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Rapid Development of High-Performance Stable Mammalian Cell Lines for Improved Clinical Development

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Introduction

Since the first approval for human use of a recombinant protein therapeutic, this sector of the pharmaceutical market has grown rapidly.[1] The first approved protein therapeutics were small, non-glycosylated proteins such as insulin and human growth hormone; they were produced in bacterial systems. With the advent of mammalian cell-based production systems, it became possible to produce more complex, glycosylated proteins for use as recombinant therapeutics.

For two decades, the primary techniques for development of mammal cell production cell lines involved the use of DNA amplification, a labor-intensive process that takes 12–18 months. Using DNA amplification for the development of a mammalian cell production cell lines involves several steps. These include the transfection of a host cell with the recombinant protein transgene, screening thousands of transfectants, selection of clones that have undergone gene amplification, and selection of final stable clones.

While the need for improvements in the entire process of protein production has been long recognized, the focus for decades has been on optimizing media development and downstream production processes.[2] This focus on downstream processing has resulted in improved productivities, and there has been little effort made, until recently, to address the need for cell lines that stably maintain expression throughout production. Loss of expression due to the instability of cell lines leads to lower yields and results in increased time and money needed for regulatory approval of products.

The selection of a cell line that has sustained productivity throughout a full process cycle has not been easy using the more traditional approaches of cell line generation.[3] Efforts to improve the first steps of the cell line development process, such as in the areas of vector construction and cell line selection, are now a renewed focus within the industry, and are aimed at creating more reliable and speedy therapeutic protein production.

Causes of Instability

To better understand how these newer approaches to cell line development can aid in increasing the stability of cell lines, it is helpful to revisit some of the issues that lead to instability of protein production in mammalian cells: gene amplification, loss of genetic material, methylation and the location of integration. Both the DHFR and GS methods of amplifying transgenes can result in genetic instability.

It is commonly reported that recombinant protein production can drop significantly within the two months following high-producing clone selection, particularly if the selection pressure is removed.[4-7] Even in the presence of selective pressure, Jun *et al*.[5] found that the stability of antibody-producing subclones was very poor. Furthermore, the specific secretion rate decreased by 50% after 100 passages, even with selective pressure. These data suggest that instability could be a concern in the development of CHO cells with DHFR and GS-mediated gene amplification.[4,5,8,9]

In addition to the potential loss of the transgene following amplification, the large number of repetitive gene sequences that result from this process has been shown to induce methylation of DNA sequences, preventing tran-

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scription.[10] These observations perhaps explain why expression following amplification does not increase proportionally with copy number.

The fact that the chromosomal location can affect the expression of eukaryotic genes has been known for a long time.[11,12] The combined effect of this random transgene incorporation results in only a small percentage (<1%) of the initially isolated cells remaining capable of producing reasonable amounts of the desired protein.[13] Therefore, lengthy selection and screening procedures must be undertaken to select and identify those cells with the proper growth and transgene expression properties required for large-scale production.

Use of Genomic Elements

Matrix attachment region (MAR) genomic elements have been shown to lead to increases in specific productivity that can be as high as 20-fold when compared to transfected cells without these elements.[14,15] The use of the MAR elements also increases the proportion of high-producing clones, thus reducing the number of clones that need to be screened. MARs, then, have been used to improve the expression of transgenes in cells cultured *in vitro* and *in vivo*.[15-19]

Recently, human genomic elements have been isolated.[16] Protein production results were published and described based on these novel human elements identified using bioinformatics. The bioinformatics analysis uses motifs that adopt a curved DNA structure that positions nucleosomes and bind specific transcription factors to identify novel MAR elements present within the human genome. This systematic approach to identify computer-recognizable structural and functional MAR has identified potent elements within the human genome that mediate high, consistent, and stable gene expression in cells in culture.[16] The uses of these elements overcome the need for amplification, and assure that all copies of the transgenes are actively expressed.

This article also discusses the use of these unique vectors with a reiterative transfection protocol that results in cell lines that express high levels of recombinant antibodies without the need for amplification. These genetic modifications, used for the initial cell line development, are used in concert with more traditional process development tools such as temperature shifts to boost expression levels.[20,21] Finally, we show data at 100–liter scale for an antibody and non-antibody protein alike.

Methods

Vector Construction

The vectors used here differ from those cited previously[16] in that the human GAPDH promoter[22] is used to direct transcription of the transgenes. Two novel MAR elements (called SGE1 and SGE2) are present on these vectors, one each flanking the transgene, and the human gastrin terminator is located 3' of each transgene[23] (Figure 1). Extraneous bacterial plasmid sequences were removed to prevent an interference of expression by putative "poison sequences."[24] In addition to the expression vectors for the heavy and light chains for antibody production, two other expression vectors were constructed. These vectors are used to produce two human recombinant proteins that are not antibody proteins. Recombinant protein-1 (rp-1) is a protein that we have historically found difficult to produce; recombinant protein-2 (rp-2) has been, in our hands, relatively simple to produce.

Transfections

CHO-K1 cells are cultured as described by Girod *et al.*[18] One day before the first transfection, cells are seeded in 24-well tissue culture plates at a density of 1.2 x 10^5 cells/well, and are used after 18 hours further incubation, when they are 40–60% confluent. On the day of transfection, the complexes of vectors and cationic liposomes are made according to the manufacturer's instructions (Invitrogen), by mixing DNA and LipofectAMINE 2000.

Increasing Copy Number of Transgene Without Amplification

We used an iterative DNA transfection process combined with the targeting of MARs in the cell genome.

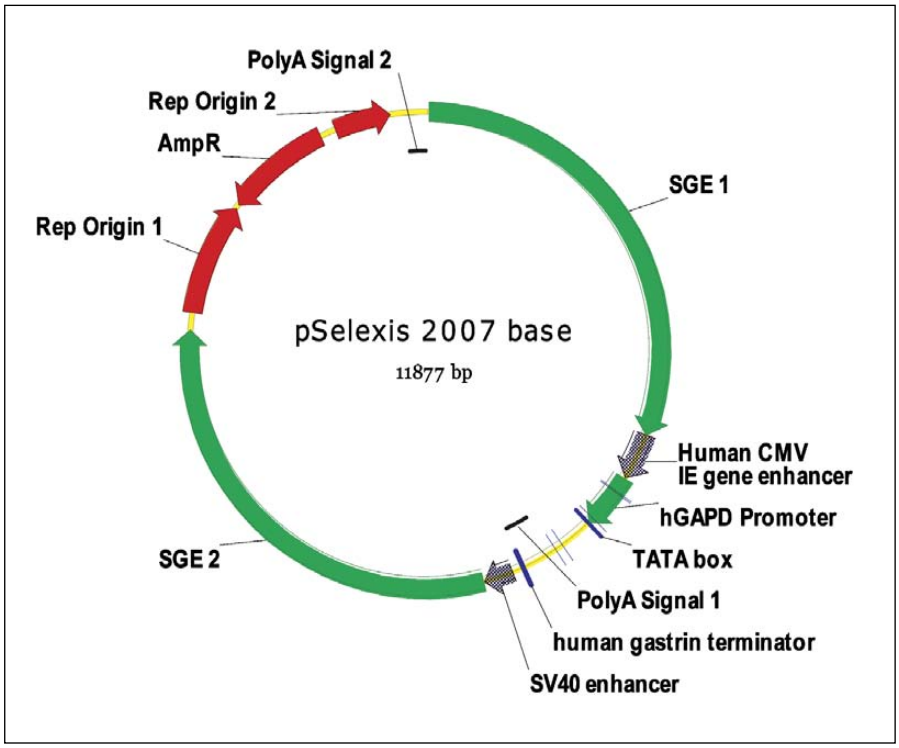

FIGURE 1. Schematic representation of expression vector containing two MAR (SGE) elements.

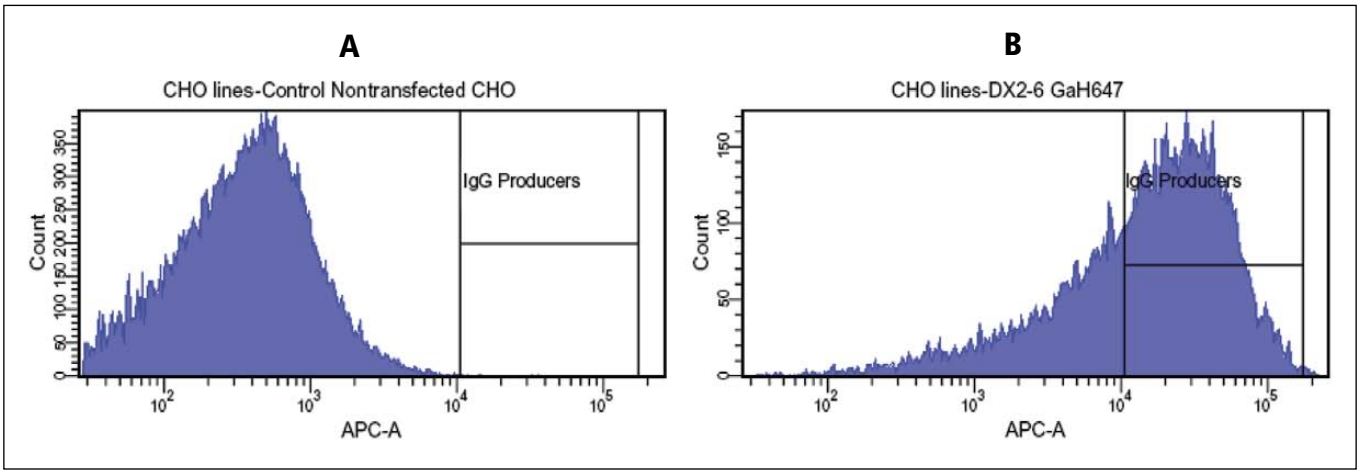

FIGURE 2. Analysis of cells transfected with a MAR expression vector producing an IgG antibody.

The FACS sorts following this iterative approach are shown in Figure 2.

Selection, Screening, and Scale-Up

Post-transfection cells undergo a selection process that takes 3–4 weeks. At this stage, 100–150 colonies are screened and the top 30 colonies are scaled-up for further productivity evaluation. From the 30 colonies, the top ten are scaled-up into shaker flasks. Cells are passaged twice, and they undergo a productivity and growth evaluation in suspension fed batch mode. The top two colonies are selected, cryopreserved, and cloned by limiting dilution. Clones go through the same screening and selection process as described previously. The entire cell line development process, from transfection to final clone selection, has now been shortened from months to a matter of 15 weeks. Clones are then ready for scale-up, production, stability studies, and process development.

Temperature Shift

Temperature shift data was obtained using Sartorius BioStat A-Plus bench-top reactors and standard cell culture techniques. Bioreactors were seeded at 0.5–1.0 x 10^6 cells/ml in 1 liter of fresh commercially-available serum-free media. The experiment was performed in fed batch mode using commercially-available feed formulation. Cell counts were acquired using a Beckman Coulter Vi-Cell XR cell viability analyser. Antibody titer values were determined using a POROS (Applied Biosystems) protein A chromatography column run on an Agilent Technologies 1200 series HPLC system. Titers for non-antibody proteins were determined using an ELISA assay.

Results

Transfection Efficacy and Productivity

As a control for transfection efficiency, cells that had been transfected with a MAR containing expression vector and an antibody gene were analyzed by FACS sorting. The expression of recombinant antibody was monitored with the use of a fluorescently labeled anti-IgG antibody.[25] Figure 2A is a FACS analysis of the untransfected parent CHO-K1 cell line. The FACS display in this panel shows a population of cells that do not express the transgene; the peak to the left of the grid shows a typical pattern for cells that do not express a transgene and provide data on background fluorescence. Figure 2B shows the shift in the population of florescent cells following transfection. Over 75% of the transfected population is expressing antibody protein, seen by the shift of the peak towards the far right. When a mini-pool of transfected cells (cells taken from one well of a 96-well dish) is analyzed for production levels of antibody protein, this mini-pool was seen to produce 0.3 PCD. Cells isolated from the far right peak of the FACS distribution produce 15–20 times more than cells isolated from a single mini-pool (Table 1).

TABLE 1. Specific productivities for a mini-pool of CHO-K1 cells transfected with an antibody protein gene.

ID	Seven Day Endpoint PCD (picogram/cell/day)
Mini-pool P1E1	0.3
FACS P5F11	5.0
FACS P1G4	7.0

These levels are compared to the increased productivities seen when cells isolated from the right hand range of the FACS sort (seen in Figure 2) are assayed.

Stability Data

To assure that the clones selected for further development had continuous stable expression of the recombinant proteins, we analyzed productivity over several generations. To do this we harvested cells producing the antibody protein at successive generations from gen-

eration 0 to generation 30. The cells from each of the 10-generation cycles were frozen for future analysis. To monitor the stability of these cells over time, cells from generation 0, generation 10, generation 20 and generation 30 were analyzed in fed batch shaker flasks over a period of 12 days. The histogram in Figure 3 shows the relative titers obtained for each generation of cells, and the line graph shows the growth in culture during the 12-day production cycle. Cells from generation 0 appear to have lags in the growth of cells once they are placed within the shaker flask. The subsequent generations assessed, generation 10, generation 20 and generation 30, all show similar doubling times in the shaker flasks and similar relative productivities over time.

Production of Non-Antibody Proteins

The transfection techniques described above are applicable to antibody and non-antibody proteins. We have also produced non-antibody proteins for clinical trials. In Figure 4 we compare the production for two of these non-antibody proteins, rp-1 and rp-2 proteins, with the production of the IgG antibody shown in Figure 3. Historically we have found that when we use our traditional vectors without any genomic elements, cell lines producing rp-1 result in quite low titers while rp-2 cells lines produce reasonable protein titers.

To monitor productivity, production cell lines for these three proteins were grown in 1-liter benchtop bioreactors. The seeding densities were 0.5–1.0 x 10^6 cells/ml. The cell density for each of these three production lines begins to plateau between 5–7 days in the bioreactor. The production cell line for rp-2 reached a density of 10 x 10^6 cells before it began to plateau, with a titer of 1.5 grams/liter, while the antibody cell line reached a maximum density of nearly 6.4 x 10^6 cells, with a titer of 2.2 grams/liter. The production cell line for rp-1 reached a plateau at 7 x 10^6 cells. Though the productivity for rp-1 is lower than that seen with the other two proteins, the titer obtained with the MAR vector system (of 0.5 gram/liter) is higher than yields we have obtained using more traditional expression vectors. For all three proteins we were able to generate the production cell lines and develop a downstream scale-up process; these can be used to produce sufficient amounts of the therapeutic protein needed to meet clinical needs in a time frame of less than nine months.

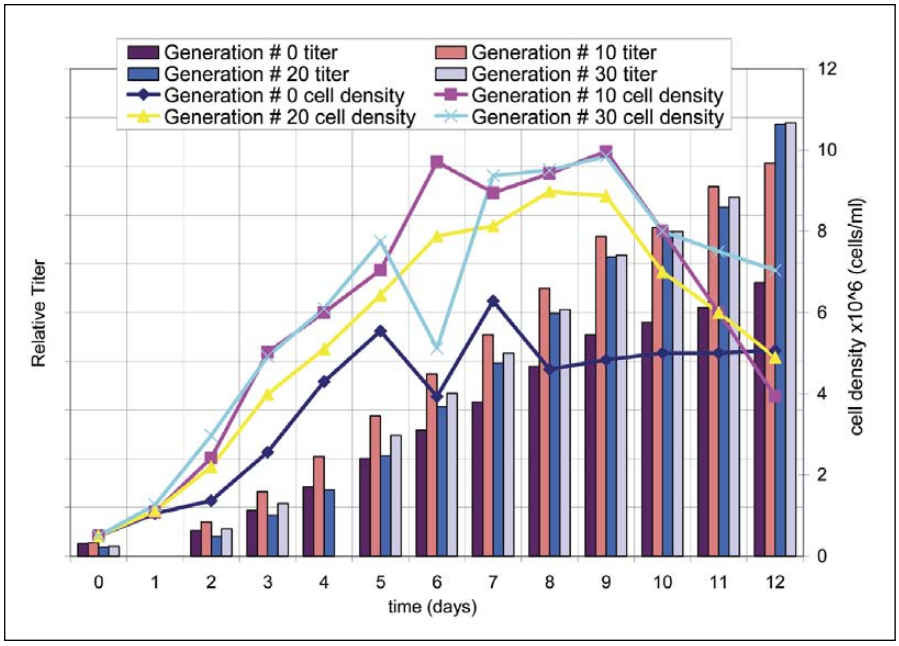

FIGURE 3. Cell growth and productivity over 30 generations in culture.

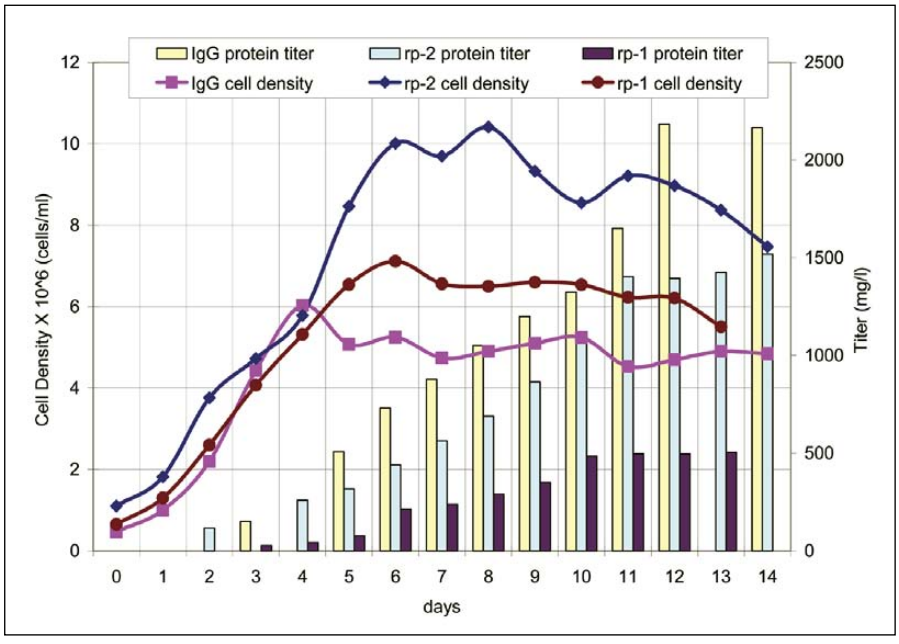

FIGURE 4. Data are from 1-liter benchtop bioreactors seeded at a target seed density between 0.5 x 10^6 cells/ml and 1.0 x 10^6 cells/ml.

Temperature Shift Data

There are a growing number of reports on the sub-physiological temperature culturing (<37°C) of mammalian cells for increased recombinant protein yield.[26] Low temperature also causes growth arrest, meaning that the cell density is significantly lower at 32°C. Nutrients are consumed at a slower rate, and the batch culture can be run for a longer period of time prior to the onset of cell death. In Figure 5, we test the relative productivity levels as a function of temperature. In this experiment, antibody producing cells are grown in a 1-liter bench top bioreactor seeded at an average density of 0.5 x 10^6 cells/ml.

In the experiment shown in Figure 5, the shift of cultivation temperature from 37°C to 30°C leads to an increase in specific productivity of 75%. Cultivation at low temperature provided higher overall product yield compared to a standard cultivation at 37°C, similar to the results seen by Kaufmann *et al.*[27] This group also showed that the expression pattern of total protein from the CHO-K1 cells is altered after decrease in cultivation temperature to 30°C. These changes in the proteome suggest that mammalian cells respond actively to low temperature by synthesizing specific cold-inducible proteins, Kaufmann *et al.*[27]

To ensure maximal yield of recombinant proteins during the production cycle, it is important to determine the appropriate time to shift the temperature. An initial growth phase of rapid cell proliferation is needed to achieve high biomass. This growth period is then followed by a stationary phase where cellular energies are directed towards production of recombinant protein. One approach to alleviating the problem of slow growth at low temperature is to utilize a biphasic process, wherein cells are cultured at 37°C for a period of time in order to obtain reasonably high cell density. Then the temperature is shifted to 32°C to achieve high specific productivity.[20] Low temperature-induced growth arrest is associated with many positive phenotypes including increased productivity, sustained viability, and an extended production phase.[28]

As illustrated in Figure 6, the highest levels of specific productivity were achieved at a temperature of 32°C after the cells had been allowed to grow to a high density for a period of five days. Figures 6 and 7 further clarify the benefit of using this biphasic approach when shifting temperature. Using an IgG-producing cell line, cell growth is almost stagnant at 30°C, but it has a specific productivity almost double that of the same cells at 37°C.

We have also boosted productivity further via media development using traditional media optimization

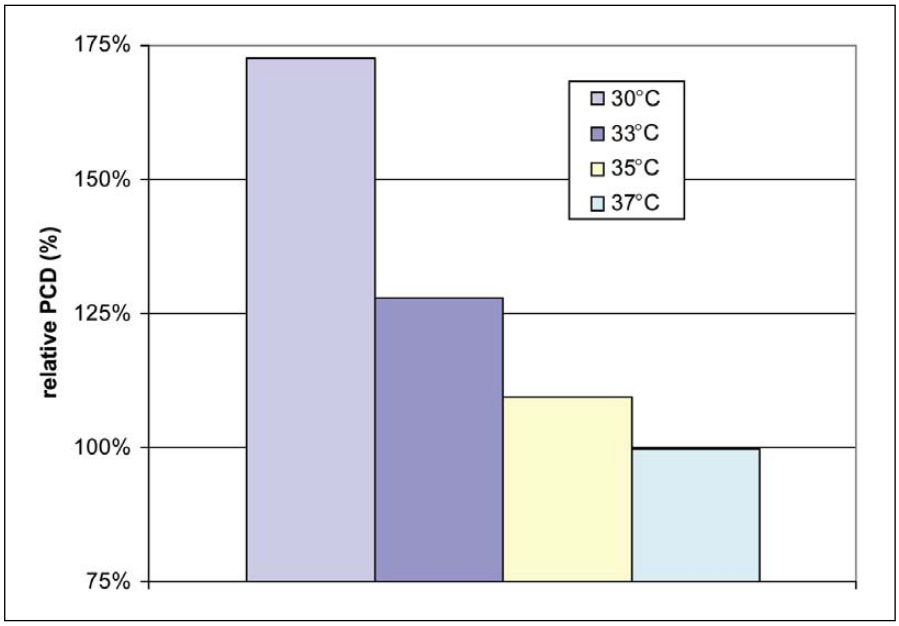

FIGURE 5. Change in specific productivity as a function of temperature. The specific productivity begins to shift as the temperature of the bioreactor is lowered. A 25% increase in productivity is seen with a moderate decrease of 4°C. With a shift of 7°C, there is an increase in productivity of 75%.

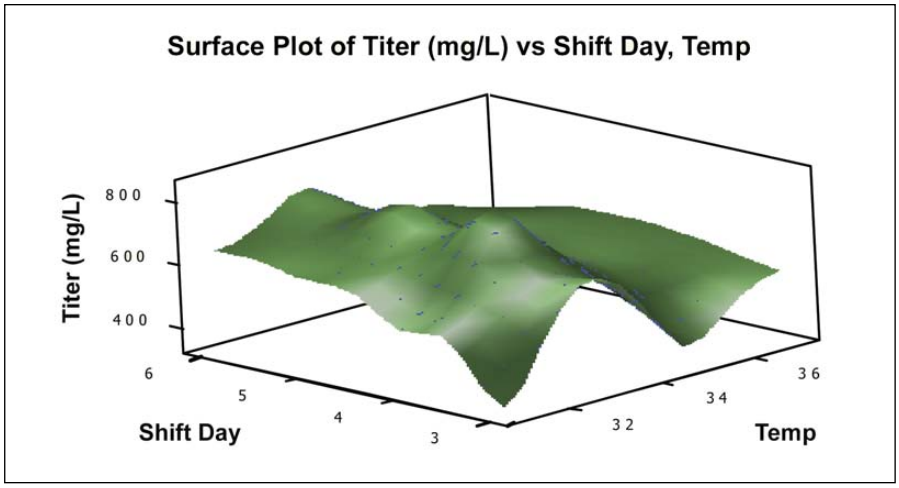

FIGURE 6. Specific productivity as a function of temperature shift and the time in culture. This is a 3-D plot of three variables: temperature; number of days grown in the shaker flasks prior to shifting the temperature; and productivities as a function of these other two variables. The highest producing cultures were achieved at a temperature of 32°C after the cells had been allowed to grow to a high density for a period of 5 days.

approaches such as optimization of amino acids, glucose, and other energy sources. Figure 8 shows the improvement gained from optimization of media components, which results in 2.2 grams/liter production.

Discussion

The development of high producer cell lines for manufacturing of recombinant proteins requires the identification of rare clones with defined specificities. We describe a method that allows the rapid generation of highly productive cell lines that express recombinant human IgG with productivities >30 PCD by placing MAR elements in the vectors. In our evaluations, not only were MAR sequences more able to promote expression, they were free of the drawbacks of amplification. Since gene amplification occurs through the mechanism of chromosomal rearrangement, which occurs through chromosomal breaks,[30,31] amplification can result in decreased stability of prolonged expression of transgenes due to these breaks and rearrangements.[29]

Typically, the stability of recombinant cell lines is determined by monitoring cell growth and protein production for several months. For some cell lines, however, protein productivity diminishes over time, usually as a result of changes in the regulation of transgene expression.[7] Regulation of higher order chromatin structure is directly coupled with the regulation of the expression and integrity of the genetic information of eukaryotes, and is likely to be a major force in the origin and evolution of genes, chromosomes, genomes, and organisms. By including these human genetics elements in the expression vectors, the chromatin structure flanking the transgene is maintained in an active configuration.

Genes located near the telomeric regions of chromosomes are more likely to be stable than those in other regions of the CHO cell genome.[29] Based on data reported by Girod *et al.*,[16] it appears that there is some preference for integration near telomeres when using the MAR-containing vectors described in this article. These human MAR DNA elements function only to boost recombinant protein production, so cells do not produce unanticipated products. The procedure for making cell lines does not require viruses and is similar to approved processes.

We describe the use of temperature shift to further increase yields of the recombinant proteins. Our data show a positive effect of low temperature on recombinant protein production. Fox *et al.*[21] have documented the

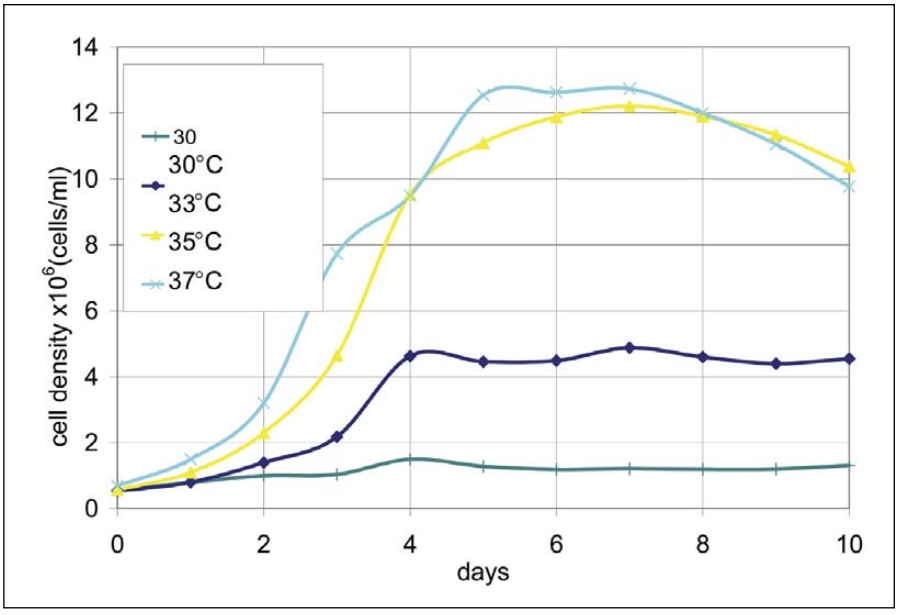

FIGURE 7.
Growth characteristics of an IgG-producing CHO-K1 cell line as a function of temperature.

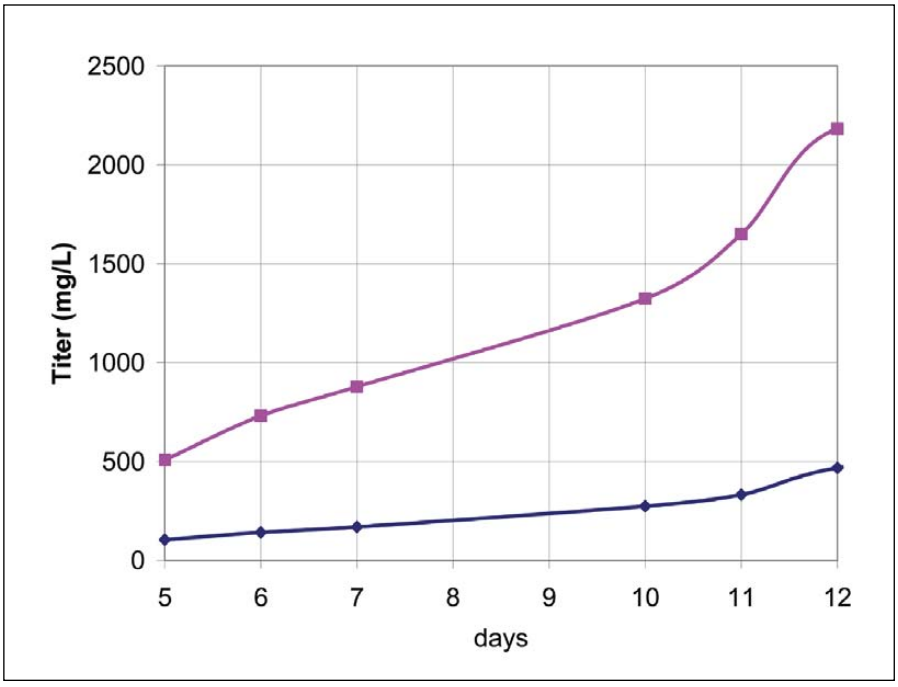

FIGURE 8. Boosted productivity via media development. This plot shows the same cell line: the lower culture with no media or process development, and the upper culture with basic optimization.

fact that such effects are due to elevated mRNA levels and not due to G0/G1-phase growth arrest, and that a cell line can still show growth-associated productivity at low temperature. The approaches used in this article blend both newer ideas of generation cell lines with improvements in process development. By improving both the molecular basis of cell line generation along with improvements in process development, production cell lines suitable for use in a GMP-controlled manufacturing process were in place in less than nine months.

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