

Control of Culture Environment for Improved Polyethylenimine-Mediated Transient Production of Recombinant Monoclonal Antibodies by CHO Cells

Douglas J. Galbraith,[†] Andrew S. Tait,[†] Andrew J. Racher,[‡] John R. Birch,[‡] and David C. James^{*,†}

School of Engineering, University of Queensland, St. Lucia, QLD 4072, Australia, and Lonza Biologics plc, 228 Bath Road, Slough, SL1 4DX, United Kingdom

In this study we describe optimization of polyethylenimine (PEI)-mediated transient production of recombinant protein by CHO cells by facile manipulation of a chemically defined culture environment to limit accumulation of nonproductive cell biomass, increase the duration of recombinant protein production from transfected plasmid DNA, and increase cell-specific production. The optimal conditions for transient transfection of suspension-adapted CHO cells using branched, 25 kDa PEI as a gene delivery vehicle were experimentally determined by production of secreted alkaline phosphatase reporter in static cultures and recombinant IgG₄ monoclonal antibody (Mab) production in agitated shake flask cultures to be a DNA concentration of 1.25 $\mu\text{g } 10^6 \text{ cells}^{-1} \text{ mL}^{-1}$ at a PEI nitrogen:DNA phosphate ratio of 20:1. These conditions represented the optimal compromise between PEI cytotoxicity and product yield with most efficient recombinant DNA utilization. Separately, both addition of recombinant insulin-like growth factor (LR3-IGF) and a reduction in culture temperature to 32 °C were found to increase product titer 2- and 3-fold, respectively. However, mild hypothermia and LR3-IGF acted synergistically to increase product titer 11-fold. Although increased product titer in the presence of LR3-IGF alone was solely a consequence of increased culture duration, a reduction in culture temperature post-transfection increased both the integral of viable cell concentration (IVC) and cell-specific Mab production rate. For cultures maintained at 32 °C in the presence of LR3-IGF, IVC and qMab were increased 4- and 2.5-fold, respectively. To further increase product yield from transfected DNA, the duration of transgene expression in cell populations maintained at 32 °C in the presence of LR3-IGF was doubled by periodic resuspension of transfected cells in fresh media, leading to a 3-fold increase in accumulated Mab titer from ~ 13 to $\sim 39 \text{ mg L}^{-1}$. Under these conditions, Mab glycosylation at Asn₂₉₇ remained essentially constant and similar to that of the same Mab produced by stably transfected GS-CHO cells. From these data we suggest that the efficiency of transient production processes (protein output per rDNA input) can be significantly improved using a combination of mild hypothermia and growth factor(s) to yield an extended “activated hypothermic synthesis”.

Introduction

Chinese hamster ovary (CHO) cells are used to produce the majority of recombinant protein therapeutics manufactured by mammalian cell culture (1). This rodent cell line is generally preferred because it has the ability to confer appropriate post-translational modifications, a variety of selection/amplification systems for cell engineering are available, and it can be adapted to growth in serum (and protein)-free environments. However, development of stably transfected CHO cell lines for manufacturing processes is a time-consuming (3–9 months), labor-intensive process that can delay the availability of product for early-stage product testing (e.g., bioassay development, biophysical characterization, formulation). In contrast, transient production systems capable of generating protein relatively rapidly may speed product development timelines by making product available in days rather than months.

Numerous reports have described methods for transfer and expression of recombinant DNA (rDNA) in mammalian cells.

Strategies for transfection of rDNA are generally based on direct introduction of rDNA into cells by processes such as electroporation, the use of engineered viruses as biological vectors, or delivery of complexed DNA particles via the endosomal pathway using inorganic salts, synthetic cationic lipids, or polymers to condense DNA (2). Despite relatively inefficient rDNA delivery and cytotoxicity, the latter have the advantage of simplicity and cost-effectiveness and are inherently more suited to large-scale use in cell culture systems. The low-cost cationic polymer polyethylenimine (PEI) has been shown to be a particularly effective gene delivery vehicle. Condensation of DNA with PEI yields cationic particles capable of traversing and escaping from the endolysosomal compartment (3, 4) to deliver transcriptionally active rDNA into the nucleus during mitosis (5).

To date, most examples of the use of synthetic gene delivery vehicles such as PEI to manufacture recombinant proteins at scale have utilized suspension-adapted human embryonic kidney (HEK293) cells engineered to express the Epstein–Barr nuclear antigen-1 (EBNA-1). In combination with plasmid vectors harboring the oriP origin of replication, this system enables both nuclear retention and autonomous replication of episomal

* To whom correspondence should be addressed. Ph: +61 7 33654638. Fax: +61 7 33654199. E-mail: davidj@cheque.uq.edu.au.

[†] University of Queensland.

[‡] Lonza Biologics plc.

plasmid DNA, yielding volumetric titers of recombinant protein up to approximately 20 mg L⁻¹ (6–8). However, HEK cells are not the optimal choice of host cell line for transient production of therapeutic proteins. For example, HEK293 cells are known to differ from CHO cells with respect to *N*-glycan processing (9), which may affect product bioactivity in vitro (10). Consequently, to rapidly produce protein product in the same cell type that would be employed for large-scale production processes, there have been recent examples of optimized, scalable transient production systems employing PEI-mediated transfection of nonengineered CHO cells (11, 12). However, these studies report a volumetric titer of monoclonal antibody product of only approximately 5–8 mg L⁻¹.

In this study, we describe a novel and simple approach to significantly improve the production of recombinant monoclonal antibody by transiently transfected CHO cells maintained in a fully chemically defined culture environment. Using a simple reduction in culture temperature to control cell proliferation, we show that transcriptionally active episomal rDNA can be maintained in the cell population for an extended period. Cell-specific productivity can be substantially increased under these conditions by simultaneous inclusion of a recombinant growth factor in the culture environment. This strategy markedly improves recombinant protein yield from transfected DNA with no adverse effect on product glycosylation.

Materials and Methods

Cell Culture. Suspension-adapted parental CHOK1SV cells were supplied by Lonza Biologics (Slough, U.K.) and were adapted for growth in a proprietary DMEM-based chemically defined, protein/animal component-free (CDACF) medium supplemented with 6 mM glutamine. Cell cultures were routinely maintained at 37 or 32 °C with an overlay of 5% CO₂ in glass shake flasks agitated on an orbital rotator at 100 rpm in a Heraeus Heracell incubator. Small-scale transfection experiments were performed in ultra-low-binding 24-well plates (Corning, Lindfield, NSW, Australia) to ensure that cells remained in suspension that were seeded in fresh medium at 2 × 10⁵ cells mL⁻¹ at 1 mL well⁻¹. Breathe Easy membranes (Diversified Biotech, Boston, MA) were applied to plates to ensure even gaseous exchange and minimal evaporation from all wells. For all transfections in shaken shake flasks, cells were seeded in fresh medium at a viable cell concentration of 1 × 10⁶ cells mL⁻¹ at the specified culture volume. Where specified, a stock solution of LR3-IGF (GroPep Ltd., Adelaide, Australia) was prepared in 0.1 M HCl (according to manufacturer's instructions) and added to the appropriate culture samples immediately after transfection. GS-CHO cells stably expressing cB72.3 recombinant IgG₄ Mab were maintained in agitated shake flasks in CD-CHO medium (Invitrogen) under standard conditions (37 °C, 5% CO₂). Viable cell concentration and culture viability were routinely determined with the ViaCount assay using a GUAVA personal cell analyzer (PCA, Guava Technologies, California, U.S.A.) or a ViCell automated Trypan blue counting device (Beckman Coulter, Fullerton, U.S.A.).

Plasmid DNA Constructs. pEE6.4 eGFP encoding enhanced green fluorescent protein (5846 bp), pcB72.3 encoding the recombinant chimeric IgG₄ Mab cB72.3 (12030 bp) have been described previously (13–15). The gWIZ vector encoding secreted alkaline phosphatase (SEAP; 6617 bp) was obtained from Aldevron (Fargo, ND). All reporter and Mab genes were under the control of human CMV promoters.

Plasmid DNA Production and Transfection. Plasmid DNA was prepared as described previously (11) using either an Aurum

plasmid maxiprep kit (BioRad, Hercules, CA) or GigaPrep kits (Qiagen, Doncaster, VIC, Australia) according to manufacturers instructions. All plasmid DNA was eluted in deionized water, filter sterilized (0.22 μm), quantified spectrophotometrically by measurement of absorbance 260 and 280 nm (ensuring A₂₆₀/A₂₈₀ > 1.8), and checked for purity by agarose gel electrophoresis (0.7% (w/v), 50 V constant voltage) prior to use.

Transfection of CHO-S Cells with PEI/DNA Complexes. Polyethyleneimine (PEI 25 kDa; branched) was obtained from Sigma (Castle Hill, NSW, Australia). A stock PEI solution (0.9 mg mL⁻¹) in deionized water was neutralized to pH 7.2 with HCl and filter sterilized. Preparation of PEI/DNA complexes and transfection of CHO cells were performed as described previously (11). Briefly, purified plasmid DNA was added to an aliquot of prewarmed (37 °C) culture media equivalent to 5% of the final culture volume and left at room temperature for 2 min. PEI was then added and the PEI-DNA mixture was incubated at room temperature for 10 min (Schlaeger and Christensen, 1999). This solution, containing PEI/DNA complexes, was then added directly to the cell culture. Unless otherwise stated, cells were seeded at a viable cell density of 2 × 10⁵ or 1 × 10⁶ cells mL⁻¹ in fresh medium immediately prior to transfection.

Reporter Gene Assays and ELISA of Recombinant IgG₄ Mab. Chemiluminescent SEAP assays and recombinant Mab ELISA were performed as described previously (11). eGFP content of transfected CHO cells was determined by flow cytometry using an EPICS Altra flow cytometer (Becton Coulter, Fullerton, U.S.A.). Cell samples (1 mL) were harvested by centrifugation (200 × g, 3 min), washed with 1 mL phosphate-buffered saline (PBS), then fixed in 1 mL of 4% (w/v) paraformaldehyde followed by incubation at -20 °C for 5 min. Cells were then washed twice with PBS and stored in PBS at 4 °C. Excitation of eGFP containing cells was achieved at 488 nm, with emission measured at 515–535 nm. eGFP positive cells were then defined as those displaying higher fluorescence intensity than 99% of control untransfected cells. At least 5000 events were collected per assay.

Analysis of Recombinant IgG₄ Mab N-Glycosylation. Cell-free supernatant was concentrated 10-fold by ultrafiltration using a centrifugal device with 30 K cutoff membrane (Millipore, Billerica, MA) and recombinant Mab was purified from cell-free supernatant by affinity chromatography using Protein A Fast Flow Sepharose (GE Healthcare, Castle Hill, NSW, Australia). Briefly, 10 μL bed volume matrix was loaded into a 0.22 μm filter microcentrifuge tube (Millipore) followed by equilibration with 100 μL of 20 mM sodium phosphate, pH 7.0. After removal of equilibration buffer by centrifugation, 200 μL of 10 × concentrated cell culture supernatant was diluted 1:1 with phosphate buffer and added to matrix, followed by incubation for 1 h at room temperature with continuous gentle inversion. After removal of the liquid phase by centrifugation, the matrix was washed twice with 200 μL of phosphate buffer, and purified Mab was eluted with 200 μL of 50 mM Gly-HCl, pH 2.7 into 20 μL of 1 M Tris-HCl, pH 8.0 for immediate neutralization. Analysis of Asn₂₉₇-containing tryptic glycopeptides prepared from purified Mab was then performed essentially as described previously (11).

Results

Optimization of PEI-Mediated Transfection of CHOK1SV Cells in CDACF Medium. As the optimal basic conditions for PEI-mediated transfection may vary with each combination of host cell line and culture environment, we determined these

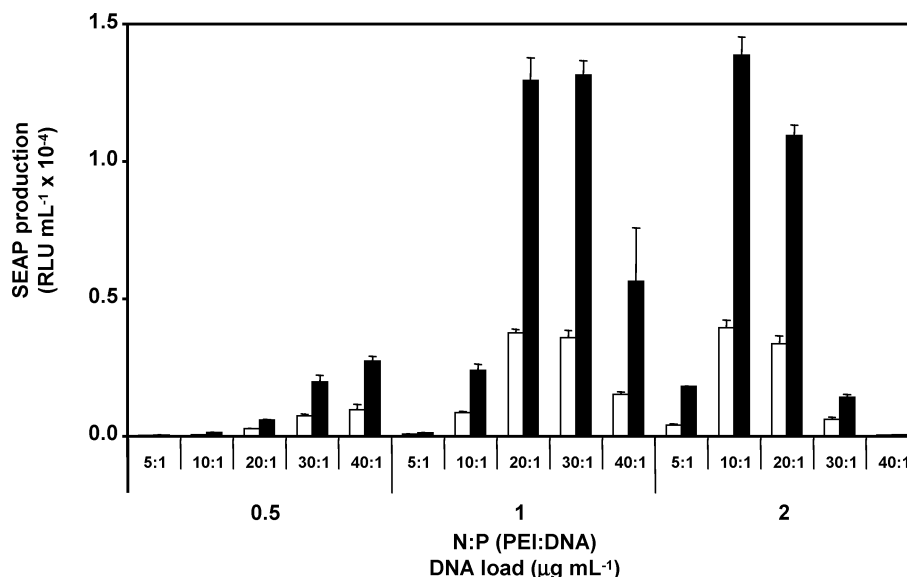


Figure 1. Initial optimization of PEI25-mediated transient transfection of CHOK1SV cells in static culture. CHO cells in ultra-low-binding 24-well plates (1 mL well^{-1} at $2 \times 10^5 \text{ cells mL}^{-1}$) were transfected with PEI/DNA complexes prepared in CDACF medium to a final volume of $60 \mu\text{L}$. Varying amounts of gWIZ plasmid DNA encoding secreted alkaline phosphatase (SEAP) were complexed at the indicated PEI nitrogen:DNA phosphate ratios for 10 min prior to transfection. White and black bars represent SEAP activity measured by chemiluminescent assay in cell-free supernatant at 24 and 48 h post-transfection, respectively. $n = 3 + \text{SD}$.

parameters experimentally for CHOK1SV host cells maintained in CDACF medium. Transfection conditions were initially screened using static cultures of unattached CHOK1SV cells in 24-well plates at a working culture volume of 1 mL well^{-1} and an initial viable cell concentration of $2 \times 10^5 \text{ cells mL}^{-1}$. Complexed plasmid DNA (6617 bp) encoding secreted human alkaline phosphatase as a reporter was added to cultures (in $50 \mu\text{L}$ of fresh media) at varying overall DNA load ($0.5\text{--}2 \mu\text{g mL}^{-1}$) and molar PEI nitrogen:DNA phosphate ratios (5–40:1).

SEAP activity in cell-free culture supernatant at 24 and 48 h post-transfection is shown in Figure 1. These data demonstrate maximum reporter expression at PEI N:DNA P ratios between 10:1 (1.35:1 w/w) and 40:1 (5.4:1 w/w), although this varied with DNA load such that there was an apparently inverse relationship between DNA load and optimal PEI N:DNA P ratio. This implies a single optimal PEI concentration for transfection, independent of DNA load (i.e., at $0.5 \mu\text{g mL}^{-1}$ DNA, the optimal PEI N:DNA P ratio was 40:1, whereas at $2 \mu\text{g mL}^{-1}$ DNA, the optimal PEI N:DNA P ratio was 10:1). Although there was some evidence of a positive relationship between DNA load and SEAP output at low PEI N:DNA P ratios, this was not the case at higher PEI N:DNA P ratios, a likely consequence of PEI-induced cytotoxicity at increased DNA load. We previously reported similar relationships between PEI N:DNA P ratio and reporter output at varying DNA load,¹¹ and as for this study, we found that PEI cytotoxicity was also independent of DNA concentration (data not shown). The optimal transfection conditions for these static cultures, yielding maximal cumulative SEAP output at minimal rDNA and/or cytotoxic PEI input, were $1 \mu\text{g mL}^{-1}$ DNA at a PEI N:DNA P ratio of 20:1 (2.7:1 w/w) or $2 \mu\text{g mL}^{-1}$ DNA at a PEI N:DNA P ratio of 10:1.

To further optimize conditions for transfection of higher cell concentration suspension cultures, the optimal basal conditions identified by transfection of static cultures in 24-well plates were utilized to transfect CHOK1SV cell cultures maintained in agitated Erlenmeyer flasks ($30 \text{ mL working volume}$). For this experiment, cells were transfected with plasmid DNA encoding a recombinant humanized IgG₄ monoclonal antibody (a single

plasmid construct, pcB72.3, also encoding glutamine synthetase used to create stably transfected cells (15)). Mab titer in cell-free supernatant was monitored by ELISA.

Initial experiments with the higher cell concentration suspension cultures showed clearly that utilization of the same DNA:cell ratio employed to optimally transfect static cultures ($5\text{--}10 \mu\text{g mL}^{-1}$ $10^6 \text{ cells mL}^{-1}$) was prohibitively cytotoxic during the first 24 h of suspension culture (data not shown). These data concur with our previous report that PEI cytotoxicity is increased in suspension culture (11). However, a reduction in DNA load at optimal PEI N:DNA P ratios permitted production of Mab without a maximal initial reduction in viable cell density exceeding 60%. As shown in Figure 2, although maximum Mab titer ($\sim 1.5 \text{ mg L}^{-1}$) after 4 days of culture was obtained with a DNA load of $2.5 \mu\text{g mL}^{-1}$ at a PEI N:DNA P ratio of 20:1, we concluded that the optimal compromise between product titer at minimal recombinant DNA consumption with a limited effect on cell viability occurred with a DNA load of $1.25 \mu\text{g mL}^{-1}$ at a PEI N:DNA P ratio of 20:1, and these conditions for PEI-mediated transfection were employed for all subsequent experiments. However, as also shown in Figure 2, although addition of PEI initially diminished the viability of the host cell population in a dose dependent manner, accumulated Mab titers may be limited by a more general decline in viable cell concentration.

Manipulation of CHO Cell Culture Environment Increases the Duration of Transient Mab Production at an Elevated Cell-Specific Production Rate. To increase transient Mab production, we sought to increase the ability of the culture as a whole to productively utilize introduced recombinant DNA. We tested simple strategies to (i) stimulate mitotic cell division/biomass accumulation post-transfection to increase nuclear rDNA uptake, (ii) increase the functional longevity of transcriptionally active rDNA in the cell population by increasing culture duration, and/or (iii) reduce PEI-induced cytotoxicity (16).

To achieve these objectives using simple bioprocess manipulations, we evaluated (i) addition of insulin-like growth factor, (ii) a mild reduction in culture temperature, and (iii) a combina-

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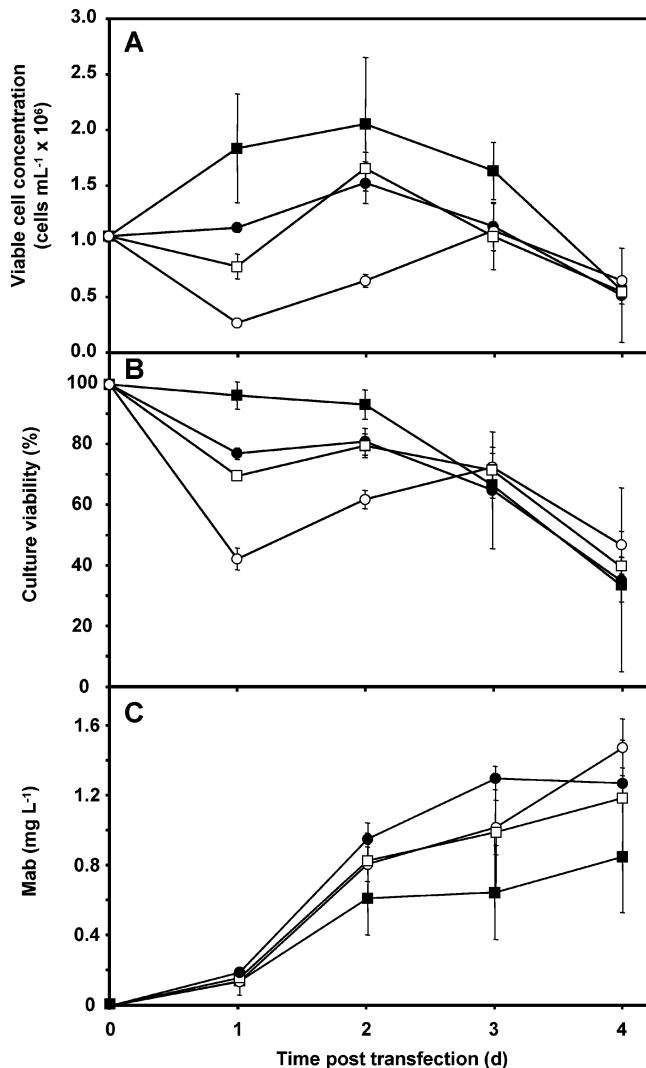


Figure 2. Optimization of PEI25-mediated transient production of recombinant Mab by CHOK1SV cells in high-density suspension culture. Cells were seeded in CDACF medium at a viable cell concentration 1×10^6 cells mL⁻¹ and cultured in 100-mL shake flasks at a working culture volume of 30 mL. Cultures were then transfected with the plasmid vector pcB72.3 as PEI/DNA complexes with a PEI N:DNA P ratio of either 20:1 (open symbols) or 10:1 (closed symbols) added to culture at a final DNA concentration of either $1.25 \mu\text{g mL}^{-1}$ (squares) or $2.5 \mu\text{g mL}^{-1}$ (circles). (A, B) Viable cell concentration and culture viability determined by Guava flow cytometry. (C) Recombinant Mab concentration in cell-free supernatant determined by ELISA. $n = 3 \pm \text{SD}$.

tion of the above. Insulin and insulin-like growth factor have been shown to promote cell growth, survival, and resistance of CHO cells to apoptosis in serum-free media (17, 18). We used recombinant LR3-IGF as it has been previously demonstrated to be more effective than insulin at enhancing recombinant protein expression by CHO cells (19) and can be utilized at concentrations much lower than insulin (typically at $0\text{--}50 \mu\text{g L}^{-1}$ as compared with $0\text{--}10 \text{ mg L}^{-1}$). Although mild hypothermia ($30\text{--}33 \text{ }^\circ\text{C}$) has been shown to suppress CHO cell proliferation (20), it has been reported to protect CHO cells against apoptosis (21), as well as increase culture duration and recombinant protein production in both stable (22) and (virus-mediated) transient formats (23).

As shown in Figure 3, although culture viability initially declined by approximately 40% during the first 24 h post-transfection under all conditions examined, addition of LR3-IGF ($20 \mu\text{g L}^{-1}$), a reduction in culture temperature to $32 \text{ }^\circ\text{C}$,

or a combination of the above delayed the longer-term reduction in culture viability previously observed for transfected cultures maintained at $37 \text{ }^\circ\text{C}$ in CDACF medium alone (Figure 2). LR3-IGF addition clearly promoted the proliferation of transfected cells cultured at both 37 and $32 \text{ }^\circ\text{C}$, although its effects were diminished at $32 \text{ }^\circ\text{C}$. Using a final culture viability of approximately 40% as a cutoff (or 12 days post-transfection for cultures at $32 \text{ }^\circ\text{C}$), addition of LR3-IGF to $37 \text{ }^\circ\text{C}$ cultures resulted in a 2.5-fold increase in culture duration, with a ~ 6 -fold increase in the integral of viable cell concentration (IVC) to 5.8×10^8 cell h⁻¹ mL⁻¹. A reduction in culture temperature alone increased IVC ~ 2 -fold (2.3×10^8 cell h⁻¹ mL⁻¹) and ~ 4 -fold in the presence of LR3-IGF (to 4.4×10^8 cell h⁻¹ mL⁻¹).

Mab titer at the end of each culture was determined by ELISA and was used to calculate an averaged (across culture) cell-specific production rate. These data are shown in Figure 3C. For the $37 \text{ }^\circ\text{C}$ culture stimulated with LR3-IGF, although final product titer was increased approximately 2-fold, qMab, relative to that of the untreated $37 \text{ }^\circ\text{C}$ culture, was reduced 3-fold (averaged qMab of $37 \text{ }^\circ\text{C}$ culture without LR3-IGF = $0.28 \text{ pg cell}^{-1} \text{ day}^{-1}$; Figure 2), indicating that the elevated product titer was solely a consequence of extended culture duration.

By contrast, although both cultures maintained at $32 \text{ }^\circ\text{C}$ also exhibited increased Mab product titer at the end of culture (~ 3 -fold at $32 \text{ }^\circ\text{C}$ alone); this was apparently a function of both extended culture duration and increased qMab, yielding elevated Mab titers. Importantly, a synergistic effect of reduced culture temperature and addition of LR3-IGF was clearly evident. Final Mab titer (13.2 mg L^{-1}) increased 11-fold over that observed for the untreated culture at $37 \text{ }^\circ\text{C}$, with a ~ 2.5 -fold increase in averaged qMab.

CHO Cells Transiently Transfected under Optimized Conditions Exhibit an Elevated and Uniform Transfection Efficiency. We compared the efficiency with which CHO cells were transfected under optimized ($32 \text{ }^\circ\text{C}$ plus $20 \mu\text{g L}^{-1}$ LR3-IGF) or control ($37 \text{ }^\circ\text{C}$ without LR3-IGF) culture conditions. Cells were transfected with a plasmid encoding eGFP and then analyzed by flow cytometry over the first 3 days of culture to determine the percent of eGFP-positive cells in transfected populations. These data are shown in Figure 4. Under optimized conditions the PEI-mediated transfection process was clearly more effective. In this case we observed a progressive increase in the proportion of cells with transcriptionally active rDNA over the first 2–3 days post-transfection, with $>80\%$ of cells expressing eGFP. In contrast, cells maintained at $37 \text{ }^\circ\text{C}$ exhibited a peak transfection efficiency of $\sim 40\%$ 24 h post-transfection, followed by a steady decline in the proportion of cells with transcriptionally active rDNA. Furthermore, the flow cytometric data (Figure 4A) revealed that cells transfected under optimized conditions were far more homogeneous with respect to eGFP content than cells cultured under control conditions.

Transfected CHO Cells Maintained at $32 \text{ }^\circ\text{C}$ Can Produce Recombinant Mab from Episomal rDNA for Extended Periods. From our previous data we inferred that the synergistic effect of mild hypothermia and addition of LR3-IGF on recombinant Mab production could be harnessed to maintain uniformly transfected CHO cells in a proliferation-controlled yet active state, capable of recombinant Mab production over extended periods in culture.

To test this hypothesis transfected CHO cells were simply subcultured (centrifugation and resuspension) into the same volume of fresh CDACF medium (at $32 \text{ }^\circ\text{C}$ with $20 \mu\text{g L}^{-1}$ LR3-IGF) approximately every 3–5 days, with daily monitoring

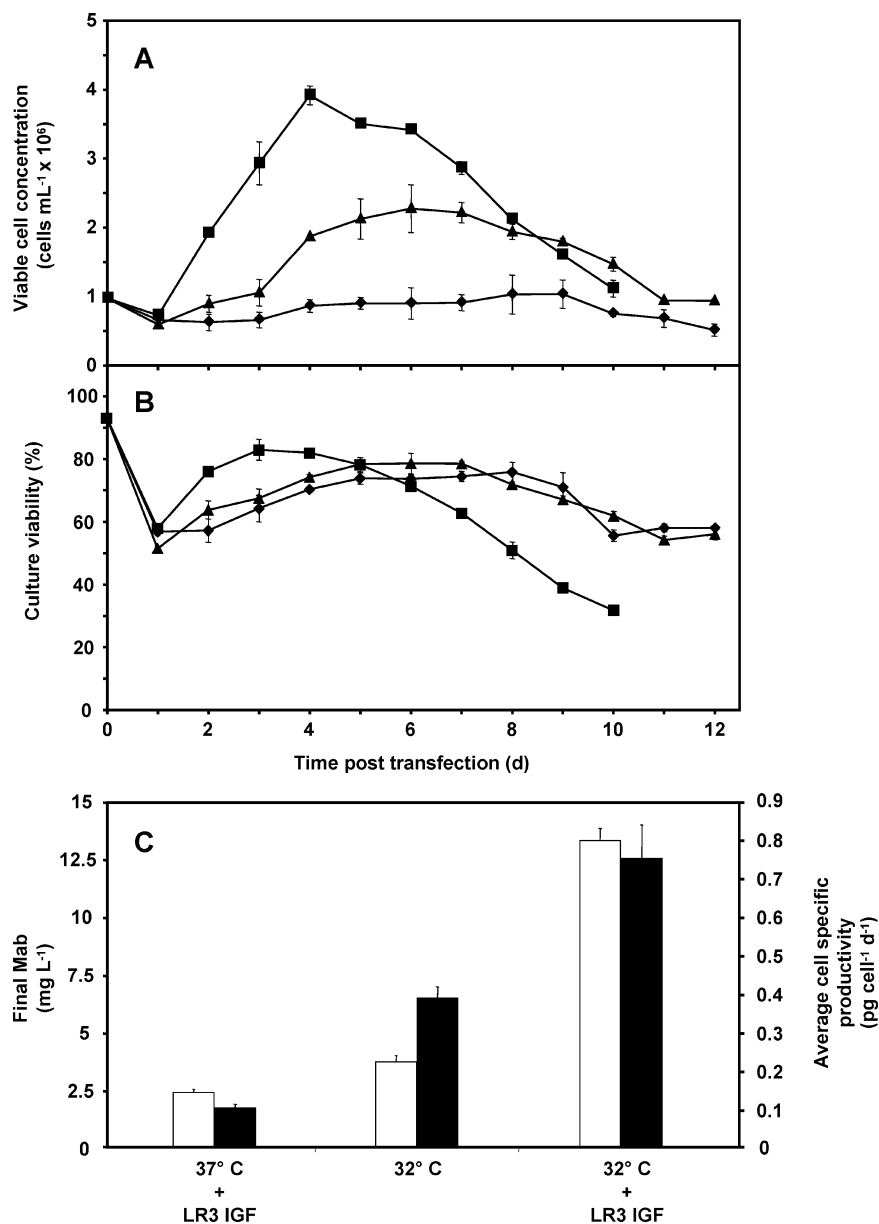


Figure 3. Effect of Long (R3) insulin-like growth factor (LR3-IGF) and/or mild hypothermia (32 °C) on transiently transfected CHO cell growth, viability, and recombinant Mab production. CHOK1SV cells were seeded in CDACF medium at a viable cell concentration 1×10^6 cells mL⁻¹ and cultured in 1000-mL shake flasks at a working culture volume of 100 mL. Cells were then transfected at an optimal PEI N:DNA P ratio of 20:1 at a final DNA concentration of $1.25 \mu\text{g mL}^{-1}$ and cultured either at 37 °C in the presence of $20 \mu\text{g L}^{-1}$ LR3-IGF (■), at 32 °C in the presence of $20 \mu\text{g L}^{-1}$ LR3-IGF (▲), or at 32 °C without LR3-IGF (◆). (A, B) Viable cell concentration and culture viability. (C) Final recombinant Mab concentration in cell-free supernatant determined at 10 days (37 °C plus LR3-IGF culture) or 12 days (32 °C cultures) post-transfection by ELISA (open bars); average cell-specific production rate over culture duration (closed bars). $n = 2 \pm \text{SD}$.

of secreted Mab titer by ELISA. As shown in Figure 5A and B, up to day 5 post-transfection the kinetics of cell growth and viability were similar to those observed previously (Figure 3). After the first medium change we observed a transient maintenance of viable cell concentration, followed by a subsequent decline in cell viability 9–10 days post-transfection. However, this was followed by an extended 11-day period of lower yet relatively constant viable cell concentration at approximately 40% cell viability. Importantly, with every passage into fresh medium, we observed continued production of recombinant Mab over the entire 21-day culture period, indicative of the maintenance of transcriptionally active rDNA in the cell population. Cell-specific production rates during the latter 11-day period of culture ranged from 1.4 to 2.3 pg cell⁻¹ day⁻¹, yielding a cumulative Mab yield at 21 days post-transfection of 31 mg Mab per mg rDNA transfected (Figure 5C).

N-Glycosylation of Recombinant Mab Derived from Extended Transient Production at 32 °C Is Similar to That of Mab Produced by Stably Transfected CHO Cells. While it has previously been observed that a reduction in culture temperature either does not affect or even improves protein product glycosylation (20, 24), this may not apply to recombinant Mabs produced by a PEI-mediated transient process under mild hypothermic conditions. Changes in Mab glycan profile can occur as a result of bioprocess changes, e.g., culture time (25), dissolved oxygen concentration (26), or pH (27). These may be relevant to function as alteration in the glycosylation microheterogeneity of monoclonal antibodies in the Fc domain at Asn²⁹⁷ has been shown to modulate their bioactivity, specifically effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC). For example, for IgG₁ Mabs a reduction in

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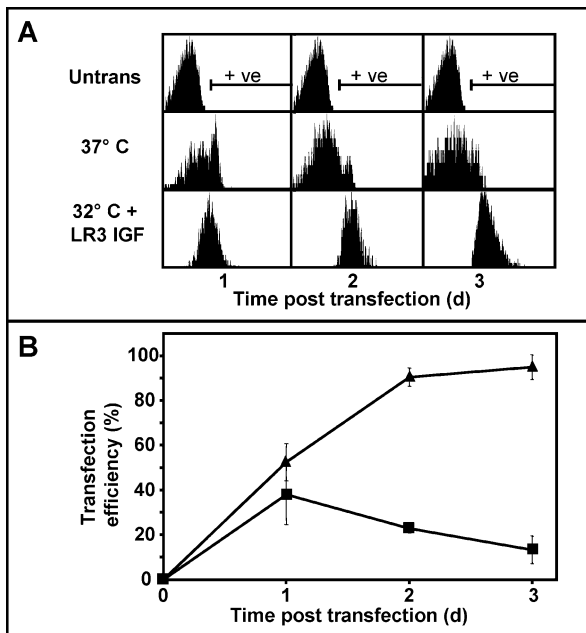


Figure 4. Characterization of transiently transfected CHO cells. CHOK1SV cells were transfected with a plasmid vector encoding enhanced GFP (pEE6.4 eGFP) as described in Figure 2 using the optimized conditions for Mab production (1×10^6 cells mL^{-1} , $1.25 \mu\text{g DNA mL}^{-1}$ at a PEI N:DNA P ratio of 20:1 in medium), followed by culture either at 37°C without LR3-IGF (■ in B) or at 32°C in the presence of $20 \mu\text{g L}^{-1}$ LR3-IGF (▲ in B). The eGFP content of transfected cells was determined daily by flow cytometry (A, ex 488 nm, em 515–535 nm; 5000 cells per analysis), and this was used to calculate transfection efficiency (B, defined as the percent of transfected cells displaying a higher fluorescent intensity than 99% of control untransfected cells). $n = 3 \pm \text{SD}$.

core α 1,6-linked fucose content is associated with elevated ADCC (28, 29), whereas reduced terminal galactose content may adversely affect CDC (30).

We compared the glycosylation of recombinant IgG₄ Mab produced by the optimized PEI-mediated transient transfection of CHOK1SV cells (described in Figure 5) with that of Mab produced by stably transfected GS-CHO cells producing the same protein product. Recombinant Mab was purified from cell-free culture supernatant at 9, 16, and 21 days post-transfection and Asn²⁹⁷-containing tryptic glycopeptides were prepared and then analyzed by MALDI-MS as described previously (11). We note that Mab samples taken from the transiently transfected cultures were not protein product that had progressively accumulated over the entire culture period but were samples derived from cells periodically resuspended in fresh medium, as described above.

As shown in Figure 6, Asn²⁹⁷ of Mab's produced by transiently transfected CHO cells was predominantly associated with N-glycans having masses that corresponded to complex biantennary oligosaccharides with a core α 1,6-fucose and varying terminal galactose content (G0F, G1F, G2F). A small proportion of nonfucosylated, nongalactosylated N-glycan (G0) was evident in each case. Importantly, we observed no significant difference in the glycosylation of Mabs produced at any stage post-transfection, and the glycosylation of Mabs derived from the transient process was also not significantly different from that produced by stably transfected GS-CHO cells.

Discussion

We experimentally determined the optimal basic conditions for PEI-mediated transfection of CHOK1SV cells in a CDACF medium to be $1.25 \mu\text{g DNA}$ complexed with 25 kDa branched

PEI (PEI25) at a PEI N:DNA P ratio of 20:1 (2.7:1 w/w) per 10^6 cells mL^{-1} . These conditions are generally similar to those also experimentally optimized for linear-PEI-mediated transfection of CHO DG44 cells in chemically defined medium recently reported by Derouazi et al. (12) ($1.25 \mu\text{g DNA}$ complexed with 25 kDa linear PEI at a PEI N:DNA P ratio of $\sim 15:1$ (2:1 w/w) per 10^6 cells). Our data therefore confirms the requirement for a molar excess of PEI over DNA to support efficient transfection of DNA. Free polycations may promote release of DNA from endosomes (the “proton sponge effect”) and/or mediate increased nuclear uptake, as previously discussed (4, 31).

Our data demonstrate the potential utility of simple bioprocess manipulations to increase the survival and productivity of nonreplicating yet transcriptionally active episomal rDNA within a transfected CHO cell population while controlling the overgrowth of nonproductive cells. This approach extends recombinant protein product output significantly. For example, on the basis of the data in Figure 5, we calculate a rDNA input:rProtein output ratio of 1:31 using our system, which could easily be improved (see below). This issue is important as the cost of production of recombinant DNA for transfection of larger volume cultures may itself be a constraint (32). Overall, the volumetric Mab productivity we observed compares very favorably with HEK293 cell based transient production systems employing the EBNA-oriP system to enable replication of episomal rDNA (8) and is an improvement on previously reported protocols for PEI-mediated transfection of nonreplicating rMab DNA into CHO cells, which yield maximal Mab titers of approximately $5\text{--}8 \text{ mg L}^{-1}$ (11, 12). All components of our transfection system are compatible with cGMP production.

Although our data showed that death of transfected cells may occur during extended culture (e.g., Figure 5, days 8–10 post-transfection), a significant proportion of cells also remained viable for an indefinite period. We have no clear explanation for the observed cell death, other than to speculate that a combination of nutrient limitation and any remaining intracellular PEI may have induced an apoptotic cascade in a proportion of cells. This could potentially have been averted by an earlier medium replacement. In our experiments, mechanical damage to cells during medium replacement (centrifugation, resuspension) also contributed to cell death during extended (sub)culture.

Although it would be possible to generate protein product by a simple batch or fed-batch culture process maintained at reduced temperature, we suggest that use of an extended culture process may be an attractive option to further increase productivity. On the basis of our data (Figure 5) it may, for example, be feasible to maintain transfected, transcriptionally active CHOK1SV cells in a perfusion bioreactor operated at low-dilution rate (33) to generate a uniform product for extended periods from previously transfected episomal DNA.

Our data show clearly that LR3-IGF and mild hypothermia act synergistically to improve qMab. This was a consequence of a significant continued improvement in measured transfection efficiency under these conditions (percent of cells determined to be eGFP positive by flow cytometric analysis) post-transfection. In general terms, although we speculate that LR3-IGF and mild hypothermia may prevent longer-term PEI-mediated apoptotic death of transfected cells (17, 18, 21) and therefore to maintain “active” rDNA in the cell population, the observed increase in transfection efficiency and qMab are a likely consequence of LR3-IGF and mild hypothermia acting on discrete cellular mechanisms in concert. Theoretically, the

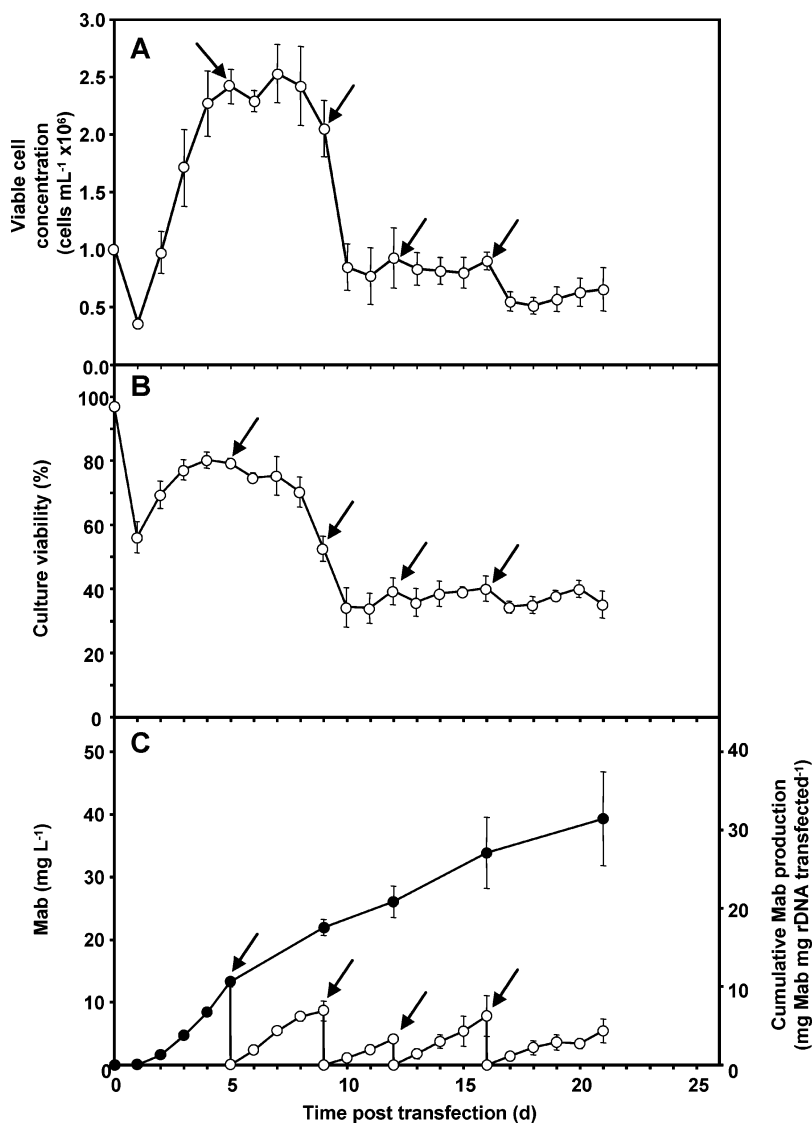


Figure 5. Extended transient production of recombinant Mab at 32 °C in chemically defined medium containing LR3-IGF. CHO K1SV cells were transfected with the plasmid vector pcB72.3 encoding a recombinant chimeric IgG₄ Mab as described in Figure 2 using the optimized conditions for Mab production (1×10^6 cells mL⁻¹, 1.25 μ g DNA mL⁻¹ at a PEI N:DNA P ratio of 20:1), followed by culture at 32 °C in medium containing 20 μ g L⁻¹ LR3-IGF. At 5, 9, 12 and 16 days post-transfection transfected cells were recovered by centrifugation (200g, 5 min) and resuspended in an equivalent volume of fresh medium (indicated by arrows). (A, B) Viable cell concentration and culture viability (○). (C) Recombinant Mab production. Volumetric Mab concentration in cell-free supernatant determined by ELISA (○) and cumulative Mab production expressed as mg Mab produced per mg rDNA originally transfected (●). $n = 3 \pm$ SD.

following may be affected: (i) stability and abundance of nuclear rDNA in the cell population, (ii) transcriptional activity of nuclear rDNA, (iii) translation of recombinant mRNA, or (iv) a combination of the above.

An increase in nuclear rDNA content may derive from altered intracellular trafficking of rDNA/PEI complexes (e.g., rate of release from endosomes) in combination with an increased frequency of mitotic cell division to enable nuclear uptake. However, with respect to the latter, even though addition of LR3-IGF to 37 °C cultures markedly stimulated cell proliferation per se, qMab was actually reduced. Therefore, we infer that the ability of LR3-IGF to (indirectly) stimulate mitosis is not relevant to its synergistic effect on qMab with mild hypothermia. Although we cannot entirely rule out the possibility that mild hypothermia affects the cellular uptake, trafficking, or fate of rDNA, it is unlikely that this is specifically elevated at 32 °C.

On the basis of previous reports, it is plausible to infer that episomal rDNA may exhibit an increased transcriptional activity at reduced temperature, relative to homologous cellular mRNA. Mild hypothermia has been shown to increase the recombinant

mRNA content of stably transfected CHO cell lines (34), and while this may be related to elevated mRNA stability at reduced temperature (35), Fox et al. have argued that recombinant genes exhibit less transcriptional suppression at 32 °C than do host cell genes (36). Therefore, in conjunction with the typical inhibition of mammalian cell proliferation observed with mild hypothermia (37), elevated recombinant mRNA could explain the increased qMab of cells maintained at 32 °C both with and without LR3-IGF, relative to cells cultured at 37 °C. In this respect, although cell-specific recombinant Mab production is ultimately limited at a post-transcriptional level (38), production rate may be proportional to cellular recombinant mRNA content at lower qMab's (39). Therefore, as transiently transfected cells are likely to have a relatively low recombinant mRNA content, with a correspondingly low qMab (up to 2 pg cell⁻¹ day⁻¹ in this study), increased recombinant mRNA content may be expected to yield an increase in qP. In support of this, although Yoon et al. have reported that the degree to which reduced culture temperature increased the specific productivity of different CHO cell lines stably expressing a recombinant Mab

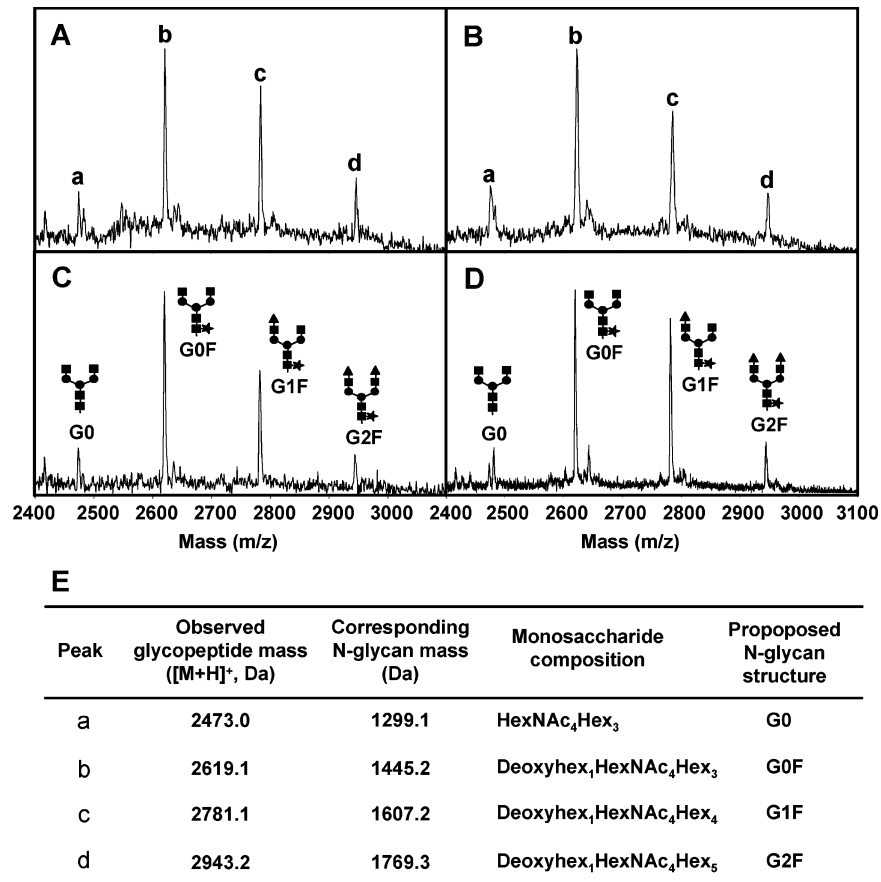


Figure 6. Comparative analysis of the glycosylation of recombinant Mabs derived from extended transient production with Mab produced by stably transfected CHO cells. Recombinant IgG₄ Mab was purified from cell-free supernatant of transiently transfected cultures of CHOK1SV cells at 9 (A), 16 (B), and 21 (C) days post-transfection (produced as described in Figure 5) or from the supernatant of stably transfected GS-CHO cells producing the same Mab (D). Tryptic glycopeptides derived from purified Mabs were purified by C₁₈ reverse-phase chromatography and then analyzed by MALDI-MS. Panels show representative MALDI-TOF MS analyses of Asn₂₉₇-containing Fc glycopeptides analyzed in linear, positive ion mode ([M + H]⁺ molecular ions). To identify discrete glycoforms (peaks a–d), the core tryptic peptide mass (1173.5 Da) was subtracted from the average mass of an observed glycopeptide species to yield an N-glycan mass (listed in E). Calculated N-glycan masses were used to infer an N-glycan structure based on a corresponding monosaccharide composition consistent with mammalian biosynthetic principles. Monosaccharide symbols as follows: (■) *N*-acetylglucosamine, (●) mannose, (▲) galactose, (★) fucose.

is cell line specific (40), it was generally the case that there was an inverse relationship between the extent of the increase in qMab at 32 °C and initial cell line qMab at 37 °C.

Accompanying any transcriptional effects, we would also expect cells maintained at 32 °C to have (accompanying reduced transcriptional activity (35)) a generally reduced rate of protein synthesis (41, 42). The reasons for this are unclear at present. It may be a consequence of reduced transcriptional activity per se, or there may be cell signaling pathways more directly controlling protein synthetic rate. As cellular mRNA's exhibit a longer half-life at reduced temperature (36), the latter is likely to be the case. In this respect, hormones such as insulin or LR3-IGF may counteract reduced protein synthesis under mild hypothermia by stimulating mRNA translation directly (43, 44).

In conclusion, the favorable combination of mild hypothermia and LR3-IGF on transient Mab production may be explained by both general and specific effects. First, both conditions may generally promote the longer-term survival of transfected cells (and thus active rDNA in the culture) by inhibiting apoptosis. Second, mild hypothermia and LR3-IGF may specifically activate complementary cellular processes: recombinant mRNA transcription and translation, respectively. Very recently, Fox et al. have also reported that mild hypothermia and insulin act synergistically to increase production of recombinant protein by stably transfected CHO cells (36, 45). They describe the combination of growth-associated productivity promoted by

insulin combined with elevated recombinant mRNA levels generated at reduced temperature as “active hypothermic growth”. In our case, cell growth is a *downstream consequence* of LR3-IGF addition (as cells have to synthesize proteins to accumulate biomass and divide) and not strictly necessary per se for maximal recombinant protein production. We would describe the optimal combination of mild hypothermia and LR3-IGF as “activated hypothermic synthesis”.

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