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Exploration of BAC versus plasmid expression vectors in recombinant CHO cells

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Abstract Vector engineering approaches are commonly used to increase recombinant protein production in mammalian cells, and among various concepts, bacterial artificial chromosomes (BAC) have been proposed to serve as open chromatin regions to omit chromosome positional effects. For proof of concept, we developed stable recombinant Chinese hamster ovary (CHO) cell lines using different expression vector systems: the plasmid vectors contained the identical expression cassette as the BAC constructs. Two anti-HIV1 antibody derivates served as model proteins (3D6scFc and 2F5scFc) for generation of four stable recombinant CHO cell lines. The BAC-derived clones showed three to four times higher specific productivity, and therefore, gene copy numbers and transcript level were quantified. The active chromatin region provided with the BAC environment significantly improved transcription evidenced with both model proteins. Specific transcription was approximately six times higher from BAC-based vectors compared to the corresponding plasmid vectors for both single-chain fragment crystallizable (scFc) proteins. Our accurate investigations elucidated also differences between translational activities related to the protein of choice. 3D6scFc expressed specifically three to four times more product than 2F5scFc indicating that the product by itself also contributes to enhanced productivity. This study indicated comparable increase of transcription level for both scFc proteins when using the BAC system, but translation, maturation, and secretion of individual proteins seem to be protein specific.

Keywords BAC · CHO · Antibody expression · gcn · Transcript level

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Introduction

Recombinant protein and especially monoclonal antibody (mAb) production in Chinese hamster ovary (CHO) cells is of immense interest for the biopharmaceutical industry (De Jesus and Wurm 2011). From 2006 to 2010, half of 25 genuinely, newly approved biopharmaceuticals were mAb products and the main workhorse for production was mammalian (mainly CHO) cells (Walsh 2010). CHO cells are regarded as safe host by the regulatories, can be cultured as serum-free suspension in large-scale bioreactors, and have the capacity for efficient posttranslational modifications. To improve low specific productivities (qp), cell lines are typically subjected to gene amplification (Kim et al. 2012), but high gene copy numbers (gcn) do not necessarily result in proportionally higher qp (Lattenmayer et al. 2007b; Reisinger et al. 2008) and methotrexate-treated CHO cells might result in clonal instability regarding gcn, transcript level, and qp (Chusainow et al. 2009). Therefore, the development of stable cell lines requires time- and labor-intensive screening processes to select clones with adequate transcription rates influenced by the chromatin surrounding the transgene (Wilson et al. 1990). The so-called positional effect is considered in different vector engineering strategies including site-specific integration and cis-acting elements (S/MAR, UCOEs) (Kim et al. 2012). These strategies facilitate transcription either by integration of a transgene in a predefined position (hot spot) or introduction of open chromatin regions adjacent to the transgene. Another method to enhance transcription is the use of

large vectors such as bacterial artificial chromosomes (BACs) which contain open chromatin loci. Blaas and coworkers showed that a BAC containing the Rosa26 locus (*Rosa26* BAC) is suitable as expression vector for recombinant protein production in HEK293 cells. Expression using the Rosa26 BAC is copy number-dependent with an increase in qp by a factor of 10 compared to a conventional vector (Blaas et al. 2009).

In this study, we evaluated the use of the *Rosa26* BAC expression vector for the production of two anti-HIV1 single-chain fragment crystallizable (scFc) antibody derivates of human IgGs (mAb2F5 and mAb3D6) (Kunert et al. 1998) in CHO cells. Both antibodies target an epitope on the HIV-1 glycoprotein gp41. mAb2F5 is able to broadly neutralize HIV-1 isolates and might be suitable in a systemic passive administration combined with anti-retroviral drugs in a prophylactic or therapeutic setting (Burton et al. 2012). mAb3D6 is non-neutralizing and can be used as a research antibody to probe the structure of gp41.

Stable recombinant CHO cell lines were established based on clones exhibiting high qp and growth rate. Transgene copy number as well as transcriptional level was analyzed to evaluate the effect of *Rosa26* BAC expression vectors in CHO cells compared to recombinant CHO cells transfected with conventional expression vectors containing the identical expression cassette.

Material and methods

Antibody constructs

The scFc antibody derivates were constructed by fusion of the single-chain fragment variable (scFv) antibody to the human IgG1 Fc region (GenBank: CAA49866). In the case of 3D6scFc, the scFv sequence of 3D6 (GenBank: CAA01551) was used. For 2F5scFc, the scFv region was constructed by combining the variable heavy chain and the variable light chain of mAb2F5 by a $(GGGGS)_3$ linker according to the 3D6scFc. The sequence of mAb2F5 was retrieved from the protein database (PDB: 2F5A). Both constructs were synthesized with CHO codon optimization (Geneart, Regensburg).

Plasmids, BACs, and cell lines

The p3D6scFc and p2F5scFc mammalian expression plasmids served as shuttle vector for integration of GOI into the $Rosa26^{BAC}$ DNA. The expression plasmids were generated by conventional cloning methods using the pBluescript KS plasmid (Stratagene) as backbone. The expression cassette is composed of the CAGGS promoter (Niwa et al. 1991), the gene of 2F5scFc or 3D6scFc, and a neomycin resistance cassette. The whole expression cassette is flanked by two attB sites. The $Rosa26^{BAC\ 3D6scFc}$ and $Rosa26^{BAC\ 2F5scFc}$ BAC vectors were assembled by homologous recombination as previously described (Blaas et al. 2007). Briefly, the p3D6scFc or p2F5scFc expression cassette from the mammalian expression plasmid was inserted into a BAC containing *Rosa26* locus using ϕC31-mediated cassette exchange.

For the generation of stable cell lines, CHO DUKX-B11 cells (ATCC CRL-9096) (Urlaub and Chasin 1980) were used. The cells were maintained in suspension under protein- and antibiotic-free conditions.

Cell line development

To establish stable recombinant CHO cell lines, either the expression plasmids (p3D6scFc or p2F5scFc) or the NotI linearized BAC vectors ($Rosa26^{BAC\ (3D6scFc\ or\ 2F5scFc)}$) were transfected using the cationic polymer PEI (Polysciences, Inc.) at a DNA/PEI ratio of 1:10 or by applying nucleofection (Lonza) according to the manufacturer's protocol. For all transfections, 2–5 pg DNA/cell was used. One or two days after transfection, selection pressure was applied (0.5 mg/mlG418 (PAA)) in ProCHO5 medium (Lonza) supplemented with 4 mM glutamine (PAA) and hypoxanthine/thymidine (Invitrogen). Monoclonal recombinant cell lines were established by limited dilution technique. The clone with the highest qp from each cell line was selected and clone homogeneity was confirmed by flow cytometric analysis. Therefore, 10^6 ethanol-fixed cells were stained with anti-huIgG-γ-chain-R-phycoerythrin antibody (Sigma) and analyzed with Gallios™ flow cytometer (Beckman Coulter).

Determination of specific productivity

The qp of recombinant CHO cell lines was calculated as picograms per cell per day (pg $cell^{-1}$ day^{-1}) as described elsewhere (Lattenmayer et al. 2007a). The cell number was determined using a Z2™ Coulter Counter® (Beckman Coulter), and 3D6scFc and 2F5scFc protein concentration was analyzed by ELISA; 96-well microtiter plates (Nunc) were coated overnight at 4 °C with 0.5 μg/ml of anti-huIgG-γ-chain-specific antibody (Sigma). Plates were incubated with serial twofold dilutions of recombinant CHO cell culture supernatant (RT, 1 h). Protein A purified 3D6scFc was used as a standard. Afterwards, plates were incubated with peroxidase-conjugated anti-huIgG antibody (Invitrogen) and reactions were visualized with *o*-phenylenediamine and H_2O_2 (Merck). Plates were measured with a microplate reader (Tecan) at 492 nm with a reference wavelength of 620 nm.

Gene copy number and transcript analysis

Preparation of genomic DNA and cDNA

Genomic DNA (gDNA) was prepared from stable recombinant CHO cell lines; 2×10^6 cells were harvested and washed with PBS. gDNA was isolated and purified using the QIAamp® DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions.

Total RNA was isolated from 2×10^6 cells using Ambion TRI Reagent Solution (Life Technologies, CA) according to the manual. DNA contaminations were removed from extracted RNA by digestion with DNase (Qiagen, Netherlands) in the presence of RNase inhibitor (Life Technologies, CA). Purified RNA was stored in RNAse-free water at −20 °C. Total RNA was reverse-transcribed with random primers (Promega) using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Purity and concentration of the gDNA and cDNA were determined with the ND-1000 Spectrophotometer (Nano-Drop®).

qPCR

qPCR was performed with the MiniOpticon™ system (Bio-Rad) utilizing the TaqMan method. All primers were designed using the Primer3 web application (Untergasser et al. 2007) and synthesized by Sigma-Aldrich. Primers were designed to amplify a 100-bp fragment in the Fc part of the two scFc antibody derivates and a 78-bp fragment in the housekeeping gene β-actin which served as an internal control and to normalize the data. qPCR was either performed with 3 ng of gDNA or cDNA including non-template controls and negative controls by denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min. Fluorescence signal and Cq values were calculated by the CFX manager software 2.1 (Bio-Rad) by baseline subtraction and linear regression. For relative quantification, the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001) was used. To obtain statistically significant data triplicates of three biological samples per cell, clones were analyzed in two technical runs. Statistical analysis was performed with the SPSS 18 software package (IBM).

Results

Recombinant cell lines

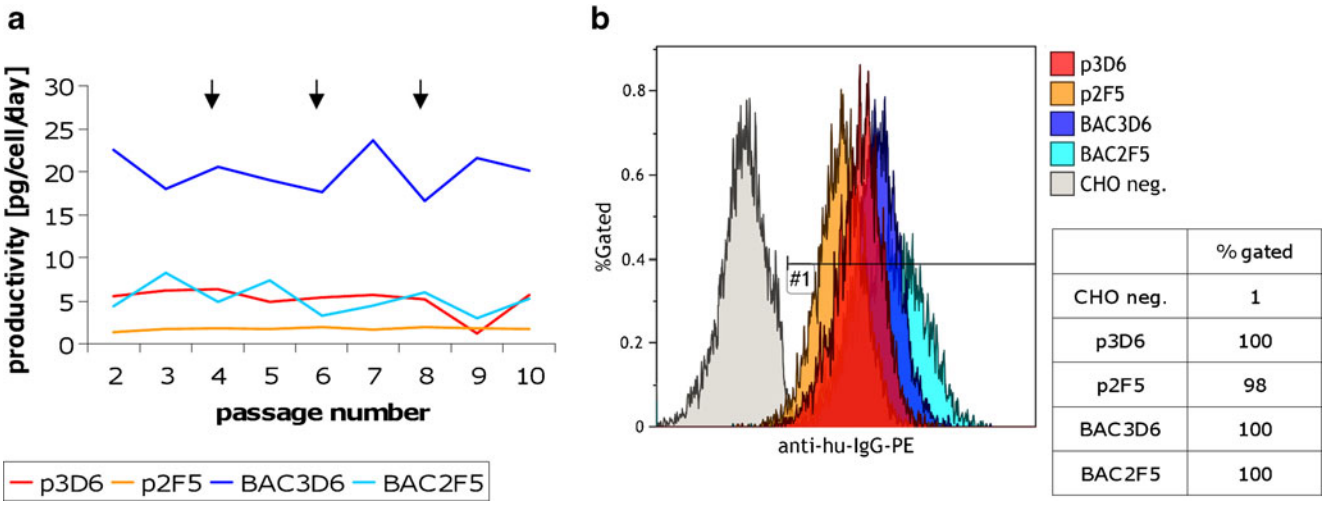
The feasibility of BAC-based production of antibody derivates in CHO cells was evaluated by generation of four individual cell lines expressing two different scFc antibody derivates under the CAGGS promoter.

CHO DUKX B11 cells cultivated under protein-free conditions were transfected with the scFc expression plasmids resulting in cell lines p3D6 and p2F5 or the *Rosa26*^{BAC} BAC vectors resulting in cell lines BAC3D6 and BAC2F5. Stable clones isolated by limited dilution subcloning in the presence of G418 were screened for production of scFc antibody derivates. Twelve best producing clones of each cell line were selected and further expanded into T-flasks and screened for high production clones in a second screening round. The qp of these clones was 10–30 pg cell^{-1} day^{-1} for BAC3D6, 1.4–7.7 pg cell^{-1} day^{-1} for the BAC2F5, 0.6–9.5 pg cell^{-1} day^{-1} for the p3D6, and 0.3–3.9 pg cell^{-1} day^{-1} for the p2F5 CHO clones. The best performing clone of each cell line was transferred to a spinner vessel, cultured, and monitored over ten passages. Figure 1a shows the qp over time indicating stable production of scFc antibody derivates. Arrows indicate the sampling points for qPCR and flow cytrometric analysis. Flow cytrometric analysis of intracellular scFc content revealed homogenous distribution of product in individual recombinant cell line populations (Fig. 1b). In Table 1, the mean values of qp and specific growth rate (μ) are summarized showing three to four times higher specific productivity for the BAC clones resulting in two to three times higher volumetric titers.

Transgene and transcript analysis

To evaluate the reason for the better productivity of BAC-transfected CHO clones, genomic DNA and mRNA were isolated from individual scFc-producing clones and gcn and transcript level were analyzed. The transgene and the housekeeping gene β-actin were amplified using Fc- or β-actin-specific primers (Table 2) and analyzed by qPCR. The relative amount of transgene copies and transcriptional level is summarized in Table 3. In general, the gcn of all BAC and plasmid clones are one to two transgene copies relative to β-actin.

Specifically, gcn of BAC3D6 were two times higher than for β-actin (Table 3) while 2F5scFc BAC and plasmid clones contained basically the same gcn as β-actin. Both plasmids are transcribed approximately with half the efficiency while BACs are transcribed three times more efficient compared to β-actin (Table 3, column 4). Direct comparison of BAC and plasmid clones is shown in Fig. 2. A significant higher amount in transcript level and specific productivity was found in both BAC clones. Specific productivity was four times higher for the 3D6scFc and three times higher for the 2F5scFc when expressed via BAC constructs. The transcript level showed the most impressive enhancement. BAC3D6scFc clones developed 16 times more transcript than the p3D6scFc clone and BAC2F5scFc developed six



	% gated
CHO neg.	1
p3D6	100
p2F5	98
BAC3D6	100
BAC2F5	100

Fig. 1 Stable recombinant monoclonal CHO cell clones expressing antibody derivates. **a** Productivity of spinner cultures expressing either plasmid (p3D6, p2F5)- or BAC (BAC2D6, BAC2F5)-transfected scFc antibody derivates over ten passages. *Arrows* indicate sample points for subsequent analysis. **b** Intracellular product analysis by flow cytometry. Gate *#1* indicates scFc-positive cells

times more transcript than the p2F5scFc clone without considering gcn. These results demonstrated, with two independent antibody derivates, that transcription efficiencies are improved with the BAC system. Superior transcription was evaluated by correlation of transcription level to gcn. In particular, we found a sixfold higher amount of specific mRNA per gcn in both scFc antibody clones than in the corresponding plasmid clones (Fig. 3). Correlation analysis between qp per gene copy showed that BAC-transfected CHO clones had a 1.5- to 2.6-fold higher qp per gcn compared to the plasmid-transfected cells. This means that the BAC-transfected clones showed superior performance in transcription rate (six times higher than plasmids) resulting also in a better but more variable specific productivity. However, the overrepresentation of transcripts cannot be translated into secreted product evidenced by correlation of qp to transcript level. The formidable amounts of transcript in BAC clones cannot be linearly transferred to specific productivity since plasmid-transfected CHO clones had a 2.3- to fourfold higher qp relative to its transcript level compared to the BAC-transfected cells (Fig. 3).

Table 1 Summary of cell culture results for plasmid and BAC cell lines

Cell line	μ (1 day^{-1})	qp (pg cell^{-1}day^{-1})	Volumetric titer (μg ml^{-1})
p3D6	0.49 (0.06)	5.61 (0.48)	16.93 (5.18)
p2F5	0.55 (0.08)	1.73 (0.17)	5.78 (1.97)
BAC3D6	0.37 (0.05)	20.13 (2.30)	45.49 (8.59)
BAC2F5	0.35 (0.08)	4.90 (1.82)	11.89 (3.07)

Mean values with standard deviation in brackets are shown

Discussion

This is the first study where recombinant CHO cell lines were developed, once with a conventional expression plasmid and secondly with the *Rosa26* BAC system being generated with the identical expression cassette as the plasmid vector. By screening of 300–400 initial transfectants, we ensured that for each cell line the best clone was selected for further characterization.

The single-chain antibody format was chosen to keep the expression system simple and to insure comparability of nucleic acid quantification. With this strategy, only one gene needs to be integrated, transcribed, and translated and misinterpretation due to unbalanced antibody chain expression is avoided. The biological activity of the 2F5scFc has been confirmed in a previous study (Mader and Kunert 2012) indicating that the scFc format had a slightly lower affinity for its linear epitope compared to mAb2F5 (data not shown).

Transfection with the *Rosa26*$^{\text{BAC (3D6scFc)}}$ and *Rosa26*$^{\text{BAC (2F5scFc)}}$ BAC expression vector and the conventional p3D6scFc and p2F5scFc plasmids showed higher qp levels for the BAC-transfected clones (Fig. 1a). In a previous protein production study using HEK293 cells

Table 2 Primer for qPCR analyses

scFc_s	5′-ACGAGGACCCTGAAGTGAAG-3′
scFc_as	5′-CGGTAGGTGGAGTTGTACTGTTC-3′
scFC_probe	5′-[6FAM]AAGTGCACAACGCCAAGACCAAGC[TAM]-3′
β-actin_s	5′-TGAGCGCAAGTACTCTGTG-3′
β-actin_as	5′-TTGCTGATCCACATCTCCTG-3′
β-actin_probe	5′-[6FAM]CCATCCTGGCCTCACTGTCCACCT[TAM]-3′

Table 3 Summary of qPCR results for plasmid and BAC cell lines

Cell line	Gene copy number (related to β-actin)	Transcript level (related to β-actin)	Transcript/gene copy number
p3D6	0.77 (0.14)	0.39 (0.05)	0.5
p2F5	0.69 (0.07)	0.33 (0.12)	0.5
BAC3D6	2.03 (0.60)	6.26 (2.40)	3.1
BAC2F5	0.64 (0.11)	2.11 (0.38)	3.3

Mean values with standard deviation in brackets are shown

transfected with the *Rosa26* BAC vector, a proportional increase of qp with increasing gcn was shown in a family of subclones expressing the same protein (Blaas et al. 2009). Furthermore, a BAC-based vector containing the DHFR locus has been shown to confer high expression levels in NIH 3T3 cells (Bian and Belmont 2010).

We expanded this question in the CHO system and analyzed the relation of gcn and transcript level as well as transcript level and productivity to get more insights about bottlenecks of the protein production machinery. We confirmed that BAC clones are able to improve transcriptional activity resulting in higher transcript to gcn ratios compared to plasmid expression. In both pairs of recombinant cell lines, the transcript level per gcn revealed a sixfold higher specific transcript amount in BAC-transfected CHO clones, and so our experiments confirm BAC vectors as open chromatin region with efficient transcription potential. Correlating the qp with transcript level leads to the conclusion that more transcript does not generate a proportionally higher qp (Fig. 3). In consistence with the literature (Lattenmayer et al. 2007b; Mohan et al. 2008; Reisinger et al. 2008), we found the bottleneck in protein maturation and/or secretion machinery. Since the amount of transcript is

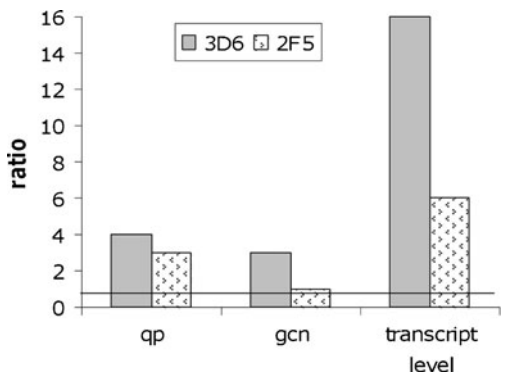


Fig. 2 Characteristics of BAC-transfected monoclonal CHO cell lines relative to plasmid derived cell lines. Gene copy number (gcn), transcript level, productivity (qp), and volumetric titer of BAC-transfected clones in relation to plasmid-transfected clones analyzed for 3D6scFc and 2F5scFc. Highest distinctions were found in transcription levels

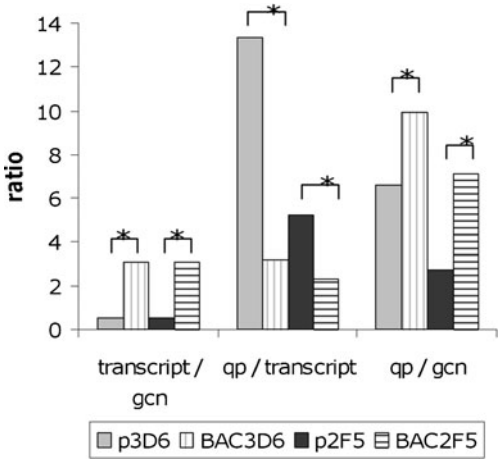


Fig. 3 Comparison of BAC and plasmid-transfected monoclonal CHO cell line. Correlation of transcript and production level to gcn indicates that BACs are preferable expression systems but qp/transcript demonstrates that high transcription rates do not correlate with productivity. *$p<0.001$, Student's t test statistical significance

rather high in the BAC clones, the specific productivity related to transcript amount lags behind plasmid clones despite the higher specific productivity in BAC clones. We also found higher specific growth rates in plasmid derived clones which might also indicate that the better producers overstrain the overall physiology of the cells.

Beside the improvements of recombinant protein expression with the BAC system, the maximal qp differed three- to fourfold between 3D6scFc and 2F5scFc independent of the applied expression vector. This is quite interesting because only the variable regions of the two molecules differ. By flow cytometric analysis, we investigated the intracellular amount of recombinant proteins of p3D6scFc and BAC2F5, characterized by nearly the same specific productivity. BAC2F5 has higher amounts of intracellular product (Fig. 1b) which does not reflect the secretion rate. We assume that this is a sign of decelerated processing of the 2F5scFc protein in the endoplasmatic reticulum and therefore leads to reduced production rates. Similar observations have been made for other clones expressing different proteins (unpublished data). In further investigations, we will define specific markers indicating the ER stress which might be related to the recombinant protein production in CHO cells.

Taken these results together, we could successfully adapt the Rosa26 BAC expression system to CHO production cell lines. Two anti-HIV1 scFc antibody derivates could be expressed in stable recombinant CHO cell clones with increased productivity compared to conventional plasmid systems. This increase is rather contributed to enhanced transcriptional levels than to elevated transgene copies even though the higher transcript level does not reflect in proportionally increased qp.

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