linshiz et al., 2013; Linshiz et al., 2014), both developed at the Joint BioEnergy Institute (JBEI), and the gene synthesis capability at the Joint Genome Institute, a library of 240 promoter sequences were designed, 154 constructed and tested. The profiles of these regulatory systems are described in this report.

2 | MATERIALS AND METHODS

2.1 | Strains and media

Escherichia coli and *S. cerevisiae* strains, along with their associated information (including annotated sequence files) and sequences of all plasmids constructed are provided through the JBEI public registry (Ham et al., 2012; <https://public-registry.jbei.org/folders/277>) and in Table S1 in the Supporting Information.

To produce ZDy1, the construct P_{ADH1}-LexA-hER-VP16 was integrated into *S. cerevisiae* BY4741 {MATa; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*} *S. cerevisiae* strain at locus YPRCΔ15 (Flagfeldt, Siewers, Huang, & Nielsen, 2009; Reider Apel et al., 2017). A complete description of this strain is available at <https://public-registry.jbei.org/entry/9623>. ZDy1 was transformed with promoter-reporter library plasmids using the conventional lithium acetate method (Gietz & Woods, 2002), modified for large-scale transformation.

Experiments were conducted in synthetic defined (0.67%, w/v) yeast nitrogen base without amino acids (VWR International), 0.2% (w/v) complete supplement mixture w/o yeast nitrogen base (Sunrise Science Products) or standard rich media (YP, 1% w/v Bacto yeast extract, 2% w/v Bacto peptone) with 1 or 2% w/v sugar. *E. coli* DH10b, used for cloning and plasmid amplification, were grown in LB supplemented with 100 µg/mL carbenicillin.

2.2 | Design and construction of synthetic promoters

Designs for the hybrid promoters were constructed using the j5 program (Hillson et al., 2012), by entering one, two or three operator elements followed by the basal promoters. The number of possible combinatorial variants was reduced by specifying Eugene rules (Bilitchenko et al., 2011) so that only one type of operator sequence was present in any given design. The j5 software generated sequences for 240 hybrid promoters resulting from a combination of 3 operator configurations × 4 operator sequences × 10 basal promoters × 2 promoter lengths. Sequences of all hybrid promoter parts are listed in Table S2.

For each hybrid promoter, oligomers for DNA synthesis were designed using the GeneDesign suite (Richardson, Nunley, Yarrington, Boeke, & Bader, 2010); however, for 60 promoters the resulting sequences were deemed too difficult to synthesize owing to the presence of multiple DNA synthesis constraint violations (Oberortner, Cheng, Hillson, & Deutsch, 2017) and were abandoned. For the remaining 180 promoters, ultramer oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA), and DNA

synthesis was performed using a 2-step PCR (Reisinger, Patel, & Santi, 2006) method as described in Heins et al. (2014). Assembled fragments were cloned into the pRS426-yeGFP vector by chew-back cloning and transformed into *E. coli* TOP10 cells. Plating and picking were performed using a QPix 400 system (Molecular Devices). Eight colonies per construct were sequence verified using the PACBIO RSII system (Pacific Biosciences), and variant calling was performed using the GATK software package (McKenna et al., 2010). One-hundred and fifty-four successful constructs were recovered from *E. coli* and used to transform ZDy1 strains.

2.3 | Promoter characterization

Growth and fluorescence measurements in the presence of estradiol were conducted in 96-well plates on a Synergy H4 plate reader equipped with a Bio-Stack 3 Microplate Stacker (BioTek, Winooski, VT, USA).

Selected yeast clones harbouring the promoter reporter constructs were grown overnight in 24-well plates in YNB, 1% dextrose and CSM-Ura medium. Cells were diluted into 8 mL of the same medium in six-well plates and allowed to grow until reaching an OD₆₀₀ of ~0.2–0.3. Cells were then inoculated into the final 96-well plates for promoter characterization: 100 µL of each clone was inoculated into each of 24 wells, so that six estradiol concentrations (0, 1, 5, 10, 50 and 100 nM) could be tested in technical quadruplicate. Additionally, this procedure was repeated for three biological clones of each promoter-reporter construct. Estradiol was added individually to each well at the specified concentrations just prior to the start of the experiment. Plates were covered with breathable adhesive plate seals (Thermo, NY, USA).

Prior to growth, plates were incubated at 23°C, without shaking. To measure growth each plate was cycled by shaking for 30 s to resuspend the cells and aerate the culture, and the fluorescence (excitation 485 ± 20 nm; emission 528 ± 20 nm) and OD₆₀₀ were immediately acquired. Each plate was read every 30 min, and the cycling was repeated 72 times for a total run time of ~36 h.

2.4 | Data analysis and methods to generate promoter profile plots

The plate reader data was normalized as follows: (a) the baseline fluorescence of each well was normalized by setting the average of the first five time points after the first two to zero (the first two time points often gave artificially high fluorescence readings and so were ignored); (b) the background fluorescence was measured in a nonfluorescent BY4741 wildtype strain at each time point was subtracted from the readings of each strain in the same condition to eliminate the contributions of autofluorescence to overall fluorescence readings. Analysis of variance and regression analyses were conducted using R [R Core Team (2015), R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org/>]. Data used for these plots are provided in the Supporting Information.

3 | RESULTS AND DISCUSSION

3.1 | Design of hybrid promoters and synthetic-chimeric TF

Since we wanted to generate a set of novel regulatory elements to enable the precise control of different genes and pathways using the same TF, the key variable in our study was the chimeric promoter sequences. We selected upstream sequences from 10 different native *S. cerevisiae* genes to act as basal promoter scaffolds: *GAL1p*, *LEU2p*, *SPO13p*, *TEF1p*, *HHF2p*, *GCN4p*, *CUP1p*, *HEM13p*, *ZRT1p* and *SSL1p*. Promoter scaffolds were chosen with hopes of representing both constitutive and inducible profiles at both low and high expression levels. Generally, 100 bp upstream of a gene is considered the core promoter sequence, which is the minimal stretch of DNA needed to initiate transcription, while 250 bp is thought to contain other *cis*-acting elements that are involved in transcriptional regulation (Butler & Kadonaga, 2002; Lubliner et al., 2015; Lubliner, Keren, & Segal, 2013). We selected a shorter (100 bp) and a longer (250 bp) sequence length for each of these promoters to serve as the promoter scaffold.

We replaced the upstream operator region of each promoter with one of four different known LexA operator sequences: consensus, *uvrA*, *umuDC* and *colE1* (Brent & Ptashne, 1985) using 1×, 2× or 3× repeats of these different LexA binding sequences. In total, we designed 240 plasmid-based promoter constructs driving a yeGFP that served as the reporter. All 240 promoters can be controlled by the same chromosomally integrated synthetic-chimeric TF. Our hypothesis was that these promoters would induce the gene of interest, in this case yeGFP, to different levels and have different inducibility, two important parameters for the design of biological circuits.

3.2 | Building the promoter library

Out of an initial set of 240 hybrid promoters that were designed, 60 constructs contained DNA synthesis constraint violations that precluded their synthesis and were abandoned at the design stage. DNA synthesis constraints violations are commonly encountered in synthetic biology designs and can be particularly severe when designing regulatory sequences, as they cannot be easily removed by codon shuffling. A strategy to overcome this limitation is to design experiments with sufficient redundancy such that biological

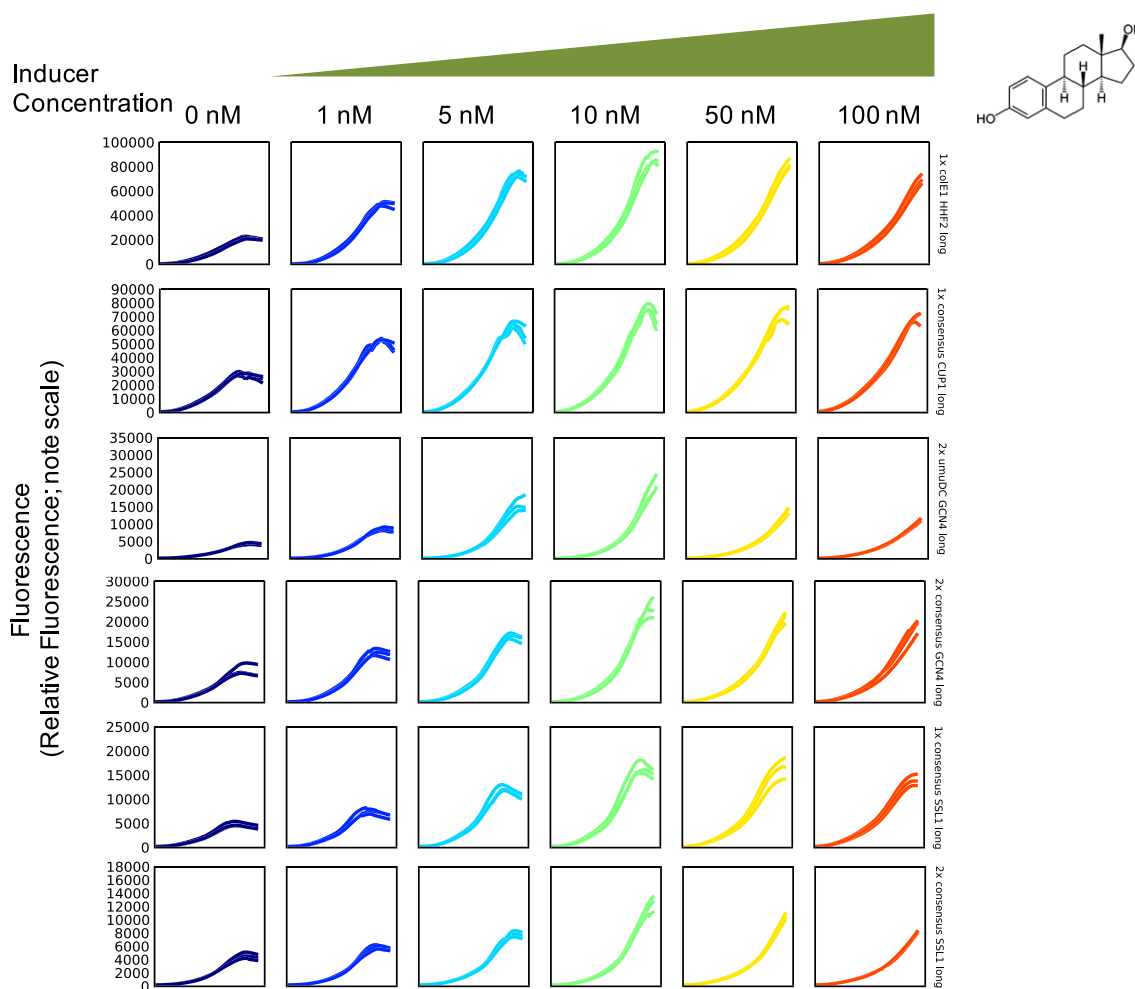


FIGURE 2 Subset of plots showing interesting hybrid promoter candidates. Fluorescence output from a subset of yeGFP expressing hybrid promoter strains (promoter names are labelled along right-hand side) induced at various estradiol concentrations (0, 1, 5, 10, 50 and 100 nM) over a 36 h time course. The plots show the range in maximum levels to which the promoters can be used for protein expression using the same level of estradiol and the range in inducibility. For the complete set of plots see Figure S2 in the Supporting Information [Colour figure can be viewed at wileyonlinelibrary.com]

insights can be obtained with a subset of the data. Out of the 180 promoters that could be synthesized, 154 were successfully constructed and cloned into the *E. coli*-*S. cerevisiae* shuttle vector (pRS426-yeGFP) in order to characterize their functional parameters. This set of 154 promoters covered most of the biological space that we wanted to capture for this experiment (Figure S1 in the Supporting Information).

3.3 | Induction and gene regulation using the promoter library

In order to easily test a large number of promoter constructs, we chose to chromosomally integrate a native ADH1 promoter driven copy of the LexA-hER-VP16 TF for stable expression. A similar, plasmid expressed, GAL4 estrogen responsive hybrid TF was previously shown to activate reporter genes exclusively in the presence of estradiol (Louvion et al., 1993). Replacement of the GAL4 DBD with the heterologous LexA prevents binding to native GAL4 promoters, thereby minimizing cross-talk.

For the promoter series, with the exception of TEF1, HHH2 and GCN4, most combinations of basal promoters in two lengths could be built with 1×, 2× or 3× repeats of the four operator sequences. The plasmids with these hybrid promoter:yeGFP cassettes were transformed into the yeast strain with the chromosomally encoded synthetic TF. Estradiol concentrations from 0 to 100 nM were tested for each variant in the promoter library and a profile was generated for each strain. The complete set of plots for all hybrid promoters is provided in the Supporting Information (Figure S2). While good inducibility was observed from 5 to 50 nM of the inducer, 10 nM estradiol was chosen as the inducer concentration that shows the maximal dynamic range for the promoter series (Figure 2).

Some studies have reported toxicity of VP16 and LexA-hER-VP16 in the presence of high levels of inducer (Gari et al., 1997; McIsaac et al., 2011). Both studies that reported toxicity did not observe growth inhibition in constructs with centromeric plasmids or chromosomally integrated versions with low levels of inducer. Consistent with this, with our experimental setup, we did not observe major toxicity at inducer concentrations of 10 nM or less, possibly owing to the use of a chromosomally integrated ADH1-driven synthetic TF in combination with a hybrid promoter containing plasmid.

The parameters of interest that we quantified for each promoter were the inducibility and response, measured in terms of the fold change in induction from 0 to 100 nM and the maximal level of induction at 10 nM, respectively. Analysis of variance was performed to statistically confirm the impact of each parameter and assess significance in the dataset (Figure 3). Overall, the promoter set with short upstream regions (100 bp) was significantly ($p < 0.001$) less responsive and did not display a wide range in either maximum fluorescence or inducibility regardless of promoter and operator sequences (Figures S1 and 3: left panels). While only 20% of yeast genes contain TATA elements, a genome-wide study of their locations revealed that some genes contain TATA boxes further upstream than 100 bp before the beginning of translation (Basehoar, Zanton, & Pugh, 2004). Therefore, it is possible that some of the short promoter scaffolds lack activity owing to the absence of TATA boxes or similar TATA protein-binding elements. For future design improvements, the lengths of the shorter promoter scaffolds could be individually customized to include functional TATA elements, which could result in better inducibility and range of induction.

Therefore, we focused our analysis on the set of promoters constructed using the longer promoter regions (250 bp). For this set, 84 total designs were constructed and tested. The main

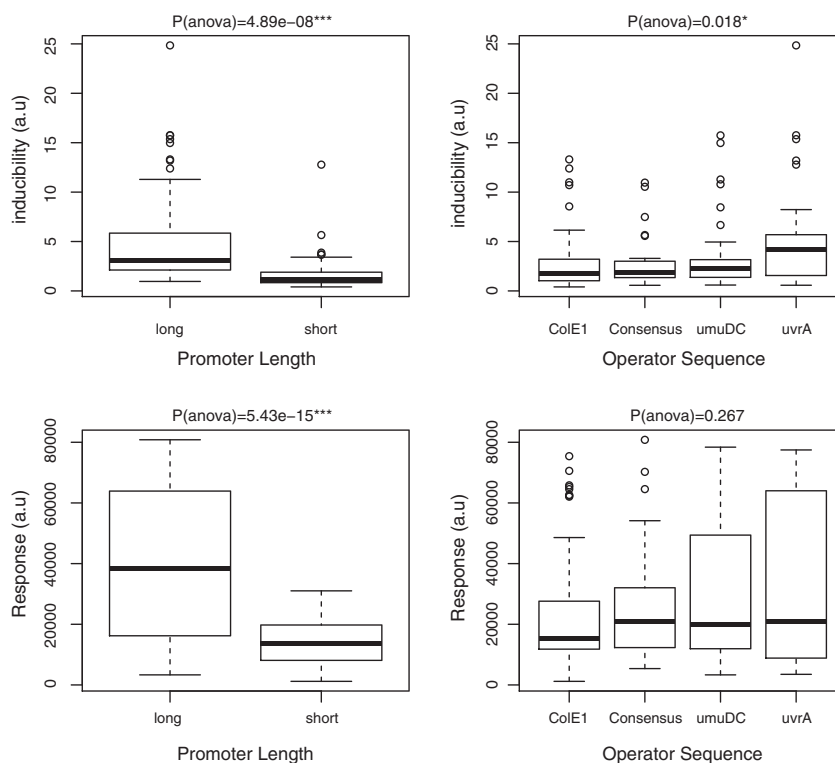


FIGURE 3 Parameters influencing promoter performance. Analysis of variance was conducted using R to establish inducibility fold (top) and maximum expression (bottom) significance for the dataset. p -value significance: * < 0.05 , *** < 0.001

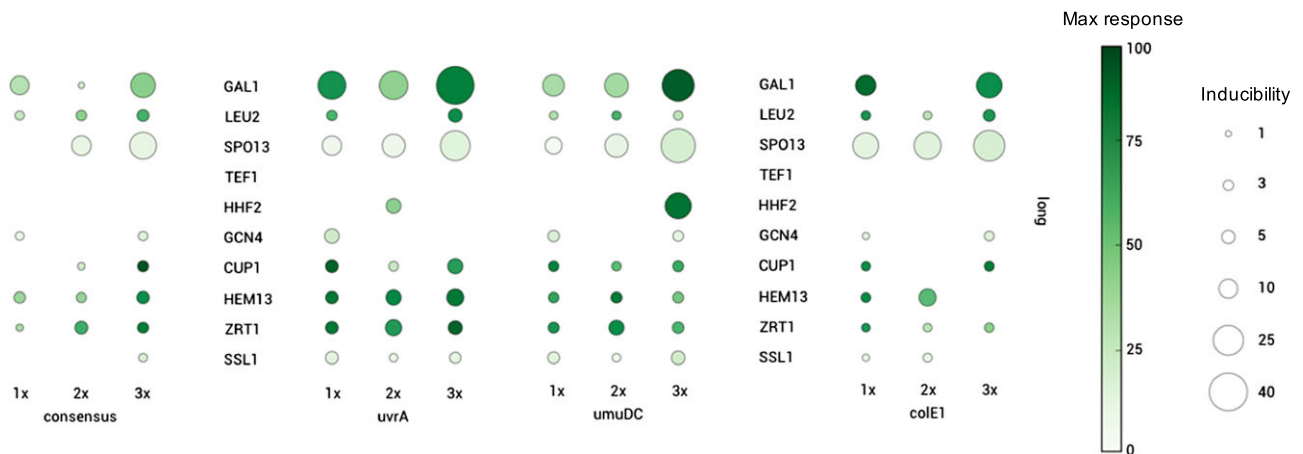


FIGURE 4 Long promoter scaffolds display a range of inducibility and responsiveness. Bubble plots showing inducibility fold (size of bubble) and maximum responsiveness level (colour of bubble) of yeGFP expression for each of the 250 bp (long) constructed hybrid promoters [Colour figure can be viewed at wileyonlinelibrary.com]

parameters, inducibility (fold change in fluorescence with the addition of estradiol) and response (maximal induction of GFP) displayed by the hybrid promoter library are summarized in Figure 4. Of the promoters tested, GAL1 and SPO13 showed the greatest inducibility. The GAL1 promoter also produced the highest fluorescence (response), with the 3× *uvrA* GAL1 construct being the highest in our entire dataset. A subset of promoters, such as HEM13 and ZRT1 with the *uvrA* and *umuDC* operators, gave very high fluorescence but at a constitutive level, that is, high response but low inducibility. Conversely, the SSL1 promoter with the *uvrA* and *umuDC* operators, and the LEU2 promoter with the *umuDC* operator, showed low fluorescence at constitutive levels, that is, both low response and low inducibility. Among the operator sequences, *uvrA* displayed significantly higher ($p < 0.05$) inducibility than the consensus and other operator sequences (Figure 3: right panels), without changing maximum expression level for each promoter scaffold. Design improvements can also be envisioned for the longer promoters in this study. Domains in the promoter regions such as the upstream activating sequence could be altered and optimized, especially in the cases where the promoters were not highly inducible. The hybrid promoters based on GAL1 and SPO3 showed the greatest inducibility, but still contained some native upstream activating sequence elements that may allow factors other than the inducer to influence the induction under a subset of growth conditions (Buckingham et al., 1990; Flick & Johnston, 1990).

4 | CONCLUSIONS

Drawing inspiration from several reported synthetic transcription factors used in yeast, we have constructed a synthetic transcription factor that consists of a chimera of a bacterial DNA binding domain, a mammalian ligand binding domain, and a viral transcriptional activation domain. The gene encoding this transcription factor was integrated into the chromosome and expressed from a constitutive promoter. The synthetic promoters were constructed from the

operators that bind the selected DNA binding domain and a native *S. cerevisiae* basal promoter that will recruit the basal transcription machinery. Transcription is activated by addition of the small molecule ligand, estradiol, which binds the mammalian ligand-binding domain. We tested the hypothesis that a series of hybrid promoters with varying promoter scaffolds and operator regions will lead to different profiles in gene expression in response to a given level of the estradiol.

The synthetic promoters tested in our study in conjunction with the synthetic TF provide a suite of control systems we set out to compile, wherein in response to the same inducer and inducer concentration, the synthetic promoters can be used to achieve different gene expression profiles such as highly inducible and highly responsive, highly inducible but lowly responsive, constitutively on and highly active as well as constitutively on and lowly activity (Figures 2 and 4). With additional characterization, these and other similar libraries allow a strain engineer to drive many genes and pathways in a cell to different levels using the same TF and same inducer added at a given level. This system has the potential to be useful for engineering heterologous enzymatic pathways in *S. cerevisiae* and for expressing multiple genes, or finding optimum levels for each gene product in a given pathway.

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CONFLICT OF INTEREST

J.D.K. has financial interests in Amyris, Lygos, Constructive Biology, Demetrix and Napigen.

ORCID

Aindrila Mukhopadhyay  <http://orcid.org/0000-0002-6513-7425>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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