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Research Article

Polysome profiling of mAb producing CHO cell lines links translational control of cell proliferation and recombinant mRNA loading onto ribosomes with global and recombinant protein synthesis

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mRNA translation is a key process determining growth, proliferation and duration of a Chinese hamster ovary (CHO) cell culture and influences recombinant protein synthesis rate. During bioprocessing, CHO cells can experience stresses leading to reprogramming of translation and decreased global protein synthesis. Here we apply polysome profiling to determine reprogramming and translational capabilities in host and recombinant monoclonal antibody-producing (mAb) CHO cell lines during batch culture. Recombinant cell lines with the fastest cell specific growth rates were those with the highest global translational efficiency. However, total ribosomal capacity, determined from polysome profiles, did not relate to the fastest growing or highest producing mAb cell line, suggesting it is the ability to utilize available machinery that determines protein synthetic capacity. Cell lines with higher cell specific productivities tended to have elevated recombinant heavy chain transcript copy numbers, localized to the translationally active heavy polysomes. The highest titer cell line was that which sustained recombinant protein synthesis and maintained high recombinant transcript copy numbers in polysomes. Investigation of specific endogenous transcripts revealed a number that maintained or reprogrammed into heavy polysomes, identifying targets for potential cell engineering or those with 5′ untranslated regions that might be utilized to enhance recombinant transcript translation.

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Abbreviations: CHO, Chinese hamster ovary; **GS**, glutamine synthetase; **HC**, heavy chain; **IgG**, immunoglobulin G; **IVC**, integral of viable cell concentration; **LC**, light chain; **M**, monosome; **mAb**, monoclonal antibody; **P**, polysome; **qP**, cell specific productivity; **S**, sub-polysome; **UTR**, untranslated region

1 Introduction

Cultured Chinese hamster ovary (CHO) cells have become the most widely used mammalian cell expression system for the production of valuable recombinant biotherapeutic proteins [1]. Over a period of several decades, the ability of this expression system to produce high quality recombinant protein at ever increasing amounts has been considerably enhanced. However, although the fed-batch culturing of these cells to produce monoclonal antibodies (mAbs) might be considered to be approaching maturity with yields in excess of 5 g/L [2–4] and up to 10 g/L [5] reported, some mAbs remain difficult to express [6]. Further, many of the novel format antibody based molecules

in development and a number of non-mAb based recombinant biotherapeutics remain challenging to express at the required amounts and with the appropriate quality and homogeneity [7, 8].

The yield of recombinant protein from cell expression systems is defined by the cell specific productivity of the cell (referred to as qP, usually expressed as pg/cell/day) and the biomass or integral of viable cell concentration (IVC) achieved in the cell culture system [1]. One of the key processes that determines the growth, proliferation and duration of the culture (and hence the IVC), and that of the cell specific recombinant protein synthesis rate, is mRNA translation [9–14]. mRNA translation is a key regulatory step in the control of gene expression in eukaryotes and it has been reported that the cellular abundance of proteins is predominantly controlled at the level of translation [15]. Indeed, a number of reports now demonstrate that recombinant mAb production from CHO cells is limited by translational efficiency [12, 13, 16, 17] and the enhancement of recombinant protein yields can be achieved by manipulating ribosome biosynthesis [18]. Further, before a cell can grow and divide it must double its protein mass, an energy-expensive process which involves extensive protein synthesis. Thus, control of both global mRNA translation and recombinant mRNA translation are key in determining the yield of recombinant proteins obtained from cultured mammalian cell systems.

In the cell, a single mRNA may be translated by one ribosome or multiple ribosomes that form polysomes (polyribosomes). When the translational processes of initiation, elongation and termination are in equilibrium, the number of ribosomes on each transcript can be considered at a steady state [19]. The number of ribosomes per transcript can be used to estimate the translation efficiency of a transcript, with a higher number of ribosomes indicative of enhanced protein synthesis from a given transcript [20]. The technique of polysome profiling can, therefore, be used to determine the relative amounts of polysomal mRNA associated ribosomes compared to monosome (80S) and individual 60S and 40S ribosomal subunit peaks as an indicator of ribosome amounts and global translational efficiency of the cell [21]. An increase in the polysome fraction is indicative of enhanced global translational activity that might for example underpin cell growth and proliferation. Additionally, such translational profiling can also be used to investigate the translational efficiency of individual mRNAs, with mRNAs located in polysomes considered to be more translationally active than those found in sub-polysomes [22]. Such approaches can be used to identify ribosome signatures of cell stress and reprogramming of cells related to observed phenotype [23].

Despite the widely applied approach of polysome profiling to determine translational control and reprogramming in cells, only three studies to our knowledge have been reported that use this method to investigate recombinant protein expression from mammalian cell systems. The first reported on the analysis of a CHO DG44 mAb producing cell line and concluded that maximal global translational activity occurred during exponential growth phase. The highly translated mRNAs at exponential phase were growth related and for 95% of investigated genes, transcript amounts and translational efficiency were uncoupled [24]. The second study showed that upon treatment of CHO cells with rapamycin, mRNAs shifted from polysomes to monosomes as would be expected when mTOR signalling is inhibited leading directly to a slowing of global translation. On-the-other-hand, feeding of cells resulted in an increase in global translational activity and an increase in specific heavy and light chain translation [25]. The third found that recombinant mRNAs are translated as efficiently as host cell transcripts and occupied up to 15% of total ribosome capacity [26]. Collectively these studies conclude that a better understanding of global and target specific translational efficiency and reprogramming in CHO cells could aid in the development of feeding strategies to prolong growth and recombinant protein production and identify targets for cell engineering strategies to prolong or enhance recombinant gene synthesis.

In view of the importance of global mRNA translation in controlling global protein synthesis and hence growth and proliferation of recombinant CHO cell lines, and of mRNA specific translational efficiency of recombinant and endogenous transcripts influencing recombinant protein synthesis, we have applied polysome profiling to the analysis of a panel of recombinant mAb producing CHO cell lines grown under batch-culture conditions. We report on the relationship between the relative amounts of ribosomes in the model cell lines and the polysome to monosome ratio, how this changes throughout batch culture and the relationship to cell growth. We also show that the distribution of recombinant mRNAs across the polysome profile differs between cell lines and relates to protein synthesis rates as determined by ^{35}S radiolabelling. Finally, we report on the translational reprogramming of CHO cell lines throughout batch culture by profiling key mRNA markers across the polysome profiles and relate this to cell performance. The study demonstrates that polysome profiling allows the unravelling of both global and recombinant mRNA translational efficiency across batch culture, and between cell lines, and shows how this relates to the recombinant protein yields obtained from CHO cells, cell growth and ultimately product yields from cultured CHO cell systems.

2 Materials and methods

2.1 Cell culture

The suspension CHO cell lines used in this study include a host CHO cell line and three CHO cell lines stably

expressing a model recombinant immunoglobulin G1 kappa (IgG) molecule whereby the glutamine synthetase (GS) selection system was used to generate the mAb producing cell lines. Routine culturing and experimental conditions are reported in Supporting information, File S1.

2.2 Antibody quantitation

The secreted antibody concentrations from recombinant cell lines were analyzed by protein A HPLC from samples taken from the single batch culture flasks, as previously reported [27].

2.3 Polysome profiling

For polysome profiling, 1×10^7 viable cells were collected from the appropriate culture in DNase/RNase-free microcentrifuge tubes on days 4, 6 and 8 of batch culture (note that total viability remained greater than 90% in all cultures and hence the total cell number taken was similar across sampling points and cell lines). A detailed explanation of polysome profiling experiments can be found in Supporting information, File S1.

2.4 Extraction of total RNA from cells and RNA from polysome profile fractions

RNA was extracted from 2×10^6 viable cells from the 30 mL cultures for each cell line for total RNA analysis, as well as from the fractions collected across the polysome profiles on days 4, 6 and 8 of batch culture as detailed in Supporting information, File S1. Normalization to the two housekeeping genes was undertaken using a method adapted from the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/) [28].

2.5 [^{35}S]-methionine nascent polypeptide labelling

For radiolabel experiments, the three recombinant CHO cell lines were grown in batch culture conditions in 25 mL CD-CHO medium for nascent polypeptide synthesis analysis. On days 4, 6 and 8, 1 mL of cells was transferred to a 24-well plate, and labelled for 1 h at 37°C with 2 µL of [^{35}S]-methionine (401 kBq/µL, Perkin Elmer) essentially as previously described [29] and as detailed in Supporting information, File S1.

2.6 Immunoprecipitation experiments

From the [^{35}S]-methionine labelled recombinant CHO cell lysates, 100 µL was taken for immunoprecipitation studies. Following centrifugation at 16 200 ×*g* for 10 min at 4°C, the cell lysate supernatants were mixed with a 30 µL mixture consisting of ¼ protein A beads/20 mM HEPES pH 7.2 (with 0.02 g NaN_3) overnight at 4°C. The samples were then reduced in 2x Laemmli loading buffer and subjected to electrophoresis on self-cast 12% SDS-polyacrylamide gels (SDS-PAGE). The gels were stained with Coomassie blue stain overnight, dried and exposed to Amersham Hyperfilm ECL (GE Healthcare) to visualize the radioactively labelled proteins via autoradiography.

3 Results and discussion

3.1 Batch culture growth and productivity characteristics of the model host and recombinant mAb producing CHO cell lines

The model cell lines investigated in this study included a host CHO cell line and three recombinant CHO cell lines engineered to stably express the same mAb, selected as they had previously shown different growth and/or cell specific productivity profiles from each other. Prior to performing polysome profiling, the growth and productivity characteristics of the model cell lines were analyzed in biological triplicate over an eight-day batch culture. Cultures were then sampled on days 4, 6 and 8 to reflect different stages of the culture (Fig. 1). The cell lines exhibited different growth profiles, with a range of maximum viable cell concentrations (VCC) and cell specific growth rates (Fig. 1A, 1B, 1D). The three recombinant mAb cell lines all maintained a culture viability over 90% across the eight-day batch culture, whilst the culture viability dropped close to 80% in the triplicate host cell line cultures after eight days. The host and recombinant cell line 3 achieved the highest, and very similar, viable cell concentrations and this was mirrored by these two cell lines having the fastest cell specific growth rate and highest integral viable cell concentrations (IVCs). Cell line 1 was the slowest growing and had the lowest IVC, approximately two thirds that of cell line 3, whilst the IVC achieved by cell line 2 was approximately mid-way between that of 1 and 3 (Fig. 1).

In addition to a range of growth characteristics, the recombinant cell lines exhibited a range of final IgG titers from 103 to 236 mg/L, with a 2.3-fold change from cell line 1 to 3 respectively (Fig. 1B). As such, the final titers at the end of the batch culture on day 8 mirrored the IVCs of the individual cell lines. When comparing the specific productivities (qP), cell line 3 did not have the highest qP (Fig. 1C) even though it had the highest titer and IVC (Fig. 1B). The qP for cell line 2 was marginally higher than cell line 3, but with a reduced IVC the final titer was substantially less than that of cell line 3 (Fig. 1B). When the qP data was calculated over days 0–4, 4–6 and 6–8, cell line 1 again had the lowest qP for each interval whilst cell line 2 was highest over days 0–4 and 6–8 but cell line 3 was highest over the day 4–6 period (Fig. 1C). The specific growth rates (μ) of the recombinant cell lines (Fig. 1D) confirmed cell line 3 had the fastest growth rate of the recombinant cell lines whilst when µ was calculated over

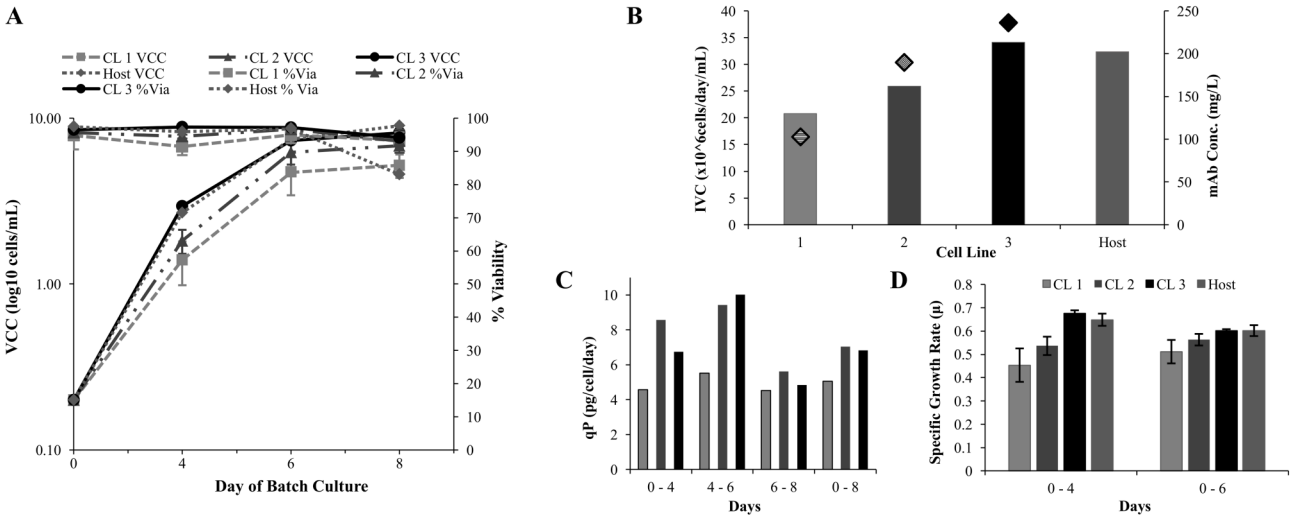

Figure 1. Growth and productivity characteristics of CHO cell lines in batch culture. (**A**) Growth curves of the stable recombinant mAb producing cell lines (1, 2 and 3) and the host cell line ($n = 3$), error bars show standard deviation (SD). All cell lines were sampled on days 4, 6 and 8 of batch culture. Figure shows log10 viable cell concentration (VCC, left axis, bottom lines) and percentage viability (right axis, top lines). (**B**) Integral viable cell concentrations, (IVC, bars) for all cell lines and mAb concentrations (mg/L, diamonds) of the recombinant stable cell lines 1, 2 and 3. (**C**) Cell specific productivity (qP, pg/cell/day) for the three recombinant cell lines from days 0 to 4, 4 to 6 and 6 to 8, and the overall productivity from day 0 to 8. (**D**) Cell specific growth rate (μ) for the four cell lines from day 0 to 4, and day 0 to 6.

days 0 to 6 of batch culture the host and cell line 3 had more-or-less equivalent growth rates.

Collectively, the growth and productivity data show that the model cell lines present a range of growth and productivity characteristics. The faster growth rate and higher IVC of cell line 3 suggests a greater translational efficiency to drive protein synthesis and hence proliferation than the other cell lines whilst, although cell line 2 has a higher cell specific productivity, reduced growth and proliferation limits the final mAb yield. The panel of cell lines therefore provides the opportunity to investigate global and transcript specific translational efficiency via polysome profiling throughout the batch process and between the cell lines to determine how this may impact on the growth and productivity characteristics.

3.2 Polysome profiling of the model host and recombinant mAb producing CHO cell lines reveals differences in the polysome to monosome ratio early in batch culture and the timing of a shift from polysomes to sub-polysomes across cell lines

Polysome profiling is a widely used technique to investigate translational differences in a variety of cell types looking at ribosomal loading of mRNAs and translation efficiencies [25, 30]. A shift in the distribution of ribosomes from polysomally-associated to monosomally-associated is indicative of reduced translational efficiency and translational reprogramming [25]. When the cell is actively translating mRNA, most of the actively translating ribosomes can be found as polysomes in the cell whereby a single transcript has many ribosomes loaded onto it [31–33]. An exception to this is if the cell is subjected to a stress or limitation that results in a slowing or block in translation elongation (the rate at which the ribosome decodes the mRNA and elongates the growing polypeptide) resulting in an increase in polysomes despite slowed or less active translational activity [32]. Here we apply polysome profiling to investigate differences in translational activity between cell lines and translational reprogramming during batch culture. This was achieved by isolating the ribosomes present in cell lysates from the same number of viable cells on different days of culture, for each cell line. Polysomes (P) were then separated from monosomes (M) by sucrose density centrifugation (Fig. 2A) [34]. For the purpose of this investigation we defined the monosome as the 80S peak (one ribosome) and the sub-polysome fraction as including the monosome (80S peak) and 60S and 40S peaks.

Polysome profiling of the four model cell lines showed that the peaks representing the number of ribosomes in sub-polysomes and polysomes changed over time for all cell lines with an increase in the monosomal (80S) and ribosomal subunit (sub-polysome) peaks which increased in size relative to the polysome peaks as the cultures progressed from day 4 through day 6 to day 8 (Fig. 2B). Thus, as growth and proliferation slows, an increase in the sub-polysome peaks was observed for all cell lines indicative of a slowing of global translation. Cell line 2 had the most striking change in the profile of the recombinant cell lines with an apparent increase in polysomes as culture progressed from day 4 to day 6, although the sub-polysome peaks also increased (Fig. 2B). An increase in the poly-

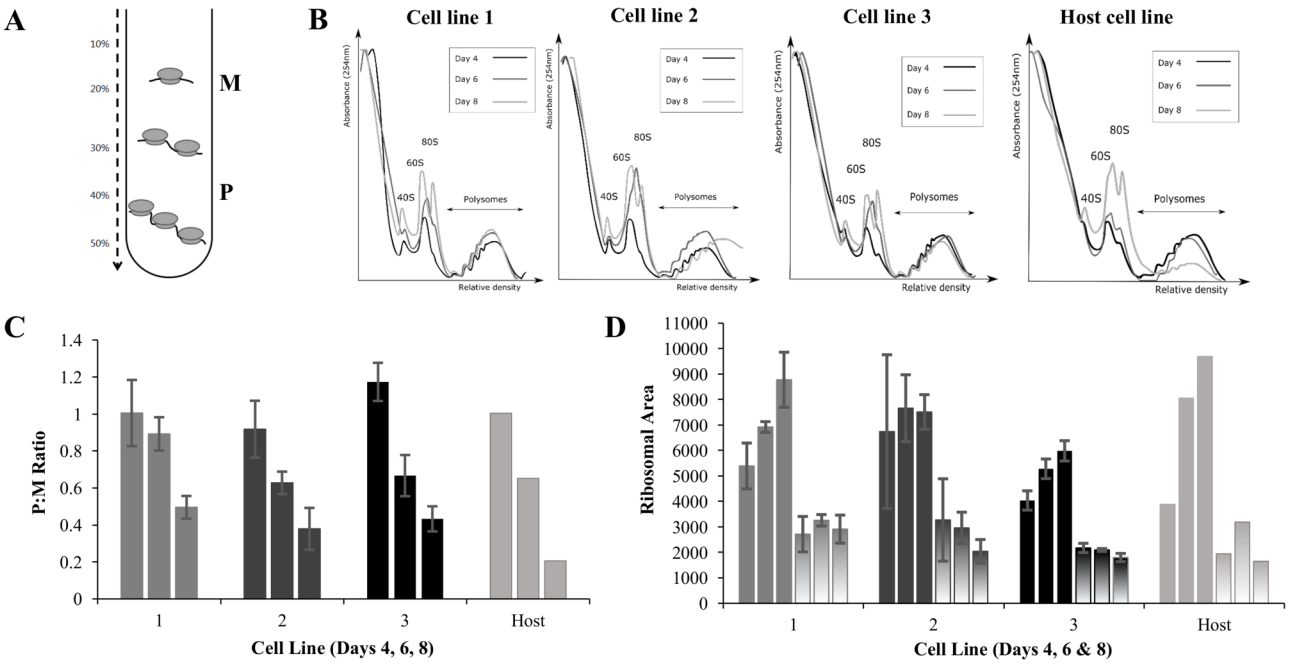

Figure 2. Polysome profiling of CHO host and stable mAb-producing recombinant cell lines 1, 2 and 3 on days 4 (exponential phase), 6 (start of plateau phase) and 8 (start of decline phase) of batch culture – shown by bars left to right. All error bars show SD ($n = 3$) for 1, 2 and 3, $n = 2$ for host. (**A**) Schematic showing the 10–50% sucrose gradient used to separate the ribosomes into monosomes (M) and polysomes (P). (**B**) Representative polysome profiles from cell lysate samples centrifuged in a sucrose gradient at 181 942 ×*g* for 2.25 h, and absorbance read at 254 nm. (**C**) Polysome/P to monosome/M ratios, calculated from the polysome profiles. (**D**) Total (solid/patterned bars) and polysomal (gradient bars) ribosomal area estimated from the polysome profiles.

some fraction can be indicative of enhanced global protein synthesis supporting faster cell growth and proliferation, or alternatively, can be the result of slowed elongation leading to ribosomes residing longer on mRNAs. As cell growth for cell line 2 at day 6 was no better than that observed in cell line 3, the increase in the polysome fraction is likely to be the result of an elongation limitation resulting in an accumulation of polysomes on mRNAs. The host cell line showed the biggest decrease in polysome content on day 8, in line with the drop in viability observed, showing a reprogramming of translation and decreased translational activity. Interestingly, when comparing the cell lines, cell line 3 had the lowest 80S monosome peak and sustained polysome peaks throughout culture compared with the other cell lines investigated (Fig. 2B).

When the total ribosome content was estimated from the areas under the curve, cell line 3 had fewer total ribosomes as determined from these data than the other cell lines (Fig. 2D). This was unexpected as cell line 3 had the highest recombinant cell line IVC and specific growth rate, and a qP similar to that of cell line 2 (Fig. 1). However, a similar number of polysomes were observed for cell line 3 compared to the other cell lines, showing that despite a lower number of total ribosomes, cell line 3 is able to use these more efficiently to maintain a high protein synthetic capacity (Fig. 2C). Interestingly, the total ribosome area from the same number of cells increased across culture for all cell lines, particularly the host cell line and between days 4 and 6, suggesting that ribosome synthesis is increased by, and to support, high demand for protein synthesis during rapid exponential growth phase. In recombinant cell line 2, this increase was less pronounced although a higher amount of ribosomes was already present at day 4 compared to the other cell lines.

We also analyzed the ratios between the areas under the polysomal peaks (P) relative to the sub-polysomal/monosomal peaks (M), or P to M ratio (Fig. 2C). This calculation was undertaken for each time point for each cell line with the baseline taken as the lowest point in the profiles for measuring the areas under the peaks. The calculated P : M ratios show that there were more actively translating ribosomes/polysomes on day 4 of culture during rapid exponential growth for all four of the cell lines (Fig. 2C). At this point in culture (day 4), higher levels of translation are required to support efficient cellular growth, division and normal cellular processes [9, 13]. On day 4 of batch culture, cell line 3 had the highest P : M ratio of approximately 1.2 whilst the other two recombinant cell lines and the host had approximately equal amounts of polysomes and sub-polysomes with a ratio of approximately 1 (Fig. 2C). These data show that when cells are in rapid growth, ribosome availability is not limiting for protein synthesis in any of the cell lines investi-

gated and suggests that additional ribosomal capacity and hence protein synthetic capacity is available. The data also suggest that recombinant cell lines with the fastest cell specific growth rates are those with the highest global translational efficiency, as determined by the polysome to monosome ratio from the polysome profiling.

When the polysome to monosome ratio data at days 6 and 8 of culture were analyzed, the ratio for cell line 3 was no longer obviously different from that of the other cell lines (Fig. 2C). This can be explained by the fact that by day 6 the rapid exponential growth phase has ceased and cell numbers are approaching a maximum for all cell lines (Fig. 1A). As such, the demand for protein synthesis to support more rapid growth is no longer required and this is reflected in the data for cell line 3. Indeed, on day 6 cell line 1 had the higher polysome to monosome ratio but has a lower growth rate (Fig. 1D), this potentially reflecting stalled ribosomes or slowed elongation in cell line 1. These data collectively support the hypothesis that the faster cell specific growth rate of cell line 3 during exponential growth phase (day 4) and the subsequent higher IVC is supported by the ability to better utilize the available ribosomal machinery during exponential growth compared to the other recombinant cell lines. Thus, in order to identify cell lines capable of achieving high IVC, at least under batch culture conditions, we suggest that host or recombinant cell lines with the highest polysome to monosome ratio could be isolated. We note that the production of recombinant proteins from mammalian cells has been referred to as a biphasic process whereby maximum recombinant protein production occurs after the end of exponential growth phase [35]. Our data confirms that as the cells enter stationary growth phase, the polysome to monosome ratio decreases reflecting a lower global demand for protein synthesis and hence more protein synthetic machinery is presumably available for the translation of recombinant mRNA transcripts. We therefore investigated whether more recombinant mRNA transcripts were present in polysome fractions on days 6 and 8 and whether the amounts of transcript found in polysome fractions related to cell line specific and culture productivity.

3.3 The number of recombinant transcripts in actively translating polysome fractions changes across batch culture and differs between recombinant cell lines

To determine whether there were changes in the active translation of the recombinant mRNAs both across the model cell lines and throughout batch culture, the mRNA copy numbers associated with ribosomes were determined by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) of the mAb heavy chain, light chain and the glutamine synthetase (GS) selection marker (Fig. 3). Fractions collected across the polysome profiles were pooled into four pools of interest to determine the mRNA copy number located in the sub-polysomes (S), and the light (L), mid (M) and heavy (H) polysomes (Fig. 3A). A shift in the number of transcripts from the sub- and light polysomes to the heavy polysomes would be indicative of increased translational activity. Conversely, a change in transcript copy numbers from the heavy to lighter or sub-polysomes would indicate translational reprogramming to less target specific translational activity [21, 24].

The majority of the transcript copies for all recombinant genes were located in the mid and heavy polysome fractions in all cell lines. It was also immediately obvious that the highest titer cell line (cell line 3) had the highest copy numbers in the heavy polysomes for all recombinant gene transcripts when comparing the three cell lines (Fig. 3). We also calculated the total copy numbers (not those just associated with ribosomes as reported in Fig. 3) on the different days from 2×10^6 cells (Supporting information, Fig. 1). Although this showed that there were generally higher total copy numbers for cell line 3, for the HC in particular the increase in copy numbers in the polysomes relative to the other two cell lines was greater than that accounted for simply by the increase in total HC copy number in this cell line (compare Fig. 3B with Fig. 1A in Supporting information). We note, that for the HC in cell line 3, an elevated amount of total transcript compared to the other two cell lines was not observed on day 8 as might be expected from the higher copy numbers observed in the polysomes of this cell line in the triplicate cultures on day 8. Collectively however, these data suggest that cell line 3, although not having the greatest amount of ribosomal machinery as previously described, was able to more efficiently load ribosomes onto recombinant HC transcripts, particularly once exponential growth phase was over on days 6 and 8 and more ribosomal machinery is presumably freed from the demands of the cell for endogenous protein synthesis. Thus, this fast growing, high IVC and good qP cell line is more translationally efficient than the lower yielding and growing recombinant cell lines.

Interestingly, the number of heavy chain mRNAs in the mid- and heavy polysomes of cell line 3 more than doubles from day 4 to day 6 to day 8 (Fig. 3B) (whereas for cell lines 1 and 2, the number of copies in the various fractions remains more-or-less constant). This again suggests that cell line 3 is able to load more available ribosomes onto heavy chain transcripts after rapid exponential growth phase when more translational machinery is available. On-the-other-hand, although more ribosomal capacity is available in cell lines 1 and 2 at the completion of the growth phase, this additional capacity is not utilized for translation of additional recombinant heavy chain transcript as in the case of cell line 3. A similar pattern between the cell lines is observed for the recombinant mAb LC transcript although the increase in transcripts in the mid and heavy polysomes from day 4 to 6 to 8 in cell

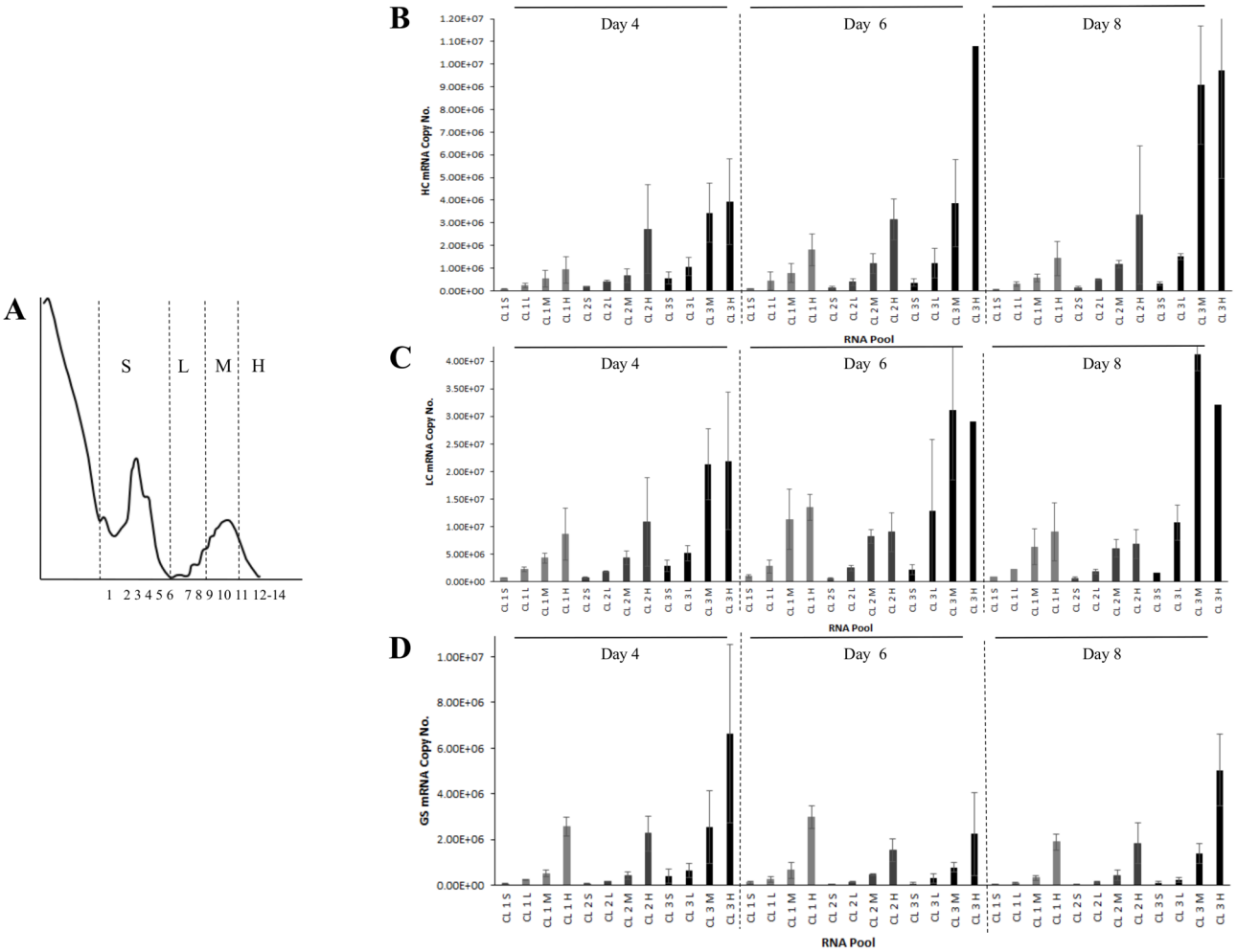

Figure 3. qPCR analysis of mRNAs across the polysome profiles of the recombinant CHO cell lines. (**A**) Schematic of how the fractions were pooled into sub-polysome (S) and light (L), mid (M) and heavy (H) polysome pools for the qRT-PCR analysis. (**B**) Amounts of mAb heavy chain (HC), (**C**) mAb light chain (LC) and (**D**) glutamine synthetase (GS) in the four pools, in the stable mAb-producing recombinant cell lines 1 (lightest bars), 2 (mid bars) and 3 (darkest bars) across days 4, 6 and 8 of batch culture. All copy numbers determined from a standard curve ($n = 3$), error bars show standard deviation.

line 3 is not as obvious as for the heavy chain mRNA (Fig. 3C). There was also an increase of both LC and HC transcript into the mid-polysomes compared to that in the heavy polysomes from day 6 to day 8 in cell line 3 that is not apparent in the other cell lines.

Previous reports have suggested that heavy chain mRNA translation can be a limitation on mAb yields from CHO [36] and NS0 cells [12]. Although both cell lines 2 and 3 have similar cell specific productivities, the heavy chain copy numbers in the polysome fractions differ, particularly on days 6 and 8 of batch culture (Fig. 3B). However, whilst there is a similar profile of LC transcript numbers between the lowest qP and titer cell line 1, and cell line 2 with a higher qP and titer, there is a clear difference in the number of ribosomes on the available HC transcripts between cell lines 1 and 2 (Fig. 3B). More HC transcripts are located in the heavy polysomes of cell line 2 than cell line 1 and hence the difference in the translational activity of the HC between cell line 1 and 2 may account for the differences in qP and productivity, in line with previous studies suggesting HC translation can be limiting with regard to productivity. However, although cell line 3 has the highest number of HC and LC transcripts in the heavy and mid-polysome fractions, the qP is similar to that of cell line 2, with the higher titer of cell line 3 being due to enhanced IVC as previously discussed. In the case of cell line 3, the enhanced global polysome to monosome ratio during rapid growth phase relates to enhanced growth and IVC, but the larger number of transcripts with more ribosomes loaded (heavy polysomes) does not enhance the qP of the cells. Interestingly, there is less variation in the copy number of the GS transcripts across the cell lines and throughout culture (Fig. 3D). The majority of the GS transcripts are found in

the heavy polysomes for all cell lines on all days investigated.

The polysome profiles and associated transcript data presented here provide evidence of differences in translational activity between the recombinant cell lines that corresponds to, and explains the observed differences in growth and productivity. However, to further confirm this and validate the polysome profiling it was necessary to investigate the nascent polypeptide synthesis rates across the cell lines and batch culture and compare this to the polysome data.

3.4 Global and recombinant nascent polypeptide synthesis corresponds to polysome profiling fingerprints of recombinant CHO cell lines

The overall rate of global polypeptide synthesis and translation in the recombinant mAb producing cell lines was measured by determining the incorporation of [^{35}S]-methionine into nascent polypeptides after 1 h of pulse-labelling. This approach was taken to determine whether the changes observed in the polysome profiling fingerprints and at the mRNA level by qRT-PCR were linked to polypeptide synthesis. SDS-PAGE analysis of samples and autoradiography was therefore conducted following the pulse-labelling, which revealed that the peak nascent polypeptide synthesis was not the same for all three recombinant cell lines (Fig. 4A). Whilst the analysis suggests that cell lines 2 and 3 had the most active nascent polypeptide synthesis on day 6 of batch culture at the end of exponential growth phase, for cell line 1 this was on day 8, as shown by the higher intensity protein bands relating to greater incorporation of the label into the newly synthesized polypeptides (Fig. 4A). This was confirmed by scintillation counting of incorporated radioactivity which showed that the greatest amount of radiolabel was incorporated into new protein on day 6 for cell lines 2 and 3 and day 8 for cell line 1 (Fig. 5A). This data also showed that there was a large drop in new polypeptide synthesis for cell line 2 between days 6 and 8 (Fig. 4A and 5A). The autoradiograph data (Fig. 4A) also showed that when the total amount of protein analyzed per sample was the same for all cell lines (to account for potentially a difference in cell number when sampling), cell line 3 was synthesizing more heavy and light chain polypeptide than the other recombinant cell lines.

When recombinant antibody nascent polypeptide synthesis was specifically investigated by immuno-precipitating the labelled cell lysates with protein A, the amount of heavy and light chain on the different days of culture and between the cell lines mirrored that of the global nascent polypeptide analysis (Fig. 4B). It is noted

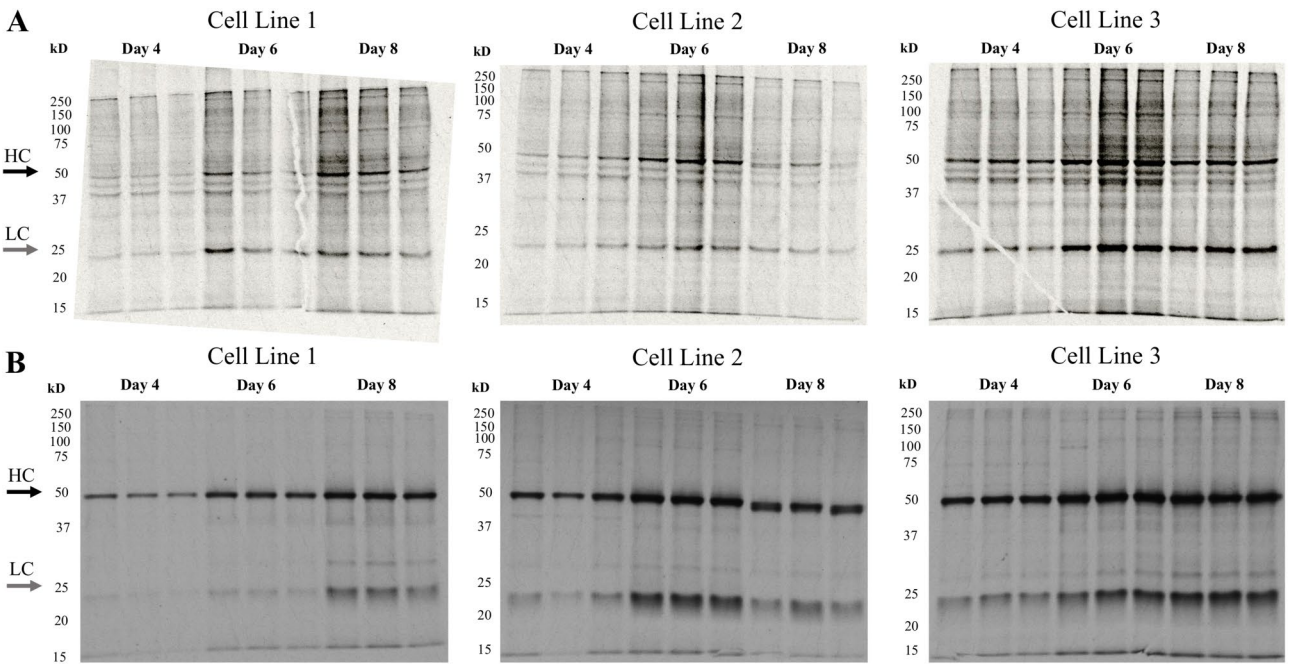

Figure 4. ^{35}S Polypeptide labelling of the stable mAb-producing recombinant CHO cell lines 1, 2 and 3 on days 4 (exponential phase), 6 (start of plateau phase) and 8 (start of decline phase) of batch culture, $n = 3$. (**A**) Autoradiographs showing the labelled proteins produced in reduced cell lysates from all recombinant cell lines, after three days exposure to the dried SDS-PAGE gels. (**B**) Immunoprecipitation studies with protein A beads mixed with the labelled reduced cell lysates showing the pulled down heavy chain (HC, 50 kDa) and associated light chain (LC, 25 kDa) – both indicated. The autoradiographs shown here were after exposure for 53 days to the dried SDS-PAGE gels with the labelled reduced cell lysates. Three lanes for triplicate cultures on each day for each cell line.

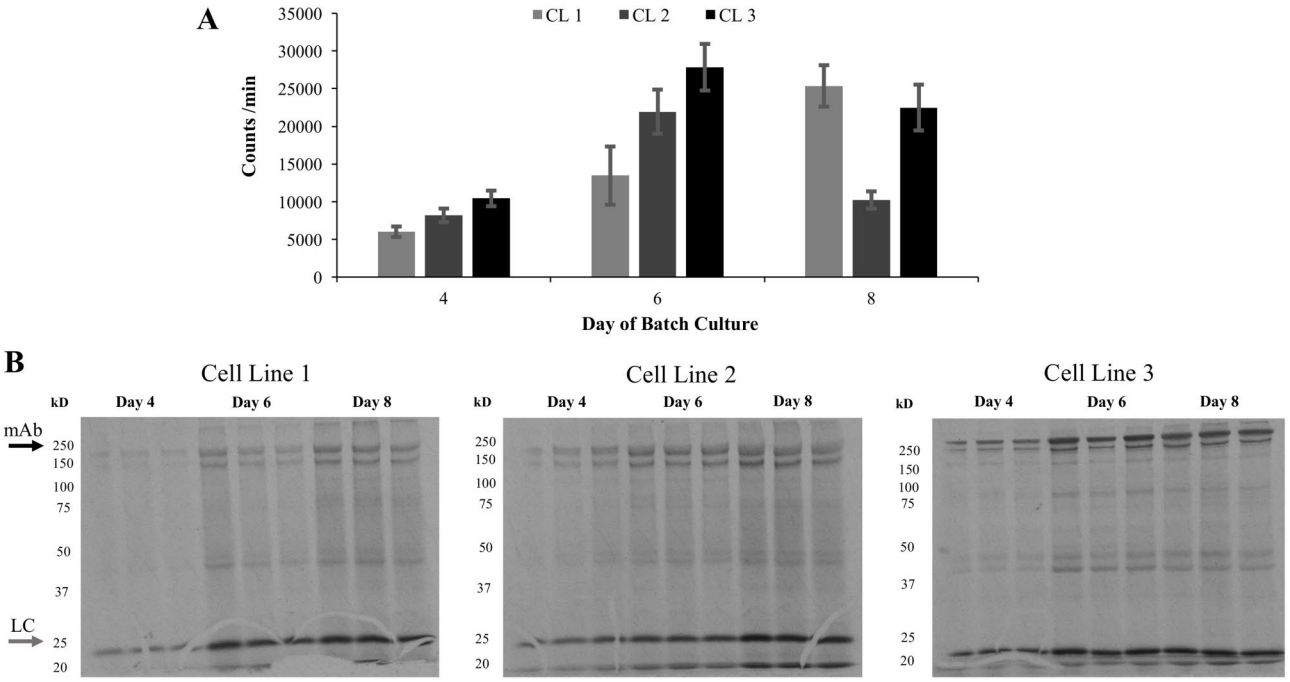

Figure 5. ^{35}S Polypeptide labelling of the stable mAb-producing recombinant CHO cell lines 1, 2 and 3 on days 4 (exponential phase), 6 (start of plateau phase) and 8 (start of decline phase) of batch culture. Error bars show standard deviation ($n = 3$). (**A**) Scintillation counting from the ^{35}S-labelled cells after 1 h of labelling and precipitation with trichloracetic acid. (**B**) Autoradiographs showing the labelled proteins in non-reduced supernatant samples from all recombinant cell lines, after 41 days exposure to dried SDS-PAGE gels. Three lanes for triplicate cultures on each day for each cell line.

that protein A will only pull down heavy chain and light chain associated with heavy chain and hence this is not a direct measure of total light chain, which can be estimated from the global analysis reported in Fig. 4A. We also note that the lysate pulldown will not account for secreted mAb and increased intracellular amounts of label may simply reflect a secretory block. We therefore also investigated heavy and light chain in the supernatant (Fig. 5B). These data showed a similar pattern to that intracellularly in that cell line 3 had the most secreted intact mAb present.

The peak mAb polypeptide synthesis for cell line 1 was therefore on day 8, as for global synthesis, whereas for cell line 2 the peak was on day 6 in line with the global protein synthesis. However, for cell line 3 the nascent antibody polypeptide synthesis was sustained at a similar level across both days 6 and 8 with higher intensity heavy and light chain bands observed than for the other cell lines (Fig. 4 and 5). This prolonged antibody synthesis relates to the sustained and increased transcript numbers in the heavy polysomes for cell line 3 and hence explains the increased translational activity that is maintained. These data again confirm that the polysome profiling and ribosome distribution is predictive of global protein synthesis and growth characteristics whilst the presence of more recombinant transcripts in heavy polysome fractions is indicative of high producing cell lines.

3.5 Investigating translational activity and reprogramming of key endogenous target transcripts during batch culture

Alongside the investigation of global and recombinant transcript translational activity we investigated translation activity and reprogramming of potential key target endogenous transcripts during batch culture (eIF3a, translation initiation; RagC, mTOR signalling; ATF1, transcription factor; XBP1, unfolded protein response; Caspase3, apoptosis; Cox11, cellular metabolism; thioredoxin reductase, oxidative stress; thioredoxin, oxidative stress). To do this we determined the relative mRNA levels of these targets by qRT-PCR. Relative levels were determined by either comparing the normalized amounts to the lowest sample on day 4 for each target individually (Supporting information, Fig. 2) or by comparing all samples to the cell line 1 day 4 sub-polysome fraction (Supporting information, Fig. 3). These analyses reveal further differences in the requirements for specific cellular processes in these cell lines, as indicated by the translational activity of these targets defined by their relative abundance in sub-polysome, light, mid or heavy polysome fractions (Supporting information, Fig. 2 and 3).

The most striking difference in the distribution of transcripts across the polysome profile with time of culture between the cell lines when the relative amount for

each transcript in each cell line was compared to the day 4 sub-polysome fraction was of the RagC transcript (Supporting information, Fig. 2). There were elevated transcript amounts of the RagC mRNA in cell line 3 samples located in the mid and heavy polysome fractions on all three days compared to the other cell lines investigated. RagC is involved in mTOR signalling as a heterodimer with other Rag proteins to recruit mTOR to lysosomes for activation and downstream signalling [37, 38]. The mTOR signalling pathway is a master regulator of ribosome biogenesis, cell growth and protein synthesis [37, 39] and a number of studies have linked enhanced mTOR signalling to enhanced growth and recombinant product yields from CHO cell expression systems [40]. The enhanced amounts of RagC transcript in the heavy polysome fraction in the fastest growing and most productive recombinant cell line is in agreement with up-regulated translational activity in fast growing, highly productive recombinant CHO cell lines. This polysome fingerprint could therefore act as a marker of such cell lines and suggests engineering of components of the mTOR signalling pathway, such as RagC, may be attractive targets for the generation of host cell lines with enhanced growth and productivity characteristics, in agreement with previous findings where mTOR was exogenously over-expressed [40].

The majority of the other transcripts investigated showed subtler changes in distribution across the polysome profile across the days of culture and between cell lines (Supporting information, Fig. 2). When the data were presented relative to the cell line 1 day 4 transcript amounts by normalizing across the plates to the day 4 amounts, a shift in transcript amounts for the thioredoxin reductase transcript into heavy polysomes is observed in all cell lines at day 8 (Supporting information, Fig. 3). This is particularly interesting as thioredoxin reductase has been implicated in the reduction of recombinant mAbs in CHO cell cultures, resulting in the formation of reduced mAb [41]. The data here suggests that towards the end of batch culture the transcript amounts of this enzyme are shifted to reside in translating polysomes, presumably in response to oxidative stress late in culture. Notably, RagC is maintained in the heavy polysome fractions by this analysis in the highest producing and growing cell line 3 (Supporting information, Fig. 3), providing further evidence that this may be important for sustaining growth and productivity in this cell line.

4 Conclusions

The data presented here show that polysome profiling, and hence translational efficiency, in model mAb producing CHO cell lines changes throughout batch culture and reflects global and transcript specific translation. The distribution of ribosomes between polysomes and sub-polysomes relates to protein synthesis rates and reprogramming of specific transcripts as they are found in either polysome or sub-polysome fractions reflecting the growth state of the cell. The identification of those transcripts found preferentially in polysomes at different stages of culture highlights these as potential targets for either over-expression or down-regulation, depending upon the role and impact of the encoded proteins on the desired phenotype (growth or productivity). These data will inform development of cell engineering strategies and/or screens to identify novel hosts and recombinant cell lines with enhanced growth and productivity characteristics based upon their translational efficiency and ability to load specific transcripts preferentially onto ribosomes to enhance protein synthesis of these targets. As such, the 5′ untranslated regions (UTRs) of those transcripts found preferentially in polysomes could potentially be used to help 'load' recombinant transcripts onto ribosomes alongside the 'optimization' of codon usage in the translated regions of recombinant transcripts (e.g. [42]) to improve recombinant protein expression later in culture. Such preferential loading of transcripts with particular 5'-UTRs to enhance protein synthesis has been reported in other contexts [21, 43, 44] and is an attractive strategy to pursue in order to enhance recombinant protein synthetic capacity of CHO and other mammalian expression systems.

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