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RESEARCH ARTICLE

Cell Culture and Tissue Engineering

# CHO synthetic promoters improve expression and product quality of biotherapeutic proteins

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## Abstract

When expressing complex biotherapeutic proteins, traditional expression plasmids and methods may not always yield sufficient levels of high-quality product. High-strength viral promoters commonly used for recombinant protein (rProtein) production in mammalian cells allow for maximal expression, but provide limited scope to alter their transcription dynamics. However, synthetic promoters designed to provide tunable transcriptional activity offer a plasmid engineering approach to more precisely regulate product quality, yield or to reduce product related contaminants. We substituted the viral promoter CMV with synthetic promoters that offer different transcriptional activities to express our gene of interest in Chinese hamster ovary (CHO) cells. Stable pools were established and the benefits of regulating transgene transcription on the quality of biotherapeutics were examined in stable pool fed-batch overgrow experiments. Specific control of gene expression of the heavy chain (HC):light chain (LC) of a Fab, and the ratio between the two HCs in a Duet mAb reduced levels of aberrant protein contaminants; and the controlled expression of the helper gene XBP-1s improved expression of a difficult-to-express mAb. This synthetic promoter technology benefits applications that require custom activity. Our work highlights the advantages of employing synthetic promoters for production of more complex rProteins.

KEYWORDS

DTE, duet mAb, fab, precise promoter activity, synthetic promoter

## 1 | INTRODUCTION

With the exponential increase in the number of more complex and non-traditional recombinant proteins (rProteins) entering therapeutic pipelines for the treatment of a range of diseases, the biopharmaceutical industry is facing new challenges in the manufacturing and purification of these novel modalities.[1] Some of these new molecular formats are difficult-to-express (DTE) and

pose challenges in achieving suitable product yields and quality.[2] Furthermore, expression of some formats, such as antigen binding fragments (Fabs) and Duet monoclonal antibodies (Duet mAbs), can generate partially- or incorrectly assembled by-products, which are often difficult to remove using standard purification processes.[3,4] The current Chinese hamster ovary (CHO) protein expression platforms do not appear to be optimal to produce these rProteins and require the implementation of new tools and technologies.

One of the key strategies to improve the expression of complex biotherapeutics containing heterologous subunits is the optimal relative expression of the corresponding product coding genes. In the example of an antibody-based molecule, enhanced transient expression can be achieved by modulating the molar ratio of heavy chain (HC):light chain (LC) genes, which can be realized via co-transfection of varying proportions of single gene expression vectors.[5] However, the molar ratio achieved from this co-transfection approach is not predictable in stable cell lines, due to the variation in transfection efficiency and chromosomal integration, and it does not allow fine-tuning of relative gene expression levels. Recent advances in CHO synthetic biology have led to the development of libraries of gene components, such as promoters, secretory peptides, 5′ and 3′ UTRs, which offer new ways to precisely regulate rProtein gene transcript and protein levels.[6–13]

CHO cell engineering approaches to improving rProtein production include the co-expression of effector genes involved directly or indirectly in biosynthetic pathways, such as the secretory pathway or cell metabolism, to alleviate post-transcriptional bottlenecks for rProtein production.[14] Studies assessing the impact of a variety of effector genes on transient and stable rProtein expression show that the optimal stoichiometry of these effector and product genes is needed.[7] Driving transcription of the effector genes from synthetic promoters offers a simple approach to enable the required control of expression levels.

We previously developed a panel of synthetic promoters that are specific for CHO cell culture (sequences of in silico designed synthetic promoters: DNA Data Bank of Japan, accession numbers LC270626–LC270639), by rationally combining transcription factor regulatory elements (TFREs) from CHO cells to create novel promoters and demonstrated highly predictable transient and stable transcriptional activities and expression of the reporter secreted alkaline phosphatase (SEAP) protein.[15,16] In this new study, we sought to evaluate the ability of these synthetic promoters to precisely control stable expression of industrially relevant rProteins in CHO cells, to improve protein production yield and enhance product quality of three different molecular formats. We used synthetic promoters to fine-tune the HC transcript expression levels for a Duet mAb to investigate reduction of the levels of a half-antibody contaminant; modulate the overexpression level of the host cell helper gene X-Box binding protein (XBP-1) to improve expression of a DTE mAb and to establish a more elegant control of HC and LC expression levels of a Fab to reduce unfavorable light chain dimer formation.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and culture conditions

Suspension adapted CHO-K1 host cells (AstraZeneca) were cultured in CD CHO medium (Life technologies, UK) supplemented with 6 mM L-glutamine (Life technologies, UK). Stably and transiently transfected CHO cells were grown in CD CHO or AstraZeneca proprietary medium supplemented with either methionine sulfoximine (MSX; Sigma–Aldrich, UK) or with hygromycin (Roche Diagnostics GmbH, Germany). Cells were cultured in a humidified incubator at 36.5°C, 6% $CO_2$ with agitation at 140 rpm.

### 2.2 | Expression plasmids

The synthetic promoters were developed in silico by Brown, Gibson, Hatton and James.[15] They vary in relative promoter units (RPUs) of transcriptional activity and were positioned upstream of the human cytomegalovirus major-intermediate early (hCMV-MIE) core promoter (Figure 1a).

#### 2.2.1 | Expression plasmids for transient gene expression

The mAb transient expression plasmids (Figure 1b) were derived from constructs described by Persic, et al.[17] by insertion of the Epstein–Barr virus (EBV) origin of replication (*OriP*). Subsequent cloning steps replaced the routinely used EF-1α promoter in transient expression system in-house, with different synthetic promoter sequences.

#### 2.2.2 | Expression plasmids for stable gene expression

Single chain Fc-fusion (Figure 1c) and Duet mAb (Figure 1d) expression plasmids—the sequences of the different synthetic promoters were inserted upstream of the coding sequences of the Fc-fusion/Duet mAb polypeptides by standard restriction enzyme digestion and ligation methods, to replace the routinely used CMV promoter in stable expression.

DTE mAb & XBP-1 multi-gene expression plasmid—Multi-gene expression vectors (MGEVs) encoding the DTE mAb HC & LC and spliced XBP-1 (XBP-1s) were constructed by Golden Gate assembly as described in Patel, et al[18] In brief, each MGEV was constructed by cloning the relevant transcription units (promoter, signal peptide, protein coding DNA and polyadenylation sequences) into a destination expression vector pEXP-Vec-GG, by Golden Gate assembly (New England Biolabs, UK) according to manufacturer's protocol. Successful MGEV construction was confirmed by restriction enzyme digestion and

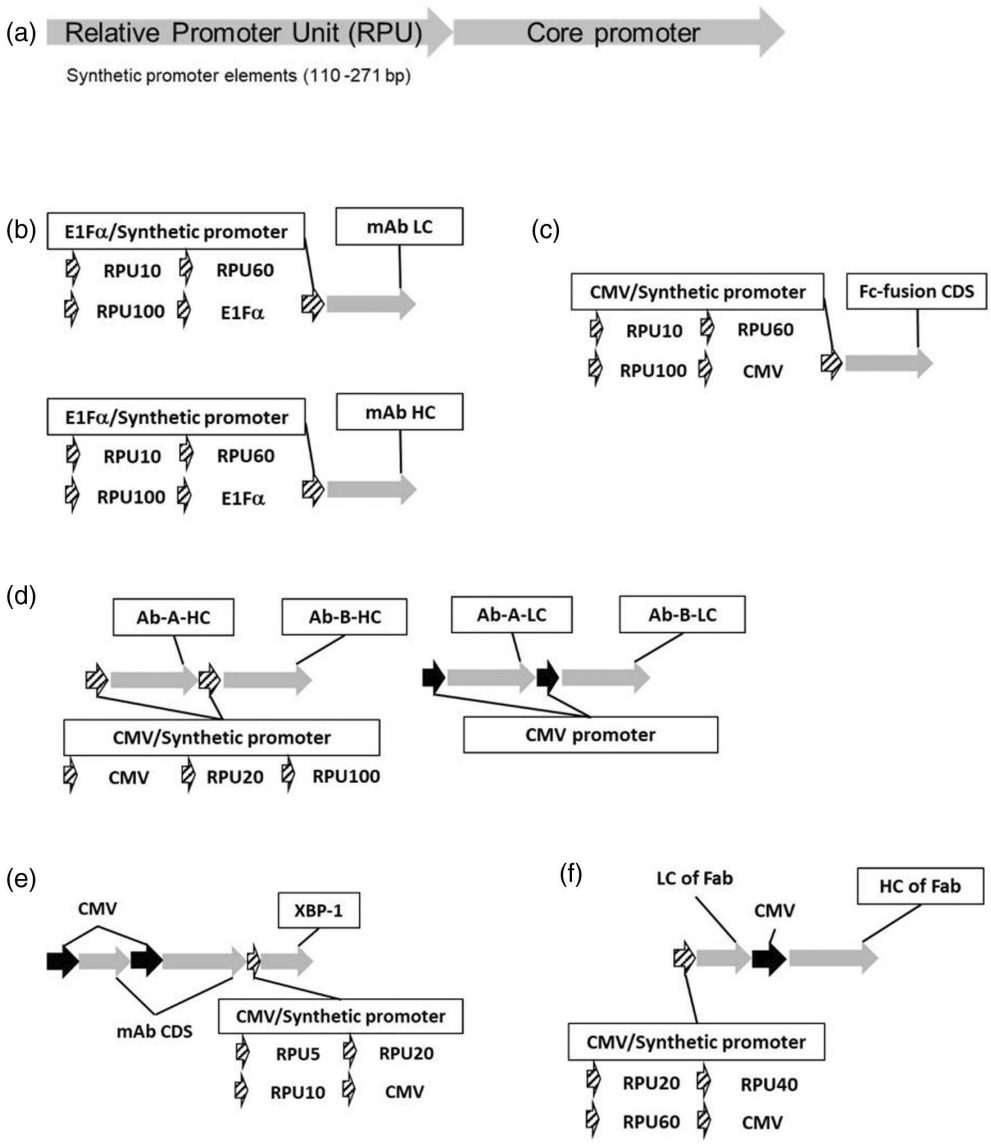



**FIGURE 1** Schematic depicting the design of a synthetic promoter and all expression constructs used in this study. (a) A synthetic promoter comprises of a relative promoter unit that is situated upstream of a core promoter. Figure adapted from Brown, Gibson, Hatton and James.[15] Structure of constructs used for (b) transient gene expression of a mAb, (c) stable gene expression of an Fc-fusion protein, (d) Duet mAb expression, (e) overexpression of XBP-1 along with a DTE mAb and (f) expression of a Fab molecule. CDS, coding sequence

DNA sequencing. In the final DNA plasmids, the LC and the HC were expressed by the hCMV-MIE promoters, while the expression of XBP-1s was driven by synthetic promoters of varying strengths (Figure 1e). Each expression cassette contains its 5′UTR, promoter, target gene coding sequence, polyA tail and a 3′UTR, regardless of it being a single or a tandem expression vector.

Fab expression plasmid (Figure 1f)—the expression plasmids for a Fab were constructed by Gateway® cloning (Invitrogen, USA). Sequences of the proximal region of various synthetic promoters (RPU 20, 40, and 60) and the hCMV-MIE core promoter were cloned upstream of the Fab LC expression cassette using standard restriction enzyme digestion and ligation methods. The HC promoter sequences remained unchanged. Each expression cassette contains its 5′UTR, promoter, target gene coding sequence, polyA tail and a 3′UTR, regardless of it being a single or a tandem expression vector.

## 2.3 | Transient production of mAb

Expression plasmid DNA was transfected into a transient CHO host cell line (AstraZeneca) as described in Daramola, et al.[19] Supernatant samples were collected on harvest day for mAb titre quantification, using a protein A high-performance liquid chromatography (HPLC) on an Agilent HP1100 or HP1200 (Agilent Technologies, Santa Clara, CA) by comparing peak size from each sample with a calibration curve, where Protein A binds to the CH3 region of the Fc-domain of the mAb, as described in Daramola, Stevenson, Dean, Hatton, Pettman, Holmes and Field.[19]

## 2.4 | Production of rProtein by stable CHO pools

Stable CHO pools expressing the rProtein were generated by transfecting CHO host cells with an expression plasmid(s) encoding the rProtein and at least one selectable marker using an Amaxa

nucleofector and reagents (Lonza, Germany). Fab and Duet mAb stable pools were generated by random integration of expression plasmid DNA, while DTE mAb-XBP-1 pools were generated using a targeted integration approach. The transfected cells were selected and maintained in CD CHO in the presence of MSX, and with the addition of puromycin for pools expressing the Duet mAb. For DTE mAb-XBP-1, an expression plasmid encoding a Cre-recombinase was co-transfected with the rProtein expression plasmid, where the expression cassette was inserted into a pre-defined CHO locus by Cre-Lox recombination via a pre-established landing pad in the genome. Transfectants were selected and maintained in CD CHO supplemented with hygromycin (Roche Diagnostics GmbH, Germany).

Cell density and viability of stable pools were assessed regularly by trypan blue exclusion (Vicell XR, Beckman, UK) following transfection and selection. Cultures were expanded and used for rProtein production in a 13- or 14-day fed-batch process using AstraZeneca proprietary medium and bolus additions of nutrient feed. Glucose and lactate were monitored throughout (YSI 2900D, YSI Inc). The cell culture medium was clarified by centrifugation and rProteins were quantified by using a protein A high-performance liquid chromatography (HPLC) on an Agilent HP1100 or HP1200 (Agilent Technologies, Santa Clara, CA) by comparing peak size from each sample with a calibration curve,[19] in which Protein A binds to the CH3 region of the Fc-domain of the mAb, a Duet-mAb or a Fc-fusion protein; or the CH1 region of the Fab molecule.

## 2.5 | Quantification of LC dimerization levels in Fab stable pools

LC dimerization levels were quantified in un-purified supernatant samples by performing reduced and non-reduced capillary gel electrophoresis separations using a LabChip GXII with a Protein Express LabChip (PerkinElmer, MA) according to the manufacturer's protocol. This method separates intact Fab from LC dimer. The respective software was used for run control and data analysis.

## 2.6 | Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) analysis for identification and relative quantitation of mis-paired species in Duet mAb stable pools

Supernatant samples were purified on Tecan Freedom EVO platform using PreDictor RoboColumn MabSelect Sure Affinity Capture. Purified samples were eluted in 25 mM sodium acetate, pH 3.6 −5CV, before they were neutralized in Trizma® hydrochloride solution, 1 M (Sigma-Aldrich, USA) to pH 5.5. Samples were de-glycosylated using PNGase-F at 1:20 ratio digestion at 37°C overnight. 1 µg of each sample was injected into a LC-ESI-MS system that comprises of reverse phase-C4 Acquity UPLC coupled to Synapt-XS MS (Waters, UK). The loaded sample was eluted using a linear gradient of LC–MS grade acetonitrile with 0.1% formic acid and 0.05% trifluoroacetic acid from 5% to 90% over 30 min at a 0.2 mL/min flow rate. The aqueous mobile phase was LC–MS grade water with 0.1% formic acid and 0.05% trifluoroacetic acid. The flow from LC was directed to ESI-MS data acquisition in positive and sensitivity modes. Other critical parameters were capillary voltage of 3.0 kV, source temperature of 150°C, sampling cone voltage of 120 V, cone gas flow of 50 L/h, desolvation temperature of 450°C, desolvation gas of 1000 L/h, and mass range of 500–4500 *m/z* that was calibrated by sodium iodide. For tuning and calibration, leucine enkephalin (Waters, UK) was used when acquiring ESI-MS data. The acquired data set was analyzed using Byos v4.4 intact mass analysis workflow (Protein Metrics, USA) for identification and relative quantification of different species detected in each sample.

## 2.7 | Gene copy number analysis

Genomic DNA was extracted from CHO cells using the PureLink™ Genomic DNA Kit (Invitrogen, USA) according to the manufacturer's protocol. qPCR was carried out on 1 ng of genomic DNA from each sample using the TaqMan Assay system (Thermo Fisher Scientific, UK) on the QuantStudio 12 K Flex Real-time PCR System (Applied Biosciences, USA). Serial dilutions of linearized plasmid DNA encoding the rProtein in genomic DNA from CHO host cells were performed and used to prepare calibration curves for the qPCR. Absolute quantification of gene copies was calculated using the calibration curves of each molecule examined. qPCR probes are detailed in Supplementary Table 1.

## 2.8 | RNA analysis

RNA was extracted from CHO cells using the Qiagen RNeasy isolation kit according to the manufacturer's instructions. cDNA was generated by reverse transcription using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, UK) according to the manufacturer's instructions; qPCR probes are detailed in Supplementary Table 1. qPCR was performed using the TaqMan Assay system (Thermo Fisher Scientific, UK) on the QuantStudio 12 K Flex Real-time PCR System (Applied Biosciences, USA). Relative gene expression was calculated using the 2(-Delta C(T)) method.[20]

## 2.9 | Western blot analysis

CHO cell pellets from each condition were treated with Radio Immuno Precipitation (RIPA) buffer supplemented with protease inhibitor (Thermo Fisher, USA). Protein bands were separated on 4%–12% Bis-Tris gels by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto PVDF membranes using an iBlot (Thermo Fisher, USA) and blocked in 5% BSA in PBS-Tween. Membranes were probed against primary antibodies (Anti-XBP1 Rabbit polyclonal, Cat. # ab37152; Recombinant Anti-beta Actin antibody [SP124] Rabbit monoclonal, Cat. #ab115777, Abcam Inc., UK) and 800CW goat anti-rabbit IgG secondary antibody (Li-Cor, USA). Protein blots were imaged on a ChemiDoc MP (BioRad, USA).

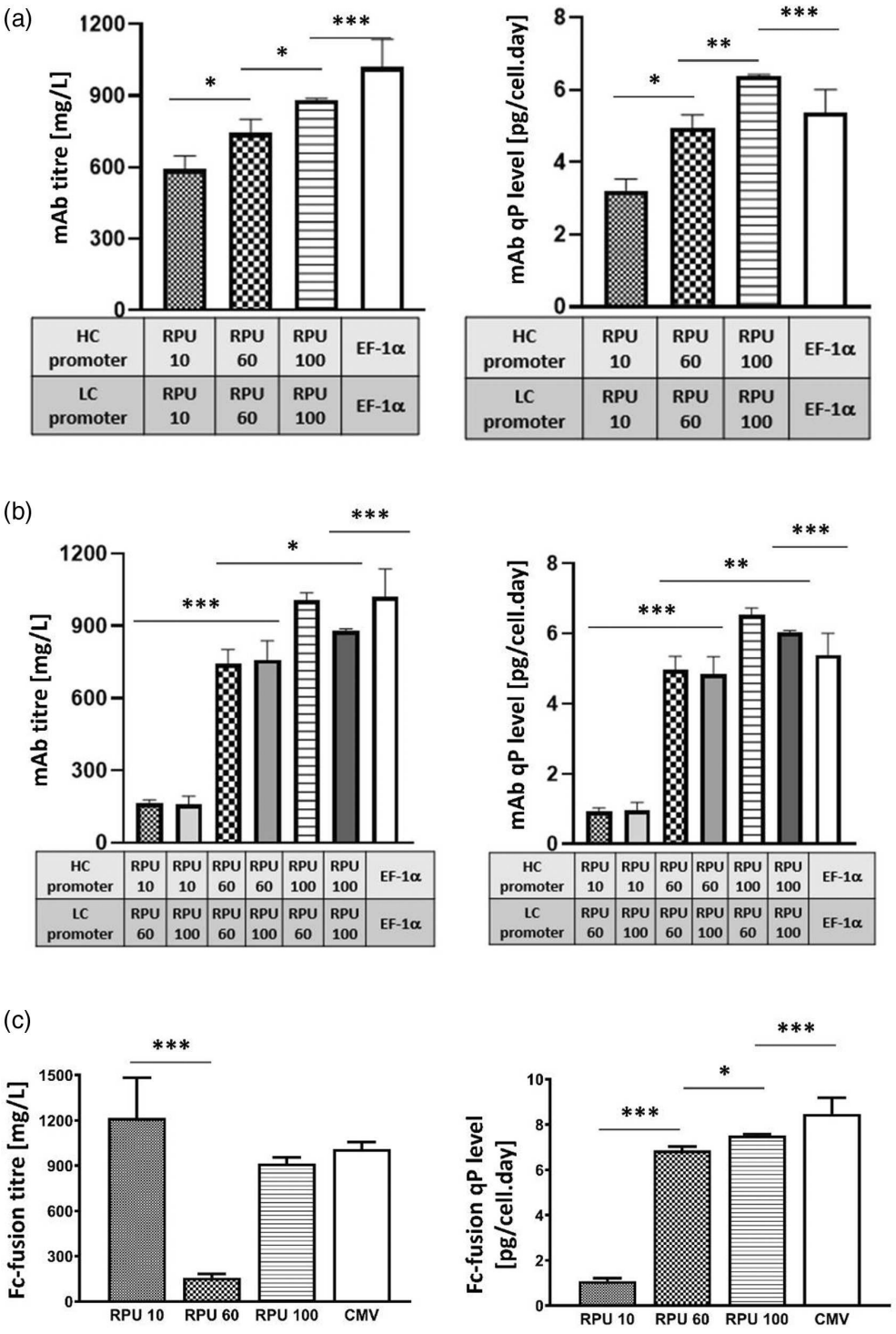
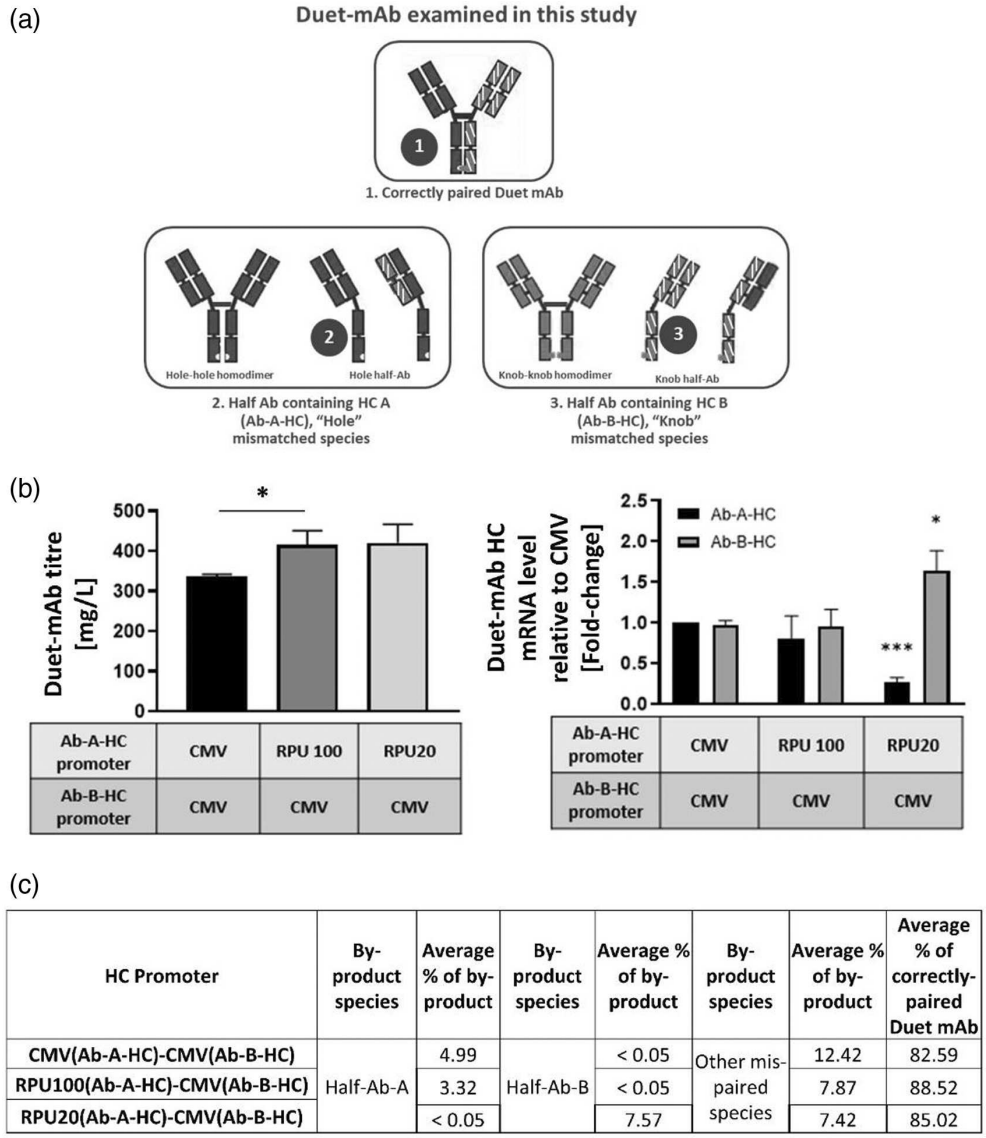



**FIGURE 2** Modifying rProtein expression levels by using different strength promoters. Transient CHO cultures expressing an easy-to-express (ETE) mAb and stable CHO pools expressing a single-chain Fc-fusion molecule were assessed in a fed-batch culture. (a) Transient mAb protein harvest titers and specific productivity, with both HC and LC expressed by the same set of synthetic promoters of various strengths, were compared to those expressed under the EF-1α promoter. EF-1α promoter is the standard promoter used for transient gene expression. (b) Transient mAb protein harvest titers and specific productivity, where HC and LC were expressed using a combination of synthetic promoters of various designed transcription activities, were compared to those expressed by EF-1α promoter. (c) Comparison of harvest titre and specific productivity of stable expression of a single-chain Fc-fusion protein by synthetic promoters of various transcription activities to that expressed using the CMV promoter. CMV promoter is the standard promoter used for stable gene expression. The rProtein expression levels in harvest samples were quantified by a protein A chromatography-based method. Synthetic promoters: RPU 10, 60, and 100. In all cases $n = 3$. Error bar represents the mean of values with standard deviation. All statistical analysis was performed by student *t*-test. *p*-Value: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

## 3 | RESULTS

### 3.1 | Synthetic promoters demonstrate tunable expression of industrially relevant rProteins in transient and stable gene expression

Synthetic promoters of various strengths (relative promoter units; RPUs) that were previously characterized using a reporter gene, were employed in this study to assess their suitability to drive transcription of genes encoding an industrially relevant mAb and an Fc-fusion protein, in transient and stable CHO expression systems, respectively. Results from the fed-batch experiments in both expression systems show a positive relationship between rProtein titre and the predicted synthetic promoter strength (Figure 2). In transient gene expression, an increase in mAb titre was observed when promoter pairs of increasing promoter strength were used to drive both the HC and LC (Figure 2a). When the HC and LC were expressed using different promoter strengths, an uplift in mAb production was observed when HC




(c)

| HC Promoter | By-product species | Average % of by-product | By-product species | Average % of by-product | By-product species | Average % of by-product | Average % of correctly-paired Duet mAb |
|---|---|---|---|---|---|---|---|
| CMV(Ab-A-HC)-CMV(Ab-B-HC) | Half-Ab-A | 4.99 | Half-Ab-B | < 0.05 | Other mis-paired species | 12.42 | 82.59 |
| RPU100(Ab-A-HC)-CMV(Ab-B-HC) | | 3.32 | | < 0.05 | | 7.87 | 88.52 |
| RPU20(Ab-A-HC)-CMV(Ab-B-HC) | | < 0.05 | | 7.57 | | 7.42 | 85.02 |

**FIGURE 3** The product quality of a Duet mAb expressed via synthetic promoters. (a) Schematic of some of the aberrant protein species that can be co-expressed with the correctly paired Duet mAb and those specifically investigated in this study (labeled 1–3). Stable CHO pools expressing a Duet mAb were assessed in a fed-batch culture, the expression constructs transfected contained the two HCs expressed from different combinations of promoters. (b) Protein expression levels of Duet mAb were quantified by a protein A chromatography-based method. Titers are relative to the CMV-CMV pools. CMV promoter is the standard promoter used for stable gene expression. (c) Relative transcript levels of Ab-A-HC and Ab-B-HC as measured by qPCR and normalized to CMV-CMV pools. (d) Half-Ab by-product species of the Duet mAb. Identity and relative levels of mis-paired species as measured by a quantitative mass spectrometry assay. All values in Figure D have p-value of <0.05. The percentage of total correctly paired Duet mAb was calculated based on the relative abundance, accounting for all the mis-paired species detected after deconvolution of the MS spectra. In all cases $n = 3$. Error bar represents the mean of values with standard deviation. All statistical analysis was performed by student *t*-test. *p*-Value: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

expression was driven by higher strength RPU promoters (Figure 2b). The observed increases in mAb titre correlated with uplifts in mAb specific productivity (qP) despite slight variations in the growth of the cells following transient transfection (Supplementary Figure 1). In stable gene expression, the level of a single chain Fc-fusion protein also positively correlates to the RPU of the synthetic promoters evaluated (Figure 2c). It is noteworthy that the stable titre differences resulted from differences in qP (Figure 2c) rather than from changes in cell growth and viability.

## 3.2 | Synthetic promoters can reduce a half-Ab contaminant of a Duet mAb by precise HC transcriptional control

Duet mAbs are monovalent bispecific antibodies consisting of two LCs and two HCs, in which each set of LC and HC are responsible for binding to different targets. This novel antibody format uses knobs-into-holes (KIH) technology for the heterodimerization of two distinct HCs and an alternative interchain disulphide bond to promote pairing

of the cognate HC and LC in one of the Fab arms.[21] Depending on how the four chains assemble, they can form the correctly paired molecule or different forms of mis-paired species (Figure 3a), consequently making Duet mAbs difficult to manufacture and purify.[22] During the development of one Duet mAb, the main contaminant was identified to be the half-antibody (half-Ab) containing Ab-A-HC. We sought to reduce the transcript level of Ab-A-HC by replacing the CMV promoter with synthetic promoters (RPU20 & 100). Figure 3b shows similar levels of total Duet mAb expression titre regardless of promoter strength used to express Ab-A-HC or Ab-B-HC and a similar trend was seen in qP (data not shown). Results from qPCR (Figure 3c) show that the HC transcript levels of Ab-A expression are comparable when the promoter strength was reduced from CMV to RPU100; whereas use of the RPU20 synthetic promoter resulted in a significant reduction in the Ab-A HC mRNA level accompanied by an elevation in the Ab-B-HC mRNA level. Using a quantitative mass spectrometry assay the relative amounts of half-Ab by-product in each condition were measured (Figure 3d). A modest but significant reduction in the level of the half-Ab-A contaminant was achieved by RPU100 compared with the dual CMV promoter construct, which results in a small percentage increase in correctly paired Duet mAb expression (Figure 3d). By further reducing Ab-A-HC expression using the RPU20 promoter, the mis-paired species had switched from half-Ab-A to the half-Ab-B, showing both the amount and type of mis-paired species can be modulated by the synthetic promoters.

### 3.3 | Synthetic promoter technology can improve expression of a DTE molecule by controlling the level of XBP-1s overexpression

The overexpression of biological accessory genes has previously been shown to improve expression of DTE molecules in CHO transient and stable platforms.[23–25] However, high levels of helper gene overexpression are not always necessary to achieve phenotypic improvements and can introduce a cellular biosynthetic burden that can counteract the benefits. Moreover, promoter squelching might affect the overexpression of multiple target genes if their expression is driven by promoters using the same TRFEs. XBP-1s is a transcription activator involved in regulation of both protein secretion and the unfolded protein response and it is well studied in rProtein expression,[24,26,27] therefore in this study we sought to finely control the overexpression level of XBP-1s, using synthetic promoters of varying strengths and determine the impact on DTE mAb expression in CHO cells. The synthetic promoters contain TFREs for transcription factors that are abundant in CHO and are therefore not likely to be limited in their capacity to drive expression.

Four multigene DNA constructs (Figure 1e), including four different promoter strengths (RPU5, 10, 20, and CMV) driving the overexpression of XBP-1s, were tested and compared to a no-XBP-1 control. Increasing levels of XBP-1s mRNA (Figure 4a) and protein (Supplementary Figure 2) expression were measured when XBP-1s expression was driven from promoters with increasing strength. The overexpression of XBP-1s resulted in an increase in volumetric yields of the DTE mAb (up 1.5-fold; Figure 4b). This increase in rProtein expression was as a result of increased total integral viable cell density (IVCD) of these pools (Figure 4c) and not as a result of increased specific productivity (Figure 4d). Interestingly, when XBP-1s overexpression was driven from the high strength CMV promoter, a consistent increase in IVCD was observed, however 2 of the 3 pools generated had much lower DTE mAb titre-observed at both the protein (Figure 4d) and mRNA level (Figure 4e). When synthetic promoters were used to drive XBP-1s overexpression, no significant negative impact on DTE HC and LC transcript levels was observed. Our results show increased consistency in higher DTE mAb expression levels from stable pools when XBP-1s overexpression is driven from synthetic promoters, when compared to a strong CMV promoter.

### 3.4 | Synthetic promoters reduce LC dimer contaminant levels in Fab production by precise control of HC:LC ratio

Some advantages of Fabs over traditional IgG formats are that, they can eliminate non-specific Fc-mediated binding, and their smaller size allows increased penetrance and enhanced target binding.[1,28] Fabs can be produced in mammalian cells using similar expression plasmid designs as for mAbs, but overexpression of LC can result in the formation of LC dimers.[3,4,28] These dimers can reduce the purified product yield as they are often difficult to remove due to the similarity in size to the Fab molecule. It was previously demonstrated in transient expression for both Fab and mAbs that increasing the ratio of HC to LC genes reduced the amount of LC dimers.[4,5] Here, we aimed to reduce the presence of LC dimers when stably expressing a Fab molecule, by increasing the HC:LC ratio using synthetic promoters.

No significant change in the specific productivity (qP) and titre of Fab across all stable pools regardless of the LC promoter strength (Figure 5d, e) was observed, however results generated from a non-reduced and reduced capillary gel electrophoresis separation-based method shows that, by replacing the CMV promoter driving LC expression with a synthetic promoter of lower strength (RPU20), while keeping the CMV promoter for the HC, our results demonstrated a 37.3% reduction in LC-dimer levels in stable pools (Figure 5a). As the strength of the synthetic promoter driving LC expression increased from RPU20 to RPU60, so did the LC-dimer levels. The levels of free LC species were also reduced by 0.8%–2.4% when the synthetic promoters RPU40 and RPU60 were used (Figure 5b). The reduced LC dimer led to a significant 32.4% increase in the level of intact Fab when LC expression was driven by the RPU20 promoter (Figure 5c).

Results from the mRNA quantification of HC and LC (Figure 5f) confirm the ability of the synthetic promoters to precisely modulate gene transcription. Expected reductions in LC mRNA levels relative to the CMV promoter were achieved with respect to the synthetic promoter strengths, with 0.2- to 0.5-fold decreases in LC mRNA levels. Furthermore, the intracellular LC:HC polypeptide ratios correlate well

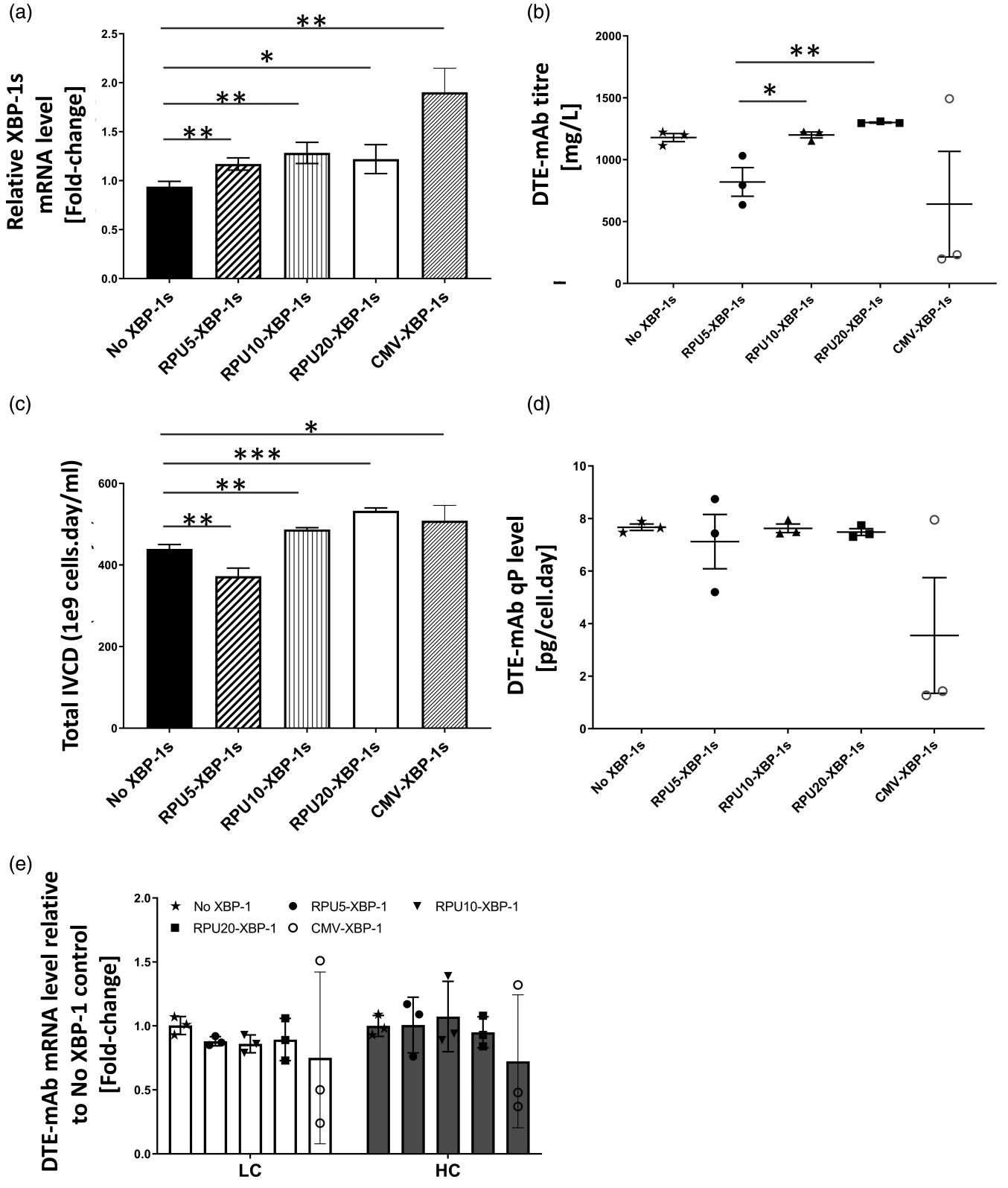



**FIGURE 4** Expression of a DTE mAb by precise control of XBP-1 overexpression via synthetic promoters. Stable CHO pools expressing a DTE mAb alone or with XBP-1s were assessed in a fed-batch culture. The expression constructs contained one LC and one HC expressed from CMV promoters, and one XBP-1s gene with different strength synthetic promoters. (a) Relative transcript levels of XBP-1s gene under each condition, as measured by qPCR and normalized to no XBP-1s control pools. (b) Protein expression level of DTE mAb under each condition, quantified by a protein A chromatography-based method. Titers are relative to no XBP-1s control pools. (c) The total integral viable cell density for the cultures over a time-course of 13 days. (d) Specific productivity of DTE mAb expression relative to no XBP-1s control pools. (e) Relative transcript levels of HC and LC of DTE mAb under each condition as measured by qPCR and normalized to the no XBP-1s control. In all cases $n = 3$. Error bars represents the mean of values with standard deviation. All statistical analysis was performed using a student *t*-test. *p*-Value: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

with the changes observed in LC mRNA levels (Supplementary Table 2). This indicates that changes in the LC-dimer levels are as a direct result of the successful control of LC mRNA expression by the synthetic promoters. In all conditions, levels of any free HC and HC-dimer were constantly low (between 1 and 2%, data not shown).

### 3.5 | Transcriptional activity of the synthetic promoters is stable and consistent in Fab-expressing stable CHO pools over 32 PDLs

To enable large scale production, it is important that the level of expression driven from the synthetic promoters is stable over a prolonged cell culture period. Here, we evaluated expression stability of the Fab and LC dimer level from stable CHO pools over 7, 18, and 32 population doubling levels (PDLs). This duration mimics the inoculum expansion phase required for a larger scale bioreactor, which is often used to supply rProtein from stable CHO pools for development-enabling activities and to support toxicology studies. Our results show no statistically significant change in Fab titre and qP in all cell ages examined for each HC and LC promoter combination (Figure 6a, b), despite a downward trend in their levels observed from stable pools generated with CMV-HC & RPU60-LC pairing, which could be the result of variations in cell growth within these replicate pools. The small fluctuation in the LC-dimer level of the CMV-control observed with time was associated with an increase in free-LC species (Figure 6d). Most importantly, there is minimal variation in LC-dimer (Figure 6c) and intact Fab levels (Figure 6e) across all PDLs where synthetic promoters were used, which is supported by no significant change in transcript and polypeptide levels of HC and LC (Figure 6f, g and Supplementary Table 2). In all conditions, levels of any free HC and HC-dimer were constantly low (between 1% and 2%, data not shown). This suggests a high level of stability of the transcriptional activity of the synthetic promoters over a prolonged cell culture period in stable CHO pools.

## 4 | DISCUSSION

We have evaluated a panel of characterized synthetic promoters in our gene constructs used for transient and stable expression of therapeutic proteins and demonstrated the ability of these promoters to exhibit tunable protein expression in both systems. This significantly extends the previous observations of Brown, Gibson, Hatton and James[15] from a reporter protein to industrially relevant mAb and Fc-fusion molecules. Also, a good dynamic range of protein expression levels (12%–85% of the Fc-fusion expression from a viral promoter) is achievable with this set of synthetic promoters in stable pools. By varying the HC expression level of a mAb in transient transfections using synthetic promoters, our data shows that the HC was the primary influence on mAb expression levels, which aligns well with other studies.[29] The comparable or slightly lower promoter performances of RPU100 than the CMV promoter in our stable expression study, is contrary to the observations by Brown, Gibson, Hatton and James[15] for SEAP production. This may reflect the differences in the complexities of SEAP compared to biotherapeutic rProteins in combination with the biosynthetic limitations of CHO cells. SEAP is a comparatively easy protein to translate, fold and secrete, and therefore when transcript levels are increased a corresponding uplift in protein expression is observed, suggesting that the bottleneck for SEAP expression is at the level of transcription. In contrast, for mAbs and other complex rProteins, in additional to variations in transgene copy number and influences from their integration sites, and other possible transcriptional constrains (e.g., transcript stability), the cellular capacity for post-transcriptional biosynthetic processing, such as folding and secretion, can potentially become the next limiting factor once transcription reaches a threshold level.[30–32] Hence, when transcription is driven using the powerful CMV or RPU100 synthetic promoters, the increased transcription does not result in a further increase in rProtein expression. This suggests that lower strength synthetic promoters can be beneficially deployed to balance chain ratios and improve product quality, as shown in this publication, rather than pushing transcription beyond that driven by strong viral promoters.[18]

Meanwhile in the XBP-1s overexpression study, we found that fine-tuning the levels of the recombinant XBP-1s transcript enabled significant improvement in DTE mAb expression levels without the need for strong overexpression of an accessory gene of interest. XBP-1s plays a crucial role in the activation of the unfolded protein response (UPR) which enhances transient expression of mAbs.[33,34] Our results suggest that by carefully modulating expression levels of XBP-1s, tunable increases in IVCD were observed in CHO cells. We speculate that driving the XBP-1s expression using a synthetic or CMV promoter dialed up the UPR to a consistent beneficial level, in which the increase in CHO cell growth observed in this study could be related to improved protein homeostasis and increased CHO cell survival via better protein folding capacity and higher resistance to cellular stresses induced by XBP-1 activation.[35,36] However, only cultures with XBP-1s overexpressed by synthetic promoters displayed a consistent improvement in DTE mAb expression. By quantifying the mRNA levels of HC and LC of the DTE mAb in Figure 4e, we can conclude that when transcription of all genes (LC, HC, and XBP-1) is driven by CMV promoters a reduction in LC and HC mRNA is observed, which could be caused by promoter squelching,[37] resulting in more variable rProtein expression profiles. In contrast, synthetic promoters that drive XBP-1s expression did not appear to affect the activity of other promoters within the gene expression cassette, where these synthetic promoters are designed to display minimal competition of transcription factors with surrounding promoters. In addition, the expected titration of XBP-1s mRNA and protein levels was observed when the promoter strength was varied (Figure 4a and Supplementary Figure 2), in pools that were generated from the targeted integration approach in which a consistency in gene copy number is ensured and any chromosomal positioning effect is maintained. This further demonstrates the potential of this synthetic promoter technology under different molecular settings.

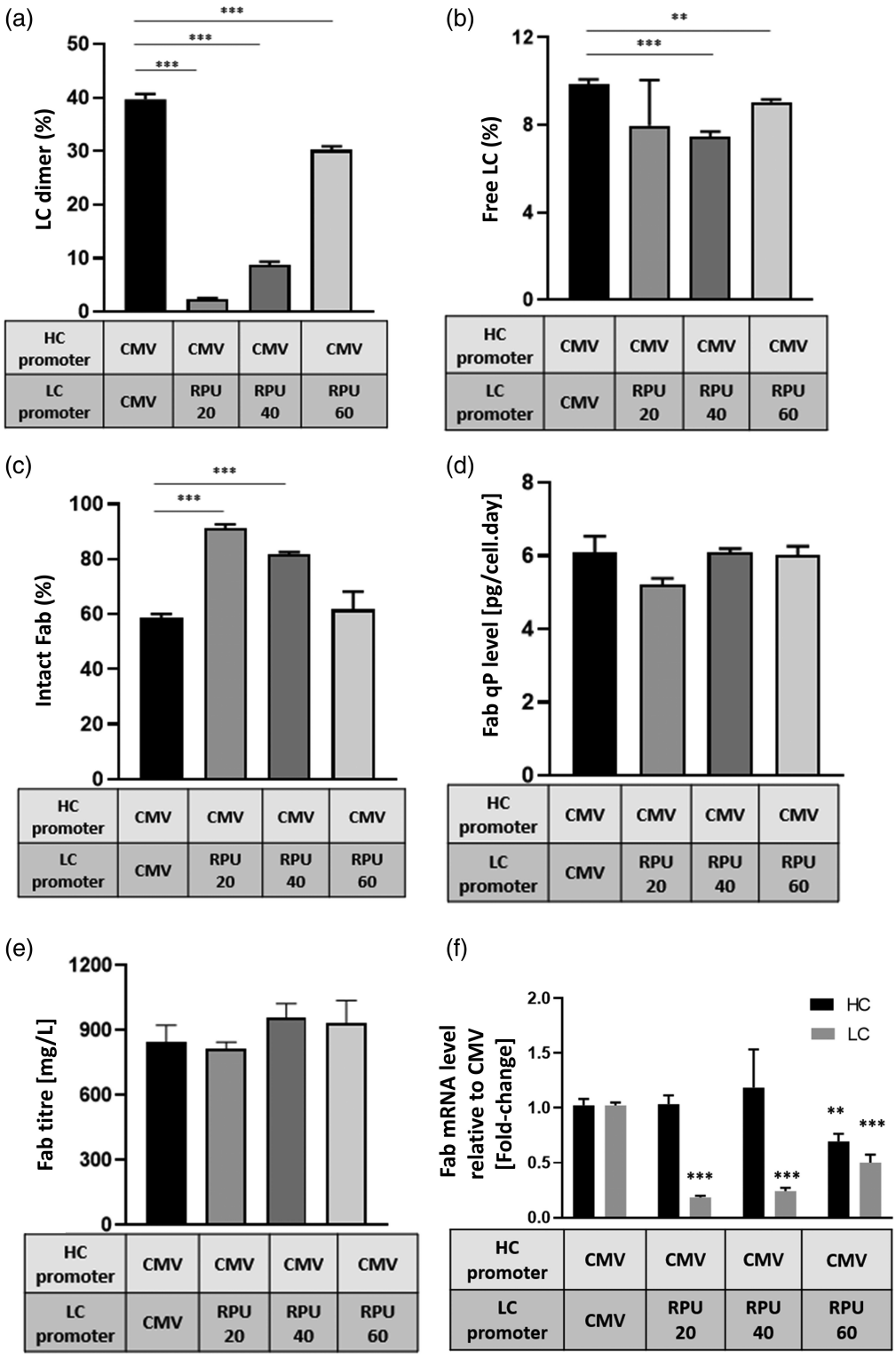



**FIGURE 5** The level of LC-dimer impurity of a Fab molecule when transgenes were expressed by synthetic promoters. Stable CHO pools expressing a Fab were assessed in a fed-batch culture, the expression constructs contained a LC downstream of different strength promoters while the HC expression was driven by the CMV promoter. CMV promoter is the standard promoter used for stable gene expression. Percentage of LC-dimer species (a), free LC species (b) and intact Fab (c) in culture supernatants were quantified using a LabChip GXII with a Protein Express LabChip. (d) Specific productivity of Fab expression in stable pools. (e) Protein expression titers of Fab in stable pools. (f) mRNA expression of HC and LC of Fab molecule, relative to CMV-CMV control. In all cases $n = 3$. Error bar represents the mean of values with standard deviation. All statistical analysis was performed using a student $t$-test. $p$-value: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

Multimeric biotherapeutic proteins comprise of multiple polypeptide chains that are typically stabilized by disulphide bonds. Depending on the assembly kinetics of the molecule, different expression levels of each polypeptide chain may be required to alleviate cellular bottlenecks and failure to optimize this can result in compromised product yields and product quality.[38,39] The synthetic promoter technology can also be applied to optimize the production of multimeric therapeutic proteins by reducing the levels of contaminating undesirable protein species expressed. As demonstrated by the Duet mAb and Fab expression in this study, it is clear that the modulation of relative product gene transcript levels by synthetic promoters was key to achieving desirable purified rProtein yields and product quality. Our results show the substantial reduction in aberrant product-related protein species in a Duet mAb (Figure 3) and a Fab molecule

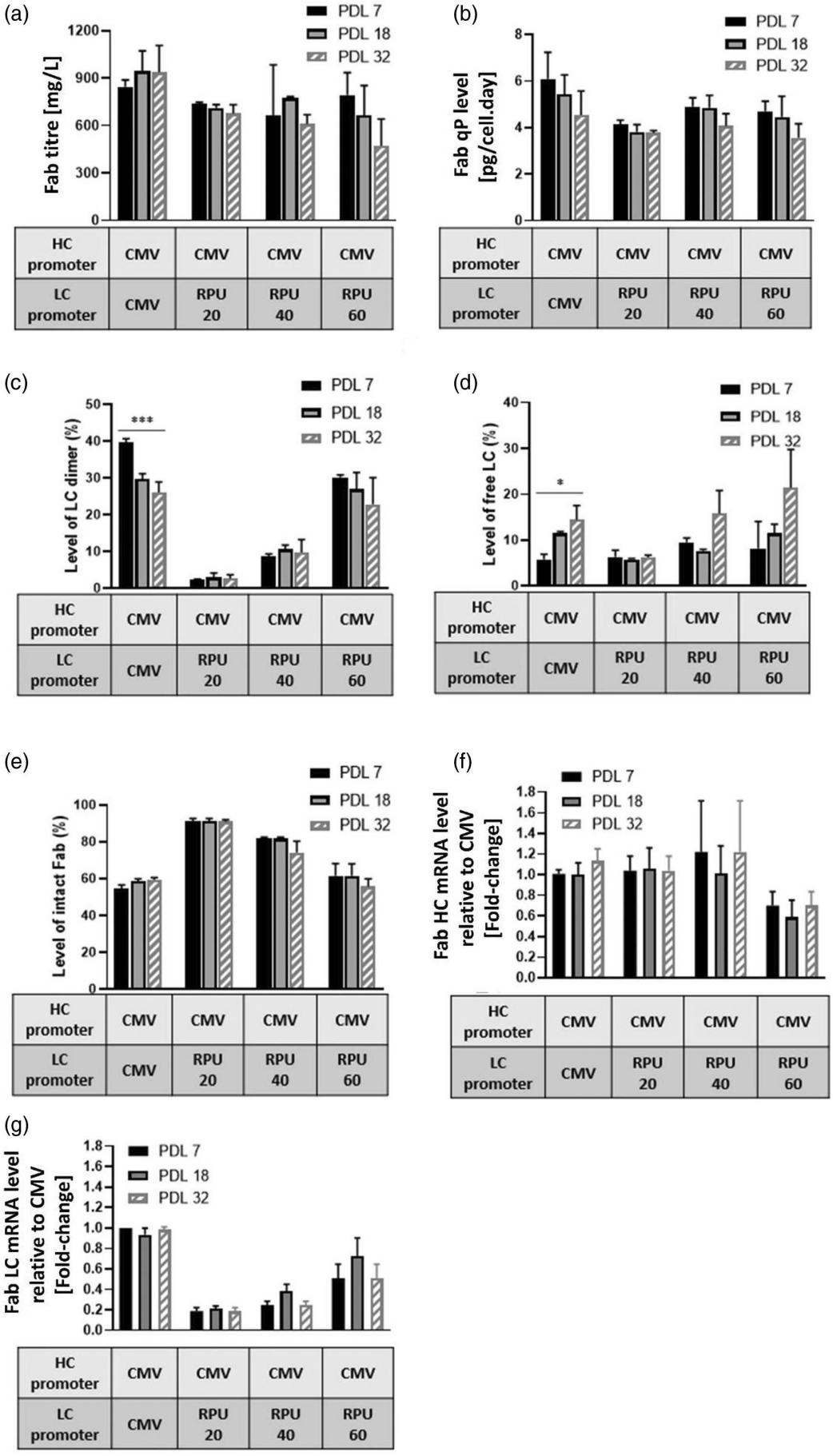



**FIGURE 6** Legend on next page.

(Figure 5). In comparison to the complex, labor- and cost-intensive downstream processes required to remove these aberrant proteins, a relatively simple plasmid engineering approach of replacing traditional viral-based promoter sequences with synthetically developed alternatives has been demonstrated here to achieve better outputs in their rProtein quality levels with fewer contaminants.

Previously, a 40% reduction in Fab LC-dimer by adjusting the ratio of the HC-containing plasmid to the LC-containing plasmid was demonstrated in transiently transfected CHO cells.[4] Here in our study, a 94% reduction in LC-dimer level of the same Fab molecule was achieved in stable CHO pools, where the HC and LC gene copy numbers remained constant on a single plasmid (Supplementary Figure 3) and the HC:LC expression ratio was adjusted solely based on the transcriptional activity of each promoter, which enabled more precise HC:LC expression ratio modulation. Similarly, the rebalancing of the relative HC gene expression for our Duet mAb using the synthetic promoter reduced the level of a half-antibody species. The HC transcript level of Ab-A was reduced slightly when driven by the RPU100 promoter and very significantly reduced when driven by the RPU20 promoter compared to the CMV control. The observed changes at the transcript level correlate well with the levels of half-Ab-A by-product measured, with a slight reduction when RPU100 was used and a significant reduction when RPU20 was used. Changing the promoter driving the expression of Ab-A HC also impacted the transcript levels of Ab-B HC even when the promoter for this gene was unchanged. This demonstrates the complex interplay between promoters in multicistronic expression systems. Different TFREs in the RPU20 promoter sequence compared to the RPU100 promoter sequence will bind different transcription factors, changing the pool of transcription factors available to bind the TFREs in the CMV promoter and drive transcription of the downstream coding sequence. The ability to modulate the level of half Ab by-products by using promoters with pre-defined transcriptional activity can ensure the optimal stoichiometry of the two HCs is achieved for correct pairing of Duet mAbs and other multi-chain rProteins.[18]

Upon demonstrating successful improvement in rProtein expression, in order for these synthetic promoters to be beneficial for large-scale production processes, it is essential to confirm their stability. Stable CHO pools are often used to supply large quantities of material to support early-stage development activities and non-GLP toxicology studies. Here, product titers and LC-dimer levels were assessed for a Fab molecule expressed by aged, stable CHO pools, where the LC expression was driven from promoters of differing strengths (RPU20, 40, 60, and CMV). Our results demonstrated a high degree of consistency of the transcriptional activity of these promoters, with no significant changes in growth phenotype, Fab productivity and LC-dimer levels, or in LC and HC mRNA levels over 32 PDLs (Figure 6). The stability of expression from the synthetic promoters across 32 PDLs demonstrates their capability for generating stable CHO pools for material supply to support early development work. These results in transfectant pools are very favorable, but further confirmatory work is required to demonstrate long term expression stability of the synthetic promoters in clonal cell lines.

In this study, stable CHO pools have been used to evaluate the impact of synthetic promoters on product quality and quantity of a number of different biotherapeutically relevant rProteins. An important consideration is how these observed trends will translate into rProtein production from stable CHO clones as a clonally-derived cell line is an important regulatory requirement for biotherapeutic manufacture. The stable CHO pools generated by random integration represent an average population, with each cell having a different gene copy number and different chromosomal loci. Where we have observed enhanced product quality, a bigger cell population in the CHO pool are expected to have the desired attributes and therefore upon single cell cloning, a higher proportion of the clones will also have those same desired attributes. It is however possible that not all very high producing clones would possess improved product quality observed in a stable pool population, but this product quality enrichment to a larger proportion of cell in a stable pool will have the benefit of reducing the number of clones that need to be screened to identify suitable candidate production cell lines.

## 5 | CONCLUSION

To conclude, we have demonstrated the benefits of engineering expression plasmids with synthetic promoters to enhance the production and product quality of three different industrially relevant rProtein formats. The data presented here focus on the application of these synthetic promoters and indicate their ability to precisely modulate gene expression for customizable functions, with consistent activity dynamics over a period of 32 generations in stable pools. This study highlights the wide range of gene targets and expression settings where synthetic promoters can be applied. It is also possible to combine these synthetic promoters with other synthetic elements, for example, synthetic leader sequences[6,40] to further regulate and

**FIGURE 6** Transcriptional activity of the synthetic promoters over 32 PDLs. Stable CHO pools expressing a Fab using various combinations of promoter strengths were routinely cultured across 32 PDLs, cells were cryopreserved at PDL 7, PDL 18 and PDL 32. Aged stable pools were revived and assessed in a fed-batch culture. (a) Fab titre under each condition, titers are relative to the CMV-CMV control at PDL 7. CMV promoter is the standard promoter used for stable gene expression. (b) Fab qP levels. (c) Percentage of LC-dimer species in culture supernatant, quantified using a LabChip GXII with a Protein Express LabChip. (d) Percentage of free LC species in culture supernatant quantified using a LabChip GXII with a Protein Express LabChip. (e) Percentage of intact Fab in culture supernatant, quantified using a LabChip GXII with a Protein Express LabChip. (f) mRNA expression of the Fab HC, relative to CMV-CMV control. (g) mRNA expression of the Fab LC, relative to CMV-CMV control. In all cases $n = 3$. Error bars represents the mean of values with standard deviation. All statistical analysis was performed by student *t*-test. *p*-Value: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

optimize expression and biosynthetic pathways in CHO cells. Finally, this synthetic biology technology offers solutions to challenges posed for the developability and manufacture of novel protein formats in the biopharmaceutical industry.

## AUTHOR CONTRIBUTIONS

**Si Nga Sou:** Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); writing – original draft (lead). **Claire L. Harris:** Data curation (supporting). **Rebecca Williams:** Data curation (supporting); formal analysis (supporting); investigation (supporting). **Dorota Kozub:** Data curation (supporting); formal analysis (supporting); investigation (supporting). **Fabio Zurlo:** Formal analysis (supporting). **Yash D. Patel:** Investigation (supporting). **Praveen Kallamvalli Illam Sankaran:** Formal analysis (supporting). **Olalekan Daramola:** Supervision (supporting). **Adam Brown:** Resources (supporting). **David C. James:** Supervision (supporting). **Diane Hatton:** Editing and proofreading (supporting); supervision (supporting). **Sarah Dunn:** Conceptualization (equal); supervision (equal); writing – review and editing (equal). **Suzanne J. Gibson:** Conceptualization (equal); supervision (equal); writing – review and editing (equal).

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## CONFLICT OF INTEREST STATEMENT

Si Nga Sou, Claire L Harris, Rebecca Williams, Dorota Kozub, Fabio Zurlo, Maurizio Muroni, Sarah Dunn, Olalekan Daramola, Diane Hatton and Suzanne J. Gibson are employees of AstraZeneca and may own stock or stock options.

## PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/btpr.3348.

## DATA AVAILABILITY STATEMENT

All data that are presented here and support the findings in this study are available in this article and in the supplementary material. Additional data available on request from the authors.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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