

Tuning Gene Expression in *Yarrowia lipolytica* by a Hybrid Promoter Approach^{∇†}

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Received 6 June 2011/Accepted 7 September 2011

The development of strong and tunable promoter elements is necessary to enable metabolic and pathway engineering applications for any host organism. Here, we have expanded and generalized a hybrid promoter approach to produce libraries of high-expressing, tunable promoters in the nonconventional yeast *Yarrowia lipolytica*. These synthetic promoters are comprised of two modular components: the enhancer element and the core promoter element. By exploiting this basic promoter architecture, we have overcome native expression limitations and provided a strategy for both increasing the native promoter capacity and producing libraries for tunable gene expression in a cellular system with ill-defined genetic tools. In doing so, this work has created the strongest promoters ever reported for *Y. lipolytica*. Furthermore, we have characterized these promoters at the single-cell level through the use of a developed fluorescence-based assay as well as at the transcriptional and whole-cell levels. The resulting promoter libraries exhibited a range of more than 400-fold in terms of mRNA levels, and the strongest promoters in this set had 8-fold-higher fluorescence levels than those of typically used endogenous promoters. These results suggest that promoters in *Y. lipolytica* are enhancer limited and that this limitation can be partially or fully alleviated through the addition of tandem copies of upstream activation sequences (UASs). Finally, this work illustrates that tandem copies of UAS regions can serve as synthetic transcriptional amplifiers that may be generically used to increase the expression levels of promoters.

The process of developing and establishing a comprehensive suite of promoter elements in organisms with poorly defined genetic tools is essential for enabling metabolic and pathway engineering applications. To this end, the oleaginous yeast *Yarrowia lipolytica* has received attention as a potential biofuel-producing host, yet genetic tools for tunable and high-level gene expression are lacking. For other organisms, prior attempts at promoter engineering relied on modifying the expression range of endogenous promoters through point mutations. For example, error-prone PCR of the native *Saccharomyces cerevisiae* TEF promoter yielded a library of mutant promoters with a nearly 17-fold range in relative expression levels (35). A similar approach in *Escherichia coli* led to a nearly 18-fold range in relative expression levels based on the heterologous Pl-lambda promoter (1). An alternative strategy aimed at tuning intergenic regions in heterologous *Escherichia coli* operons enabled a 7-fold improvement in pathway throughput and was used to prevent the accumulation of a toxic intermediate (37). While successful, these methods and applications are quite limited by (i) their reliance on a strong, well-defined starting core promoter and (ii) the tendency of alterations to result in expression levels lower than these baselines. These limitations present a challenge for creating a dy-

namic range of promoters with similar regulations in a novel organism without highly characterized strong promoter elements.

Beyond point mutations, the generation of hybrid promoters was previously successful in significantly augmenting promoter architecture and function (11, 31, 39). Most of these approaches attempted to create synthetic hybrid promoters by fusing an upstream activation sequence (UAS) to a separate (often minimal) core promoter region. The result is a functional UAS-core promoter chimera. In this construct, the UAS regulatory site enhances gene expression by localizing *trans*-acting regulatory elements (transcription factors). As a result, this approach raises the possibility of uniquely engineering promoters as two independent, synthetic parts: activating regions and core regions. In this work, we present a generalizable approach to hybrid promoter engineering for the construction of both very-high-strength promoters and tunable promoter libraries in the nonconventional, oleaginous yeast host *Y. lipolytica*.

Y. lipolytica is a unique host for biochemical production and heterologous protein excretion on account of its abilities to accumulate high levels of lipids (4–6), utilize hydrophobic and waste carbon sources (2, 3, 15), and secrete native and heterologous proteins at high levels (12, 27, 32). The availability of *Y. lipolytica*'s genome sequence (13, 42) along with basic genetic tools such as transformation methods (8, 10, 17), gene knockouts (16), and both episomal (17, 29, 45, 46) and integrative (21, 24, 44) expression cassettes enable metabolic engineering approaches. However, many of the methods using this organism rely on ill-defined genetic elements (3), especially in the area of promoters. One of the strongest promoters in *Y. lipolytica*, the XPR2 promoter (pXPR2), has complex

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 16 September 2011.

TABLE 1. List of promoter elements used in this study^a

| Promoter element | Open reading frame regulated | YALI no. | bp range | Reference |
|------------------|--|--------------|--------------|------------|
| EXP1 | Export protein | YALI0C12034p | −999 to −1 | 9 |
| GPAT | Glycerol-3-phosphate dehydrogenase | YALI0C00209p | −1130 to −1 | 9 |
| GPD | Glyceraldehyde-3-phosphate dehydrogenase | YALI0C06369p | −931 to −1 | 9 |
| TEF | Translation elongation factor EF-1α | YALI0C09141p | −406 to −1 | 9 |
| YAT1 | Ammonium transporter | YALI0E27203p | −775 to −1 | 9 |
| FBA | Fructose-bisphosphate aldolase | YALI0E26004p | −830 to +171 | 9 |
| XPR2 | Alkaline extracellular protease | YALI0E26719p | −947 to −1 | 28 |
| TEF(136) | Translation elongation factor EF-1α | YALI0C09141p | −136 to −1 | This study |
| TEF(203) | Translation elongation factor EF-1α | YALI0C09141p | −203 to −1 | This study |
| TEF(272) | Translation elongation factor EF-1α | YALI0C09141p | −272 to −1 | This study |
| TEF(504) | Translation elongation factor EF-1α | YALI0C09141p | −504 to −1 | This study |
| TEF(604) | Translation elongation factor EF-1α | YALI0C09141p | −604 to −1 | This study |
| TEF(804) | Translation elongation factor EF-1α | YALI0C09141p | −804 to −1 | This study |
| TEF(1004) | Translation elongation factor EF-1α | YALI0C09141p | −1004 to −1 | This study |
| Leum | β-Isopropylmalate dehydrogenase | YALI0C00407p | −92 to +25 | 28 |
| UAS1B | Alkaline extracellular protease | YALI0E26719p | −805 to −701 | 28 |

^a The elements used in this study are listed with their names, open reading frames regulated, YALI accession numbers, and base pair ranges.

requirements for induction that hinder its industrial applications (28). Nevertheless, this promoter has been functionally analyzed to reveal a 105-bp distal UAS fragment named UAS1B (7, 26). Previously, between one and four tandem UAS1B copies were fused to a core minimal LEU2 promoter to create four increasingly strong hybrid chimera promoters, named hp1d to hp4d (28). As a result, the hp4d promoter has become a commonly used tool for heterologous protein expression in *Y. lipolytica*. A further reanalysis of these four promoters revealed a linear increase of promoter strength as a function of the number of tandem UAS1B elements, which raises the possibility of further improvements.

Specifically, we report here the development of a generic, hybrid promoter approach for creating synthetic, tunable promoters. Furthermore, these results demonstrate that the activating regions and core regions can act as independent synthetic parts to create a hybrid promoter. In particular, we constructed and characterized two libraries of synthetic hybrid promoters: a UAS1B-Leum library, in which between 1 and 32 UAS1B elements are fused to the minimal LEU2 promoter region, and a UAS1B_{8/16}-TEF library, in which 8 or 16 tandem UAS1B elements are fused to TEF promoter regions of various sizes. In doing so, this work creates the strongest characterized promoters in *Y. lipolytica* and the first-ever reported capacity for tunable gene expression in this system. Moreover, this work establishes synthetic hybrid promoter construction as a generic approach for promoter engineering to expand and enhance the strength of engineered promoters in a manner not accessible through traditional promoter engineering applications.

MATERIALS AND METHODS

Strains and media. *E. coli* strain DH10β was used for all cloning and plasmid propagations. DH10β cells were grown at 37°C with constant shaking in Luria-Bertani (LB) broth (Teknova) supplemented with 50 μg/ml ampicillin for plasmid propagation. *Yarrowia lipolytica* strain PO1f (ATCC MYA-2613), a leucine and uracil auxotroph devoid of any secreted protease activity (28), was used for all studies. *Y. lipolytica* PO1f containing plasmids was routinely cultivated at 30°C with constant agitation in yeast synthetic complete (YSC)-LEU medium, consisting of 20 g/liter glucose (purchased from Fisher Scientific), 0.69 g/liter complete amino acid supplement mixture (CSM)-leucine supplement (purchased from MP Biomedicals), and 6.7 g/liter yeast nitrogen base (purchased from

Becton Dickinson and Company). Solid medium for *E. coli* and *Yarrowia lipolytica* was prepared by adding 15 g/liter agar (Teknova) to liquid medium.

Cloning procedures. All restriction enzymes were purchased from New England BioLabs, and all digestions were performed according to standard protocols. PCRs were set up under the recommended conditions by using Phusion high-fidelity DNA polymerase (Finnzymes). Ligation reactions were performed overnight at 16°C by using T4 DNA ligase (Fermentas). Gel extractions were performed by using the GeneClean gel extraction kit, purchased from MP Biomedicals. Purifications of small DNA fragments (<200 bp) generated during plasmid construction were performed by using the MERmaid Spin kit (Qbiogene). *E. coli* minipreps were performed by using the Zippy plasmid miniprep kit (Zymo Research Corporation). *Y. lipolytica* minipreps were performed by using a Zymoprep yeast plasmid miniprep II kit (Zymo Research Corporation). The transformation of *E. coli* strains was performed by using standard electroporation protocols (40). The transformation of *Y. lipolytica* was performed by using Zymogen Frozen EZ yeast transformation kit II (Zymo Research Corporation). Genomic DNA (gDNA) was extracted from *Y. lipolytica* by using the Wizard genomic DNA purification kit (Promega).

Calculation of the codon adaptation index. Codon adaption indices were calculated for the humanized *Renilla* green fluorescent protein (hrGFP), mStrawberry, enhanced green fluorescent protein (EGFP), and yECitrine (1) genes by using the CAIcal server (38) and the codon usage table for *Y. lipolytica* available in the Codon Usage Database (34).

Plasmid construction. (i) Construction of endogenous promoter fluorescence cassettes. Primer sequences can be found in the supplemental material. All plasmids employed for gene expression in *Y. lipolytica* were centromeric, replicative vectors based on plasmid pSI16-Cen1-1(227) (48), which was initially modified to include a new multicloning site and redubbed pMCS-Cen1. The cycl terminator (cycl1t) was amplified from p416-TEF-yECitrine (1, 33) and inserted into pMCS-Cen1 with an EcoNI/BspI digestion to form pMCS-cycl1t. Endogenous promoters defined previously (9), EXP1 (primer pair JB096/JB097), GPAT (JB094/JB095), GPD (JB088/JB089), TEF (JB104/JB105), YAT1 (JB090/JB091), XPR2 (JB275/JB276), and XPR2fus (JB277/JB276) (Table 1), were amplified from *Yarrowia lipolytica* PO1f genomic DNA and ligated into pMCS-cycl1t by using XmaI/AscI for FBA and BstBI/AscI for the rest of the promoters to form pMCS-promoter serial constructs. Reporter genes, including yECitrine (1) (primer pair JB083/JB084), mStrawberry (pmstrawberry; Clontech) (JB153/JB155), EGFP (41) (JB156/JB158), hrGFP (pIRES-hrGFP; Agilent) (JB160/JB161), and lacZ (23) (JB312/JB313), were amplified by using the indicated primer pairs and inserted into appropriate pMCS-promoter constructs to form different pMCS-promoter-reporter constructs. Additionally, hrGFP and lacZ were inserted into pMCS-cycl1t to form plasmids pMCS-hrGFP and pMCS-lacZ (Fig. 1a).

(ii) Constructions of UAS1B₁-Leum through UAS1B₃₂-Leum expression cassettes. Primer pair JB162/JB163 amplified a 140-bp minimal leucine promoter, Leum (Table 1), from plasmid pMCS-Cen1 that was inserted into pUC19 with SphI/HindIII to form pUC-Leum. Additionally, Leum was amplified by using primer pair LQ19/LQ20 and inserted into pMCS-hrGFP with BstBI/AscI to form plasmid pLeum-hrGFP (Fig. 1b).

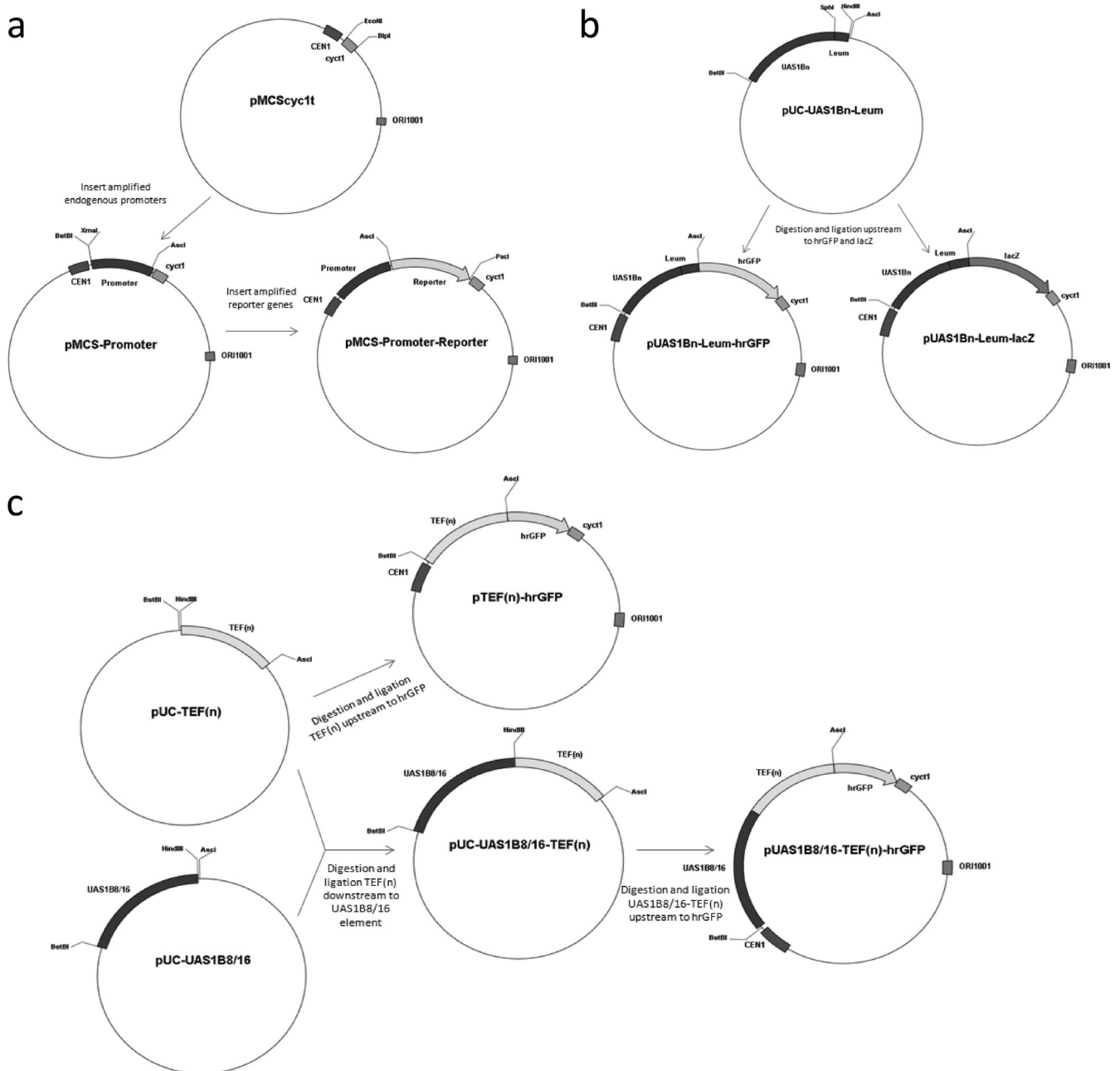


FIG. 1. Construction of plasmids for this study. A simplified schematic picture is provided, detailing the construction of endogenous promoter fluorescence cassettes (a), the construction of UAS1B₁-Leum to UAS1B₃₂-Leum expression cassettes (b), and the construction of TEF-based promoters and expression cassettes. Precise details on the construction of these plasmids are provided in the supplemental material.

The template for UAS1B was created by annealing primer pair JB177/JB178. Primer pair JB164/JB165 amplified a UAS1B oligonucleotide that was inserted into pUC-Leum with **SacI**-HF/**SphI**-HF to form pUC-UAS1B₁-Leum-No5'3'. Additionally, primer pair JB174/JB165 amplified a UAS1B oligonucleotide that was inserted into pUC-Leum with **SacI**/**SphI** to form pUChp1dins. pUC-1dins contained only **EcoRI** and **SacI** sites 5' of the UAS1B to allow for the future insertion of four tandem UAS1B sequences.

Primer pair JB167/JB168 amplified a UAS1B oligonucleotide for **BamHI**-HF/**Sall**-HF insertion into pUC-UAS1B₁-Leum-No5'3' to form pUC-UAS1B₂-Leum-No5'3', while primers JB174 and JB168 were used to create plasmid pUC-2dins from pUC-UAS1B₁-Leum-No5'3'. Primer pair JB169/JB170 amplified a third UAS1B for **XbaI**/**BamHI**-HF insertion into pUC-UAS1B₂-Leum-No5'3' to create pUC-UAS1B₃-Leum-No5'3', while primers JB174 and JB170

were used to create plasmid pUC-3dins from pUC-UAS1B₂-Leum-No5'3'. Primer pair JB171/JB172 amplified a fourth UAS1B for **XbaI**/**SacI**-HF insertion into pUC-UAS1B₃-Leum-No5'3' to create pUC-UAS1B₄-Leum-No5'3', while primers JB173 and JB172 were used to form pUC-4d5'ins from pUC-UAS1B₃-Leum-No5'3'. pUC-4d5'ins was edited with a **Sall**/**SphI**-mediated UAS1B replacement (from primer pair JB163/JB166) to form pUC-4dins.

Plasmid pUC-4dins was digested with **EcoRI**/**SacI** to extract a 444-bp fragment containing four sequential UAS1Bs that was ligated into digests of pUC-1dins, pUC-2dins, pUC-3dins, and pUC-UAS1B₄-Leum-No5'3' to form plasmids pUC-UAS1B₅-Leum-No5'3' to pUC-UAS1B₈-Leum-No5'3'. An **Ascl** restriction enzyme site was added 3' of Leum in these eight plasmids with primer pair JB251/JB252, and a **BstBI**-**PstI**-**KpnI** sequence was added 5' of the **EcoRI** site of the **Ascl**-altered plasmids by using primer pair JB249/JB250 to form plasmids

pUC-UAS1B₁-Leum to pUC-UAS1B₈-Leum. pUC-UAS1B₈-Leum-No5'3' was modified to include a 5' (of EcoRI) PstI site and a 3' (of Leum) KpnI site using primer pairs JB253/JB249 and JB254/JB252, respectively, to create plasmid pUC-8dins. pUC-8dins was PstI/KpnI digested to extract a 902-bp fragment containing eight UAS1Bs that was inserted into pUC-UAS1B₁-Leum to pUC-UAS1B₈-Leum to create plasmids pUC-UAS1B₉-Leum to pUC-UAS1B₁₆-Leum.

Plasmid pUC16dblank was created by annealing together primer pair JB289/JB290 and inserting this 74-bp oligonucleotide into pUC19 with NdeI/HindIII. Plasmid pUC-UAS1B₈-leum was digested with KpnI and then SphI-HF, and an 895-bp fragment containing "KpnI-EcoRI-UAS1B₈-SphI" was extracted and inserted into pUC16dblank to form pUC16d8dins. pUC-UAS1B₈-leum was digested with EcoRI-HF, and a 901-bp UAS1B₈ fragment was extracted and inserted into pUC16d8ins to create pUC16dins. A 1,808-bp UAS1B₁₆ fragment was BstBI/PstI digested and gel extracted from pUC16dins and inserted into vectors pUC-UAS1B₁-Leum to pUC-UAS1B₁₆-Leum to form plasmids pUC-UAS1B₁₇-Leum to pUC-UAS1B₃₂-Leum.

UAS1B_n-Leum promoter elements were cut out by using BstBI/AscI and inserted 5' of the hrGFP and lacZ reporter genes in pMCS-hrGFP or pMCS-lacZ constructs in which the hrGFP and lacZ genes lacked their native ATG start sites.

(iii) **Construction of TEF-based promoters and expression cassettes.** The 1,004-bp region upstream and including the TEF promoter was amplified from PO1f gDNA by using primers LQ13 and JB105 and inserted into a pMCS-HrGFP expression cassette in which the hrGFP gene included its native ATG start site using BstBI/AscI to form plasmid pMCS-TEF(1004)-HrGFP. The promoters TEF(804) (primer LQ12), TEF(604) (LQ10), TEF(504) (LQ9), TEF(272) (LQ16), TEF(203) (LQ15), and TEF(136) (LQ14) were amplified from plasmid pMCS-TEF(1004)-HrGFP by using the indicated primer and JB105. These seven promoters replaced TEF(1004) in pMCS-TEF(1004)-HrGFP to form the pMCS-TEF(n)-HrGFP core TEF promoter series (Table 1 and Fig. 1c).

Plasmid pUC19-8d was formed by the insertion of a BstBI/HindIII-digested, gel-extracted UAS1B₈ fragment from pUC16d8dins in place of UAS1B₁₆-Leum in the digested pUC-UAS1B₁₆-Leum vector. Plasmid pUC19-16d was formed by the insertion of a BstBI/HindIII-digested, gel-extracted UAS1B₁₆ sequence from pUC16dins in place of UAS1B₁₆-Leum in the digested pUC-UAS1B₁₆-Leum vector. The TEF series of promoters was reamplified by using primer pairs LQ29/LQ17 for TEF(1004), LQ28/LQ17 for TEF(804), LQ26/LQ17 for TEF(604), LQ25/LQ17 for TEF(504), LQ18/LQ17 for TEF, LQ32/LQ17 for TEF(272), LQ31/LQ17 for TEF(203), and LQ30/LQ17 for TEF(136) and inserted into plasmids pUC19-16d and pUC19-8d by using a HindIII/AscI digest to form pUC-UAS1B_{8/16}-TEF(n) vectors. UAS1B_{8/16}-TEF(n) promoters were cut out with BstBI/AscI and inserted in place of TEF(136) in the pMCS-TEF(136)-hrGFP vector to form pMCS-UAS1B_{8/16}-TEF(n)-hrGFP vectors.

Promoter characterization with flow cytometry. *Y. lipolytica* PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stocks (in biological duplicates or triplicates) in YSC-LEU medium for 48 h at 30°C with shaking and then normalized to an optical density at 600 nm (OD₆₀₀) of 0.03 in 2 ml fresh YSC-LEU medium and incubated for another 48 h at 30°C in a rotary drum (CT-7; New Brunswick Scientific) at speed 7. A time course of fluorescence values showed 48 h to be an optimal incubation time for high expression levels from native and hybrid promoters (data not shown). For harvesting, the cultures were spun down at 500 × g for 5 min, washed in cold water, and resuspended in 5 ml ice-cold water before testing with a FACSCalibur instrument (BD Biosciences), using a 488-nm excitation wavelength, an FL1 detector, and a 10,000-cell count for hrGFP detection. Standard, optimized protocols were used for other reporter proteins tested in this study. The samples were kept on ice during the test, and the data were analyzed by using FlowJo software (Tree Star Inc., Ashland, OR) to compute mean fluorescence values.

Promoter characterization through β-galactosidase assays. *Y. lipolytica* PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stocks (in biological triplicates) in YSC-LEU medium for 48 h at 30°C in a rotary drum (CT-7; New Brunswick Scientific) at speed 7 and then normalized to an OD₆₀₀ of 0.03 in 2 ml fresh YSC-LEU medium and incubated for another 48 h under the same conditions. The cultures were washed twice in 1 ml Z buffer and resuspended in 1 ml of Z buffer, and their OD₆₀₀ readings were recorded (18, 30). β-Galactosidase assays were performed as described previously by Miller (30), using 10 μl of chloroform-permeabilized cells with a reaction time of 17 min.

Promoter characterization through quantitative reverse transcription-PCR (qRT-PCR). *Y. lipolytica* PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stocks (in biological triplicates) in YSC-LEU

medium for 48 h at 30°C with shaking, normalized to an OD₆₀₀ of 0.03 in 2 ml fresh YSC-LEU medium, and incubated for another 48 h at 30°C in a rotary drum (CT-7; New Brunswick Scientific) at speed 7. The cells were pelleted, and total RNA was extracted by using the RiboPure-Yeast kit (Ambion). One thousand nanograms of RNA from each sample was used for a reverse transcription reaction with the High-Capacity cDNA reverse transcription kit (Applied Biosystems). A 1.2-μl sample from each reaction mixture was used to set up quantitative PCRs (qPCRs) (in triplicate) with FastStart SYBR green master mix (Roche) using primers 5'-TCAGCGACTTCTTCATCCAGAGCTTC-3' and 5'-ACACGAACATCTCCTCGATCAGGTTG-3', as described in the manual, with a nontemplate control. The reactions were run with an Applied Biosystems 7900HT Fast real-time PCR system using Fast 96-well plates (Applied Biosystems). The data were analyzed with ABI 7900HT sequence detection systems (version 2.4; Applied Biosystems).

Plasmid stability test. *Y. lipolytica* PO1f strains carrying plasmid pUAS1B₁₂-Leum-hrGFP or pUAS1B₁₆-Leum-hrGFP were grown for 48 h from glycerol stocks and thereafter subcultured in fresh YSC-LEU medium at an OD₆₀₀ of 0.01 every 48 h. After a total continuous culture time of 192 h, corresponding to 36 cell doublings, yeast cells were miniprepred to extract the plasmid. Individual plasmids were isolated by transformation into *E. coli* cells, and sequencing and restriction enzyme digests of isolated plasmids were used to confirm the stability of the UAS1B₁₂-Leum and UAS1B₁₆-Leum promoters over this time frame.

RESULTS

Characterization of endogenous promoters at the single-cell level. Previous studies of promoter strength in *Y. lipolytica* have relied on assaying whole cultures for protein expression levels (using reporters such as β-galactosidase) (28, 32). It is commonly known that these methods can mask potential bimodal "on/off" distributions within the population. Thus, to avoid this complication, we sought to utilize a fluorescence-based assay using *Y. lipolytica* plasmid pSI16-Cen1-1(227) (48). All results generated in this study, except where indicated, employed derivatives of this replicative, origin of replication-centromere (ORI-CEN)-based plasmid. Since codon biases are known to limit translation in *Y. lipolytica* (19), and no fluorescent reporter protein has been previously used with *Y. lipolytica* to gauge promoter strength, we initially evaluated several available fluorescent reporter proteins. The TEF promoter was coupled to four different fluorescence genes, yECitrine, EGFP, hrGFP, and mStrawberry, and flow cytometry was performed to determine reporter functionality. Of these variants, only hrGFP imparted detectable fluorescence (Fig. 2a). This gene, optimized for expression in mammalian cells, has the highest codon adaptation index for *Y. lipolytica* of the four fluorescence genes (20), indicating the closest compatibility with codon usage frequencies for this organism.

As a result, the hrGFP reporter gene was used to evaluate the promoter strengths of seven previously identified endogenous *Y. lipolytica* promoters: TEF, EXP, FBA, GPAT, GPD, YAT, and XPR2 (Table 1 and Fig. 2b) (9, 32, 36). Based on this analysis, the relative ordering of promoters strengths is EXP > TEF > GPD > GPAT > YAT > XPR2 > FBA. The low mean fluorescence values of even the strongest of these native promoters, EXP and TEF, highlight that the levels of even strong endogenous promoters in *Y. lipolytica* may be too low for metabolic engineering purposes. When each of these promoters was used in a plasmid-based construct, a bimodal fluorescence distribution was seen. Based on previous reports, to further improve the expression level, a consensus four-nucleotide "CACA" sequence 5' of the +1 ATG codon was included (14, 19, 43, 47); however, this inclusion was found to be detrimental to expression levels in most cases (data not

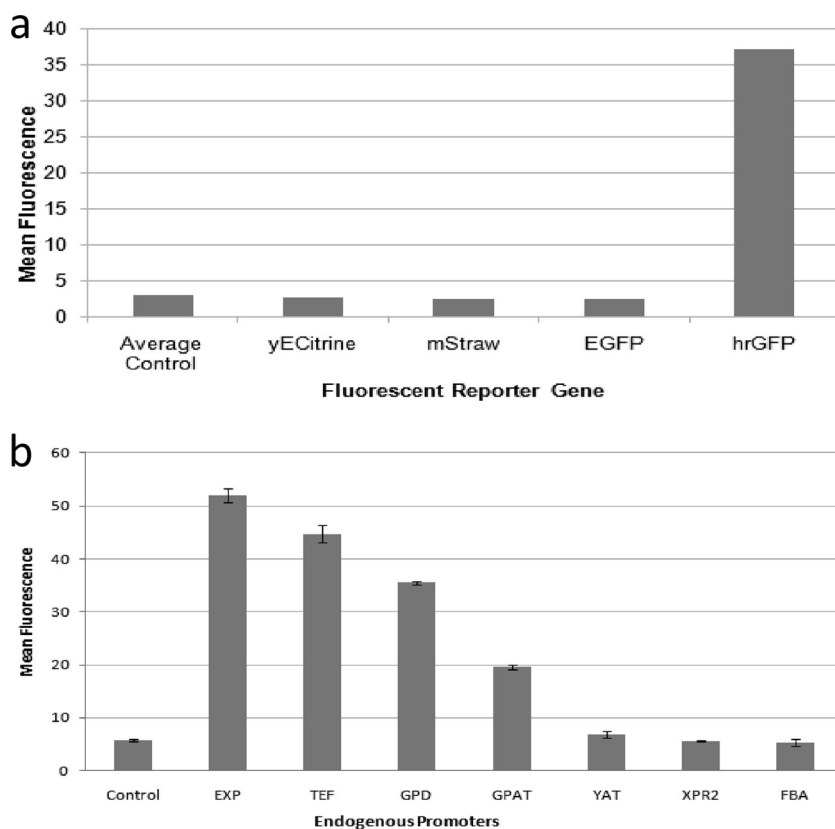


FIG. 2. Development of a fluorescence-based assay for endogenous promoter characterization. (a) The relative fluorescence levels of yE-Citrine, mStrawberry, EGFP, and hrGFP driven by the TEF promoter in *Yarrowia lipolytica* were tested by flow cytometry, and mean fluorescences from different fluorescent proteins are presented for comparison. These results indicate that hrGFP was the only functional fluorescent protein for this system. (b) Endogenous promoters, including EXP, TEF, GPD, GPAT, YAT, XPR2, and FBA, were used to drive the expression of hrGFP. The mean fluorescence data were collected by flow cytometry analysis for comparison at a time point of 48 h in minimal medium. Error bars represent standard deviations from biological replicates. The control for these experiments was *Y. lipolytica* strain PO1f transformed with blank, replicative plasmid pMCSCen1. The EXP and TEF promoters were identified as the strongest among this tested set.

shown). The differential regulation patterns and small dynamic range of these endogenous promoters require a novel approach to enable metabolic engineering applications using this organism.

Creation and characterization of a hybrid promoter series using the UAS1B element and minimal leucine core promoter. To bypass the limitations of endogenous promoters in *Y. lipolytica*, we evaluated the generalizable nature of hybrid promoters comprised of upstream activation sequences fused with a minimal core promoter. In previous work, a nearly perfect positive linear correlation was detected between the numbers of tandem UAS1B sequences and promoter outputs in the promoter series of hp1d to hp4d (28). This observation led to our hypothesis that these minimal core promoters and potentially other *Y. lipolytica* full-length, native promoters are enhancer limited. To address this limitation, we created an expanded series of hybrid promoters by fusing between 1 and 32 tandem UAS1B enhancer sequences to the leucine minimal promoter (Leum) to form the promoters UAS1B₁-Leum to UAS1B₃₂-Leum (Fig. 3a). The newly constructed UAS1B-Leum promoter series was tested by hrGFP-based flow cytometry analyses. The fluorescence data displayed several domains for correlation between output fluorescence and the number of

UAS elements. Initially, an exponential increase in fluorescence was seen as the UAS1B sequence count increased from 1 to 8. This trend became linear through 19 tandem repeats (a total of 1,995 bp of upstream activation sequences upstream of the core promoter). Finally, the output fluorescence seemed to be saturated through 32 tandem UAS1B repeats (Fig. 3b). These data strongly conformed to a Hill cooperative binding model (correlation coefficient of 0.95) and exhibited a high Hill constant value (3.889), which indicates a strong amount of binding cooperativity of the enhancer elements (Fig. 3b). Specifically, these data were fit to the following equation:

$$\text{mean FL} = \min \text{ mean FL} + (\max \text{ mean FL} - \min \text{ mean FL}) \times a \times \frac{\text{no. of UAS}^{\text{Hill coefficient}}}{c^{\text{Hill coefficient}} + \text{no. of UAS}^{\text{Hill coefficient}}} \quad (1)$$

where FL is fluorescence, resulting in an a value of 0.794, a Hill coefficient of 3.889, and a c value of 10.146. As with prior tests using endogenous promoters, these plasmid-based expression cassettes showed a bimodal distribution of fluorescence levels that was independent of the promoter being evaluated. However, when these cassettes were inte-

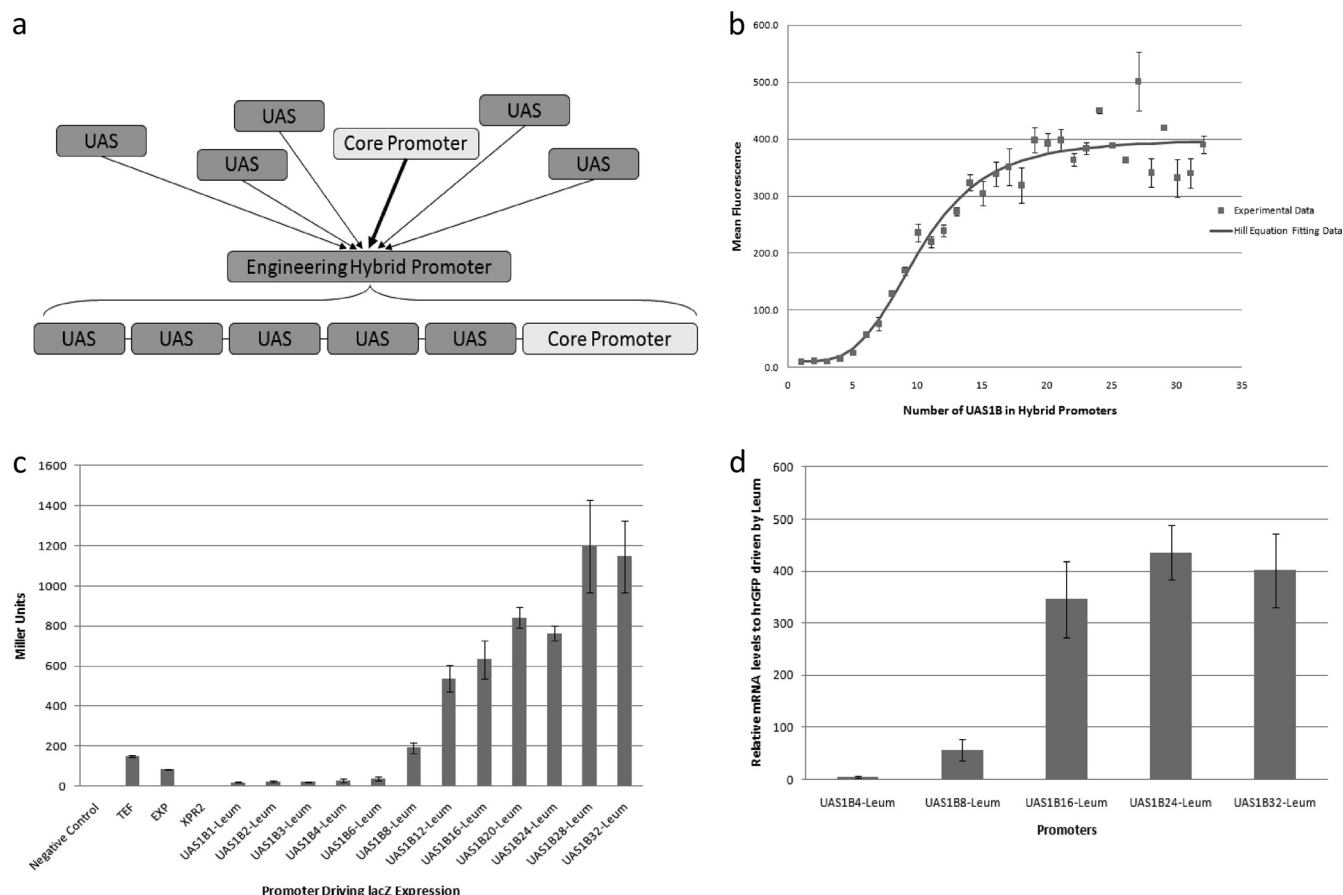


FIG. 3. Development and characterization of a UAS1B-Leum hybrid promoter set. (a) Schematic diagram for UAS-enabled hybrid promoter engineering illustrating that these promoters were created from two distinct parts: the core promoter and upstream activation sequence (UAS) elements. By inserting tandem copies of UASs upstream of the core promoter, fine-tuned promoter sets with the highest expression levels seen for *Y. lipolytica* were created. (b) The tandem insertion of 1 to 32 copies of UAS1B sequences upstream of a minimal LEU promoter enabled the construction of the UAS1B₁-Leum to UAS1B₃₂-Leum promoters. Error bars represent standard deviations from biological triplicates. The relative promoter strengths of UAS1B-Leum promoters were fit to the Hill equation (see equation 1), resulting in an *a* value of 0.794, a Hill coefficient of 3.889, a *c* value of 10.146, and an *r*² value of 0.9495 using Polymath (Willimantic, CT) software. (c) Transcriptional profiling of select promoter constructs was used to calculate mRNA levels relative to those of the minimal Leum promoter. Expression profiles matched fluorescence data. Error bars represent standard deviations from biological triplicates. (d) Endogenous and hybrid promoters were tested with a β -galactosidase reporter gene, yielding results similar to those of the hrGFP assay. Error bars represent standard deviations from biological triplicates.

grated into the genome, these promoters gave a singular, high expression peak, which indicates that the bimodal nature was conceivably due to an episomal expression issue related to this organism. These integrative expression cassettes generated roughly 2-fold-higher fluorescence values than their corresponding replicative-based cassettes, and the lack of a bimodal distribution was seen for all promoters tested and was not dependent on the UAS1B copy number (data not shown).

Transcriptional analysis of the UAS1B-Leum hybrid promoter series. A transcriptional analysis was performed to confirm that the observed effect on fluorescence was indeed manifested at the transcriptional level. To do so, qRT-PCR analysis was employed by using the hrGFP mRNAs of select promoter constructs (Leum, UAS1B₄-Leum, UAS1B₈-Leum, UAS1B₁₆-Leum, UAS1B₂₄-Leum, and UAS1B₃₂-Leum) (Fig. 3c). Expression values were normalized to the mRNA level seen with only the minimal leucine promoter used to drive hrGFP. In-

deed, the increase in mean fluorescence levels was strongly correlated with the increase in relative mRNA levels. The relative mRNA levels increased and likewise plateaued for constructs with a high number of UAS1B repeats. Moreover, these results demonstrate an extraordinary range of promoter strengths in this series, with more than a 400-fold dynamic range of transcript levels between the minimal promoter and the strongest promoters in this set.

Utility and stability of the UAS1B-Leum hybrid promoter series. To ensure that the observed effect was independent of the reporter gene, we sought to provide a further characterization of the UAS1B-Leum promoter series with a separate reporter gene, the β -galactosidase gene, carried by *E. coli lacZ*. Select promoter constructs (including the endogenous TEF, EXP, and XPR2 promoters as well as hybrid UAS1B-Leum constructs containing 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 28, and 32 UAS1B copies) were used to construct expression cassettes with *lacZ* in place of hrGFP. β -Galactosidase assays were per-

formed as described previously (18, 30), with a maximum value of 1,198 Miller units generated by the UAS1B₂₈-Leum construct (Fig. 3d). The promoter strength increased with increasing UAS1B copy numbers up through 32 UAS1B repeats, unlike the hrGFP assay. However, the β -galactosidase assay results matched well with the hrGFP analysis, and the data showed a strong positive statistical correlation ($r^2 = 0.85$). These results demonstrate that the UAS1B-Leum hybrid promoter series developed here is a generic tool for obtaining tuned gene expression in *Y. lipolytica*.

These hybrid promoters rely on a high number of tandem repeats; thus, genetic stability was evaluated. To accomplish this, selected promoter constructs (with 12 or 16 repeated UAS1B elements) were tested on the basis of sequence fidelity after nonselective serial subculturing. These strains were subcultured for a total of 36 generations. After this process, cells were harvested, and plasmids were isolated and sequenced to assess gene construct stability. In total, 20 separate plasmids carrying the UAS1B₁₂-Leum promoter and 20 plasmids carrying the UAS1B₁₆-Leum promoter were evaluated. Seventeen out of 20 UAS1B₁₂-Leum and 20 out of 20 UAS1B₁₆-Leum constructs were positively confirmed by sequence and restriction enzyme digestion after 36 doublings. Only 3 out of 20 UAS1B₁₂-Leum constructs were unstable through 36 generations, as they were truncated down to UAS1B₃-Leum. Thus, these promoters are suitably stable in *Y. lipolytica* for long-term expression and use. Collectively, these data suggest that the expression output from hybrid promoters can be altered by changing the number of activating regions with a given core promoter region. Next, we sought to address the ability to alter the core promote region of this construct.

Generalizing the hybrid promoter approach by switching the core promoter region. As discussed above, a hybrid promoter has two potential independent elements: activating regions and the core promoter region. The data described above suggest that tandem UAS elements may serve as movable, synthetic expression amplifiers for a given promoter. Next, we sought to test the hypothesis that even native promoters in *Y. lipolytica* are enhancer limited and can be strengthened by the addition of additional UAS elements. To do so, we constructed new hybrid promoters containing either 8 tandem UAS1B sequences (UAS1B₈) or 16 tandem UAS1B sequences (UAS1B₁₆) inserted 5' upstream of a series of different TEF-based core promoters. Specifically, we amplified eight different regions of the TEF promoter spanning 136 bp and 1,004 bp upstream of the ATG starting site from PO1f (28) genomic DNA (Table 1). Included in this set are the consensus 404-bp TEF promoter for *Y. lipolytica* as well as lengthened and truncated versions of this promoter. These eight core TEF promoters and their corresponding UAS1B₈ and UAS1B₁₆ hybrid promoters were tested and compared with the Leum, UAS1B₈-Leum, and UAS1B₁₆-Leum constructs.

This new series of hybrid promoters was assayed via hrGFP fluorescence by flow cytometry. In the absence of UAS elements, it can be seen that the fluorescence value decreases for truncated promoters below the consensus TEF size, as expected. Moreover, the full-length (and larger) TEF promoters have more strength than the minimal leucine promoter (Fig. 4a). The UAS1B₈ and UAS1B₁₆ enhancer fragments in isolation do not confer any promoter activity (data not shown).

When these enhancer fragments were fused with the TEF promoter elements, a substantial increase in the net promoter strength was seen regardless of the TEF variant utilized. The enhancement provided by UAS1B₈ was roughly half the value obtained by using UAS1B₁₆. Moreover, these enhancements were seen for both more minimal and full-length TEF promoter elements, even with the existence of naturally occurring UAS elements in the consensus and longer TEF promoters. Thus, these data suggest that even strong endogenous promoters like TEF are enhancer limited in *Y. lipolytica*, and their expression capacity can be increased through additional UAS elements.

The amplification of expression imparted by the UAS1B₈ and UAS1B₁₆ enhancer elements was not even for all promoters. The fold increase of the constructs relative to the UAS-free TEF core promoters is plotted in Fig. 4b. In general, the greatest improvement was obtained when the UAS elements were placed most closely 5' of a core promoter region. This fold improvement trend is rationalized in terms of a mechanism of proximity and localization of enhancer elements. However, it is interesting that the total promoter size (with 16 activating sequences and a core region) totals upwards of 3 kbp for many of these promoters, a region that is quite large for typical yeast constructs. In addition, many of these larger constructs were the best-performing promoters. Specifically, nearly all of these promoters proved to be stronger than the corresponding UAS1B₈-Leum and UAS1B₁₆-Leum promoters, demonstrating the fitness of the TEF-based core promoter regions for strong hybrid promoter engineering. In this regard, this work demonstrates that tandem UAS elements serve to synthetically amplify the expression level imparted by the core promoter element chosen.

DISCUSSION

This study establishes a synthetic approach for tuning gene expression by using a hybrid promoter approach. Moreover, this approach is unique in its capacity to upregulate the expression of the baseline starting promoter, unlike most traditional approaches that typically generate promoters of decreased expression levels. In doing so, this work created the strongest known promoters in the oleaginous yeast *Yarrowia lipolytica* and allows for fine-tuned gene expression in this organism. The general strategy developed in this study employing tandem UAS repeat elements could potentially be applied to other organisms and further generalized by using other UAS regions. Finally, these results have the biological implication that the expression capacity for promoters (at least in *Y. lipolytica*) is enhancer limited. In this regard, this approach expands the quantity and quality of parts available for systems biology research (25, 49).

Using the hrGFP-enabled single-cell analysis, this work observed bimodal fluorescence distributions for all the data collected by using plasmid-based expression. These results strongly suggest an "on/off" switch mechanism resulting from the low-copy-number ORI-CEN-based plasmid used in the study. It is interesting that this bimodal distribution was absent when an integrated expression cassette was used instead. As a result, this work highlights an important consideration for using plasmids in a *Y. lipolytica* system. Nevertheless, the hrGFP

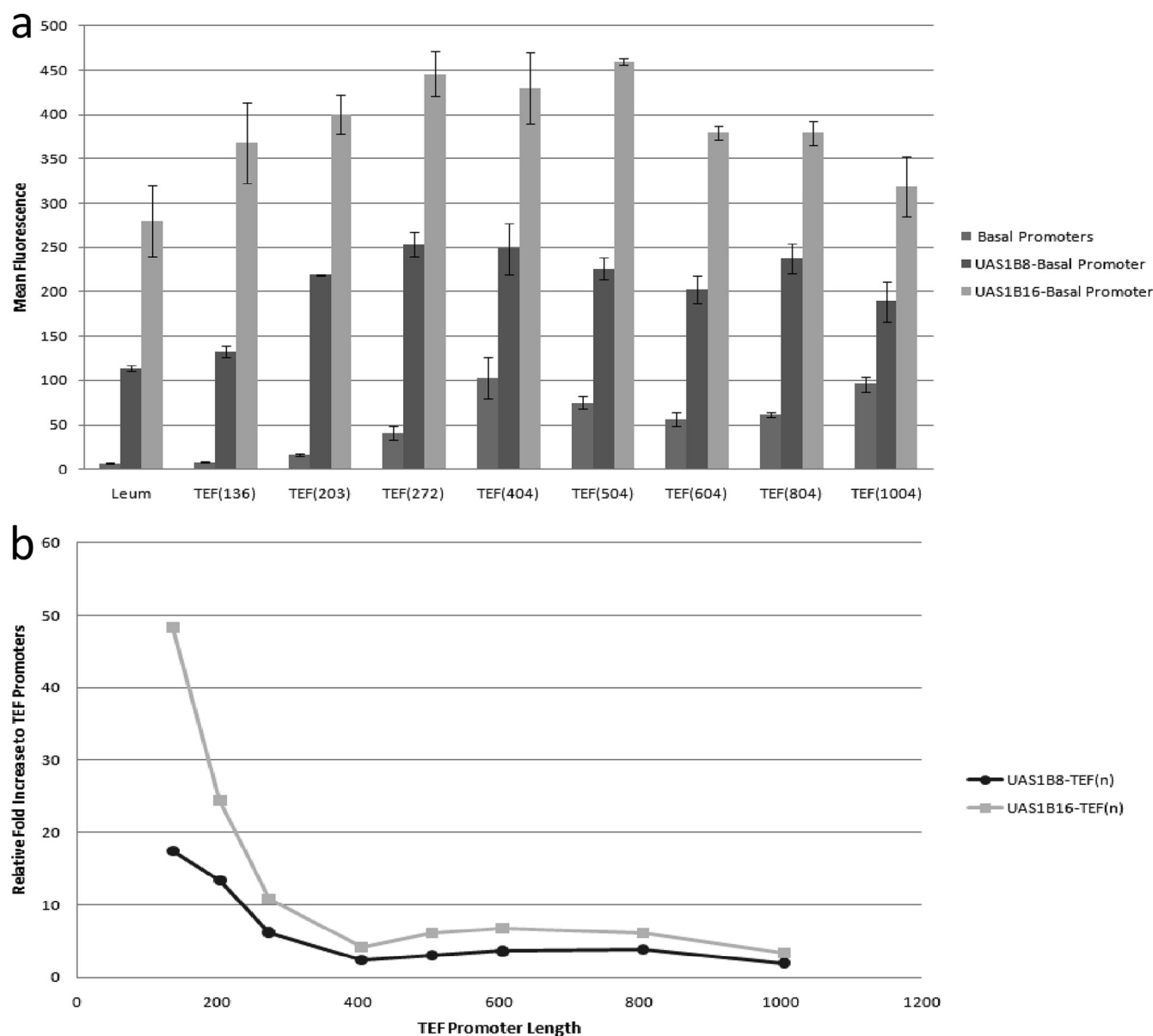


FIG. 4. Expanding the hybrid promoter approach by altering the core promoter element. (a) Characterization of relative promoter strengths for the core TEF promoters, UAS1B₈-TEF promoters, and UAS1B₁₆-TEF promoters using flow cytometry. These data were compared with data for the UAS1B₈-Leum and UAS1B₁₆-Leum promoters. (b) Tuning ability of UAS1B₈ and UAS1B₁₆ decreases as a function of core promoter length.

analysis allowed the characterization of endogenous *Y. lipolytica* promoters and identified EXP and TEF as strong promoters among the set tested. Furthermore, this analysis led to the creation of several series of promoter elements capable of high-level expression.

The magnitude of these hybrid promoters can be seen by the relative mRNA level range of more than 400-fold between the core promoter (Leum) and the maximum of UAS1B₂₄-Leum. The strongest UAS1B-Leum hybrid promoter exhibited a more-than-8-fold increase in promoter strength in terms of Miller units compared to the strong endogenous promoters tested in this study. It should be noted that the levels of ex-

pression of the UAS1B₄-Leum construct reported here were substantially lower than those previously reported. These comparisons are difficult due to the slight differences in the restriction sites used to create these hybrid promoters, the use of rich media in previous reports, and the use of replicative plasmids here compared with the integrated plasmids used in other studies. Even with these differences and discrepancies, this work still presents up to a 4-fold increase in performance compared with the best reported endogenous promoters or previously constructed hybrid promoters (22, 28). This illustrates that multiple tandem repeats of the UAS1B enhancer element activate transcription to levels far higher than those

previously described and that this enhancer activation can occur at regions more than 2,000 nucleotides upstream of the start codon (for UAS1B_{16–32}-Leum).

The UAS1B₈ and UAS1B₁₆ elements were shown to behave as synthetic amplifiers when tested with various TEF-based promoters. In this regard, we demonstrated that the ability of a UAS1B element to amplify expression is independent of the core promoter element. However, the magnitude of this amplification was dependent on the core promoter used. Thus, the choice of both the tandem enhancer element and the core promoter element contributes to the collective strength of hybrid promoters. This observation raises the possibility of rationally designing hybrid promoters with a specified expression strength. The drastic increase in expression levels with both the UAS1B₈ and UAS1B₁₆ elements across this series demonstrates that these genetic elements are portable, modular components that can generically alleviate native enhancer limitation without disrupting endogenous regulation. The modularity of the UAS1B insert and the strength of the UAS1B-Leum and UAS1B-TEF series advocate for the use of hybrid promoter engineering as a generic approach to building stronger, fine-tuned promoter libraries with interchangeable, modular components.

Collectively, these results give credence to the theory that *Y. lipolytica* promoters are enhancer limited, and we have shown that this limitation can be effectively overcome through hybrid promoter engineering. Tandem UAS elements help bypass the enhancer-limited nature of promoters by serving as transcriptional amplifiers. Cells containing the strong hybrid promoters developed in this study did not exhibit any growth defects. Therefore, transcription factor availability does not seem to be limiting for these cells, as these promoters did not seem to deplete or starve cells of transcription factors. In this regard, transcription factor binding is posited to serve as a major rate-limiting step for transcription at promoter sites, with the addition of upstream activation sites alleviating this limitation. Thus, it is possible for us to control transcriptional activity by indirectly modulating transcription factor localization or affinity through the choice of enhancer and core elements in a hybrid promoter.

The generic approach of hybrid promoter engineering described here is an important, generic synthetic biology tool enabling the construction of high-level and fine-tuned promoters with interchangeable promoter parts. This approach is one of the first to rationally amplify the expression output of a given promoter element. By utilizing this approach, we have expanded the metabolic engineering toolbox in *Y. lipolytica* and developed several novel promoter series: UAS1B_{1–32}-Leum, UAS1B₈-TEF, and UAS1B₁₆-TEF. Heterologous protein expression requires strong promoters to obtain high protein expression levels, while metabolic pathway engineering necessitates strictly controlled, fine-tuned promoters set to optimize pathways. The generic hybrid promoter approach described here accomplished both of these tasks. Moreover, these results demonstrate that the expression from *Y. lipolytica* native promoters is enhancer limited, which may be a generic phenomenon across other yeast organisms. Finally, given the results of this work, it is possible to conceive of novel combinations of upstream activation sequences and various promoter elements

to achieve both fine-tuned expression and controlled regulation.

ACKNOWLEDGMENTS

Portions of this work were funded by a DuPont Young Professor Grant.

We thank Masayoshi Matsuoka for the generous gift of plasmid pSI16-Cen1-1(227). We also acknowledge the helpful discussion and technical support of Amanda Lanza to this work.

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