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Advances and current limitations in transcript-level control of gene expression

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Gene expression control is critical to increase production of recombinant proteins, fine-tune metabolic pathways and reliably express synthetic pathways. The importance of transcriptional control seems to be most important in eukaryotic systems. In this review, we highlight recent developments in the field of transcriptional engineering with an emphasis on the opportunities and challenges. We discuss the engineering of 'parts' that influence transcriptional throughput including promoters, terminators, and transcription factors as well as the genetic context of the expression cassette. While great strides have been made in the area, the robustness of these parts has been largely untested. This review highlights the importance of considering robustness in biological systems and the limitations that current synthetic parts possess.

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Introduction

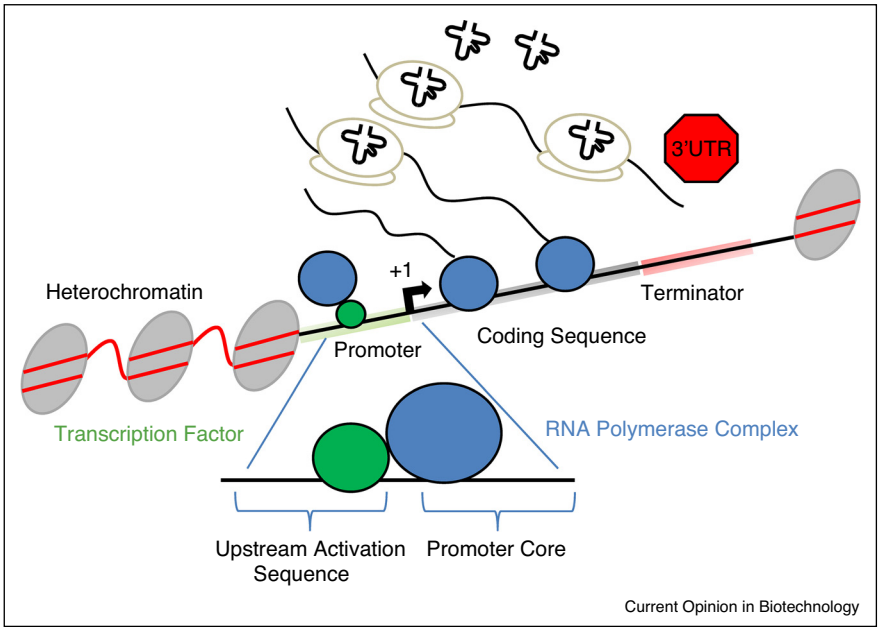
Controlling gene expression is a paramount, and often foremost, goal of most biological endeavors — from therapeutic antibody production [1] to the production of industrial enzymes [2] to the expression of heterologous metabolic pathways [3,4]. While most of these efforts initially focus on the need for high expression, further work (especially in optimizing these processes) requires a more sophisticated, tighter control of gene expression. The need for control at many levels obviates the necessity of libraries of synthetic parts capable of controlling transcript levels. However, not all parts are created equal and not all have been tested adequately enough to ensure function in a new system. Specifically, the current synthetic biology 'parts on a shelf' model seemingly necessitates interoperability and robustness of parts, yet relies on community sourced databases to assemble experimental tools [5,6]. This reality provides both opportunities for rapid advancement as well as a limitation in the field. In this review, we highlight the advances in synthetic parts for controlling transcript levels and address inherent challenges and considerations in more accurately defining their robustness.

Two major processes contribute to protein expression level: transcriptional rates and translational rates. Translation-level control (especially through tools such as ribosomal binding site calculators [7–9] and codon optimization) allow users to forward engineer the ribosomal efficiency for their gene of interest. This approach has been successfully demonstrated in prokaryotic systems where strong, orthogonal viral promoters and simpler translational mechanisms exist. In this context, translation-level control can span a 10^5-fold range [7] by editing a relatively small sequence space (such as the 5′UTR containing an RBS). Recent work on translational control in eukaryotes has focused on codon optimization to allow for improved protein expression, but the level of control of translation is not nearly as high. As an example, by optimizing the codon usage of the heterologous catechol 1,2-dioxygenase gene to be better expressed at stationary phase in *Saccharomyces cerevisiae*, a 2.9-fold increase in titer was achieved [10]. In contrast, for yeast and higher eukaryotes, tuning transcription rates through promoters imparts a higher level of control and can achieve between a 10^2-fold dynamic range [11•] and 10^4 range for orthogonal transcription factors [12••]. By comparison, the native range in transcript levels for yeast spans a roughly 10^3–10^4 dynamic range [13]. A similar range is also achievable in prokaryotic systems. The mRNA level of a transcript is controlled by many factors including the promoter, terminator, plasmid/expression cassette copy number, and the surrounding DNA context of the plasmid or genomic locus of integration (Figure 1). Given the success of transcription-level control (esp. in eukaryotic systems), we focus the rest of this review on this area by first considering the synthetic parts that lead to control and then addressing the issues of robustness.

Promoters

Promoters have one of the largest impacts on gene expression and were among the first synthetic parts to be studied and diversified via random mutagenesis [14]. These initial efforts were marked by a more robust definition of promoter strength taking into account

Figure 1



Factors influencing transcriptional control of genes. The above expression cassette is color coded to represent promoter (green), coding sequence (gray) and terminator regions (red). Heterochromatic regions are used as an example of potential complications provided by the genetic context in which the cassette is located. Ribosomes moving along the mRNA and movement are limited by factors such as tRNA availability. In the expanded region of the figure, a simplified promoter diagram is provided that depicts upstream activation sequence containing a bound transcription factor (green) along with RNA polymerase complex (blue) bound to the promoter core.

dilutions by growth, the promoter's ability to impact multiple proteins, measurement of mRNA levels, and utility in heterologous pathway expression. More recent efforts aim at creating more novel promoters (independent of a native scaffold) to increase the range of transcriptional capacity (Table 1). Developing synthetic promoters for eukaryotic systems will increase the number and diversity of promoter parts available in these systems. Comparable efforts to increase part diversity in *Escherichia coli* have been undertaken using genome mining [15] and screening of promoter and translation initiation libraries [16].

The galactose inducible promoter (GAL) is the strongest yeast inducible promoter; however it suffers from complete repression by glucose. Liang and coworkers developed a novel gene switch that coupled the inductive strength of the GAL promoter with the tight binding affinity of estradiol for the estrogen receptor protein. This ultimately led to a series of parts capable of inducing a multistep pathway using 10 nM estradiol in the presence of glucose and resulting in a 50-fold improvement in zeaxanthin production over previous efforts using constitutive promoters [17•].

The strongest yeast promoters have been constructed through a hybrid approach by coupling upstream activating sequences (UAS) with a core promoter. Adjusting the composition of the UAS elements enables upwards of 50–300-fold dynamic range in expression strength, reaching the highest reported strength of a promoter in *S. cerevisiae* [11•,18]. Among these, the strongest constitutive hybrid promoter exhibits a 2.5-fold improvement over the TDH3 promoter with respect to mRNA levels making this promoter as strong as the GAL promoter [11•]. However, it should be noted that there is no real statistically significant improvement in fluorescent protein production, illustrating the limitations of relying on reporter proteins alone without more robust, comprehensive measurements like mRNA levels. Improved core promoters could lead to even greater transcriptional control in these systems. Core promoters were investigated in the yeast *Pichia pastoris* and synthetic core promoters were designed using common sequence motifs and transcription factor binding sites. These synthetic core promoters were combined with the methanol inducible promoter pAOX1 to generate diverse activity between 10% and 117% of the wild-type promoter, however only fluorescent protein expression was reported [19].

The field is also quickly moving to de novo synthetic promoters that lack homology to anything else in the genome. In the case of Chinese Hamster Ovary (CHO) cell lines, Brown and coworkers used an enrichment

Table 1

Characterization of synthetic parts. Various synthetic parts controlling transcription have been discussed in the text. We highlight these parts, the dynamic ranges achieves and the conditions tested

Organism	Part	Expression cassette location	Conditions tested	Dynamic range	Citation
S. cerevisiae	Promoter	Plasmid	β-Gal, yECitrine, qRT-PCR	50-Fold fluorescence	[11•]
Yarrowia lipolytica	Promoter	Plasmid	hrGFP, β-Gal and qRT-PCR	400-Fold mRNA	[18]
E. coli	Promoter	Plasmid	GFP, lycopene production, qRT-PCR, and chloramphenicol resistance	196-Fold fluorescence, 325-Fold mRNA, 26-fold chloramphenicol	[14]
S. cerevisiae	Promoter	Plasmid	yECitrine	20-Fold fluorescence	[14]
E. coli	Insulated promoter	Plasmid	GFP	100-Fold fluorescence	[45]
S. cerevisiae	Promoter and switch	Plasmid and Integration	GFP, zeaxanthin, qRT-PCR	8-Fold range of induction, 50-fold higher zeaxanthin than constitutive	[17•]
P. pastoris	Core promoter	Plasmid	GFP	10–117% of WT fluorescence	[19]
CHO	Promoter	Transfection	SEAP and GFP	2.2-Fold improvement over CMV, 140 promoters with 10^2-fold range	[20•]
S. cerevisiae	Promoter	Plasmid and Integration	yECitrine, LacZ	3.2-Fold over wt CYC, 16-fold improvement when integrated, 20-fold dynamic range	[21••]
E. coli	Terminator	Plasmid	Transcriptional read-through with GFP, RFP	>100-Fold range in termination efficiency	[22]
S. cerevisiae	Terminator	Plasmid	yECitrine, qRT-PCR and mRNA half-life, Xylose Growth Rate	11-Fold increase in fluorescence between terminators, 35-fold without terminator	[24•]
S. cerevisiae	Terminator	Integration and Plasmid	opGFP or mKO2 Fluorescent reporters, qRT-PCR and cellulase expression	2-Fold range in secretory protein production	[25]
E. coli	Polymerase	Plasmid	GFP and Lycopene Production	Orthoganol promoters induce target 8–75-fold more than off target	[27]
HEK293T	Transcription-activator like effector (TF)	Transfection	Luciferase, qRT-PCR	10^4 total dynamic range achieved	[12••]
HEK293T	Transcription factor	Transfection	YFP	56× activation with 3× operator sites	[28]
S. cerevisiae	Transcription factor	Integration and Plasmid	GFP	70-Fold activation with 12× operator sites	[28]

screen to identify synthetic promoters including one with activity twice as high as the cytomegalovirus (CMV) promoter, but in half the size [20•]. In this case, transient transfection was used for analysis, but two reporters (SEAP and GFP) were tested. In the case of yeast, Curran and coworkers used a computational design based on nucleosome occupancy to re-design native and de novo design synthetic promoters. Following an iterative design process, they were able to increase expression up to 6-fold of the original promoter when maintained on a plasmid and 16-fold following integration [21••]. This recognized difference between plasmid and integration provides an important demonstration of the role of the surrounding DNA context on the numbers reported in the literature, which will be addressed in more depth below.

Terminators

Whereas promoters aim to increase the rate of transcript formation, terminators (and 3′UTR design) serve as a way to augment half-life and thus mRNA persistence (Table 1). However, terminators also serve an essential role—to terminate transcription. Moreover, their sequence often looks similar to the features of a promoter. Thus, the 'strength' of a terminator can be evaluated based on read-through capacity, the impact on mRNA half-life, the structure of the 3′UTR, or even latent promoter activity. This complexity has given rise to confusion within the field. A recent survey of 582 terminators in *E. coli* by Chen and coworkers identified 39 strong terminators which reduce downstream gene expression by over 50-fold [22]. A set of commonalities between these 'strong' terminators led to design parameters which may inform future forward-engineering attempts at generating synthetic terminators with desirable strength in *E. coli*.

In eukaryotic systems (such as yeast), synthetic terminator projects have attempted to identify improved

terminators based on mRNA stability via protein readouts. Work by Munchel and coworkers supports the idea that mRNA stability natively regulates genes in *S. cerevisiae* and higher organisms as significant half-life shifts occur following growth and stress conditions [23]. Recent studies suggest that gene expression can be tuned by nearly 11-fold by swapping common terminators [24•] whereas other studies have indicated that for a strong promoter, this value may be two-fold for the case of secreted proteins [25]. However, it is difficult to compare the synthetic parts in these two studies as distinct terminators and loci (one used plasmid, the other used integration) were used. Nevertheless, a more global study in yeast by Yamanishi and coworkers suggests that native 3′UTR activity can span 2 orders of magnitude [26] thus showing the importance of terminators in transcript-level control.

Trans-acting factors

Each of the DNA constructs described above were characterized independent of trans-acting factors that may be used to further augment transcription control. Moreover, trans-factors can be engineered to be orthogonal to the native transcriptional machinery allowing for a synthetic separation of pathways and regulation (Table 1). As examples, T7 RNA polymerase variants were generated for *E. coli* that recognize unique promoter sequence 8–75-fold more than off target promoters leading to the ability to control multiple pathways [27]. CrisprTF's developed by Farzadhad and co-workers based on the CRISPR/Cas system from *Streptococcus pyogenes* use an endonuclease deficient Cas9 combined with an activation domain to enable up to 70-fold activation of desired promoters in HEK293T cells and *S. cerevisiae* [28]. Another demonstration of orthogonal-TF's are TALE-TF's that contain a 33–35 amino acid repeat leading to a protein-DNA code capable of targeting almost any DNA sequence [29]. TALE-TFs were shown to provide a range of induction from 10 to 10^4-fold induction in mRNA concentration in mammalian cells following transfection [12••].

Genetic context

Regardless of the approach taken, transcriptional control is ultimately influenced by genetic context. Thus, all characterization of strength is subject to this caveat. Small changes to 5′UTR sequence can have a significant impact on expression levels [30]. Thus, a promoter cloned to a gene via restriction sites versus the same promoter cloned to the same gene via seamless assembly may give different expression levels. In short, the genetic context of the expression cassette matters, even down to the single basepair level. Previously, it had been believed regulatory elements only exist within the 500 bp 5′ of the gene for *S. cerevisiae* and other yeast, with even shorter distances in bacterial systems. However, Shetty and coworkers recently demonstrated that the *SNA3* gene was subject to regulation by the UAS_{INO} element downstream of its genomic locus [31•]. These long distance interactions and context dependences could play a larger role when attempting to create large, poly-cistronic messages.

Furthermore, gene expression comes at a cost. A recent study using a PURE cell-free system using a multicistronic message found that while promoters had an impact on RNA levels, the greatest impact on protein levels occurred as a result of the operon positioning, with an average 6-fold drop between the first and second positions and a 12-fold drop between first and third [32]. In a final example demonstrating the importance of context and orientation, a URA and GAL genetic circuit was integrated into *S. cerevisiae* in multiple orientations [33]. In doing so, Lee and coworkers found that relative promoter position had a significant impact on the activity of the other promoter, as GAL induction led to a reduction in nucleosome positioning on pURA3 [33]. Integration of the expression cassette into a location of the genome can also lead to changes in transcriptional rates. In fast replicating bacterial species, integration location relative to the origin of replication can have a significant impact on gene expression, with those closer to the origin being up to 2-fold more highly expressed than one closer to the terminus [34]. In eukaryotes, there have been shown to be 'hot spot' loci which are ideal targets for cassette expression. Genomic loci dependent effects have been investigated in *S. cerevisiae* using the LacZ reporter, demonstrating an 8.7-fold difference in expression across genomic loci [35]. In CHO and HEK293 cell lines, work has shown that gene expression in specific integration sites was sensitive to the promoters used, with a 10–30-fold difference in antibody production between SV40 and MPSV/CMV promoters at the same locus [36]. These complex and position specific changes in gene expression suggest a need for clearer, more comprehensive demonstrations of activity within the literature.

Robustness

Further complicating issues of expression is the reliance on a cell's transcriptional and translational resources. It is possible that our highest expression systems are already touching the ceiling of transcriptional capacity and having an impact on the expression of other native promoters at the same time by titrating away key transcription factors and polymerases. Through the use of tandem repeats of transcription factor binding sites, Lee and coworkers were able to competitively bind and turn off the tetOff system through the use of a titrating decoy [37]. This study remains as a reminder that transcription factors and polymerases are a scarce resource within the cell and abundance and competition may influence the performance of synthetic parts (especially for systems with multiple copies of the same elements).

It is tempting to assume that transcription rates per DNA copy remains constant regardless of whether considering a plasmid or a genomic copy. However, much work needs to be done in order to understand the impact of context on gene expression in order to provide a robust part. Robustness is a confounding issue limiting our ability to claim field standardization. Even for the case of parts tested in a plasmid, there is an assumption of some burden when a cell has to expend resources simply to maintain the plasmid. Recent work to characterize this metabolic burden demonstrates the vast differences that can exist when changing promoters, selection markers, and origins of replication [38]. When layered together with heterologous genes, more complex interactions can take place and simply increasing copy number does not necessarily increase yields and even lower yields [39]. Further complicating the matter is that individual pathway elements can modulate the total output of the network [40]. Recent work which integrates computational modeling and synthetic biology has demonstrated that improved connections between pathway elements and balanced expression can lead to improvements in net pathway performance [41,42].

Given the importance of robustness and difficulty in defining 'robust' parts, an alternative solution is to create new elements such as insulators that may reduce the impact of genomic context. In eukaryotic systems, insulator proteins function as enhancer blockers or heterochromatin barriers [43]. In higher eukaryotes the CCCTC-binding factor (CTCF), a zinc finger protein, mediates interactions between distant genomic sequences helping to maintain chromosomal organization and control gene expression [44]. Recent work has shown the promise of producing *E. coli* promoters across two orders of magnitude range that function consistently across several genomic context through the use of an insulation site [45]. In doing so, the insulator and core promoter must be combined together as a new synthetic part that will be referred to as the 'promoter'. In doing so, the size of the bacterial promoter was increased over 3-fold in sequence space. However, the trade-off is a higher level of robustness and guarantee that a part will work in a new context.

Conclusions

Transcriptional control is critical for synthetic biology applications. However, these parts are much more prone to failing 'off-the-shelf' as a result of a lack of robustness testing and context specific behavior. As a result, we would recommend that these parts should be studied and reported in a variety of context, namely alternating/flipping the context within a plasmid, integrating the construct into multiple loci and/or changing copy number. To provide a final case for robustness, it would be ideal to observe these parts respond to change while increasing oxygenation and cell density, moving up from culture tube to shake flask, and even into a bioreactor. Some excellent examples of work validating promoter expression has been performed using endogenous expression in *E. coli* and *S. cerevisiae* [46••], but this needs to be a top priority especially for validating the improvements of new synthetic parts over existing parts. Without these types of tests (and careful consideration of base-pair resolution of genetic context), it is hard to pull a part 'off-the-shelf' and guarantee it will work as advertised. Nevertheless, transcriptional control of expression is critical in synthetic biology. Current work and advances continue to be made which allow for an ever expanding range of gene expression across a wide array of organisms. This is indeed the first step to creating the synthetic parts we need to rewire organisms.

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