Novel bidirectional promoter AG971 doubles the yield of bispecific T-cell engager Blinatumomab produced in CHO cells

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Abstract

A substantial proportion of recombinant biotherapeutic proteins are currently manufactured using Chinese hamster ovary (CHO) cells. Maximizing both yield and product quality remains a complex and critical challenge. Optimization strategies typically focus on advanced cell line engineering approaches (e.g., enhancing secretory pathway capacity or tightly regulating cell proliferation) as well as process optimization (e.g., precise control of culture conditions and nutrient supply). However, significant opportunities remain to improve the DNA regulatory elements, particularly promoters, that govern transgene expression. The development of more potent and stable promoters is essential to increase biotherapeutic yields, especially for difficult-to-express proteins. In this study, we employed a high-throughput functional screening assay based on the Survey of Regulatory Elements (SuRE™) platform (proprietary to Annogen) to systematically identify CHO genomic fragments with high promoter activity. Using the most promising candidate sequences, we engineered a novel bidirectional promoter, AG971. We evaluated the suitability of AG971 for biotherapeutic production in CHO cells using the model difficult-to-express CD3- and CD19-targeting bispecific T-cell engager (BiTE) Blinatumomab. Expression performance was assessed following electroporation and subsequent fed-batch cultivation. In both transient and semi-stable cell pools, the AG971 promoter consistently outperformed the industry-standard CMV promoter. Notably, in semi-stable cell pools, AG971 achieved a twofold increase in yield. Collectively, our findings establish AG971 as a valuable regulatory element for optimizing manufacturing-grade CHO cell pools and cell lines. Furthermore, this work highlights the capability of the Annogen's SuRE™ platform to generate novel promoters that surpass current industry benchmarks, thereby enabling improved titers and product quality, particularly for difficult-to-express proteins.

Introduction

CHO cells remain the predominant host system for the production of recombinant biotherapeutic proteins. Despite substantial progress in optimizing CHO-based protein expression, there is a continued need for further improvements, particularly for difficult-to-express therapeutics. A key challenge is to achieve maximal titers of high-quality target proteins within the shortest possible development timeline. To address this need, considerable efforts are focused on developing advanced regulatory elements, including optimized promoters, to enable robust and stable transgene expression. These approaches complement parallel strategies such as engineering intracellular protein synthesis and secretion pathways, designing bioreactor systems that allow precise control of culture conditions, and improving gene delivery technologies [McGraw *et al.*, 2020].

Promoters commonly used for protein expression in CHO cells can be broadly classified into several categories: (i) viral promoters (e.g., CMV and SV40), (ii) endogenous promoters (e.g., EF-1α), (iii) heterologous eukaryotic promoters (e.g., mouse PGK and human UBC), (iv) hybrid promoters composed of elements from different origins (e.g., CAG), (v) *in silico*-designed or synthetic promoters, and (vi) inducible promoters (e.g., Tet-On) [Romanova and Noll, 2018]. Although certain viral promoters can drive high levels of transgene expression, particularly in transient transfection settings, they are susceptible to transcriptional silencing over time, especially after stable integration of the transgene into the CHO genome [Zúñiga *et al.*, 2019]. In contrast, endogenous promoters generally provide more stable but comparatively moderate expression levels. Hybrid and selected synthetic promoters, however, have shown considerable promise in achieving enhanced and sustained expression [Brown *et al.*, 2014; Tossolini *et al.*, 2022; Sou *et al.*, 2023]. An additional consideration is that the presence of multiple strong promoters within the same vector construct (e.g., independently driving the gene of interest and a selection marker) can lead to transcriptional interference [Shearwin *et al.*, 2005; West, 2014], which may ultimately compromise overall productivity.

The aim of this study was to develop a novel bidirectional promoter designed to enhance the production of difficult-to-express biotherapeutic proteins. This promoter was engineered to possess properties that surpass the current industry standard, the widely used CMV promoter. It is intended to serve as the foundation for an optimized gene expression system in CHO cell pools and cell lines employed for biomanufacturing.

Results

To identify novel promoter elements capable of driving high and stable expression of biotherapeutic proteins in CHO cells, we performed a large-scale functional screen using the Survey of Regulatory Elements (SuRE™) platform (proprietary to Annogen) [van Arensbergen *et al.*, 2017]. For the initial screen, high molecular weight CHO genomic DNA was randomly sheared to an average length of ~400–600 bp using NEBNext dsDNA Fragmentase (New England Biolabs, M0348). The resulting fragments were subcloned into a barcoded, promoterless plasmid vector upstream of a GFP open reading frame (ORF), generating a library of approximately 1.7×10^9 unique clones. Next-generation sequencing (NGS)-based analysis of barcoded GFP reporter transcript abundances in transiently transfected CHO cells provided a genome-wide, high-resolution profile of autonomous promoter activity among the sampled CHO genomic sequences (**Figure 1**). Overall, the library achieved ~150-fold coverage of the complete CHO genome.

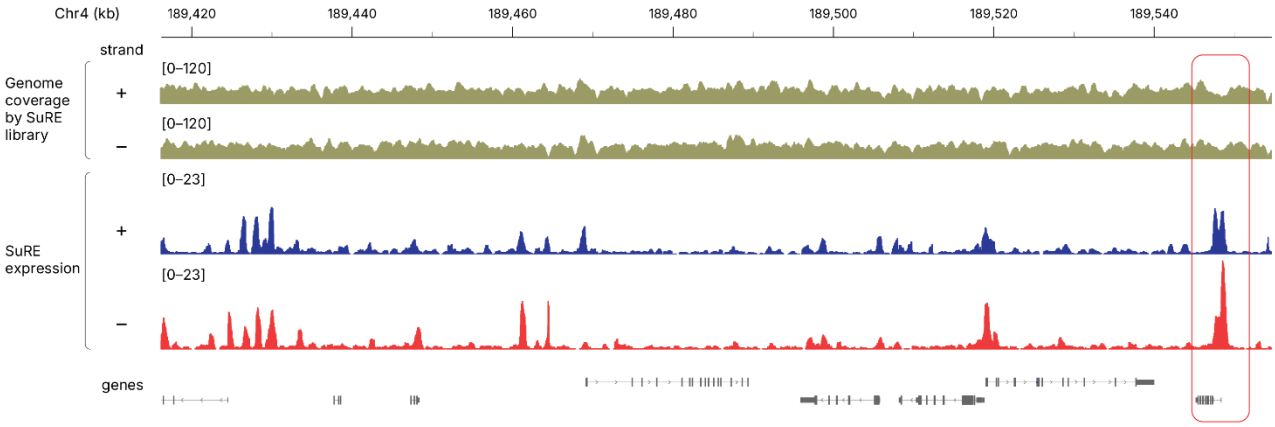

Figure 1. Genome-wide analysis of autonomous promoter activity in CHO genomic sequences. A representative ~138-kb genomic region is shown, illustrating the coverage of the SuRE™ plasmid library (input, top tracks) and the corresponding SuRE™ expression signals (bottom tracks), displayed as fold enrichment over input. Data are presented separately for the plus and minus DNA strands. Notably, most identified active genomic elements exhibit bidirectional promoter activity, driving reporter gene expression from both strands. The SuRE™ expression peaks highlighted on the right (red box) correspond to the promoter region of the *Eukaryotic translation elongation factor 1 alpha 1* gene (*Eef1a1*, also known as *EF-1α*, *CHEF-1α* or *NM_001244402.1*). This endogenous promoter is widely used for recombinant protein expression in CHO cells [Wang et al., 2017].

We subsequently identified peaks of SuRE™ expression and selected 192 promising genomic fragments, each averaging approximately 400 bp in length, for further characterization. These fragments were selectively PCR-amplified from genomic DNA and combined in all possible pairwise arrangements. The resulting products were further fused to some standard regulatory elements. These final hybrids were used to generate a second barcoded GFP-encoding plasmid library (using the same method described above) comprising approximately 25×10^6 unique clones. The ability of these hybrid elements to autonomously drive transcription was assessed in CHO cells following transient transfection, mirroring the initial SuRE™ screening. The hybrid promoter that produced the highest transcript levels was designated AG971. Its activity was further validated using a dual GFP/mScarlet-I plasmid vector, which allowed simultaneous assessment of bidirectional promoter activity by measuring expression of both fluorescent proteins. This analysis confirmed that, in addition to driving high GFP expression, the AG971 promoter also directs substantial mScarlet-I expression via its minus-strand activity in CHO cells. The final bidirectional AG971 promoter has a total length of 1607 bp.

To evaluate the suitability of the AG971 promoter for biotherapeutic protein production, we generated a series of plasmids expressing the CD3- and CD19-targeting bispecific T-cell engager (BiTE) Blinatumomab. Blinatumomab was selected due to its established classification as a difficult-to-express protein [Maltais *et al.*, 2023; Kim *et al.*,

2025]. In these constructs, a bicistronic mRNA was engineered to co-express a GFP reporter alongside the BiTE, serving as a visual readout of promoter activity (**Figure 2**). The minus-strand activity of AG971 was utilized to drive expression of the neomycin resistance (Neo^R) selection marker. For comparison, a standard 655-bp CMV promoter was used to express the BiTE-GFP cassette in combination with a separate, conventional SV40-driven Neo^R selection marker.

Finally, for each promoter system, a plasmid enabling piggyBac transposon-mediated integration of the transgene into the CHO genome was also prepared.

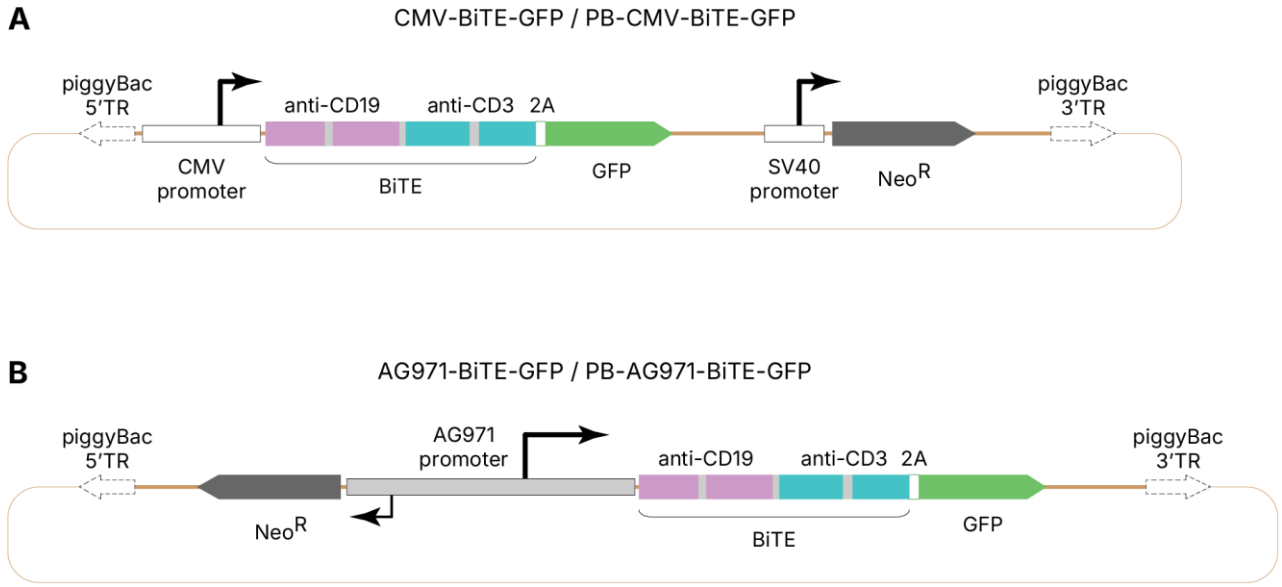

Figure 2. Schematic representation of the plasmid constructs used in this study. (**A**) Conventional CMV/SV40 system (control). This configuration comprises two separate expression cassettes. The first cassette, driven by the CMV promoter, encodes the Blinatumomab bispecific T-cell engager (BiTE) linked to green fluorescent protein (GFP) via a self-cleaving 2A peptide. The second cassette, under the control of the SV40 promoter, drives expression of the Neomycin resistance (Neo^R) gene, conferring resistance to G418. (**B**) Novel bidirectional AG971 system. A single bidirectional AG971 promoter drives expression of both the Blinatumomab-2A-GFP cassette and the Neo^R selection marker. (**A–B**) In both systems, GFP serves as a proxy for the expression level of the therapeutic transgene. To enable efficient and stable genomic integration in CHO cells, all 'PB-' plasmid constructs contain piggyBac transposon terminal repeats flanking the entire expression cassette(s).

Blinatumomab production was evaluated in fed-batch culture under three distinct experimental conditions: (i) following transient transfection with the standard CMV-BiTE-GFP or AG971-BiTE-GFP plasmid, which lacked piggyBac terminal repeats; (ii) immediately after co-transfection of a plasmid carrying the transgene cassette flanked by piggyBac terminal repeats, PB-CMV-BiTE-GFP or PB-AG971-BiTE-GFP, together with piggyBac transposase-encoding mRNA; and (iii) in stable cell pools established from cells co-transfected with the PB-AG971-BiTE-GFP plasmid and piggyBac transposase mRNA.

We first analyzed fed-batch yields of Blinatumomab following transient transfection of CHO cells with CMV-BiTE-GFP and AG971-BiTE-GFP plasmids. CHO cells were maintained in suspension culture using CD OptiCHO™ medium (Thermo Fisher Scientific) and expanded to a density of 4×10^6 cells/ml. Twenty-four hours prior to transfection, the culture was diluted to 1×10^6 cells/ml. On the day of DNA delivery, cell density and viability were determined by Trypan blue exclusion. For each construct, 2×10^8 cells with viability exceeding 99% were combined with 200 µg of plasmid DNA. Cells were collected by centrifugation at 200×*g* for 5 min in a swing-bucket rotor, the supernatant was removed, and the pellet was resuspended in electroporation buffer to a final density of 2×10^8 cells/ml. Aliquots were then prepared containing either no DNA (mock control), a GFP-expressing control plasmid (positive control), or the respective BiTE-encoding plasmid, followed by gentle mixing. The mixtures were transferred to electroporation cuvettes and electroporated. After treatment, cells were allowed to recover for 30 min at 37°C. Fed-batch cultivation was subsequently initiated by resuspending the recovered cells in 30 ml of fresh medium in 125-ml shake flasks, yielding an approximate starting density of 6×10^6 cells/ml.

Several key parameters were monitored throughout the culture period, including cell viability, the proportion of GFP-positive cells, GFP expression levels, and BiTE titers (**Figure 3**). No significant differences were observed between the CMV and AG971 samples with respect to cell viability or the proportion of transfected cells. However, the AG971 promoter consistently outperformed the CMV control in terms of expression performance. Specifically, AG971 drove a ~1.6–2.9-fold increase in GFP expression between days 3 and 6 post-transfection. Importantly, this enhancement resulted in a ~1.4-fold increase in the final Blinatumomab titer measured at the end of the fed-batch culture (day 14).

Next, we assessed antibody titers obtained using the semi-stable piggyBac transposon-mediated expression system. CHO cells were co-transfected, as described above, with either the PB-CMV-BiTE-GFP or PB-AG971-BiTE-GFP plasmid together with piggyBac transposase-encoding mRNA.

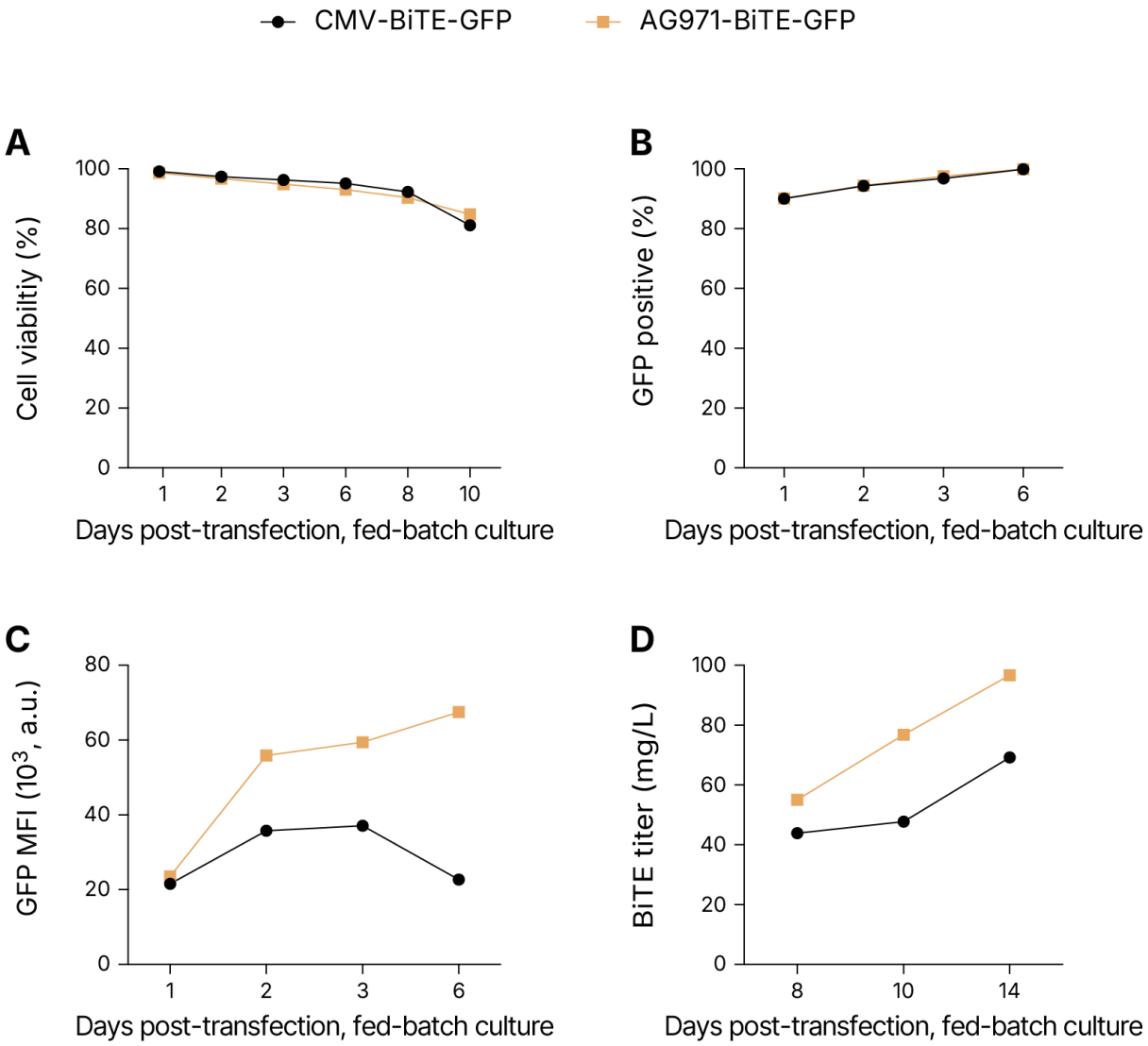

Figure 3. Enhanced transient episomal expression of Blinatumomab BiTE using the novel AG971 vector system. CHO cells were transiently transfected with either the CMV-BiTE-GFP or AG971-BiTE-GFP plasmid. Fed-batch cultures were initiated immediately after electroporation without applying selection pressure. Cells were supplemented with feed equivalent to 10% of the total culture volume on days 1, 4, and 7. Cultures were harvested prior to substantial dilution of episomal plasmid DNA. (**A**) Cell viability. Both transfected cell pools maintained high viability following electroporation and throughout the fed-batch production phase until day 10. (**B**) Transfection efficiency. Flow cytometry analysis performed on day 6 post-electroporation demonstrated high transfection efficiency (~99%) for both plasmids, as determined by the percentage of GFP-positive cells. (**C**) Mean fluorescence intensity (MFI). Flow cytometric analysis of average GFP expression levels (MFI) showed that the AG971-BiTE-GFP plasmid mediated higher protein expression from day 2 post-transfection onward compared with the CMV-BiTE-GFP plasmid. (**D**) Blinatumomab titer. The AG971-BiTE-GFP construct resulted in a higher yield of secreted antibody between days 8 and 14 post-transfection relative to the CMV control. BiTE titers were quantified using an in-house assay, with purified Blinatumomab used as the reference standard.

In this semi-stable configuration, no selection pressure was applied; consequently, the cell population comprised a heterogeneous mixture of cells harboring stably integrated transgenes and cells retaining episomal plasmids, with the relative proportions of these subpopulations changing over the course of cultivation. Under these conditions, the early enhancement conferred by AG971 was modest, resulting in only a ~1.3-fold increase in GFP expression on day 3 of the fed-batch culture compared with the CMV control. Nevertheless, by the end of the culture (day 9), the final Blinatumomab titer was 2-fold higher in the AG971 sample (**Figure 4**).

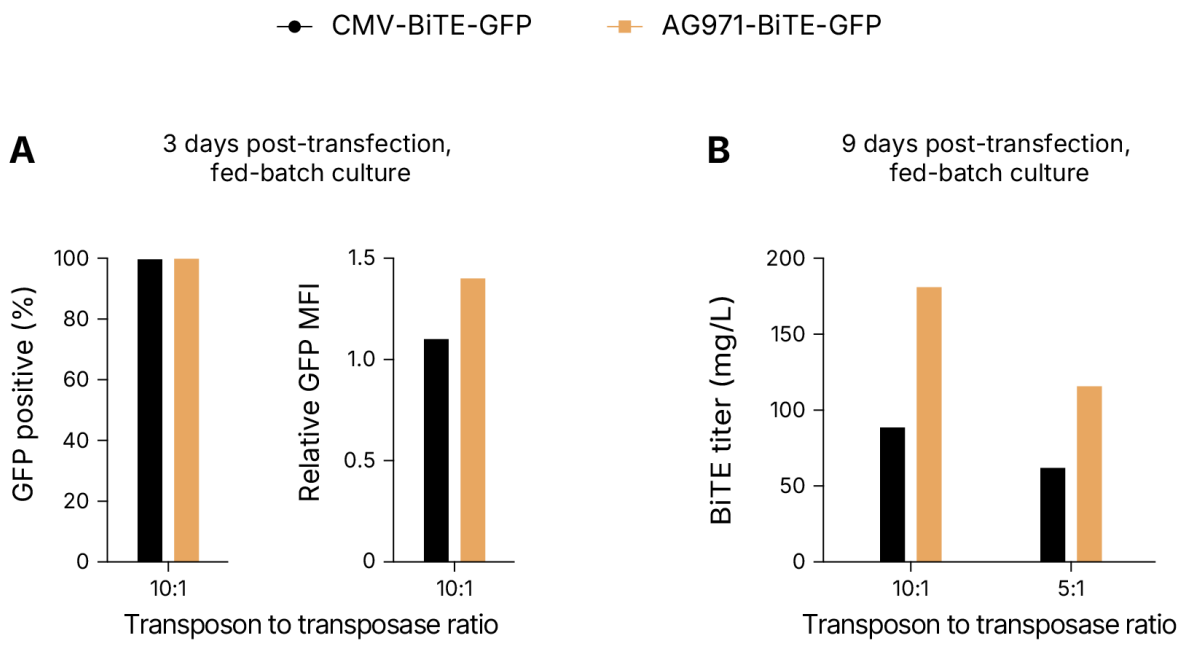

Figure 4. AG971 vector system enhances Blinatumomab expression by ~2-fold in semi-stable piggyBac transposon-mediated expression. CHO cells were co-transfected with (i) either the PB-CMV-BiTE-GFP or PB-AG971-BiTE-GFP plasmid and (ii) 20 µg or 40 µg of in vitro-synthesized mRNA encoding the piggyBac transposase. No selection was applied. Fed-batch cultures were initiated immediately after electroporation, and cells were supplemented with feed equivalent to 10% of the total culture volume on days 1, 4, and 7. (**A**) Transfection efficiency and GFP expression. Co-transfection achieved high efficiency, with ~99% GFP-positive cells on day 3 post-electroporation (left panel). Flow cytometry measurements of mean fluorescent intensity (MFI) indicated that PB-AG971-BiTE-GFP samples exhibited slightly higher GFP expression than PB-CMV-BiTE-GFP starting on day 3 (right panel). Proportions of GFP-positive cells and average GFP levels (MFI) were determined by flow cytometry. Data shown are representative of samples generated using a 10:1 plasmid DNA to mRNA (weight:weight) ratio. (**B**) Blinatumomab titer. Use of the PB-AG971-BiTE-GFP construct resulted in an approximately 2-fold increase in secreted antibody yield on day 9 post-transfection compared with PB-CMV-BiTE-GFP. This effect was consistent across both plasmid DNA to transposase mRNA ratios tested. BiTE titers were quantified using an in-house assay with purified Blinatumomab as the reference standard.

These results highlight the substantial potential of the AG971 system for biotherapeutic production, particularly in semi-stable CHO pools (also sometimes called transient/stable hybrid expression systems), which are increasingly recognized for offering an optimal balance between production speed and yield.

Given that the AG971 promoter consistently outperformed the CMV promoter in both transient transfection and semi-stable expression systems, we next evaluated the production capability of stable AG971 cell pools. To this end, CHO cells were co-transfected, as described above, with different ratios of the PB-AG971-BiTE-GFP plasmid and piggyBac transposase mRNA. Following G418 selection, fed-batch cultures were initiated (**Figure 5**). Under these optimized conditions, the titer of secreted Blinatumomab reached up to 280 mg/L by day 17 of the fed-batch process.

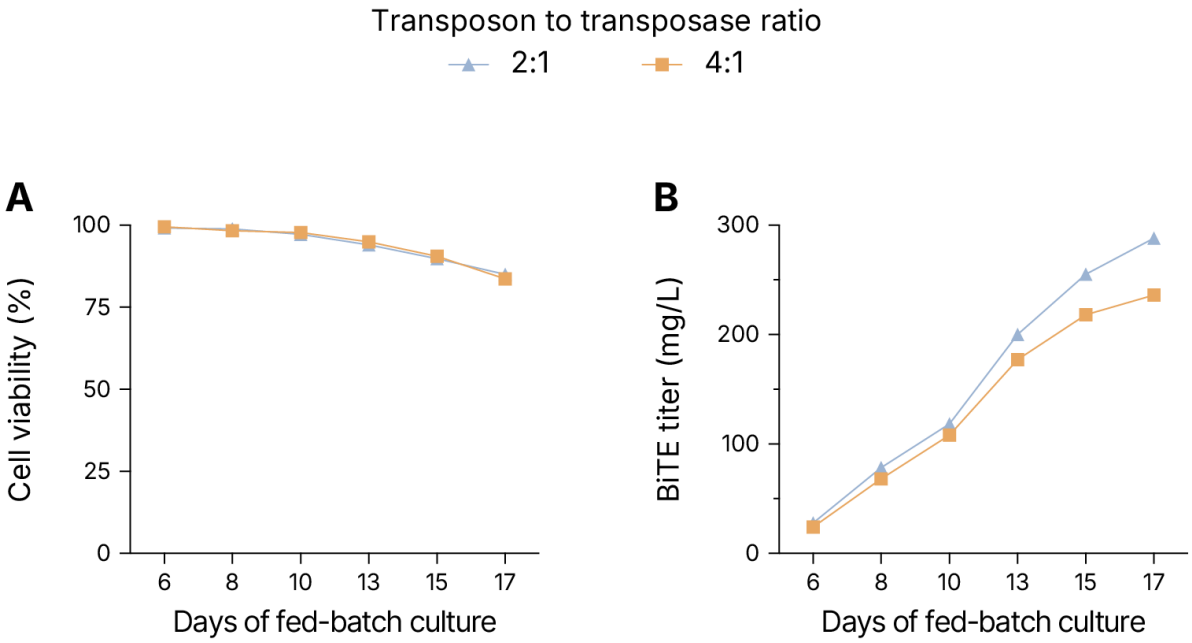

Figure 5. High-titer production of Blinatumomab in stable cell pools established with the novel AG971 vector system. Stable CHO cell pools were generated by co-transfection with (i) PB-AG971-BiTE-GFP plasmid and (ii) 50 µg or 100 µg of in vitro-synthesized mRNA encoding the piggyBac transposase. Transfected cells were subjected to G418 selection (1 mg/ml) for 10 days, starting 24 h post-transfection. Selected stable pools were used to initiate fed-batch cultures, with cells supplemented with feed equivalent to 10% of the total culture volume on days 1, 4, and 7. (**A**) Cell viability. Both stable cell pools displayed similar viability dynamics, gradually declining from ~99% at culture initiation to ~83% on day 17. (**B**) Blinatumomab titer. Final antibody yield depended on the amount of piggyBac transposase used during transfection. The pool generated using a 2:1 (weight:weight) ratio of plasmid DNA to transposase mRNA achieved the highest secreted BiTE concentration, reaching 280 mg/L by the end of the fed-batch culture. BiTE titers were quantified using an in-house assay with purified Blinatumomab as the reference standard.

Conclusions

In summary, we successfully developed the novel AG971 expression vector, which substantially enhances the production of the model difficult-to-express biotherapeutic protein Blinatumomab in both transient transfection and semi-stable cell systems. Notably, in semi-stable cell pools, the AG971 promoter doubled the yield compared with the industry-standard CMV promoter. Furthermore, the bidirectional activity of AG971 offers a valuable advantage. It can be utilized for the co-expression of a selection marker, thereby mitigating the undesirable phenomenon of transcriptional interference that often occurs between different promoters within a transgene cassette [Shearwin *et al.*, 2005; West, 2014] and tightening the association between selective pressure and transgene expression. This bidirectional capacity may also be leveraged for the co-expression of multiple components, such as different antibody chains.

Overall, this study demonstrates the capability of Annogen's SuRE™ platform to develop novel promoters that drive expression beyond current industry standards, opening new opportunities to enhance titers and quality for both difficult-to-express proteins and other recombinant products. We are currently leveraging these capabilities to achieve further improvements over these benchmarks.

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