Application of Factorial Design to Accelerate Identification of CHO Growth Factor Requirements

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To accelerate recombinant CHO media and process development, we describe a simple approach to integrating multiple tasks associated with these processes including initial media design, serum-free adaptation, stability analysis and first generation scale-up. Factorial design techniques and normal probability chart representation of the results were first applied to identify potent parental CHO cell growth factors in a lean basal medium. These results were then applied to identify a suitable manufacturing medium from a panel of commercial and proprietary media formulations. When this approach was applied to recombinant CHO cell line, rapid adaptation of the cell line to an appropriate production medium occurred during culture expansion in the presence of the identified growth factor(s). This approach allows media component screening to be naturally integrated into the adaptation and scale-up processes since components that have little or no relative effect on cell proliferation are selected against as the "best" cultures are moved forward. The rapidity of the adaptation process allowed cell line stability studies to be initiated relatively early in the development process, thus providing preliminary stability information by the time the "outgrowing" culture could be scaled to 100-L reactors some 30 days after adaptation commenced. The application of full factorial design techniques allowed us to calculate the maximum number of interaction effects, the interpretation of which we believe can provide insights into growth factor biology.

Introduction

Recent advances in industrial biotechnology and genomics have led to an increase in the number of potential protein therapeutics under development, and efforts are now underway to increase the throughput through the development process. While increased throughput of any unit operation would be of value, it is generally accepted that cell line development consumes a majority of the needed development time. This intricate process requires that a number of time-consuming steps, including genetic manipulations, cell line selection, serum-free adaptation and stability studies, are successfully completed and often involves iterative procedures. During development it is not uncommon for time to be lost as seemingly excellent candidate cell lines drop out of development for failure to achieve certain required benchmarks. Accordingly, a number of recent studies aimed at reducing the time and manpower required to complete the process can be found. Such studies have generally sought to either accelerate cell line development (*1*, *2*) or the early process development steps associated with media adaptation, stability and scale-up.

Reduction in the uncertainty associated with serum-free media adaptation can dramatically reduce the time and manpower requirements associated with process development. The time associated with adapting a serum- and anchorage-dependent cell line is generally on the order of months, lasting as much as half a year in some cases (*1*). A common approach to effecting such adaptation involves sequentially passaging the cell line of interest in a basal medium of interest in decreasing concentrations of serum. Since serum contains many growth and survival factors, it is common to supplement the medium under investigation with growth factors (*3*). The cell culture being adapted generally exhibits massive death or a "crisis" at some stage in the weaning process, and the culture resuscitation process typically involves increasing the serum concentration in the media to pre-crisis levels. Such cycles of culture crisis followed by resuscitation result in an iterative process that is both time-consuming and uncertain from a development perspective. To eliminate or reduce such uncertainties, several complementary approaches have been adopted. One approach involves pre-adapting the parental cell line of interest in the "final" development serum-free medium in order to facilitate the serum-free adaptation process once a production cell line has been generated using the pre-adapted host. This approach has led to a reduction in the time to serum-free adaptation under circumstances where the final production media is known very early in the adaptation process. More recently, researchers have applied genetic engineering techniques to create cell lines harboring constitutively active intracellular signaling pathways to alleviate exogenous growth supplements (*2*). Such an approach has the potential to deliver desirable parental cell lines for future use. However, such approaches are generally inapplicable in situations where it is desired to rapidly switch between different, possibly

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unknown, production media so that a methodology applicable to such situations would be of value.

In this article we report on our approach to reducing the uncertainty associated with the serum-free media adaptation process. Specifically, we have increased throughput by integrating multiple development steps including initial media design, serum-free adaptation, stability analysis and first generation process scale-up. Starting with a CHO DG44 cell line adapted to grow in a commercial serum-free medium of unknown composition, a process was developed that allowed the rapid adaptation of recombinant progeny cell lines from a serum-containing medium to defined medium suitable for rapid transfer to manufacturing. Statistical experimental design techniques were applied to identify insulin or IGF-1 as potent parental cell line growth factors. The use of normal probability chart for results presentation was found to provide robustness to these conclusions, in light of our lack of replicate data, and we also found that such plots provided insights into the significance of multifactor interactions. Our parentally derived growth factor requirement information was found to be transferable to derived recombinant cell lines. The integration of growth factor requirement information with a subsequent adaptation through outgrowth process allowed recombinant cell lines to be transferred from serum containing media to chemically defined media with concomitant scale-up to 100-L reactor fermentations within 30 days. Stability analysis was also performed within this time frame.

Materials and Methods

Cell Lines and Media. The parental cell line was obtained by weaning CHO DG44 off of serum according to standard cell culture techniques. The base medium employed during the weaning process was commercial serum-free medium X known to contain animal-derived proteins and hydrolysates. The process of weaning DG44 of its serum dependence lasted approximately 5−6 months, and the resulting cell line was used as the starting point for all subsequent development efforts including recombinant cell line generation, as well as in our parental growth factor screening studies. Recombinant cell lines were derived from our parent line using standard molecular biology techniques (*4*). The basal protein-free medium consisted of a DMEM/F12 base supplemented with 0.01% (v/v) pluronic F-68 (Gibco/Invitrogen Gaithersburg, MD), 4 mM L-glutamine (JRH Biosciences, Lenexa KS), GHT supplementation (glycine 0.5 g/L, hypoxanthine 0.25 g/L, and thymine 0.75 g/L; Sigma Chemicals, St. Louis, MO) and 200 mg/L TC-yeastolate (Becton Dickenson, Sparks, MD). Human transferrin was obtained from Sigma Chemicals. bFGF was obtained from Gibco/Invitrogen. IGF-1 (LongR3) and recombinant insulin were obtained from JRH Biosciences. All growth factors were prepared and stored according to the manufacturers recommended conditions.

Assays. Cell cultivation was performed in 37 °C humidified incubators supplemented with 5% carbon dioxide. Cell counts were performed using a Cedex automated cell counter (Innovative Directions, Rodeo CA). Protein titer was determined using a protein-A-based Ig assay implemented on a Waters HPLC. Specific productivities were determined using 24-h differential expression data. Briefly, 20−40 million cells were harvested from media X or E at the indicated time points, washed, and then resuspended in 20 mL of fresh media, either medium X or E respectively, at a concentration between 1−2 million viable cells/mL. After 24 h, the cells were counted and the broth was assayed by HPLC.

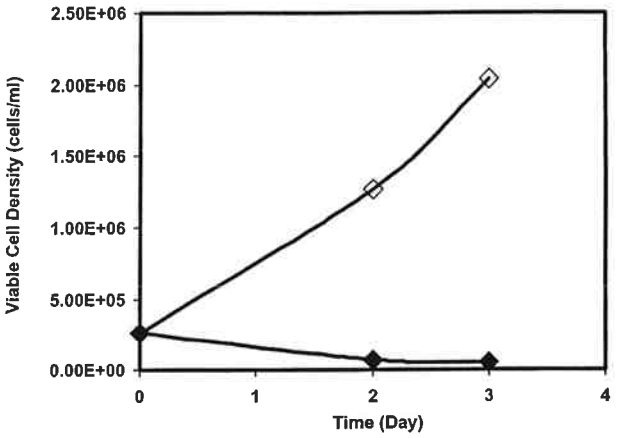

Figure 1. Time course output from the proliferation assay for the parental DG44 CHO culture growing in medium X (positive control; open symbol) and in DMEM/F12 basal medium (negative control; closed symbol).

Specific production rate data was determined by dividing the titer data by the average cell concentration and the time.

Cell proliferation assays were initiated in six-well plates using 4 mL final culture volume. After 3 days in culture, the cell counts were performed and the wells containing the highest viable and total cell densities, relative to the positive control, were further expanded using 125-mL shaker flasks with approximately 12−15 mL culture volume. The 125-mL shaker flasks were scaled to 250-mL shaker flasks containing 40−50 mL culture volume. The scale-up process was continued through the use of flasks (e.g., 100 mL, 500 mL, 1 L, to 3 L; Belco Glass) depending on the required material needed for bioreactor inoculation (e.g., 5-L glass versus 150-L stainless steel vessel). Unless otherwise indicated, the output from the proliferation assay was always evaluated on day 3 post-inoculation.

Results

Proliferation Assay. To assess the proliferative activity of growth factors without the ambiguities associated with the previous culture medium, we developed an assay that called for a brief growth factor "starvation" period to precede component addition. Suspension CHO DG44 cells growing in medium X were harvested during exponential growth, when the cell density reached approximately 1×10^6/mL, washed once in prewarmed DMEM/F12 basal medium devoid of growth factors, and then resuspended in the same medium at a concentration of approximately 4×10^5/mL. The culture was allowed to incubation for 20 h in a spinner flask. Following this starvation phase, the cells were washed once in prewarmed basal medium, and then 2 mL was plated into a six-well at a concentration of 4×10^5 viable cells/mL. Each well was then supplemented with 2 mL of prewarmed basal medium containing twice the final component concentration, and the growth of the culture was monitored over the next 3 days. A representative output from this assay is found in Figure 1 where the time courses of the positive (medium X) and negative (DMEM/F12 basal) controls are shown. The data indicate that the no spurious growth-promoting activity is associated with the negative control. In the remaining experiments, we adopted a single 3-day time point representation of the data for comparative simplicity.

Growth Factor Screening and Normal Probability Plot Representation. IGF-1 or insulin alone

Table 1. (A) Full Factorial Design Matrix for the Four Growth Factors Screened; (B) Output from Proliferation Assay[a]

	A				B
expt no.	1 transferrin (5 mg/L)	2 recomb insulin (5 mg/L)	3 IGF-1 (50 μg/L)	4 bFGF (2.5 μg/L)	DG44 viable cell density (× 10^{-5} cells/mL)
1	−	−	−	−	1.64
2	+	−	−	−	1.30
3	−	+	−	−	9.91
4	+	+	−	−	9.38
5	−	−	+	−	12.64
6	+	−	+	−	13.59
7	−	+	+	−	11.80
8	+	+	+	−	11.01
9	−	−	−	+	2.85
10	+	−	−	+	2.58
11	−	+	−	+	9.14
12	+	+	−	+	10.92
13	−	−	+	+	11.32
14	+	−	+	+	11.80
15	−	+	+	+	14.62
16	+	+	+	+	13.79
positive					22.87

[a] Cells were counted on day three post-inoculation

was found to be sufficient to promote sustained proliferation of our in-house CHO cell line in a minimal basal medium. Proliferation assays were used to screen a panel of growth factors including insulin, IGF-1, transferrin and basic fibroblast growth factor using a full factorial experimental design. These growth factors were chosen as a result of their documented roles in both media design and cell cycle regulation (*5*−*8*). Table 1 part A shows the design matrix for our four factor factorial design experiment. Also shown are the raw viability data obtained as the output from the assay (part B). Compared to the negative control (experiment 1), the results indicate that experiments 3−8 and 11−16 exhibit good proliferation. On the basis of the factors present in these wells, the results indicate that either IGF-1 or insulin alone greatly stimulate CHO cell proliferation in our minimal basal medium. These results are consistent with those of a numbers of laboratories where it has been reported that IGF-1 (*5*, *9*, *10*) and insulin (*5*) are potent CHO growth and survival factors. The results also show that no combination of growth factors with our basal media yields more cells than our positive control, which highlights the "lean" nature of our basal formulation.

Normal probability chart representations of the results confirm the potent effect of IGF-1 and insulin on our CHO cell line. A shortcoming associated with the use of factorial design techniques without replicates is the possibility of data corruption. While in theory it is possible to include sample replicates, as the number of factors grows practical considerations limit this option. To address this issue, we adopted the use of normal probability charts for the analysis of effects. The reader to referred to various texts on the subject of effects computation (*11*) and on the use of normal probability charts in development (*11*−*13*). Briefly, the theory behind the use of normal probability charts is that random effects can be expected to cluster along a line when plotted on normal probability paper. Effects of significant magnitude can be expected to fall off the "line of chance." Such a plot is constructed by plotting the magnitude of the effects on the abscissa versus the cumulative probability function (see legend). Figure 2 shows a graphical representation of the effects including main, two-factor, three-factor and four-factor interactions (Table 2) as calculated from the data in Table 1 column B, when plotted on normal probability paper. Our results show that three effects fall significantly off the "line of chance." Two of the effects are main effects and correspond to insulin and IGF-1. The third effect is the two-factor interaction term characterizing the insulin−IGF-1 interaction. The data representing the insulin and IGF-1 effects lie in the upper right-hand corner of the graph and are associated with large positive values and provide "statistical" confirmation for our earlier conclusions. The two-factor interaction term lies in the lower left-hand corner of the graph and is characterized by a large negative value. The simplest interpretation of this result is that insulin and IGF-1 do not act independently on our CHO cell cultures. Other possible interpretations of this value are addressed in the discussion.

Adaptation through Outgrowth. The successful adaptation of a cell line to a particular medium is generally characterized by the cells' ability to be repeatedly grown in such a medium. If a cell line does not meet this criterion, then the behavior of the cells reflects, in part, the cells' previous history. To address the possibility that the proliferation exhibited by our CHO cultures in six-well plates represented a transient artifact, we assessed whether the culture identified by experimental condition 5 (i.e., the IGF-1 culture) was capable of

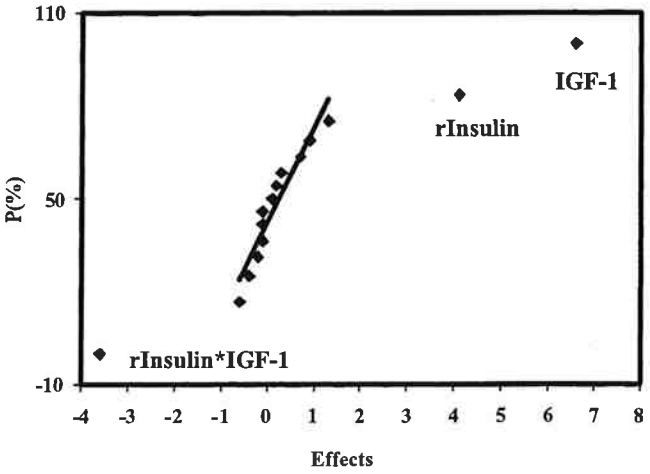

Figure 2. Normal probability chart representation of the effects obtained from our growth factor screen (*11*). The data points plotted are identical to those shown in Table 2 and were obtained using our parental DG44 CHO cell line. As discussed in the text, insulin and IGF-1 represent the significant single factor effects and the only significant multifactor effect is the insulin−IGF-1 pair.

Table 2. Ordering of Effects for Normal Probability Chart Presentation[a]

	I														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
P [$= 100(I - 0.5)/15$]	3.3	10.0	16.7	23.3	30.0	36.7	43.3	50.0	56.7	63.3	70.0	76.7	83.3	90.0	96.7
identity of effects	23	123	134	1234	12	13	34	1	14	124	4	24	234	2	3
effect (estimate)	−3.64	−0.61	−0.36	−0.23	−0.15	−0.10	−0.10	0.06	0.24	0.34	0.72	0.87	1.30	4.11	6.61

[a] The bottom row represents the calculated effects listed from lowest to highest into magnitude where each effect is identified by a number code (third row). Main effects are identified by single digits (e.g., 1 = transferrin, 2 = recombinant insulin, 3 = IGF-1 and 4 = bFGF), and two- and three-factor interaction effects are identified by the concatenation of their respective single components (e.g., 134 denotes the three-factor interaction between transferrin, IGF-1 and bFGF). The second row represents the cumulative probability for each of the newly ordered effects, which was plotted against the effects to construct our normal plot.

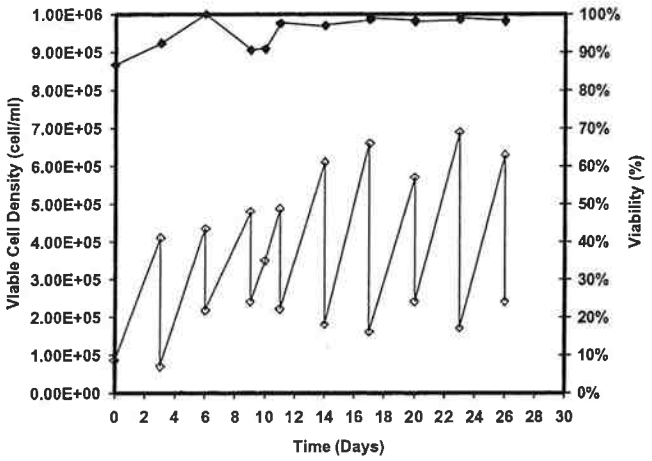

Figure 3. Parental DG44 CHO time course viability (open symbol) and viable cell number (closed symbol) profiles upon repeated passages and scale-up following its initial adaptive outgrowth in a six-well plate. Following six-well static growth, the culture was transferred to suspension growth in spinner flasks of various sizes.

sustained proliferation following its initial outgrowth in our growth assay. IGF-1 was chosen over insulin because of internal raw materials considerations. At the conclusion of our proliferation assay, CHO cells growing in our DMEM/F12 base supplemented with IGF-1 were transferred to a shaker flask (passage 2; Figure 3). Three days later, the culture was transferred to a spinner flask where it could be repeatedly subcultured (Figure 3). On the basis of the consistently high cell viability and the cells ability to continuously grow in our basal formulation, we conclude that the cells have been rapidly adapted to growth in our basal medium supplemented with IGF-1.

IGF-1 Promotes Parental Cell Growth in Various Production Media. From an applications viewpoint, we were interested in knowing whether our growth factor requirement information could be applied to identify a robust medium formulation for manufacturing purposes. To address this question, we screened our parental CHO cell line for growth using a panel of commercial and proprietary serum-free media formulations with and without the presence of IGF-1. Similar to previous experiments, CHO DG44 cells growing in medium X in suspension culture were starved for 24 h, washed, and resuspended in various test media. The results of this assay are shown in Figure 4. The data indicate that the performance of our cell line in many of the media (B, E, F and ABX-CHO1) is greatly enhanced by the addition of IGF-1. The results also suggest that many of the media (A, C and D) possess components that obviate the beneficial effects of IGF-1 supplementation. A simple explanation is that these media contain insulin or IGF-1.

IGF-1 Facilitates Adaptation and Scale-Up of Recombinant CHO Cultures. IGF-1 potentiates the rapid adaptation of our recombinant culture growing in medium E. On the basis of internal manufacturing considerations medium E was selected as our manufacturing medium for recombinant protein production. A growth factor screen similar to that described in Table 1 was performed using medium E as our basal formulation. The resulted indicated that the dependence of our recombinant culture on IGF-1 was sustained (data not shown). Similar to our parental cell culture, we adopted an adaptation through outgrowth approach to accelerate scale-up and stability studies. The results of continuously passaging our recombinant CHO cell line in medium E supplemented with IGF-1 following transfer from medium X containing 5% serum are shown in Figure 5A. Also shown are the associated cell viability and specific productivity data as determined using 1-day differential measurements (Figure 5B; see Methods). The results indicate that our recombinant cell line can be easily transfered/adapted to our production medium in the presence of IGF-1. Additionally the rapid adaptation of the culture to the final production media also permitted an early initiation of stability studies. By the time the culture was expanded to provide an innoculum for our first 100-L fermentation, somewhere around passage 10–11, stability information had already been attained. It was also possible to integrate cell banking into the adaptation and outgrowth process because cells banked at passage 5 could be successfully revived (data not shown).

Discussion

We have described a simple approach to screening growth factors that has allowed us to accelerate development timelines via a rapid adaptation process. The approach combined elements of statistical experimental design with growth factor biology to identify potent growth factor responsive in our in-house CHO cell lines. The use of normal probability plots confirmed the experimental results in a statistical sense when no replicates were available and provided a simple means of analyzing and prioritizing the consequences of our experimental results. The finding that IGF-1 was the critical growth factor is not surprising as ample evident exists in the literature (*5*, *9*, *10*). Pietrzkowski (*9*) first reported on the genetic engineering of the CHO autocrine IGF-1/IGF-1R loop, and this work has recently been extended (*10*). The fact that our approach could be applied to recombinant CHO cell cultures suggests that it represents a plausible path in which to initiate early process development.

A full factorial experimental design allowed for a more thorough analysis of the phenomena at hand. Specifically, the redundancy in the data set with respect to calculating the main effects allowed us to calculate many interaction

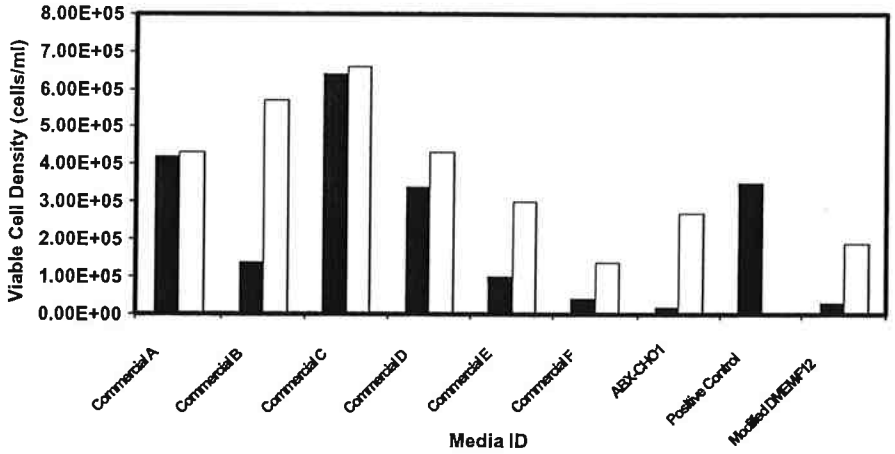

Figure 4. CHO media screen results obtained using the parental DG44 CHO cell line in the presence (open symbol) or absence (closed symbol) of IGF-1. Cells were transfered from medium X to the respective media as described in the text.

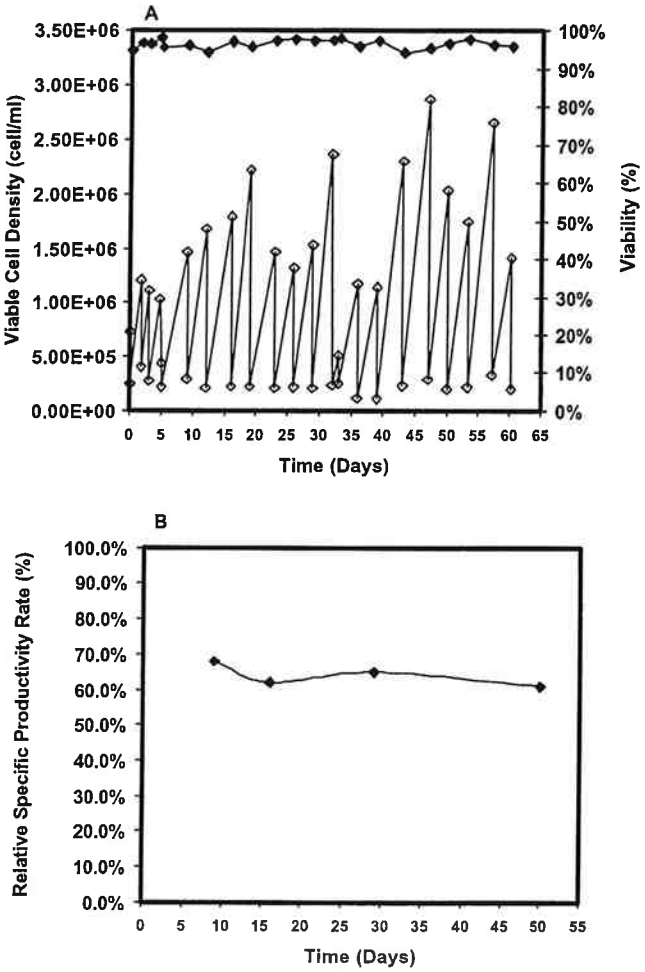

Figure 5. (A) Time course viability (open symbol) and viable cell density (closed symbol) of a recombinant CHO DG44 following its adaptive outgrowth in medium E supplemented with IGF-1. (B) Relative specific productivity of cells as monitored during the course of its adaptive outgrowth (100% is defined as the SPR for the recombinant DG44 CHO growing in medium X supplemented with 5% FBS).

effects that would not have been possible had a fractional factorial design approach been applied. These results (Table 2) when plotted on normal probability charts allow us to identify at least two "classes" of interactions. The first class represents interactions whose magnitudes are indistinguishable from "noise" and are identified by their clustering along the "noise" line on normal probability charts. Also embedded in this locus of "insignificance" are the magnitude of the effects from transferrin and bFGF. Surprisingly, we saw no significant effect from these components. Since bFGF is believed to play a role in the G0-G1 transition, via the upregulation of IGF-1R (*14*, *15*), a simple explanation for this observation are that our cells never re-enter G0. Such a phenomenon is not uncommon for cells in culture, particularly anchorage-dependent cells that have been "adapted" for growth in suspension. Alternatively, our cells might already possess sufficient IGF-1R. The latter explanation is consistent with IGF-1R receptor numbers on DG44 CHO cells (*16*). It is also possible that the cells express bFGF through autocrine loops as it has been reported that many cell cultures appear to express bFGF (*17*). The finding that transferrin exhibited no positive effect on growth in our lean basal medium might simply reflect the fact that iron metabolism is not limiting under the circumstances. Apparently the trace amount of iron in DMEM-F12 and presumably in yeast extract is sufficient to promote proliferation under these assay conditions. In more complete or robust media, we have found a beneficial effect of transferrin in this assay format (data not shown).

The second class of interaction effects represents those that fall off the "line of chance". Specifically the insulin–IGF-1 interaction term was found have a large negative value. The simple mathematical interpretation of the large "magnitude" exhibited by the insulin–IGF-1 interaction is that both components exert their influence in a dependent manner. However, the negative sign associated with the effect can lead to several interpretations. One might be tempted to conclude that the IGF-1–insulin pair exhibits competitive dependence. This interpretation agrees well with what has been reported about the insulin–IGF-1 signaling system in CHO cells. Specifically, evidence exists indicating that insulin may act through the IGF-1 receptor in CHO cells if present in high concentration (*6*). More recently researchers have found that CHO DG44 does not contain detectable levels of the insulin receptor, providing further support for the conclusion that insulin's action is mediated through the IGF-1 receptor (*16*). However this interpretation need not hold if "optimal" concentrations of insulin and/or IGF-1 were used. Under these circumstances, any increase in concentration would lead to a smaller response, if not a decrease, which would also be represented mathematically by the presence of a negative interaction term. Insights into which of these possibilities reflect reality require detailed dose–response titration curves of the

components of interest. Had the sign on this interaction effect been positive, synergy as opposed to competition would be implied. Interestingly, our results indicate that no other dependencies, as would be reflected by significant interaction effects, influence the behavior of our system. In view of the fact that only insulin and IGF-1 registered any significant component effect, these results are not surprising.

We believe that our approach to integrating component screening and adaptation offers great advantageous when adaptation to a different production medium is desired. The assay format allows for numerous components and combinations of components to be assayed, while possessing an inherent selection mechanism against unsuccessful screens. The end result is that negative results are identified early in the screen so that resources are not wasted on futile efforts. These attributes are particularly relevant when compared to the weaning approach to serum-free media adaptation. This process is generally characterized by continuously passaging cells in media of decreasing concentration of the undesired component, typically serum. Such experiments often require weeks before they can be scored. An obvious shortcoming of our approach is that we cannot rule out the possibility that the early passages of the cultures are artifacts or whether relative rank effects are preserved between conditions following subsequent passages. In the case of growth factor identification for screening purposes, we believe that our starvation step is sufficient since the control data indicate that proliferation has ceased by the time of growth factor addition. With respect to preserving rank relations among the conditions, the fact that the culture appears to perform better in medium E following subsequent passages (Figure 5A and unpublished results) indicates that rank relationships should not be expected to hold when comparing "positives". However, in all cases examined, the growth factor requirement information was conserved. In contrast to growth factors, when screening non-growth factor media components, it is possible that intracellular stores might be sufficient to promote proliferation in the early passages. To eliminate such uncertainties altogether, it would be necessary to continue to passage the cultures of concern. In microbiology, it is common to grow cells for five to seven divisions to eliminate "carry over" effects through the dilution of intracellular components. Our process can accommodate such cell generation requirements because the volume of culture that we start with is small, providing ample opportunity for culture expansion.

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References and Notes

(1) Sinacore, M. S.; Charlesbois, T. S.; Brennan, S.; Richards, T.; Hamilton, M.; Scott, S.; Brodeur, S.; Oakes, P.; Leonard, M.; Switzer, M.; Anagnostopoulos, A.; Foster, B.; Harris, A.; Jankowski, M.; Bond, M.; Martin, S.; Adamson, S. R. CHO DUKX cell lineages preadapted to growth in serum-free suspension culture enable rapid development of cell culture procsses for the manufacture of recombinant proteins. *Biotechnol. Bioeng.* **1996**, *52*, 518–528.

(2) Reddy, P.; Rasmussen, B.; Davis, R.; Aldrich, T.; Morris, A. Altered growth factor signaling involved in serum-free adaptation of CHO cells. In *Cell Culture Engineering VIII*, Snowmass, CO; United Engineering Foundation, Inc., 2002.

(3) Barnes, D.; Sato G. H. Methods for growth of cultured cells in serum-free media. *Anal. Biochem.* **1980**, *102*, 255–270.

(4) Sambrook, J., Fritsch, E. F., Maniatis, T., Eds.; *Molecular Cloning: A Laboratory Manual*, 2nd ed; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(5) Fung, V. P., Ed. *Replacement of Bovine Insulin with Recombinant LongR3 IGF-I in CHO Cells. Animal Cell Technology: Basic and Applied Aspects*; Kobayashi, T., Ed.; Kluwer Academic Publishers: Netherlands, 1994; Vol. 6, pp 337–343.

(6) van Obberghen-schilling, E.; Pouyssegur, J. Mitogen-potentiating action and binding characterisitcs in insulin and insulin-like growth factors in Chinese hampster fibroblasts. *Exp. Cell Res.* **1983**, *147*, 369–378.

(7) Armelin, H. A. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*(9), 2702–2706.

(8) Jiang, F.; Frederick, T. J.; Wood, T. L. IGF-I synergies with FGF-2 to stimulate oligodendrocyte progenitor entry into cell cycle. *Dev. Biol.* **2001**, *232*, 414–423.

(9) Pietrzkowski, Z.; Lammers, R.; Carpenter, G.; Soderquist, A. M.; Limardo, M.; Phillips, P. D.; Ullrich, A.; Baserga, R. Constitutive expression of insulin-like growth factor 1 and insulin-like growth factor receptor abrogates all requirements for exogenous growth factors. *Cell Growth Differ.* **1992**, *3*, 199–205.

(10) Sunstrom, N.-A.; Gay, R. D.; Wong, D. C.; Kitchen, N. A.; DeBoer, L.; Gray, P. P. Insulin-like growth factor-I and transferrin mediate growth and survival of chinese hampster ovary cells. *Biotechnol. Prog.* **2000**, *16*, 698–702.

(11) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building*; John Wiley and Sons: New York, 1978.

(12) Daniel, C. *Applications of Statistics to Industrial Experimentation*; John Wiley & Sons: New York, 1976.

(13) Daniel, C. Use of half-normal plots in interpreting factorial two-level experiments. *Technometrics* **1959**, *1*(4), 311–341.

(14) Hernandez-Sanchez, C.; Werner, H.; Roberts, C. T., Jr.; Woo, E. J.; Hum, D. W.; Rosenthal, S. M.; LeRoith, D. Differential regulation of insulin-like growth factor-I (IGF-I) receptor gene expression by IGF-I and basic fibroblastic growth factor. *J. Biol. Chem.* **1997**, *272*(8), 4663–4670.

(15) Scheidegger, K. J.; Du, J.; Delafontaine, P. Distinct and common pathways in the regulation of insulin-like growth factor-1 receptor expression by angiotensin II and basic fibroblast growth factor. *J. Biol. Chem.* **1999**, *274*(6), 3522–3530.

(16) Yandell, C. A.; Wade; Sheehan, A. J.; Simula, A. P.; Goddard, C. Receptor expression and effects of insulin and LongR3 insulin-like growth factor-I on chinese hampster ovary cells. In *Cell Culture Engineering VIII*, Snowmass, CO; United Engineering Foundation, Inc., 2002.

(17) Rifkin, D. B.; Moscatelli, David Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* **1989**, *109*, 1–6.

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