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Towards combinatorial transcriptional engineering

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• The modular nature of the transcriptional unit makes it possible to design robust modules with predictable input-output characteristics using a 'parts- off a shelf' approach. Customized regulatory circuits composed of multiple such transcriptional units have immense scope for application in diverse fields of basic and applied research. Synthetic transcriptional engineering seeks to construct such genetic cascades. Here, we discuss the three principle strands of transcriptional engineering: promoter and transcriptional factor engineering, and programming inducibilty into synthetic modules. In this context, we review the scope and limitations of some recent technologies that seek to achieve these ends. Our discussion emphasizes a requirement for rational combinatorial engineering principles and the promise this approach holds for the future development of this field.

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Abbreviations: ARE, androgen responsive element; aTF, Allosteric Transcription Factor; BSR, base specifying residue; CMV, Cytomegalovirus; CR, chromatin regulator; DBD, DNA binding domain; dCas9, endonuclease deficient Cas9; G6PDH, Glucose-6 phosphate dehydrogenase; gRNA, guide RNA; HBGS, homology based gene silencing; HPL, hybrid promoter libraries; IPTG, Isopropyl β-D-1-thiogalactopyranoside; KRAB, Krüppel-associated box; OPEN, oligomerized pool engineering; PAM, protospacer adjacent motif; RE, response elements; RNAP, RNA Polymerase; RVD, repeat variable di-residues; SPL, synthetic promoter library; sTF, synthetic TF; TALE, Transcription activator-like effector; TALOR, TALE orthogonal repressor; TEF, translation elongation factor; TF, Transcription Factor; TFBS, TF binding site; TSS, transcription start site; UAS, upstream activating sequences; ZNF, Zinc Fingers..

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1. Introduction

In recent years, transcription in eukaryotes has been examined in great detail as a component of protein synthesis. This has uncovered a highly complex machinery that regulates transcription of individual genes. *cis*-Acting promoter elements including suppressors, silencers and enhancers provide sites for the binding of *trans*-acting activators and repressors (Bhattacharjee et al., 2013). These elements form discrete transcriptional modules, which may be combined to form a complete transcriptional regulatory unit. Synthetic biologists now seek to exploit the modular feature of transcriptional units to design customized regulatory circuits.

Eukaryotic transcriptional programs are highly integrated networks that wire together multiple promoter elements to specific cellular pathways. These transcription modules are implicated in the tuning of molecular noise, recruitment of transcription factor (TF) complexes and in controlling nucleosomal remodeling, among others (Hahn and Young, 2011; Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2005). The details of the regulatory pathways are yet to be understood clearly, however, the design of synthetic circuits using a 'parts-off a shelf' approach will expedite our understanding of these basic frameworks *in-vivo* (Guido et al., 2006; Vilar, 2006). Additionally, custom transcriptional networks may be employed to advance metabolic engineering and optimization, which are useful in industry, therapeutics and crop improvement (Alper and Fischer, 2005; Jensen and Hammer, 1998a; Le Bec and Douar, 2006; Nandagopal and Elowitz, 2011; Smolke and Silver, 2011).

The future of transcriptional network engineering demands building a repository of *cis*-regulatory modules that can be knit into synthetic promoters. Although a large library of native promoters is available, this resource does not encompass a wide range of promoter strengths over a continuous range (Mehrotra et al., 2011). Sometimes, this basic library may itself be limited as in new or obscure model organisms (Blazeck et al., 2012; Siegl et al., 2013). The modular promoter elements required for construction of novel expression cassettes are sufficiently large in *Escherichia coli* (Andrianantoandro et al., 2006; Nandagopal and Elowitz, 2011). These standard biological parts have also been opened up for public access (Bio FAB Group et al., 2006). However, promoter engineering efforts have only been partially successful in eukaryotes, despite the availability of strong native promoters in organisms like *Saccharomyces cerevisiae* (Da Silva and Srikrishnan, 2012).

In some eukaryotic systems like the budding yeast (*S. cerevisiae*), the repeated use of the same promoter modules is problematic due to homologous recombination events that decrease the stability of the artificially introduced expression cassettes (Gibson et al., 2008). In plant systems, the introduction of multiple promoters or transgenes bearing homology to host *cis*-regulatory modules leads to homology based gene silencing (HBGS) at the transcriptional or post-transcriptional level (Meyer and Saedler, 1996). Native promoter modules also use the natural TF population in the cell, which increases the probability of inducing off-target genes, whose promoters contain the binding sites for the same TF (Siegl et al., 2013). Such transcriptional noise overburdens the cell and adversely affects its survival. Therefore, this necessitates the development of modules with novel TF binding sites (TFBS) and of synthetic TFs (sTF) with low off-target binding. In synthetic biological terms, this concept is called orthogonality (Rao, 2012).

In complex multicellular systems like humans or plants, systemic effects are undesirable, particularly in the fields of therapeutics and transgenic technology. Thus, synthetic transcriptional networks with intended application in these areas will need to have high spatiotemporal resolution for targeted action in specific tissues. Prolonged expression of target genes from potent activator systems with supra-optimal regulatory activity may burden the cell and reduce its viability. In other cases, it may be desirable to express a particular gene for a limited duration only. Therefore, there is need for high spatial and temporal control of expression from synthetic transcriptional modules. This can be achieved by placing synthetic modules under control of external inducers whose concentration in the extracellular environment may be tightly regulated by the user.

Efforts towards developing new transcription modules have focused on addressing these requirements by one or more of the following methods: (a) creating promoter libraries with reduced homology to native *cis*-regulatory elements; (b) design of novel expression cassettes either stronger (for promoting high output) or weaker than wild-type promoters (for reducing the expression of gene products that could be toxic); (c) achieving orthogonality by designing custom-made TFs that are specific to the target promoters; and, (d) making synthetic promoters or TFs responsive to physical and chemical inducers.

Ultimately, the aim is to design complete transcriptional networks that are easily tunable, dynamic, robust, orthogonal and simple to handle with predictable input-output characteristics. This requires rational engineering that combines designer promoters, transcription factors and the ability to regulate them externally. In this review, we address these three strands of synthetic transcriptional engineering. We initially present some recent tools for creating synthetic promoter libraries followed by a discussion of three major technologies that have revolutionized TF engineering: Zinc Fingers (ZNFs), Transcription Activator-like Effectors (TALEs) and CRISPR/Cas9. In each of these sections we separately discuss the property of inducibilty to underscore the differences in the required principles. In our discussion, we make the case for the utility of rational combinatorial engineering. The latter is important for overcoming the limitations of individual technologies and for designing transcriptional networks with improved future performance.

2. Promoter engineering

The basic eukaryotic promoter is the seat for assembly of TFs. It consists of two regions – the core promoter and the upstream promoter elements. The core promoter element lies about 40 bp upstream of the transcription start site (TSS) and contains −10 TATA box (Molina et al., 2005), which is bound by the basal TFs that recruit RNA Polymerase (RNAP) II (Lee and Young, 2000). Promoters lacking a TATA-box are also known; these promoters use upstream or downstream activating sequences to assemble TFs a few base pairs (bps) upstream of the TSS. The basal transcription rate from the core promoter is minimal and varies depending upon the nature of core promoter motifs (Blazeck et al., 2013). However, transcription rate may be enhanced or suppressed through the presence of additional regulatory elements, like enhancers or repressors. The core TATA box motif itself promotes gene transcription independent of the upstream regulator regions (Mogno et al., 2010), and without altering the gene specificity conferred by them. An experimenter may thus combine these motifs with other activator or repressor modules to produce a chimeric promoter that caters to his/her given requirements.

Proximal or distal *cis*-regulatory elements may be used to develop either constitutive or inducible promoters. Constitutive promoters do not require additional factors such as inducers, enhancers, repressors *etc*. Thus, they can offer relatively stable transcriptional outputs in diverse conditions. Among eukaryotes, many constitutive promoters have been characterized in yeast; most of them are associated with

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