

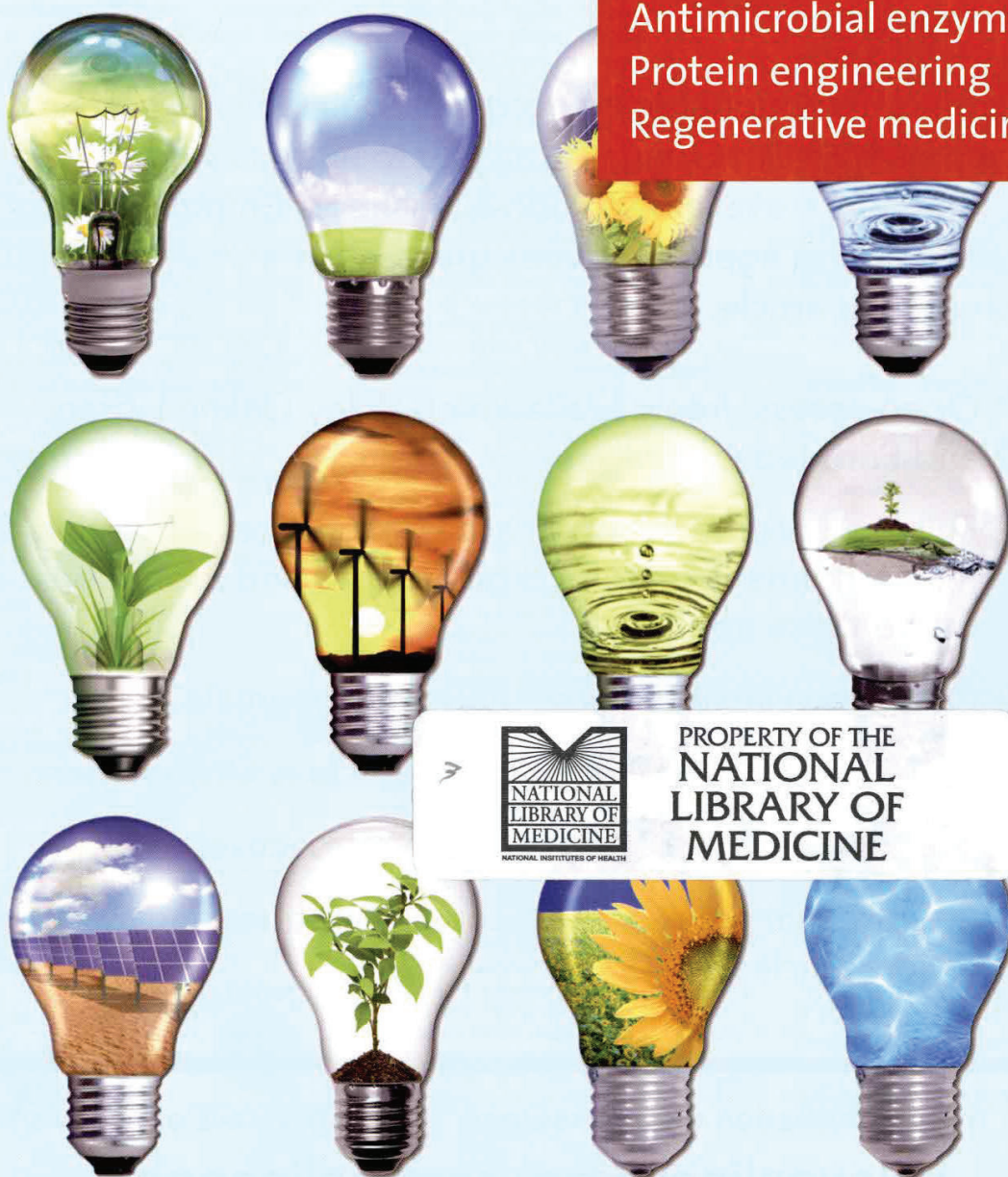
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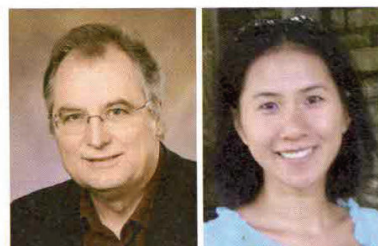
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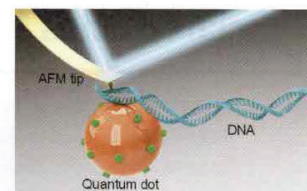
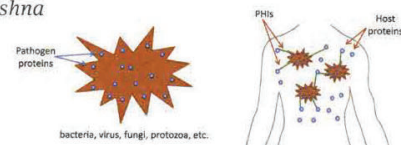
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Review

Promoter engineering: Recent advances in controlling transcription at the most fundamental level

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Synthetic control of gene expression is critical for metabolic engineering efforts. Specifically, precise control of key pathway enzymes (heterologous or native) can help maximize product formation. The fundamental level of transcriptional control takes place at promoter elements that drive gene expression. Endogenous promoters are limited in that they do not fully sample the complete continuum of transcriptional control, and do not maximize the transcription levels achievable within an organism. To address this issue, several attempts at promoter engineering have shown great promise both in expanding the cell-wide transcriptional capacity of an organism and in enabling tunable levels of gene expression. Thus, this review highlights the recent advances and approaches for altering gene expression control at the promoter level. Furthermore, we propose that recent advances in the understanding of transcription factors and their DNA-binding sites will enable rational and predictive control of gene expression.

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1 Introduction

Intracellular metabolic flux is regulated by a series of distinct, yet interwoven, levels of regulatory control – occurring at the transcriptional, translational, and protein levels [1]. One of the fundamental access points to alter this metabolic flux is to control transcript production at the promoter level. Hence, metabolic engineering applications have long relied on effective promoter discovery and characterization. Specifically, a wide array of expression capacities is required since the optimal expression level is likely gene specific and can vary by several orders of magnitude. While identifying an existing promoter represents a path forward, the field of promoter engineering attempts to modulate promoter transcriptional capacity by mutating, enhancing, or otherwise altering promoter

DNA sequence. In doing so, promoter engineering can help generate the dynamic range necessary to enable fine-tuned gene expression for metabolic engineering applications.

Simple promoter-gene cassettes have been an essential component of the metabolic engineering paradigm since the field was first described [2], and since that time promoters have become focal points as enabling “parts” for synthetic biology applications [3]. Promoters can either be isolated from endogenous sequences within the host organism or isolated from a virus that infects the host. Viral or phage-derived heterologous promoters can generate unregulated levels of transcription that are too high for many of the fine-tuned controls required in metabolic engineering applications. Thus, a number of endogenous promoters are typically employed as a set to enable a range of gene expression. Endogenous promoter isolation and utilization is limited by a variety of difficulties, e.g. (i) promoter isolation and characterization can be tedious and genetic-context specific, (ii) isolated promoters only sample the continuum of gene expression at a few

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Abbreviations: bp, base pair; Ep-PCR, error-prone polymerase chain reaction; PIC, pre-initiation complex; TFBS, transcription factor-binding site; TSS, transcription start site; UAS, upstream activation sequence

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discrete points and may be plagued by disparate regulation patterns, and (iii) isolated endogenous promoters are unable to maximize the true transcriptional capacity attainable within the host. While high-strength heterologous viral promoters alleviate this last concern in prokaryotes and metazoans, there is no such recourse in yeast. The field of promoter engineering has sought to overcome all of these above listed difficulties, and allow efficient optimization of pathway flux for metabolic engineering uses. Other methods for fine-tuning gene and/or protein expression at the translational level have been successful at producing large ranges in gene expression, including design of synthetic ribosome-binding sites [4] and directed RNase III cleavage of mRNA transcripts [5, 6], but we do not discuss these here as they have been well-covered in other reviews.

The purpose of this review is to provide an overview of promoter engineering techniques and recent successes, especially in the area of designing promoters for metabolic engineering applications. This review focuses predominantly on pertinent advances in the two most-commonly utilized model organisms, *Saccharomyces cerevisiae* and *Escherichia coli*. First, we introduce promoter architecture and function and give examples of traditional promoter utilization in metabolic engineering. We then discuss recent advances in the field of promoter engineering and novel approaches to alter gene expression control at the promoter level. Finally, we highlight recent advances in the understanding of transcription factors and their DNA-binding sites, and propose how this

knowledge sets the stage for de novo promoter design that could enable tunable gene expression in any system.

2 Overview of promoter structure and function

A promoter can be defined as any sequence of DNA that can independently facilitate the binding of transcription factors and enable transcription initiation. Such interactions between promoter DNA and transcription factors aid in the recruitment of the cellular machinery necessary for transcription of an open reading frame [7–9]. Consensus *E. coli* promoter structure includes a –35 “TTGACA” motif and a –10 “TATAAT” motif, relative to a +1 transcription start site (TSS) (Fig. 1A). These motifs are separated and surrounded by nucleotide spacer regions in which little or no nucleotide homology has been deduced; however, the nucleotide spacer region between the –35 and –10 motifs has a consensus length of 17 base pairs (bp) [10, 11]. In prokaryotes, the σ factor of the RNA polymerase is sufficient for promoter recognition and transcription initiation [12]. The α subunit of RNA polymerase can also recognize UP element DNA, a very rare A+T-rich upstream region of promoter consensus regions that can increase basal promoter transcription 1.5- to 90-fold [13] (Fig. 1A).

Eukaryotic transcription initiation is far more complex, requiring DNA sequence-specific transcription factors to bind within a promoter element and interact with transcriptional coactivators that help localize the basic

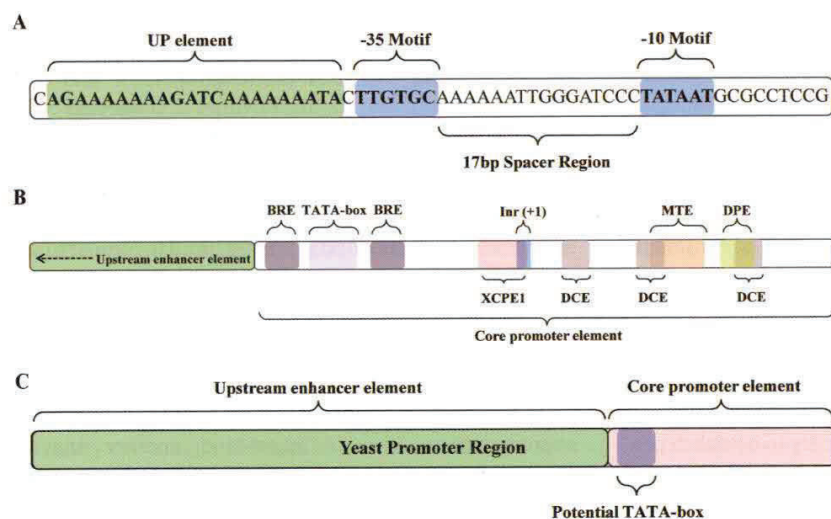


Figure 1. Consensus promoter motifs and architecture for prokaryotic, yeast, and metazoan promoters. (A) A diagram of the *rmD P1 E. coli* promoter modified from [13], illustrating prokaryotic promoter structure, including the very rare UP element. ‘Consensus’ –35 “TTGACA” and –10 “TATAAT” motifs are present and separated by a 17-bp nucleotide spacer region. (B) A diagram of the ‘consensus’ metazoan promoter, modified from [20], illustrating potential conserved elements including the TATA box, Inr (initiator), BREs (transcription factor for RNA polymerase IIB recognition element), MTE (motif ten element), DCE (downstream core element), DPE (downstream core promoter element), and XCPE1 (X core promoter element 1). (C) Understanding of yeast promoter structure is lacking for defined consensus motifs, although modularity has been demonstrated between the enhancer and core elements.

transcriptional machinery [14]. For the most common form of RNA polymerase (Pol II), up to 30 protein-based elements comprising the five general transcription factors are required to assemble RNA Polymerase II into the pre-initiation complex (PIC) at the core promoter element [15, 16]. Transcription initiation is then triggered as promoter DNA is unwound around the PIC. The PIC undergoes a conformation change into the open complex [17], scans for a suitable TSS, and initiates active elongation [15, 18].

Routinely, eukaryotic promoters are thought to contain two distinct regions: (i) a core element and (ii) an upstream enhancer element. Transcriptional direction and start site are determined by the core promoter element, and the upstream enhancer element helps determine transcriptional frequency, or promoter strength. The core promoter is the minimal promoter region required to initiate transcription [14–16], and is typically a succinct stretch of less than 80 nucleotides, extending roughly 35 bp upstream or downstream from the +1 site [16, 19]. Metazoan core promoter structure has been recently reviewed [19, 20] and may contain a variety of distinct DNA motifs that modulate core promoter activity (Fig. 1B). Metazoan core elements are typically not conserved in *S. cerevisiae* or *E. coli*. In *S. cerevisiae*, only ~20% of genes contain a TATA box, and little is known about other potential consensus motifs (Fig. 1C) [21]. In TATA-box-containing promoters in *S. cerevisiae*, RNA polymerase II initiates transcription at a site 45–120 nucleotides downstream from the TATA element [22]. Zhang et al. [23] aligned the flanking sequences of 4637 TSSs to identify the consensus $A(A_{rich})_5NPYA(A/T)NN(A_{rich})_6$ pattern, confirming and expanding the previously reported $PyA(A/T)Pu$ sequence [22, 24]. Yeast promoter structure, promoter function, and transcriptional regulation have also recently been thoroughly reviewed [14]. As the core promoter's function is to enable basal transcription, a suitable TSS and the capacity to recruit PIC components are equally essential.

Eukaryotic upstream enhancer elements (similar to prokaryotic UP elements) localize trans-acting regulatory elements (transcription factors) as a means of controlling transcriptional frequency or imparting regulation to a core promoter. Within the enhancer element, concise and specific DNA sequences serve as transcription factor-binding sites (TFBSs) or "docking points" for transcriptional activators or repressors [25–30]. Promoter-bound transcription factors interact with one another locally and with the basal transcriptional machinery to establish promoter regulation and promoter strength, or frequency of PIC formation and subsequent transcription initiation [9, 31, 32]. DNA regions prone to increase transcriptional frequency of a core promoter are commonly referred to as upstream activation sequences (UAS). Similarly, an upstream repressive sequence localizes transcription factors that reduce transcription rate [14]. Promoter regulation (induction or repression dependent on varying con-

ditions) is also a result of transcription factor-mediated interactions in the enhancer element [33–36]. Promoter engineering techniques alter both core and enhancer elements to modulate overall promoter expression capacity.

3 Examples of promoter selection in metabolic engineering applications

Promoter selection is often a key component of the metabolic engineering design cycle. Often, *E. coli* is selected as a host for the overexpression of heterologous proteins. In such applications, high strength and tightly controlled promoters are generally required to maximize protein production and reduce toxicity during growth phase [37]. The end result is that only a few promoters are used for protein production despite the hundreds of *E. coli* promoter sequences that have been elucidated [38]. Among these select promoters are two very high strength phage-derived promoter systems based on the T7 RNA polymerase and the P_L temperature-regulated phage promoter systems [37, 39–41]. The abnormally high transcriptional capacity of these systems creates an excessive metabolic load on the *E. coli* host that decreases product formation in other metabolic engineering applications [42]. Hence, several lower strength, but still strong promoters, such as the *lac*, *tac*, *trc*, $P_{BAD'}$, or $rhaP_{BAD}$ promoter systems are more commonly utilized to maximize product formation [43–46]. Blending gene overexpressions with these promoters into *E. coli*'s natural metabolism enabled the production of 1,4-butanediol [47], 1-butanol [48], isobutanol and other branched-chain higher alcohols [49], and polylactic acid and its copolymers [50]. Synthetic promoter regulation has been imparted to *E. coli* promoters utilizing the *tetA* promoter/operator and *tetR* repressor system [34, 35, 51]. Reviews of these and other advances have been recently published [52–54].

In yeast, strong endogenous constitutive promoters (including P_{TEF} [55], P_{HXT7} [56], and P_{GPD} [57, 58]) or galactose-inducible promoters [33, 59] are typically employed for metabolic engineering purposes [60, 61]. Similar to the case of *E. coli* discussed above, these promoters have enabled metabolic engineering successes in yeast. Constitutive overexpression of the pentose-phosphate pathway enzymes transketolase and transaldolase enabled yeast fermentation of xylose [62]. In separate studies, ethanol yield from xylose was further increased by modulating overexpression level of these and another enzyme through multiple-gene-promoter shuffling [63], and the bacterial L-arabinose degradation pathway was overexpressed to enable arabinose fermentation [64]. Inducible promoters offer a complementary method for recombinant protein expression in yeast and, as such, the *GAL* promoters have been widely employed in pathway engineering applications including the production of artemisinic acid [65], isoprenoids [66, 67], and n-butanol

[68], and various other metabolites and products [61]. Thorough reviews of promoter utilization for metabolic engineering purposes have been recently published [60, 61].

4 Promoter engineering strategies

As described above, strong overexpression is not always optimal for a given gene, thus a range of promoter strengths is necessary. Promoter engineering is becoming an enabling technology to facilitate this optimization and provide more synthetic promoter elements. The basis for much of this engineering is related to promoter architecture. As examples, prokaryotic promoters contain two essential motifs surrounded by variable spacer regions of DNA. Thus, introducing variation into these spacer regions can modulate expression potential in these promoters. Visualizing eukaryotic promoters as a fusion of core and enhancer facilitates engineering hybrid promoters, in which modular enhancer and core combinations can determine promoter regulation and transcriptional capacity. At the more basic level, both enhancer and core elements possess specific TFBSs that determine overall promoter function. Randomized promoter mutagenesis through error-prone PCR (Ep-PCR) alters TFBSs, thus altering promoter strength. Since binding site mutations are far more likely to reduce transcription factor interactions, these random mutagenesis approaches often produce promoter variants with lower strengths than the template sequence. In this section, we discuss each of these promoter engineering strategies in detail and give examples of their effectiveness and utility. Instances of each of these strategies are summarized in Table 1.

4.1 Ep-PCR

Ep-PCR introduces random mutations into a DNA sequence of choice (Fig. 2A). When applied to an entire promoter region, mutations occur throughout the consensus and spacer regions, and lead to disparate function. This approach is guaranteed to yield novel promoter variants (with sufficient library sizes), and proper selection techniques allow the isolation of promoters driving a wide variation in gene expression. For example, mutagenesis of the bacteriophage-derived P_L - λ constitutive *E. coli* promoter yielded a library of engineered promoters of varying strengths spanning a 196-fold range with identical regulation [69]. Individual promoters displayed uniform expression on the single-cell level, and library application enabled identification of optimal expression levels of phosphoenolpyruvate carboxylase (*ppc*) and deoxy-xylulose-P synthase (*dxs*) to maximize the desired growth and lycopene-production phenotypes, respectively. Moreover, optimal expression levels were revealed to be dependent on strain genetic background, thus demonstrating the ne-

cessity of promoter libraries to sample ranges of expression [69]. Ep-PCR of the strong constitutive *S. cerevisiae* *TEF1* promoter generated a similarly diversified promoter library spanning a 15-fold range [69, 70]. Utilizing these promoters in knock-in promoter replacement cassettes revealed a linear relationship between glycerol-3-phosphate dehydrogenase (*GPD1*) expression on glycerol yield, which saturated at the higher activity of the mutant *TEF1* series [70]. Additionally, a recent application of the *TEF1* promoter series enabled a graded dominant mutant approach that provided novel insight into the catalytic function of global yeast GCN5p [71]. Finally, random mutagenesis of the oxygen-responsive *S. cerevisiae* *DAN1* promoter enabled the isolation of two mutants induced under less-stringent anaerobiosis than the wild-type promoter, enabling induction of gene expression simply by oxygen depletion during cell growth [72].

Ep-PCR of an endogenous constitutive promoter is likely to decrease promoter activity by mutating TFBSs and reducing transcription factor-promoter affinity [69, 70, 73]. In this manner, it is possible to discover and characterize TFBSs through random Ep-PCR-mediated TFBS [74]. Promoter mutagenesis and characterization allowed identification of functionally important mutations with the P_L - λ promoter in *E. coli* and in sugar cane cells [73, 74]. Through the same mechanism, Ep-PCR of a highly regulated promoter is likely to temper strict regulation by decreasing promoter-transcription factor affinity [72]. Interestingly, successive mutation of a randomly chosen inactive eukaryotic DNA sequence from the HeLa genome through four rounds of Ep-PCR generated a strong *E. coli* promoter [75], indicating that it is relatively easy to generate or improve a prokaryotic promoter. Likely, this is due to the simplicity and conservation of prokaryotic promoter function, allowing highly selective targeting for promoter function.

4.2 Saturation mutagenesis of nucleotide spacer regions

More directed promoter engineering efforts focus on retaining consensus regions of the promoter structure while mutating only variable regions. Prokaryotic promoters contain consensus -35 and -10 motifs separated and surrounded by variable nucleotide spacer regions [10] (Fig. 1A). Hence, the saturation mutagenesis of these nucleotide spacer regions (while keeping consensus motifs intact) represents a somewhat rational methodology to modify prokaryotic promoter strength (Fig. 2B). Jensen et al. [76] demonstrated that saturation mutagenesis of a *Lactococcus lactis* promoter drastically modulates expression, generating a synthetic promoter library spanning a 400-fold range in expression. Mutations within the consensus motifs or alterations of spacer length greatly reduced promoter function, complementing and increasing library coverage to a range of three to four logs [76].

Table 1. Improvement/diversification of gene expression afforded by the examples discussed^{a)}

Promoter engineering strategy	Promoter or biological parts utilized	Expression range (fold)	Fully sampled range?	Predominantly increased/decreased promoter strength	Reference
Ep-PCR	Phage P _L -λ	196	Yes	Decreased	[69]
Ep-PCR	<i>S. cerevisiae</i> TEF1 promoter	15	Yes	Decreased	[70]
Saturation mutagenesis of spacer regions	<i>L. lactis</i> 'consensus' promoter – 31 conserved, 2 semi-conserved (W or R) and 22 random nucleotides (Fig. 1A)	6833	Yes	N/A	[76]
Saturation mutagenesis of spacer regions	<i>E. coli</i> 'consensus' promoter – 24 conserved, 13 semi-conserved (W, R and D) and 20 random nucleotides	349	Yes	N/A	[78]
Saturation mutagenesis of spacer regions	<i>Lb. plantarum</i> 'consensus' promoter – 16 conserved, 3 semi-conserved (W, R and D) and 20 random nucleotides	160	Yes	Decreased	[79]
Saturation mutagenesis of spacer regions	Mammalian 'Jet' promoter – 61 conserved and 69 random nucleotides	10	Yes	Decreased	[82]
Saturation mutagenesis of spacer regions	<i>S. cerevisiae</i> core promoter – two CT boxes, two RPG boxes, one TATA box, and 83 random nucleotides	5286	Yes	N/A	[83]
Saturation mutagenesis of spacer regions	<i>S. cerevisiae</i> PFY1p core promoter – Reb1p TFBS, poly-dT element, 26 conserved and 48 random nucleotides	9	Yes	Decreased	[87]
Hybrid promoter engineering	<i>Y. lipolytica</i> UASTB enhancer and LEU2 minimal core promoter	32	No	Increased	[97]
Hybrid promoter engineering	<i>Y. lipolytica</i> UASTB enhancer, LEU2 minimal core promoter, TEF core promoter series	400	Yes	Increased	[99]
Hybrid promoter engineering	<i>S. cerevisiae</i> UAS elements UAS _{CLB} , UAS _{CIT1} and UAS _{TEF} and core promoters P _{LEU1} , P _{GPD} , P _{TEF} , P _{CYC} and P _{CYC158}	90	Yes	Increased	[100]
Hybrid promoter engineering/TFBS modification	<i>S. cerevisiae</i> Gal4p TFBSs and P _{LEU1} core promoter	50	Yes	N/A	[100]
Hybrid promoter engineering	Mammalian apoE and ABP enhancers and the ADH6, hAAT, CYP, SV40, and FIX core promoters	975	No	Increased	[102]
TFBS modification	Mammalian TFBS for activating protein-1 (AP-1), nuclear factor κB (NF-κB), CArG-binding factor A (CBF-A), and a TATA Box	~40	No	N/A	[104]
TFBS modification	<i>Pichia pastoris</i> AOX1 promoter	28	Yes	Decreased ^{b)}	[105]

a) Several initial promoters employed for saturation mutagenesis of spacer regions were composite 'consensus' promoters derived from available literature and sequence data. Thus, there is no reference promoter to determine if mutant promoters had increased or decreased expression capacities. N/A, not available.

b) Several modified AOX1 promoters displayed increased activity compared to wild type, but the majority did not.

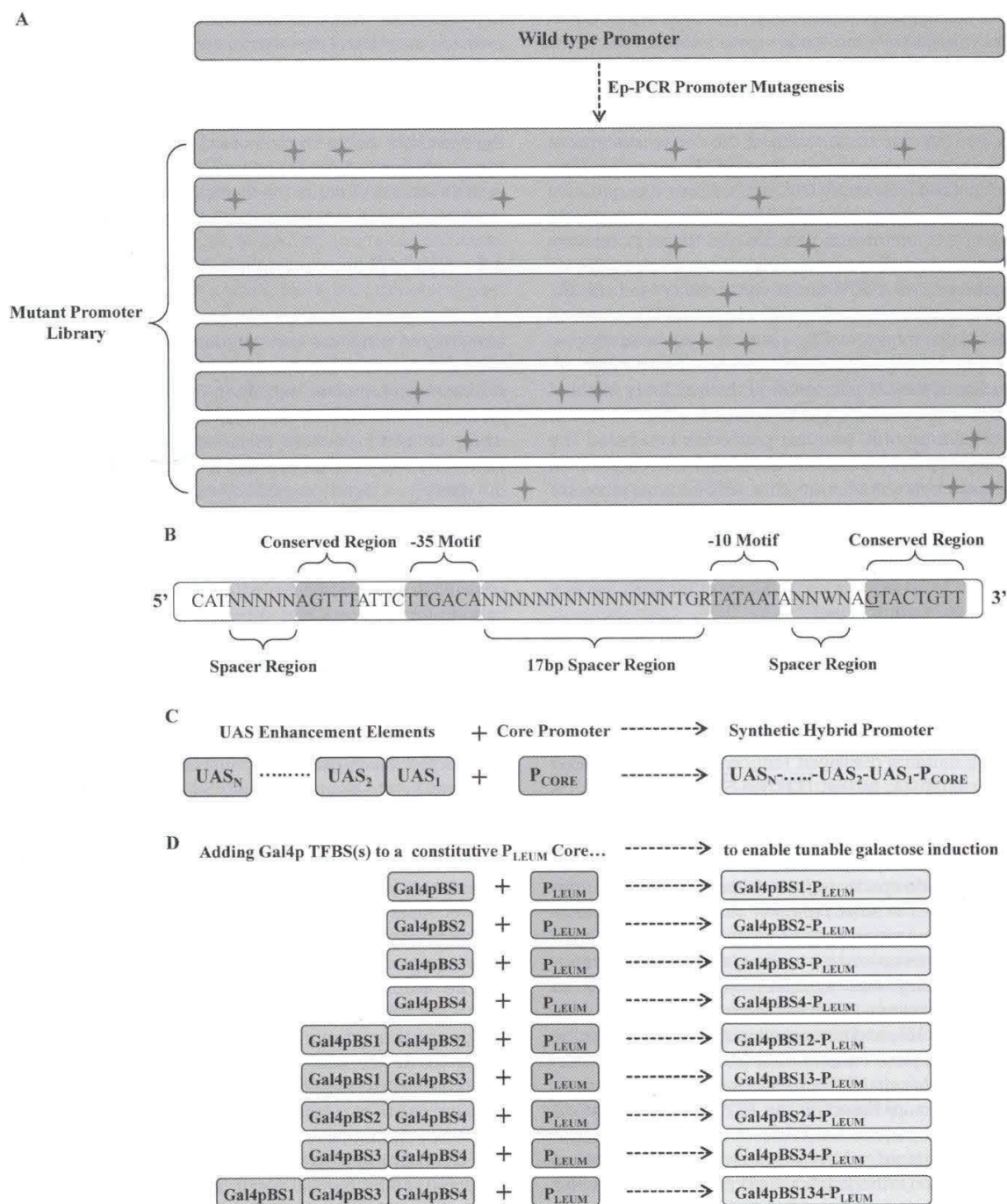


Figure 2. Overview of promoter engineering methods. (A) Ep-PCR introduces random mutations (depicted as red stars) into a wild-type promoter element that alter promoter sequence, modified from [69]. Large-scale characterization of mutated promoters facilitates isolation of a promoter library that retains endogenous regulation but spans large expression ranges. (B) Saturation mutagenesis of nucleotide spacer regions diversifies non-consensus nucleotides within a promoter to enable wide ranges in promoter library strength. A schematic of the saturation mutagenesis of three nucleotide spacer regions within four conserved regions of a consensus *L. lactis* promoter has been modified from [76]. Underlined 'G' denotes the +1 TSS. (C) Hybrid promoter engineering utilizes tandem upstream activations sequences to modulate core promoter expression to construct synthetic hybrid promoters with novel strength or regulation, modified from [100]. (D) Directed introduction, deletion, or modification of TFBSs rationally alters promoter strength of regulation. Addition of one to three distinct Gal4p TFBSs to a constitutive core promoter enabled tunable galactose-induction over a 50-fold range, as shown in [100].

Application of this synthetic library successfully modulated individual and multi-gene operon expression levels [77], and utilizing the promoter library in *E. coli* revealed that promoter strength is not conserved between prokaryotic hosts [76]. Interestingly, the drastic decrease in promoter activity due to mutation of the consensus spacer length was not observed in *E. coli* [76], a finding confirmed in a separate study [78]. Accordingly, elongation of the 16-bp spacer region of the *E. coli* hybrid *tac* promoter by one or two bp (creating the *trc* and *tac* promoters, respectively) decreased in vivo activity only slightly; the *trc* promoter exhibited 90% *tac* in vivo activity, and the *tac*, 65% [44]. Application of spacer region mutagenesis to *E. coli* and *Lactobacillus (Lb.) plantarum* successfully created high-coverage synthetic promoter libraries [78, 79]. Utilization of the *Lb. plantarum* promoter library enabled production of PepN protein at approximately 10–15% of total cellular protein, and the promoters possessed the same activity level in another *Lactobacillus* species [79]. These examples demonstrate that semi-rational promoter diversification via saturation mutagenesis repeatedly produces robust synthetic promoter libraries in prokaryotes [80, 81]. Such libraries represent ideal tools for fully examining how gene expression levels alter intracellular flux and product accumulation.

Saturation mutagenesis of nucleotide spacer regions has also been successfully applied to metazoan and yeast promoter architectures for synthetic library construction [82, 83]. Mutagenesis of the hybrid Jet promoter yielded a mammalian promoter library with a 10-fold range, in which all mutants displayed reduced activity compared to the original Jet hybrid [82]. As *S. cerevisiae* lacks a strict consensus structure, Jeppsson et al. [83] pieced together a hybrid promoter containing two Gcr1p TFBSs, two Rap1p TFBSs, and a TATA box, separated by degenerate nucleotide spacer regions following spacial guidelines elucidated in other promoter studies [84–86]. Variations in the spacer regions allowed isolation of 37 synthetic promoters covering a range of three orders of magnitude. Utilizing this synthetic promoter library to down-regulate native *ZWF1* expression increased ethanol production by 16% and decreased xylitol accumulation by 55% in yeast xylose fermentation [83]. Spacer region diversification also enabled construction of a yeast synthetic promoter library based on the *PFY1* promoter scaffold [87]. Specifically, introduction of tetR regulation and binding sites for customized transcription activator-like effectors [88] enabled orthogonal regulation of this promoter for synthetic biology purposes [87]. As a final application in yeast, spacer-region diversification, combined with tet-based and UAS_{GAL}-based [36] control, enabled model-guided construction of a synthetic gene network that controlled yeast-sedimentation timing [89].

As demonstrated in this section, directed diversification of non-consensus promoter sequences has enabled promoter library constructions across species, most often

in prokaryotes. These libraries benefit from very large expression ranges and represent an enabling technology for pathway flux optimization. Saturation mutagenesis efforts have often utilized synthetic, composite promoter scaffolds that are stitched together using motifs and binding sites from disparate promoters. As a result, establishing a base line is difficult as there is no reference promoter to determine whether the diversified promoter libraries tend to reduce or increase expression capacity.

4.3 Hybrid promoter engineering

A hybrid promoter engineering approach entails the assembly of enhancer element–core promoter fusions to rationally enhance basal core transcriptional capacity or enable novel promoter regulation (Fig. 2C). Basic hybrid promoter work in *E. coli* led to the formation of many commonly utilized promoters, including the *tac* promoter (a fusion derived from the *trp* and *lac* promoters) [43], and the *rhaP_{BAD}*, a tightly regulated arabinose and rhamnose promoter fusion [46]. Traditionally, hybrid promoters have been utilized to dissect promoter function and regulation in *S. cerevisiae* [43, 90–95]. In this light, essential DNA sequences are identified in part due to the modularity of hybrid promoter core and enhancer elements. Upstream enhancer elements contain TFBSs that enable native regulation and expression activation or repression to be maintained independently of core promoter region. Minimal regions of these DNA regions can identify specific regions essential for transcriptional activation or induction control (i.e. upstream activation sequences). Constructing hybrid promoters composed of tandem repeating UAS elements can radically increase core promoter expression capacity, as each additional UAS increases overall hybrid promoter strength. Thus, hybrid promoter engineering presents the dual advantage of (i) generating large-coverage promoter libraries, and (ii) enhancing the transcriptional capacity of even the strongest endogenous core promoters.

The oleaginous yeast, *Yarrowia lipolytica*, has served as a recent platform for hybrid promoter engineering technology stemming from a dissection of the strong, highly regulated XPR2 promoter that revealed a 105-bp upstream enhancer element, dubbed UAS1B [96]. Fusion of between one to four tandem UAS1B copies to a core minimal LEU2 promoter created four hybrid promoters of increasing strength, named hp1d through hp4d [97]. As *Y. lipolytica* suffers from a dearth of strong characterized promoters, the hp4d hybrid has become a commonly used tool for heterologous protein expression in *Y. lipolytica* [98]. An analysis of the hp_d promoter series revealed a linear increase of promoter strength as a function of number of tandem UAS1B copies. Hence, expanding hybrid promoter library construction to fuse between 1 and 32 UAS1B elements to the minimal LEU2 promoter region enabled far higher expression levels, creating the

strongest characterized promoters in *Y. lipolytica* that spanned a 400-fold range in expression capacity [99]. Furthermore, characterization of a hybrid promoter library consisting of 8 or 16 tandem UAS1B elements fused to variously sized TEF core promoter regions demonstrated that the enhancer and core regions act as independent, synthetic parts within the hybrid promoter. Hybrid promoters constructed in this manner are immediately useable to optimize pathway gene expression in a pathway engineering application. Moreover, promoter enhancement via hybrid construction represents the only known methodology to systematically increase promoter-driven transcriptional capacity with an organism.

The synthetic hybrid promoter approach has recently been employed in the model yeast *S. cerevisiae* for the creation of both strong promoter libraries and regulated gene expression. Fusion of disparate UAS elements to endogenous promoters and core promoter elements established a dynamic library of constitutive expression that ranged 90-fold. Moreover, this approach increased the transcriptional capacity of the strongest constitutive yeast promoter, P_{GPD} , by 2.5-fold based on mRNA level [100]. Hybrid promoter engineering also produced a library of galactose-inducible promoters that fully sampled a nearly 50-fold range of galactose-induced expression levels and additionally increased the transcriptional capacity of the Gal1 promoter by 15% [100]. These results demonstrate that endogenous promoters in yeast, and potentially all eukaryotes, can be enhanced simply through the addition of upstream activation elements. To this end, basic hybrid promoter engineering efforts have even generated strong promoters for mammalian cell lines [101, 102], and rational design of a composite metazoan 'super-core' promoter, constructed from several high-activity core promoter motifs, enabled high gene expression levels compared to native core promoters [103]. It is expected that this paradigm for promoter engineering will be used more commonly in the future and will expand to other host systems.

4.4 Direct modification of TFBSs

In the end, promoter strength and regulation are the cumulative effect of short, distinct nucleotide sequences that mediate binding of cellular transcriptional machinery. Hybrid promoter engineering relies on this assumption by fusing tandem UAS elements (that contain TFBSs to localize specific transcriptional activators) to enhance, fine-tune, or regulate promoter transcriptional capacity. Ep-PCR mutagenesis enables promoter engineering by randomly mutating DNA around or within TFBSs, and mutagenesis of nucleotide spacer regions enables promoter control by only mutating DNA around conserved DNA motifs. Thus, all promoter engineering approaches rely on the addition, abrogation, or modification of TFBSs and TFBS genetic context to alter promoter transcription-

al capacity. In this light, the future of transcriptional control at the promoter level will entail the refinement of promoter expression capabilities through the direct, systematic modification of TFBSs.

This technique is already being applied across different species and basic promoter architectures. In *S. cerevisiae*, combinatorial promoter design enabled understanding of how the position and number of tet repressor-binding sites within the GAL1 promoter affect gene expression levels [34]. In *E. coli*, a combinatorial promoter library containing up to three inputs from four different TFs modulated promoter strength over five decades [35]. These combinatorial promoter systems allowed for the rational prediction of gene expression from combinatorial promoters with some success. Moreover, the galactose-induced yeast hybrid promoter library mentioned in the hybrid promoter engineering section above was in fact generated by the fusion of known Gal4p-binding sites to a minimal core promoter [100] (Fig. 2D). In metazoans, fusing activating TFBSs to a core promoter created two strong mammalian promoters [104]. Finally, in *Pichia pastoris*, removal of putative repressive TFBSs and addition of putative activating TFBSs into the AOX1 promoter enabled construction of a promoter library for fine-tuned gene expression [105]. Application of this library under industrial settings increased yield and quality of heterologous protein production, illustrating that promoter engineering via direct modification of TFBSs can enable metabolic engineering applications.

5 The future of transcriptional control

5.1 High-throughput characterization of TFBSs

High-throughput bioinformatic technologies have accumulated massive data sets to analyze cell wide genome, transcriptome, proteome, interactome, and fluxomic states [106]. High-throughput technologies have recently been developed to characterize the effect of mutating any DNA nucleotide within a promoter element. By synthesizing and uniquely tagging enhancer elements with a slight level of degeneracy, single nucleotide polymorphisms (SNP) were investigated for their effect on promoter function in vivo using massively parallel assays [107, 108]. This manner of dissection applied to three enhancer elements in liver cells yielded highly reproducible models for describing the impact of every possible SNP on enhancer activity [108]. Analysis of mutation effect agreed well with predicted TFBSs in most instances, and disagreements observed only served to illustrate the necessity of enhancer experimental characterization [108]. A similar dissection of two well-characterized inducible enhancers, a cAMP-regulated enhancer and the virus-induced interferon- β enhancer, accurately defined TFBSs at single-nucleotide resolution and allowed cus-

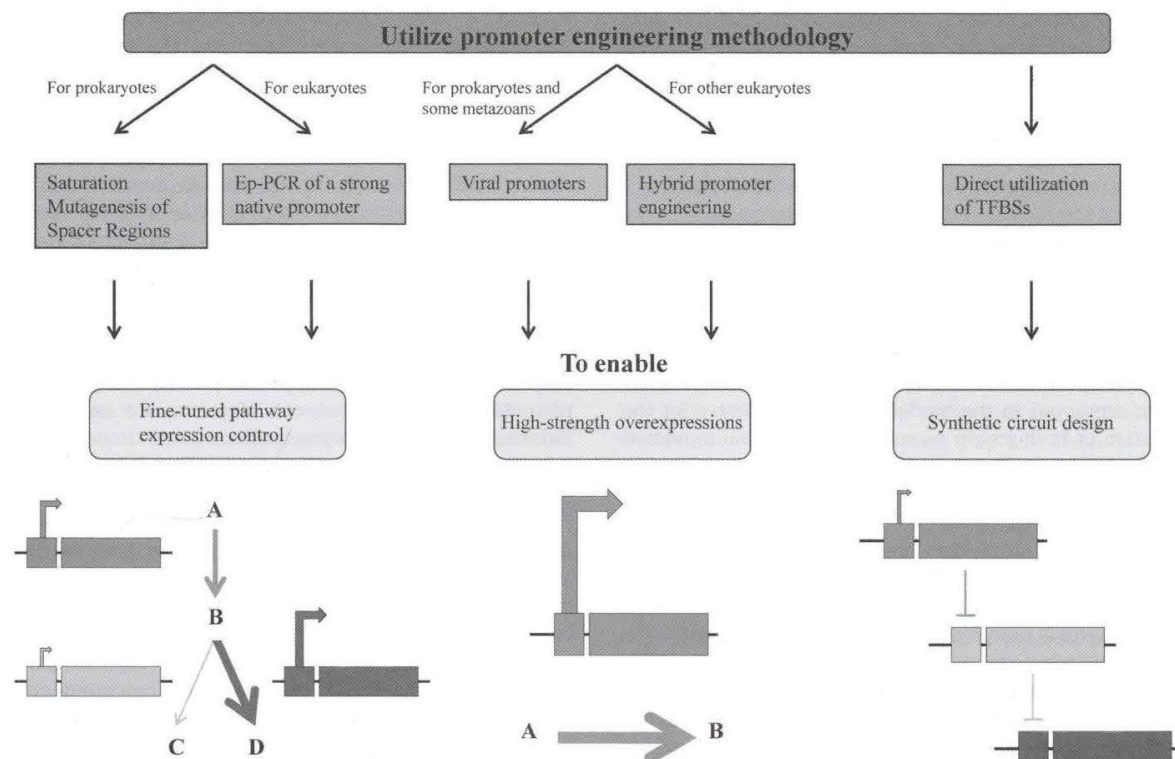


Figure 3. Utilizing promoter engineering methods. Metabolic engineering applications require precise control of pathway gene expression to optimize product formation, easily achieved through the use of expansive, well-sampled promoter libraries. In prokaryotes, saturation mutagenesis of spacer regions facilitates construction of these tunable libraries, while Ep-PCR of a strong basal promoter is more straightforward for more complex eukaryotic promoters. In the instances that gene expression needs to be further increased, viral promoters generate high transcription levels in prokaryotes and to a lesser extent in metazoans, and the hybrid promoter engineering approach can increase transcriptional capacity within eukaryotic hosts. Synthetic circuit design requires the strict regulation of inducible genetic networks, best attained through the utilization of well-characterized TFBS in hybrid promoters.

tomizable design [107]. As high-throughput technologies are becoming cheaper and more accessible, thorough binding site and enhancer element characterizations will become more common. Data generation of this type can be utilized to construct predictive models of promoter function to fine-tune promoter expression capabilities in a rational manner [107].

5.2 Future applications: Utilizing rational promoter design to enhance metabolic engineering

Metabolic engineering attempts to optimize flux towards product formation, typically by controlling transcript production at the promoter level. Distinct metabolic engineering applications require different promoter properties, in terms of expression range and inducibility. Promoter engineering is an evolving field that has developed diverse technologies aimed towards generating this wide-spread transcriptional control (Fig. 3). Pathway engineering applications require fully sampled expression ranges to enable determination of optimum gene expres-

sion levels. In this regard, saturation mutagenesis, Ep-PCR, and hybrid promoter engineering methodologies complement each other well across promoter architectures to create robust promoter libraries. Only hybrid promoter engineering easily increases promoter strength, but each of these methods is able to generate low strength mutant promoters. These low strength promoters are useful for genetic knockdowns of essential genes. Recombinant protein production necessitates inducible high-expression to maximize protein output while avoiding toxic product growth inhibition. Ideally, induction of heterologous protein expression would occur without the need of expensive inducers, and only when cells are fully grown. As an example, the T7 polymerase could be incorporated into a quorum sensing synthetic circuit in *E. coli* to enable cell-density-based, high-level protein production. Recently, Zhang et al. [109] altered *E. coli* promoter regulation through the incorporation of a FadRp TFBS to enable synthetic induction by fatty acids. Utilization of these enhanced promoters triggered the transcription of complex gene combinations and enabled a 3-fold increase

in the production of fatty acid ethyl esters. While *E. coli* and mammalian hosts are typically utilized for protein production, a yeast platform could be advanced as stronger promoters facilitate increased protein expression.

As TFBS characterization becomes more widespread, the future of promoter engineering could entail rationally designing promoters that enable discrete, advantageous expression amounts. In fact, a recent analysis of combinatorial cis-regulation in synthetic and genomic yeast promoters enabled prediction of mig1 site interaction and glucose-mediated repression through thermodynamic modeling [110]. At the most fundamental level, it should be possible to correlate transcription factor activation of an enhancer element solely through the change in free energy upon transcription factor binding to its target DNA sequence. Hence, a thermodynamic understanding of protein-DNA interactions could enable de novo prediction of promoter expression capacity, rational enhancement of promoter properties, or rational construction of promoter regions with defined expression and regulation properties in any organism. Moreover, more elaborate models of genetic circuits can help link genetic context with gene and protein expression capacity [111]. When these capacities are coupled, the true potential of metabolic engineering can be unlocked in which rationally designed promoters can optimally and precisely control pathways of engineering.

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