Biotechnology Advances xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Precision control of recombinant gene transcription for CHO cell synthetic biology

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ARTICLE INFO

Article history:
Received 5 October 2015
Received in revised form 11 December 2015
Accepted 22 December 2015
Available online xxxx

Keywords:
CHO cells
Synthetic biology
Biopharmaceutical production
Transcriptional regulation
Cell engineering

ABSTRACT

The next generation of mammalian cell factories for biopharmaceutical production will be genetically engineered to possess both generic and product-specific manufacturing capabilities that may not exist naturally. Introduction of entirely new combinations of synthetic functions (e.g. novel metabolic or stress-response pathways), and retro-engineering of existing functional cell modules will drive disruptive change in cellular manufacturing performance. However, before we can apply the core concepts underpinning synthetic biology (design, build, test) to CHO cell engineering we must first develop practical and robust enabling technologies. Fundamentally, we will require the ability to precisely control the relative stoichiometry of numerous functional components we simultaneously introduce into the host cell factory. In this review we discuss how this can be achieved by design of engineered promoters that enable concerted control of recombinant gene transcription. We describe the specific mechanisms of transcriptional regulation that affect promoter function during bioproduction processes, and detail the highly-specific promoter design criteria that are required in the context of CHO cell engineering. The relative applicability of diverse promoter development strategies are discussed, including re-engineering of natural sequences, design of synthetic transcription factor-based systems, and construction of synthetic promoters. This review highlights the potential of promoter engineering to achieve precision transcriptional control for CHO cell synthetic biology.

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Abbreviations: CHO, Chinese hamster ovary; Cirp, cold-inducible RNA-binding protein; CMV-IE, cytomegalovirus immediate early; CRISPR, clustered regularly interspaced short palindromic repeats; DBD, DNA binding domain; DTE, difficult-to-express; EF1α, elongation factor 1 alpha; ER, endoplasmic reticulum; gRNA, guide RNA; hCMV-IE1, human cytomegalovirus immediate early 1; HT, high-throughput; LTR, long terminal repeat; MAb, monoclonal antibody; MAR, matrix attachment region; PIC, pre-initiation complex; SV40E, simian virus 40 early; TALE, transcription activator-like effector; TF, transcription factor; TFRE, transcription factor regulatory element; UPR, unfolded protein response; ZF, zinc finger.

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http://dx.doi.org/10.1016/j.biotechadv.2015.12.012
0734-9750/© 2015 Published by Elsevier Inc.

Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012

1. Introduction

The majority of new biopharmaceuticals brought to market are recombinant monoclonal antibodies (MAbs) utilizing Chinese hamster ovary (CHO) cells as a production host (Walsh, 2014). Over recent years, the creation of production CHO cell lines has ostensibly matured into a streamlined, rapid process. This is largely a consequence of the introduction of new platform technologies that permit rapid selection, isolation and testing of clonally derived subpopulations (Kuystermans and Al-Rubeai, 2015; Lai et al., 2013), and underpinning this, new genetic engineering technologies have been developed that offer general improvements in transgene expression and stability, e.g. matrix attachment regions (MARs), site-specific genomic integration, codon optimization algorithms (Kotsopoulou et al., 2010; Lee et al., 2015b; Saunders et al., 2015). Of course, recent advances in genome editing now offer new possibilities for rapid, high throughput knock-in and knock-out of functional genes (Lee et al., 2015a; Ronda et al., 2014). We also have access to CHO genomic tools and resources that are beginning to impact cellular engineering strategies (Datta et al., 2013; Hammond et al., 2012).

Despite this, bioindustrial CHO cell factory development still relies heavily upon blind screening of genetic/functional heterogeneity in parental CHO cell populations to derive a phenotypic variant capable of the core manufacturing process objective: synthesize and secrete a complex protein product whilst maintaining rapid cellular biomass accumulation. Moreover, the cell factory should maintain this functionality over many generations. Certainly we may be able to more accurately integrate the transgene in the host cell genome, and include some sequences that predispose the local genetic environment or some synthetic processes towards stable, higher-level expression, but ultimately we do not design and therefore precisely control the variable ability of the host cell environment to manufacture a specific protein. We can understand, as observed during transient gene expression, that nearly all CHO cells in a parental population can manufacture many protein architectures to some extent. However, when the cell factory is required to achieve both high-level proliferation *and* production, we require a cell factory with a set of manufacturing machinery and associated synthetic and regulatory processes particularly attuned to the idiosyncratic requirements of a given protein product.

There is one major caveat to the above where cellular product processing is *not product specific*. For years we have demonstrated control of synthetic processes involved in post-translational modification of recombinant proteins, most obviously N-glycosylation (Beck, 2013; Jedrzejewski et al., 2013). Importantly, these molecular modifications (the reason that CHO/mammalian cell factories are utilized in the first place) often govern the bioactivity and pharmacodynamics of the product in vivo (Jefferis, 2012). However, in these examples, generic CHO cell engineering yields engineered host cells that may be employed to produce many different protein products. Most often a single reaction has been eliminated (e.g. α1,6-fucosyltransferase (Yamane-Ohnuki et al., 2004)) or incorporated (e.g. β1-4-N-acetylglucosaminyltransferase III (Davies et al., 2001)), with more recent examples of co-expression of up to three processing enzymes (Yin et al., 2015). Glycosylation engineering, as an example of CHO cell engineering, has been very successful. However, this is the low hanging fruit. We generally know what to engineer in a digital on/off sense, and we do not have to deal with the biological variability of protein product architecture and regulation of transfected cell clone synthetic processes — an interface that largely defines product manufacturability.

We argue that the future of CHO cell engineering has to be based on an ability to interactively design (i.e. with respect to a specific product and specific CHO host cell genotype) and create new CHO cell functional phenotypes that do not exist naturally. We will need to extend the CHO cell "design space" beyond the natural boundaries created by random genetic mutation and chromosomal instability. Historically, through iterative improvement we have succeeded in improving the design of an external, multi-component synthetic environments for CHO cells (e.g. media/feed/process composition), which have massively improved functional performance (Wurm, 2004; Zhu, 2012). To control complex multigenic phenotypes (e.g. increased cell growth rate) and product-specific manufacturing capability, we now need to create a technology platform that enables an internal, multi-component synthetic cell environment for knowledge-based control of cell factory manufacturing performance.

This will require a CHO cell engineering platform that has one core practical attribute: the coordinated expression of multiple transgenes at precise relative stoichiometry. For instance, how do we co-express eight functional proteins in a host cell at a relative stoichiometry optimal for a new metabolic pathway function that we wish to introduce? This is a fundamental operational requirement of any biological or indeed engineered system — try making a cake using a random proportion of known ingredients! Although recent reports describe new methods for synthetic multigene vector construction for mammalian cell synthetic biology (Guye et al., 2013; Kriz et al., 2010), the synthetic parts or positional combinations that may be utilized on the vector to achieve a given stoichiometry of encoded proteins are typically not dealt with. We will need to create bespoke, synthetic mammalian cell vectors that not only harbour multiple genetic components, but also enable them to operate at an optimal, designed stoichiometry.

In a bioproduction context, embedding the relative stoichiometry of multiple transgenes is entirely desirable. The simplest example would be expression of heavy and light chain genes at an optimal ratio for synthesis of a given MAb, where the optimal ratio may be very MAb specific (Ho et al., 2013; Pybus et al., 2014; Schlatter et al., 2005). For more advanced applications, to achieve a significant change in cell factory production capability may require unnatural combinations of functional genes (Le Fourn et al., 2014; McLeod et al., 2011; O'Callaghan et al., 2010; Xiao et al., 2014) that may be designed to introduce a single concerted function (e.g. a new metabolic pathway) or used to simultaneously engineer different functional modules of a cell. Indeed, as industrial pipelines fill with engineered protein products it is entirely likely that protein-specific solutions may be necessary. For example, we recently showed for both IgG1 MAbs exhibiting variation in production titre and an engineered difficult-to-express fusion protein that different combinations of functional proteins known to modulate cellular folding/assembly capacity could, in a protein-specific manner, increase production titre significantly (Johari et al., 2015; Pybus et al., 2014). Moreover, the background host cell or production process context may significantly alter required functional genes and their relative stoichiometry. To enable significant reductions in development times for these new products, there is an urgent need to shift from screening-led to design-led technologies; embedding prediction and design of product manufacturability at an earlier stage in bioprocess development to speed products into the clinic.

1.1. *Optimized stoichiometry of genetic components in synthetic CHO cell factories can be achieved via promoter engineering*

CHO cell engineering still relies upon the widespread use of a very limited set of complex, functionally ill-defined genetic components. Currently available technologies generally employed in industry only enable gross control of recombinant gene expression. We require new design and engineering technologies that will enable us to equip cells with new machinery and processing capability optimally suited for a specific intended purpose. In essence this is a statement of the synthetic biology paradigm (Church et al., 2014; Silver et al., 2014), applied as context-dependent retro-fitting. This process will be dominated by two key questions, which new functionalities are required (for a given product/cell line/process) and how do we embed optimal performance?

Transcriptional-control tools are an attractive route to achieving the essential optimal stoichiometry of biological parts as i) transcription is

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the first step in protein expression and represents the first functional interaction between the host cell and the recombinant gene, ii) it is relatively easy to manipulate transcription rates by altering the synthetic DNA sequence, and iii) transcriptional activity can be controlled over broad dynamic ranges. Precise gene expression control could also be achieved by developing translational-control tools, for example by engineering novel untranslated regions, ribozyme switches, or coding sequences (Ferreira et al., 2013; Quax et al., 2015; Wei et al., 2013). Ultimately, CHO cell engineering requires well-characterized, mutually-compatible genetic palettes of functional components, including both transcriptional and translational control elements. Transcription control tools are the focus of this review.

Whilst transcription can be specifically controlled by engineering terminators or coding sequences, by far the most common method is promoter modulation (Bauer et al., 2010; Curran et al., 2015). Promoters are the primary regulators of transcription, and changing a gene's promoter is a simple, common, and effective method to rationally alter its expression level. However, as naturally occurring promoters are evolved, rather than designed, they are unlikely to exhibit the controllable functionality required for divergent synthetic applications. Controlling the expression level of multiple effector genes simultaneously in CHO cells will require highly-context specific promoter design criteria, such as long-term expression stability, predictable activity over several orders of magnitude, inducibility, and co-ordination of promoter function with cellular and bioproduction processes. Accordingly, natural promoters are of limited use in the context of CHO cell engineering, necessitating the design of novel promoters that are specifically optimized-for-purpose.

The purpose of this review is to discuss how promoter engineering strategies can be used to achieve precision control of gene expression in CHO cells, in order to provide a key enabling toolkit technology for CHO cell synthetic biology. First, we introduce promoter structure and present the mechanisms of transcriptional regulation that specifically affect promoter function during recombinant protein expression in CHO cells. We then outline the specific promoter functionalities that are required in the context of CHO cell engineering, before reviewing available promoter engineering strategies; presenting recent developments and evaluating the relative advantages and disadvantages of each approach. Finally, we conclude by summarizing how diverse promoter engineering techniques can be utilized cooperatively to construct promoters that are specifically designed for use in CHO cell engineering.

2. Promoter engineering design space

Promoters are defined as DNA sequences that function to specifically control the transcription rates of individual genes. They are comprised of two discrete structural components, the core and proximal regions, containing sequence-specific binding sites for general transcription factors (i.e. components of the pre-initiation complex (PIC)) and regulatory transcription factors (i.e. activators and repressors) respectively (Fig. 1) (Lenhard et al., 2012). Transcription factors (TFs) bind at cognate sites (or transcription factor regulatory elements, TFREs) within promoters and mediate regulation via co-regulators that act to increase or decrease the rate of rate-limiting steps in the transcription process, including chromatin opening, PIC formation, initiation, promoter escape, elongation and termination (Fuda et al., 2009). The nucleotide composition of a promoter therefore determines the frequency, intensity and duration of transcription in order to generate a gene-specific pattern of mRNA synthesis. Accordingly, there is considerable interest from promoter engineers in the creation of novel sequences with novel transcriptional outputs in order to specifically control the expression of genes of interest. The design space available to achieve this is vast, given that i) a typical mammalian cell contains thousands of TFs, corresponding to hundreds of discrete TFREs (Vaquerizas et al., 2009), ii) synthetic TFs recognizing synthetic TFREs can be engineered relatively easily (Lienert et al., 2014), and iii) transcription factor function can be regulated by binding site sequence, orientation, copy number and position (with respect to both the transcriptional start site and neighbouring TFREs) (Gertz et al., 2009; Jolma et al., 2013; Sharon et al., 2012; Todeschini et al., 2014; Weingarten-Gabbay and Segal, 2014). However, promoter engineering is not an exercise in pure abstraction and both the host cell background and the intended purpose of promoters needs to be carefully considered.

2.1. Transcriptional regulation in CHO cells is highly context-specific

Promoter functionality is highly context-dependent, exemplified by gene-specific expression profiles across different tissues and cell-types

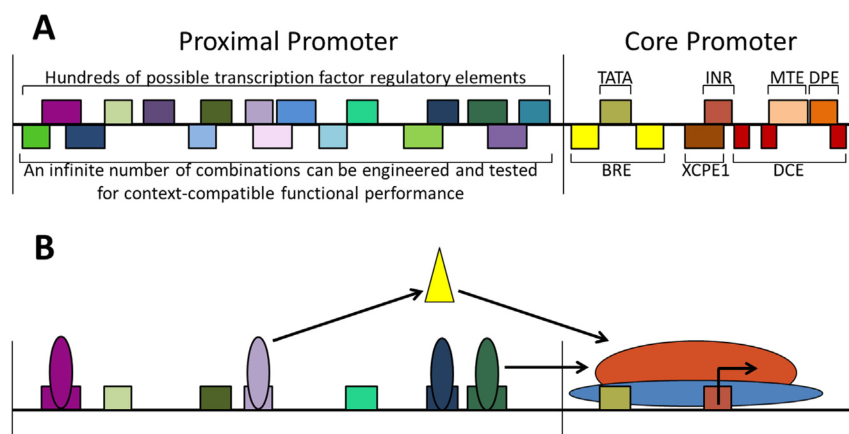

Fig. 1. Promoters with novel functionalities can be engineered by altering the composition of transcription factor binding sites. There is a limited design space to engineer core promoter regions, which contain binding sites for RNA polymerase II and general initiation factors, as there are relatively few well-characterized regulatory elements available (Lenhard et al., 2012). In contrast there are hundreds of known transcription factor regulatory elements (TFREs) that can be used to construct proximal promoter regions, and this design space is further expanded by the ability to design synthetic TFs with cognate synthetic TFREs (Garg et al., 2012; Mathelier et al., 2013). By using these TFREs in varying combinations (upstream of a core promoter), promoters with any desired functionality can be engineered (A). Panel B depicts how engineered promoters interact with components of the cellular transcriptional machinery. General transcription factors (shown as a complex; blue ellipse) bind to regulatory elements in the core promoter and facilitate binding of RNA polymerase II (red ellipse). TFs (varying coloured ellipses) bind specifically to cognate TFREs and function to regulate transcription by increasing or decreasing the rate of rate-limiting steps, such as pre-initiation complex formation, initiation and promoter escape. Regulation is mediated either by direct interaction with the general transcriptional machinery or via co-regulators (yellow triangle). *BRE = transcription factor for RNA polymerase IIB recognition element*; TATA = TATA *box*; *XCPE1 = X core promoter element 1*; *INR = initiator*; *DCE = downstream core element*; *MTE = motif ten element*; *DPE = downstream core promoter element.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012

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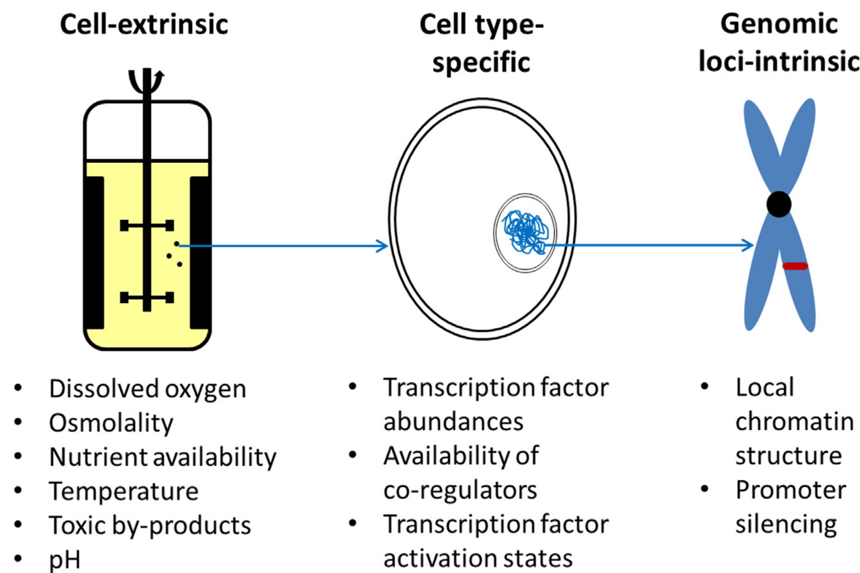

Fig. 2. Promoters are subject to highly context-specific sources of regulation during protein production in CHO cells. Promoter functionality is highly context-dependent, necessitating that promoters must be engineered specifically for their intended-purpose. In the context of recombinant protein expression in CHO cells, promoter performance is a function of i) dynamic changes in the extracellular environment (cell-extrinsic, examples shown), ii) CHO cells unique repertoire of transcriptional machinery components (cell type-specific), and iii) the local chromatin structure at the site of transgene integration (genomic loci-intrinsic). These three sources of transcriptional regulation need to be carefully considered when designing novel promoters for use in biomanufacturing.

(Consortium, 2014). Cell and condition-specific promoter activities are a function of three sources of transcriptional regulation — cell-extrinsic, cell type-specific and genomic loci-intrinsic (Voss and Hager, 2014). Fig. 2 illustrates how these regulatory levels specifically apply in the context of recombinant protein production in CHO cells. Cell-extrinsic regulation is caused by dynamic changes in the extracellular milieu. Bioreactor-grown CHO cells are subject to fluctuations in multiple interacting variables including dissolved oxygen, osmolality, nutrient availability, toxic by-product accumulation, temperature and pH (Li et al., 2010). Indeed, some changes are specifically employed, for example the utilization of hypothermic conditions to arrest cell growth, increase culture longevity, and enhance specific productivity (Masterton and Smales, 2014). Cellular stress imposed by these environmental changes can activate signal transduction pathways and subsequently alter the abundance and activation state of TFs within the nucleus (de Nadal et al., 2011). Accordingly, the activity of promoters containing cognate binding sites for these TFs will fluctuate, leading to increased or decreased gene expression. CHO cell promoter engineers therefore have to consider the impact of biomanufacturing processes on promoter function. For example cell-extrinsic noise could be minimized by removing appropriate TFREs from promoters, or utilized by designing promoters that specifically respond to parameter changes (Sumitomo et al., 2012).

Cell type-specific promoter regulation is a function of the cells unique repertoire of transcriptional machinery components. Regulation of promoter activity is highly cell type-specific due to different cells containing varying complements of TFs and co-regulators and, unsurprisingly, novel promoters developed for use in one mammalian cell type display highly variable activities in others (Schlabach et al., 2010; Vaquerizas et al., 2009). Accordingly, promoters should be engineered to specifically interact with the machinery available within the CHO cell factory. Following the sequencing of the CHO cell genome in 2011, numerous studies have started to decode the CHO cell transcriptome and proteome (Kumar et al., 2014; Vishwanathan et al., 2015; Xu et al., 2011). As the volume and quality of CHOmics data continues to increase we are beginning to decipher which TFs are present in CHO cells and their relative abundances. Although we are still far from understanding the complex post-translational modifications that control the activation states of these TFs (e.g. subcellular localization, DNA binding, regulatory function) (Filtz et al., 2014), this catalogue of transcriptional components enables construction of promoters that are specifically designed to exploit the machinery available in CHO cell factories.

Genomic loci-intrinsic promoter regulation is determined by the chromatin structure at the promoter. Formation of repressive chromatin conformations over a promoter prevents TFs from binding at target sites, thereby altering the promoter activity state (Keung et al., 2015). Transcriptionally inactive chromatin structures can form either transiently, as part of a dynamic cycle between 'open' and 'closed' configurations, or permanently (Voss and Hager, 2014). The latter is the primary consideration for CHO cell promoter engineers, as promoter silencing is a common cause of production instability (Kim et al., 2011). Genomic loci-intrinsic regulation is predominantly controlled in CHO cells by either using non-promoter elements, such as MARs, or specifically targeting transgenes into desirable genomic sites (Harraghy et al., 2015; Lee et al., 2015b). However, promoters can be engineered to enhance expression stability by removal or addition of sequence features that promote or prevent silencing respectively. For example, Curran et al. recently described the redesign of endogenous promoter sequences in silico in order to decrease nucleosome affinity (Curran et al., 2014).

2.2. Specific promoter design criteria are required for CHO cell engineering

Precise titration of functional effector gene expression levels will require the ability to fine-tune transcriptional rates over several orders of magnitude, and accordingly, promoters with activities spanning a broad dynamic range will be needed. In addition to this, promoters intended for use in CHO cell engineering will also require further, highly context-specific, design criteria. For example, promoter function must be co-ordinated with cellular and bioproduction processes. Any promoter introduced into CHO cells relies on components of the cellular transcriptional machinery (e.g. polymerases, TFs, co-regulators) for function. Where these components are in limited supply, heterologous promoters may potentially sequester them away from endogenous genes, causing changes in their expression levels (Brewster et al., 2014; Karreth et al., 2014). This is a particular concern in cell engineering, where multiple new promoters are being introduced into the host cell. Alterations in the cellular transcriptome could have significant

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effects on key cellular processes that underpin protein production. Accordingly, the impact of engineered promoters upon the cell needs to be carefully considered, and tested, to ensure that they are compatible with desirable cellular functionalities, such as proliferation and cell survival.

It is likely that, for the majority of effector genes, it will be desirable to not only control the level of expression, but also the timing. For example, biphasic bioreactor processes are often employed, whereby cell biomass is rapidly accumulated (proliferation phase) and subsequently maintained (production phase); sometimes associated with a shift to hypothermic conditions (37–32 °C) to increase culture longevity and enhance specific productivity (Masterton and Smales, 2014; Nam et al., 2009). Accordingly, it will presumably be optimal to express genes involved in proliferation during the proliferation phase (to maximize cell biomass accumulation), and switch them off during the production phase (to maximize productivity). Conversely, it is reasonable to assume that it would be optimal to express other effectors, such as anti-apoptotic genes, or genes involved in lactate metabolism, with the opposite expression kinetics (Le et al., 2013). For other accessory genes, such as ER molecular chaperones, it may be advantageous to adjust expression continually throughout culture according to the cells' need, in order to remove the metabolic burden of their expression when they are not required. The ability to synchronize promoter activity with other desirable bioproduction and cellular processes is therefore a necessary design criterion.

Recombinant gene expression in CHO cells is commonly unstable as a result of epigenetic silencing (Dahodwala and Sharfstein, 2014). As it has been shown that silencing can be mediated by the promoter driving recombinant gene expression, any promoter intended for use in CHO cells must be specifically designed to prevent this issue (Kim et al., 2011; Yang et al., 2010). Gene silencing can also be caused by changes in the chromatin structure at the transgene integration site (Bannister and Kouzarides, 2011; Harraghy et al., 2015). These positional effects can be minimized by i) using boundary elements to prevent the spread of silencing heterochromatin (Harraghy et al., 2015) or ii) targeting the transgene to desirable genomic sites known as ‘hotspots’ (Lee et al., 2015a). However, the function of both barrier elements and specific hotspots can vary when used in combination with different promoters (Ho et al., 2015; Hou et al., 2014; Nehlsen et al., 2009). Accordingly, promoters should be engineered to function cooperatively with other expression stability-enhancing technologies. Finally, gene silencing can also be caused by deletion of DNA segments via homologous recombination (Jasin and Rothstein, 2013; Moynahan and Jasin, 2010). Genes that are bordered by homologous sequence repeats are susceptible to genomic excision (Lambert et al., 1999; Read et al., 2004). Accordingly, the use of multiple copies of identical or similar sequences in multi-gene constructs, such as using the same promoter, or promoters that contain highly similar regions, to control expression of multiple effector genes, may predispose synthetic genetic circuits to failure. Therefore, multi-gene engineering strategies in CHO cells will require the design of diverse promoters that have minimal sequence homology. Indeed, promoters used cooperatively for cell engineering would ideally have few TFREs in common in order to both i) minimize the risk of TFs being titrated away from endogenous genes and ii) prevent promoter-promoter interference between recombinant transgenes competing for the same TFs.

Given the highly context-specific mechanisms of transcriptional regulation and required promoter design criteria, we may reasonably assume that specifically designed, built-for-purpose novel promoters will be required for CHO cell synthetic biology. These promoters can be constructed by either re-engineering natural sequences or designing completely synthetic elements. With respect to the latter, synthetic promoters can be designed to function either with or without cognate synthetic TFs. We now turn to discussing how promoters can be constructed using these three distinct routes of promoter engineering.

3. Engineering natural promoters

Natural promoters are typically isolated for use in a particular host cell background based on a single functionality (e.g. activity level). As they have evolved to function in a specific context (i.e. to control the expression of natural genes in whole organisms) it is highly unlikely that they will be optimal for use in unnatural processes, such as recombinant gene expression in CHO cells. However, natural promoters can be engineered to improve their performance. This is an inherently less comprehensive approach than constructing synthetic promoters from the bottom-up that are specifically designed-for-purpose. On the other hand, this strategy is simpler and quicker to implement, requires minimal a priori knowledge, and begins with some desirable promoter properties already ‘built-in’. In this regard, engineering natural promoters is somewhat analogous to using directed evolution, rather than large-scale genetic engineering, to develop improved cell factories.

With respect to CHO cells, known natural promoters available for engineering fall into two categories, being either endogenous or of viral origin. Multiple viral promoters have been utilized to drive recombinant gene expression in CHO cells, including the Human Cytomegalovirus immediate early (CMV-IE) 1, Mouse CMV-IE1, Rat CMV-IE1, Mouse CMV-IE2, Myeloproliferative sarcoma virus long terminal repeat (LTR), Rous sarcoma virus LTR, and Simian virus 40 early (SV40E) promoters (Chatellard et al., 2007; Spenger et al., 2004; Xia et al., 2006). Amongst these, the Human CMV-IE1 (hCMV-IE1) and SV40E promoters have been most widely employed in industry, commonly used to control transcription of product and selection marker genes respectively. Most viral promoters were originally used in CHO cells based on their ability to drive constitutive high levels of recombinant gene expression. However, this property makes them likely to induce downstream bottlenecks in translation and ER folding/assembly processes. Moreover, they have also been shown to be prone to epigenetic silencing by cytosine methylation within CpG islands (Kim et al., 2011; Yang et al., 2010).

CHO cells express thousands of genes, meaning that there are likely thousands of corresponding endogenous promoters with unique regulatory properties that have evolved to utilize the pre-existing transcriptional activation machinery of the host cell (Xu et al., 2011). However, it is a significant challenge to determine the genomic regulatory sequences that control expression of specific genes. Consequently, very few CHO endogenous promoters have been characterized to date. However, it is likely that the number of available promoters will increase significantly in the near-future as the availability of transcriptomic datasets enables the identification of promoters with diverse activities and expression dynamics (Becker et al., 2014; Bort et al., 2012; Doolan et al., 2013; Kang et al., 2014; Rupp et al., 2014). For example, using this approach, promoters have recently been identified that are preferentially active in late stage culture and under hypothermic conditions (Le et al., 2013; Thaisuchat et al., 2011). The best characterized endogenous promoter to date uses regulatory sequences from the highly expressed elongation factor 1 alpha (EF1α) gene (Deer and Allison, 2004). Whilst this promoter enables high levels of stable gene expression, it requires the use of two distinct multi-kb elements. Indeed, endogenous promoters are typically very large, which can potentially reduce transfection efficiencies, and limits their use in multigene vectors. Ultimately, like viral promoters, endogenous promoters typically have a range of both desirable and undesirable functionalities, making them prime candidates for improvement by engineering.

3.1. Strategies to engineer natural promoters

As shown in Fig. 3, natural promoters can be engineered by deleting, inserting, mutating, or combining sequence elements. The simplest of these methods is sequence truncation in order to either i) reduce promoter size without affecting activity via deletion of non-functional regions or ii) create promoter variants with altered activities by removing functional elements (e.g. TFREs that regulate promoter activity).

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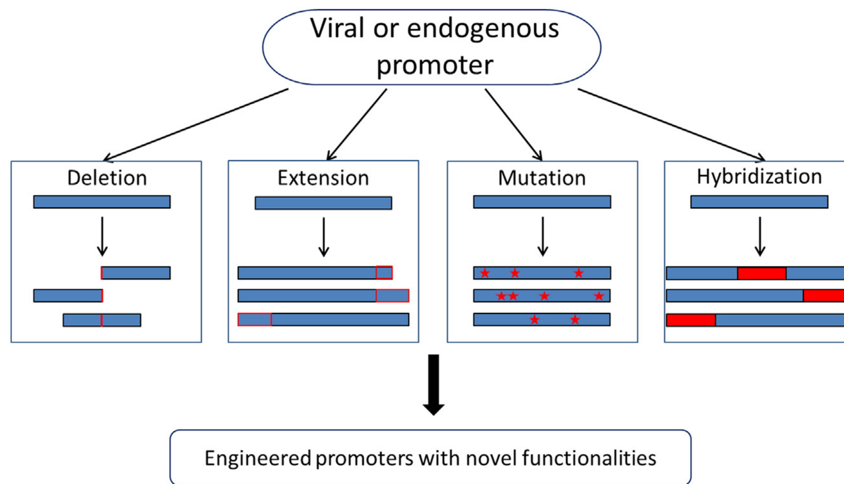

Fig. 3. Naturally occurring promoters can be engineered to improve their functionality. The function of a natural promoter can be altered by i) deleting sequence regions to remove functional or non-functional elements, ii) extending the naturally occurring sequence from either terminus to incorporate additional regulatory elements, iii) introducing random or specific sequence mutations, or iv) hybridizing it with regulatory regions from distinct natural promoters to incorporate multiple discrete functionalities into a single sequence.

With respect to the former, CHO S100a6, ferritin heavy chain and CHEF1α promoters could be truncated by 1.3 kb, 3.9 kb and 7.6 kb respectively without reducing their activities in CHO DG44 cells (Deer and Allison, 2004; Prentice et al., 2007; Thaisuchat et al., 2011). With respect to the latter, Fan et al. constructed SV40E promoter variants exhibiting 61% and 44% of wild-type SV40E activity in CHOK1SV cells by removing both 72-bp repeats and one or two 21-bp repeats from the promoter respectively (Fan et al., 2013). Conversely, Chen and colleagues were able to increase the activity of a CHO endogenous promoter sequence (isolated by a promoter trap strategy) over 4-fold in CHO-K1 cells via a 708 bp 5′ truncation; suggesting that unidentified repressive elements had been deleted (Chen et al., 2013). An analogous method to truncation is extension, whereby natural sequences are extended to incorporate additional regulatory regions in order to enhance promoter function. For example, Mariati et al. utilized 542 bp 5′ and 824 bp 3′ extensions of the hCMV-IE1 promoter, known to function as boundary/insulator and translation-enhancing elements respectively, to increase stable gene expression over 3-fold in CHO-K1 cells (Lashmit et al., 2004; Mariati et al., 2010; Skoko et al., 2011; Stinski and Isomura, 2008).

Mutagenesis can be employed to generate multiple promoter variants that exhibit variable activity compared to the wild-type sequence. ‘Negative’ mutations (e.g. mutations within TFREs that reduce affinity for cognate activators) will occur much more frequently than ‘positive’ ones, and accordingly mutated promoters are usually weaker than the original promoter (Kwasnieski et al., 2012; Melnikov et al., 2012; Patwardhan et al., 2012). However, by selecting a ‘parent’ promoter with high activity, mutagenesis can be used to isolate promoters with a wide-range of useful novel activities. This can be achieved via targeted mutations within known regulatory elements; for example we recently mutated NFκB, CRE and YY1 binding sites in the hCMV-IE1 promoter to create variants with a 5-fold range of activities in CHO-S cells (Brown et al., 2015). Alternatively, random mutagenesis can be utilized, using methods such as error-prone PCR, to introduce mutations randomly throughout the sequence. Ferreira and co-workers used this strategy to construct a library of hCMV-IE1 and human EF1α promoter variants exhibiting a 40-fold expression range (Ferreira et al., 2011).

Natural promoters are commonly isolated based on their exhibition of an individual desirable functionality, such as activity level, expression dynamics or expression stability. In order to incorporate multiple desired functions into single sequences, regulatory regions from multiple discrete promoters can be rationally combined to construct novel promoter-hybrids. Sumitomo et al. used this strategy to construct a promoter capable of conditional high activity under hypothermic conditions by combining a regulatory region from the cold-inducible RNA-binding protein (cirp) promoter with the highly active hCMV-IE1 promoter (Sumitomo et al., 2012). Identification of the element within the cirp promoter responsible for conferring the 32 °C-response enabled design of a hybrid promoter that exhibited a 2.6 fold increase in transcriptional activity at 32 °C compared to 37 °C in CHO-K1 cells (where wild-type hCMV-IE1 showed no difference between the two temperatures). A similar approach has also been used to create a promoter hybrid capable of maintaining high levels of gene expression under hypoxic conditions in CHO cells, by combining a regulatory element from the hypoxia-inducible erythropoietin promoter with the hCMV-IE1 promoter (Moon et al., 1997). Finally, Mariati and colleagues used a promoter combination strategy to construct a highly active promoter with enhanced resistance to promoter silencing (Mariati et al., 2014). A sequence element from the hamster adenine phosphoribosyltransferase promoter that is known to provide protection against DNA methylation was inserted into varying positions within the hCMV-IE1 promoter. Amongst the resulting hybrids, promoters were isolated that exhibited significantly enhanced expression stability compared to wild-type hCMV-IE1 in both CHO-K1 stable clones and CHO-DG44 stable pools.

4. Constructing synthetic promoters

Synthetic promoters are artificially constructed assemblies of sequence elements that function cooperatively to drive and modulate transcription. Specifically developed using ‘natural’ and/or ‘unnatural’ building blocks, the design space is theoretically unlimited, enabling construction of promoters with any desired functionality. However, whilst it is therefore advantageous to construct specifically designed promoters from the bottom-up, it is also typically a far more complex and time-intensive process than re-engineering natural promoters. Nevertheless, given the continuing rapid advances in both promoter engineering methodologies and our understanding of transcriptional control, synthetic promoters have enormous potential for CHO cell synthetic biology. Diverse synthetic promoter engineering strategies can include varying combinations of DNA (e.g. TFREs), RNA (e.g. clustered regularly interspaced short palindromic repeats (CRISPR) guide RNAs (gRNAs)) and protein (e.g. synthetic DNA binding domains (DBD)) building blocks; here, we separate them into two broad categories, depending on whether they are used with or without co-expression of a cognate synthetic TF.

Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012

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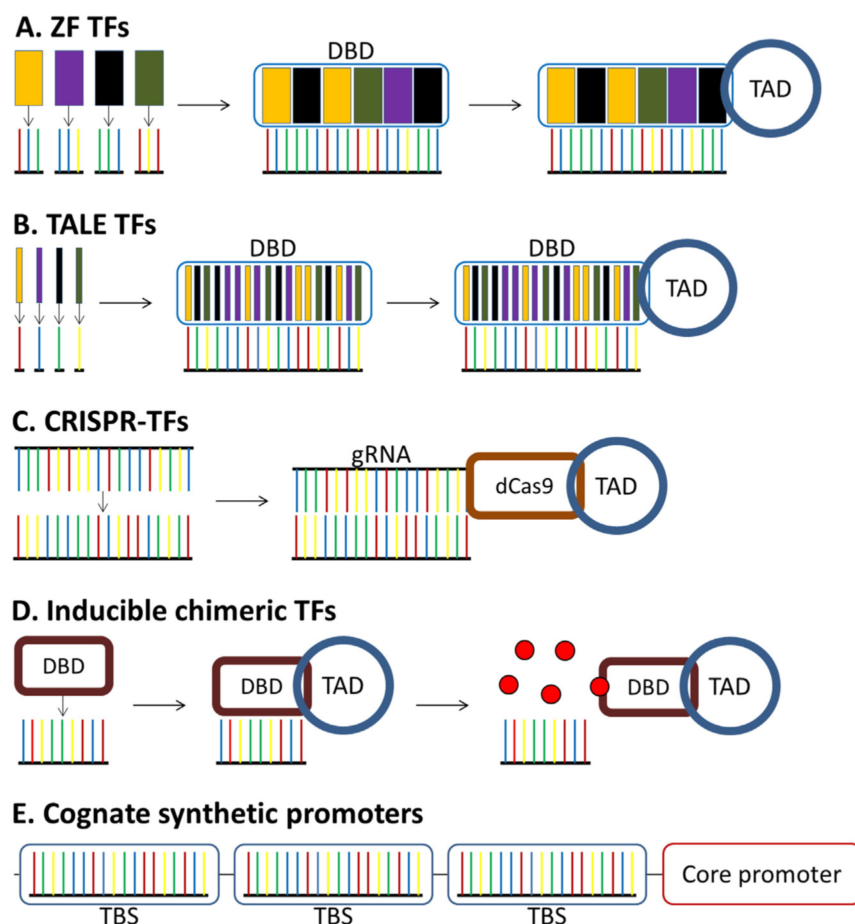

Fig. 4. Synthetic transcription factors can be designed to specifically regulate transcription from cognate synthetic promoters. Synthetic DNA-binding domains (DBD) can be constructed to target any DNA sequence by assembling discrete combinations of either A) zinc finger (ZF) domains or B) transcription activator-like effector (TALE) domains, that recognize 3 bp or 1 bp motifs respectively. These custom DBDs can then be functionalized with transactivation domains (TAD) to construct sequence-specific synthetic transcription factors. CRISPR-TFs (C) can be created by fusing an endonuclease-deficient cas9 (dcas9) to a TAD, and targeted to a user-defined sequence by co-expression of a complementary guide RNA (gRNA). Finally, chimeric-TFs (D) can be constructed by fusing TADs to naturally occurring DBDs. These systems typically utilize trigger-responsive DBDs, such that activity of the synthetic TF can be regulated by addition of inducer molecules (shown as red circles). Synthetic TFs can be used to specifically regulate the activity of target cognate synthetic promoters. Whilst varying promoter architectures can be created, the most common design consists of multiple copies of the target binding site (TBS) upstream of a minimal core promoter (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Synthetic promoters used in conjunction with cognate synthetic TFs

A major advantage of synthetic TF-based approaches is their orthogonality, meaning that their function has minimal effect on the cell, and the cell has minimal effect on their function (Blount et al., 2012; Stanton et al., 2014). As depicted in Fig. 4, Synthetic TFs can be constructed via four distinct routes, namely CRISPR-TFs, zinc finger (ZF) TFs, transcription activator-like effector (TALE) TFs, or chimeric TFs. Of these, the latter has been most commonly utilized in CHO cells to date, typically assembled using a bacterial DNA binding protein and a mammalian transactivation/transsilencing domain (Ausländer and Fussenegger, 2013). Given the large number of naturally evolved trigger-responsive DNA binding proteins available, chimeric TFs are particularly useful for designing inducible gene-expression systems (Bacchus et al., 2013; Rössger et al., 2014; Weber et al., 2008). Such systems typically use synthetic promoters containing multiple copies of the synthetic TF binding site either i) upstream of a minimal core promoter or ii) downstream of a strong viral promoter, such as hCMV-IE1. By using these two promoter architectures in association with either transsilencing or transactivating chimeric TFs, systems can be constructed that tune transcriptional output 'up' or 'down' in response to the inducer in a dose-dependent manner (Ausländer and Fussenegger, 2013).

In order for these systems to have application in biomanufacturing, the nature of the inducer molecule must be carefully considered to avoid potential regulatory issues, prohibitively high costs and/or cytotoxic side effects. For example, Gitzinger et al. recently described the development of a system utilizing the 'regulatory-friendly' licenced food-additive vanillic acid as an inducer, and showed that it had no effect on CHO cell physiology (Gitzinger et al., 2011). Moreover, multiple light-controlled systems have been designed, avoiding the inherent issues in downstream processing and product validation associated with chemical inducers (Müller et al., 2013; Müller et al., 2014; Ye et al., 2011). However, as these systems use bipartite synthetic TFs, and therefore require the expression of two hybrid proteins, they require more complex vectorology with the risk of increased metabolic burden on the cell factory and instability.

Synthetic DNA-binding domains enable a massively expanded design space, compared to chimeric TFs, allowing construction of synthetic TFs that can recognize any user-defined sequence. ZF proteins and TALEs both have modular structures where single repeat domains consisting of either ~30 or ~34 amino acids recognize 3 bp or 1 bp sequences respectively (Gaj et al., 2013). Accordingly, repeat modules can be fused together to construct DBDs with novel binding specificities. Functionalizing such domains with a eukaryotic transactivation domain creates a sequence-specific synthetic TF that can be utilized to

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transactivate synthetic promoters containing the designed recognition sequence (Garg et al., 2012; Lohmueller et al., 2012). Tunability can be achieved by adjusting the specificity of DNA binding, the transactivation domain, or the number of recognition sites within the promoter, to create promoter variants with activities ranging over multiple orders of magnitude (Khalil et al., 2012; Li et al., 2012; Perez-Pinera et al., 2013b). However, construction of novel ZF and TALE-based TFs can be a difficult, time and labour intensive operation, involving multi-step DNA assembly, selection, screening and optimization processes.

CRISPR-TFs, adapted from bacterial CRISPR/Cas systems, offer programmable, tuneable DNA binding without necessitating complex protein engineering. A mutagenized endonuclease-deficient cas9 (Qi et al., 2013) fused to a transcriptional activation domain can be targeted to function as a sequence-specific transactivator at any DNA sequence via a customizable gRNA) (Perez-Pinera et al., 2013a). By placing 'target sites' (i.e. sequences complementary to the gRNA) upstream of a minimal core promoter, CRISPR-TFs can be utilized to specifically transactivate a synthetic promoter designed in silico (Cheng et al., 2013; Gilbert et al., 2013). The ability to construct orthogonal, robust, and tuneable synthetic TF-promoter systems via simple alteration of an RNA sequence, without requiring laborious design and development stages, offers significant advantages over protein engineering-based approaches. Despite this, the application of CRISPR-TFs in mammalian cells was initially limited by the very low levels of transcriptional output achievable (Cheng et al., 2013; Hu et al., 2014; Maeder et al., 2013). However, a number of recent developments have substantially increased activities by designing systems that mimic the natural transcription process, where multiple TFs binding at a single promoter function synergistically to significantly enhance transactivation. These include i) increasing the number of target sites in the synthetic promoter (Farzadfard et al., 2013), ii) fusing multiple activation domains in tandem to cas9 (Chavez et al., 2015), iii) expressing multiple discrete gRNAs from a single transcript to target multiple cognate target sites in the synthetic promoter (Nissim et al., 2014), and iv) functionalizing cas9 with a polypeptide scaffold to recruit multiple copies of a separately expressed transactivator (Tanenbaum et al., 2014).

4.2. Synthetic promoters used without synthetic TFs

Synthetic promoters built for use in isolation (i.e. without a complementary synthetic TF) are designed to interact with the host cells existing repertoire of transactivators and transsilencers. Harnessing the cells endogenous transcriptional machinery obviates the need to design and express accessory TFs, and enables relatively rapid construction of diverse promoters whose function is co-ordinated with cell physiology. However, due to the complexity of cell systems, it can also lead to undesirable effects on the function of both the promoter (e.g. fluctuations in abundance/activity of intracellular TFs affecting promoter activity) and the cell itself (e.g. titration of TFs away from endogenous genes affecting their expression levels) Brewster et al., 2014; Hansen and O'Shea, 2013; Karreth et al., 2014). Accordingly, to enable rational design of promoters with predictable functions, synthetic promoters should be constructed using building-blocks that have been well-characterized by modelling and testing (Silver et al., 2014). However, thorough testing of parts can be time and labour intensive, and, as such, synthetic promoters often have to be built using poorly-characterized components.

Whilst multiple synthetic promoter libraries have been developed for use in prokaryotes (Rytter et al., 2014; Sohoni et al., 2014; Yim et al., 2013), yeast (Blazeck et al., 2012; Vogl et al., 2013) and plants (Lehmeyer et al., 2015; Liu et al., 2014), here we focus on mammalian cell-based strategies. As shown in Fig. 5, mammalian synthetic promoters are commonly constructed by randomly assembling TFRE building-blocks that possess desirable characteristics, such as activity in the intended host cell or responsiveness to a specific trigger, upstream of a minimal core promoter. Using this methodology, promoter libraries have been designed that i) respond to specific stimuli, such as radiation (Ogawa et al., 2013), oxidative stress (Watanabe et al., 2009), and glucose levels (Han et al., 2011), and ii) function specifically in diverse cell types, including macrophages (He et al., 2006), hepatocytes (Han et al., 2011), neurons (Kawashima et al., 2013), and myocytes (Jianwei et al., 2012; Li et al., 1999). As an example, in order to construct promoters for use in liver-directed insulin gene therapy, Han et al. utilized liver-active (HNF-1, C/EBP) and glucose-responsive (GlRE) TFREs to build a library of hepatocyte-specific, glucose-inducible synthetic promoters with a >20 fold range of activities (Han et al., 2011). We recently utilized the TFRE-assembly technique to construct the first libraries of synthetic promoters designed specifically to function in CHO cells (Brown et al., 2014). By identifying CHO-active TFREs we were able to construct 140 synthetic promoters that exhibited variable activity over two orders of magnitude in transiently transfected CHO-S cells. Despite this, the application of TFRE-assembly methods are currently limited in most cell-types (including CHO) by the availability of well-characterized TFRE-blocks that have been tested for properties

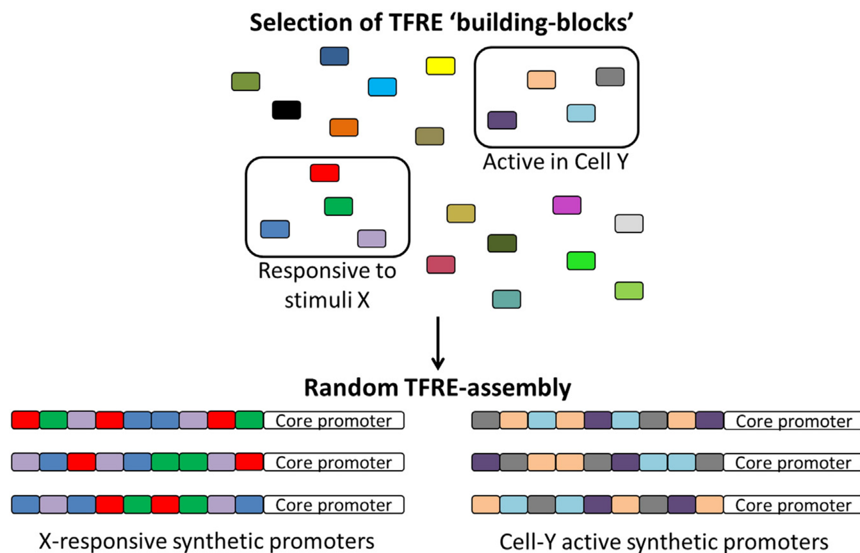

Fig. 5. Context-specific synthetic promoters can be constructed from characterized building blocks. Transcription factor regulatory elements (TFREs) can be characterized through modelling and testing to determine TFRE-specific activity-profiles. TFREs with appropriate properties can then be selected as building blocks and randomly assembled to construct synthetic promoters with user-specified functionalities.

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such as stimuli-responsiveness, TF binding affinities, TF–TF interactions, and spatiotemporal regulation. However, via in silico analyses of diverse transcriptomic datasets, and in vitro use of massively parallel high-throughput (HT)-screening techniques, hundreds of TFRE parts can now be characterized simultaneously, enabling identification of novel synthetic promoter construction design spaces (Kheradpour et al., 2013; Melnikov et al., 2012; Mogno et al., 2013; Nishikata et al., 2014).

Synthetic promoters can also be constructed without bias for known TFRE sequences. Rationalizing that TFRE consensus motifs are suboptimal representations of actual functional TF binding sites, Grabherr et al. built synthetic promoters based on the nucleotide composition (defined as "*the frequency patterns of mono-nucleotides*, *di-nucleotides*, *tri-nucleotides*, *etc*") of 1746 highly active endogenous human promoters (Grabherr et al., 2011). Identification of promoter-like features (e.g. CpG content) enabled in silico design of a transcriptionally active synthetic promoter library. Employing a completely random approach, Schlabach and colleagues screened the transcriptional activity of >50,000 100-mer sequences, comprising every possible 10-mer sequence in 10× repeat copies upstream of a minimal core promoter, and isolated synthetic promoters that were as potent as hCMV-IE1 in HeLa cells (Schlabach et al., 2010). The drawback with such approaches is the difficulty in identifying features underpinning promoter behaviour, preventing rational improvement or confident prediction of their functionality. However, as unbiased methods explore the entire design space they theoretically enable isolation of the 'ideal' promoter for any purpose, and, given the availability of HT screening techniques, finding such promoters may indeed be tractable (Mogno et al., 2013).

5. Concluding remarks and future perspectives: utilizing promoter engineering strategies to enable CHO cell synthetic biology

In summary, there are three divergent routes available to engineer promoters for use in CHO cell synthetic biology. New promoter technology can be developed by i) optimizing endogenous promoters that have been identified by transcriptomics profiling, ii) designing transcriptional control systems that use synthetic TFs to regulate the activity of target synthetic promoters, or iii) constructing synthetic promoters from characterized building-blocks. Each of these strategies could be used to design promoters that have the specific functionalities required in the context of CHO cell engineering. For example, transcriptional control over a broad dynamic range in CHO cells could be achieved by engineering endogenous promoters of genes that have varying expression levels, modifying promoter architecture and/or protein domains in synthetic TF-based systems, or constructing libraries of synthetic promoters with varying building-block compositions. Similarly, the timing of gene expression could be controlled by engineering endogenous promoters of genes with appropriate spatiotemporal expression profiles, designing trigger-inducible synthetic TFs, or constructing synthetic promoters from TFRE blocks that have variable activity during bioproduction processes. Homologous recombination-mediated silencing in multigene circuits could be avoided by engineering endogenous promoters that have minimal sequence homology or constructing libraries of synthetic promoters with unique TFRE-complements. This would also minimize the risk of altering the host cell transcriptome via TF-titration, although of course the ultimate defence against this potential problem would be achieved by designing synthetic TF-based systems. Finally, protection against heterochromatin silencing may be improved by (re)designing endogenous and synthetic sequences in silico to optimize both their CpG content and nucleosome affinity (Curran et al., Li et al., 2014). By rationally utilizing the available design space in this way, promoters that exhibit all desired functionalities for CHO cell engineering can be constructed.

Ultimately, the most successful approaches will likely use diverse promoter engineering strategies in cooperation. For example, endogenous promoters contain a rich resource of useable sequence features, including i) elements that enable their long-term expression stability and ii) regulatory regions that mediate the discrete expression dynamics of co-regulated genes (Clarke et al., 2012; Deer and Allison, 2004; Roider et al., 2009). These features could be utilized as building blocks, together with elements designed in silico, to construct optimized synthetic promoter libraries using block-assembly methods. Alternatively, they could be used in the design of synthetic promoters for use in synthetic TF-based systems, to help prevent silencing of the target promoter during long-term culture. With respect to these systems, they typically use a strong viral promoter such as hCMV-IE1 to drive expression of the synthetic TF. Given the problems associated with using such viral promoters (e.g. cell stress, promoter silencing), these systems could likely be improved for use in the context of CHO cell engineering by replacing them with specifically designed synthetic, or engineered endogenous, promoters.

In order to fully realize the potential of promoter engineering in CHO cells, a number of associated enabling technologies will also likely need to be developed. For example, development of compatible translational control elements will help ensure that engineered promoters behave predictably and reliably when assembled into complete expression cassettes (Mutalik et al., 2013). Further, the establishment of standardized units of promoter activity would enable robust functional comparison of promoters that have been constructed in different academic and industrial laboratories worldwide (Radeck et al., 2013). Moreover, given that it is intractable to characterize the performance of hundreds of promoters in stable cell lines, it will be desirable to design HT microtitre plate-based screening platforms that enable rapid prediction of promoter function in bioreactor-based stable expression systems. Finally, it is critical that complementary boundary elements and/or site specific integration technologies are developed to ensure that specifically designed promoter functionalities are not lost as a result of genomic loci-intrinsic transcriptional regulation (Harraghy et al., 2015; Lee et al., 2015a).

In conclusion, precise control of transcription is a key enabling technology required for CHO cell synthetic biology. The ability to precisely tailor the expression dynamics of multiple effector genes simultaneously will allow construction of synthetic CHO cell factories in order to enable both (i) reverse engineering of existing stable cell factories to render failed products manufacturable and (ii) forward engineering of new cell factories and products with predictable manufacturing properties. However, context-specific mechanisms of transcriptional regulation, and highly-specific promoter functionality requirements, necessitate that promoters must be carefully designed for use in CHO cell synthetic biology.

There are multiple distinct routes available to create novel built-for-purpose promoters and, given their relative associated advantages and disadvantages, it is likely that the most successful approaches in CHO cells will use multiple strategies cooperatively to construct sophisticated gene expression control systems.

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Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012

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Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012

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Please cite this article as: Brown, A.J., James, D.C., Precision control of recombinant gene transcription for CHO cell synthetic biology, Biotechnol Adv (2015), http://dx.doi.org/10.1016/j.biotechadv.2015.12.012