Active hypothermic growth: a novel means for increasing total recombinant protein production by CHO cells

by

Stephen Richard Fox

M.S.C.E.P., Massachusetts Institute of Technology, Cambridge, MA, 2003
B.S. Chemical Engineering, Carnegie Mellon University, Pittsburgh, PA, 1998

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Author: _____
Department of Chemical Engineering
April 2005

Certified by: _____
Daniel I. C. Wang
Institute Professor
Thesis Supervisor

Accepted by: _____
Daniel Blankschtein
Professor of Chemical Engineering
Chairman, Committee for Graduate Students

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ABSTRACT

Recombinant human glycoproteins produced by Chinese Hamster Ovary (CHO) cells are an important class of therapeutic molecules and investigating means of improving the production rate and product quality of these glycoproteins is therefore of great interest. Culturing CHO cells under mild hypothermia (30-33 °C) leads to growth arrest in the G_0/G_1 phase of the cell cycle and, in some cases, causes an increase in specific productivity of recombinant protein, as was shown here for the model CHO cell line producing human interferon-gamma (IFN-γ).

Controlled proliferation, achieved by inducing growth arrest in the G_0/G_1 phase by chemical, environmental or genetic means, is commonly used to increase CHO specific productivity and thus there is speculation that enhanced hypothermic productivity is due to growth arrest. However, it was proven here that the positive effect of hypothermia on recombinant protein production is due to elevated IFN-γ mRNA levels instead. At both 32 °C and 37 °C, specific productivity is growth-associated, increasing as the percentage of cells in the S phase increased, demonstrating that a cell line can be both a growth-associated producer and have enhanced productivity under hypothermic conditions.

It was hypothesized that the best production platform would be cells actively growing at low temperature and this was proven to be the case using two different methods, namely growth factor supplementation and selection of cells capable of hypothermic growth. Both methods gave multi-fold increases in total IFN-γ production compared to the 32 °C and 37 °C controls, thereby validating the novel culture strategy of active hypothermic growth. Cells capable of achieving significant hypothermic growth were also isolated for the non-recombinant CHO-K1 cell line and are now available for the future production of any recombinant protein.

Glycoprotein quality is partially assessed by the level of glycosylation and IFN-γ contains two potential N-linked glycosylation sites. This thesis gives the first report of a detrimental effect of hypothermic culture on glycosylation, showing a 4-5% decrease in the end-of-batch percentage of 2-sites occupied glycoforms relative to the 37 °C control. However, this negative effect is completely eliminated by culturing under perfusion conditions.

Thesis Supervisor: Daniel I. C. Wang
Title: Institute Professor

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1. INTRODUCTION

1.1 BACKGROUND

Researchers have long realized that cells must be able to quickly adapt to sudden decreases in temperature. For example, bacteria residing in the intestines of animals living in cold environments confront sudden and drastic temperature downshift following excretion (Thieringer et al, 1998). Some mammals have also adapted to deal with low temperatures, as evidenced by adaptive hypothermia, or hibernation. *Cricetus griseus*, more commonly known as the Chinese hamster, is native to the cold deserts of Northern China and Mongolia and is one such hibernating animal. Hibernation occurs when the environmental temperature falls below 5 ^{o}C (ProVet, 2003). The hamster's metabolic rate falls dramatically, resulting in as few as one heart beat every few minutes and slow breathing. During hibernation, the hamster's body temperature drops to just 1-2 ^{o}C above the environmental temperature. They do not eat for weeks and unsuspecting owners may think their hamster is "dead", which unfortunately results in pet Chinese hamsters being discarded during hibernation, or worse, being buried alive! Pet hamsters undergoing hibernation can be revived by placing on a warming pad set to 30 ^{o}C for 30-60 minutes, but should **never** be warmed in cooking ovens or microwaves (ProVet, 2003).

The production of recombinant human glycoproteins is routinely carried out using the Chinese Hamster Ovary (CHO) cell line. Although bacterial and yeast cell lines are capable of producing much larger quantities of recombinant proteins, only mammalian cells are capable of correctly carrying out complex post-translational modifications such as proper folding and glycosylation, which has been correlated with specific activity and circulatory half life of therapeutic glycoproteins (Goochee et al, 1991; Kaufman, 1989; Kaufman et al, 1988). CHO cells have emerged as the mammalian cell line of choice for industrial-scale production of many recombinant proteins owing to their ability to perform post-translational modifications, their high

stability of chromosomally integrated heterologous transgenes (Schimke, 1984), their relative ease of adaptation to protein-free suspension culture (Meents et al, 2002) and the availability of dihydrofolate reductase mutants (DHFR-) that enable amplification of recombinant product-encoding regions of chromosome (Kaufman et al, 1983; Urlaub and Chasin, 1980) and subsequent high expression levels.

Animal cell culture is typically conducted at human physiological temperature, namely 37 °C, long viewed as a constant physical parameter by the bioengineering community (Fussenegger and Bailey, 1999). However, given the fact that the original animal source of the cell line is a hibernator, CHO cells should be quite capable of handling low temperatures and one may speculate there would be bioprocess benefits to growing under hypothermic conditions. This speculation has borne fruit, as within the last five years, several research groups have found that certain CHO cell lines are capable of improved productivity at low temperature, prompting interest in further understanding and optimizing hypothermic culture.

1.2 MOTIVATION

In the next few years, the number of FDA-approved protein pharmaceutical products is expected to increase substantially, with many products currently under development or in the clinical trial phase (Kretzmer, 2002). Production of these products will become a major bottleneck for bringing them to market, as current manufacturing capacity will not be sufficient. Bringing these products to the consumer will be delayed, as new and costly facilities will need to be constructed or existing production facilities will need to be revamped for higher productivity. Thus, any operational improvements to the cell culture process that cause significant increases in productivity without compromising product quality will have a positive impact on meeting the

upcoming manufacturing challenge and providing humans with sufficient amounts of medicine in the shortest time frame.

1.3 THESIS OBJECTIVES

The central goal of this thesis was to study and optimize the application of mild hypothermia for recombinant protein production. The model system used was a CHO cell line, referred to as CHO-γ, producing recombinant human interferon-gamma (IFN-γ). Within this central goal, the thesis has four major objectives. First, demonstrate that the CHO-γ line has enhanced productivity at low temperature under a variety of culture conditions. Second, determine the basis for the hypothermic enhancement in productivity. Specifically, it is speculated in the literature that growth arrest is the cause of the enhanced productivity during hypothermia and this claim needed to be verified or refuted before determining how best to optimize hypothermic culture. Third, verify whether active hypothermic growth, a novel culture strategy, can be implemented by either growth factor supplementation or mutant cell line generation and whether it causes the hypothesized increase in total IFN-γ production. Fourth, determine whether hypothermic culture has a significant impact on IFN-γ glycosylation.

1.4 THESIS ORGANIZATION

This thesis is divided into ten chapters. Chapter 2 provides a review of the literature on hypothermia and other topics pertinent to the thesis. In Chapter 3, the materials and methods used are given in detail. Chapter 4 demonstrates the effect of mild hypothermia on growth and productivity in batch and fed-batch cultures of the CHO-γ cell line. In Chapter 5, a model is proposed for optimizing the trade-off between high growth and low productivity at standard

culture temperature (37 °C) and low growth and high productivity at low temperature (32 °C) by using a temperature shift strategy. Chapter 6 deals with the mechanistic effect of low temperature by studying the combined effect of hypothermia and growth arrest on IFN-γ mRNA levels. The results of Chapter 6 form the basis for the work of Chapter 7, which considers the effects of growth factors on hypothermic culture and demonstrates that the most suitable production platform for maximizing total IFN-γ production is active growth at low temperature. These findings formed the motivation to produce a CHO-γ cell capable of active hypothermic growth without the use of growth factors, and this work is discussed in Chapter 8. Also in Chapter 8, the development of a non-recombinant CHO-K1 cell capable of active hypothermic growth and envisioned as a generic production platform for enhanced productivity of any recombinant protein, is discussed. Chapter 9 covers the effect of low temperature on IFN-γ glycosylation, a key indicator of recombinant protein product quality. Chapter 10 presents concluding remarks and recommendations for future research in the hypothermic cell culture field. A nomenclature section and a list of the cited references follow chapter 10.

2. LITERATURE REVIEW

The response of cells to hyperthermia, or heat shock, is a well-studied phenomenon. From bacteria to mammals, an increase in temperature results in a heat shock response, characterized by the synthesis of a set of well-conserved heat shock proteins (HSP). These proteins have been identified as molecular chaperones, responsible for assisting protein folding and enhancing protein stability, two crucial requirements for maintaining protein function under elevated temperatures (Thieringer et al, 1998). In contrast, the response of cells to hypothermia, or cold shock, is not well characterized at the molecular level. The scant evidence available suggests that, much like the heat shock response, there is a conserved cold shock response that involves the active response of the cell by synthesizing a set of cold shock proteins (CSP). The known elements of the cold shock response will be considered in more detail later.

Although little is known about the molecular level response of cells to hypothermia, it has been known for many years that the growth rate of mammalian cells decreases as temperature is lowered from the conventional cultivation temperature of 37 °C (Rao and Engelberg, 1965; Watanabe and Okada, 1967). Cell cycle distribution analysis has shown that by and large, the G_0/G_1 phase of the cell cycle is most affected by low temperature and cells exposed to mild hypothermic conditions for a long enough period of time will tend to arrest in this phase (Fujita, 1999). This is the case for Chinese hamster cells, at least within the mild hypothermic range of 25-32 °C (Moore et al, 1997; Shapiro and Lubennikova, 1968). Within the 6-15 °C range, Chinese hamster cells arrest during mitosis instead (Shapiro and Lubennikova, 1968). However, because active metabolism such as ATP production and macromolecular synthesis is not observed at such extreme hypothermic conditions (Fujita, 1999), these very low

temperatures probably have limited application for mammalian cell culture and were not considered in this thesis.

Given that growth arrest is a hallmark of the mammalian hypothermic response, and that cells can actively induce growth arrest via various cell cycle controls, it is appropriate to review the regulation of the cell cycle and some of the key molecules involved, prior to considering the cold shock response.

2.1 THE CELL CYCLE: AN OVERVIEW

Progression through the various stages of the cell cycle is a tightly controlled, complex event meant to ensure that cells do not grow and divide when conditions are unfavorable. Given the growth-arresting effect of hypothermia, a review of the cycle and its control mechanisms is pertinent here. This review is not comprehensive, but provides an overview of cycle progression and control and also introduces some of the key molecular players involved, several of which will be discussed in the context of hypothermia as well as biotechnology applications later.

The cell cycle is divided into four phases, one associated with DNA synthesis (S Phase), one with mitosis (M Phase) and two gap phases, G_1 and G_2, during which time cells prepare for S and M Phase, respectively (Alberts et al, 1994). The drivers of the cell cycle are protein complexes that are activated and trigger the initiation of key events, such as DNA replication, nuclear envelope breakdown, spindle formation and chromosome segregation. These protein complexes are subjected to tight control via signaling cascade pathways, ensuring that events occur in the proper order. The transduction pathways are initiated by extracellular signals. For example, mitogenic growth factors bind their specific receptors and initiate a cascade of events. This cascade will lead to the production of holoenzymes composed of cyclin and a catalytic

subunit called cyclin dependent kinase (CDK) (Hartwell and Weinert, 1989). These kinase enzymes are responsible for phosphorylation reactions, which in turn lead to gene expression and the completion of various cell cycle tasks, such as DNA replication. Several different cyclins and CDK exist in mammalian cells and owing to their critical role in the cell cycle, they are often referred to as the "cell cycle engine" (Graňa and Reddy, 1995). Figure 2-1 gives an overview of the points of control in the cell cycle and the specific cyclins/CDK complexes involved at each checkpoint. Note that major control occurs at the two most critical points: that of DNA synthesis (S) and cell division (M). Owing to the fact that CHO cells exhibit arrest in the G_0/G_1 phase of the cycle, the discussion here will center on the control events at this progression point. Figure 2-2 gives an overview of the signals and signaling pathways involved in the control at the G_1 to S progression point.

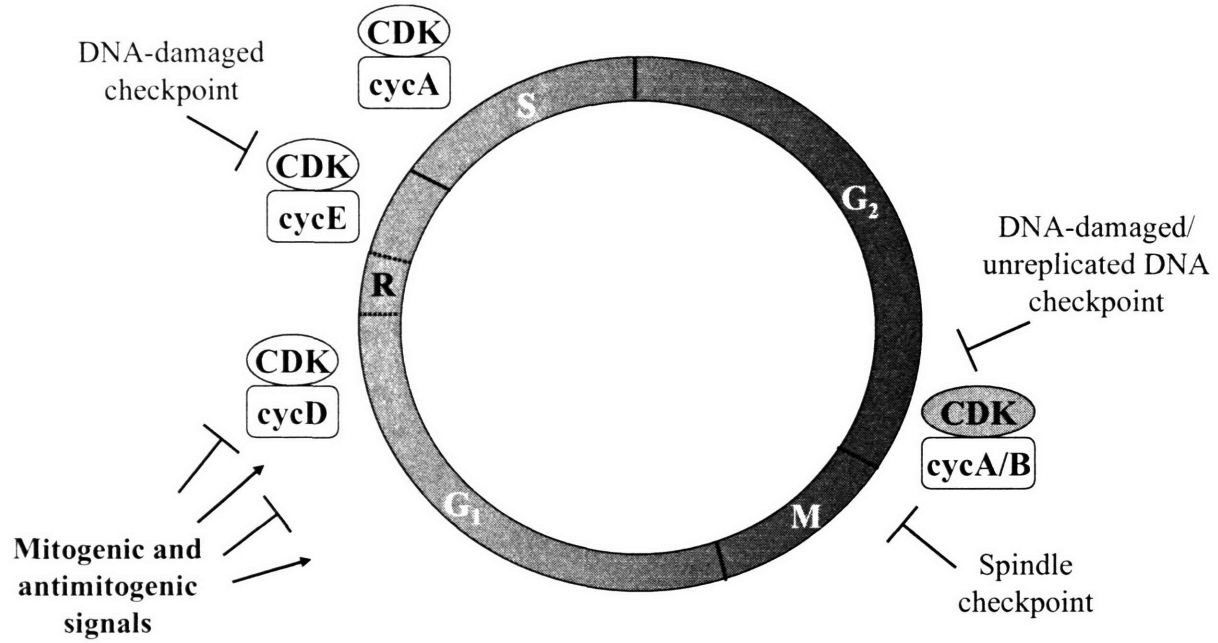

Figure 2-1. Cell cycle control and checkpoints. Progression through the cell cycle is mediated by cyclin/CDK complexes, which exert their positive effects at various checkpoints. Arrows represent activation and bars represent inhibition. R indicates a restriction point. Refer to the text for further details (adapted from Graña and Reddy, 1995).

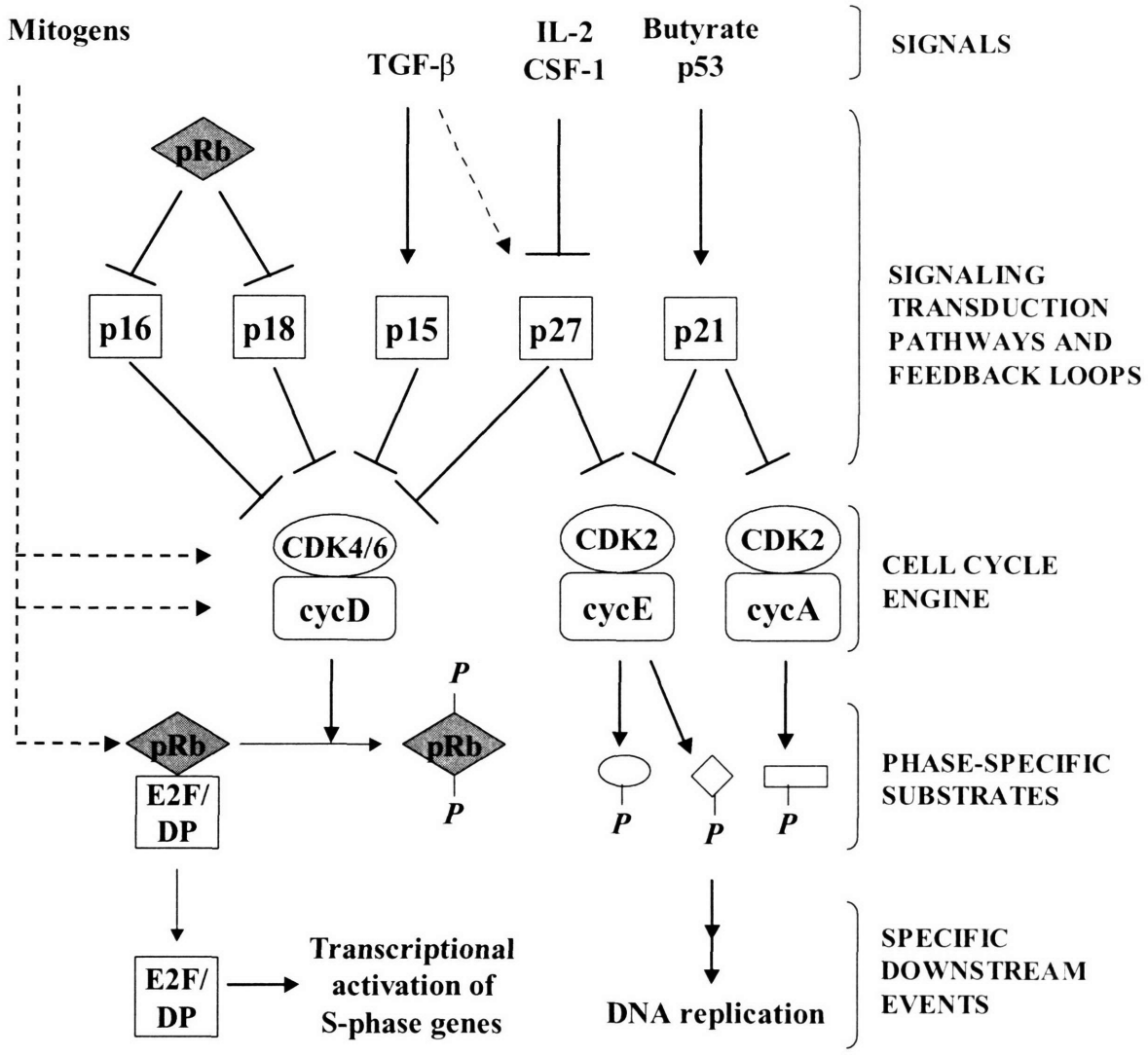

Figure 2-2. Control of the cell cycle engine at the G_1 to S phase transition. Positive and negative signals are propagated to the cyclin/CDK complexes, which then lead to phosphorylation of specific substrates and ultimately to the specific downstream events of the cycle. Signals listed are meant to serve as examples and are not a comprehensive set. Arrows represent activation and bars represent inhibition. Dashed lines represent hypothesized action. Refer to text for details (adapted from Graña and Reddy, 1995).

The signaling cascade begins with the input of extracellular signals, including growth promoters such as interleukin-2 (IL-2), colony stimulating factor-1 (CSF-1), and other mitogens, growth inhibitors such as transforming growth factor-β (TGF-β) and environmental signals such as γ-irradiation, which in turn activates the p53 protein (Graňa and Reddy, 1995). Different cell types require different combinations of growth factors. For example, T Lymphocytes can be induced to proliferate by antigen stimulation and IL-2 (Graňa and Reddy, 1995). Owing to the industrial relevance of CHO, the growth factor requirements of these cells in serum-free medium have been quite well studied. Insulin (Bailey et al, 2002; Sanfeliu et al, 2000), insulin-like growth factor (IGF-I) in combination with transferrin (Sunstrom et al, 2000) and basic fibroblast growth factor (bFGF) (Bailey et al, 2002) have all been shown to stimulate CHO proliferation. Growth factors are required for progression through the G_1 phase to a specific point called the restriction point (R; see Figure 2-1), after which point, growth factors are no longer necessary for cell cycle progression (Pardee, 1989).

Extracellular signals play a role in regulating the level of cyclins. For example, cyclin D is absent in quiescent cells and its expression is stimulated by growth factors. In the presence of growth factors, cyclin D levels are maintained quite constant throughout the cell cycle. In contrast, cyclin E levels oscillate periodically, with activity peaking in late G_1, followed by degradation of cyclin E once S phase begins, allowing CDK2 to associate with cyclin A (Graňa and Reddy, 1995). The levels of both of these G_1 cyclins are rate-limiting for the progression of cells through the cell cycle, as shown by the fact that ectopic expression of the cyclins shortens the length of the G_1 phase (Sherr, 1994). Over-expression of cyclin E in CHO cells was shown

to give the same morphology and active growth as control cells supplemented with bFGF, suggesting that the bFGF exerts its effect by controlling the level of cyclin E (Bailey et al, 2002).

In many cases, extracellular signals also exert their effect by changing the levels or activity of the cyclin-dependent kinase inhibitors (CKIs), examples of which are shown in Figure 2-2 (p15, p16, p18, p21, and p27). The first CKI identified, p21, was discovered as a protein that forms complexes with a cyclin/CDK pair (Xiong et al, 1992), preventing the CDK from carrying out downstream phosphorylation and thereby preventing DNA replication. This CKI is induced by p53 but also appears to be involved in p53-independent growth arrest (Halevy et al, 1995).

Another major CKI is p27, which has been shown to exert negative control on the activity of CDK2/CyclinE and CDK4/6/CyclinD (Graňa and Reddy, 1995). This CKI is present in quiescent cells, but addition of growth factor (e.g. IL-2 to T lymphocytes) results in a decrease in the p27 levels which eventually leads to the activation of the relevant CDK complexes (Firpo et al, 1994; Nourse et al, 1994). This CKI also plays a role in the growth-arresting effect of the potent growth inhibitor, TGF-β. In this case, TGF-β inhibits CDK4 synthesis, leading to release of p27 from CDK4/cycD complexes and the subsequent inhibition of CDK2/cycE complexes by p27 (Ewen et al, 1993). Other CKIs have been identified, such as p15, p16, and p18, which play similar roles as p21 and p27 (Graňa and Reddy, 1995). In summary, in the presence of negative extracellular signals, the CKIs have a vital role in maintaining cells in G_0/G_1.

When the CDK/cyclin complexes are present in an activated form, they propagate growth signals via phosphorylation of various substrates. Multiple proteins have been proposed as putative downstream targets of different cyclin/CDK holoenzymes. One target protein in particular, the retinoblastoma protein (pRb), has been well studied and will be used here to illustrate the elegant cell cycle control and feedback system. The proposed model for the

interaction of pRb with the cell cycle machinery is shown in Figure 2-2. In this model, hypophosphorylated pRb exists in quiescent or early G_1 cells. In this form, the pRb binds several other proteins, including E2F/DP, a transcription factor complex. As cells progress through G_1, CDK/cycD activity increases and acts to phosphorylate pRb. In the more phosphorylated form, pRb no longer binds E2F/DP, freeing the transcription factor to activate a variety of S Phase genes. In addition, the hypophosphorylated pRb is believed to act as a repressor of p16 and p18, resulting in CDK/cycD levels increasing (see Figure 2-2). Phosphorylation of pRb by the CDK/cycD complex eliminates pRb repression of p16 and p18, resulting in increased p16 and p18 levels, which in turn reduces CDK/cycD activity. Thus, the CDK/cycD activity is turned off after it has completed its function. Prior to the next pass through G_1, the pRb will become hypophosphorylated again (Graňa and Reddy, 1995).

In summary, progression through the mammalian cell cycle is a complex and tightly regulated process that takes into account both positive and negative environmental cues and translates these cues into signal transduction pathways, culminating in the expression of genes required for the specific events of each phase of the cycle. Within the framework of the events discussed here, mild hypothermia is a negative extracellular signal, causing the arrest of CHO in the G_0/G_1 phase. The mechanism and molecular players involved in the hypothermic response, and their link to the cell cycle controls in the case of mammalian cells, are discussed next.

2.2 CELLULAR RESPONSE TO HYPOTHERMIA

It has become increasingly clear that the observed effects of hypothermia are not simply a passive result of reduced kinetic rates but rather are the result of an active response on the part of the cell to the cold shock (Fujita, 1999). Given the growth-arresting effect of low temperature on

mammalian cells, the cold shock response must interact with the cell cycle machinery in some way and evidence supporting this link is beginning to emerge, as will be discussed later. First, it is pertinent to review the key aspects of the cold shock in microorganisms, because, as is the case with heat shock, evidence exists showing that cold shock is conserved at some level from bacteria to mammals.

2.2.1 Prokaryotic Cold Shock Response

Bacteria have shown a remarkable ability to adapt to various temperatures, existing in subfreezing to 110 °C environments (Thieringer et al, 1998). The most extensive cold shock research has been conducted with *Escherichia coli*, which belongs to the psychrotroph species, composed of bacteria that grow optimally between 15 and 40 °C (Thieringer et al, 1998). In *E. coli*, a reduction in temperature to the 8-15 °C range causes a transient inhibition of protein synthesis and growth lag, after which growth resumes (Thieringer et al, 1998), albeit at a lower rate. It is during the lag period that the cold shock proteins are produced, after which they exert their effect on the cell and growth resumes. The currently accepted model for the events in the *E. coli* cold shock response (Thieringer et al, 1998) is shown in Figure 2-3 and described here.

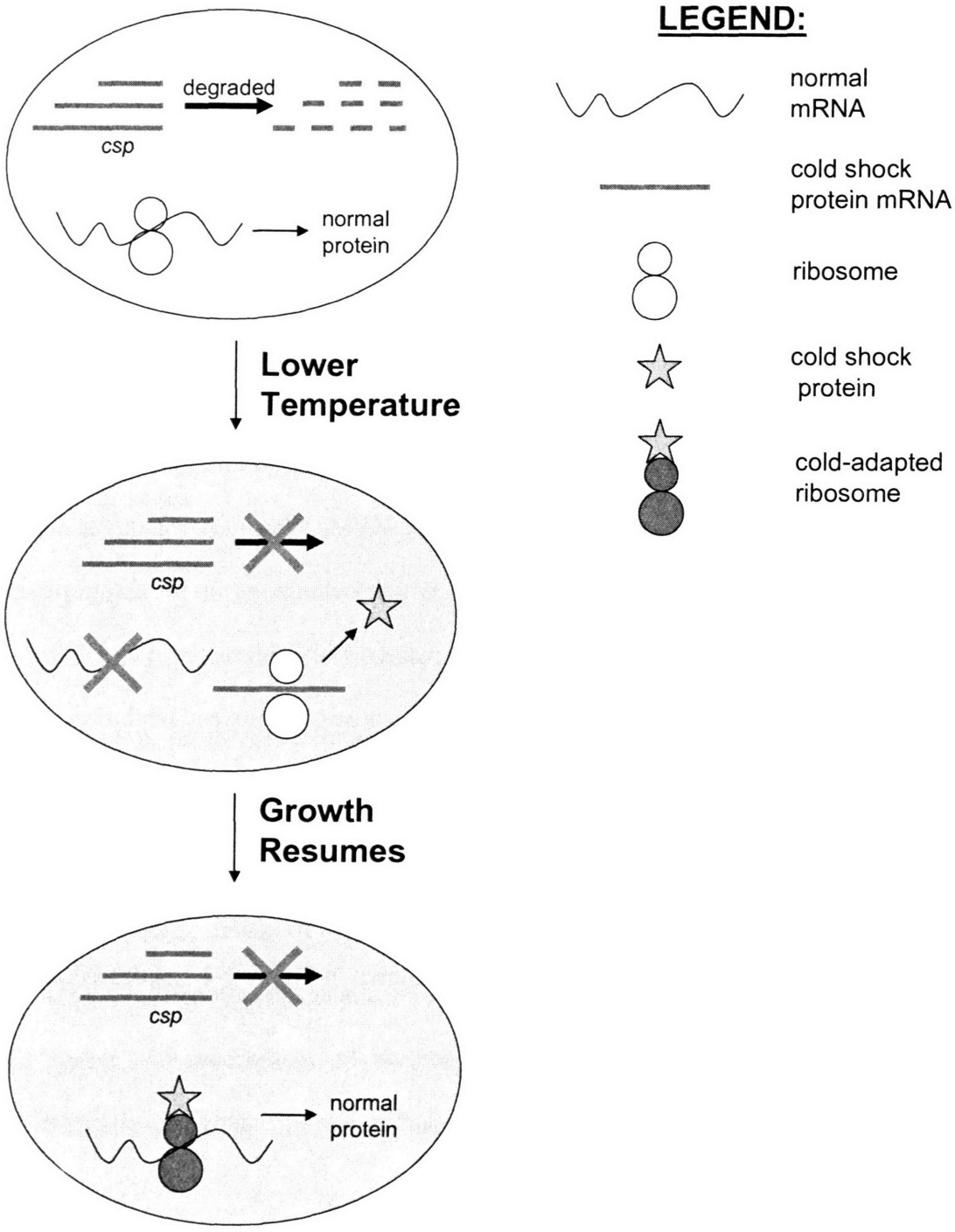

Figure 2-3. Model of the *E. Coli* Cold Shock Response. Under normal conditions, csp mRNA are rapidly degraded and no CSP are produced. Cold shock is sensed by the inactivation of ribosome function and consequent inhibition of protein synthesis and growth arrest. Simultaneously, csp mRNA stability is greatly enhanced. Only csp mRNA can still be translated by the inactivated ribosomes and CSP are synthesized. The CSP then stabilize the ribosome and normal protein synthesis and growth resumes. See text for details.

An abrupt decrease in temperature results in a temporary inhibition of protein synthesis (Jones and Inouye, 1996). The ribosome is composed of two subunits, termed 50S and 30S, which form the 70S ribosome. Several 70S ribosomes can bind to and translate a single mRNA and the resulting structure of multiple ribosomes and one mRNA is called a polyribosome or simply a polysome (Alberts et al, 1994). When *E. coli* are shifted from 37 °C to 5-15 °C, there is a transient decrease in the number of polysomes and a transient increase in the number of 70S monomers and 50S / 30S subunits (Broeze et al, 1978; Jones and Inouye, 1996), showing that polysome structure is altered by the low temperature. This is the reason that overall translational efficiency is greatly compromised.

In this state, only mRNA encoding csp genes can be translated by the 70S ribosome (Thieringer et al, 1998). This trait is due to the presence of an extra ribosome-binding site called the downstream box (DB) in the coding region of the csp mRNA (Mitta et al, 1997). These mRNA are constitutively expressed in *E. coli* at normal growth temperature, but are highly unstable, meaning that they are degraded rapidly and consequently there is negligible protein production. At low temperature however, the stability is greatly improved and the mRNA are translated. The precise mechanism for the enhancement of csp mRNA stability remains unknown, but because mRNA degradation in *E. coli* is tightly coupled with the ribosome, it is possible that the instability of the polysomes and ribosomes at low temperature plays a role in the dramatic increase in mRNA stability (Py et al, 1996). Other hypothesized reasons for the enhanced stability include transcriptional attenuation and cold-induced changes in csp mRNA secondary structure, rendering them less susceptible to RNase (Thieringer et al, 1998).

The enhanced stability and presence of the DB sequence in the csp mRNA results in production of CSP under cold shock. At least three of these proteins then interact with the

ribosomal subunits, converting the nontranslatable ribosome into a cold-resistant translatable state (Jones and Inouye, 1996), and allowing normal protein synthesis and cell growth to ensue. In addition to ribosomal binding function, other CSPs have been implicated in various DNA and RNA binding functions and thus may function as molecular chaperones for more efficient DNA replication, RNA transcription and mRNA translation (Jiang et al, 1997; Thieringer et al, 1998).

A second facet of the prokaryotic cold shock response is a change in the composition of the cell membrane, known as homeoviscous adaptation. Membranes are normally in a liquid crystalline form but will undergo a transition to a gel phase when temperature is reduced (Thieringer et al, 1998). It is critical for the cell to maintain proper membrane form, so in order to compensate for the effect of low temperature on membrane fluidity, the microorganisms will change the degree of saturation of the hydrocarbon chains of the phospholipids that make up the membrane. Phospholipids with unsaturated fatty acids have lower melting points and a higher degree of flexibility and thus are favorable at low temperatures (Thieringer et al, 1998). Increasing unsaturation is accomplished in several different ways, such as increasing synthesis and stability of membrane-bound desaturase enzymes (Fulco and Fujii, 1980) and increasing the activity of synthase enzymes responsible for synthesizing unsaturated fatty acids (Garwin and Cronan, 1980; Garwin et al, 1980).

2.2.2 Eukaryotic Cold Shock Response – Analogy with Prokaryotes

The eukaryotic cold shock response is far less understood than that for prokaryotes. However, the two characteristics of cold shock adaptation in bacteria, namely protein synthesis adaptation and homeoviscous adaptation, are present in eukaryotes as well. The disruption of protein synthesis at reduced temperature that was discussed previously for bacteria also occurs in

eukaryotes. For example, in a variety of plants, there is evidence showing changes in ribosomal proteins (Bixby and Brown, 1975), polysome quantity, polymerization and melting points (Laroche and Hopkins, 1987) and polysome translation efficiency (Johnson-Flanagan and Singh, 1987) occur at low temperatures. Likewise, hibernating mammals demonstrate moderate ribosome disaggregation (Frerichs et al, 1998; Knight et al, 2000).

As one known case of protein synthesis adaptation to counter the ribosome disruption, levels of elongation factor 1 (EF1), a protein involved in translation, have been shown to increase significantly in the liver of cold-adapted fish (Nielson et al, 1977). EF1 is believed to help the cells overcome a reduction in protein synthesis immediately following a shift to lower temperatures. Higher EF1 levels lead to higher rates of protein synthesis, suggesting that a new steady state level of enzymes may exist in response to cold shock (Nielson et al, 1977). Also, in the yeast *S. cerivisae* the cold-induced protein, NSR-1, has been shown to be involved in ribosome biogenesis (Kondo et al, 1992).

A recently identified mammalian cold-shock gene, RBM3, has been shown to have a cold-shock adaptation response with parallels to the prokaryotic csp genes. As discussed earlier, the prokaryotic csp genes contain DB sequences that allow for high-level translation during hypothermia. The 5' leader sequence of the RBM3 gene contains an internal ribosome entry site (IRES) that allows for cap-independent translation to occur. By unknown mechanisms, this IRES has up to 5-fold higher activity at 33 °C compared to 37 °C (Chappell et al, 2001). A striking feature of the RBM3 5' leader sequence is that it contains many complementary matches to 18S rRNA, leading to speculation that it plays a role in directly binding 40S ribosomal subunits by interacting with the 18S rRNA (Chappell et al, 2000). This suggests that internal

initiation of translation may be a key component of the cold shock response in mammals, allowing for high-level translation of certain proteins under hypothermic conditions.

The response of mammalian cells to extreme hypothermia appears to involve a near complete arrest of cellular activity, rather than adaptation for continued protein synthesis. For example, cells strongly suppress transcription (van Breukelen and Martin, 2002) but enhance mRNA stability (Knight et al, 2000), resulting in unchanged mRNA levels (Frerichs et al, 1998), and then also suppress protein synthesis (Frerichs et al, 1998; Knight et al, 2000). Even at the low temperatures of hibernation, suppressed protein synthesis is not merely a passive thermodynamic phenomenon, as seen by measuring synthesis rates in cell-free extracts from hibernating brain at 37 ^{o}C, which showed 3-fold reduction compared to extracts from non-hibernating brain (Frerichs et al, 1998). However, the fact that cell activity is negligible during extreme hypothermia means that this condition is probably not very useful for recombinant protein production, and therefore this thesis focused on mild hypothermia.

There are numerous examples to demonstrate that eukaryotic cells also undergo the second hallmark of the bacterial cold shock response, namely homeoviscous adaptation. For example, hibernating animals change their membrane composition in a variety of ways to control membrane fluidity (Aloia et al, 1974; Goldman, 1975). A striking example of the active nature of the cold response was demonstrated in carp fish, which show an eight to ten-fold increase in stearoyl coenzyme A desaturase activity after cold exposure (Tiku et al, 1996). Plants also exhibit increases in desaturase activities at lower temperatures (Nozawa and Kasai, 1978; Thompson and Nozawa, 1984). The need for homeoviscous adaptation is apparent in *Arabidopsis*, which require polyunsaturated lipids in the membrane to survive low temperature (Miguel et al, 1993). Homeoviscous adaptation also affects microsomal membranes, showing

that the adaptation process is not limited to the cell membrane (Dickens and Thompson, 1982). In addition to altering the level of hydrocarbon saturation, some eukaryotic organisms stabilize their membrane structure by using low molecular weight protectants, such as trehalose in yeasts, nematodes and insects and proline in plants and insects (Storey and Storey, 1988). As a final note, homeoviscous adaptation has positive implications for biotechnology, because the membrane changes of baby hamster kidney (BHK) cells at low temperature impart added resistance to shear stress encountered in bioreactors (Ludwig et al, 1992).

2.2.3 Mammalian Cold Shock Actively Causes Growth Arrest

In addition to the similarities between eukaryotic and prokaryotic cold shock discussed above, eukaryotes have an additional complexity owing to the link between cold response and the cell cycle control system. As noted earlier, a hallmark of hypothermia in mammalian cells is growth arrest. This growth arrest is now known to be an actively controlled part of the mammalian cold shock response (Fujita, 1999), rather than a passive response to depressed metabolism, as will be shown in this section. The protein-level connection between hypothermia and growth arrest has been established by two recent findings, namely the linking of the cold shock response to increased p53 activity and the discovery of a cold-inducible gene responsible for causing growth arrest. The proposed mechanism for how these two proteins are affected by low temperature and exert their effect on the cell cycle is shown in Figure 2-4 and discussed here.

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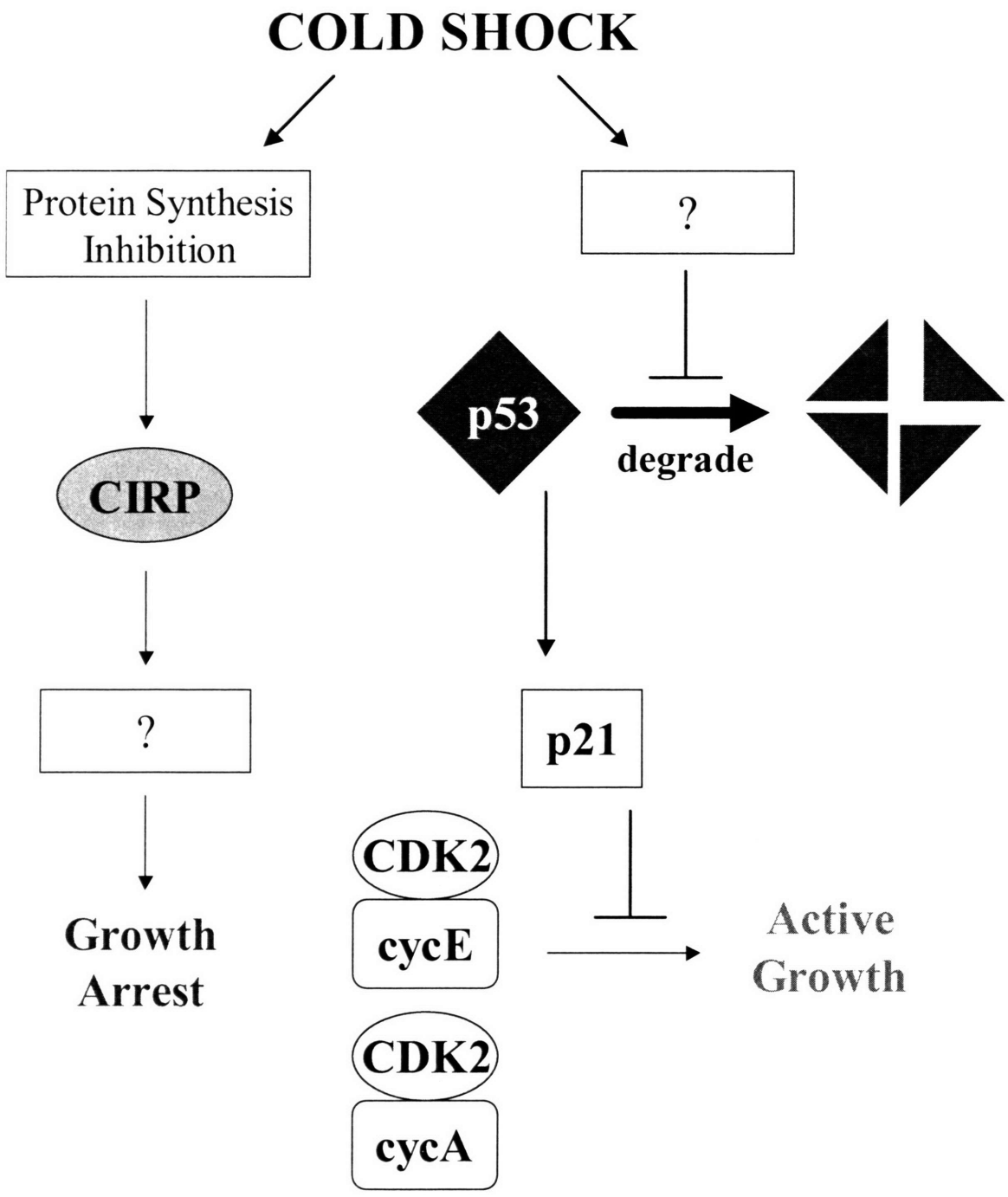

Figure 2-4. Model for cold-induced growth arrest in mammalian cells. The growth-arresting p53 gene is stabilized under cold shock, resulting in induction of p21 and subsequent inhibition of CDK/cyc activity, arresting growth in G_0/G_1. In another path, cold shock disruption of protein synthesis causes the induction of CIRP, which causes growth arrest via an unknown mechanism. Arrows represent activation and bars represent inhibition.

In the earlier review of the cell cycle (Section 2.1), one of the proteins identified as playing a role in the G_0/G_1 phase growth arrest was p53, so named because it has an apparent molecular weight of 53 kDa (Lodish et al, 2000). This protein is a potent growth inhibitor and mutations in p53 are evident in about 50% of human cancers (Alberts et al, 1994). This protein exerts its effect as a transcription factor, binding to DNA and inducing the transcription and subsequent expression of the CKI, p21, which in turn inhibits CDKs and causes cells to arrest in G_0/G_1 (refer to Figure 2-2). Under normal conditions, p53 is very unstable and is rapidly degraded. However, upon exposure to stresses such as γ-irradiation, p53 degradation ceases and the protein then exerts its effect as a transcription factor (Alberts et al, 1994). When mammalian cells are exposed to cold shock (4-20 °C was tested), p53 protein levels increase significantly, followed by an increase in p21 mRNA and then p21 protein (Ohnishi et al, 1998). The level of p53 mRNA is unchanged during this sequence of events, indicating that, as with γ-irradiation, temperature exerts a post-transcriptional, stabilizing effect on p53 (Ohnishi et al, 1998). The mechanism by which cold shock stabilizes p53 remains unknown (Ohnishi et al, 1998).

The second link between cold shock and active growth arrest in mammalian cells comes from the cold-inducible RNA binding protein (CIRP) (Nishiyama et al, 1997b). The CIRP cDNA was first isolated from a screen for RNA-binding proteins expressed in mouse testis, which are maintained at 32 °C by the animal. As noted in Section 2.2.1, several cold shock proteins in prokaryotes exhibit RNA-binding properties (Jones and Inouye, 1996) and this was the basis for screening for the RNA-binding property. Since then, CIRP has also been identified in humans (Nishiyama et al, 1997a) and, interestingly, in CHO cells during a screen for UV-inducible genes (Sheikh et al, 1997). CIRP is induced rapidly under mild hypothermia (25-32 °C), with detectable protein levels 1 hour after a shift from 37 °C, a peak in protein levels at 12-

hour post-shift and maintained high levels for at least 24 hours post-shift, but showed decreasing levels at 36 hours (Nishiyama et al, 1997b; Danno et al, 1997). CIRP is neither induced under extreme hypothermia (<15 °C) nor under heat shock (39-42 °C) (Nishiyama et al, 1997a; Nishiyama et al, 1998; Sheikh et al, 1997).

CIRP function has been directly linked to growth arrest by knockdown and over-expression experiments conducted by Nishiyama and colleagues (1997a). Using antisense oligonucleotides (ODN) homologous for a region of CIRP transcript near the translation initiation site, they were able to partially reduce CIRP levels, as shown by Western blot. The knockdown cells exhibited reduced growth arrest, reaching double the cell density after two days of growth at 32 °C compared to the control cells. Next, Nishiyama and colleagues tested the effect of over-expressing CIRP at 37 °C and found that the presence of the CIRP protein at 37 °C caused partial growth arrest. The doubling time at 37 °C was increased from 17.6 to 23.5 hours as a result of CIRP over-expression. For comparison, doubling time at 32 °C was 28.6 hours. The length of the various cell cycle phases was measured and CIRP over-expression was found to increase the time required for the G_1 phase at 37 °C from 6.1 hours to 10.5 hours. The G_1 phase takes 12.7 hours for the same cells at 32 °C. In contrast, the remaining phases of the cell cycle were not significantly affected by CIRP over-expression, showing that CIRP exerts its effect primarily in G_0/G_1, the phase in which mammalian cells tend to arrest when exposed to mild hypothermia.

The molecular mechanism by which CIRP exerts its effect remains unknown. The protein contains an amino terminal consensus sequence RNA-binding domain (CS-RBD) and a carboxyl-terminal glycine-rich domain, and due to these motifs, fits into the glycine-rich RNA-binding protein (GRP) family (Nishiyama et al, 1997b). Other members of the GRP family are

induced by cold stress in plant cells (Bergeron et al, 1993; Carpenter et al, 1994; Heintzen et al, 1994). Proteins containing the CS-RBD are involved in post-transcriptional regulation of gene expression, such as regulating mRNA stability, splicing, export and translation (Burd and Dreyfuss, 1994). Antibody staining shows that CIRP resides in the nucleoplasm (Nishiyama et al, 1997b). Also, Northwestern blot analysis showed that CIRP preferentially binds to poly(U) RNA. Other proteins known to reside in the nucleoplasm and preferentially bind poly(U) have been shown to function in mRNA regulation, including 3'-end cleavage, polyadenylation, 5'-splice site selection and transport (Mayeda and Krainer, 1992; Pinõl and Dreyfuss, 1992; Swanson and Dreyfuss, 1988; Wilusz et al, 1988). Therefore, the evidence suggests that CIRP is involved in gene regulation at the post-transcriptional level, although this is just a hypothesis (Nishiyama et al, 1997b).

Likewise, the mechanism by which CIRP is induced following cold shock remains unknown. However, evidence exists suggesting that CIRP, like the bacteria cold shock genes (see Figure 2-3), is induced by a disruption in protein synthesis. This was shown by the induction of CIRP transcription following translation inhibition using cycloheximide or puromycine (Danno et al, 1997; Fujita, 1999).

Recently, a second mammalian cold shock protein, called RBM3, has been identified (Danno et al, 1997). This protein has strong homology with CIRP (64% identical amino acid sequence) and also contains a CS-RBD, suggesting a role in post-transcriptional regulation of gene expression during cold shock. Following a shift from 37 ^{o}C to 32 ^{o}C, the cold shock induction profile of RBM3 differs from CIRP, with RBM3 showing a higher induction ratio and persisting for at least 36 hours post-shift, a point in time at which CIRP levels have begun to

decrease (Danno et al, 1997). RBM3 function has not been determined, but, unlike CIRP, RBM3 *does not* appear to play a role in hypothermic growth arrest (Danno et al, 2000).

In summary, the cold shock response is an active, genetically regulated program that shows conservation from bacteria to mammals. The state of the ribosome appears to be the physiological sensor for the cold-shock induction in all organisms (Fujita, 1999). Two hallmarks of the cold response are protein synthesis adaptation and homeoviscous adaptation. In addition, mammalian cells show an active connection between the complex cell cycle regulatory mechanism and the cold shock response, as shown by the induction of p53 at low temperature and the G_0/G_1 growth-arresting ability of the cold-inducible RNA binding protein.

2.3 CONTROLLED PROLIFERATION IN BIOTECHNOLOGY

Controlled proliferation, wherein cells are actively growth arrested by chemical, environmental, metabolic and genetic means, has shown tremendous promise for improving therapeutic protein production by mammalian cells. The rationale is simple: limiting cell growth extends batch length times, due to fewer cells consuming less nutrients, and also should improve specific productivity, due to lowered metabolic burden in arrested cells and thus more resources being available for product formation (Fussenegger and Bailey, 1999). Given the growth-arresting effect of hypothermia, it is classified as one of several controlled proliferation techniques (Fussenegger and Bailey, 1999). This classification has profoundly affected how hypothermic culture is viewed and therefore the biotechnological application of controlled proliferation in general is first considered before considering the biotechnological application of hypothermia.

Interest in controlled proliferation was stimulated by the pioneering work of Suzuki and Ollis (1989, 1990), who developed a model predicting that specific antibody productivity by hybridoma cells would show an inverse dependence on growth rate. The model was based on formulating antibody mRNA balances and assuming constant mRNA transcription, translation and degradation rates throughout the cell cycle. In this model, the inverse dependence on growth rate is a result of the increased time available for slow-growing cells to build up mRNA levels before the next cell division (Suzuki and Ollis, 1990). By and large, the model predicted the general trend of inverse growth-associated productivity exhibited by the researchers' hybridoma cell line, although this trend was not universal (Suzuki and Ollis, 1990). Other researchers also showed inverse growth-associated productivity for different hybridoma lines (Al-Rubeai et al, 1992; Franek and Dolnikova, 1991). Since then, many different methods for obtaining controlled proliferation have been studied, as discussed next.

2.3.1 Methods for Obtaining Controlled Proliferation

Early methods for obtaining controlled proliferation relied on nutrient deprivation or toxic chemical additives to enforce growth arrest. For example, cells were starved of essential energy sources (Birch et al, 1984; Suzuki and Ollis, 1990), treated with DNA-synthesis inhibitors (thymidine, hydroxyurea, TGF-β and adriamycin) (Al-Rubeai, 1992; Suzuki and Ollis, 1990), selective protein synthesis inhibitors (potassium acetate and cycloheximide), or RNA synthesis inhibitor (Actinomycin D) (Suzuki and Ollis, 1990). These treatments not only caused growth arrest but also caused the cells to undergo cell death, dramatically shortening the viable lifespan of the culture.

The most popular chemical additive for obtaining proliferation control is sodium butyrate, which is widely used in biopharmaceutical manufacturing (Fussenegger and Bailey, 1999) owing to its ability to elicit enhanced productivity of a variety of industrially-relevant proteins, including tissue-type plasminogen activator (t-PA) (Arts et al, 1995; Kooistra et al, 1987), factor VIII (Palermo et al, 1991), von Willebrand factor, erythropoietin (EPO) (Dorner et al, 1989), follicle stimulating hormone (FSH) (Gebert and Gray, 1995) and monoclonal antibodies (Chevalot et al, 1995; Mimura et al, 2001; Oh et al, 1993), in a range of mammalian cells (human endothelial cells, CHO, hybridoma). Owing to its anti-proliferative properties, sodium butyrate has important implications for cancer treatment. It is produced by bacterial fermentation of colon flora and is believed to be the protective compound linking a high-fiber diet with a reduced incidence of colon cancer (Archer et al, 1998; Heerdt et al, 1997). Butyrate is a reversible inhibitor of deacetylase, an enzyme responsible for removing acetyl functional groups from lysine residues on the histones (Kijima et al, 1993). The histones are proteins that bind DNA, thereby making it less accessible to RNA polymerase and consequently less transcriptionally active. By removing acetyl groups, a positive charge is restored to the lysine and association with the negatively charged DNA is increased (Grunstein, 1997). Thus, addition of butyrate to culture medium inhibits deacetylase, making DNA more accessible, increasing transcription and ultimately increasing heterologous protein production (Grunstein, 1997). In addition, butyrate exerts a growth-arresting affect by activating the CKI p21 (refer to Figure 2-2). Butyrate causes p53-independent, p21 induction by activating the p21 promoter through Sp1 sites, gene regulatory sequences potentially masked by hypoacetylated histones (Alberts et al, 1994; Kijima et al, 1993; Nakano et al, 1997).

Other promising growth-arresting additives recently tested include nucleotides, nucleosides and bases. The advantage of these compounds is that they are naturally synthesized by the cell and are expected to be less toxic than foreign compounds (Carvalhal et al, 2003). Of the various compounds tested, adenosine 5'-monophosphate (AMP) was found to be most promising for increasing specific productivity. However, the growth arrest in this case occurred in the S phase.

Another common method for achieving G_0/G_1 growth arrest is by increasing medium osmolality, termed hyperosmotic pressure. The advantage of this method is the low cost, with osmolality being readily increased by adding cheap salts and sugars (Ryu et al, 2001). Like butyrate addition, hyperosmotic pressure routinely causes enhanced heterologous protein production in industrially-important cell lines such as CHO and hybridoma (Chen et al, 1998; Kim et al, 2000; Oh et al, 1993, 1995; Ryu et al, 2000, 2001; Takagi et al, 2000). The enhanced productivity under hyperosmotic pressure appears to be a result of increased mRNA levels (Lee and Lee, 2000; Ryu et al, 2001; Ryu and Lee, 1999), but may also involve enhancement to downstream steps in the production process (Ryu et al, 2001). There are at least four hypotheses to explain the mechanism for enhanced productivity: (1) hyperosmotic pressure causes transcriptional activation because of changes of chromatin structure, as in the case with butyrate treatment (Lee and Lee, 2000; Ryu and Lee, 1999); (2) hyperosmotic pressure induces chaperone proteins that are involved in protein processing and secretion (Lee, 1992); (3) hyperosmotic pressure enhances the transport of nutrients into the cell (Christensen, 1984; Oh et al, 1993, 1995) and increases ATP production rates (Lin et al, 1999); (4) the G_1 phase of the cell cycle is an optimal environment for production (Fussenegger and Bailey, 1999) and thus by causing arrest in this phase, specific productivity is increased.

Another method considered for controlled proliferation is by changing the major carbon source to a slowly metabolized compound. In one instance, Altamirano and colleagues replaced glucose with galactose as the major carbon source during the course of perfusion, resulting in complete growth arrest (Altamirano et al, 2001). However, the specific productivity of t-PA was unchanged in this case.

Knowledge of the proteins involved in cell cycle control and the advent of recombinant DNA technology has allowed bioengineers to achieve controlled proliferation by over-expressing growth-arresting genes. The first attempt at controlled proliferation by genetic means for biotechnology applications was by engineering BHK cells with a fusion protein between the interferon-responsive factor 1 (IRF-1) and the estrogen receptor (ER) (Kirchhoff et al, 1993; Kirchhoff et al 1995; Kirchhoff et al 1996). IRF-1 is a transcription factor that has been shown to cause growth arrest by cooperating with p53 to upregulate p21 in response to DNA damage (Tanaka et al, 1996). By fusion to ER, IRF-1 activity becomes responsive to the hormone estradiol (Fussenegger and Bailey, 1999). Contrary to what was expected, IRF-1 over-expression did not result in G_0/G_1 growth arrest but rather lengthened all phases of the cell cycle (Müller et al, 1998). In addition, specific productivity for the IRF-1 expressing BHK cells was either unchanged or lower than the controls (Kirchhoff et al, 1996; Müller et al, 1998). Finally, IRF-1 mediated growth arrest resulted in significant cell death (Carvalhal et al, 1998), making use of this growth arrestor for biopharmaceutical manufacturing highly undesirable.

Another logical gene for over-expression is the tumor suppressor p53, discussed previously (Section 2.1 and 2.2), which is known to cause G_0/G_1 growth arrest via several different pathways. However, stable over-expression of this gene resulted in rapid cell death (Mazur et al, 1998). Even when an apoptosis-deficient mutated p53 gene (Rowan et al, 1996)

still capable of achieving growth arrest was used, rapid cell death ensued (Mazur et al, 1998). Thus, p53 over-expression appears unsuitable for achieving high viability growth arrest.

Rather than over-expressing a growth-arresting gene, a different approach to achieve proliferation control genetically is to eliminate expression of growth-promoting genes. The feasibility of this approach has been demonstrated by over-expressing antisense c-jun, an early response gene rapidly induced by external mitogenic stimuli, which plays a critical role in a cell committing to proliferation (Ryseck et al, 1988). Together with c-fos, c-jun forms the transcriptional activator AP-1, which promotes several genes involved in cell cycle progression (Fussenegger and Bailey, 1998). A stable friend murine erythroleukemia (F-MEL) cell line over-expressing antisense c-jun achieved sustained and complete growth arrest, with reasonably high viability maintained (Kim et al, 1998b; Kim et al, 1998a). However, this strategy has not been tested in industrial cell lines, which express c-jun at much lower levels than the F-MEL carcinoma cell line (Fussenegger and Bailey, 1999).

The most successful endeavors into genetic proliferation control have been achieved by over-expressing the CKI molecules p21 and p27. Initially, over-expressing p21 in a stable cell line was unsuccessful for obtaining sustained growth arrest (Fussenegger et al, 1998a). However, by expressing p21 simultaneously with the CCAAT/enhancer-binding protein α (C/EBPα), which is known to both stabilize and induce p21, G_0/G_1 phase growth arrest of CHO cells was achieved, and specific productivity of recombinant secreted alkaline phosphatase (SEAP) increased 10-15 fold (Fussenegger et al, 1998b). The ability of p21 to cause growth arrest and enhanced productivity has also been demonstrated in the industrially-important nonsecreting myeloma (NS0) cell line (Watanabe et al, 2002). Over-expression of p27 in CHO

was the most successful, requiring no additional stabilizing proteins, resulting in growth arrest and causing 15-fold higher specific productivity (Fussenegger et al, 1998b).

To summarize, controlled proliferation, by chemical additives, environmental changes or genetic engineering, can be achieved in animal cell culture. Using the techniques described above, cells arrest in G_0/G_1 and, in numerous cases, have higher specific productivity of heterologous protein than the non-arrested controls.

2.3.2 Potential Drawbacks of Controlled Proliferation

Despite the higher specific productivities often achieved, controlled proliferation has several potential drawbacks that could limit its applicability in an industrial manufacturing setting. First, the use of growth arresting agents is often plagued by significant cell death and low viability, since blocking growth often diverts cells into programmed cell death, or apoptosis (Fussenegger and Bailey, 1998). In the case of sodium butyrate addition or hyperosmotic pressure, the effect on viability is usually apparent within one day (Kim and Lee, 2001; Kim and Lee, 2002). Due to this major problem, researchers have over-expressed anti-apoptotic genes, such as bcl-2, in an attempt to prevent or delay cell death. These efforts have shown merit, causing increased viable culture lifespan and improved total production, but cell death still ensues relatively quickly (Kim and Lee, 2001; Kim and Lee, 2002). Growth arrest by p27 does not appear to be susceptible to apoptosis (Fussenegger and Bailey, 1999). However, over-expressing bcl-x_L, another anti-apoptotic protein, improves the specific productivity of a p27-expressing CHO cell line by an additional 2-fold over the already 15-fold improvement relative to the control (Fussenegger et al, 1998b). The mechanism for this additional improvement is unknown, but may derive from the tendency of bcl-x_L to induce polyploidy (Minn et al, 1996).

Thus, genetically-regulated growth control seems far superior to chemical additives for maintaining high viability during growth arrest.

A major drawback of genetically regulated proliferation control is the tremendous selection pressure on arrested cells (Fussenegger and Bailey, 1998). A single cell that mutates to escape the growth arrest will eventually overgrow the entire population, making the operation unstable and diminishing the productivity with time. This phenomenon is readily observed in p27-based proliferation control, with mutant growth detectable after only four days of culture (Mazur et al, 1999). In these mutants, the dicistronic expression unit appears to be absent, as the cell neither expresses p27 nor the model glycoprotein, SEAP. Efforts to prevent the emergence of fast growers include self-regulated genetic systems, such as including an antibiotic resistant marker downstream of the growth-suppressing gene that allows selection against mutants (Müller et al, 1998; Rees et al, 1996), using chemical additives that target actively dividing cells (Fussenegger and Bailey, 1998) and using low temperature culture to slow mutant growth rates (Kaufmann et al, 2001). However, mutants remain a major problem for genetically-controlled growth arrest (Fussenegger and Bailey, 1999).

Controlled proliferation results in lower cell density, which will diminish the potential for maximizing volumetric productivity. Whereas a large improvement in specific productivity, in and of itself, is a desirable outcome, the *volumetric* productivity is most important in industrial manufacturing and ultimately depends on the cell concentration as well as specific productivity. Thus, the implementation of controlled proliferation in a manufacturing process involves a tradeoff between high growth and high productivity. Examples of this disappointing tradeoff abound in the literature. Kim and Lee (2001) found 5-10 mM butyrate treatment significantly improved specific productivity, but the lower cell density meant total batch production was

unchanged relative to untreated control. Likewise, increasing hyperosmotic pressure over the range 302-620 mOsm kg^{-1} caused progressively higher specific productivity (up to 7-fold higher) but had an insignificant effect on total production (Ryu et al, 2001), due to progressively reduced growth rates. Using p27-mediated growth arrest in CHO cells caused up to 5-fold improvements in specific SEAP productivity but resulted in a 5% lower total product concentration (Meents et al, 2002). By conducting optimization studies, it is possible to find a suitable strategy for using controlled proliferation for maximizing volumetric productivity. For example, treatment with 1 mM butyrate improved total IFN-γ production, whereas 5-10 mM caused a decrease relative to the untreated control (Lamotte et al, 1999). However, such optimizations are ultimately a compromise between high productivity and high cell density, and the low densities achieved by controlled proliferation are recognized as a drawback by conducting these optimization studies.

Finally, the success of controlled proliferation depends on productivity being increased during the G_0/G_1 phase of the cell cycle. It is not clearly understood why productivity should increase during G_0/G_1. A commonly cited hypothesis is that in the absence of growth, intracellular resources and energy are conserved and can be used for product formation (Fussenegger and Bailey, 1998). A second theory is that G_1 represents an optimal environment for heterologous protein production because the cell replenishes its metabolic precursors and repairs genetic damage (Ko and Prives, 1996; Linke et al, 1996). A positive correlation has been shown between G_0/G_1 and productivity (Section 2.3.1 productivity references; Batt et al, 1990; Kromenaker and Srienc, 1991; Lee et al, 1998; Miller et al, 1988; Ozturk and Palsson, 1991a, 1991b; Ramirez and Mutharasan, 1990). However, in just as many other cases, other cell cycle phases, most often the S Phase, have been shown to be preferable for achieving high specific productivity (Aunins and Henzler, 1993; Banik et al, 1996; Gu et al, 1994; Kimura and Miller,

1996; Lee et al, 1998; Leelavaatcharamas et al, 1994; Lloyd et al, 1999; Robinson and Memmert, 1991; Scott et al, 1987; Yuk, 2001). One hypothesis explaining the cell cycle dependence of productivity is that the viral promoter used to drive expression of the recombinant gene plays a major role. There are several studies showing that two of the most widely used viral promoters, namely the simian virus 40 (SV40) and the cytomegalovirus (CMV) promoters, show enhanced transcription during S phase (Gu et al, 1993; Mariani et al, 1981; Kubbies and Stockinger, 1990) whereas the adenovirus major late (AML) promoter enhances transcription primarily during G_1 (Kubbies and Stockinger, 1990). The work of Kubbies and Stockinger (1990) is particularly compelling, as they tested the effect of SV40 and AML each promoting a different gene on the same plasmid, thereby presumably eliminating any effect of the chromosomal integration site. This hypothesis is not entirely satisfactory though, as another study, using the same cell line (and thus same promoter) for all conditions tested, showed maximum productivity during different phases of the cell cycle, depending on the culture conditions (Carvalhal et al, 2003). In another study, the researchers found that the positive association between S phase and specific productivity in their cell line is subordinate to the effect of medium condition (Lloyd et al, 1999). In summary, specific productivity depends not only on cell cycle but on other factors as well and controlled proliferation in the G_0/G_1 phase will not cause enhanced specific productivity for many cell lines and culture conditions.

2.4 HYPOTHERMIC ANIMAL CELL CULTURE IN BIOTECHNOLOGY

Mild hypothermia is classified as another means for obtaining G_0/G_1 controlled proliferation in animal cell cultures. Lowering the temperature has several advantages over some of the other controlled proliferation techniques. First, it is far more cost effective than buying

chemical additives. Second, it is much easier to implement than other strategies. In the case of chemical growth-arrestors, these must be added to the culture at a chosen point in time, requiring another feed stream to the bioreactor, increasing cost and contamination potential. In the case of genetically-controlled proliferation, these systems are generally under the control of tetracycline responsive transactivators, meaning tetracycline must either be added to the culture at the chosen point in time (TET_{ON} system) or calculations must be performed to ensure tetracycline has fully degraded by the chosen point in time (TET_{OFF} system) (Mazur et al, 1999). Third, low temperature seems to overcome the problem discussed earlier of non-producing mutant cells rapidly escaping genetically controlled growth arrest, as shown by studies comparing p27-arrested cells to low-temperature-arrested cells (Kaufmann et al, 1999, 2001; Mazur et al, 1999). Fourth, unlike some of the other controlled proliferation techniques, mild hypothermia does not adversely affect cell viability, and in fact cells remain viable for longer periods of time at low temperature, recognized early as a key advantage of low temperature culture (Fussenegger and Bailey, 1998). The improved viability doesn't appear to be due to temperature *per se*, but rather is due to slower consumption of nutrients or generation of toxic metabolites, as CHO cells have been shown to die at the same rate at 30 °C or 37 °C once the apoptotic program is triggered (Moore et al, 1997).

Given these merits, low temperature has been evaluated for use in biomanufacturing processes. When considering process changes, bioengineers evaluate the impact of the change on growth, metabolic and production rates as well as product quality. This section will cover what is known about the effect of mild hypothermia in animal cell culture on these four parameters.

2.4.1 Impact of Mild Hypothermia on Mammalian Cell Growth

It has been known for many years that the growth rate of mammalian cells decreases as temperature is lowered from the conventional cultivation temperature of 37 °C (Rao and Engelberg, 1965; Watanabe and Okada, 1967). The decrease in growth rate is caused by an increase in the time needed to progress through each phase of the cell cycle, although this increase is not uniform and is cell line dependent (Rao and Engelberg, 1965; Watanabe and Okada, 1967). For CHO cells, a decrease in temperature causes an increase in the percentage of G_0/G_1 cells, indicating that this phase is most strongly affected by hypothermia (Hendrick et al, 2001; Kaufmann et al, 1999; Moore et al, 1997; Yoon et al, 2003). For the temperature range considered in these papers (30 – 37 °C), G_0/G_1 cells are never 100% of the population and the cells continue to increase in number, albeit at a slower rate.

The activity of enzymes has often been found to vary with temperature according to Arrhenius' law (Kretzmer et al, 1998):

$$v(T) = v_0 \exp\left(-E/RT\right) \qquad (2\text{-}1)$$

where v(T) is the enzyme activity at a given temperature, T, v_0 is the enzyme activity at the temperature providing maximal activity (usually a few degrees above 37 °C), E is the activation energy and R is the ideal gas constant. Above the temperature providing maximal activity, this equation is no longer valid, and activity will tend to *decrease* with increasing temperature, owing to protein denaturation.

Mammalian growth is not satisfactorily explained by an Arrhenius-like dependence on temperature. For example, Rao and Engelberg (1965) showed that HeLa cells only undergo exponential growth between the temperature range of 33 – 37 °C and within this range the

growth rate is approximately linear with temperature. In the range 26 - 31 °C, cells could not be grown exponentially. Likewise for some CHO cell lines grown at 30 °C, cells almost fully cease growing, with cell numbers remaining stationary with time (Furukawa and Ohsuye, 1998; Kaufmann et al, 1999). Thus, there appears to be a critical temperature below which growth ceases completely. These growth characteristics are likely due to the fact that low temperature growth arrest is an active response by mammalian cells, as discussed in Section 2.2.3, rather than simply a manifestation of a kinetic limitation in key enzyme activities.

2.4.2 Impact of Mild Hypothermia on Mammalian Cell Metabolism

The effect of low temperature on mammalian cell metabolism varies from cell line to cell line. In general, consumption rates decrease with low temperature, but this is not always the case. Table 2-1 summarizes the effect of low temperature, relative to 37 °C, on some key metabolic parameters, namely glucose consumption rate, lactate yield from glucose (moles lactate produced per moles glucose consumed), and glutamine consumption rate, for several industrially relevant mammalian cell lines.

Table 2-1. Effect of hypothermia on mammalian cell metabolism (relative to 37 °C)

Reference	Cell Line	Temp. (° C)	Specific Glucose Consumption[1]	Lactate Yield from Glucose[1]	Specific Glutamine Consumption[1]
Reuveny et al, 1986	Hybridoma	28	0.41	*Not measured*	*Not measured*
		31	0.45		
		34	0.59		
Sureshkumar and Mutharasan, 1991	Hybridoma	33	0.60	0.62	*Not measured*
		35	0.80	0.90	
Barnabé and Butler, 1994	Hybridoma	33	0.87	0.89	**1.0**
Weidemann et al, 1994	BHK-21	33	0.78	*Not measured*	*Not measured*
Chuppa et al, 1997	Hamster cells[2]	34	0.52	**1.38**	0.91
		35.5	0.75	**1.12**	0.94
Furukawa and Ohsuye, 1998	CHO	32	0.53	*Not measured*	0.77
		35	0.90		0.73
Yoon et al, 2003	CHO	30	**1.44**	**1.12**	**1.47**
		33	0.94	0.94	**1.03**

[1]Value at 37 °C set to 1.0
[2]Hamster type not given

From Table 2-1, it is apparent that metabolite consumption rates do not follow Arrhenius kinetics dependence on temperature. For example, in some cases metabolite consumption rates *increased* with decreasing temperature. Furukawa and Ohsuye (1998) conducted a thorough analysis of amino acid consumption at 32, 35 and 37 °C for CHO cells. They found that the consumption rates of most amino acids decreased with lower temperature, but there were exceptions. Aspartate and alanine consumption increased and glutamate, glycine, methionine, leucine and isoleucine were hardly changed under mild hypothermia. On the other hand, Yoon and colleagues (2003) found that the consumption of all amino acids at 33 °C was about the same as at 37 °C, but at 30 °C, there were major changes in metabolism. Six amino acids (threonine, arginine, valine, isoleucine, leucine and phenylalanine) that had previously been consumed at 37 and 33 °C were produced at 30 °C. In addition, the uptake rate of cysteine was 3.7-fold higher at 30 °C. Thus, significant metabolic changes were taking place below a certain temperature in this CHO cell line.

In contrast, a study on the effect of temperature on oxygen consumption rates (OCR) in BHK, CHO and hybridoma showed that oxygen consumption follows Arrhenius kinetics quite closely for all three cell lines for the 6-37 °C range (Jorjani and Ozturk, 1999). However, it is critical to highlight that for this study, cells were moved from 37 °C bioreactors to the respirometer and the OCR was measured within a maximum of 30 minutes, whereas the metabolic data given in Table 2-1 is long term data, reflecting consumption rates over the course of days when the cells have been cultured at lower temperature for the entire time, and thus have had sufficient duration to adapt to the hypothermic conditions. Taken together, these contrasting findings suggest that mammalian cells will adapt to compensate for the Arrhenius-type kinetic limits that would otherwise have to hold upon lowering the temperature.

2.4.3 Impact of Mild Hypothermia on Mammalian Cell Productivity

The first known cases of researchers using low temperature to improve productivity were during culture of non-transformed human fibroblasts producing interferon. These cells are primed to synthesize the endogenous interferon protein by exposure to metabolic inhibitors and protein synthesis inhibitor (cycloheximide) and then induced to produce interferon by removing the cycloheximide. This results in a rapid, but brief, period of protein synthesis, including large quantities of interferon. One group found that by using 32 °C instead of 37 °C for the entire process, the peak productivity was unchanged but the production period was sustained for significantly longer at the lower temperature, meaning total production improved (Vilĉek and Havell, 1973). The authors hypothesized that the sustained release was due to enhanced interferon mRNA stability at low temperature or due to slower degradation of another component required for protein synthesis. Giard and Fleischaker tested the effect of different temperatures on production and found that total interferon was optimal at 34 °C, followed by 37 °C then 30 °C. The mechanism for this effect is unknown, but they hypothesized that 34 °C represents the optimum of a tradeoff between enhanced mRNA stability and reduced metabolic rates at low temperature (Giard and Fleischaker, 1980). Another study showed maximal interferon production by carrying out the initial induction at 37 °C and then shifting to 30 °C (better than either 34 °C or 25 °C) one hour post-induction (Giard et al, 1982). Again, mRNA stability was assumed to be the cause for the prolonged production period, but no evidence was given.

Initial investigations into the effect of low temperature on specific productivity in industrially relevant mammalian cells were not promising, with specific productivity either being practically unchanged (Borth et al, 1992; Chuppa et al, 1997; Ryll et al, 2000; Weidemann et al,

1994) or decreased (Barnabé and Butler, 1994; Bloemkolk et al, 1992; Reuveny et al, 1986; Ryll et al, 2000; Sureshkumar and Mutharasan, 1991). Within the last five years, however, several researchers have found that specific productivity of a variety of recombinant proteins produced by CHO cells can be enhanced by culturing at temperatures in the 30-35 ^{o}C range (Ducommun et al, 2002; Furukawa et al, 1998; Hendrik et al, 2001; Kaufmann et al, 1999; Yoon et al, 2003). The effect of mild hypothermia on heterologous protein production is summarized in Table 2-2.

Table 2-2. Effect of hypothermia on heterologous protein productivity (relative to 37 °C)

Reference	Cell Line	Promoter	Temperature (° C)	Change in Specific Productivity[1]
Reuveny et al, 1986	Hybridoma	N/A	28 31 34	0.1 0.3 0.4
Sureshkumar and Mutharasan, 1991	Hybridoma	N/A	33 35	0.6 0.9
Bloemkolk et al, 1992	Hybridoma	N/A	34	0.9
Borth et al, 1992	Two distinct Hybridomas	N/A	30 33	Decreased for both cell lines[2]
Barnabe and Butler, 1994	Hybridoma	N/A	33	0.8
Weidemann et al, 1994	BHK-21	Unknown	33	**1.1**
Chuppa et al, 1997	Hamster cells[3]	Unknown	34 35.5	**Slight increase at both temperatures[2]**
Furukawa and Ohuye, 1998[4]	CHO	SV40	30 32 33.5 35 36	**2.0** **2.4** **1.7** **1.6** **1.1**
Kaufmann et al, 1999[4]	CHO	CMV	30	**1.7**
Ryll et al, 2000	Two distinct CHO	SV40	33	Unchanged in one line and reduced in the other[2]
Hendrick et al, 2001	CHO	SV40	32	**Slight increase[2]**
Ducommun et al, 2002	CHO	Unknown	32 33.5	**~6** **~2-3**
Yoon et al, 2003	CHO	Unknown	30 33	**5.4** **3.9**

[1]Value at 37 °C set to 1.0
[2]Numerical values for specific productivity not given
[3]Hamster type not given
[4]Producitivity varied with time; value given is the maximum at listed temperature relative to maximum at 37 °C

Clearly, the effect of low temperature on specific productivity is cell line dependent. It is interesting to observe that no hybridoma lines with improved productivity under mild hypothermia have been identified, despite the belief that hybridoma generally exhibit highest productivity during G_0/G_1 (Lee et al, 1998), whereas CHO cells appear to frequently have higher productivity under hypothermic conditions. There are currently no hypotheses in the literature as to why these two cell lines show such distinct behavior.

The mechanism by which mild hypothermia leads to enhanced productivity in CHO cells is not fully known. As discussed above, early hypotheses of researchers working on interferon production in induced fibroblasts focused on increased mRNA stability as the reason for the improvement, although no evidence supporting these hypotheses was given. Furukawa and Ohsuye (1998) provided the first evidence that recombinant mRNA levels are increased at low temperature. This finding was followed by similar results from two other research groups (Kaufmann et al, 2001; Yoon et al, 2003), suggesting that this is a consistent finding in CHO cells exhibiting enhanced productivity at low temperature. However, the Northern blotting technique used for quantifying mRNA in these studies is not accurate, so it was not possible to ascertain that the increase in mRNA levels corresponded directly with the increase in specific productivity. Also, no evidence was given as to whether the stability of the mRNA had been increased, only that the steady state level was higher. This may have been due to higher transcription following growth arrest by low temperature.

Thus, mild hypothermia has shown potential for significantly improving specific productivity in CHO cells. The enhanced productivity appears related to increased mRNA levels, but whether increased mRNA stability is the cause and whether there is a direct correlation between the mRNA level and protein productivity increase remains unknown.

2.4.4 Impact of Mild Hypothermia on Heterologous Protein Quality

Before implementing a change in a bioprocess, such as reducing the temperature, it is critical to ensure that the product quality will not be significantly compromised. Many recombinant therapeutic proteins undergo asparagine-linked (N-linked) glycosylation, which is the most extensive post-translational modification performed by mammalian cells (Kornfeld and Kornfeld, 1985). Glycosylation is an indicator of recombinant protein quality, owing to the fact that the glycan structures have a significant impact on specific activity, folding, immunogenicity and circulatory half life (Goochee et al, 1991; Jenkins and Curling, 1994; Kaufman, 1989; Kaufman et al, 1988). It is well known that glycoproteins exhibit heterogeneity, meaning that for a given protein, a mixture of different glycoforms will exist (Kornfeld and Kornfeld, 1985). This heterogeneity can be classified as two types, called macroheterogeneity or microheterogeneity.

Macroheterogeneity, or variable site occupancy, is the result of incomplete transfer of oligosaccharide to the protein inside the endoplasmic reticulum (ER). As a glycoprotein is translated and passes into the ER, it encounters the enzyme oligosaccharyltransferase, which is responsible for transferring an oligosaccharide structure from the lipid dolichol phosphate to an asparagine within the Asn-X-Ser/Thr consensus sequence on the protein, where "X" refers to any amino acid except proline. For a given protein, this transfer reaction does not always take place, resulting in a mixed glycoprotein population.

Microheterogeneity is the result of the oligosaccharide branch having different specific sugar structures. This processing occurs as the protein is passed from the ER to the golgi complex (Kornfeld and Kornfeld, 1985). During this process, a variety of exoglycosidase- and glycosyltransferase-catalyzed reactions take place and generate the different types of oligosaccharides, known as the high-mannose, hybrid and complex structures. The variability in

these steps causes the microheterogeneity of the glycoprotein. One key parameter in microheterogeneity is the presence or absence of sialic acid as the terminal sugar on complex or hybrid type structures. The presence of sialic acid is desirable as it increases *in vivo* circulatory lifetime, owing to inhibition of clearance by the asialoglycoprotein receptors on hepatocytes (Weiss and Ashwell, 1989).

The model protein used in this thesis, IFN-γ, is a secretory glycoprotein that plays an important role in immune system function. Produced by T lymphocytes and natural killer cells in response to various stimuli, IFN-γ has antiviral, antiproliferative and immunomodulatory functions (Devos et al, 1982; Farrar and Schreiber, 1993). IFN-γ is a 138 amino acid glycoprotein that serves as a good model for studying N-linked glycosylation because it contains two potential glycosylation sites, at Asn^{25} and Asn^{97}, both of which exhibit variable site-occupancy as well as a significant degree of microheterogeneity (Curling et al, 1990; Gu, 1997; Gu et al, 1997; Hayter et al, 1993; Nyberg, 1998). A schematic representative of IFN-γ heterogeneity is shown in Figure 2-5.

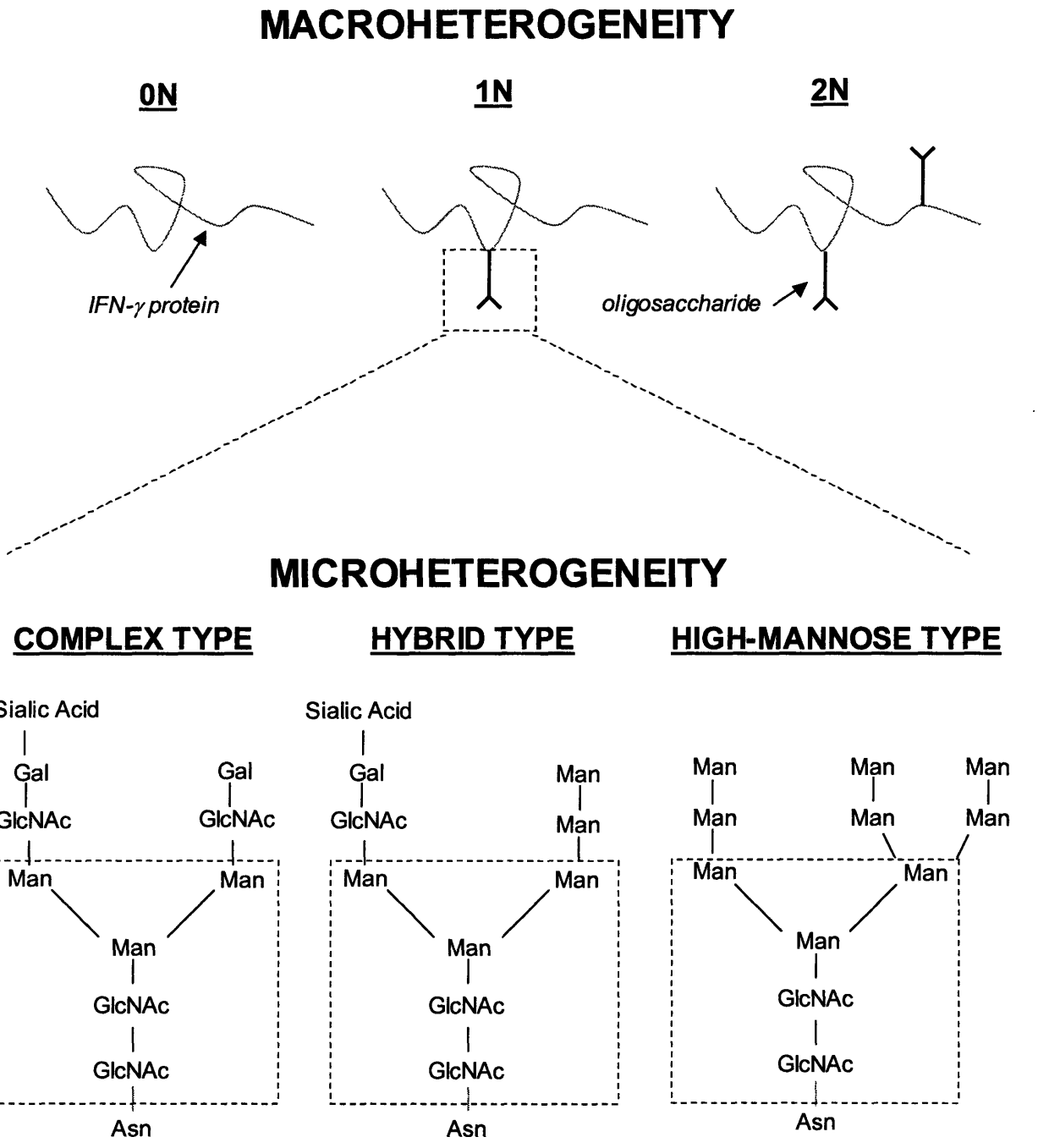

Figure 2-5: IFN-γ Glycosylation Heterogeneity. IFN-γ is produced with 0, 1 or 2 oligosaccharide branches. Each of these branches can be one of three types, complex, hybrid or high-mannose. All three types are derived from a common pentasaccharide core, which is shown in the large, dashed boxes. Each type can have up to four terminal branches, although only 2-3 branches are shown here.

IFN-γ can have zero (0N), one (1N) or two (2N) of the possible two N-linked consensus sequences occupied. The 1N species consists exclusively of Asn^{25}-occupied glycoforms (Hooker et al, 1999). For an occupied site, further microheterogeneity is possible, as discussed previously. The branching shown in Figure 2-5 is for illustration purposes only and is not comprehensive (see Gu, 1997 for a comprehensive list). In theory, there can be up to four terminal branches on the three oligosaccharide types shown. However, for IFN-γ, the complex, biantennary type shown in Figure 2-5 predominates (Gu, 1997). Owing to the importance of sialylation discussed above, one can calculate the theoretical maximum number of sialic acid residues that a single IFN-γ molecule can possess. This would occur for the case of 2N site-occupancy with complex tetraantennary structures, each containing a sialic acid residue, for a theoretical limit of 8 molecules sialic acid per molecule of IFN-γ. In practice, site occupancy is variable, biantennary branches predominate and sialylation is incomplete, meaning a typical value for IFN-γ produced by CHO-γ is 2.3 – 3.1 sialic acids per molecule of IFN-γ (calculation based on values reported in Gu, 1997).

There is very little data on the effect of hypothermia on recombinant glycoprotein glycosylation. When cells die and lyse, they release degradative enzymes, such as proteases or glycosidases, into the culture medium, where they can degrade the recombinant protein and its attached oligosaccharides. It has been shown that these extracellular degradative processes adversely affect recombinant protein glycosylation, in particular by the action of sialidase removing sialic acid residues (Gramer et al, 1995; Gu et al, 1997). Given that enzymes exhibit decreased activity as temperature is lowered, one would expect the sialidase enzyme to cause less product degradation, but this has yet to be reported.

The above stated advantage has been reported for another class of degradative enzymes, namely proteases, where it was found that proteolytic degradation of rhesus thrombopoietin (TPO) produced by CHO cells in perfusion culture was reduced when the temperature was shifted from 37 °C to 33°C (Ryll et al, 2000). This result is consistent with another group's finding that protease activity decreases by approximately 25% when reducing the temperature of a hamster cell perfusion from 37 °C to 34°C (Chuppa et al, 1997).

Intracellular glycosylation requires the concerted action of a series of enzymes and the presence of various substrates, all of which could be affected by lower temperature. The impact of hypothermia on the overall glycosylation reaction network remains quite unknown. One group addressed the glycosylation issue by using lectin staining of protein gels and found glycosylation to be unchanged in batch CHO culture producing t-PA when the temperature was changed from 37 °C to 32 °C (Hendrik et al, 2001). However, lectin staining is too crude to detect subtle changes in glycosylation and more accurate techniques should be employed.

Recently, a group has determined the effect of low temperature culture on the sialylation and *in vivo* biological activity of EPO (Yoon et al, 2003). During the viable portion of the culture, there was very little difference in sialylation of EPO produced at 30, 33 or 37 °C. However, during the death phase of culture, the sialic acid content of EPO at 37 °C decreased by about 40% from the maximum value whereas that at 30 and 33 °C decreased by only 20 and 10%, respectively. In addition, the *in vivo* activity of the EPO produced at the low temperatures was slightly higher than for the EPO produced at 37 °C.

In an earlier study on the effect of hypothermia on sialylation, Kaufmann and colleagues (Kaufmann et al, 2001) used HPAEC-PAD to separate differentially sialylated forms of SEAP produced by CHO cells grown at 37 °C and 30 °C and found that disialylation increased from

70% to 80% for the lower temperature culture. This is considered a positive change as increased sialylation is considered beneficial for *in vivo* efficacy, as discussed earlier. One should state that SEAP is not an ideal product for studying all of the details of glycosylation because there is no macroheterogeneity issue to be considered. Although SEAP contains two N-linked sites, one site is never occupied and the other site is always occupied (Kaufmann et al, 2001). Thus, the effect of reduced temperature on macroheterogeneity could not be adequately addressed by using SEAP.

Andersen et al. (2000) addressed the effect of culture temperature on site occupancy in t-PA, which has variable site occupancy at one of the three possible N-linked glycosylation sites. In type II t-PA, two N-linked oligosaccharides exist, whereas in type I t-PA, three N-linked oligosaccharides are present. The authors found that reducing the temperature from 37 ^{o}C to 33^{o}C in CHO batch culture increased the amount of type I t-PA by over 5% (i.e. more glycosylation).

Thus, the few studies available suggest that mild hypothermia has a small but positive effect on both site occupancy and sialylation. However, given the potential of hypothermia for improving productivity, further work is warranted to understand the product quality implications of low temperature culture on a wider pool of recombinant glycoproteins.

3. MATERIALS AND METHODS

3.1 GENERAL NOTES

The hypothermic condition frequently used during this thesis was 32 °C. There were two reasons for choosing this temperature. First, the temperature optimization work of Furukawa and Ohsuye (1998) showed that 32 °C gave the highest specific productivity in the range of 30-37 °C for a CHO cell line producing a C-terminal α-amidating enzyme. Although this may not be the optimal temperature for CHO-γ specific productivity, it provided a rational basis for a starting point temperature to determine whether CHO-γ would exhibit enhanced hypothermic productivity. The second reason is a more practical one, discovered upon starting the research: the incubators used during this project (Sanyo CO_2 Incubator Model MCO 345, Sanyo Electric Company Ltd, Osaka, Japan) can reach a minimum temperature of 10 °C above room temperature. We found that 32 °C could be consistently maintained at all times in our laboratory, whereas lower temperatures (30 °C was tried for several days) tended to be difficult to control, especially for suspension cultures wherein the shaker platforms generate heat.

All chemicals used during this project were obtained from Sigma Chemical Company, Saint Louis, MO, unless noted otherwise.

3.2 CELL CULTURE

3.2.1 Cell Lines

3.2.1.1 CHO-γ

A CHO cell line expressing IFN-γ driven by the SV40 promoter was obtained several years ago from Dr. Walter Fiers (Scahill et al., 1983) and has been used extensively in this laboratory (e.g. Yuk, 2001; Nyberg, 1998; Gu, 1997). This cell line, referred to as CHO-γ, was

created from a DHFR$^-$ CHO cell line by cotransfecting the cells with genes for both DHFR and IFN-γ. The original cell line was anchorage dependent. However, the cell line has also been adapted for growth in suspension culture (Nyberg, 1998). In the course of this thesis, both adherent and suspension CHO-γ cells were utilized. The number of passages of the cell line prior to this project is unknown, but is probably several hundred.

3.2.1.2 CHO-K1

The non-recombinant CHO-K1 cell line, obtained from the American Type Culture Collection (ATCC number CCL-61), was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster (Puck et al, 1958). The number of passages of the cell line prior to being deposited with ATCC is unknown but estimated by ATCC to be approximately 400.

3.2.2 Culture Medium and Maintenance

3.2.2.1 Adherent Cell Cultures

The basal medium used for *all* adherent cultures discussed in this thesis was Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY). The pH was adjusted to 7.4 prior to vacuum filtration (0.22 μm) sterilization and then supplemented with 0.25 μM methotrexate, 20 U ml^{-1} penicillin-20 μg ml^{-1} streptomycin mix (Invitrogen) and 10% Fetal Bovine Serum (FBS) (HyClone, Logan, UT). For maintenance, cultures were incubated at 37 °C and 5-10% CO_2 overlay in a humidified incubator. Cells were grown in surface-treated 25, 75 or 150 cm^2 T-flasks or 6-well (9.62 cm^2) or 24-well (2 cm^2) surface-treated tissue culture plates.

The above procedure applies to CHO-K1 as well *except* that methotrexate was not added to the medium.

3.2.2.2 Suspension Cell Cultures

The basal medium used for *all* suspension cultures discussed in this thesis was protein-free HyQ PF-CHO (HyClone, Logan, UT). The pH was adjusted to 7.4 prior to vacuum filtration (0.22 μm) sterilization and then supplemented with 4mM L-glutamine, 0.25 μM methotrexate, a 20 U ml^{-1} penicillin-20 μg ml^{-1} streptomycin mix (Invitrogen) and 0.1% Pluronic® F-68 solution (Invitrogen). For maintenance, cultures were incubated at 37 °C and 5-10% CO_2 overlay on shaker platforms set at 100 rpm in a humidified incubator. Cells were grown in disposable 125-ml, 250-ml, 500-ml or 1L Erlenmeyer flasks.

3.2.2.3 Cell Bank Maintenance

Frozen stocks were prepared from cells with greater than 95% viability by centrifuging the cells at 950 rpm for 5-10 minutes and resuspending the cells at 10^7 cells ml^{-1} in fresh medium containing 7.5% (v/v) dimethyl sulphoxide (DMSO). Cryogenic vials containing 1ml of the cell suspension were placed into a Nalgene™ 1 °C min^{-1} freezing container and placed into a -80 °C freezer overnight. The following day, the vials were transferred to a -150 °C freezer for long-term storage.

New cultures were started from the stock vials by quickly thawing the cells by immersing the vial in a 37 °C water bath and then transferring the cell suspension into 10 ml of fresh medium, followed by centrifuging at 950 rpm for 5 minutes. The supernatant was aspirated, the cell pellet resuspended in fresh medium (15 ml for adherent cells and 25 ml for suspension cells)

and then transferred to the appropriate culture vessel (75 cm^2 T-flask for adherent cells and 125-ml Erlenmeyer flask for suspension cells).

3.2.3 Cell Enumeration

3.2.3.1 Adherent Cells

The procedure used for counting adherent cell cultures was designed to ensure that both the adherent cells and any dead or viable cells that had become detached during the course of the experiment were included in the total cell count. Medium was removed from the cells and centrifuged at 950 rpm for 5 minutes to retain detached cells for counting. After centrifugation, supernatant was removed and stored at -80 °C for future analysis. The adherent cells were treated with a 0.05% Trypsin / EDTA solution (Invitrogen) for 5-10 minutes at 37 °C. The trypsin was removed and pooled with the previously centrifuged, detached cells. The well was rinsed with fresh medium to remove any remaining cells and the rinse was combined with the trypsin pool. The cell number per well was then determined by counting the cells using a hemacytometer and multiplying the concentration by the known volume of the cell solution. Each sample was counted in duplicate and each count consisted of at least 200 total cells. If the results of the duplicate counts did not agree within 10% of each other, additional counts were conducted. The cell viability was determined using the trypan blue exclusion assay in conjunction with cell counting.

3.2.3.2 Suspension Cells

Approximately 0.5 – 1 ml samples were removed from well-mixed suspension cultures. The cell concentration was determined by counting the cells using a hemacytometer. Each

sample was counted in duplicate and each count consisted of at least 200 total cells. If the results of the duplicate counts did not agree within 10% of each other, additional counts were conducted. The cell viability was determined using the trypan blue exclusion assay in conjunction with the cell counting. After counting, samples were centrifuged at 950 rpm for 5 minutes and the supernatant was removed from the cell pellet and stored at -80 °C for future analysis.

3.2.4 Batch Suspension Cultures in Erlenmeyer Flasks

Following 2-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes and resuspended in fresh medium at a density of 2.5×10^5 viable cells ml^{-1}. The experiments were conducted in 250-ml Erlenmeyer flasks with a starting volume of 50 ml. Cells were cultured at the stated temperature on shaker platforms set at 100 rpm in humidified incubators with 8% CO_2 overlay. For temperature shift (TS) experiments, cultures were moved from the 37 °C incubator to the 32 °C incubator at the stated TS time. Cultures were conducted in duplicate. Samples were taken at least daily for cell counting and viability determination. Following counting, samples were spun at 10,000 rpm for 5 minutes and the supernatant was stored at -80 °C for future analysis. The experiments were terminated once the percent viability was less than 90% (Chapter 4 and 5 data) or 80% (Chapter 7 data).

For cultures aimed at achieving active hypothermic growth, the procedure was the same as outlined above except that 10 or 25% FBS was added to the initial culture. Volume was maintained the same as the other cultures (50 ml) by adding less medium to the FBS-containing cultures.

3.2.5 Batch Suspension Cultures in Bioreactors

Following 3-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes, resuspended in 1,500 ml fresh medium at a density of $2.5x10^5$ viable cells ml^{-1} and transferred into 2-liter bioreactors attached to Braun Biostat® B-DCU units (B.Braun Biotech International GmbH, Melsungen, Germany). The reactors were equipped with calibrated dissolved oxygen (DO), pH and temperature probes. DO was maintained at 50% of saturation while pH was controlled at 7.2 by the addition of CO_2 or 7.5% (w/v) sodium bicarbonate solution. The stir rate was fixed at 150 rpm. Temperature was either maintained at 37 °C or 32 °C for the entire run or shifted from 37 °C to 32 °C at one time point during the culture (so called "temperature shift", or TS, experiments). For the TS experiments, the jacket temperature was reduced to 25 °C and then the vessel temperature was monitored. Once the vessel temperature reached 32 °C, the automatic temperature control was turned on. Using this method, the time required to cool the culture from 37 °C to 32 °C was approximately 5 minutes.

Samples were taken at least daily for cell counting and viability determination. Following counting, samples were spun at 3,000 rpm for 5 minutes and the supernatant was stored at -80 °C for future analysis. The reactor runs were terminated once the percent viability was less than 80%.

3.2.6 Fed-Batch Suspension Culture in Bioreactors

Fed-batch bioreactor cultures used the same method as outlined above for batch bioreactor runs expect that two concentrated nutrient feeds were added on a twice daily basis for

these runs. The concentrated nutrient feeds were specially-formulated, low salt DMEM/F12 media (Hyclone), containing 10X of normal DMEM/F12 concentrations of all amino acids and either 10X (200 mM) glucose or 10X (40 mM) glutamine. The feed was also supplemented with 1 wt% HYsoy peptide mixture (Quest International, Hoffman Estates, IL) and 0.5% (v/v) chemically defined lipid solution (Invitrogen). The concentrate feed *did not* contain the following DMEM/F12 components: calcium chloride, D-calcium pantothenate, HEPES, and sodium bicarbonate. The amount of each feed medium added twice per day was calculated so as to restore glucose to 20 mM and glutamine to 4 mM following the addition of the concentrated feed solutions. The medium was added by injection through sterile, self-sealing ports on the top of the reactor.

Samples were taken at least daily for cell counting and viability determination. Following counting, samples were spun at 3,000 rpm for 5 minutes and the supernatant was stored at -80 °C for future analysis. The reactor runs were terminated once the percent viability was less than 80%.

3.2.7 Batch Suspension Cultures in Tissue Culture Plates

This procedure applies to both the CHO-γ and CHO-K1 cell lines. Following 2-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes and resuspended in fresh medium. One quarter million cells were resuspended in 3 ml of medium and added to each well of a group of 6-well plates. Cells were incubated at 31.5 °C, 32 °C, 34.5 °C or 37 °C in humidified incubators with 8% CO_2 overlay. Every day, 2 wells per condition were sacrificed for cell counting. The supernatant from the

sacrificed wells was stored at -80 °C for later analysis. The experiments were terminated once the percent viability was less than 80%.

For cultures aimed at achieving active hypothermic growth, various amounts of stock growth factor solutions were added to the medium at the beginning of the experiment. In all cases, the growth factors were dissolved in the standard basal medium containing the same supplements as the control cultures. Thus, all conditions were identical except for the presence or absence of growth factors. The insulin stock solution contained 100 $\mu g\ ml^{-1}$ insulin and the bFGF stock solution contained 25 $\mu g\ ml^{-1}$ bFGF. Insulin concentrations of 5, 15 and 25 $\mu g\ ml^{-1}$, bFGF concentrations of 13, 21, 42 and 45 $ng\ ml^{-1}$, and a mixed culture containing 15 $\mu g\ ml^{-1}$ insulin and 21 $ng\ ml^{-1}$ bFGF were tested.

3.2.8 Pseudo-perfusion Culture in Tissue Culture Plates

Following 2-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes and resuspended in fresh medium. One quarter million cells were resuspended in 3 ml of medium and added to each well of a group of 6-well plates. Cells were incubated at 32 °C or 37 °C in humidified incubators with 8% CO_2 overlay. Cultures were run in batch mode for the first 4 days, at which point the spent medium was aspirated from all plates and 3 ml of fresh medium was added per well. Medium was changed again on day 6. From day 7 forward, medium was changed daily (3 ml for 32 °C culture and 5 ml for TS and 37 °C cultures; differences due to different nutrient consumption rates). Every day, 2 wells per condition were sacrificed for cell counting. The supernatant from the sacrificed wells was stored at -80 °C for later analysis and the cells were saved for RNA analysis and fixed for cell cycle analysis, using the procedures outlined below. By day 10, the 37 °C

samples had reached full confluency and had stopped growing, at which point half of the 37 °C plates were moved to the 32 °C incubator (temperature shift or "TS" cultures). Thus, the 32 °C incubator contained two different sets of plates, namely the TS set and also a set that was placed at 32 °C from the start of the experiment. The experiment was continued for an additional 10 days, with daily medium change and sampling of the 32 °C, 37 °C and TS cultures.

On day 16, actinomycin D (5 μg ml^{-1}) was added to several wells of the 37 °C and TS cultures to inhibit transcription. The concentration used was based on the successful transcription inhibition at this level exhibited in CHO cells previously (Thekkumkara et al, 1998). Cell samples were taken in duplicate at 2, 6, 10, 18, 24 and 30 hours after actinomycin D addition for RNA isolation, quantification, and real time RT-PCR quantification of IFN-γ and β-actin mRNA levels.

3.2.9 Active Hypothermic Growth CHO-γ Cell Population

3.2.9.1 Generating the Hypothermic Growth Cell Population

Adherent CHO-γ were grown for several passages to obtain sufficient cell stocks to produce 10 confluent, Corning® 100 mm tissue culture dishes and several confluent 6-well plates. The medium was aspirated from each of the 6-well plates and the plate was placed in a biological safety cabinet. The plate lid was removed and the cells were irradiated with the in-hood UV lamp for 0, 1, 2, 3, 5, 7.5, 10, 15, 20 or 30 minutes. The following day, the cells were harvested and counted to determine the time of UV exposure required to give less than 50% of the viable cell numbers as compared to the control (0 minutes irradiation). A 1 minute UV exposure was found to give approximately 25% the viable cell count as the control, and thus 1 minute was selected as the UV dosage for the subsequent mutation.

The medium was aspirated from the 10 confluent, 100mm tissue culture dishes and the cells were exposed to 1 minute of UV irradiation. Immediately afterwards, fresh medium was added to the plates and the plates were transferred to a humidified incubator with 5-10% CO_2 overlay set at 32 °C.

The cells were then grown for 80 days with passaging and medium change as required to prevent the plates from becoming confluent or nutrient depleted. This growth period was given to allow rare mutants with an increased growth rate compared to the original cell line to comprise a significant proportion of the cell population. After this time, the cells were banked using the procedure outlined previously. Then, the growth characteristics of the mutant population at 31.5 °C, 34.5 °C and 37 °C was monitored versus the control cells using the procedures for batch adherent cultures described previously.

3.2.9.2 Single Cell Cloning of CHO-γ Active Hypothermic Growth Cell Lines

Single cell cloning was conducted on the mixed population to generate pure active hypothermic growth cell lines as follows. Cells were seeded at a density of 1 cell per 200 µl and 100 µl was added to each well of 10 x 96-well plates. The plates were incubated at 32 °C. After 10 days incubation, the plates were visually screened to eliminate the few wells that appeared to contain two colonies. The plates were then incubated for another 20 days at 32 °C. At this point, colonies that were growing well at 32 °C were identified based on the color of the phenol-red based medium. Approximately 50 colonies were chosen for further analysis. These colonies were trypsinized and transferred to 1ml fresh medium in 24-well plates and grown at 37 °C to speed up the process. After 3 days, the colonies were transferred to 3 ml fresh medium in 6-well plates and grown at 37 °C. After 4 days, the cells were trypsinized in 0.5 ml trypsin, diluted in 2

ml fresh medium and a sample was resuspended in ISOTON solution (Coulter Corporation, Miami, FL) and counted using a Coulter® Z2 Particle Counter (Coulter Corporation). Based on this count, the cells were seeded at 2.5 x 10^5 particles (assumed to be cells) per 3 ml fresh medium, and 3 ml per well was transferred to 6-well plates. The clones were grown for 4 days at both 32 °C and 37 °C, after which point they were quantitatively sampled using the previously described procedure for adherent cells and counted using the coulter counter. Twelve clones were selected for further analysis based on either having a high final 32 °C count or a high 32 °C to 37 °C cell count ratio. Both parameters were considered indicative of cells capable of reduced growth arrest at low temperature. The batch culture growth characteristics of these twelve clones at 32 °C and 37 °C were then monitored using the same seeding and sampling procedures as outlined above for attached cells. From this experiment, two different types of clones were observed. Both types were capable of improved growth at low temperature. Type 1 had similar growth at 37 °C compared to the control cell. Type 2 had reduced growth at 37 °C compared to the control cells. Clone A was chosen as representative of type 1 and Clone 1 was chosen as representative of type 2. These two cell lines were expanded in number and banked using the procedure given above. Then, the growth and IFN-γ production of these two clones was monitored in duplicate and compared to the control cells, using the procedure given above for adherent batch cultures.

3.2.10 Active Hypothermic Growth CHO-K1 Clones

An identical procedure as described above for the CHO-γ cells was used to generate pure CHO-K1 clones capable of enhanced growth under hypothermic conditions *except* for one addition to the protocol, as now discussed.

A drawback of the CHO-γ screen is the inability to distinguish between mutants generated during the UV irradiation step and those that may already exist in the cell population. To be able to distinguish between these two types, an additional control was conducted for the CHO-K1 procedure. This control consisted of cells carried through an identical selection procedure except they were not exposed to UV light to generate new mutations. A clone obtained using this procedure has the suffix "C" whereas a clone obtained using the UV procedure has the suffix "M".

3.3 IFN-γ GLYCOSYLATION ANALYSIS

Several of the glycosylation methods described below are based on a prior thesis studying IFN-γ glycosylation changes during CHO-γ culture (Gu, 1997) and this work should be consulted for details regarding the method development.

3.3.1 Immunoaffinity Column

Two milligrams of purified mouse anti-human IFN-γ clone B27 (BD Pharmingen, San Diego, CA) was coupled to Cyanogen Bromide-activated Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden) to form a wet volume of 300 μl. The beads were packed into a HR 5/2 0.5 ml column (Amersham Biosciences).

3.3.2 IFN-γ Purification

Samples containing IFN-γ from culture supernatant were filtered (0.45 μm Millex HV, PVDF low protein binding) (Millipore, Bedford, MA) and 0.02% Sodium Azide added. Twenty to 40 ml of the prepared sample was loaded at 0.2 ml min^{-1} onto the anti-human IFN-γ

immunoaffinity column that had been equilibrated with loading buffer. The purification was carried out on an AKTA Explorer chromatographic system (Amersham Biosciences). The loading buffer, comprising of 20 mM sodium phosphate buffer (Merck KgaA, Damstadt, Germany), pH 7.2 and 150 mM sodium chloride (Merck KgaA) was used to wash the column after loading. The sample was eluted at 0.02 ml min^{-1} using a low pH buffer comprising of 10 mM HCl (37% purity) (Merck KgaA) and 150 mM sodium chloride (Merck KgaA) at pH 2.5. The column was regenerated for subsequent runs using the loading buffer.

3.3.3 IFN-γ Quantification and Purity

Quantification of purified IFN-γ was done using Reverse Phase (RP) HPLC. Twenty-five to 50 µl of the purified IFN-γ was injected into a Shimadzu LC-10ADvp HPLC (Shimadzu Analytical instruments, Kyoto, Japan) and separated on a Vydac C18 1 mm x 250 mm column (Grace Vydac, Hesperia, CA). After holding the run at 5% buffer B for 10 minutes (buffer A: HPLC grade water + 0.1% trifluoroacetic acid (TFA); buffer B: HPLC grade acetonitrile + 0.1% TFA), the sample was eluted over a 90 minute linear gradient to 95% buffer B. Eluted protein was monitored at 220 nm and quantified using a calibration curve created from serial dilution of IFN-γ standards (Research Diagnostics Inc., Flanders, NJ). The purity of IFN-γ was at least 90%, as determined by integrating the IFN-γ peak and normalizing with the total area of all peaks.

3.3.4 IFN-γ Macroheterogeneity using Micellar Electrokinetic Capillary Chromatography

Site-occupancy analysis of the three IFN-γ glycoforms was done using Micellar Electrokinetic Capillary Chromatography (MECC) on a Beckman Coulter P/ACE MDQ capillary

electrophoresis system (Beckman Coulter, Fullerton, CA). A 50 μm diameter x 52 cm (40 cm length to detector) unfused silica capillary (Beckman Coulter) was used for separation. The capillary was conditioned with 0.1M NaOH and running buffer for 3 hours. Prior to a separation run, the capillary was cleaned with 0.1M NaOH for 10 minutes, flushed with HPLC grade water for 5 minutes and subsequently equilibrated with running buffer for another 10 minutes. The running buffer consisted of 30 mM sodium borate, 30 mM boric acid and 100 mM sodium dodecyl sulphate (SDS) (Merck KgaA) at pH 9. Samples were pressure injected at 5 psi over 5 seconds and then a 12-15 kV voltage was applied to the capillary over 40 minutes. The chromatograms were integrated and the percentage of 2-site (2N), 1-site (1N) and nonglycosylated (0N) peaks calculated.

3.3.5 IFN-γ Sialic Acid Content

Total sialic acid was measured using a modified version of the thiobarbituric acid assay (Hammond and Papermaster, 1976) as follows. Sialic acid was digested from constant quantities (6 μg) of IFN-γ by sialidase treatment. The sample was mixed with an equivalent volume of 50 mM acetate buffer, pH 5.2 (J.T. Baker, Phillipsburg NJ) to obtain a final acetate concentration of 25 mM. Then, 1 μl (0.0025 U) of neuraminidase (sialidase) (F. Hoffmann-La Roche, Basel, Switzerland) was added and the sample was digested for 24 hours at 37 °C. These digestion conditions were verified as sufficient for quantitative removal of sialic acid from the amount of IFN-γ used here. After digestion, an amount of Milli-Q® Ultrapure Water (Millipore, Billerica, MA) was added to obtain 500 μl of total volume and the solution was mixed with 250 μl of periodic acid reagent (25mM periodic acid in 0.125N H_2SO_4) and incubated at 37 °C for 30 minutes. Arsenite solution (200 μl of 2% sodium arsenite in 0.5N HCl) was added to destroy the

excess periodate, followed by the addition of thiobarbituric acid reagent (2 ml of 0.1M 2-thiobarbituric acid, adjusted to pH 9 with NaOH) and heating at 98 °C for 7.5 minutes. The samples were cooled on ice for 10 minutes and then mixed with 1.5 ml of acid / butanol solution (n-butanol containing 5% (v/v) 12N HCl). The samples were shaken vigorously and centrifuged at 3,000 rpm for 3 minutes. The clear organic phase was transferred to a 10 mm cuvette and the fluorescence intensity (λ_{ex} = 550 nm, λ_{em} = 570 nm) was measured on a Cary Eclipse Fluorescence Spectrophotometer (Varian Incorporated, Palo Alto, CA). Sialic acid content of each sample was then quantified by interpolating a standard curve generated from pure sialic acid dissolved in water and carried simultaneously through the above procedure (0.1 μg to 1μg curve, $R^2 > 0.999$).

3.4 OTHER ANALYTICAL METHODS

3.4.1 Glucose, Glutamine and Lactate Concentration

Glucose, glutamine and lactate concentrations in medium were measured using the YSI Model 2700 SELECT Biochemistry Analyzers (YSI Incorporated, Yellow Springs, OH). If required, samples were diluted with Milli-Q® Ultrapure Water (Millipore) prior to measurement to ensure the measurement fell within the linear range of the calibration curves for each of the three compounds.

3.4.2 Osmolality Quantification

Osmolality of medium was measured using the Vapro™ Vapor Pressure Osmometer 5520 (Wescor Inc., Logan, UT) and following the manufacturer's protocol.

3.4.3 Lactate Dehydrogenase Activity

Lactate Dehydrogenase (LDH) activity in medium was measured using a commercially available kit (Sigma Diagnostics Procedure No. 228-UV) and following the manufacturer's protocol.

3.4.4 Cell Counting using the Coulter Counter

Cell samples were diluted 20 – 100 fold using ISOTON II solution (Coulter Corporation) and cell size distribution and particle count were measured using a Coulter® Z2 Particle Counter (Coulter Corporation) and following the manufacturer's protocol. To remove artifacts caused by cell debris and cell clumps, only particles between 10 – 20 μm were considered for cell counting.

3.4.5 Cell Imaging

Adherent cells were visualized using a 20X objective on an Olympus® IX70 Inverted Microscope (Olympus Optical Company, Hamburg, Germany). The image was recorded by a digital camera and analyzed using the MicroImage Version 4.0 software (Olympus Optical Company).

3.4.6 IFN-γ Concentration in Culture Medium

IFN-γ concentration was measured using an ELISA kit (HyCult biotechnology b.v., Uden, the Netherlands) by following the manufacturer's protocol. Culture medium was thawed from -80 °C on the day of the analysis and was serially diluted using phosphate buffered saline (PBS) to obtain samples within the range of the standard curve (325 – 5,000 pg ml^{-1}). For a data

set from the same experiment, ELISA was done at the same time using the same kit and same serial dilution. Each sample was measured in duplicate or triplicate.

3.4.7 Cell Cycle Analysis using Flow Cytometry

At least one million cells per sample were collected by centrifugation (5 minutes at 950 rpm) and washed once with 5 ml of PBS. After a second centrifugation step (5 minutes at 950 rpm), cells were resuspended in 0.5 ml PBS, followed by addition of 4.5 ml of ice-cold 70% ethanol and stored at –20°C for at least 1 day and up to 1 month. The cells were centrifuged (5 minutes at 950 rpm) and the ethanol was aspirated. Then, the cells were resuspended in 5 ml PBS, held at room temperature for 60 seconds and centrifuged again (5 minutes at 950 rpm). Finally, the cells were resuspended in 1 ml of the propidium iodide (PI)/Triton X-100 staining solution [0.1% (v/v) Triton X-100; 10 μg ml^{-1} PI; and 0.2 mg ml^{-1} RNase A in PBS] and incubated at room temperature for 30 minutes prior to the flow cytometry analysis. The flow cytometry measurements were carried out on EPICS® Elite flow cytometer (Coulter Corporation, Miami, FL) equipped with a single argon ion laser (λ_{ex} = 488 nm, λ_{em} = 610 nm). DNA distribution histograms were analyzed with the WinMDI Multi-Cycler 2.7 software.

3.4.8 RNA Analysis

RNA isolation and subsequent handling was conducted in a specially-designated RNA hood using nuclease-free equipment and reagents in order to minimize RNase degradation of the RNA.

3.4.8.1 Total RNA Isolation

Total RNA was isolated using TRIzol reagent (Invitrogen) as follows. One million cells were removed from cell culture, centrifuged at 950 rpm for 5 minutes, and the supernatant was aspirated. The cells were resuspended in 1 ml of TRIzol reagent, sheared 25 times using a 1 ml syringe and 2-1/2 G needle, and frozen at -80 °C for subsequent analysis. On the day of RNA isolation, the sheared cell mixture was thawed on ice and 200 µl of chloroform was added to the mix. The tubes were shaken vigorously for 30 seconds, incubated for 2 minutes at room temperature and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The upper aqueous phase (400 µl) was transferred to a fresh tube containing 400 µl of isopropanol. The sample was mixed by vortexing and then stored at -20 °C for at least 2 hours. Following the isopropanol precipitation, the samples were centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was aspirated from the RNA pellet, which was subsequently washed with 800 µl of 75% (v/v) ethanol in water and vortexed for 30 seconds. Then, the sample was centrifuged at 12,000 rpm for 2 minutes at 4 °C. The ethanol was aspirated from the RNA pellet, which was then air dried at room temperature inside the RNA hood. Once the pellet was dry (5-15 minutes), it was resuspended in 50 µl of nuclease-free water.

3.4.8.2 *RNA Quantification and Quality Assessment*

One microliter of RNA was combined with 4 µl of water and the concentration and quality of the total RNA was estimated from the absorbance at 260nm and 280nm, measured using a GeneQuant™ Pro RNA/DNA Calculator (Amersham Biosciences, Piscataway, NJ). The RNA concentration is obtained from the absorbance at 260 nm (an assumed 40 µg ml^{-1} for 1.0 OD of single-stranded RNA is standard practice) and the RNA quality can be assessed from the

ratio of the absorbance at 260nm to the absorbance at 280nm. A ratio of approximately 1.8 or higher is considered an indicator of a high quality RNA preparation and was achieved routinely.

3.4.8.3 First-Strand cDNA Synthesis

First-strand cDNA was synthesized from 1-5 μg of total RNA using Superscript reverse transcriptase (SSII) (Invitrogen) as follows. In a nuclease-free tube, 5 μl of total RNA (1-5 μg) was combined with 1 μl of oligo(dT) mix and 5 μl of nuclease-free water. The mixture was heated to 70 °C for 10 minutes and then chilled on ice for a few minutes. The contents of the tube were collected by a quick centrifugation and the following additional reagents were added to the tube: 4 μl 5X first strand buffer, 2 μl of 0.1M DTT, 1 μl of 10 mM dNTP mix, 1 μl of RNASIN (an inhibitor of RNase) and 1 μl of SSII (200U ml^{-1}). The tube contents were mixed gently and then incubated at 42 °C for 1 hour. Following cDNA synthesis, 1 μl of RNase was added and the mix was incubated for 20 minutes at 37 °C to degrade RNA prior to the polymerase chain reaction (PCR) analysis of the cDNA quantity.

3.4.8.4 Real Time PCR Quantification of IFN-γ and β-actin cDNA Levels

A 121-bp region of the IFN-γ cDNA was amplified using primers F378 (5'-GAATGTCCAACGCAAAGCAA) and R498 (5'-CTGGGATGCTCTTCGACCTC) and a 163-bp region of the β-actin cDNA was amplified using primers F3-ACTIN (5'-AGCTGAGAGGGAAATTGTGCG) and R9-ACTIN (5'-GCAACGGAACCGCTCATT). Primer sequences were chosen using the web-based xprimer software (xprimer, 2002). Both genes used the same real time protocol described below except that the annealing temperature used for IFN-γ was 61 °C and that for β-actin was 59 °C. Annealing temperatures for the two

genes were selected based on a gradient PCR optimization by testing annealing temperatures between 50 °C and 65 °C. The temperature that provided the sharpest and most intense PCR product when run on an agarose gel was selected as the annealing temperature. Results from this analysis for each gene are shown in Figure 3-1.

The real time PCR assay is as follows. The reaction was performed in 50 μl total reaction volume of a proprietary buffer (courtesy of Professor Heng-Phon Too, Biochemistry Department, National University of Singapore) containing 1X SYBR Green I (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs mix, 2mM $MgCl_2$, 0.2 μM of each primer, 1U of Dynazyme II thermostable DNA polymerase (2 U μl^{-1}) (Finnzymes Oy, Espoo, Finland), and 1.5 μl of a 1/10 dilution of the cDNA sample in water. Real-time quantification was performed using the Rotor-Gene 2000 system (Corbett Research, Mortlake, Australia). Cycling conditions were 3 minutes at 95 °C and then 40 cycles consisting of 30 seconds at 95 °C, 30 seconds at 61 °C (IFN-γ) or 59 °C (β-actin) and 30 seconds at 72 °C. The PCR product for each set of primers was verified as a single amplicon of the correct size by gel electrophoresis (Figure 3-1).

The fluorescence in each sample is measured after each cycle and is proportional to the amount of double stranded DNA at each step owing to the presence of the double-stranded DNA binding dye SYBR green I (λ_{ex} = 494 nm, λ_{em} = 521 nm). Examples of fluorescence versus cycle number are shown in Figure 3-2. The cycle at which a given sample crosses a threshold fluorescence value (threshold cycle, C_t) is proportional to the amount of starting DNA template and was calculated using the Rotor-Gene 2000 system version 4.6 software. A standard curve was generated by simultaneously running real time PCR on serial dilutions of IFN-γ cDNA standards of known concentration. A plot of C_t versus the logarithm of concentration is linear and can be interpolated to find the concentration of unknown samples. Example standard curves

are shown in Figure 3-3. Both the samples and standards were run in triplicate and final C_t values were taken as the average of the three runs.

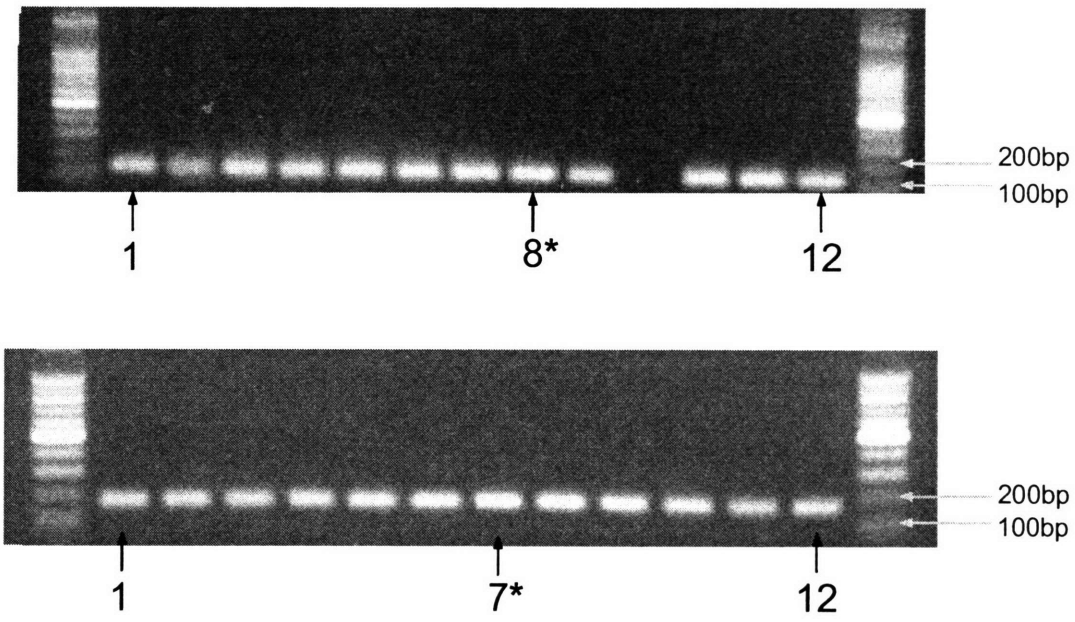

Figure 3-1. Gel electrophoresis of IFN-γ and β-actin PCR products at different PCR annealing temperatures. PCR products, from otherwise identical runs but using different annealing temperatures, were run on a 1% agarose gel (90V, 45 minutes) alongside Promega 100-bp DNA "step ladder" (Promega Corp, Madison, WI). Products from PCR using IFN-γ primers are shown in the top frame and product from PCR using β-actin primers are shown in the bottom frame. The products are verified to be single amplicons within the correct size range, namely 121-bp for IFN-γ and 163-bp for β-actin. The annealing temperature for each lane is as follows: (1) 50 °C; (2) 50.4 °C; (3) 51.2 °C; (4) 52.5 °C; (5) 54.2 °C; (6) 56.4 °C; (7) 58.9 °C; (8) 61.7 °C; (9) 62.7 °C; (10) 63.9 °C; (11) 64.7 °C; (12) 65 °C. The optimal band for each gene was identified by visual inspection and was judged to be lane (8) for IFN-γ and lane (7) for β-actin.

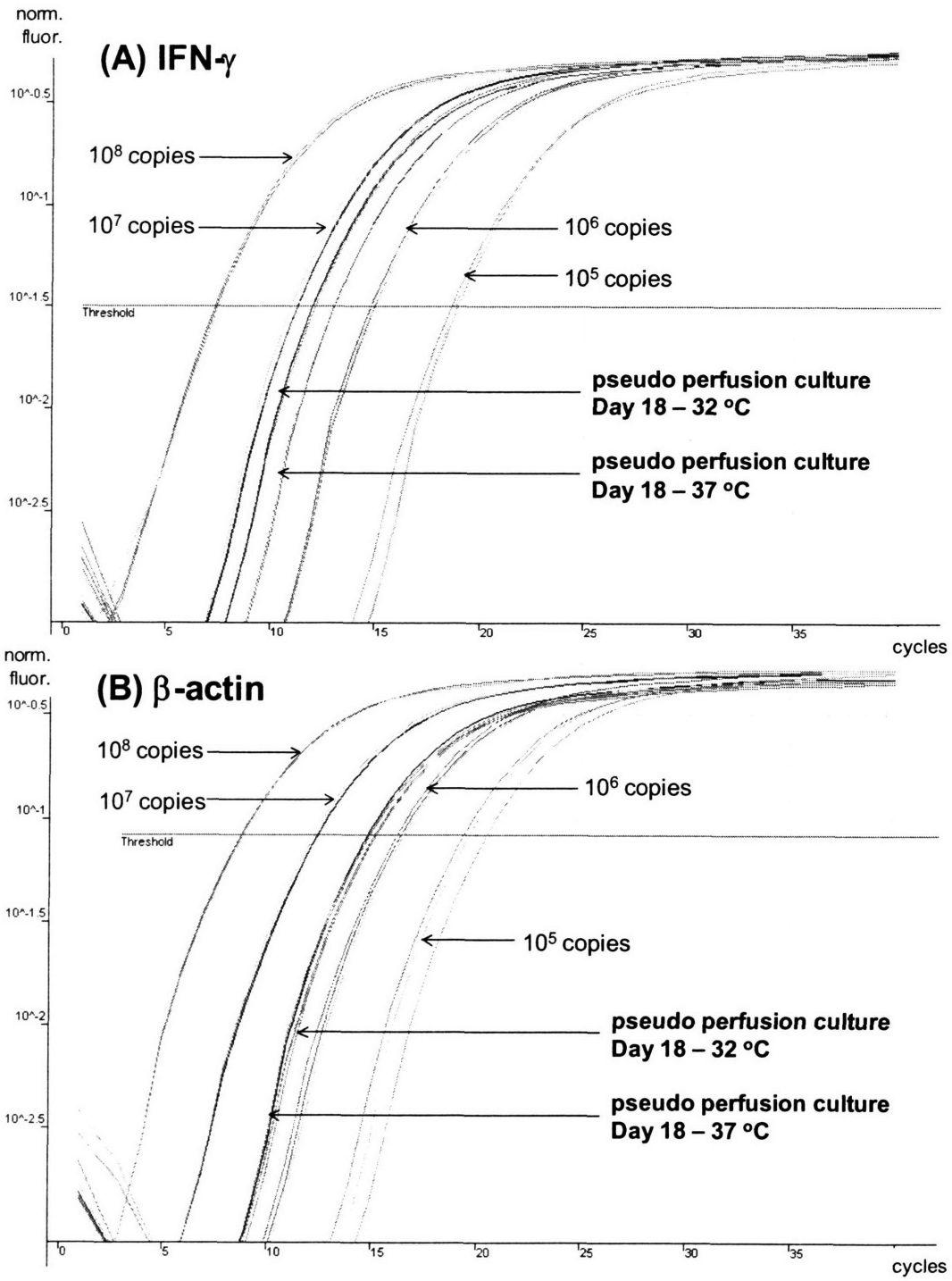

Figure 3-2. Real time PCR fluorescence versus PCR cycle number. An example of normalized fluorescence versus PCR cycle number for triplicates of (A) IFN-γ serial standards (10^5 – 10^8 copies per sample) and (B) β-actin serial standards (10^5 – 10^8 copies per sample) are shown. Threshold fluorescence was calculated by the RotorGene 2000 Version 4.6 software and is shown as a horizontal line on the figures. Two experimental samples from day 18 of the pseudo perfusion culture are also shown (refer to Chapter 6).

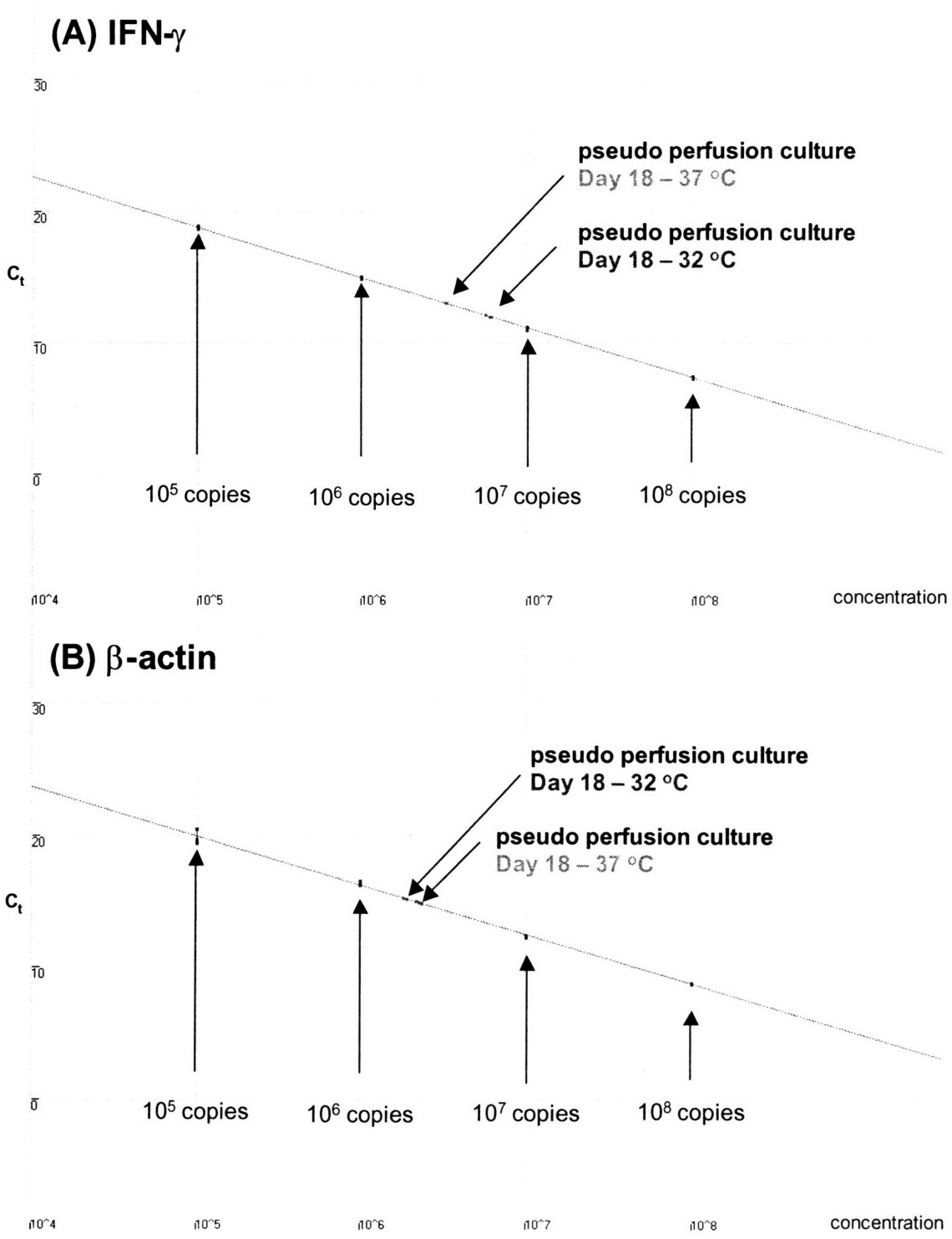

Figure 3-3. Threshold cycle (C_t) as a function of DNA copy concentration. The data from Figure 3-2 is plotted here. The concentration of the standards is known and from these values, the standard curves for (A) IFN-γ and (B) β-actin are constructed and the concentration of each gene in each sample can be interpolated from the standard curve.

4. HYPOTHERMIA INCREASES IFN-γ PRODUCTION IN BATCH AND FED-BATCH CULTURES

Given that not all CHO cell lines exhibit enhanced hypothermic productivity, the first objective was to determine whether CHO-γ is one such enhanced hypothermic producer, and to do so under a variety of culture conditions.

4.1 BATCH SHAKER FLASK CULTURE

As an initial assessment of the response of CHO-γ cells to changes in growth temperature, cells were grown in shaker flasks at 32 °C, 37 °C and 40 °C. The growth curves are shown in Figure 4-1 and the viability curves in Figure 4-2. Cells grown at elevated temperature, 40° C, stop growing and rapidly die. In addition, specific productivity of IFN-γ is very low (data not shown). Thus, elevated temperature adversely affects growth and production and was not considered further. In contrast, cells grown under hypothermic conditions, 32 °C, become growth arrested but are able to maintain high viability for an extended period of time. The growth arresting effect of low temperature is apparent by comparing the cell cycle distribution of 32 °C to 37 °C cells 1 day after cell seeding, as shown in Figure 4-3. These results show a significant shift of cells towards the stationary G_0/G_1 phase at low temperature. Thus, the prolonged period of growth arrest and high viability suggests low temperature culture may be a feasible strategy for increasing IFN-γ production.

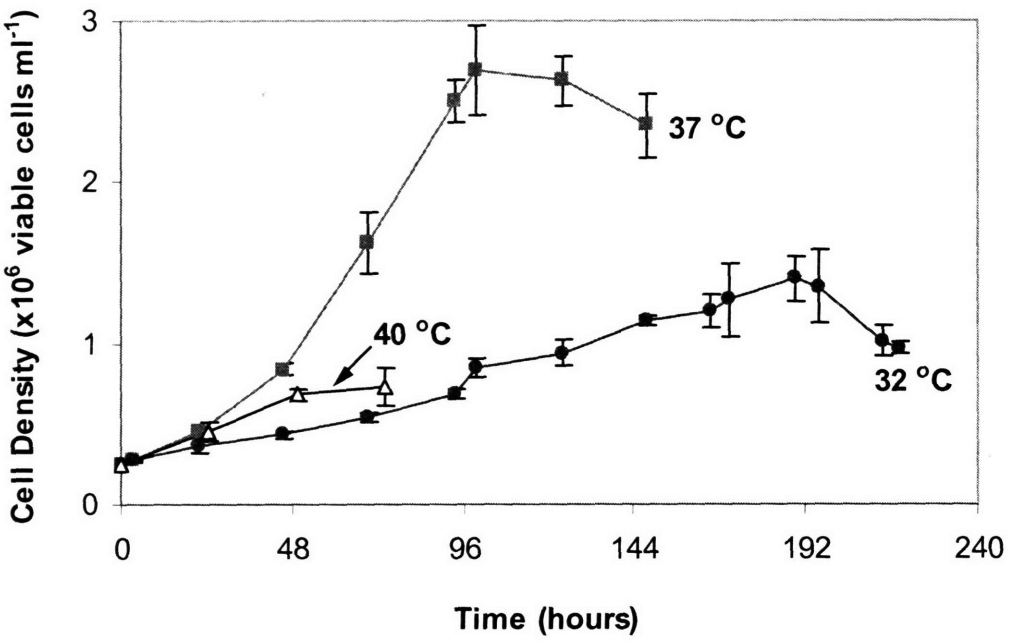

Figure 4-1. Shaker flask growth curves at different culture temperatures. Cells were grown at 32 °C (•), 37 °C (■) or 40 °C (Δ). The cultures were terminated once the percent cell viability dropped below 90%. For all figures in this thesis, the error bars are ± one standard deviation.

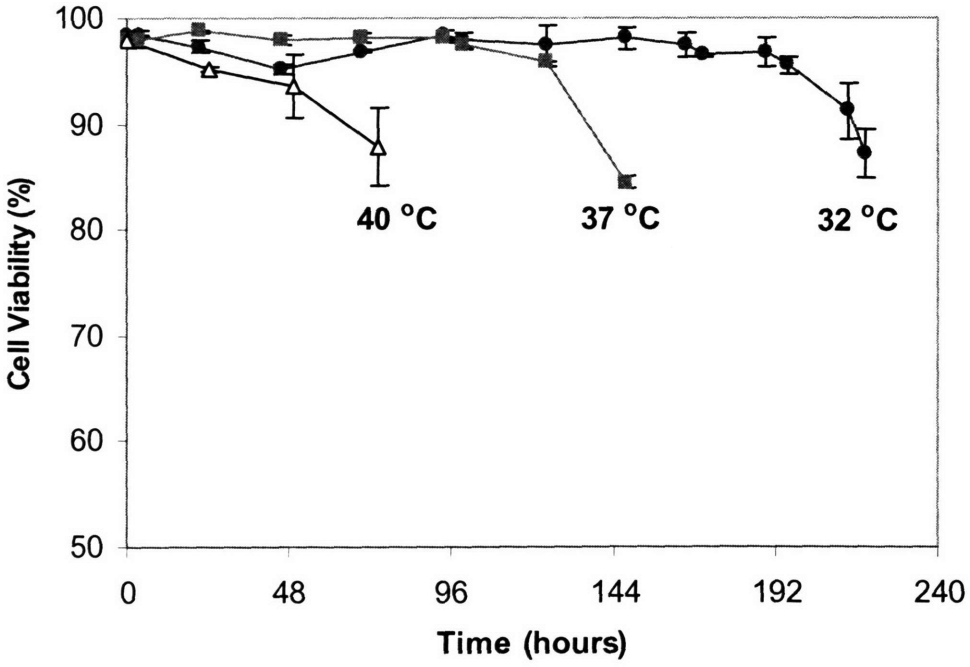

Figure 4-2. Shaker flask viability curves at different culture temperatures. Cells were grown at 32 °C (•), 37 °C (■) or 40 °C (Δ). Each culture was ended once the percent cell viability dropped below 90%.

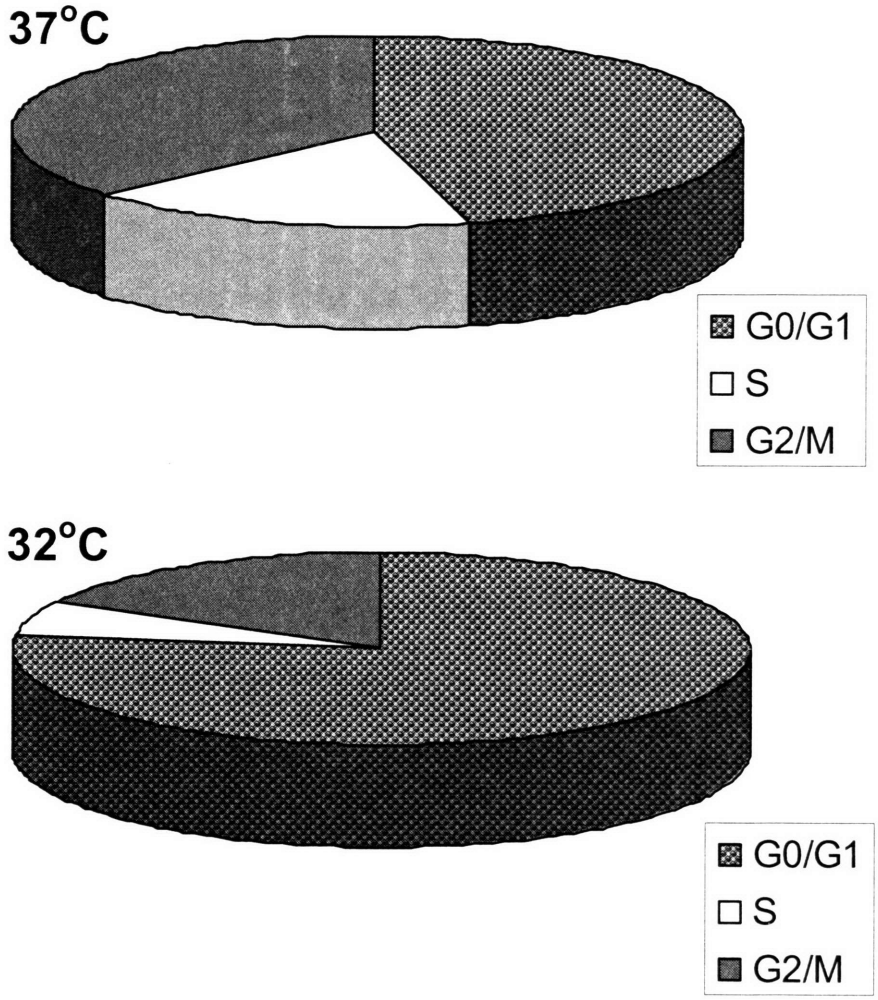

Figure 4-3. Cell cycle distribution one day post-seeding. Cells from the same cell seed stock were grown for 24 hours at either 32 °C or 37 °C and their cell cycle distribution was then measured. Hypothermia increases the percentage of G_0/G_1 cells significantly.

To test the effect of low temperature on productivity, IFN-γ concentration at various time points was measured. A plot of IFN-γ concentration versus the integral of viable cell density (IVCD) will give a line with slope equal to the average specific productivity (Renard et al, 1988), as shown in Figure 4-4. The average specific productivity of IFN-γ at low temperature is approximately twice the value at 37 °C (3.0×10^{-8} μg $cell^{-1}$ hr^{-1} compared to 1.5×10^{-8} μg $cell^{-1}$ hr^{-1}). Also, the total IVCD for the batches is approximately the same, meaning that the total IFN-γ production is doubled at low temperature. However, one should note that the low temperature process took about 50% longer to complete (Figure 4-1). This would mean that volumetric IFN-γ productivity is only marginally improved by using low temperature. The implications of this finding will be discussed in Chapter 5, wherein a model for optimizing IFN-γ volumetric productivity by shifting the temperature from 37 °C (growth phase) to 32 °C (production phase) at a certain time point in culture will be presented.

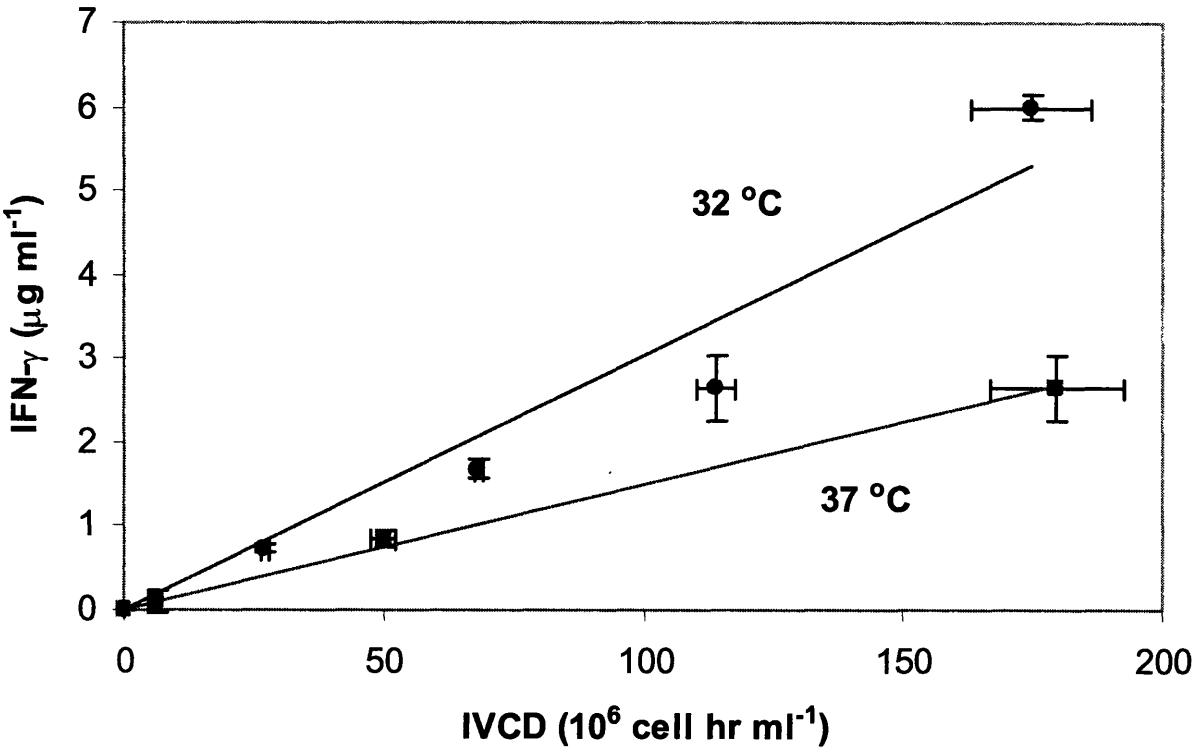

Figure 4-4. IFN-γ concentration versus IVCD for shaker flask cultures. The slope of the curve is the average specific productivity at 32 °C (●) and 37 °C (■). The lines shown are linear least-squares fits of each data set.

4.2 BATCH BIOREACTOR CULTURE

One drawback of using shaker flasks is that the culture conditions, such as pH, dissolved oxygen (DO) concentration and temperature, cannot be accurately controlled. To verify that the increase in specific productivity of CHO-γ observed at low temperature was a result of only the effect of temperature, instead of due to the uncontrolled nature of the shaker flasks, the batch cultures were repeated in bioreactors. The bioreactor temperature, pH and DO levels can be tightly controlled, meaning that any changes seen in culture behavior at the different temperatures can be more directly attributed to the temperature difference itself, instead of due to an extraneous effect. The growth and productivity curves for the batch bioreactors are shown in Figure 4-5 and Figure 4-6, respectively. As was the case with the shaker flasks, low temperature culture results in an approximate 2-fold increase in average specific productivity (4.9×10^{-8} μg $cell^{-1}$ hr^{-1} compared to 2.3×10^{-8} μg $cell^{-1}$ hr^{-1}), providing evidence that the positive effect of low temperature is not due to extraneous changes in pH or DO levels, both of which were tightly controlled during bioreactor operation.

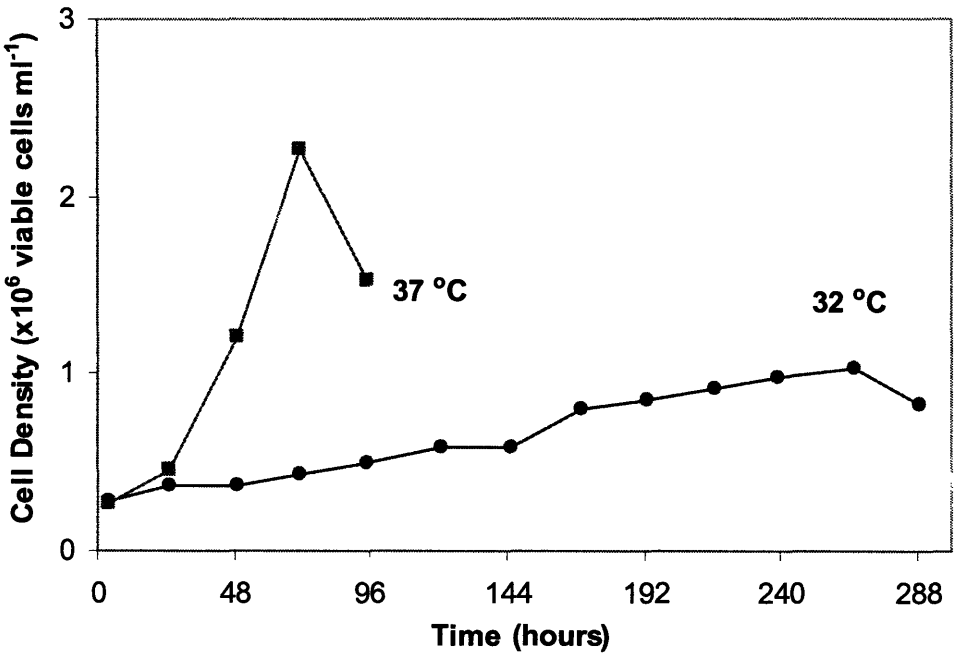

Figure 4-5. Batch bioreactor growth curve. Cells were grown at either 32 °C (●) or 37 °C (■). The cultures were terminated once the percent cell viability dropped below 80%.

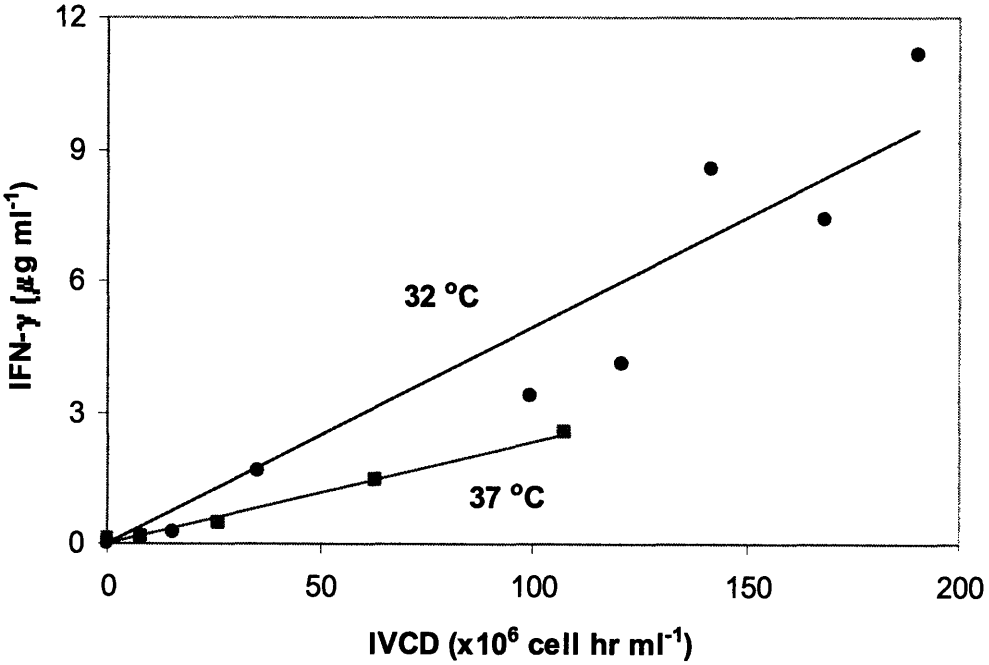

Figure 4-6. IFN-γ concentration versus IVCD for batch bioreactor cultures. The slope of the line gives the average specific productivity of IFN-γ at 32 °C (●) and 37 °C (■). The lines shown are linear least-squares fits of each data set.

4.3 FED-BATCH BIOREACTOR CULTURE

It has been previously reported that CHO cells usually exhibit reduced metabolism at low culture temperatures (Section 2.4.2). Coupled with the reduced growth rate at low temperature (Figure 4-5), the result is that the concentration of nutrients in a 32 °C batch bioreactor rapidly becomes markedly different from a 37 °C batch bioreactor, with the 32 °C culture exhibiting higher nutrient concentrations. Thus, it is feasible that the positive effect of low temperature culture on productivity is an artifact of reduced nutrient depletion, with low temperature cells being exposed to higher average nutrient concentrations during the course of the culture and consequently having more nutrient resources for IFN-γ production. To test this possibility, fed-batch bioreactors were used, wherein concentrated feed medium was added to the bioreactors twice daily to prevent nutrient depletion. The growth and productivity curves for the fed-batch runs are shown in Figure 4-7 and Figure 4-8, respectively. Again, as was the case with the shaker flasks and the batch bioreactor cultures, low temperature culture results in an increase in average specific productivity (4.1×10^{-8} μg $cell^{-1}$ hr^{-1} compared to 2.7×10^{-8} μg $cell^{-1}$ hr^{-1}), despite the fact that cells at both temperatures were in a high nutrient concentration environment throughout the culture. Thus, the enhanced productivity seen at low temperature is not due to reduced nutrient depletion.

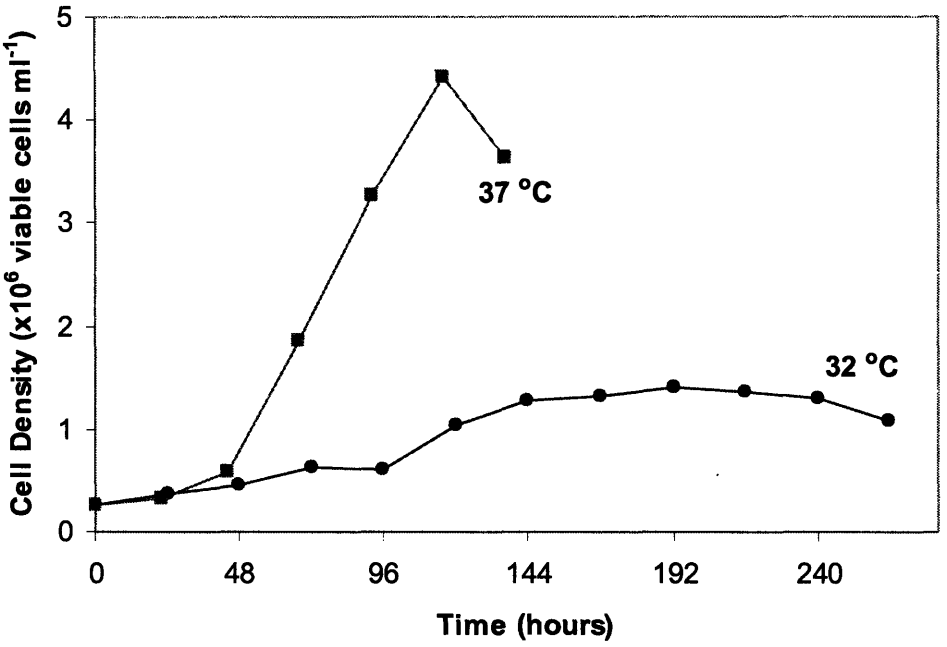

Figure 4-7. Fed-batch bioreactor growth curves. Cells were grown at 32 °C (●) or 37 °C (■) and fed a 10X concentrated feed medium twice daily. The cultures were terminated once the percent cell viability dropped below 80%.

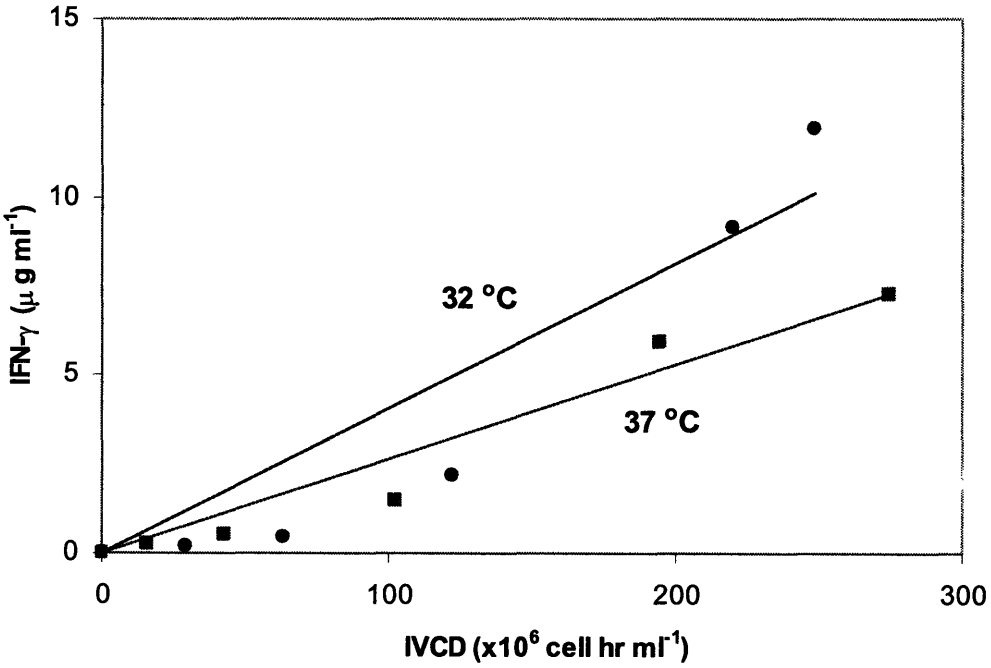

Figure 4-8. IFN-γ concentration versus IVCD for fed-batch bioreactor cultures. The slope of the curve gives the average specific IFN-γ productivity at 32 °C (●) and 37 °C (■).

Unlike for the batch cultures, the specific productivity in the fed-batch appears to be increasing as the culture progresses. This may be due to the increase in osmolality caused by adding the concentrated feed twice daily (Figure 4-9). As discussed in Section 2.3.1, hyperosmotic pressure is known to cause increased specific productivity of a variety of heterologous proteins by different cell lines, including CHO. Because hyperosmotic pressure is generally associated with increased specific productivity and because the osmolality of the 37 °C culture increased 45% whereas that of the 32 °C culture only increased 22%, hyperosmotic pressure would have a greater impact on the 37 °C culture and thus is likely not the cause of the higher specific productivity seen when comparing 32 °C to 37 °C.

4.4 CONCLUSIONS

This chapter has shown that mild hypothermic culture enhances specific IFN-γ productivity by CHO-γ (Table 4-1). This effect is evident even in fed-batch, well-controlled bioreactors, eliminating nutrient depletion and DO or pH differences as the cause for hypothermic increase in productivity seen in batch shaker flasks and batch bioreactors. However, potential drawbacks of low temperature culture, namely low cell concentrations and longer batch times, have also been identified. These drawbacks will be addressed in the next chapter, which presents a model for temperature shift optimization. In subsequent chapters, the cellular mechanism behind the increase in productivity will be studied. Based on the findings from the mechanistic work, several ways to further optimize hypothermic culture for obtaining maximum IFN-γ production will be presented.

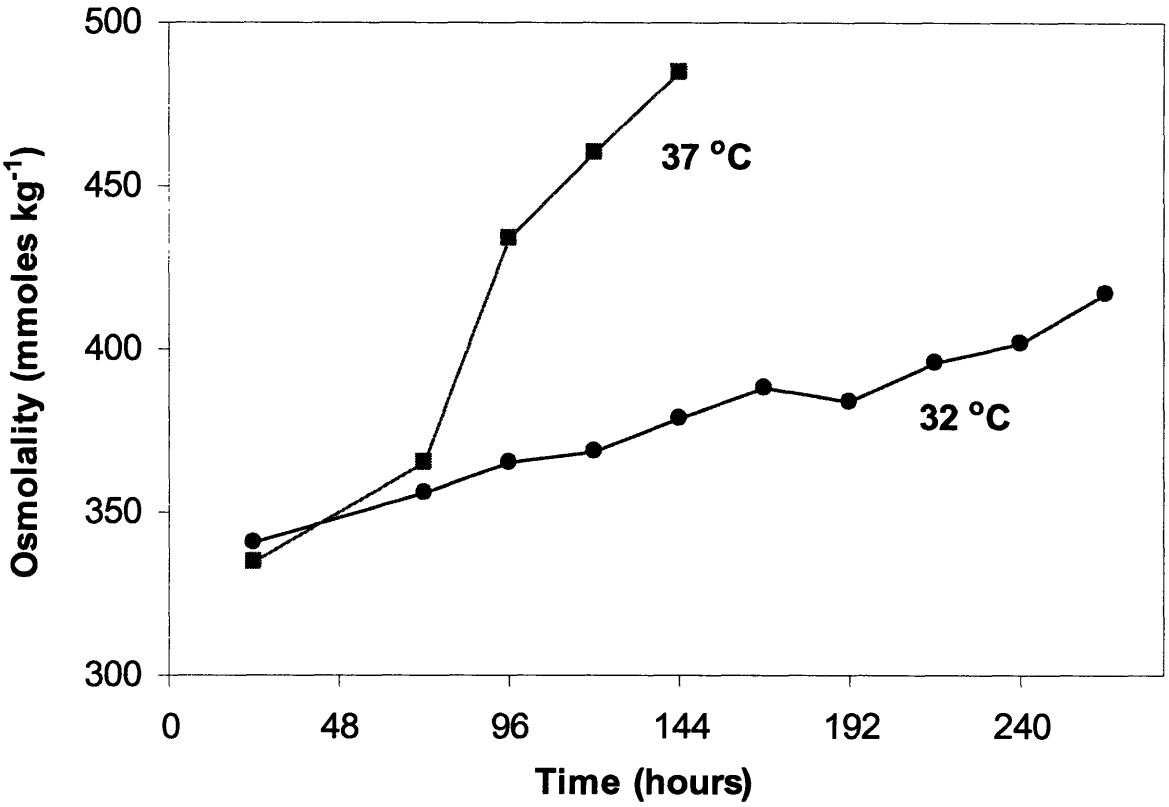

Figure 4-9. Fed-batch bioreactor osmolality curves. Cells were grown at 32 °C (●) or 37 °C (■) and fed a 10X concentrated feed medium twice daily, causing osmolality to increase with time.

Table 4-1. Effect of culture temperature on IFN-γ production

	Productivity (10^{-8} μg cell^{-1} hr^{-1})		Total Production (μg ml^{-1})	
Culture Condition	**32 °C**	**37 °C**	**32 °C**	**37 °C**
Batch Flasks	3.0	1.5	6.0	2.7
Batch Bioreactor	4.9	2.3	11.2	2.6
Fed-batch Bioreactor	4.1	2.7	11.9	7.3

5. TEMPERATURE SHIFT OPTIMIZATION MODEL FOR MAXIMIZING IFN-γ VOLUMETRIC PRODUCTIVITY

In Chapter 4, it was shown that the CHO-γ cell line has higher specific productivity of IFN-γ at 32 °C compared to 37 °C. However, the drawback of culturing at 32 °C is the extended batch times, owing to the low cell density and correspondingly slower consumption of nutrients. As a result, volumetric productivity may not be significantly increased under hypothermic conditions. In this chapter, a model is proposed and validated for optimizing volumetric productivity in hypothermic culture.

Several groups have recognized that in order to maximize the volumetric productivity, a good strategy is to use a biphasic process. In this case, the cells are grown at 37 °C for a certain period of time to obtain a suitable cell density, followed by shifting the culture to a reduced temperature, wherein the specific productivity of recombinant protein is higher, for the remaining period (Furukawa and Ohsuye, 1999; Hendrick et al, 2001; Kaufmann et al, 1999). For example, by using an arbitrary temperature shift of 48 hours following inoculation, Furukawa and colleagues improved the recombinant protein production for a 9 day culture by approximately 1.7-fold above the value obtained at 32 °C, which was already 2-fold higher than the value obtained at 37 °C.

However, despite the recognized value of the temperature shift strategy described above, there have been no attempts to optimize the point in time at which the culture temperature is shifted. There is a tradeoff between the two key parameters for determining total production: the high cell densities obtained at 37 °C and the high specific productivity obtained at 32 °C. Given this tradeoff, there exists an optimal time at which to shift the culture temperature to obtain maximum volumetric productivity.

The goal of this part of the thesis was to develop and validate a model for rationally selecting the optimal temperature shift time for maximizing recombinant protein volumetric production. The model is based on simultaneously solving cell, glucose and recombinant protein balances at 37 °C, 32 °C, or in a culture shifted from 37 °C to 32 °C at a specified time. This study was conducted using suspension cultures grown in Erlenmeyer flasks. The data from Chapter 4, namely that the positive effect of hypothermia is valid when scaling up from the flask to the bioreactor, shows that the model developed here should be applicable to the industrially-relevant bioreactor environment as well.

The first set of cultures was conducted solely at 32 °C and 37 °C and was used to generate data for the optimization model (Model Development). Following this first set of cultures, the temperature shift experiments were conducted for validating the model (Model Validation). For these cultures, the flasks were transferred from the 37 °C to the 32 °C incubator at the stated temperature shift times, which were 1, 2, 3 or 4 days following inoculation. For all experiments, cultures were terminated once the percent cell viability decreased below 90%, which signaled the onset of massive cell death.

5.1 MODELING METHODOLOGY

The objective of the model is to predict time profiles of cell, glucose and IFN-γ concentrations and the time at which the onset of massive cell death occurs during culture at either 37 °C, 32 °C or in a 37 °C to 32 °C temperature shift culture. Based on the model predictions, an optimal time to shift temperature in order to maximize IFN-γ volumetric productivity can be designated *a priori*.

The first set of experiments, conducted only at 32 °C or 37 °C, was used to generate the data needed for the model, namely specific growth rate (μ), specific glucose consumption rate (q_S) and specific IFN-γ production rate (q_{IFN}) at the two temperatures. The model consists of batch balance equations on viable cells (X), glucose (S) and IFN-γ (P) (equations 5-1 through 5-3) and assuming the Monod model is valid for predicting specific growth rate (5-4) and specific glucose consumption rate (5-5):

$$\frac{dX}{dt} = \mu X \qquad (5\text{-}1)$$

$$\frac{dS}{dt} = -q_S X \qquad (5\text{-}2)$$

$$\frac{dP}{dt} = q_{IFN} X \qquad (5\text{-}3)$$

$$\mu = \frac{\mu_{max}.S}{K + S} \qquad (5\text{-}4)$$

$$q_S = \frac{q_{max}.S}{K_S + S} \qquad (5\text{-}5)$$

The parameters in equations (5-4) and (5-5) were determined by nonlinear regression from the 32 °C and 37 °C experimental data using NLREG Version 5.3 Software (Sherrod, 2003).

An assumption included in the model is a 12-hour lag phase following culture inoculation, during which time the viable cell density was assumed to remain constant. This assumption is consistent with the 10-18 hour lag exhibited by another CHO IFN-γ cell line (Lloyd et al, 1999). It was also assumed that the various parameters (μ_{max}, K, q_{max}, K_s and q_{IFN}) changed instantaneously from the 37 °C to the 32 °C culture values following the temperature shift. This instantaneous change is not necessarily correct, but for simplicity of modeling, this approach was followed. Finally, in the batch culture, and therefore the simulation studied, it was assumed that the onset of massive cell death coincided with the depletion of glucose. The basis for this assumption will be discussed in more detail later. We defined glucose depletion as the glucose concentration reaching less than 1% of the starting concentration. Prior to the onset of glucose depletion, cell death was neglected, an assumption consistent with the high viabilities (>95%) exhibited during all cultures, as will be shown in later figures.

Equations (5-1) through (5-5), in conjunction with the assumptions outlined above, were solved simultaneously on Microsoft® Excel 2002 (Microsoft Corporation, Redmond, WA) using the finite difference method with one hour step size to obtain time profiles of cell, glucose and IFN-γ concentration. The numerical values of the various model parameters were changed from the 37 °C to 32 °C values at the modeled time of temperature shift. Shifts of 1, 2, 3 or 4 days post-inoculation were modeled. From the solution to the glucose balance equation, the time at which glucose was depleted was found. This time represents how long the batch operates prior to the onset of massive cell death, as will be supported later by the data. From the IFN-γ balance equation, the IFN-γ concentration at this point in culture time was known and IFN-γ volumetric productivity was then found by dividing the total IFN-γ concentration by the batch time (μg ml^{-1}

day^{-1}). From these results, the optimal temperature shift time, namely the time that maximizes the volumetric productivity, was found.

5.2 MODEL DEVELOPMENT

The model was developed based on the growth, glucose consumption and production kinetics exhibited by the CHO-γ cells at 32 °C or 37 °C. The parameters fitted from the 32 °C and 37 °C data are presented in Table 5-1 and the source data will be discussed in this section and shown in the accompanying figures.

The growth arresting effect of using low temperature is clear by comparing 32 °C to 37 °C growth (Figure 5-1). Specific growth rate was found to follow the Monod model (5-4) for 37 °C growth (Figure 5-2A). In contrast, the specific growth rate at 32 °C did not follow the Monod model very well and instead exhibited a more constant growth rate during most of the culture (Figure 5-2B). This is reflected by the low value of K for 32 °C compared to 37 °C (see Table 5-1).

The specific consumption rate of glucose was found to follow reasonably well the proposed Monod equation (5-5) at both culture temperatures (Figure 5-3), although at 37 °C the saturation glucose concentration has not been reached and consequently the consumption rate is quite linear for the glucose concentrations relevant in this study.

The specific IFN-γ productivity was assumed to be time invariant and was determined by the slope of IFN-γ concentration versus IVCD (Renard et al, 1988), which was determined by numerically integrating equation (5-1). The CHO-γ cell line exhibits a two-fold increase in specific productivity at 32 °C compared to 37 °C, as seen from the slopes of the lines in Figure 5-4.

Table 5-1. Temperature shift optimization model parameters

Parameter	32 °C	37 °C
μ_{max} (hr^{-1})	0.011	0.036
K (mg ml^{-1})	0.10	0.80
q_{max} (mg $cell^{-1}$ hr^{-1})	4.6×10^{-8}	2.4×10^{-7}
K_s (mg ml^{-1})	1.2	11
q_{IFN} (μg $cell^{-1}$ hr^{-1})	3.0×10^{-8}	1.5×10^{-8}

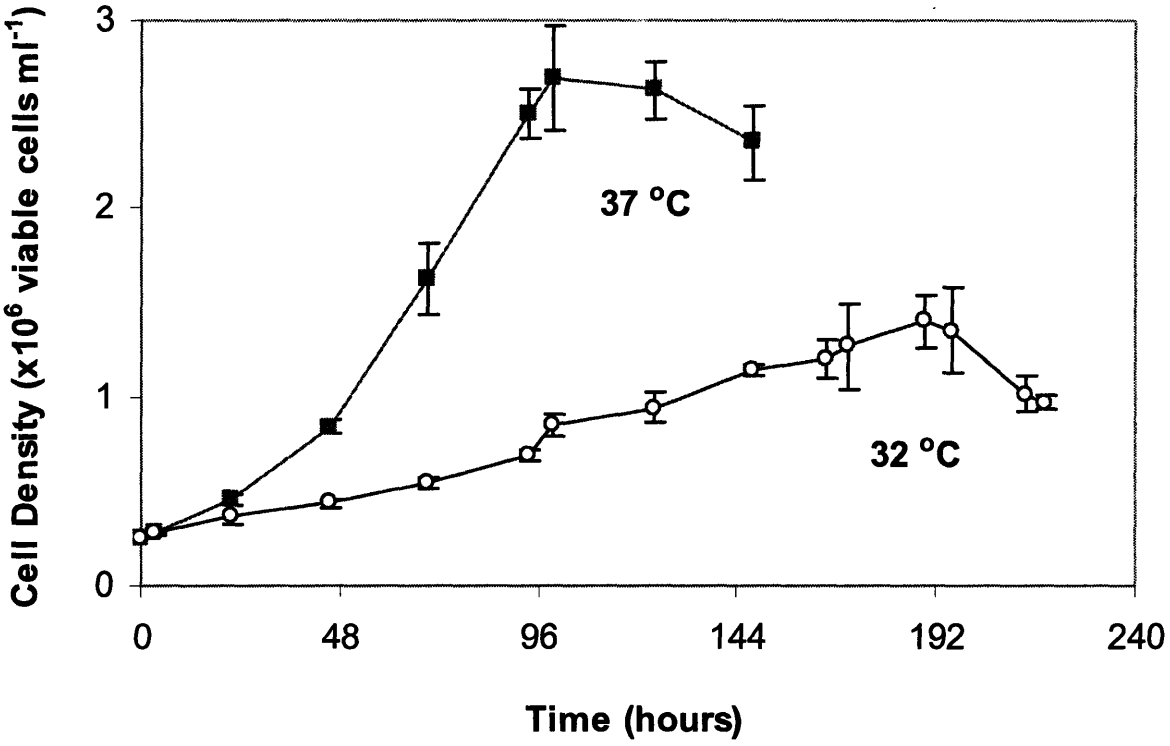

Figure 5-1. Low temperature reduces cell growth. Cell concentration profile at 32 °C (○) and 37 °C (■). The cultures were terminated once the percent cell viability dropped below 90%.

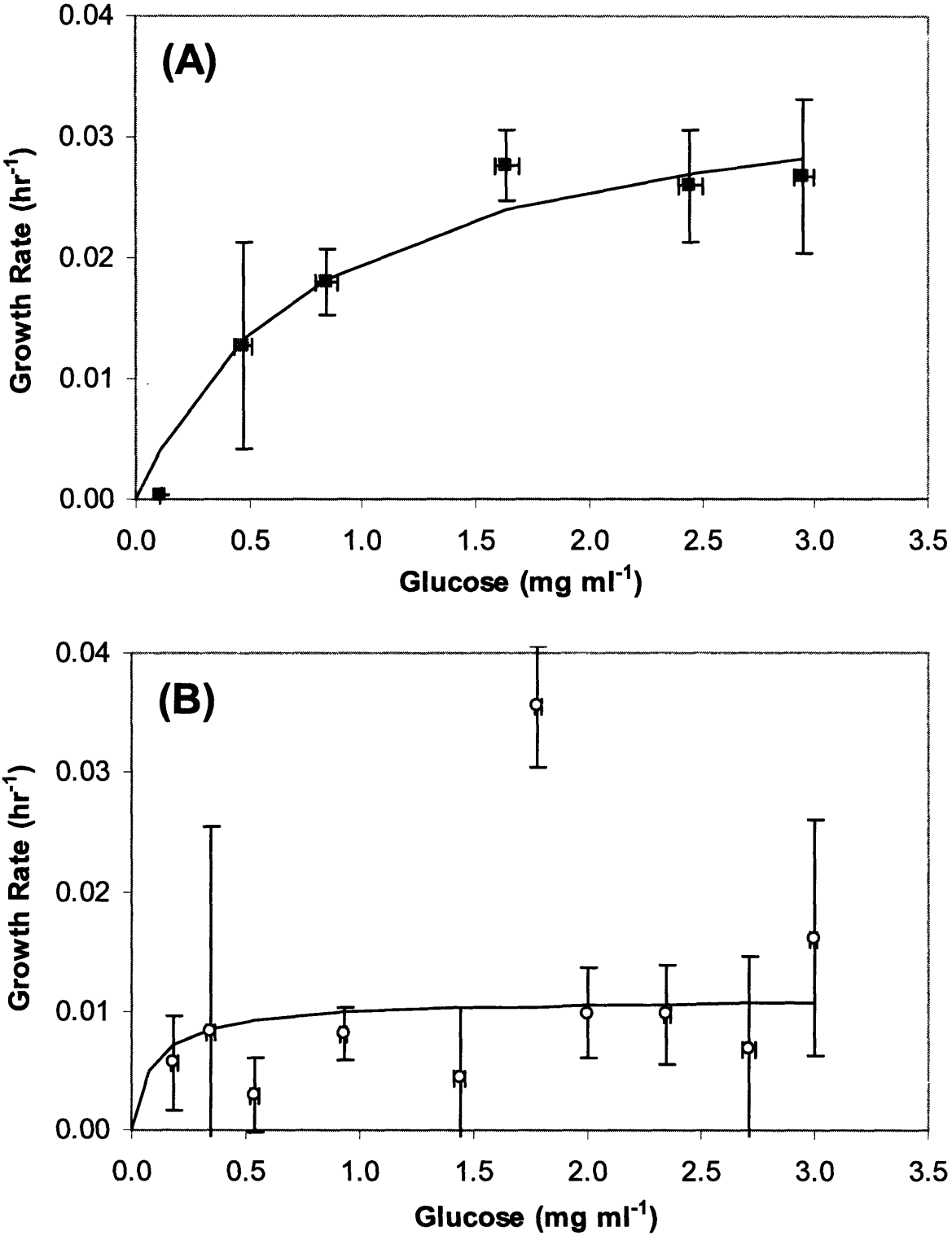

Figure 5-2. Growth rate as a function of glucose concentration. Monod model prediction (-) compared to experimental data for (A) 37 °C culture (■) and (B) 32 °C culture (○).

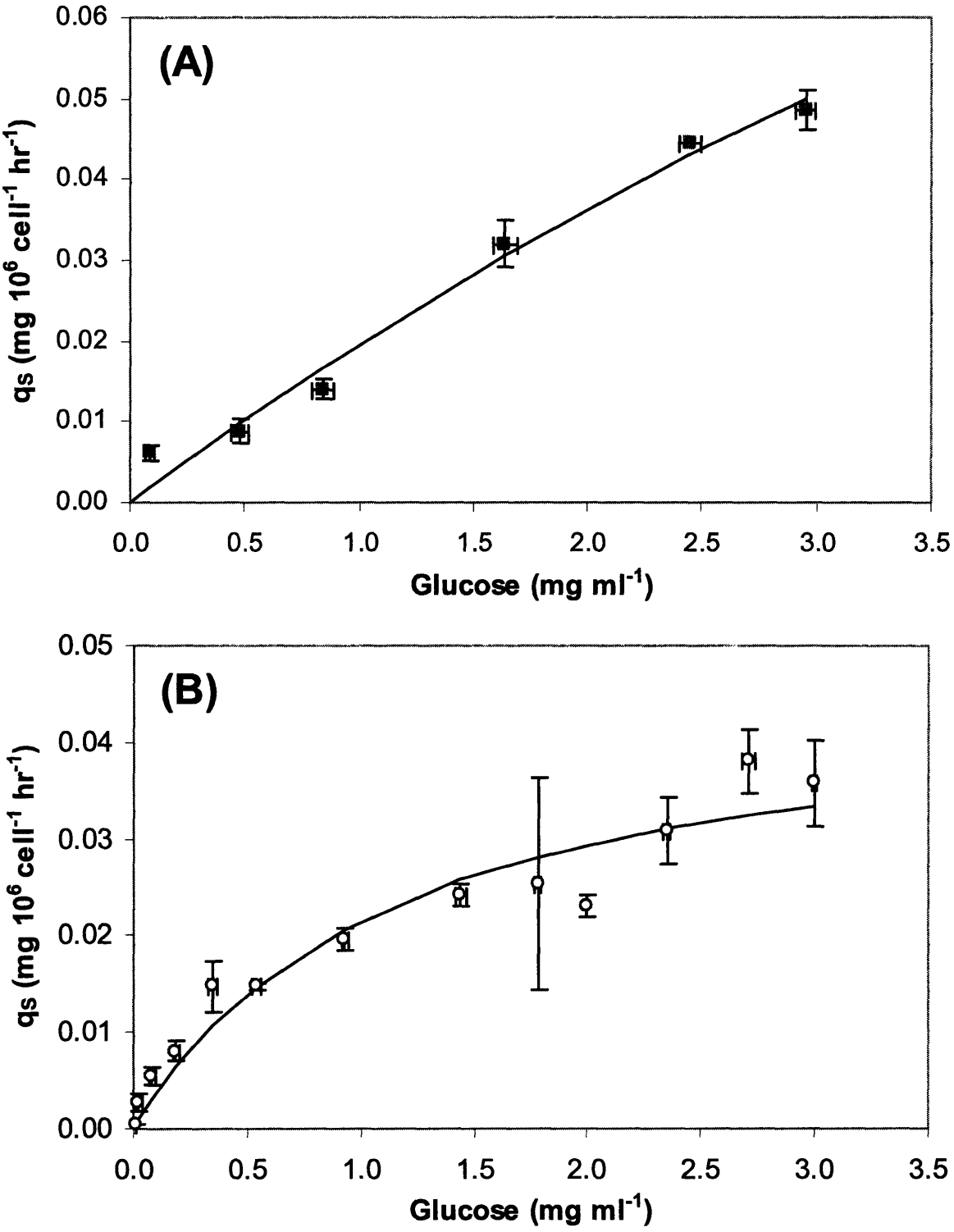

Figure 5-3. Glucose consumption rate as a function of glucose concentration. Monod model prediction (-) compared to experimental data for (A) 37 °C culture (■) and (B) 32 °C culture (○).

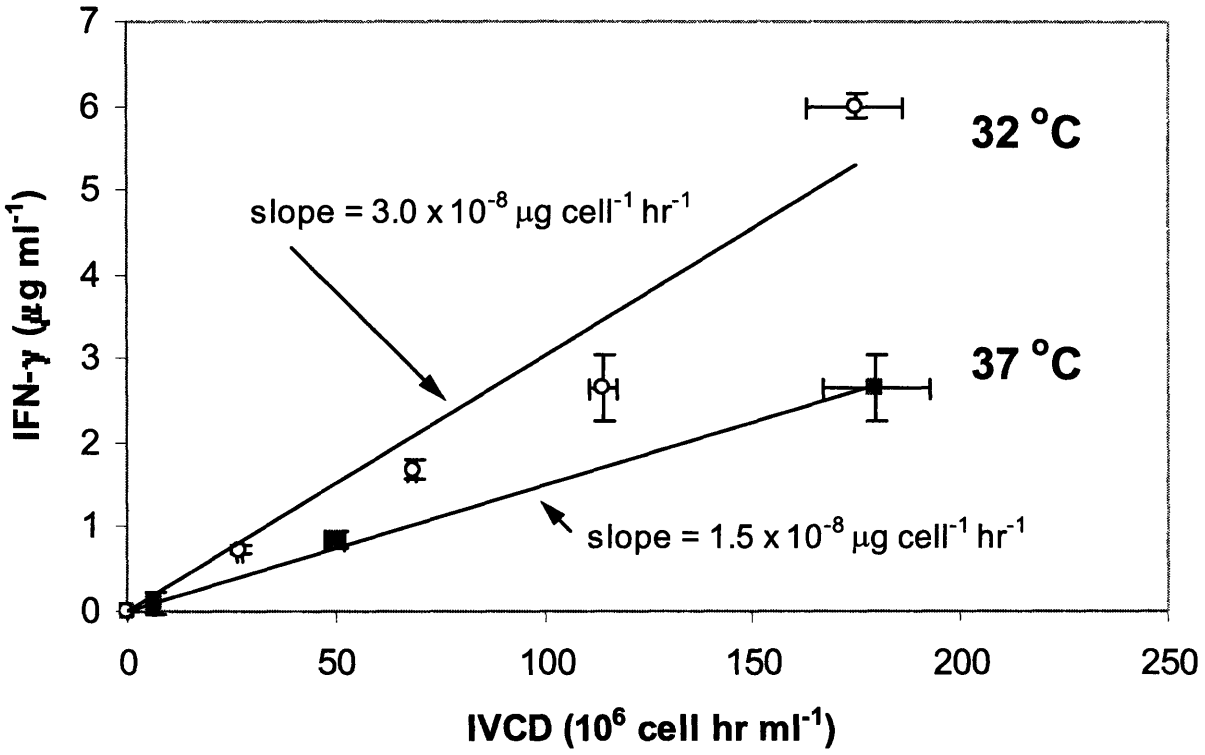

Figure 5-4. IFN-γ concentration as a function of IVCD. Average specific productivity, q_{IFN}, is the slope of the least-squared linear fit for 32 °C (○) and 37 °C (■).

The end-of-batch IVCD obtained at both temperatures is about the same, and consequently the total production at 32 °C is 125% the value at 37 °C (6.0 μg ml^{-1} at 32 °C compared to 2.7 μg ml^{-1} at 37 °C). However, the 32 °C culture took almost 9 days (215 hours) to complete whereas the 37 °C culture required only about 5 days (125 hours). This would mean that the IFN-γ volumetric productivity is only 40% higher at 32 °C (0.70 μg ml^{-1} day^{-1} at 32 °C compared to 0.51 μg ml^{-1} day^{-1} at 37 °C). This observation suggests that significant improvement of volumetric productivity can be achieved by using a temperature shift strategy, which will shorten the batch time by obtaining rapid growth in the early phase of the culture while still obtaining the high productivity characteristic of 32 °C later in the culture, which is the period of high cell density.

A key assumption in the model is that massive cell death would begin with the depletion of glucose and that the batch culture would be terminated at this point. The rationale for this assumption is that in an industrial production process, one would not want to compromise the product quality and downstream purification by allowing a significant amount of cell death prior to the end of the process, and hence the culture would be ended once the onset of significant cell death was detected. The assumed correlation between glucose depletion and the onset of cell death is validated by the data, as shown in Figure 5-5, where the last day when cell viability was greater than 90% coincides with the onset of glucose depletion.

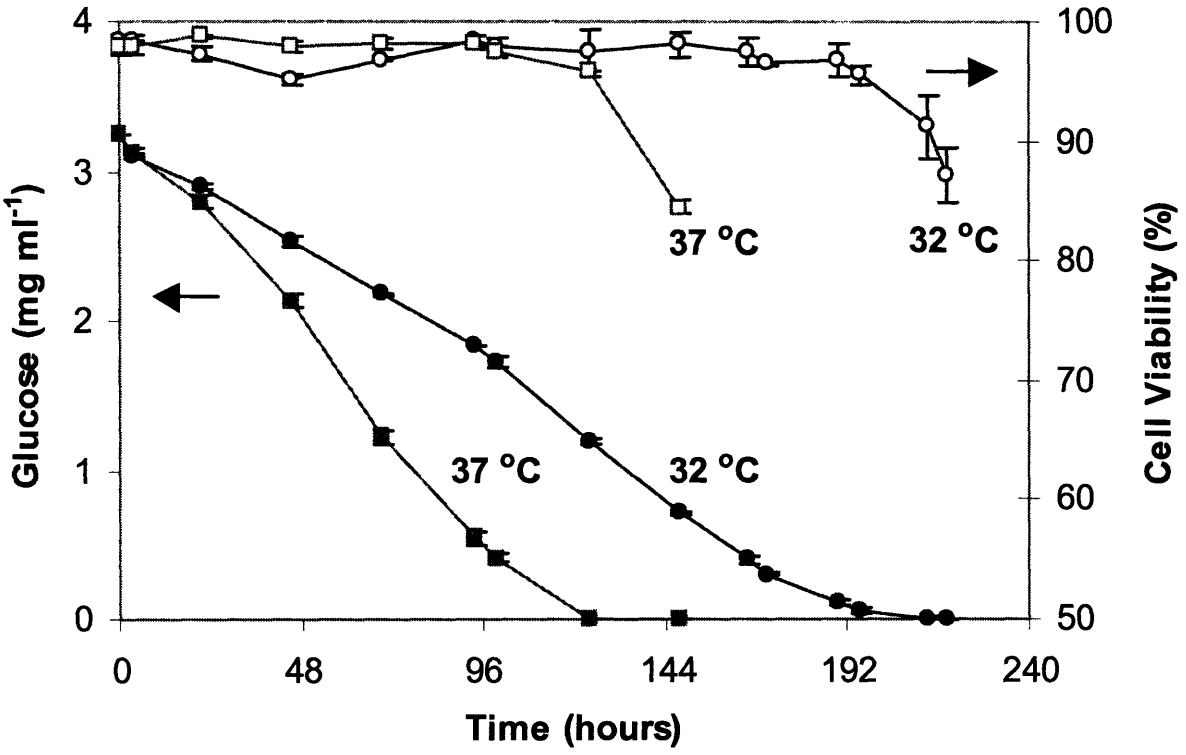

Figure 5-5. Correlation between glucose depletion and the onset of massive cell death. The drop in percent viability to below 90% at 32 °C (○) and 37 °C (□) coincides with the depletion of glucose at 32 °C (●) and 37 °C (■), respectively. The last day at which viability is above 90% occurs on the same day that glucose is depleted (<0.03 mg ml^{-1}; see text for details).

5.3 MODEL VALIDATION

The model was validated by comparing its ability to predict cell growth, glucose consumption, IFN-γ production and the onset of death for cultures shifted from 37 °C to 32 °C at 1, 2, 3, or 4 days post-inoculation. Figure 5-6 shows the predicted versus actual maximum cell densities that were obtained for each of the four temperature shifts. The model simulations matched the data reasonably well, with differences between model and experimental data of 15% or less. Thus, cell growth kinetics responds as predicted from the model equations and values in Table 5-1 when the temperature is shifted from 37 °C to 32 °C.

Figure 5-7 shows the model versus experimental glucose concentration for a 3-day temperature shift experiment. The agreement between the model and experimental data for the temperature shift is reasonably good, suggesting that CHO glucose metabolism responds quickly to a temperature shift to establish a new consumption rate similar to that of a culture run at 32 °C exclusively. The other temperature shift cultures yielded similar and satisfactory levels of agreement.

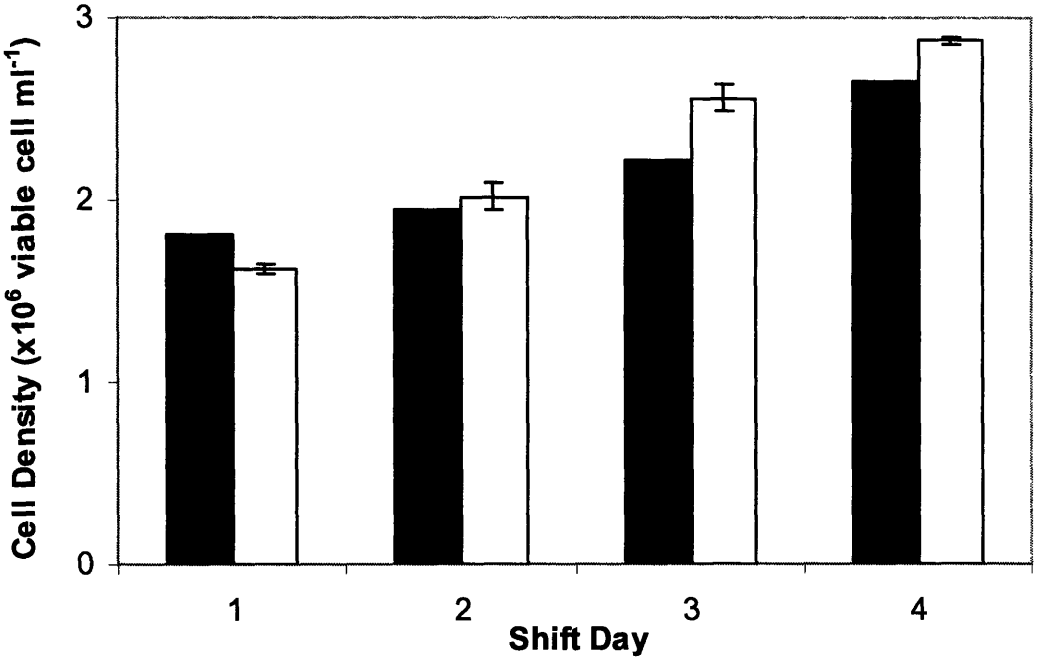

Figure 5-6. Peak cell density as a function of temperature shift day. Model prediction (■) compared to experimental data (□).

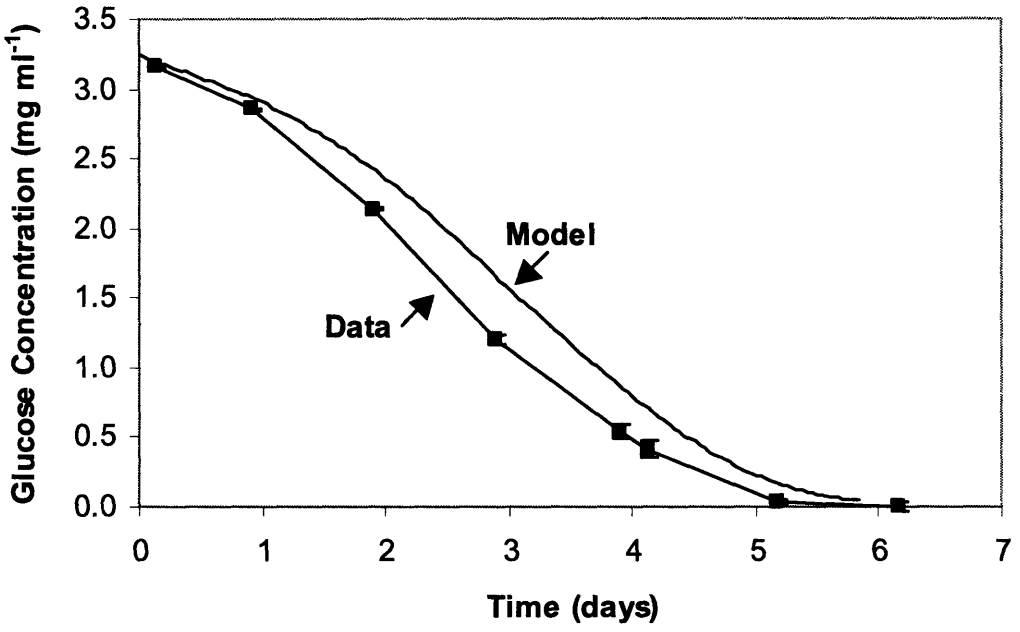

Figure 5-7. Glucose concentration profile for a shift from 37 °C to 32 °C on the third day of culture. Model prediction (-) compared to actual data (■).

As was the case with the 32 °C and 37 °C model development experiments (Figure 5-5), the onset of cell death in the temperature shift cultures coincides with the depletion of glucose (Figure 5-8). This validates that glucose can be used as an indicator of when massive cell death will begin in our culture system. This information is required to predict the duration of the batch culture, which in turn is needed to predict the volumetric productivity, as discussed previously. The predicted and actual time of glucose depletion (i.e. the batch duration) are shown in Table 5-2 for the various temperature shifts. The model does reasonably well at predicting the onset of glucose depletion, with an average difference between prediction and data of 8% and no predicted time varying by more than 20% from the actual time. It should be noted that the actual time of glucose depletion can only be known within about half a day or so, as this is the time scale of sampling and subsequent glucose measurement. In light of this fact, having the predicted versus actual batch duration varying by only 8% on average seems quite satisfactory.

The model prediction versus the experimental data for total IFN-γ produced for the various temperature shifts are shown in Figure 5-9. By dividing these values of IFN-γ concentration by the culture duration (Table 5-2), one obtains IFN-γ volumetric productivity, and this result is shown in Figure 5-10. The model predicts the general trend for IFN-γ volumetric productivity, namely that the productivity will progressively increase relative to a 32 °C culture by delaying the temperature shift up until an optimal shift time, after which volumetric productivity will decrease to the lowest value at 37 °C (Figure 5-10). Most importantly, the model is able to correctly predict the optimal shift day, namely day 3.

Table 5-2. Duration of batch culture for various temperature shifts

Temperature Shift Day[1]	Predicted Batch Time (days)[2]	Actual Batch Time (days)[2]
0	9.2	8.5
1	8.5	7.9
2	7.1	7.1
3	6.0	6.2
4	5.6	5.2
∞	6.3	5.2

[1]A temperature shift day of "0" refers to 32 °C exclusively and "∞" refers to 37 °C exclusively (no shifting)
[2]Batch duration is defined as the point in time at which less than 1% of glucose remains (refer to text for details)

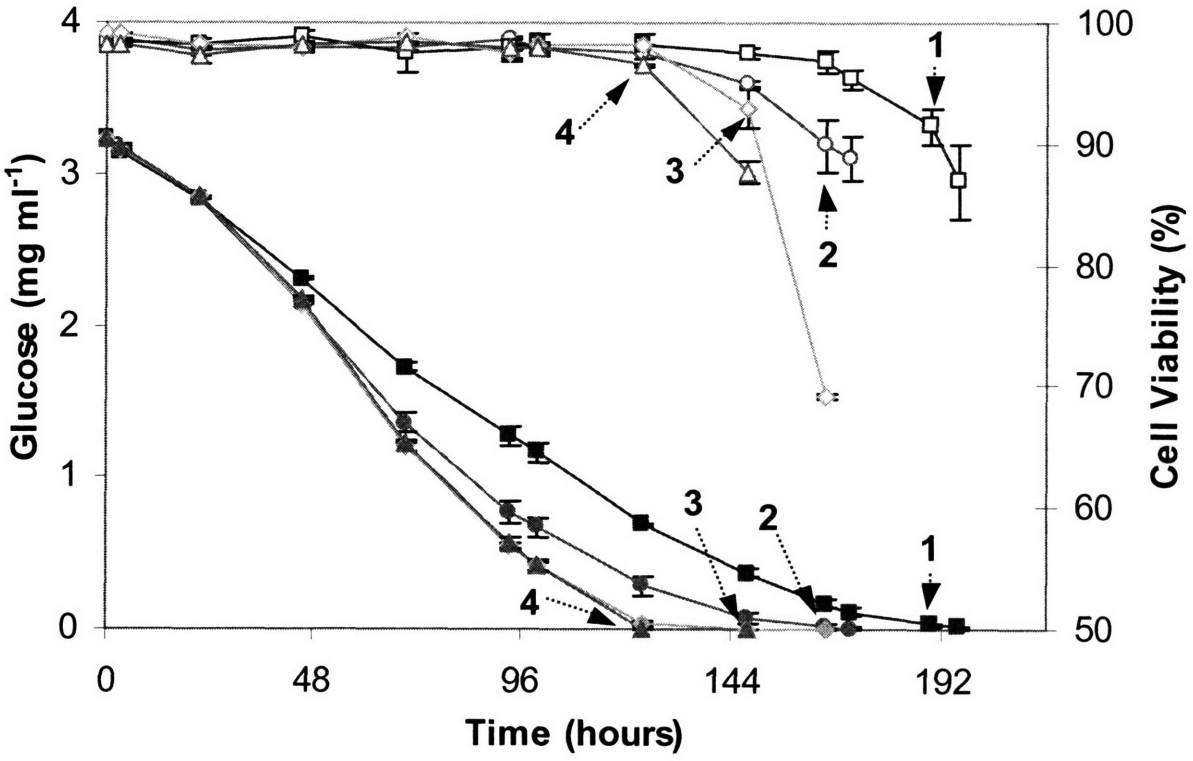

Figure 5-8. Correlation between glucose depletion and the onset of massive cell death in temperature shift cultures. The drop in percent viability to below 90% for 1-day (□), 2-day (○), 3-day (◊), and 4-day (▲) temperature shifts coincides with the depletion of glucose (solid symbols of the same shape as the corresponding viability symbol). The last day at which viability is above 90% and the time that glucose is depleted (<0.03 mg ml^{-1}; see text for details) occur on the same day and are marked with dashed arrows on the figure and labeled with the respective temperature shift day (1-4).

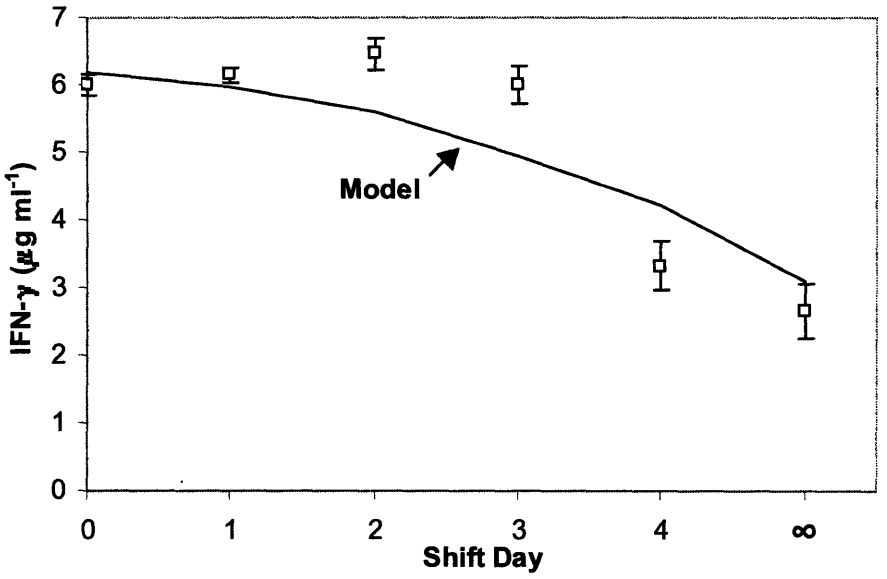

Figure 5-9. Total IFN-γ produced at the end of batch culture as a function of the temperature shift day. Samples were taken on the day glucose became depleted, which coincides with the last day of high cell viability. Model prediction (-) compared to actual data (□). A shift day of "0" means that the culture was conducted at 32 °C exclusively and a shift day of "∞" means that the culture was conducted at 37 °C exclusively.

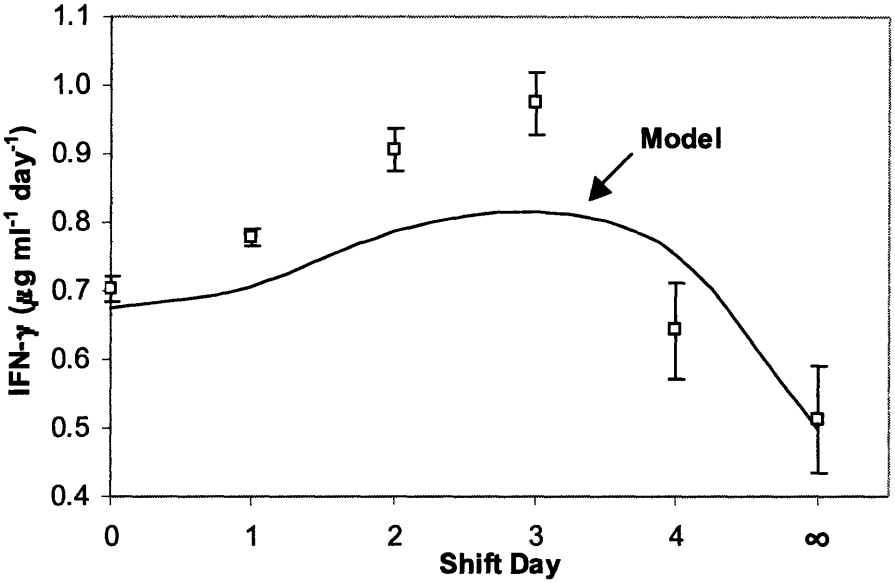

Figure 5-10. IFN-γ volumetric productivity as a function of the temperature shift day. Total IFN-γ produced for a given batch was normalized by the number of days required to complete the batch. Model prediction (-) compared to actual data (-□-). A shift day of "0" means that the culture was conducted at 32 °C exclusively and a shift day of "∞" means that the culture was conducted at 37 °C exclusively.

5.4 DISCUSSION AND CONCLUSIONS

The goal of this study was to develop a modeling framework for predicting the optimal temperature shift time *a priori*. Kinetic parameters (Table 5-1) were obtained at 32 °C and 37 °C and then used to simultaneously solve the balance equations for cell growth, nutrient consumption and recombinant protein production in cultures that had the temperature reduced from 37 °C to 32 °C at a specific time. The optimal time at which to reduce the culture temperature from 37 °C to 32 °C corresponds to the time giving the maximum IFN-γ volumetric productivity. Using the optimal temperature shift time of 3 days post inoculation, the volumetric productivity is increased by 40% above the value obtained by culturing at 32 °C alone and by 90% above the value obtained by culturing at 37 °C alone (0.97 μg ml^{-1} day^{-1} for a temperature shift on day 3 versus 0.70 μg ml^{-1} day^{-1} at 32 °C and 0.51 μg ml^{-1} day^{-1} at 37 °C). In addition, total production of IFN-γ is more than doubled compared to the value obtained at 37 °C and essentially unchanged compared to the value obtained at 32 °C (6.0 μg ml^{-1} for a temperature shift on day 3 versus 2.7 μg ml^{-1} at 37 °C and 6.0 μg ml^{-1} at 32 °C). The success of the 3 day temperature shift resulted from the fact that the vast majority of IFN-γ is produced during the second half of culture, when cell density is highest and IVCD increases most drastically, and thus trading rapid growth for high specific productivity during the first half of the culture has only a minor effect on total production, relative to 32 °C culture, but significantly improves volumetric productivity by shortening the batch time.

As seen in Figures 9 and 10, the model underpredicted the total and per day IFN-γ production by about 20-30% for temperature shifts at day 2 and day 3. This is likely due to assuming that specific productivity is a constant, with the value of q_{IFN} only depending on the culture temperature. It is known that recombinant protein productivity can exhibit growth rate

dependency. For example, it has been suggested by several authors that the SV40 viral promoter is growth-related (Kubbies and Stockinger, 1990; Lloyd et al, 1999). In fact, a positive association between active growth and specific productivity has been shown for the cell line used in this study, CHO-γ (Yuk, 2001). It is therefore possible that the temperature shift strategy is able to combine the favorable effect of low temperature on specific productivity with the favorable effect of active growth on the SV40 promoter, resulting in improved specific productivity for a brief period following temperature shift over what is obtained in a culture grown at 32 °C exclusively. Thus, the actual IFN-γ produced was higher than the model prediction, which assumed specific productivity only depends on temperature and not on growth state. This proposed separation between the hypothermic effect and growth state effect on specific productivity will become a central theme of this thesis and will be discussed in detail in the chapters following this one.

The model depends on being able to predict specific growth rate. This was shown to be feasible at 37 °C by using the Monod model (Figure 2a). However, for the low temperature data, there was only a weak dependence of growth rate on nutrient concentration (Figure 2b) and growth rate remained at approximately 0.01 hr^{-1} for the entire culture. This is not surprising when one considers that mammalian cells actively arrest growth under hypothermic conditions by expressing cold inducible genes (refer to Section 2.2.3). Under such a control system, growth presumably becomes more dependent on the action of certain cold inducible gene products than on the concentration of nutrients. Thus, to accurately predict the growth rate of CHO cells at low temperature requires a deeper understanding of the cold response of these cells.

The success of the model also depends on being able to predict when the onset of massive cell death will occur, as this information is required to predict the culture duration. For the case

of the batch culture system used here, glucose depletion turns out to be a reliable indicator of the onset of cell death. This can be seen from the data in Figure 5-5 and Figure 5-8, wherein viability begins to drop immediately following glucose depletion. It is quite feasible that glucose depletion causes apoptosis in this system. For example, it has been shown that glucose depletion can be a potent inducer of apoptosis for CHO cells growing in protein-free medium (Zanghi et al, 1999). However, the aim of this study was not to prove that glucose depletion causes apoptosis for this culture system. For the modeling purposes, one requires a means of predicting when the batch will end. In this case, glucose depletion provided the means. In a different culture system, a different nutrient or toxic metabolite, or combination thereof, may need to be modeled instead of glucose.

The model presented here was developed using shaker flasks. The drawback of this culture system is that parameters such as pH and dissolved oxygen concentration cannot be tightly controlled. Thus, before being applied in an industrial setting, the model would need to be verified using bioreactors. Owing to the fact that the enhanced effect of temperature on productivity is still maintained in the tightly controlled bioreactor environment, as demonstrated in Chapter 4, the modeling framework and proof-of-concept demonstrated here are expected to still be applicable for optimizing bioreactor operation.

For industrial application, the model can be refined to include down time for reactor shutdown, cleaning, sterilization and inoculation. By incorporating such times in the calculation of volumetric productivity, the cultivation process at 37 ^{o}C would become even more unfavorable as it would require more time to produce the same amount of product relative to the strategies that have longer culture periods at low temperature. This is due to the low temperature

cultures having longer run times and thus requiring fewer turnarounds, and consequently less down time, on an annual basis.

The general framework of the model, namely a tradeoff between a period of high growth and low productivity and a period of low growth and high productivity, is generally applicable to controlled proliferation strategies. As discussed in Section 2.3, in controlled proliferation, an agent is used to induce a G_0/G_1 phase growth arrest, resulting in a cessation of cell growth and, in some cases, a dramatic increase in specific productivity. The point in time at which to add the chemical or express the gene responsible for growth arrest in order to maximize volumetric productivity can be determined by applying the same modeling framework presented here, namely solving balance equations and normalizing the total production by the batch time.

Despite the success of temperature shift for improving volumetric productivity, the strategy has potential disadvantages. For example, changing process conditions during culture will complicate process operation. In addition, changing temperature will affect product quality of recombinant proteins (Andersen et al, 2000; Kaufmann et al, 2001). Also, the longer batch time compared to 37 °C increases the potential for contamination. However, for the same reason, temperature shift will provide less time for contamination than a 32 °C culture.

This chapter presented a model to predict the optimal culture time for temperature shift leading to maximizing the volumetric productivity for recombinant proteins in mammalian cell culture. The model was able to correctly predict the optimal day for temperature shifting in order to maximize IFN-γ volumetric productivity. By utilizing a well-chosen temperature shift strategy, volumetric productivity is increased by 90% over the value obtained at 37 °C and by 40% over the value obtained at 32 °C. Total production is unchanged, or even slightly increased, by using a temperature shift instead of growing at 32 °C for the entire culture.

6. ENHANCED HYPOTHERMIC PRODUCTIVITY IS NOT DUE TO GROWTH ARREST

The success of controlled proliferation as a means for enhancing productivity is usually attributed to the increased fraction of G_0/G_1 cells, the cell cycle phase often considered optimal for heterologous protein production (Fussenegger and Bailey, 1999). However, it has never been shown that the increase in specific productivity is due to the increase in G_0/G_1-arrested cells. The observed growth arrest may simply be a separate event and unrelated to the change in productivity. This suspicion was increased by the observation that many CHO cell lines show enhanced specific productivity at low temperature and yet the recombinant protein is under control of the SV40 or CMV promoters (refer to Table 2-2), generally considered to have higher transcription during active growth (Lee et al, 1998). Also, it is noted that no hybridoma cell line has ever shown enhanced productivity at low temperature (Table 2-2) despite this type of cell generally being regarded as a G_0/G_1 producer (Lee et al, 1998).

For the CHO-γ cell line, it has already been shown in Chapter 4 that hypothermia increases specific productivity. Yet, it has also been shown that this cell line exhibits higher productivity during active growth (Yuk, 2001). To reconcile these apparently contradictory findings, a major objective of this thesis was to determine whether the positive effect of low temperature was due to the cells becoming growth-arrested in G_0/G_1. If this were not the case, the mechanism by which hypothermia leads to enhanced productivity needed to be determined, as this would provide useful clues on further optimizing hypothermic culture. To separate the hypothermic and cell cycle effect on productivity, cells were grown as adherent cells under long-term perfusion conditions. Given the finite surface area available, the cells will become growth arrested and can then be maintained in a viable, growth arrested state for long-term analyses on

the performances of the cultures. Over the course of the entire perfusion, cells within a fairly wide range of cell cycle distribution can be obtained at both low and physiological temperature (37 °C), and the relationship between productivity, cell cycle and temperature can finally be determined.

6.1 PSEUDO PERFUSION CELL GROWTH

Cell growth over the course of the three-week perfusion culture is shown in Figure 6-1. Cells were grown under three conditions: (1) 32 °C entirely, (2) 37 °C entirely and (3) shifted from 37 °C to 32 °C at the point in time when growth at 37 °C had ceased (day 10), henceforth referred to as temperature shift (TS) culture. Cell growth at both temperatures can be seen to consist of a growth period, with cell number increasing with time, and a stationary period, wherein the cells no longer grow. The growth arrest is corroborated at the cell cycle level, as seen in Figure 6-2. After day 10, all three cultures (32 °C, 37 °C, and TS) are at least 80% G_0/G_1 phase. After the peak density was reached on day 10, the total cell concentration decreased over the remaining days of the experiment for the 37 °C and TS cultures. This decrease in cell number appears to be due to some of the cells becoming detached on a daily basis rather than due to significant cell death. Viability remained high throughout the experiment, never dropping below 80% and generally above 90% at both temperatures, and also LDH levels in the supernatant were low, indicating that very little cell death was occurring. The maximum LDH activity measured for any culture during the entire perfusion was 20 U L^{-1}. In contrast, during the death phase of a batch culture of CHO cells, LDH activity reach about 100 U L^{-1} when cells reach 80% viability and are about 400 U L^{-1} for approximately 25% viability (Yoon et al, 2003). Thus, the data shows it was feasible to (1) obtain exponential growth at both temperatures, (2)

reach similar peak cell density at both temperatures and (3) maintain the cells in a growth-arrested, viable state at both 32 ^{o}C and 37 ^{o}C for an additional 10 days by using the perfusion culture.

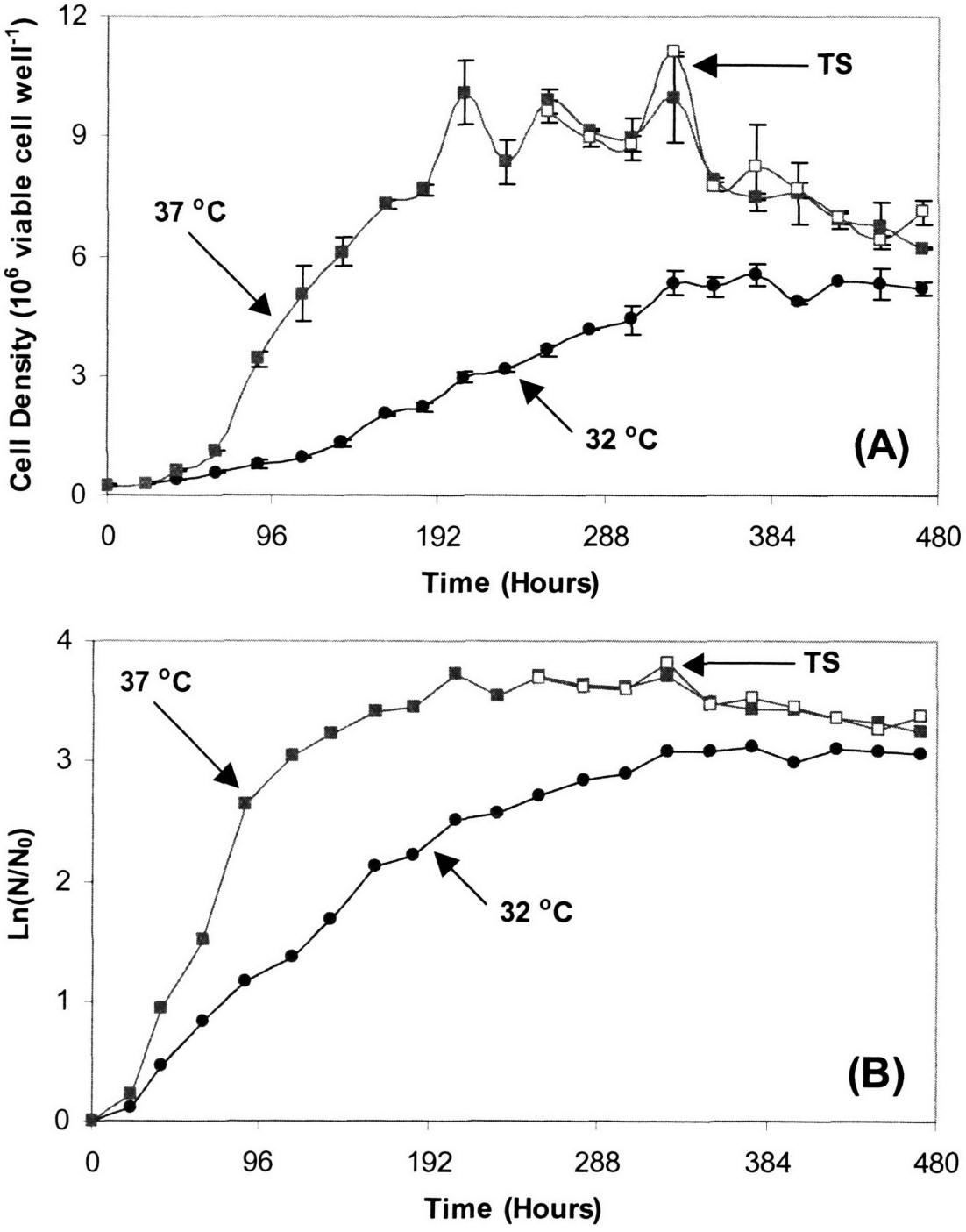

Figure 6-1. Cell growth during pseudo-perfusion culture. Cells were grown at 32 °C (•), 37 °C (■), or at 37 °C for 10 days followed by a shift to 32 °C (TS) (□). Cell density is shown as cells per well in (A) or as natural logarithm of cell density (N) divided by initial cell density (N_0) in (B).

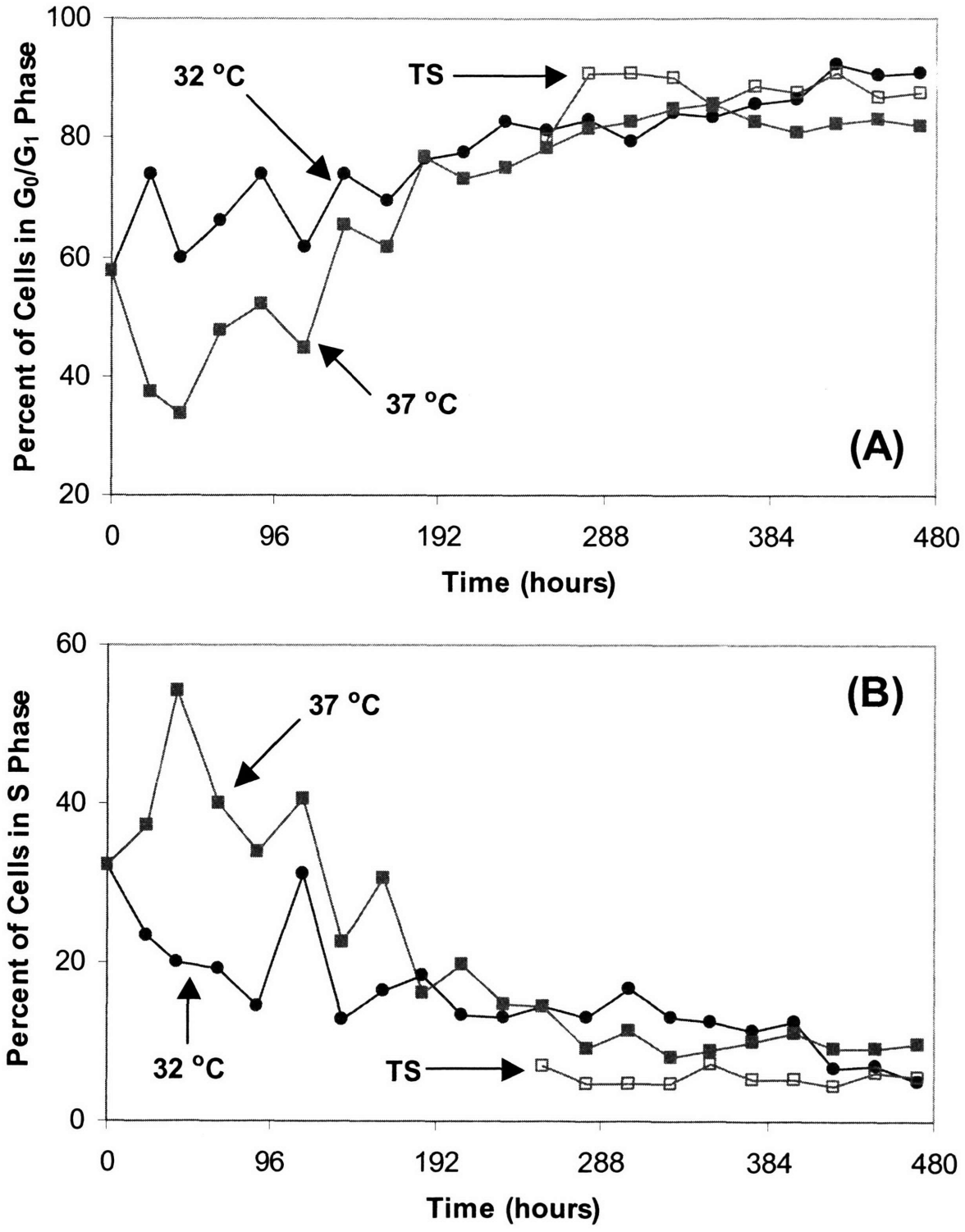

Figure 6-2. Cell cycle distribution during pseudo-perfusion culture. Cells were grown at 32 °C (●), 37 °C (■), or at 37 °C for 10 days followed by a shift to 32 °C (TS) (□). The percentages of G_0/G_1 phase (A) and S phase (B) cells are shown.

6.2 IFN-γ PRODUCTIVITY IS HIGHEST DURING ACTIVE GROWTH

Using the perfusion system, viable cells were maintained for an extended period of time and a wide range of cell cycle distribution was obtained for both temperatures. Specifically, data were obtained over a range of 60-92% in the G_0/G_1 phase at 32 °C and 34-86% in the G_0/G_1 phase at 37 °C (Figure 6-2). At the same time, IFN-γ concentration was also measured over the range of culture, and thus specific productivity versus cell cycle distribution could be determined. The percentage of cells in the S phase is a good indicator of cell growth. Thus, for an analysis of the relationship between productivity and cell cycle, the specific productivity of the cells at 32 °C and 37 °C versus the weighted average percentage of cells in the S phase during the given production period is shown in Figure 6-3.

The data support the earlier finding that CHO-γ is a growth-associated producer (Yuk, 2001) as seen by the fact that specific productivity increases as the percentage of S phase cells increase. The data also prove that the enhanced effect of hypothermia on specific productivity is *not* due to growth arrest. Even at low temperature, it was still preferable to have a higher fraction of S phase cells to maintain high specific productivity. Thus, the hypothermia effect on productivity is due to a different mechanism that is unrelated to cell cycle distribution. The assumption that the positive effect of hypothermia on productivity is due to cells arresting in G_0/G_1 is clearly wrong.

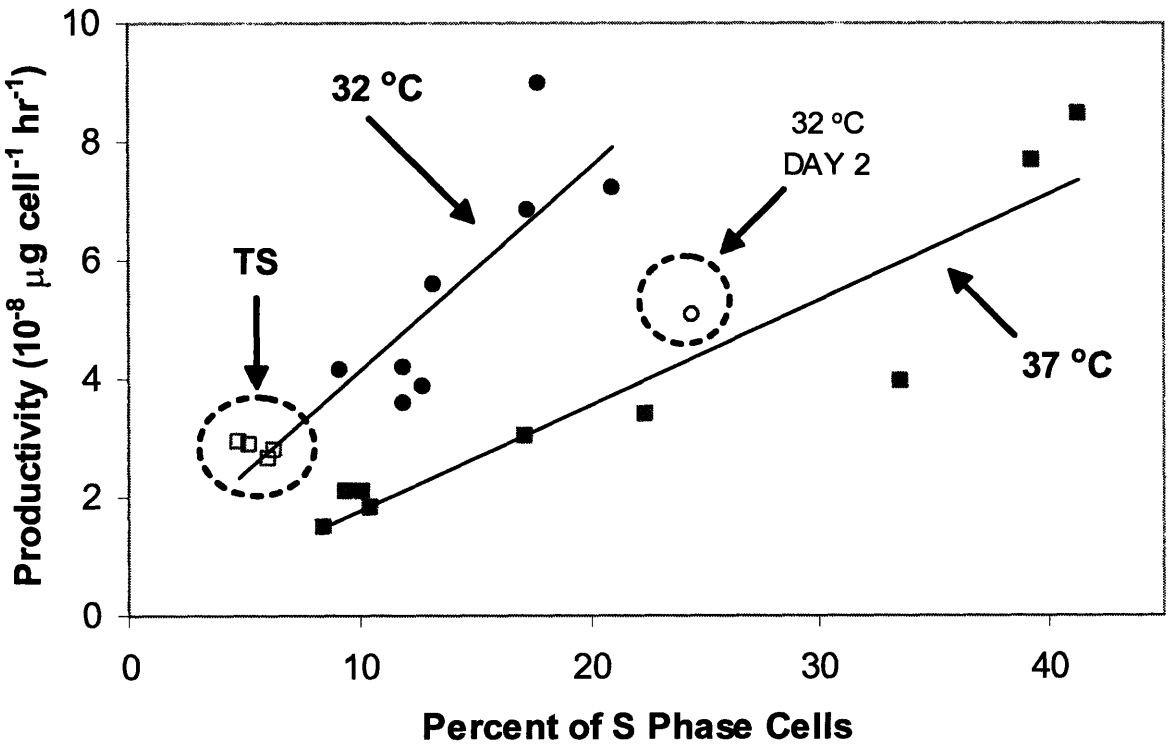

Figure 6-3. Specific productivity correlates with the percentage of cells in the S phase. The specific productivity was measured throughout the perfusion culture and the weighted average percentage of S phase cells during the same period was calculated. At both 32 °C (●) and 37 °C (■), productivity increases as the percent of S phase cells increases. The 32 °C data includes the TS (□) samples. Day 2 at 32 °C (○) appears to be an outlier. The two lines are linear, least-square fits of the 32 °C (including TS) and 37 °C data.

6.3 IFN-γ PRODUCTIVITY IS PROPORTIONAL TO IFN-γ mRNA LEVELS

Based on the hypothermic culture literature, the most probable cause for the improved productivity of the CHO-γ cells under hypothermic conditions is an increase in the IFN-γ mRNA levels. As discussed in Section 2.4.4, the ability of low temperature to improve interferon production by non-transformed cells was hypothesized to be due to increased mRNA stability, but this was never shown to be the case (Vilĉek and Havell, 1973; Giard and Fleischaker, 1980; Giard et al, 1982). In more recent years, other researchers have found recombinant mRNA levels to increase at low temperature (Furukawa and Ohsuye, 1998; Kaufmann et al, 2001; Yoon et al, 2003). However, all three groups used Northern blot hybridization, which does not allow one to conduct a good quantitative analysis, meaning increased transcript levels could only be considered one of several possible causes for increased productivity (Yoon et al, 2003).

Real time reverse-transcriptase (RT) PCR is a more reliable method for accurately quantifying mRNA levels (Bustin, 2000). A real time RT-PCR assay for measuring IFN-γ and β-actin mRNA was developed and used to quantify the amount of mRNA present in cells under various conditions. The β-actin housekeeping gene is frequently used as an internal control when normalizing mRNA levels (Bustin, 2000). In addition, for CHO cells grown at temperatures between 30 °C and 37 °C, β-actin expression has been shown to remain constant (Furukawa and Ohsuye, 1998), and thus this housekeeping gene is particularly appropriate for the analysis conducted here. The idea of housekeeping mRNA levels remaining constant when lowering temperature may seem at first to be unexpected, but another group found that four ubiquitous mRNAs maintained their levels at 1.0 ± 0.1 when comparing hibernating and active squirrels (Frerichs et al, 1998), reinforcing that housekeeping gene normalization is a valid approach.

The level of IFN-γ and β-actin mRNA was measured at several points during the perfusion culture and was found to be a reliable indicator of the specific productivity. First, the analysis was conducted for days 15-18 of culture, a stretch of relatively little cell growth for all three conditions (32 °C, 37 °C and TS). Figure 6-4 shows the correlation between an increase in mRNA levels and a near one-to-one increase in specific productivity when comparing 32 °C culture to 37 °C culture. The findings that the 32 °C culture specific productivity was about 2.2 times higher than at 37 °C whereas the TS culture was only about 1.5 times higher may at first seem contradictory (Figure 6-4). The fact that the TS culture had been at 32 °C since day 10 makes it reasonable to expect it, *a priori,* to behave much the same as the culture grown at 32 °C for the entire experiment. This contradiction can be explained by the fact that the average percent of S phase cells is higher during the day 15-18 period for the 32 °C culture than for the TS culture (11±3% versus 6±1%), as the 32 °C density was not yet as high as the 37 °C and TS cultures and thus the cells were not fully contact inhibited yet and were still undergoing slow cell growth. When this difference is considered in conjunction with the finding shown in Figure 6-3 that productivity is proportional to the percent of S phase cells, the difference between TS and 32 °C cultures during days 15-18 is explained. This data emphasizes the fact that specific productivity depends on both the temperature and the cell cycle distribution.

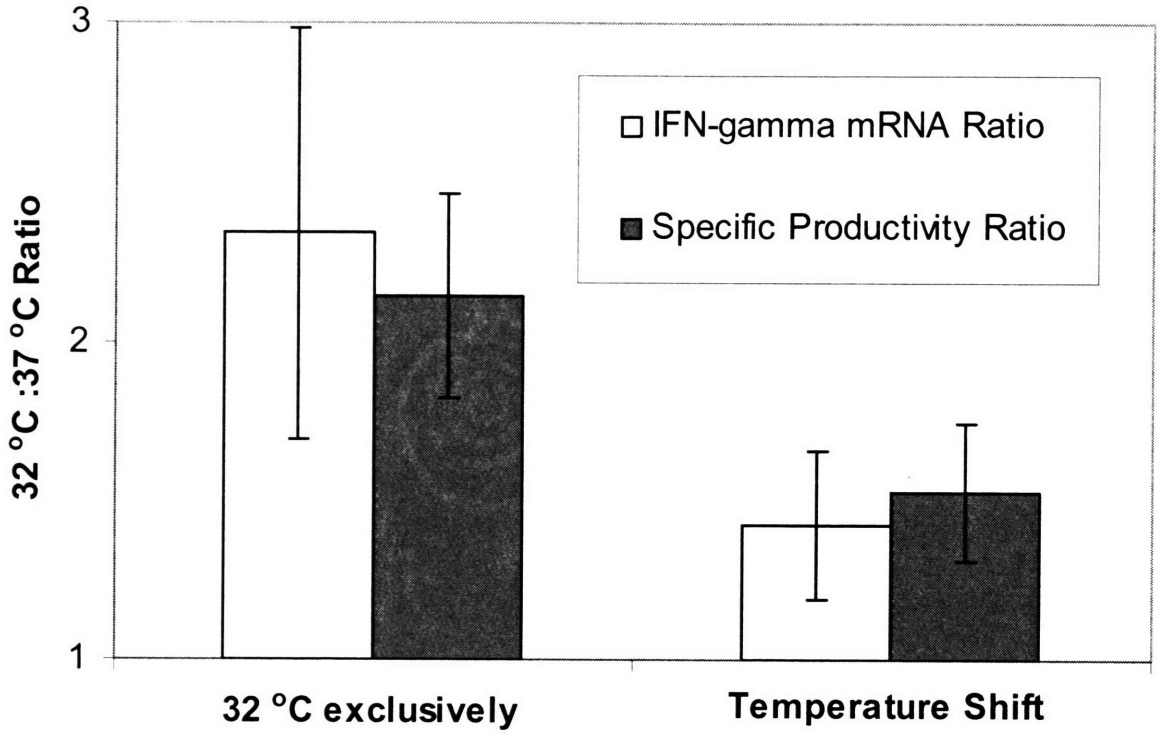

Figure 6-4. Specific productivity correlates with the level of IFN-γ mRNA during day 15-18 of culture. The specific productivity was measured daily during day 15-18 of the perfusion culture and the IFN-γ mRNA levels during the same period were determined and normalized to β-actin mRNA. Samples were measured from all three conditions (32 °C, 37 °C, TS). The ratio of productivity and IFN-γ mRNA at 32 °C to their counterparts at 37 °C (labeled "32 °C exclusively") and the ratio of productivity and IFN-γ mRNA at TS to their counterparts at 37 °C (labeled "Temperature shift") were calculated and plotted. In both cases, the increase in specific productivity under hypothermic conditions correlates with the increase in IFN-γ mRNA.

The analysis of the correlation between mRNA levels and productivity was extended to the entire culture in order to consider a wider range of cell cycle distribution than the rather narrow, growth-arrested day 15-18 period. Over the entire culture, it was found that specific productivity correlates with the normalized IFN-γ mRNA levels, as shown in Figure 6-5, which combines all of the data for all three culture conditions. Thus, the differentiator between hypothermia and 37 °C is that mRNA levels are on average higher at low temperatures, although the mRNA level also depends on cell cycle, explaining the spread of productivity data for a constant culture temperature. Thus, both hypothermia and a higher fraction of S phase cells causes IFN-γ mRNA levels to increase.

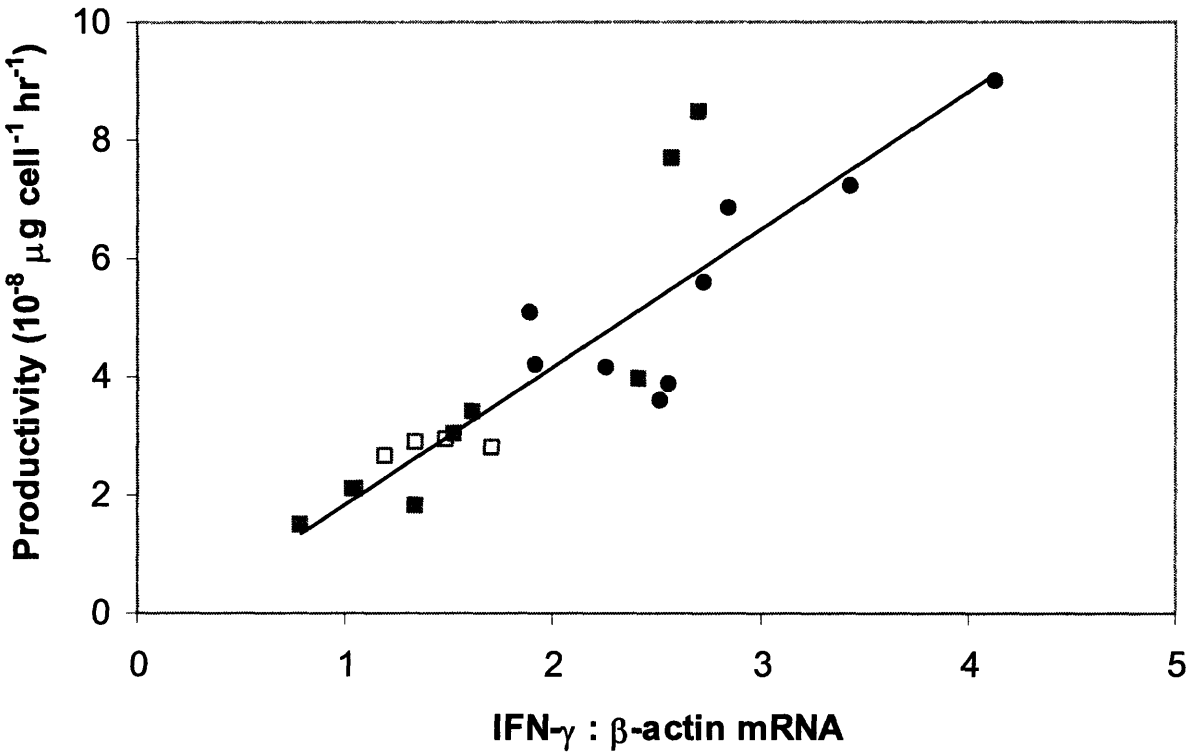

Figure 6-5. Specific productivity correlates with the level of IFN-γ mRNA throughout the entire culture period at all temperature conditions. The specific productivity was measured throughout the perfusion culture and the IFN-γ mRNA levels during the same period was determined and normalized to β-actin mRNA. At 32 °C (●), 37 °C (■) and TS (□), productivity increases as the level of IFN-γ mRNA increases. The line is a linear least-square fit for all of the data points.

6.4 mRNA STABILITY INCREASES DURING HYPOTHERMIA

The half-life ($t_{1/2}$) of IFN-γ and β-actin mRNA was measured at both 32 °C and 37 °C by measuring the mRNA levels at various time points following Actinomycin D treatment (a transcription inhibitor). In the absence of transcription and cell growth, the change of mRNA level with time is due to degradation. Degradation of mRNA is generally assumed to be first-order with respect to mRNA concentration, so the degradation rate constant, and consequently $t_{1/2}$, can be found from measurements of mRNA level versus time (Ross, 1995). The Actinomycin D method is well established (Ross, 1995) and has recently been adapted for real time PCR (Leclerc et al, 2002). In this case, the mRNA levels are normalized using total RNA concentration. The rationale for this normalization is that total RNA is composed mostly of rRNA and tRNA, which are far more stable than mRNA and their levels will not drop very quickly following transcription inhibition. Normalizing real time PCR results using total RNA is analogous to the situation used for the older Northern blot hybridization methods for quantifying $t_{1/2}$, where equal amounts of total RNA are loaded per lane on a gel and then probed for the gene of interest.

The half-life experiments were conducted on day 17 of the perfusion culture, a point when cell growth had essentially ceased. The concentration profile of IFN-γ and β-actin mRNA is shown in Figure 6-6. Although the measurements fluctuate, the slower decay at 32 °C for both genes is readily apparent. From these profiles, the $t_{1/2}$ values shown in Table 6-1 were found. It is observed that hypothermia has a remarkable effect on stabilizing mRNA, with the half-lives of both mRNAs increasing by 7- to 8-fold. The β-actin half-life at 37 °C (10 hours) is of the same magnitude as the values found for this gene in other cell lines (6-14 hours) (Villarete and Remick, 1996; Leclerc et al, 2002) and thus appear reasonable.

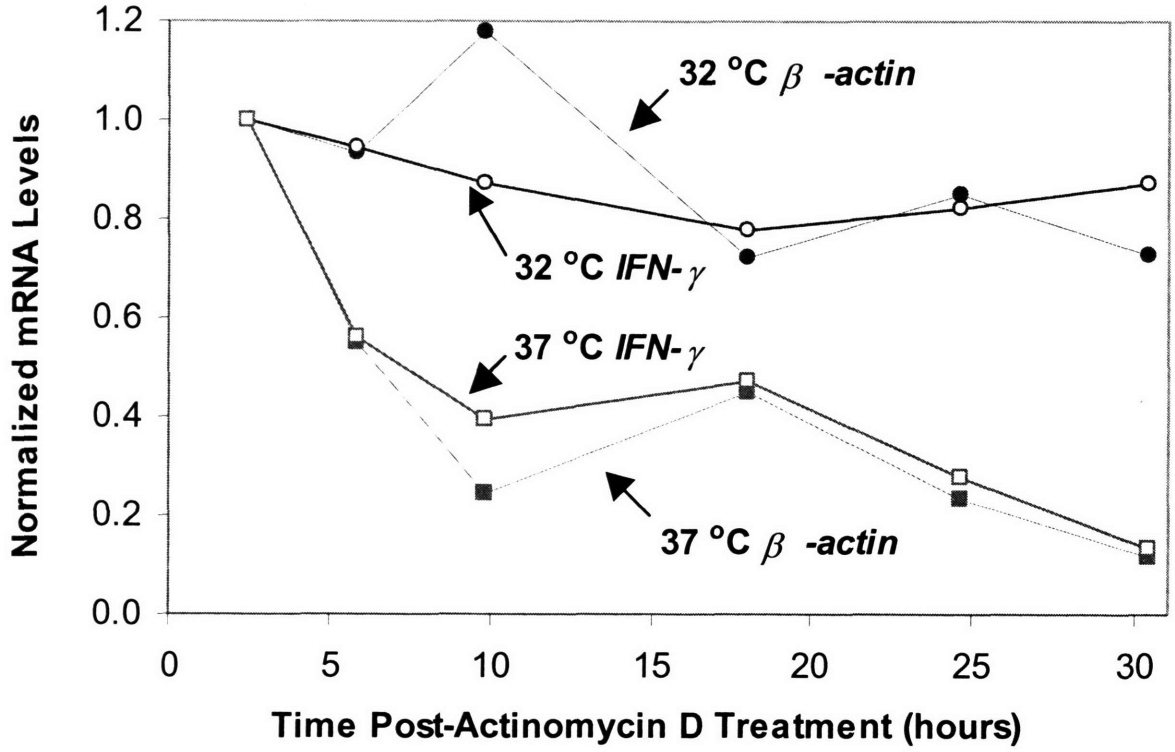

Figure 6-6. mRNA degradation profile. Cells on day 17 of the pseudo perfusion growing at 37 °C or 32 °C (TS samples) were treated with Actinomycin D to arrest transcription at 0 hours and then RNA was isolated from cells sampled at various times following Actinomycin D treatment. The β-actin and IFN-γ mRNA levels in the samples were measured using real time RT-PCR and normalized to the total RNA concentration. The levels of mRNA per total RNA were then normalized against the levels of mRNA per total RNA of the first sample (~2 hours post-Actinomycin D treatment) for each gene at each temperature to obtain the values shown in this figure. Symbol coding is as follows: 32 °C IFN-γ (○); 32 °C β-actin (●); 37 °C IFN-γ (□); 37 °C β-actin (■).

Table 6-1. mRNA half-lives in Pseudo Perfusion Culture

	IFNγ	β-actin
Half-life at 37 °C (hours)	12±1	10±1
Half life at 32 °C, (hours)	91±20	75±25

6.5 DISCUSSION AND CONCLUSIONS

The main objective of this chapter was to better understand the relationship between low temperature, cell cycle and productivity. This was motivated by the apparent contradiction for CHO-γ that the cell line was known *a priori* to be *growth-associated* (Yuk, 2001) yet had higher productivity during hypothermia (see Chapter 4), a condition shown to induce G_0/G_1 *growth-arrest.* This contradiction was resolved by showing that the CHO-γ cell line is a growth-associated producer at both temperatures and that the effect of temperature is related to increased IFN-γ mRNA levels, not due to growth-arrest. The IFN-γ mRNA levels were shown to correlate directly with the IFN-γ productivity.

It is not known for certain why the IFN-γ mRNA levels are elevated at low temperature, although enhanced transcript stability may play a role. The half-life was shown to increase substantially at low temperature. However, the change was about the same for both the IFN-γ and β-actin mRNA, suggesting that enhanced mRNA stability is a generic part of the cold shock response. This has been qualitatively observed before in hypothermic mammalian cells by Fujita, who noted that "hypothermia delays degradation of mRNAs in general" (Fujita, 1999), although quantitative data was not provided, so it remains unknown as to whether the enhancement was consistent with the 7-8 fold increase seen here. It is unlikely that such a large increase in stability can be attributed solely to a thermal effect on enzyme activity. In the absence of rate data, a general guideline used when considering the temperature effect on enzyme rates is that a 10 °C reduction in temperature halves enzyme activity (Marsh et al, 2001). Using this guideline, it is improbable that the 7-8 fold increase in stability for a 5 °C change in temperature seen here is due to reduced enzyme (e.g. RNase) activity solely as a result of lower temperatures. It is more likely that the stability is enhanced as part of the cold shock response,

either by active inhibition of RNase enzymes or by stabilization of transcripts by RNA-binding proteins, or by other means. This would be consistent with the information presented in the literature review, such as showing that a dramatic increase in cold shock protein mRNA stability is part of the prokaryotic cold response (Section 2.2.1) or that many cold shock proteins have RNA-binding properties (Section 2.2.3). In fact, there is indirect evidence that poly(A)-binding protein (PABP) plays a significant role in enhanced mRNA stabilization during ground squirrel hibernation (Knight et al, 2000).

Steady-state mRNA levels are also a function of the transcription rate. Since transcription rates were not measured during this thesis, it is not possible to say with certainty that a change in transcription played a role in the elevated mRNA levels. However, by considering a balance on mRNA molecules, one can use the calculated degradation rates to surmise some information about transcription rate changes under hypothermic conditions, as now follows. In the absence of cell growth, which is valid here as the mRNA half-lives were measured on day 17 of the pseudo perfusion culture using the 37 °C and TS cultures, the balance on an mRNA species is as follows:

$$dR(t)/dt = k_T - k_D R(t) \qquad (6\text{-}1)$$

Where R(t) is the number of mRNA molecules per cell at time t, k_T is the rate of transcription, and k_D is the degradation rate constant. As noted before, degradation is considered a first-order process and transcription is usually assumed to be zero-order (Suzuki and Ollis, 1990). The number of mRNA molecules per cell at steady-state, R_{ss}, becomes:

$$R_{ss} = \frac{k_T}{k_D} \qquad (6\text{-}2)$$

The ratio of IFN-γ to β-actin mRNA levels at steady-state is therefore given by:

$$\frac{R_{ss}^{IFN}}{R_{ss}^{actin}} = \frac{k_T^{IFN}}{k_T^{actin}} \frac{k_D^{actin}}{k_D^{IFN}} \qquad (6\text{-}3)$$

Equation (6-3) can then be applied to cells at two different temperatures, 37 °C and 32 °C, and combined to find the ratio of the increase in IFN-γ mRNA relative to β-actin mRNA when reducing temperature, which has been shown previously to be the cause for enhanced productivity (Figure 6-5) and therefore is the ratio of greatest interest:

$$\frac{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{32}}{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{37}} = \left[\frac{\frac{(k_T^{IFN})_{32}}{(k_T^{IFN})_{37}}}{\frac{(k_T^{actin})_{32}}{(k_T^{actin})_{37}}}\right]\left[\frac{\frac{(k_D^{actin})_{32}}{(k_D^{actin})_{37}}}{\frac{(k_D^{IFN})_{32}}{(k_D^{IFN})_{37}}}\right] \qquad (6\text{-}4)$$

The numerical value of the second term on the right hand side of Equation (6-4), namely a ratio of the degradation constants for the two genes at the two temperatures, is known from the $t_{1/2}$ values given in Table 6-1:

$$k_D = \frac{t_{1/2}}{\ln(2)} \qquad (6\text{-}5)$$

Using Equation (6-5) in conjunction with the values from Table 6-1, one obtains the following for the ratio of the degradation constants in Equation (6-4):

$$\left[\frac{\frac{(k_D^{actin})_{32}}{(k_D^{actin})_{37}}}{\frac{(k_D^{IFN})_{32}}{(k_D^{IFN})_{37}}}\right] = \left[\frac{\frac{75hr}{10hr}}{\frac{91hr}{12hr}}\right] = 0.989 \approx 1 \qquad (6\text{-}6)$$

Therefore, when considering the ratio of IFN-γ to β-actin mRNA levels at 32 °C to 37 °C, the differences in degradation essentially cancel out, and Equation (6-4) becomes:

$$\frac{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{32}}{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{37}} = \left[\frac{\frac{(k_T^{IFN})_{32}}{(k_T^{IFN})_{37}}}{\frac{(k_T^{actin})_{32}}{(k_T^{actin})_{37}}}\right] \qquad (6\text{-}7)$$

Thus, although the finding that mRNA stability is greatly increased at low temperature may prompt one to assume the improved specific productivity is due to increased stability, the above analysis cumulating with Equation (6-7) shows that it is differential transcription that likely plays the main role in the enhanced level of IFN-γ mRNA relative to β-actin when decreasing temperature from 37 °C to 32 °C.

The analysis can be taken a step further to predict the change in transcription of both genes when shifting from 37 °C to 32 °C. Given that β-actin levels per CHO cell do not vary when shifting from 37 °C to 32 °C (Furukawa and Ohsuye, 1998) the steady-state mRNA balance for β-actin (Equation 6-2) at each temperature can be solved in conjunction with the degradation

rate data (Table 6-1) to find the relative change in β-actin transcription when reducing temperature:

$$\frac{(k_T^{actin})_{32}}{(k_T^{actin})_{37}} = \frac{0.0092hr^{-1}}{0.066hr^{-1}} = 0.14 \qquad (6\text{-}8)$$

Thus, transcription of β-actin at 32 °C is *predicted* to be only 14% of the 37 °C value. Substituting this result into Equation (6-7) and also in conjunction with the real time PCR results showing IFN-γ mRNA levels to be 1.4-fold higher than β-actin for the 32 °C steady-state (TS) culture (see Figure 6-4 and Figure 6-5) gives:

$$\frac{(k_T^{IFN})_{32}}{(k_T^{IFN})_{37}} = \frac{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{32}}{\left(\frac{R_{ss}^{IFN}}{R_{ss}^{actin}}\right)_{37}} \left[\frac{(k_T^{actin})_{32}}{(k_T^{actin})_{37}}\right] \approx \frac{1.4}{1.0} x0.14 = 0.20 \qquad (6\text{-}9)$$

Thus, IFN-γ transcription at 32 °C is *predicted* to be only 20% of the 37 °C value. However, this level is higher than the 14% predicted for β-actin and hence the IFN-γ mRNA levels can rise relative to β-actin, due to a relatively smaller decrease in transcription, and this leads to higher specific IFN-γ protein productivity.

Given that the recombinant gene product is under the control of a viral promoter, whereas endogenous genes such as β-actin are under the control of endogenous mammalian promoters, it is possible that the recombinant product is able to circumvent the control systems that may be in place for reducing transcription during the cold response to compensate for the enhanced stability. Thus, the foreign gene continues to be transcribed at higher levels than endogenous

genes while also benefiting from the apparently generic enhanced stability of low temperature, allowing the levels to rise relative to the well-controlled endogenous mRNA levels, which, as noted before, appear to be remarkably well-controlled when shifting from 37 °C to hibernation conditions (Frerichs et al, 1998). As predicted from the analysis above, there is evidence that transcription is reduced significantly in mammals under extreme hypothermic conditions (van Breukelen and Martin, 2002). However, whether the above predictions of reduced IFN-γ and β-actin transcription in CHO-γ at 32 °C are correct remains to be determined.

The enhanced productivity obtained during controlled proliferation is often attributed to the cells being arrested in the G_0/G_1 phase of the cell cycle. For hypothermia, which is classified as an environmental means for obtaining controlled proliferation, we have shown that the enhanced productivity is not due to G_0/G_1 arrest and in fact it is preferable to have actively growing (S phase) cells, regardless of temperature, owing to the CHO-γ line being a growth-associated producer. In light of the findings presented here and the fact that some cells are non growth-associated producers (refer to Section 2.3.2), it is interesting to consider the four hypothetical production scenarios one may encounter when considering hypothermic cultivation of *any* animal cell line. These four scenarios are schematically shown in Figure 6-7.

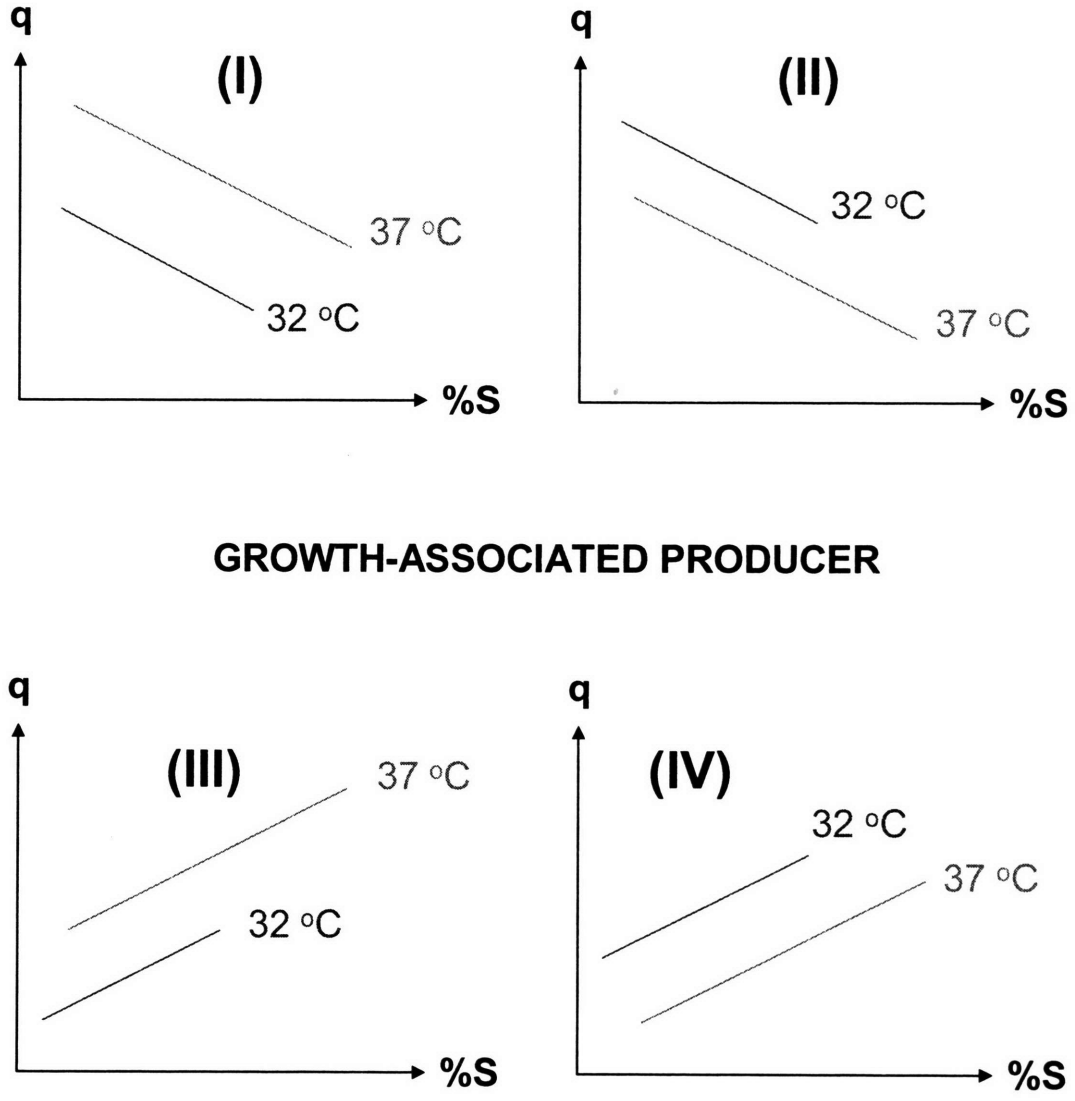

Figure 6-7: Four production scenarios when considering combined hypothermia and cell cycle effects. A cell line can be either non growth- [(I) and (II)] or growth-associated [(III) and (IV)] and also show either an increase [(II) and (IV)] or decrease [(I) and (III)] in productivity under hypothermic conditions. On the figures, "q" refers to specific productivity and "%S" refers to the percentage of cells in the S phase. Refer to text for details.

Scenario IV involves a tradeoff between the positive direct effect of hypothermia on productivity and the negative indirect effect of hypothermia, via growth arrest, on productivity and is representative of the behavior of the CHO-γ cell line (compare to Figure 6-3). This scenario may also be representative of the CHO cells listed in Table 2-2 that showed only a mild increase (Chuppa et al, 1997; Furukawa and Ohsuye, 1998; Hendrick et al, 2001; Kaufmann et al, 1999; Weidemann et al, 1994) or a small decrease (Ryll et al, 2000) in productivity at low temperature.

A cell line showing the behavior of scenario (II) will *always* benefit from hypothermic cultivation, owing to the fact that temperature will increase productivity and also decrease the percent of S phase cells, increasing productivity further. This scenario may be representative of some of the CHO cell lines listed in Table 2-2 that have shown *significant* enhanced productivity at low temperature. For example, some CHO cell lines had a 4- to 6-fold increase in productivity at low temperature, far higher than the approximately 1.5- to 2-fold increase consistently obtained for CHO-γ, and thus may be benefiting from the additive effect of hypothermia and growth arrest (Ducommun et al, 2002; Yoon et al, 2003).

On the other hand, scenario (III) will *never* benefit from hypothermic cultivation, owing to the fact that temperature will decrease productivity and also decrease the percent of S phase cells, decreasing productivity further. Whether such cell lines exist is unknown, but this scenario may be representative of the hybridoma cells in Table 2-2 that exhibit a significant decrease in productivity at low temperature (Reuveny et al, 1986; Sureshkumar and Mutharasan, 1991).

Like Scenario (IV), scenario (I) involves a tradeoff scenario, although in this case temperature has a negative direct effect on productivity but a positive indirect effect, owing to the growth arrest. This scenario may be representative of the hybridoma cell lines that showed

only a minor decrease in productivity at low temperature (Bloemkolk et al, 1992; Barnabé and Butler, 1994). After all, hybridomas are generally considered high producers during growth arrest (Lee et al, 1998) but have never been shown to have higher productivity at low temperature (see Table 2-2). If this interpretation that hypothermia and growth states are separable is valid for all cell lines, then it would be appropriate to develop curves analogous to those shown in Figure 6-7 for all commercial cell lines. Based on the resulting curve, one could make the best choice regarding the optimal culture conditions (hypothermia versus 37 °C; active growth versus growth arrest) and design culture strategies to reach these conditions.

In light of the findings here, it is worthwhile examining controlled proliferation from a fresh perspective. As was demonstrated for hypothermia, it is feasible that the other methods for obtaining controlled proliferation are also exerting their positive effect on productivity via a mechanism that is unrelated to the cells arresting in G_0/G_1. This information is important to know for the sake of optimizing the cell culture process. There is evidence in the literature to suggest that this is the case for at least one of the other popular means for obtaining controlled proliferation, namely sodium butyrate treatment. As discussed in Section 2.3.1, butyrate is postulated to have a positive effect on productivity by making DNA more accessible to RNA polymerase, thereby increasing transcription and consequently productivity. Based on this mechanism, there is no reason to believe G_0/G_1 growth arrest should be a condition for such an effect and it appears this skepticism is supported by the literature. In a different CHO-γ cell line treated with butyrate, Lamotte and colleagues found that productivity is positively correlated with growth rate for both the butyrate-treated and control culture (Lamotte et al, 1999). The focus of the study was on the effect of butyrate on IFN-γ glycosylation, so the authors did not discuss their productivity data in much detail. However, the analogy between their work and the

hypothermia effect described in this chapter is clear. In their case, the butyrate treatment is independent of growth-arrest and it is desirable to have actively growing cells in the presence of butyrate. In light of these findings and the independence of the hypothermia effect from growth arrest demonstrated in this chapter, other controlled proliferation techniques should also be reexamined in order to separate the cell cycle effect from the specific effects of the growth-arresting agent on productivity.

In conclusion, the positive effect of hypothermia on specific productivity is not due to growth arrest but rather due to increased IFN-γ mRNA levels. Consequently, it is hypothesized that the best platform for maximizing total IFN-γ production would be cells capable of actively growing and achieving high cell density under hypothermic conditions.

7. ACTIVE HYPOTHERMIC GROWTH MAXIMIZES TOTAL IFN-γ PRODUCTION

In Chapter 6, it was shown that the enhanced productivity achieved by hypothermia is not due to growth arrest. For the CHO-γ cell line, hypothermia enhances productivity whereas growth arrest diminishes productivity. Based on these findings, it was hypothesized that the best conditions for maximizing total IFN-γ production would be cells capable of actively growing at low temperature, as these cultures would maintain the benefits of hypothermia on *specific* productivity whilst also achieving the high cell densities characteristic of active growth, and consequently *total* production would be greatly enhanced. The goal of this chapter was to test this hypothesis by achieving hypothermic active growth, which is a novel culture condition.

7.1 IS ACTIVE GROWTH AT LOW TEMPERATURE FEASIBLE?

Given that growth arrest is a hallmark of the mammalian cold shock response, it was not apparent that hypothermic active growth was even achievable in the CHO-γ cell line. Perhaps the cells are already growing at their kinetic limit at 32 °C. To test whether the growth rate of the CHO-γ cell line was kinetically limited by temperature, cell growth at three different temperatures, 31.5 °C, 34.5 °C and 37 °C, was measured. The growth profiles for all three temperatures are shown in Figure 7-1. Remarkably, CHO-γ showed a negligible difference in growth at 37 °C and 34.5 °C, whereas growth at 31.5 °C was significantly reduced. This finding is consistent with the previous observation during the literature survey (Section 2.4.1) that mammalian cell growth appears to become severely arrested below a threshold temperature. An Arrhenius plot reveals that the reduced growth rate at low temperature is not due to thermal kinetic limitations (Figure 7-2). Thus, hypothermic active growth may be feasible.

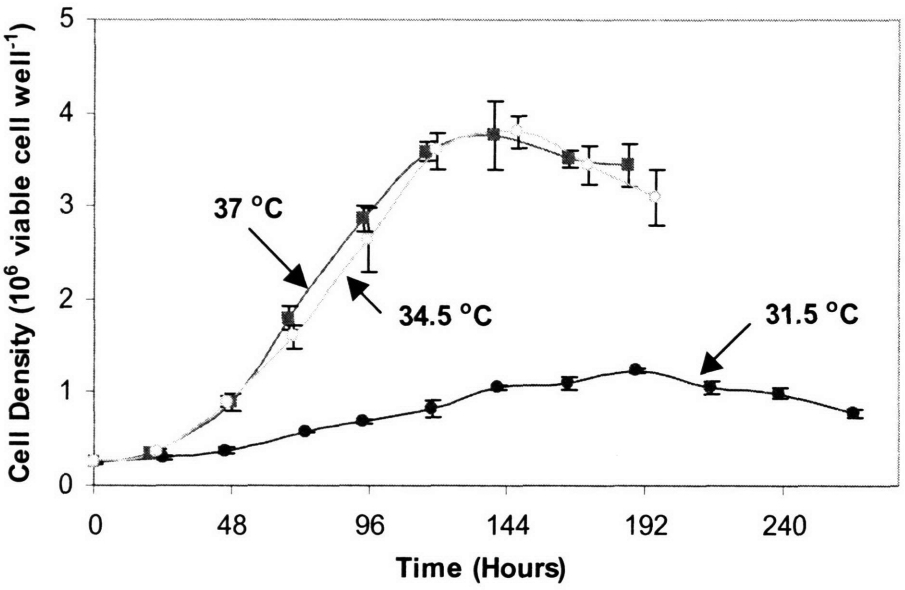

Figure 7-1. Cells are not growth arrested at intermediate temperature. Adherent cells were grown at 31.5 °C (●), 34.5 °C ( ) and 37 °C (■). Whereas cells exhibit strong arrest at 31.5 °C relative to the 37 °C control, they are hardly affected by growing at 34.5 °C. The culture was terminated once the percent viability was less than 80%.

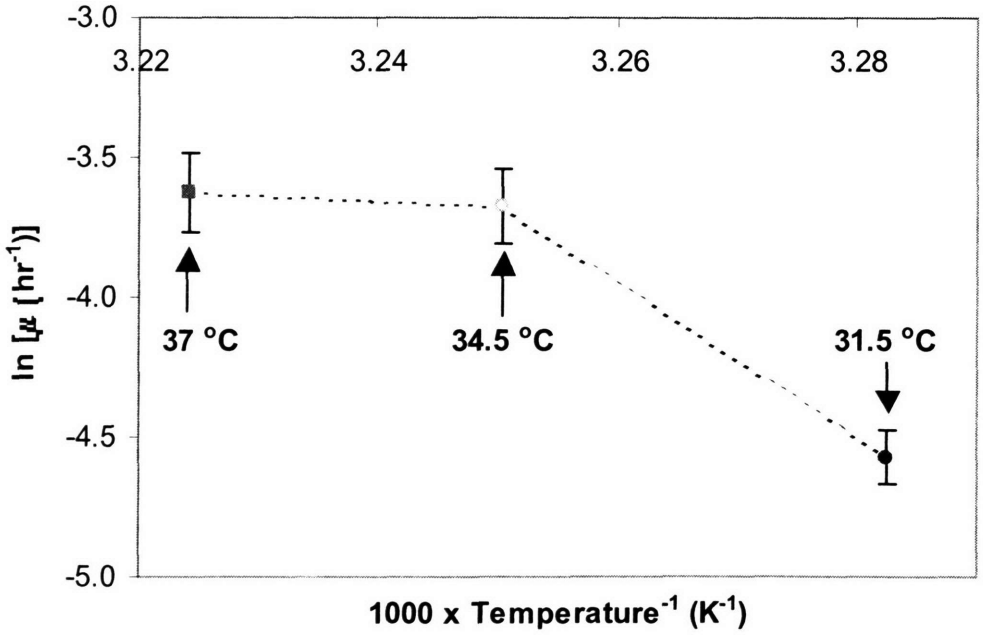

Figure 7-2. Arrhenius plot of CHO cell growth. Adherent cells were grown at 31.5 °C (●), 34.5 °C ( ) and 37 °C (■). Whereas cells exhibit strong arrest at 31.5 °C relative to the 37 °C control, they are hardly affected by growing at 34.5 °C. This plot shows that growth under hypothermic conditions is not kinetically-limited.

After obtaining surprisingly successful growth of the CHO-γ at 34.5 °C, the productivity was measured to see if the intermediate temperature had achieved the desired outcome of maintaining 37 °C growth rate whilst achieving the improved productivity characteristic of hypothermia. If this were the case, obtaining hypothermic active growth at 32 °C would no longer be useful as one could simply run the process at 34.5 °C. The specific productivity at the three temperatures is shown in Figure 7-3. Interestingly, as was the case with growth rate, specific productivity is unchanged when lowering the temperature from 37 °C to 34.5 °C. This is perhaps not all that surprising, as the growth rate shows that the cold shock response has evidently not been activated at 34.5 °C and thus the mRNA stabilizing/transcription lowering effect and consequent higher IFN-γ mRNA levels associated with increased productivity (refer to Chapter 6) is not expected to have been activated either.

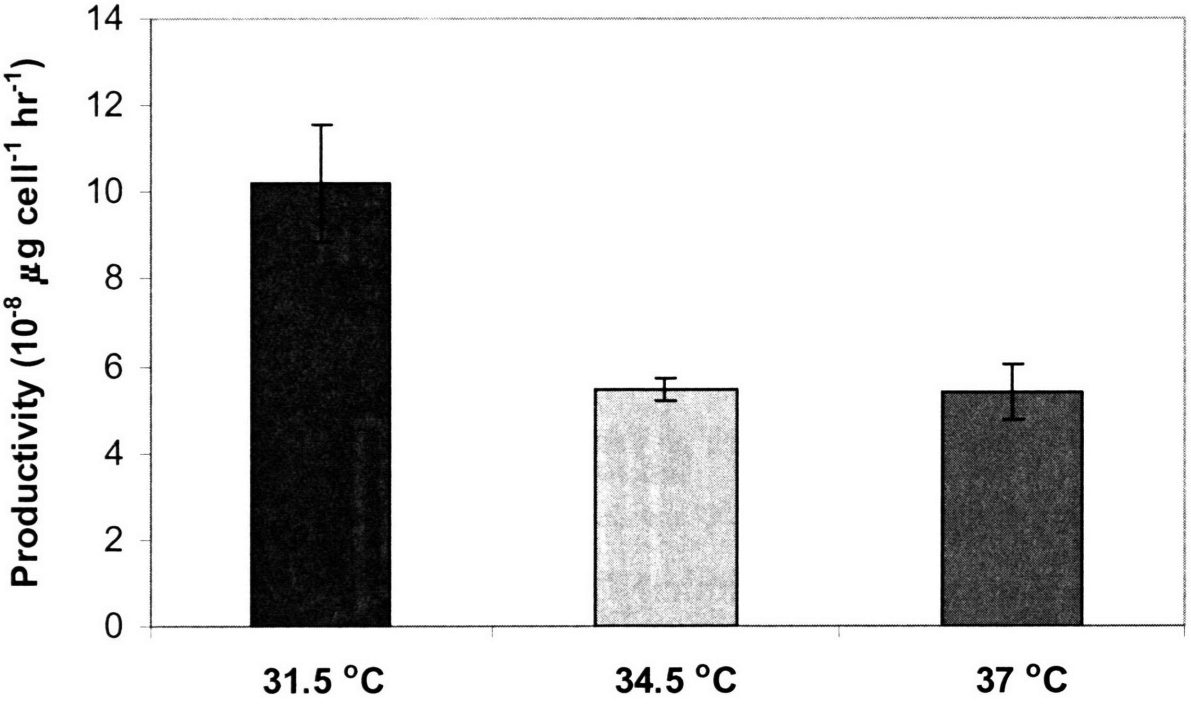

Figure 7-3. Average specific productivity is not enhanced at intermediate temperature. Adherent cells were grown at 31.5 °C (■), 34.5 °C ( ) and 37 °C (■). Whereas cells exhibit enhanced productivity at 31.5 °C relative to the 37 °C control, productivity is unaffected by growing at 34.5 °C.

7.2 GROWTH FACTORS IMPROVE CELL DENSITY AT LOW TEMPERATURE

Given that hypothermia directly interacts with the cell cycle control system as a negative growth signal (Section 2.2.3), it was hypothesized that hypothermic active growth could be achieved by providing high concentrations of growth factors to serve as positive growth signals, thereby overcoming the hypothermic arrest. To test the feasibility of this approach, the effect of different concentrations of the growth factors insulin and bFGF on cell density was determined. The concentrations tested were similar to those shown by other researchers to have a positive effect on CHO cells [5 $\mu g\ ml^{-1}$ insulin (Sanfeliu et al, 2000) and 20 $ng\ ml^{-1}$ bFGF (Renner et al, 1995)]. Figure 7-4 shows the peak cell density obtained at 32 ^{o}C for the different growth factor conditions tested. Clearly, both insulin and bFGF are able to cause higher cell densities relative to the control and some level of hypothermic active growth is achieved. The combination of insulin and bFGF gave a slightly higher cell density than either of the two growth factors alone, but the additive effect was not significant. The viability for the cultures containing insulin was consistently lower than the bFGF cultures. Insulin has previously been observed to cause cells growth-arrested by serum withdrawal to proliferate but also to undergo apoptosis (Sanfeliu et al, 2000) and it is likely that a similar event is taking place here, resulting in lower cell viability. Because of this detrimental effect and the fact that insulin did not give higher cell density than bFGF, only bFGF was considered further.

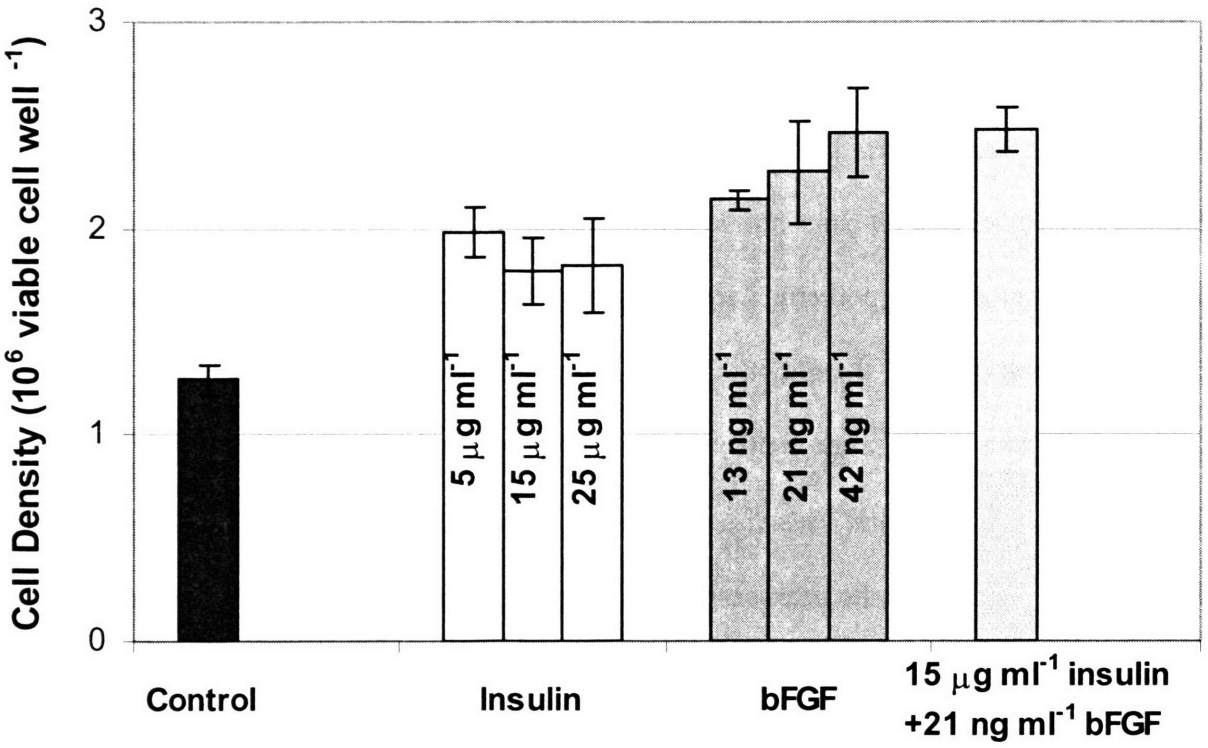

Figure 7-4. Effect of growth factors on hypothermic growth peak cell densities. Adherent cells were grown at 32 °C in the absence ("Control") or presence of insulin (5-15 μg ml^{-1}), bFGF (13-42 ng ml^{-1}), or a combination of the two growth factors (21 ng ml^{-1} bFGF and 15 μg ml^{-1} insulin) and their peak cell density was determined. Both factors are able to promote growth at low temperature although bFGF treated cells have higher peak cell density. The combination of the two growth factors did not have a significant additive effect.

7.3 bFGF DECREASES THE LEVEL OF G_0/G_1 CELLS DURING HYPOTHERMIA

Figure 7-5 shows images of cells treated with bFGF and control cells at both 32 °C and 37 °C. The growth-enhancing effect of bFGF at 32 °C is apparent from this qualitative comparison, with the hypothermic bFGF culture having a more dense appearance. The long, elongated appearance of growth-arrested CHO cells has been described previously (Kaufmann et al, 1999). The 32 °C controls are exhibiting this morphology but the bFGF-treated 32 °C cells are not, providing qualitative evidence that the growth factor has partially alleviated growth arrest.

This qualitative observation of enhanced growth is corroborated quantitatively by the cell growth curves at 32 °C and 37 °C (Figure 7-6). The growth factor causes the peak cell density to decrease slightly at 37 °C whereas it causes a significant increase in cell density at 32 °C, with peak cell density doubling relative to the untreated control.

The effect of bFGF at 32 °C is evident at the cell cycle level, as shown in Figure 7-7, which plots the percentage of S phase versus time. As early as day 2, the bFGF-treated hypothermic culture has a significantly higher percent of cells in S phase, and this trend continues for the remainder of the culture.

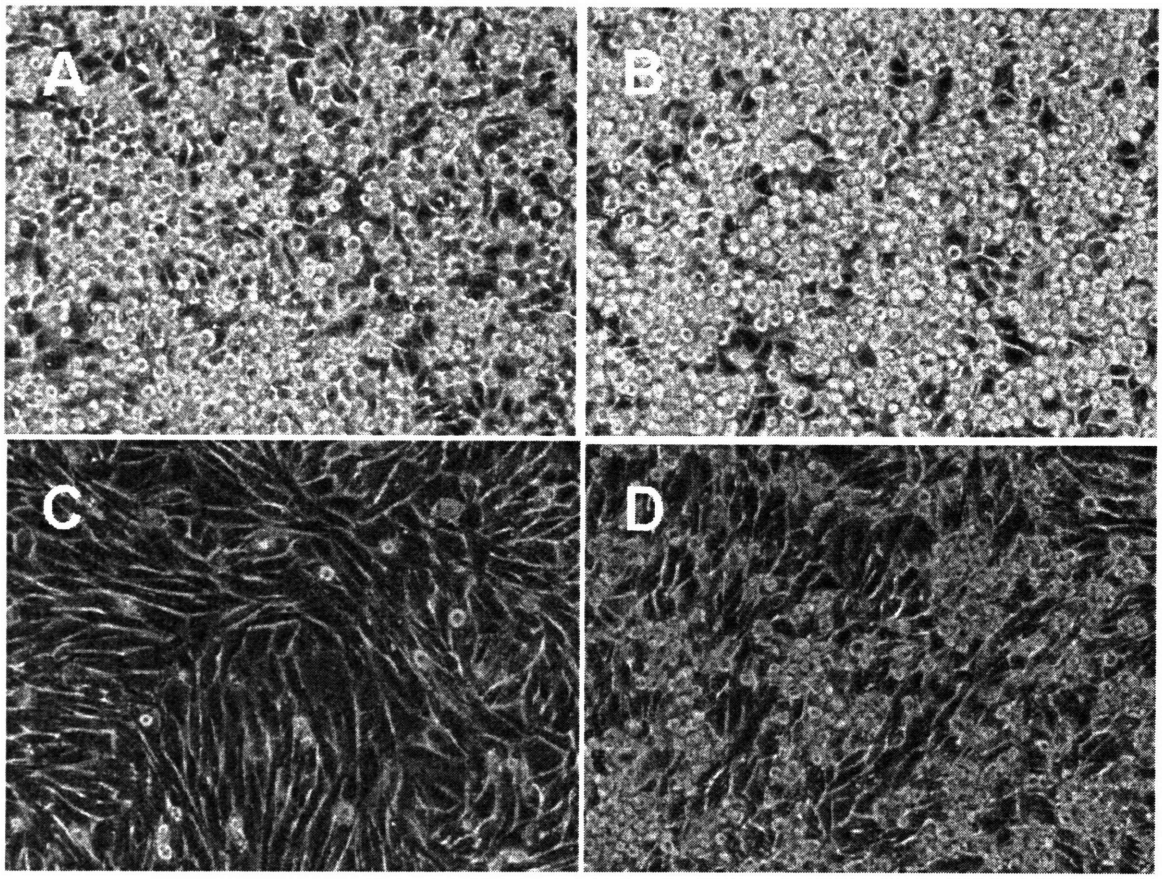

Figure 7-5. Treatment with bFGF promotes growth under hypothermic conditions. Adherent CHO-γ control and bFGF-treated cells (45 ng ml^{-1}) were seeded at the same density and grown at 32 °C or 37 °C. (A) Control cells on day 5 at 37 °C; (B) bFGF-treated cells on day 5 at 37 °C; (C) Control cells on day 8 at 32 °C; (D) bFGF-treated cells on day 8 at 32 °C.

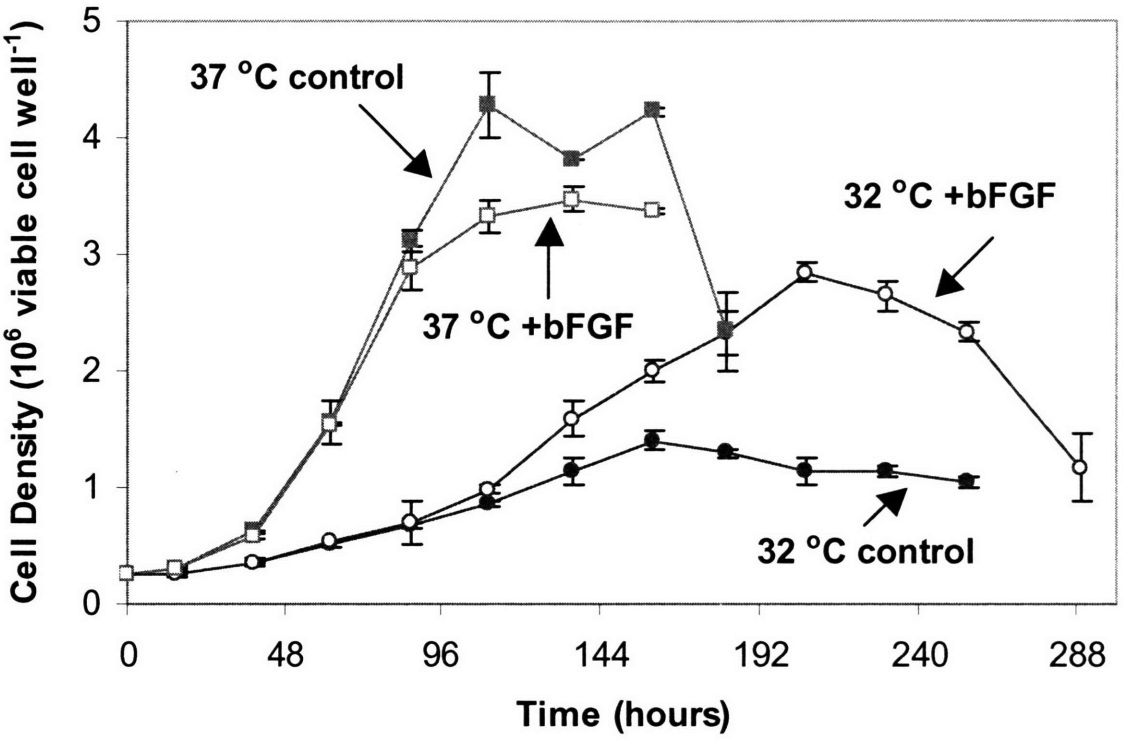

Figure 7-6. bFGF stimulates cell growth under hypothermic conditions. Adherent cells were grown at 32 °C and 37 °C in the presence or absence of 45 ng ml^{-1} bFGF. The bFGF has no beneficial effect at 37 °C (■ control; □ bFGF). In contrast, the bFGF causes the peak cell density to double at 32 °C (● control; ○ bFGF). The cultures were terminated once the percent cell viability was less than 80%.

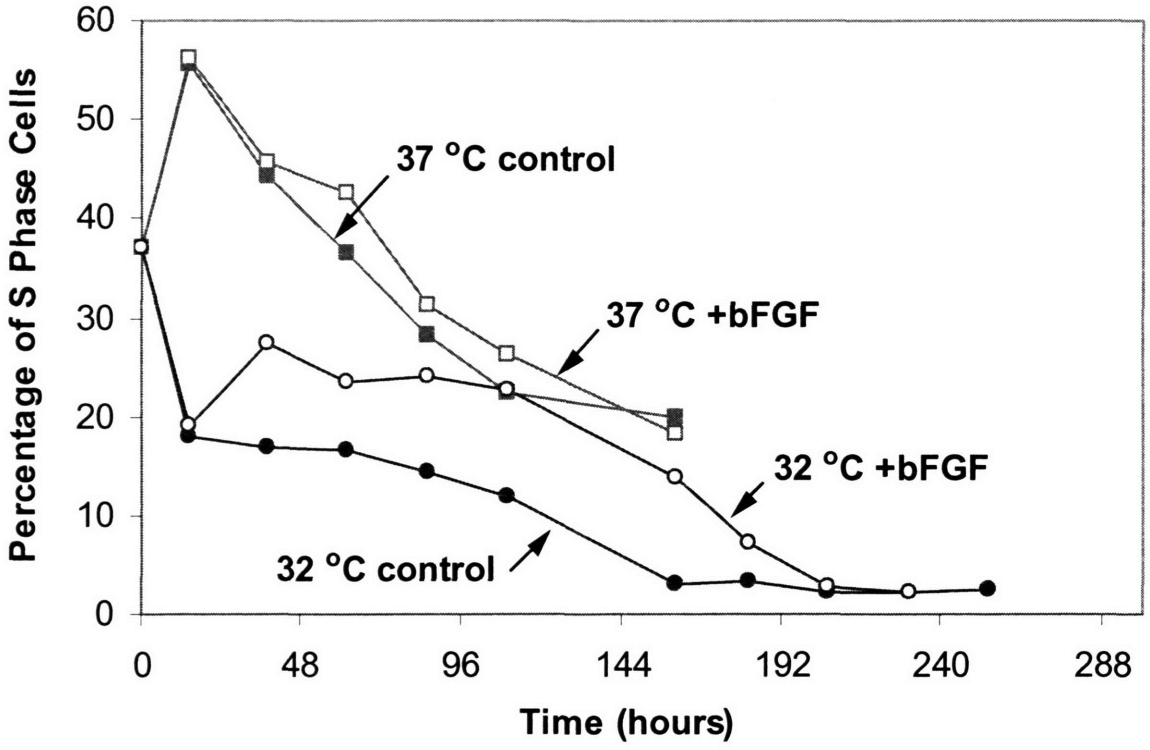

Figure 7-7. bFGF increases the percent of S phase cells throughout culture under hypothermic conditions. Adherent cells were grown at 32 °C and 37 °C in the presence or absence of 45 ng ml^{-1} bFGF. The bFGF has no significant effect at 37 °C (■ control; □ bFGF). In contrast, the bFGF causes the cells to maintain about 10% more S phase cells at 32 °C throughout the culture (● control; ○ bFGF).

7.4 HYPOTHERMIC ACTIVE GROWTH INCREASES IFN-γ PRODUCTION

The end-of-batch IFN-γ concentration is significantly higher for the bFGF-treated, hypothermic culture (Figure 7-8) compared to any of the other three conditions, increasing total production by 110% compared to the 37 °C control and by 50% compared to the 32 °C control. Thus, the novel culture concept, hypothermic active growth, is validated as a means for improving total production of recombinant glycoproteins.

The bFGF itself appears to have a negative effect on specific productivity, as can be seen by the 20-25% decrease relative to the non-treated control (Figure 7-9), regardless of culture temperature. The molecular mechanism for this detrimental effect is unknown. However, the critical point for validating *active hypothermic growth* is that the ratio of 32 °C to 37 °C specific productivity remains essentially unchanged for the non-treated and bFGF-treated samples (Figure 7-10) showing that the positive effect of hypothermia on specific productivity can be achieved simultaneously with active growth.

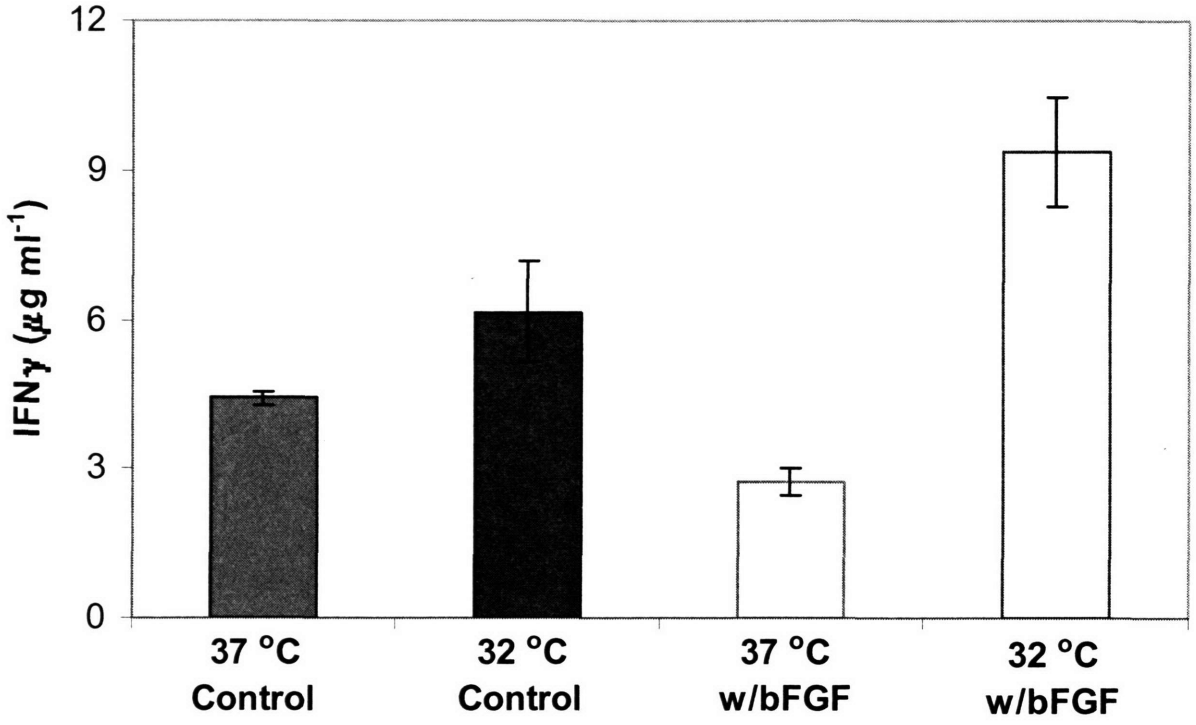

Figure 7-8. Active hypothermic growth caused increased total IFN-γ production. Adherent cells were grown at 32 °C and 37 °C in the presence or absence of 45 ng ml^{-1} bFGF and IFN-γ concentration was measured at the end of the batch culture. The bFGF causes total production to decrease at 37 °C (■ control; □ bFGF). In contrast, the bFGF causes the total production to increase by about 50% under hypothermic conditions by promoting growth (● control; ○ bFGF).

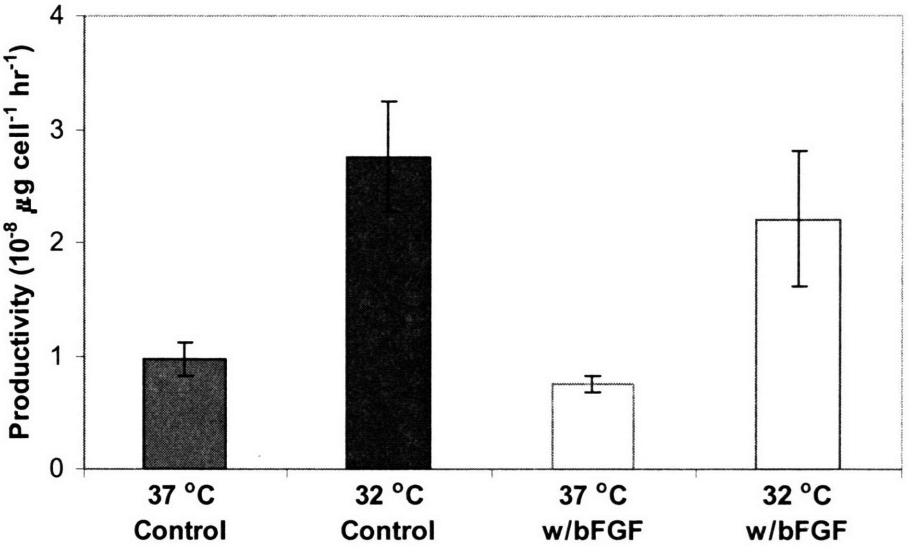

Figure 7-9. bFGF reduces average specific IFN-γ productivity at both temperatures. Adherent cells were grown at 32 °C and 37 °C in the presence or absence of 45 ng ml^{-1} bFGF and average IFN-γ specific productivity for the entire culture was calculated. The bFGF causes specific productivity to decrease both at 37 °C and at 32 °C.

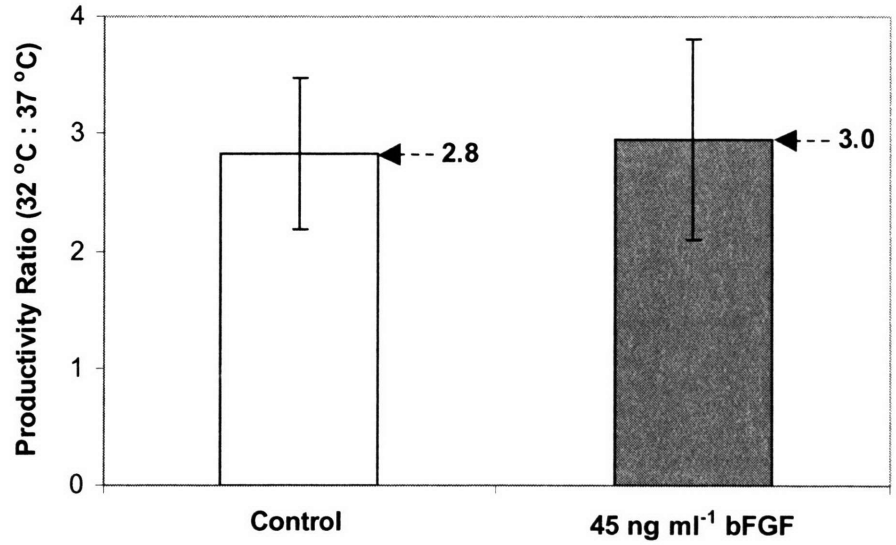

Figure 7-10. The positive effect of hypothermia on specific productivity is maintained during active growth. Adherent cells were grown at 32 °C and 37 °C in the presence or absence of 45 ng ml^{-1} bFGF and average IFN-γ specific productivity for the entire culture was calculated. The *ratio* of 37 °C to 32 °C specific productivity remains unchanged in the presence or absence of bFGF, showing that hypothermic active growth does not diminish the positive effect of low temperature on specific productivity.

7.5 HYPOTHERMIC ACTIVE GROWTH IN SUSPENSION CULTURE

For an industrial production process, cells are usually grown in suspension culture. The results presented in the first few sections of this chapter were generated using adherent cells grown in tissue culture flasks. To verify that hypothermic active growth is also feasible in suspension culture, cells were subjected to mitogen stimulation using high doses of FBS. The basal medium used was a commercially available protein-free medium. The effect of 0, 10 and 25% (v/v) FBS on cell growth at 37 °C and 32 °C is shown in Figure 7-11. Clearly, hypothermic active growth is feasible in suspension culture, as seen from the data. In fact, the hypothermic growth is far more impressive than what had been obtained for the bFGF-stimulated adherent cells, with hypothermic culture in the presence of FBS reaching the same peak density as 37 °C control.

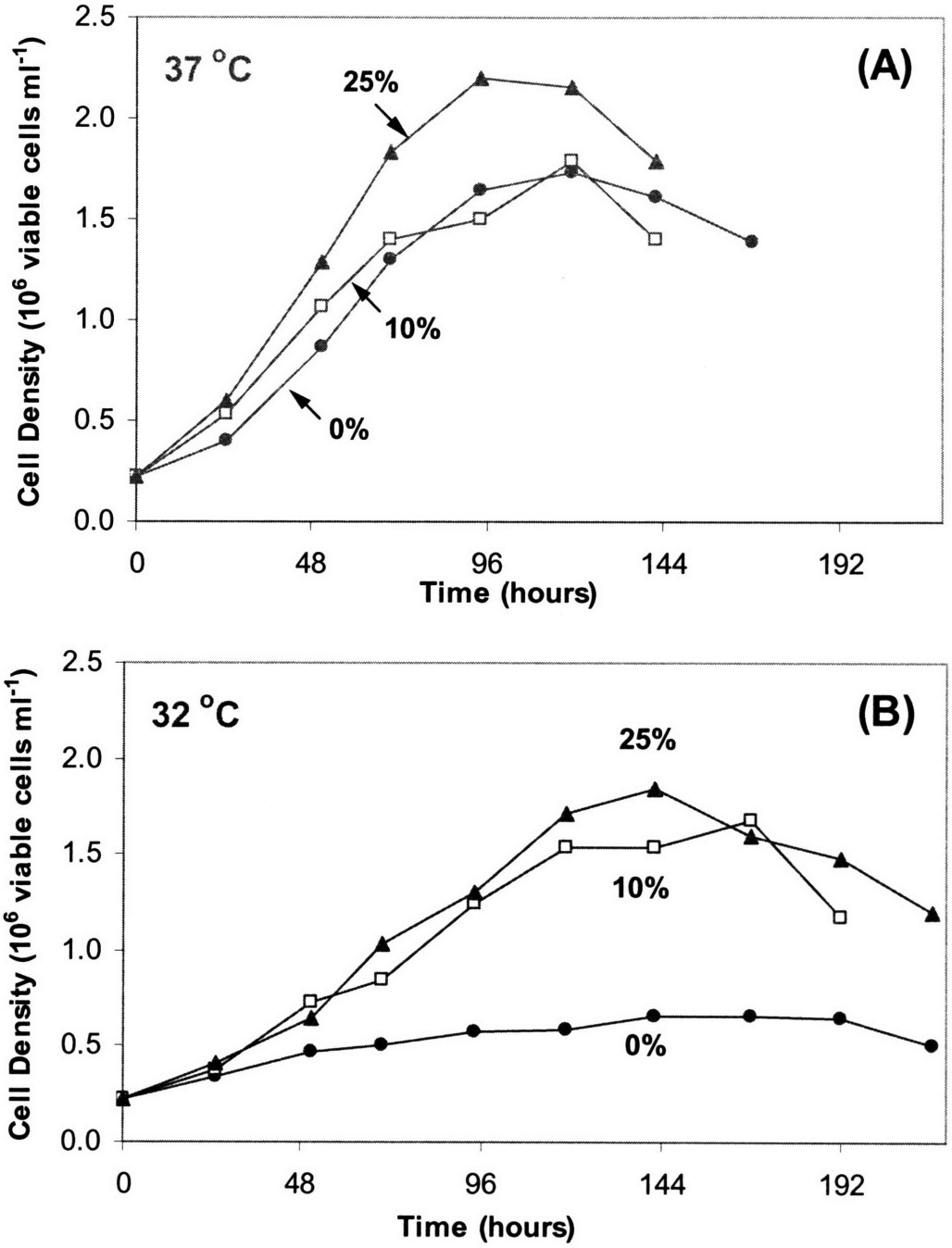

Figure 7-11. Serum supplementation significantly increases cell density at low temperature. Cells were grown in suspension culture in a protein-free medium supplemented with 0 (●), 10 (□) or 25% (▲) FBS. FBS supplementation does not significantly increase cell density at 37 °C (A) whereas FBS supplementation has a large effect on growth at 32 °C (B). The cultures were terminated once the percent cell viability was less than 80%.

7.6 HYPOTHERMIC ACTIVE GROWTH GREATLY ENHANCES TOTAL IFN-γ PRODUCTION IN SUSPENSION CULTURE

As hypothesized, the hypothermic active growth cultures were able to simultaneously maintain high productivity and achieve high cell density, meaning that total production was enhanced significantly (Figure 7-12). The 25% FBS culture at low temperature had 7.7-fold higher total production than the 37 °C control and 4.9-fold higher than the 32 °C control. The FBS had an insignificant effect on 37 °C total production, and thus the enhancements under hypothermic conditions can be attributed directly to the improved growth rather than to a secondary effect of the FBS.

The separation between the hypothermic effect on productivity and the growth state effect on productivity has been demonstrated previously (e.g. see Figure 6-3). In the absence of cell cycle distribution data, a similar effect can be seen by plotting productivity versus growth rate obtained during the exponential phase of growth (Figure 7-13). This figure shows that in suspension culture, as was the case with the previous findings using adherent cells, the hypothermic effect is not due to growth arrest, and in fact improved growth is desirable for increased CHO-γ productivity, regardless of culture temperature.

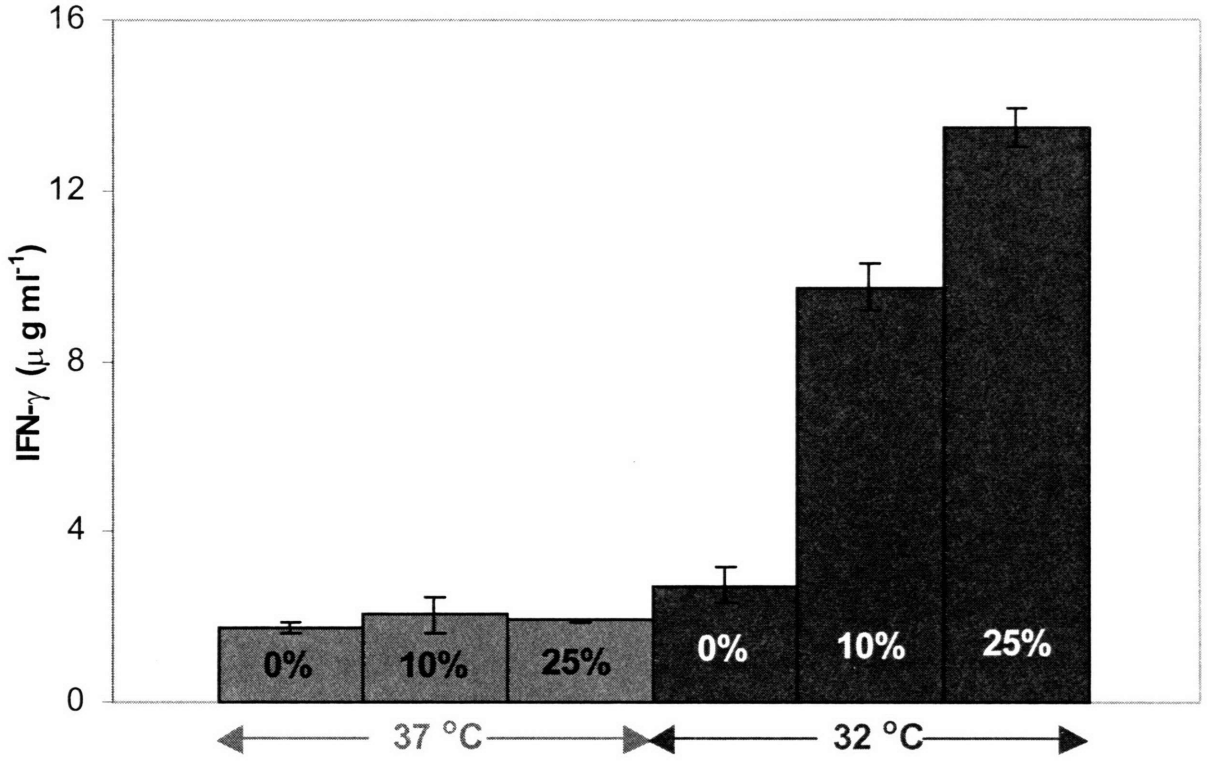

Figure 7-12. Serum supplementation significantly increases total production during hypothermic growth. Cells were grown in suspension in protein-free medium supplemented with 0, 10 or 25% FBS. Whereas FBS has an insignificant impact on production at 37 °C, serum is seen to dramatically increase total production at 32 °C, as a result of the combined improvement in cell growth (see Figure 7-11) and the high specific productivity characteristic of hypothermia.

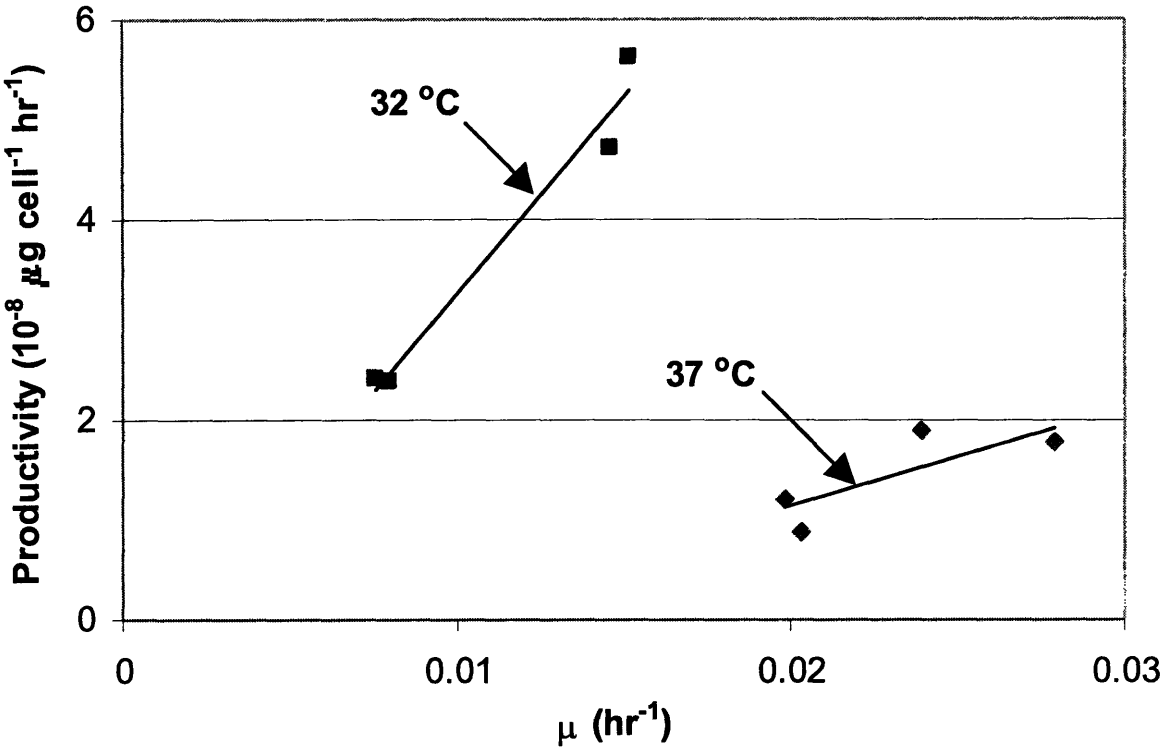

Figure 7-13. Specific productivity is higher in actively growing cells. Cells were grown in suspension in protein-free medium supplemented with 0, 10 or 25% FBS. Average specific productivities for the entire culture were found from the final IFN-γ production (Figure 7-12) and the culture IVCD (Table 7-1). The effect of temperature on productivity is separate from the effect of growth state on productivity. Both hypothermia and active growth promote increased specific productivity.

7.7 DISCUSSION AND CONCLUSIONS

In Chapter 6, it was hypothesized that cells capable of maintaining active growth at low temperature would prove to be the optimal production platform for maximizing IFN-γ production, owing to the fact that CHO-γ was a growth-associated producer that also had enhanced productivity at low temperature. In this chapter, the hypothesis was proven to be correct by stimulating hypothermic growth using mitogens. The growth factors kept the cells cycling through the cell cycle instead of arresting in G_0/G_1, resulting in much higher cell densities at low temperature without sacrificing the enhanced specific productivity. This, consequently, achieved higher IFN-γ concentrations.

The greatly improved cell densities are due somewhat to improved growth rates but are also due to exponential growth being maintained for a longer period of time. These findings are summarized in Table 7-1. The growth rate was improved by about 10-35% at low temperature. More importantly, the mitogen-stimulated cells maintained exponential growth for about 2 days longer than the control for all the hypothermic conditions tested.

Table 7-1. Culture performance parameters for various mitogen conditions

Growth condition	Temp. (°C)	μ (hr^{-1})	Days of log phase[1]	Maximum Cell Density Relative to: 32 °C Control	37 °C Control	IVCD (cell hr ml^{-1})
Suspension- Control	37	0.025	3	-	*1.00*	2.0×10^8
Suspension- 10% FBS	37	0.028	3	-	1.03	1.6×10^8
Suspension- 25% FBS	37	0.032	3	-	1.27	2.1×10^8
Suspension- Control	32	0.015	2	*1.00*	0.38	1.2×10^8
Suspension- 10% FBS	32	0.019	4	2.55	0.97	2.1×10^8
Suspension- 25% FBS	32	0.020	4	2.88	1.03	2.6×10^8
Adherent- Control	37	0.027	5	-	*1.00*	1.5×10^8
Adherent- 45 ng ml^{-1} bFGF	37	0.028	4	-	0.81	1.2×10^8
Adherent- Control	32	0.011	7	*1.00*	0.33	0.7×10^8
Adherent- 45 ng ml^{-1} bFGF	32	0.012	9	2.03	0.67	1.4×10^8

[1] Defined here as the last day on which a linear fit of a log plot of cell density versus time has an R^2 value > 0.97

The molecular mechanism by which the mitogens maintain the cells in active growth is likely by exerting a positive effect on cell cycle progression. Growth factors, such as bFGF, bind specific cell surface receptors. This binding event in turn increases the kinase activity of the receptor, which then leads to a cascade of signals promoting growth (Jones and Kazlauskas, 2000). Whereas hypothermia serves as a negative signal to the cell cycle control system, activating p53 and CIRP and thereby triggering a further cascade that ultimately prevents the CDK molecules from activating cell passage to the S phase, growth factors will have the opposite effect, promoting CDK activity or inhibiting CKIs (see Section 2) as a result of their initial binding and activation of the cell surface receptors. Thus, there appears to be a "tug-of-war" effect on the cell cycle, with hypothermia promoting arrest and mitogens promoting growth and the outcome depending on mitogen level. For example, increasing the bFGF concentration has an increasing effect on improving the peak cell density achievable in a batch culture (Figure 7-4). Whether this trend would continue beyond the 45 ng ml^{-1} bFGF concentration tested is unknown. Insulin, on the other hand, did not have such an effect, with 5-25 mg ml^{-1} causing essentially the same peak density. In this case, this may be due to the previously mentioned increasing levels of apoptosis with increasing levels of insulin, and thus the positive effect on hypothermic growth being masked by the cell death.

Compared to bFGF, the effects of FBS were far more impressive. Whereas bFGF treatment at 32 °C allowed cells to reach 44% and 67% of 37 °C growth rate and peak density, respectively, FBS treatment at 32 °C allowed cells to reach 80% and 103% of the 37 °C values of these two parameters. The end result was a spectacular, multi-fold increase in total IFN-γ production (Figure 7-12). Unfortunately, FBS cannot be used for new bioprocesses producing human therapeutics. Nevertheless, the contrasting results between bFGF and FBS should

motivate the search for a well-defined, FDA-approved growth factor or chemical formulation capable of achieving the level of hypothermic growth caused by FBS, as shown here.

As discussed in previous chapters, one of the major drawbacks of controlled proliferation strategies, including hypothermic culture, is that although specific productivity is higher, total production is not always significantly improved due to the compromise on cell density by using controlled proliferation. This is evident in the hypothermic culture literature. For example, Furukawa and Ohsuye (1998) found that *specific* productivity was highest at 32 °C, within the range 30 – 37 °C, for their recombinant CHO cell line. However, culturing at 35 °C maximized *total* production, because this temperature achieved the optimum in the tradeoff between higher productivity at low temperature and higher growth rates at elevated temperatures. This was despite the fact that 32 °C had a 50% higher specific productivity than 35 °C. A similar finding can be found in another hypothermic study, wherein specific productivity at 30 °C was 40% higher than that at 33 °C, yet total production at the slightly higher temperature was 3 times higher than at 30 °C (Yoon et al, 2003). By promoting active growth at low temperature, one no longer needs to compromise as significantly on the reduced growth rate and can operate closer to the temperature that gives highest specific productivity. From the findings in this chapter, it is predicted that the above mentioned researchers would achieve maximum total production by stimulating growth at the lower temperatures, thereby achieving the high cell density whilst simultaneously maintaining the high productivity.

The generally concept shown here, namely promoting growth under otherwise unfavorable conditions, may also be applicable and beneficial to other controlled proliferation methods. For example, it may be extremely useful to promote active growth in the presence of

sodium butyrate, the enhanced specific productivity of which does not appear to depend on growth arrest, as discussed at the end of Chapter 6.

In conclusion, this chapter introduced and validated a novel cell culture strategy, namely active hypothermic growth. This finding is envisioned to be applicable to any cell line that has growth-associated productivity and also has enhanced productivity under hypothermic conditions. The finding also applies to suspension culture and therefore is relevant for significantly improving production in industrial processes.

8. ISOLATING CHO CELLS CAPABLE OF HYPOTHERMIC GROWTH

The previous chapter validated hypothermic active growth as a novel means for achieving much higher total production of recombinant glycoproteins. This was achieved by providing high doses of mitogenic factors. There are several drawbacks associated with using this approach in an industrial production process, such as the high cost of such factors, the undefined nature of serum, and the additional components that must be removed in downstream purification steps. Clearly, the decision as to whether to include mitogenic factors in production medium in order to obtain increased production would need to be evaluated by a cost-benefit analysis on the entire process.

A better option would be to obtain a CHO cell capable of hypothermic active growth without the addition of extra mitogenic factors. If this is possible, no changes would need to be made to the production medium and the downstream purification process would not be adversely impacted. This chapter presents the findings from the isolation and characterization of hypothermic growth cells. The concept was first proven possible and validated in the CHO-γ cell line and then extended to CHO-K1, the non-recombinant CHO cell line, thereby producing a generic production platform that is suitable for producing *any* recombinant protein.

8.1 OBTAINING CELLS WITH IMPROVED GROWTH UNDER HYPOTHERMIC CONDITIONS

The details of the screen used to obtain the cells capable of improved growth under hypothermic conditions have been presented in the Materials and Methods section (Chapter 3). The philosophy of the screen is as follows: mutate the cells and then allow the population to recover under hypothermic conditions, where it is assumed that any cell with a growth advantage

over the other cells will comprise an ever increasing fraction of the population as time passes under hypothermic selection. The increase in cell number with time during exponential growth is found from the following equation:

$$N = N_0 \exp(\mu t) \qquad (8\text{-}1)$$

Where N_0 is the initial cell number, N is the cell number at time t, and μ is the growth rate.

To estimate the *minimum* time required for recovery at 32 °C for obtaining a population dominated by the fast-growing cells, several assumptions were made, as explained below. If we consider the population to consist of two distinct cell types, namely fast growers (f) at 32 °C and normal growers (n) at 32 °C, then the ratio of these two cell types at any time is given by taking the ratio of Equation (8-1) applied to each subpopulation:

$$N_f / N_n = N_{f,0} / N_{n,0} \exp[(\mu_f - \mu_n)t] \qquad (8\text{-}2)$$

In Table 7-1 of Chapter 7, the difference in growth rate between 32 °C and 37 °C control cultures is approximately 0.01 to 0.016 hr^{-1}. Based on the finding in Chapter 7 that growth rate at 34.5 °C is indistinguishable from that at 37 °C, it was assumed theoretically possible to obtain a mutant capable of achieving the 37 °C growth rate at 32 °C, and consequently the difference in growth rate term in Equation 7-1 is set equal to 0.01 to 0.016 hr^{-1}. Approximately 10^8 cells were used in the mutagenesis. Assuming only 1 cell in this population at time zero is a fast grower at 32 °C, Equation (8-2) can be rearranged and solved to find the time needed for the 1 mutant cell to have become 50% of the population:

$$t = \frac{\ln\left[\left(N_f / N_n\right)\left(N_{n,0} / N_{f,0}\right)\right]}{\mu_f - \mu_n} = \frac{\ln[10^8]}{0.01 \text{ to } 0.016\ hr^{-1}} = 1150 \text{ to } 1840\ hr = 48 \text{ to } 76\ days \qquad (8\text{-}3)$$

Based on this calculation, the cells were given 80 days recovery at 32 °C following mutagenesis before conducting an initial analysis of the mixed population. After this recovery period, it was found that the irradiated population did indeed exhibit improved growth at low temperature and the population was then characterized and compared to the control CHO-γ cells in terms of growth at 32 °C, 34.5 °C and 37 °C, as discussed below.

8.2 GROWTH CHARACTERISTICS OF AN IRRADIATED POPULATION

The irradiated population exhibited improved growth at 32 °C, proving that the screen described above was effective at enriching the population for cells that have the ability to grow under hypothermic conditions. Images of the cells taken during culture are shown in Figure 8-1. At 37 °C, there is no obvious difference between the mutant and control populations on day 4. However, at 32 °C, the mutants are clearly growing faster than the control by day 7 and have a morphology characteristic of the 37 °C cells. The mutant morphology is similar to that observed for bFGF-treated 32 °C cells (compare to Figure 7-5).

The growth curve of the control and mutant CHO-γ cells (Figure 8-2) shows quantitatively that the mutant population exhibits reduced growth arrest at 32 °C whilst maintaining nearly identical growth to the controls at 37 °C. This shows that the screen did not simply produce fast growers *in general* but specifically produced cells that are fast growers *under hypothermic conditions*. In addition, the mutants and controls were grown at 34.5 °C and the growth rate at this temperature for both cell lines was nearly identical to the 37 °C growth rate, as can be seen in the Arrhenius plot of growth rate for both cell lines in Figure 8-3.

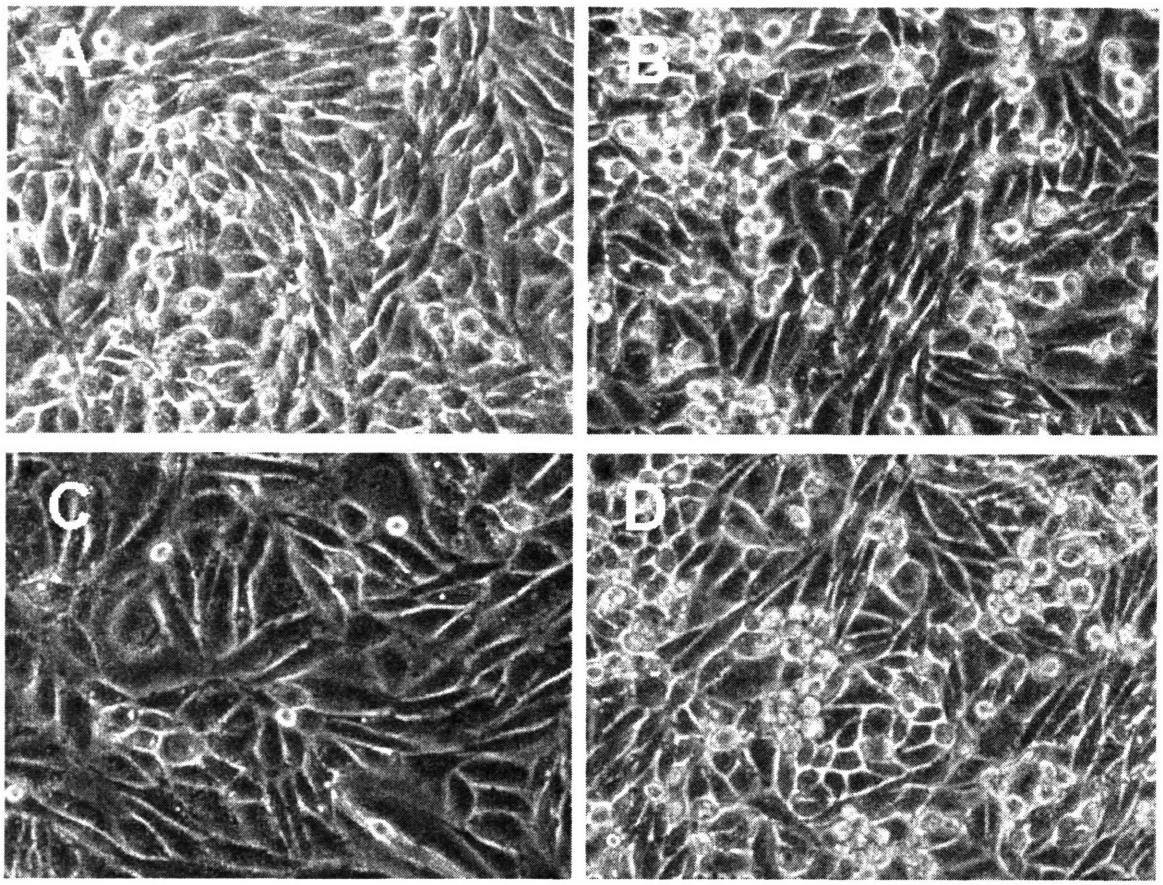

Figure 8-1. Hypothermic mutant population exhibits improved growth at low temperature. Adherent CHO-γ control and hypothermic mutant cells were seeded at the same density and grown at 32 °C or 37 °C. (A) Control cells on day 4 at 37 °C; (B) Mutant cells on day 4 at 37 °C; (C) Control cells on day 7 at 32 °C; (D) Mutant cells on day 7 at 32 °C. Note the lower surface density for "C" relative to "D" and the long, elongated morphology in "C", a characteristic of G_0/G_1 arrested CHO cells. See text for details.

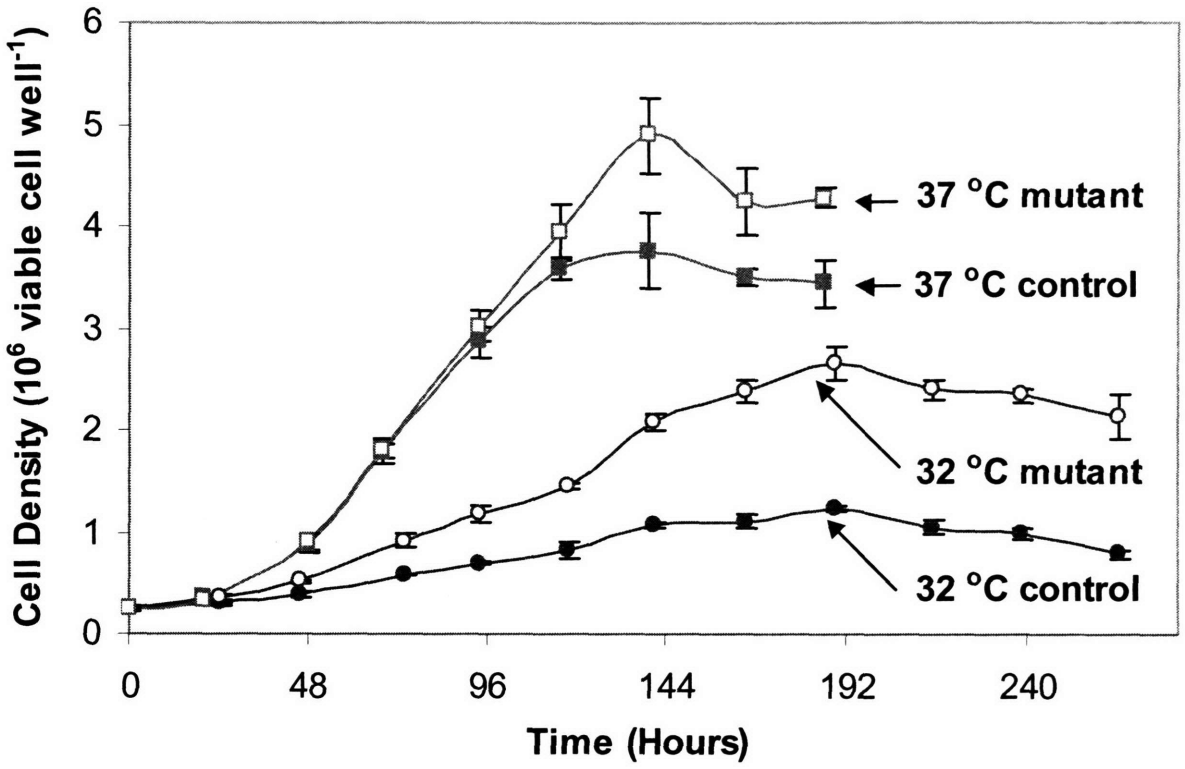

Figure 8-2. Hypothermic mutants are capable of improved growth at 32 °C. Adherent CHO-γ control and hypothermic mutant cells were grown at 32 °C (● control; ○ mutant) and 37 °C (■ control; □ mutant). At 32 °C, the mutants achieve more than double the cell density of the control, whereas growth at 37 °C is not greatly affected. The cultures were terminated once the percent cell viability was less than 80%.

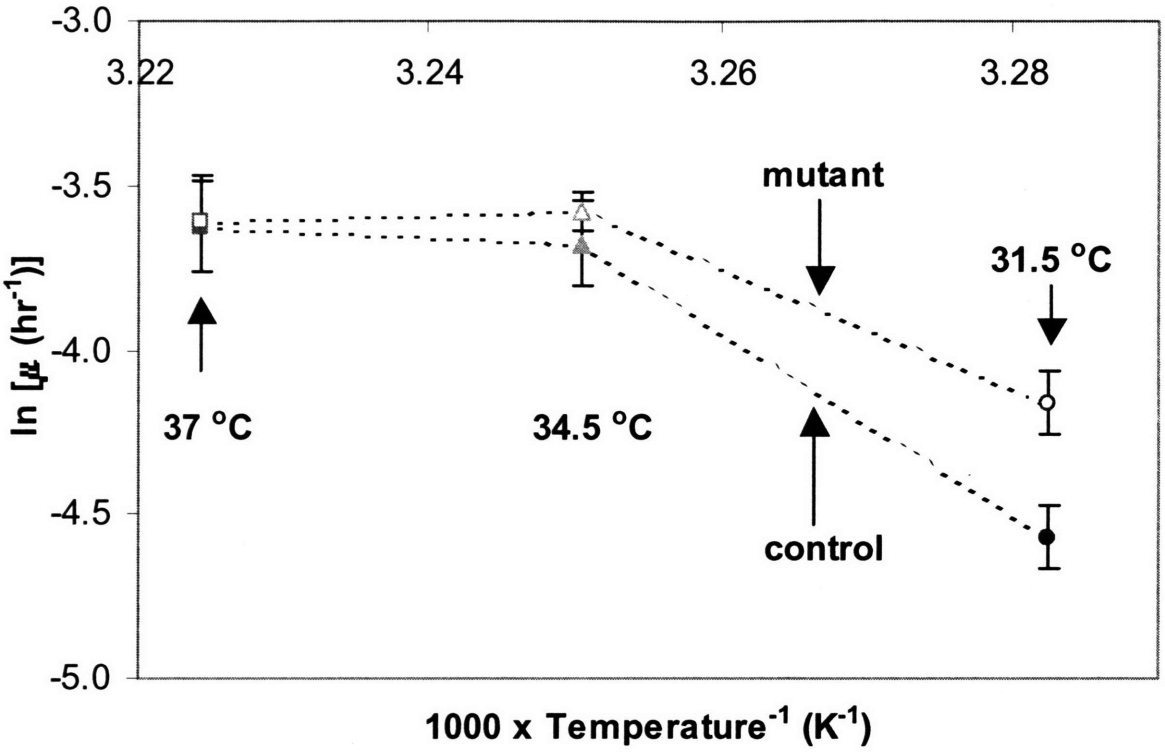

Figure 8-3. Arrhenius plot reveals that hypothermic mutants exhibit improved growth rate under hypothermic conditions. Adherent CHO-γ control and mutant cells were grown at 31.5 °C (● control; ○ mutant), 34.5 °C (▲control; Δ mutant) and 37 °C (■ control; □ mutant). Whereas both cell lines exhibit strong arrest at 31.5 °C relative to the 37 °C control, they are hardly affected by growing at 34.5 °C. The mutant has significantly higher growth rate at 31.5 °C than the control

8.3 GROWTH CHARACTERISTICS OF SINGLE CELL CLONES

The mutant population was subjected to single cell cloning to obtain pure mutant strains of the CHO-γ cell. These cells were characterized for growth at 32 °C and 37 °C, and it was found that two distinct cell types existed in the mutant population. Both cell types grow faster than the control at 32 °C, which was the desired phenotype. In contrast, the first type of mutant selected at 32 °C grew almost the same as the control at 37 °C whereas the second type of mutant selected at 32 °C grew slower than the control at 37 °C. One cell line from each type was selected for further characterization.

The growth curves of the pure mutant clones and control at 32 °C and 37 °C are shown in Figure 8-4. The mutants are capable of achieving improved growth at 32 °C and consequently reach much higher maximum cell densities and batch IVCD than the control cells. The key growth parameters are summarized in Table 8-1.

As was the case with the growth factor (serum and bFGF) supplementation, the success of the mutant growth at 32 °C is due to both a slight improvement in growth rate but also due to the ability to remain in exponential growth for a longer period of time than the controls (Table 8-1). For example, the control had ceased growing by day 6 at 32 °C whereas the two mutants continued to exhibit exponential growth until day 9. This prolonged exponential growth plays a larger role in the 2.4-2.8 fold increase in peak cell density and final IVCD than the 16% increase in specific growth rate (0.012 versus 0.014 hr^{-1}) does.

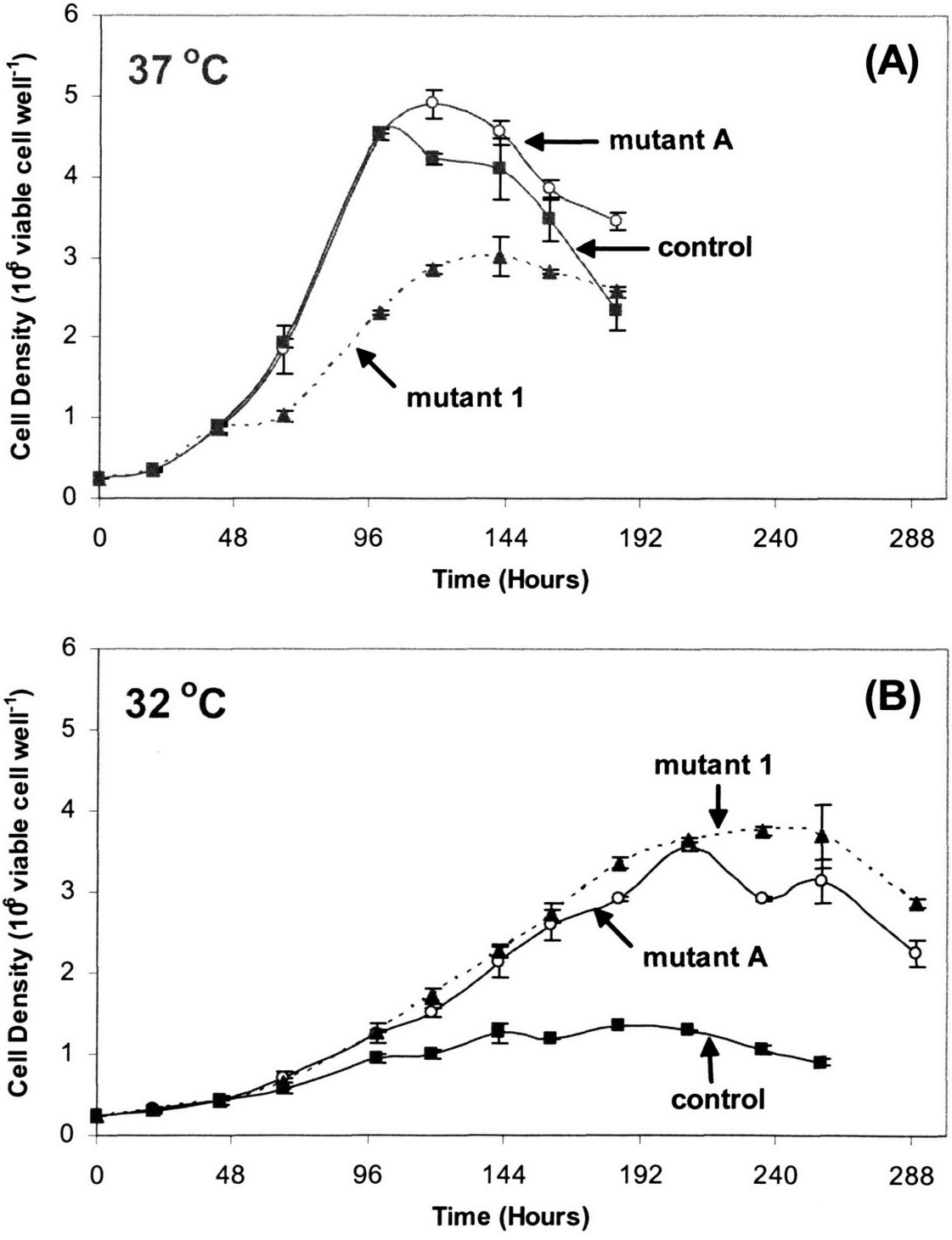

Figure 8-4. Mutant Clones are capable of significantly enhanced hypothermic growth. Adherent CHO-γ control cells and mutant clones "1" and "A" were grown at (A) 37 °C and (B) 32 °C. At 37 °C, mutant A (○) grows similarly to the control (■) whereas mutant 1 (--▲--) grows slower. In contrast, both mutants exhibit significantly improved growth at 32 °C. The cultures were terminated once the percent cell viability was less than 80%.

Table 8-1. Culture performance parameters for CHO-γ Control and Mutants

Cell Line	Temp. (°C)	μ (hr^{-1})	Length of log phase (days)[1]	Maximum Cell Density Relative to: 32 °C Control	Maximum Cell Density Relative to: 37 °C Control	End-of-Batch IVCD (cell hr ml^{-1})
CHO-γ Control	37	0.030	4	-	*1.00*	1.6×10^8
CHO-γ Mutant A	37	0.027	5	-	1.08	1.7×10^8
CHO-γ Mutant 1	37	0.021	5	-	0.67	1.1×10^8
CHO-γ Control	32	0.012	6	*1.00*	0.30	0.8×10^8
CHO-γ Mutant A	32	0.014	9	2.6	0.79	1.8×10^8
CHO-γ Mutant 1	32	0.014	9	2.8	0.83	2.1×10^8

[1] Defined here as the last day on which a linear fit of a log plot of cell density versus time has an R^2 value > 0.97

The mutants grown at 32 °C still do not reach the cell concentration obtained at 37 °C, but the cell concentration difference between 32 °C and 37 °C has been reduced significantly from what is obtained with control cells. Whereas the 32 °C control only obtains 30% of the peak density seen at 37 °C, the mutants grown at 32 °C reach about 80% of the 37 °C control peak density. In addition, the control cells at 32 °C have less than half the batch IVCD compared to 37 °C controls whereas the mutants at 32 °C actually exceed the 37 °C control by 20-30% in IVCD!

The ability of the mutants to obtain higher cell density under hypothermic growth conditions can be attributed to cell cycle level changes. The percentages of S phase cells, an indicator of active growth, are shown as a function of culture time for both 32 °C and 37 °C in Figure 8-5. Under hypothermic conditions, the mutants have a consistently elevated (5-10% higher) percent of S phase cells compared to the CHO-γ controls for most of the culture. In contrast, the situation at 37 °C is far less consistent. Mutant A has a higher percent S than the control for the first 3 days of culture but then has a lower percent S for the rest of the time. Mutant 1 exhibits very peculiar behavior, showing a large "spike" in the percent of S phase cells and then decreasing below the control by day 5. The "spike" is consistent with the growth curve of Mutant 1 at 37 °C (Figure 8-4), which shows almost no growth from 40 – 70 hours but a large increase in the percentage of S phase cells during this period (buildup of the "spike"), followed by a period from 70 – 96 hours wherein the cell number more than doubles and the percentage of S phase cells decreases significantly.

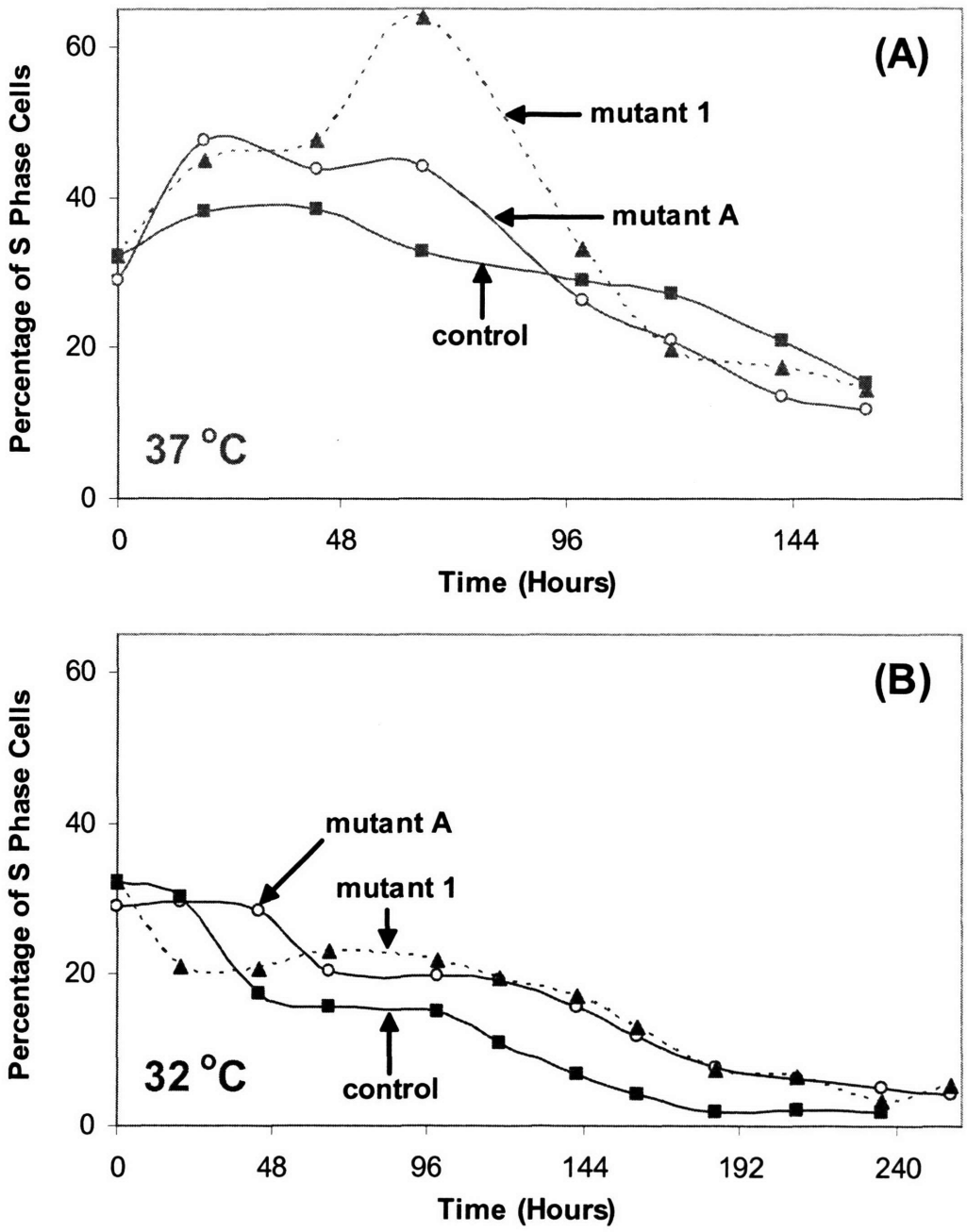

Figure 8-5. Mutant Clones have a higher proportion of S phase cells during hypothermic growth. Adherent CHO-γ control cells and mutant clones "1" and "A" were grown at (A) 37 °C and (B) 32 °C and the proportion of cells in each phase of the cell cycle was measured on a daily basis. At 37 °C, mutant A (○) exhibits a higher percentage of S phase cells relative to the control (■) during the first few days, followed by a period of slightly lower percentage of S phase cells whereas mutant 1 (--▲--) exhibits a sharp increase in S phase cells on day 3 followed by a rapid decline. In contrast, the trends are far more consistent under hypothermic conditions, with both mutants exhibit a higher proportion of S phase cells throughout the culture at 32 °C.

To gain more insight into the mutant effect on cell cycle, the time spent in each phase of the cell cycle can be estimated by a series of equations developed by Slater et al (1977) and referred to as the "homogeneous daughter" model:

$$T_S = \frac{T_d}{\ln(2)} \ln\left[1 + \frac{f_S}{1 + f_{G2M}}\right] \qquad (8\text{-}4)$$

$$T_{G1} = \frac{T_d}{\ln(2)} \ln\left[1 - \frac{f_{G1}}{2}\right] \qquad (8\text{-}5)$$

$$T_{G2M} = \frac{T_d}{\ln(2)} \ln\left[1 + f_{G2M}\right] \qquad (8\text{-}6)$$

Where T_S, T_{G1} and T_{G2M} are the time spent in the S, G_1 and G_2/M phase, respectively, during the course of one cell doubling, T_d is the cell doubling time and f_S, f_{G1} and f_{G2M} are the fractions of cells in the S, G_1 and G_2/M phase, respectively (measured by flow cytometry). This model assumes an exponential age distribution within the different cell cycle subphases and allows the determination of the duration of the cell cycle subphases in unsynchronized, exponentially growing cultures (Slater et al, 1977). These three equations were applied to days 1-4 of culture, a time period chosen because all cultures are still in exponential growth, and the results are summarized in Table 8-2.

Table 8-2. Duration of each phase of the cell cycle for CHO-γ control and mutants

Cell Line	Temperature (°C)	Doubling Time (hr)	Length of G_1 Phase (hr)	Length of S Phase (hr)	Length of G_2/M Phase (hr)
Control	37	22.3 ± 2.7	9.1 ± 1.9	**8.4 ± 1.1**	4.7 ± 1.4
Mutant A	37	22.5 ± 2.6	8.0 ± 2.1	**10.0 ± 1.5**	4.5 ± 1.3
Mutant 1	37	33.4 ± 9.6	9.9 ± 2.6	**17.8 ± 5.1**	5.7 ± 2.5
Control	32	53.3 ± 5.7	**31.2 ± 1.2**	11.7 ± 4.1	10.4 ± 0.6
Mutant A	32	43.9 ± 10.7	**22.5 ± 3.1**	12.5 ± 5.0	8.9 ± 2.6
Mutant 1	32	45.5 ± 9.9	**25.1 ± 6.4**	11.3 ± 2.1	9.2 ± 1.4

Thus, the strange behavior of Mutant 1 at 37 °C can be seen to be due to a lengthening of the time spent in S phase by about 10 hours compared to the control. The other phases are not significantly different from the control. Mutant A, on the other hand, does not differ significantly in the length of any of the phases relative to the control at 37 °C. At 32 °C, the striking effect of hypothermia on the G_1 phase is evident by observing the length of each phase at the two temperatures for the control cells. The S and G_2/M length are mildly affected by hypothermia, with each increasing by 0-6 hours. However, the G_1 phase increases by about 22 hours for the control cells under hypothermia! Looking further, it is apparent that the enhanced growth of the mutants at low temperature is due to shortening the length of the G_1 phase by about 7 hours, reducing the time required for G_1 at 32 °C from 31 hours to 22-25 hours. The length of the other phases, S and G_2/M, are insignificantly different between the control and mutants at 32 °C.

8.4 HYPOTHERMIC GROWTH MUTANTS OBTAIN THE HIGHEST TOTAL PRODUCTION

The previous section showed that it is feasible to generate cell lines capable of having reduced growth arrest under hypothermia. However, the crucial issue was whether the mutants were able to maintain the enhanced productivity at hypothermic conditions despite alleviated growth arrest. Total production of IFN-γ was measured and the mutants grown under hypothermic conditions were found to have 3.3 - 3.8 times higher total production than the 37 °C control and 2.4 – 2.8 fold higher than the 32 °C controls (Figure 8-6). Total production at 37 °C for the mutants was not significantly different from the control, indicating that the screen had not

simply selected high producing clones and that the improvement in total production during hypothermic culture was attributable to the higher cell densities.

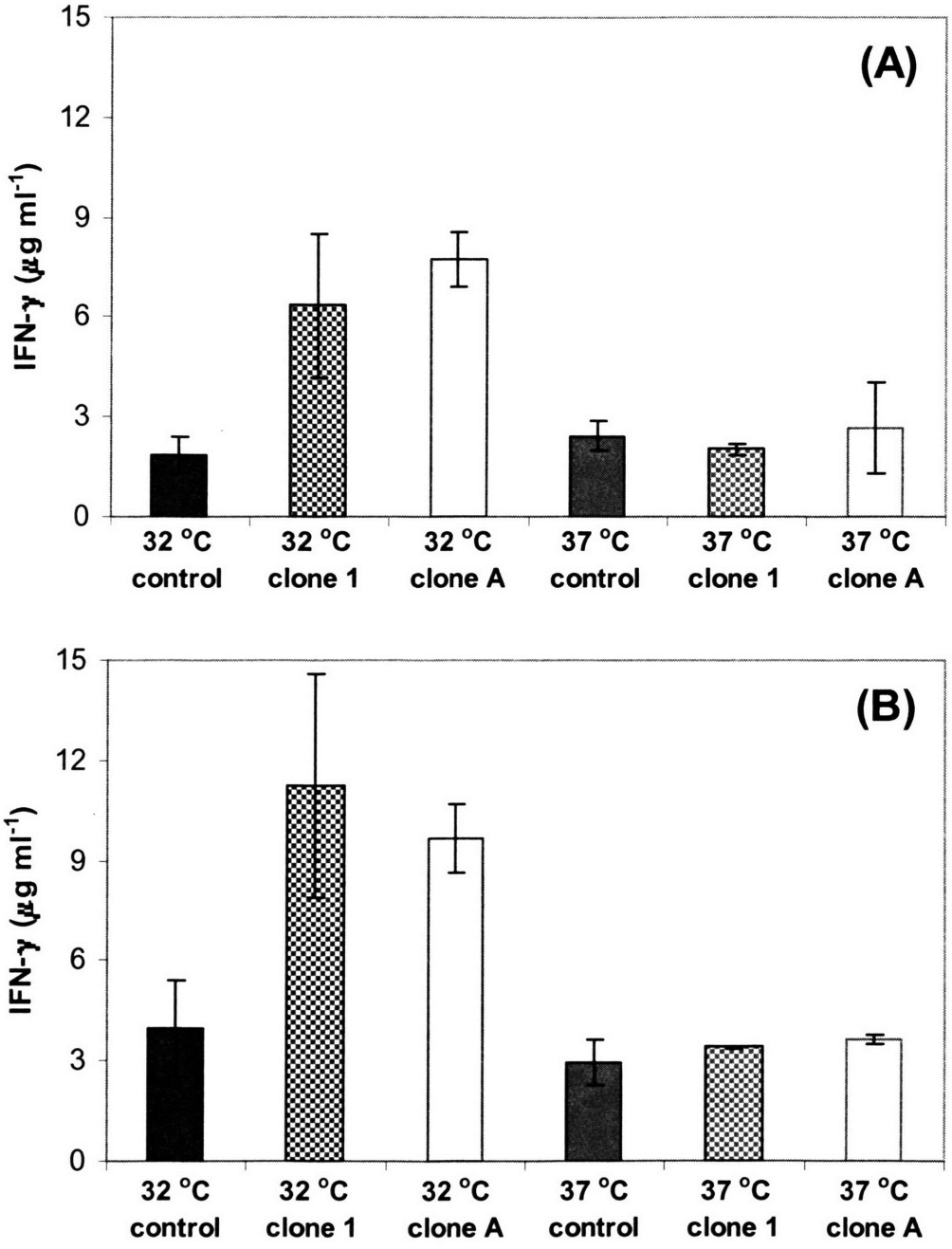

Figure 8-6. Mutants produce significantly more IFN-γ than control cells under hypothermic conditions. Adherent CHO-γ control cells and mutant clones "1" and "A" were grown at 37 °C and 32 °C and the total IFN-γ was measured on the last day cell viability was above 90% (A) and the first day cell viability was below 80% (B), which was also the point at which all cultures were terminated. At both viability points, the mutants grown under hypothermic conditions significantly outperformed any other cultures. The mutants did not show significant enhanced total production at 37 °C and thus the increase can be attributed to active hypothermic growth.

The fact that Mutant 1 has similar total IFN-γ production as the control at 37 °C despite having a lower IVCD (see Table 8-1) shows that the specific productivity is somewhat higher for this clone at 37 °C, as will now be discussed. Based on the findings in Chapter 6 showing that CHO-γ is a growth-associated producer and the fact that Mutant 1 was shown to spend a longer duration in S phase at 37 °C compared to Mutant A and the control cells (Table 8-2), it was hypothesized that the enhanced specific productivity exhibited by Mutant 1 at 37 °C is due to the relatively high proportion of S phase cells. This is seen by plotting specific productivity versus percent of weighted average S phase cells for all of the three cell lines at 32 °C and 37 °C (Figure 8-7). The highest productivity for the 37 °C group coincides with the period in which Mutant 1 had a very high percent of S phase cells (see the "spike" in Figure 8-5A). The general trend of the data is towards higher productivity when the population has a larger fraction of S phase cells, consistent with the Chapter 6 findings.

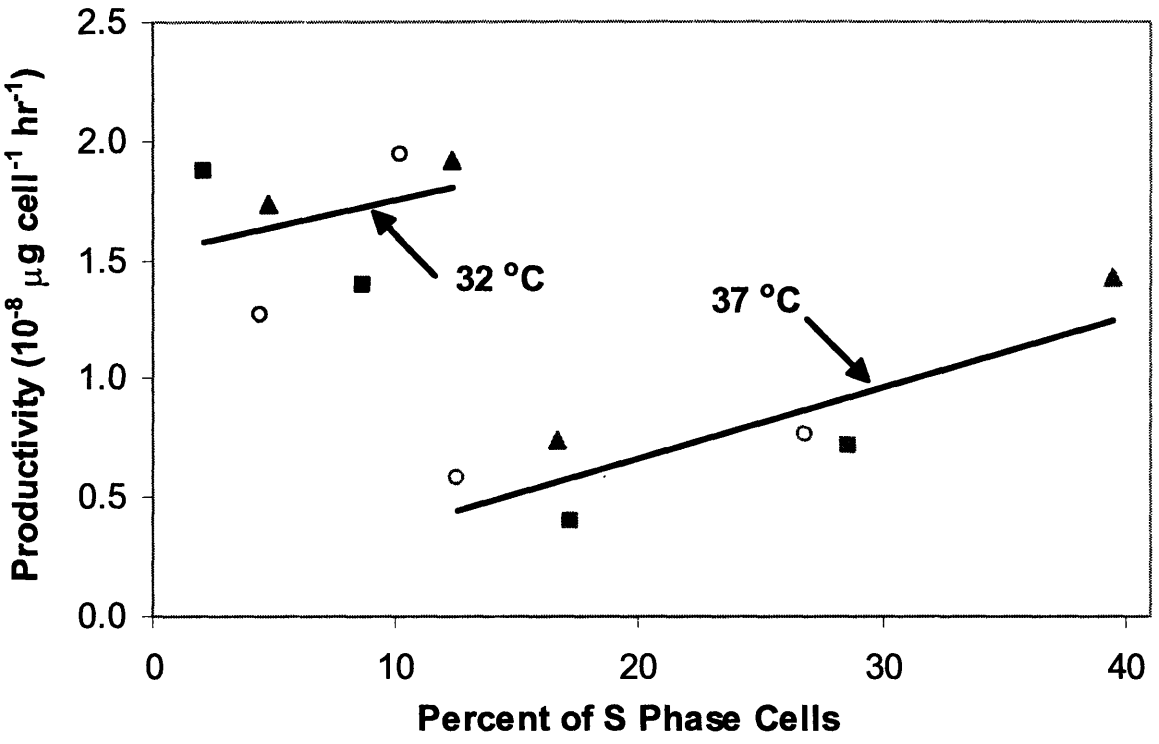

Figure 8-7. Specific Productivity of mutant and control cells at each temperature depends on cell cycle distribution. Adherent CHO-γ control cells (■) and mutant clones "1" (▲) and "A" (○) were grown at 37 °C and 32 °C. Specific productivity was calculated from the total IFN-γ concentration measured at two points per cell line (Figure 8-6). Differences in specific productivity between the mutants and control appear to be due to the dependence of productivity on cell cycle distribution.

8.5 CHO-K1 HYPOTHERMIC GROWTH CELL LINES

Based on the success of the hypothermic CHO-γ mutants, the mutagenesis screen was extended to CHO-K1, the non-recombinant parental cell line. The motivation for this study was two-fold. First, if successful, it would serve as a confirmation that the hypothermic growth CHO-γ cells were not dependent in an unknown manner on the presence of recombinant protein and that the concept of a hypothermic active growth cell was generally applicable. Second, the CHO-K1 cells can serve as future parental cell lines into which any recombinant protein can be engineered for future research into hypothermic culture as well as for future industrial production.

As discussed in the Materials and Methods (Section 3.2.10), the CHO-K1 screen included an extra control that was not included in the CHO-γ screen, namely a group of cells not UV irradiated (UV(-)) but otherwise carried through the identical screening procedure. The purpose of this control was to determine if cells capable of hypothermic growth were already present in the heterogeneous cell population prior to the mutagenesis.

Several promising clones were isolated from both the UV(+) and UV(-) screen. One of each type was selected for characterization and their growth at 32 °C and 37 °C was compared to control CHO-K1. The growth curves are shown in Figure 8-8 and the performance parameters are given in Table 8-3. Clone M25 was from the UV(+) screen and C33 from the UV(-) screen. The clone growth at 32 °C is remarkable, reaching approximately 50% higher density than any of the cells *at 37 °C* and achieving 2.5- to 3-fold higher batch IVCD than the 37 °C cells. It is apparent that hypothermic active growth cells can be isolated from the non-recombinant CHO-K1 cell line.

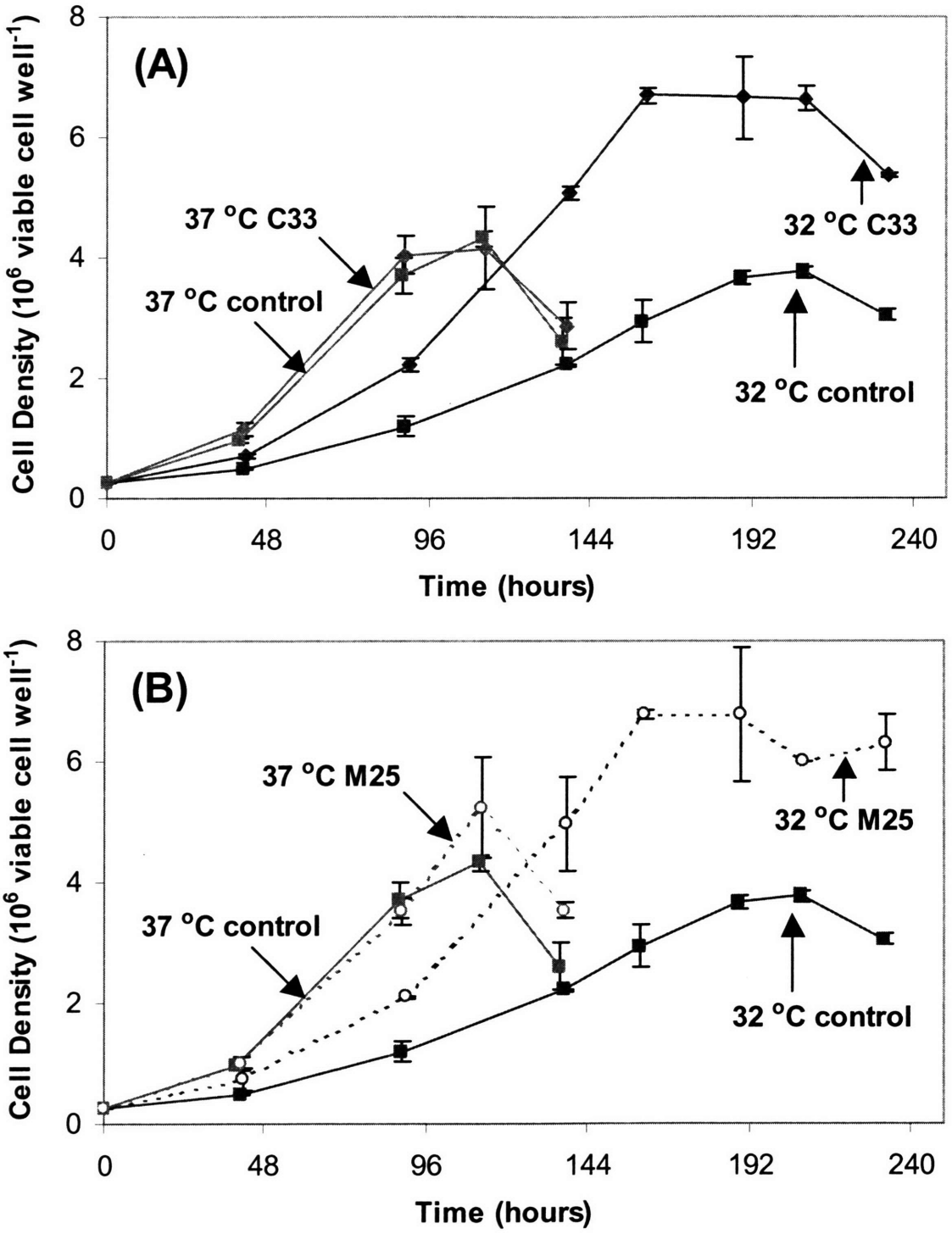

Figure 8-8. Mutant Clones are capable of significantly enhanced hypothermic growth. Adherent CHO-K1 control (■) cells and clones "C33" (♦), shown in (A), and "M25" (--○--), shown in (B), were grown at 37 °C and 32 °C. At 37 °C, the mutants grow similarly to the control. In contrast, both mutants exhibit significantly improved growth at 32 °C, with peak densities significantly surpassing the 37 °C and 32 °C control values.

Table 8-3. Culture performance parameters for CHO-K1 Control and Mutants

Cell Line	Temp. (°C)	μ (hr^{-1})	Length of log phase (days)[1]	Maximum Cell Density Relative to: 32 °C Control	37 °C Control	IVCD (cell hr ml^{-1})
CHO-K1 Control	37	0.031	4	-	*1.00*	1.0×10^8
CHO-K1 C33	37	0.032	4	-	0.96	1.1×10^8
CHO-K1 M25	37	0.028	5	-	1.21	1.1×10^8
CHO-K1 Control	32	0.015	7	*1.00*	0.87	1.5×10^8
CHO-K1 C33	32	0.022	7	1.78	1.54	2.8×10^8
CHO-K1 M25	32	0.022	8	1.80	1.57	2.8×10^8

[1] Defined here as the last day on which a linear fit of a log plot of cell density versus time has an R^2 value > 0.97

8.6 DISCUSSION AND CONCLUSIONS

In Chapter 6, it was shown that the hypothermic effect on productivity is not due to growth arrest and in fact active growth under hypothermic conditions is preferable for maximizing productivity. In Chapter 7, hypothermic active growth was shown to be possible by adding growth factors and the predicted, greatly improved total IFN-γ production was achieved. In this chapter, hypothermic active growth has been achieved and validated using a second method, namely by isolating CHO-γ cells capable of improved growth at 32 °C. As predicted from the previous chapters' findings, these mutants maintain high productivity, and coupled with the greatly improved cell densities, the total production is improved significantly.

The mutants did not exhibit significantly improved growth at 37 °C compared to the control and in some cases exhibited reduced 37 °C growth. This was expected, based on the same rationale as that behind the screen devised to isolate the hypothermic mutants. Namely, because the CHO-γ and CHO-K1 cell lines have been passaged and maintained at 37 °C for years, the fastest growers under these conditions would have already emerged and come to dominate the population. The opportunity to select hypothermic mutants is due to the fact that the cell lines are not maintained at low temperature and thus there has never been a selection pressure for hypothermic mutants to emerge.

The CHO-K1 screen added the second control of the non UV-irradiated cells, admittedly absent from the CHO-γ screen, needed to determine whether hypothermic growth cells already exist in the cell population prior to mutagenesis. The CHO-K1 screen did isolate fast growers without irradiation (see "C33" in Figure 8-8) thus showing that at least some of the hypothermic growth clones were already present in the population prior to UV exposure. Cell lines are well

known to exhibit heterogeneity (Follstad, 2000) and given that CHO-K1 has been passaged at least 400 times (see Section 3.2.1) it is not surprising if many different mutants already exist.

There is evidence showing that the clones isolated herein are not simply a homogeneous population that has adapted to growth at low temperature. In the case of the CHO-γ clones, they can be grown for at least 5 passages at 37 °C and still exhibit the same improved growth once shifted to 32 °C, suggesting that this improvement is stable and that these isolated clones are fundamentally different from the majority of the cells comprising the control population. Also, during the course of both the CHO-γ and CHO-K1 screens, other clones were isolated that showed growth characteristics at 32 °C intermediate between the control and the clones presented here. Because the goal was to obtain the maximum growth at low temperature, these intermediate clones were not analyzed in detail and only the ones showing the largest growth difference from the control were considered further. Also, CHO-γ mutant 1 behaves very differently than mutant A at 37 °C (Figure 8-4), exhibiting a dramatically lengthened S phase (Table 8-2). This shows that the clones are fundamentally different and thus the population from which they were isolated is heterogeneous, rather than a homogeneous population adapting to new conditions.

The molecular mechanism behind the improved growth of the CHO-γ and CHO-K1 clones isolated by the screen is unknown. For the CHO-γ cells, the fact that the G_1 phase was shortened relative to the control (Table 8-2) suggests that one of the proteins responsible for promoting G_0/G_1 arrest, such as p21, p27, p53 or CIRP, is no longer functioning properly or has reduced activity in the mutant cell lines. However, none of the mutants exhibited a full recovery of growth at 37 °C to levels obtained at 37 °C. Owing to the fact that 34.5 °C growth is indistinguishable from 37 °C growth, it seems plausible that a mutant can be found that exhibits

better growth at 32 °C than Mutant 1 and Mutant A did. Perhaps the complexity of the cell cycle and the many controls that are involved means that several rounds of mutagenesis and selection would be needed to obtain faster and faster hypothermic growth mutants.

The CHO-K1 mutants gave far more impressive growth results than the CHO-γ mutants, as can been seen by comparing the performance characteristics in Table 8-1 and Table 8-3. The CHO-γ mutants at 32 °C reached about 80% of the peak density of the 37 °C control, compared to the 32 °C control only reaching 30% of the density. This seems impressive until considering that the CHO-K1 mutants reached almost 50% *higher* density than the 37 °C controls! Thus, the hypothermic, growth-arrested cultures actually grew to higher peak densities than the actively growing 37 °C cells, a highly counterintuitive, but exciting, finding. Metabolic assays were not performed on the mutants. However, the color change of the phenol red based medium suggests that the 32 °C cells generate waste such as lactic acid at a slower rate than the 37 °C cells, and thus the culture is possibly able to reach higher density because nutrients aren't consumed or waste doesn't reach toxic levels as quickly. This remains to be verified. Regardless of the mechanism involved, the CHO-K1 mutants grown at 32 °C gave almost 3-fold higher IVCD than cells grown at 37 °C. Coupled with the potential for enhanced productivity of recombinant proteins during hypothermic culture, these cells appear very promising for greatly improving *total* recombinant protein production.

9. THE EFFECT OF HYPOTHERMIA ON IFN-γ GLYCOSYLATION

The previous chapters have demonstrated that mild hypothermia can be used for greatly improving recombinant protein production. Besides product quantity, another critical process parameter is product quality, as measured by the recombinant protein glycosylation.

As covered in Section 2.4.4, the literature contains scant information on the effect of culture temperature on glycosylation. The single paper on site occupancy shows a small (~5%) increase in t-PA glycosylation (Andersen et al, 2000) under hypothermia. The two papers dealing with sialylation show nil to small (~10%) increases in EPO (Yoon et al, 2003) and SEAP (Kaufmann et al, 2001) sialylation at low temperature. All three of these studies used CHO batch culture as the model system. The drawback with conducting an analysis using batch culture is that the large differences in cell growth dynamics between 37 °C and hypothermic conditions means that one is often comparing cells that are not only at different temperatures but also under very different nutrient and metabolite concentrations, owing to the faster growing 37 °C cells consuming more total nutrients and generating more metabolic byproducts.

To obtain a more diverse view on the effect of temperature on glycosylation, three different culture conditions were tested. First, the glycosylation of suspension CHO-γ cells growing in commercial, protein-free medium in batch bioreactors, the growth and productivity data of which were presented in Chapter 4, will be discussed. Next, adherent CHO-γ cells growing in 10% FBS DMEM medium in batch cultures will be covered. Lastly, the glycosylation from the pseudo perfusion system presented in Chapter 6, which also used adherent CHO-γ cells maintained in 10% FBS DMEM medium, will be presented.

9.1 IFN-γ GLYCOSYLATION DURING BATCH BIOREACTOR CULTURE

CHO-γ cells were cultured in protein-free commercial medium in well-controlled bioreactors (50% DO, pH 7.2) with the temperature set at 32 °C, 37 °C or shifted from 37 °C to 32 °C 60 hours post-inoculation (referred to as TS). Samples were taken periodically throughout the cultures for glycosylation analysis. The cultures were terminated once the percent cell viability dropped below 80%. As has been shown throughout this thesis, the 37 °C cultures lasted for a much shorter duration, which explains why the time scales for the two temperatures on the figures throughout this chapter are so different. In all cases, the final point shown for a given sample reflects the sample taken on the last day of culture.

The time profile for the percentage of 2N (2-sites) IFN-γ glycoforms is shown in Figure 9-1. The percentage of 2N appears to be lower at 32 °C, a result further substantiated by the large drop in percentage 2N for the TS culture following a shift from 37 °C to 32 °C. The percentage of 2N decreases significantly with time at 37 °C whereas the decrease with time is far less pronounced at 32 °C and TS. In fact, there is an increase in the percentage of 2N at a midpoint in the 32 °C culture.

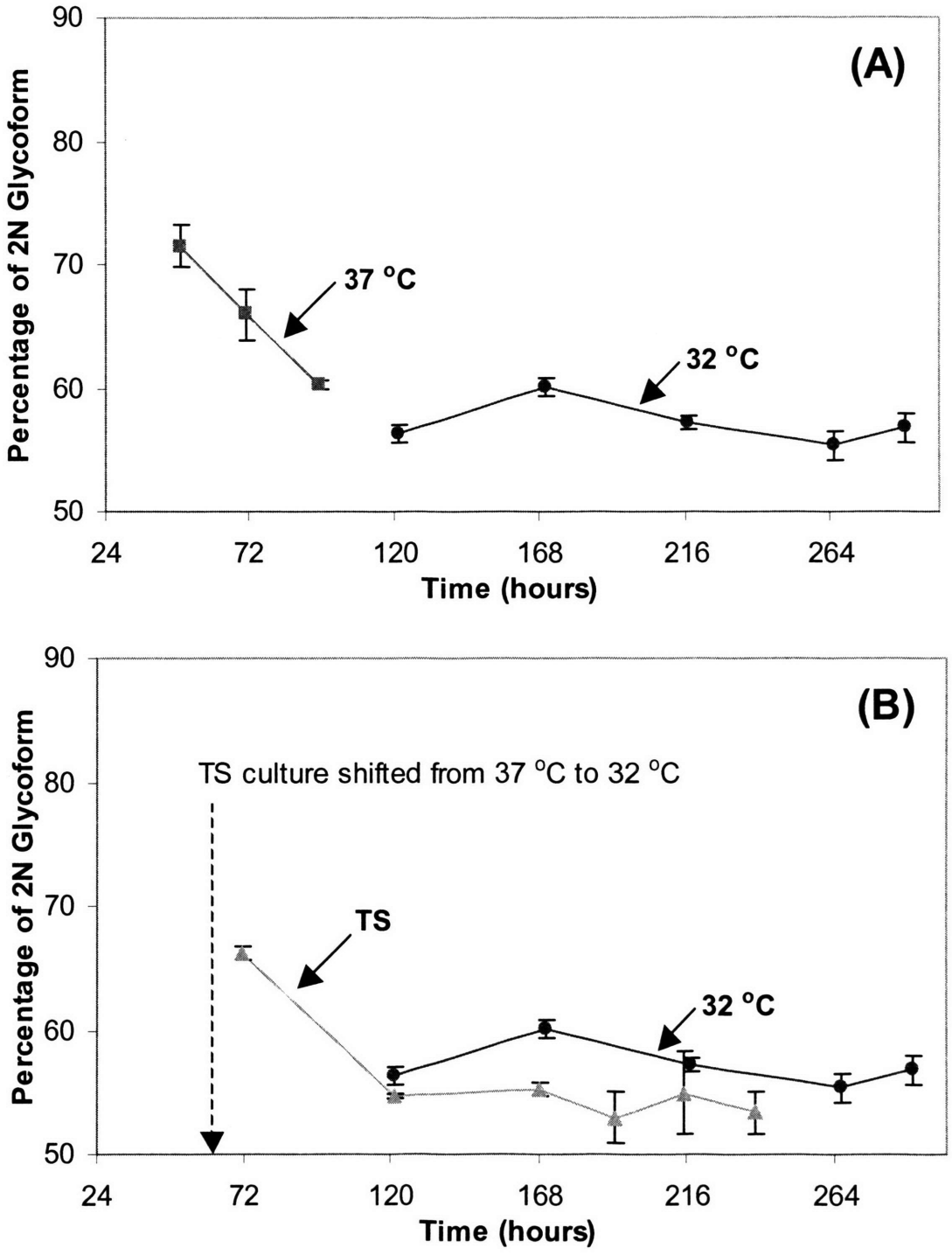

Figure 9-1. Percentage of 2N IFN-γ glycoform produced during batch bioreactor cultures. The percentage of 2N glycoform at various points during culture is shown for 37 °C (■) and 32 °C (●) culture in (A) and for TS (▲) and 32 °C (●) culture in (B). Figure Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

The sialylation of the IFN-γ produced at various points in culture was measured to obtain a ratio of moles of sialic acid per mole of IFN-γ. Next, the mole to mole ratio was normalized against the number of available N-linked sites. By conducting this normalization, one is able to directly consider the ability of the cell to sialylate an available site when exposed to various culture conditions. The formula for converting the moles sialic acid per mole of IFN-γ ratio to molecules of sialic acid per available N-linked site, henceforth referred to as *site sialylation*, is as follows:

$$\frac{Molecules\ Sialic\ Acid}{Available\ N-linked\ Site} = \frac{Mole\ sialic\ acid/Mole\ IFN-\gamma}{0.01\left(2\cdot\%2N+1\cdot\%1N+0\cdot\%0N\right)} \qquad (9\text{-}1)$$

where %2N, %1N and %0N is the percentage of 2-sites, 1-site and 0-site glycosylated IFN-γ, respectively.

The site sialylation for the batch bioreactor cultures are shown in Figure 9-2. The sialylation at 37 °C remains fairly constant during culture, whereas the 32 °C sialylation is initially quite high but later decreases significantly from the peak value. Sialylation data for the TS culture was not obtained.

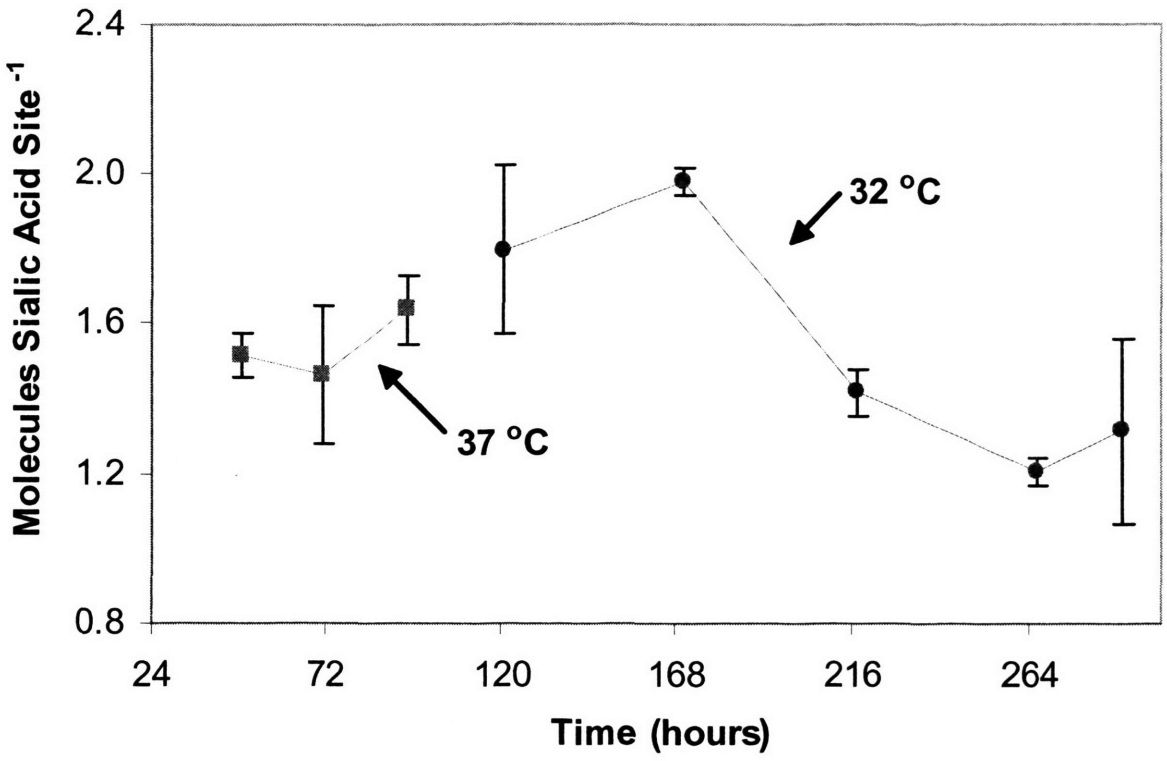

Figure 9-2. Site sialylation in batch bioreactor cultures. The site sialylation at various points during culture is shown for 37 °C (■) and 32 °C (●) culture. Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

9.2 IFN-γ GLYCOSYLATION DURING BATCH ADHERENT CULTURE

The previous section showed that culture temperature has an impact on glycosylation in the batch bioreactor culture. To verify that this effect was generic, a different culture system was tested, namely adherent cells growing in 10% FBS DMEM. The time profile for the percentage of 2N IFN-γ glycoforms is shown in Figure 9-3. As was the case with the bioreactor culture, the percentage of 2N appears to be lower at 32 °C. Also following the bioreactor trend is the decrease with time of the percentage of 2N at 37 °C. However, in this case the decrease is not as substantial (7% decrease in this case compared to 16% for the bioreactor). Finally, the slight increase in the percentage of 2N glycoforms at a midpoint in the 32 °C culture is obtained here, as was the case with the bioreactor culture.

The site sialylation was determined for the adherent cell batch cultures and the values are shown in Figure 9-4. The sialylation at both temperatures appears to decrease with time. The 32 °C data profile, in particular, looks very similar to that obtained in the batch bioreactor culture, with the first few measurements being quite high followed by a large decrease in sialylation later in culture.

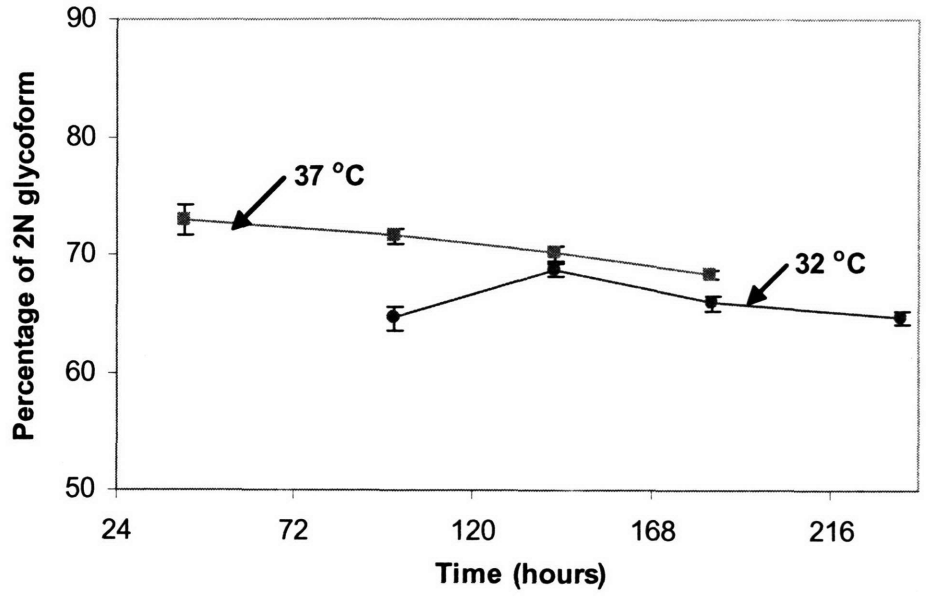

Figure 9-3. Percentage of 2N IFN-γ glycoform produced during adherent cell batch cultures. The percentage of 2N glycoform at various points during culture is shown for 37 °C (■) and 32 °C (●). Figure Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

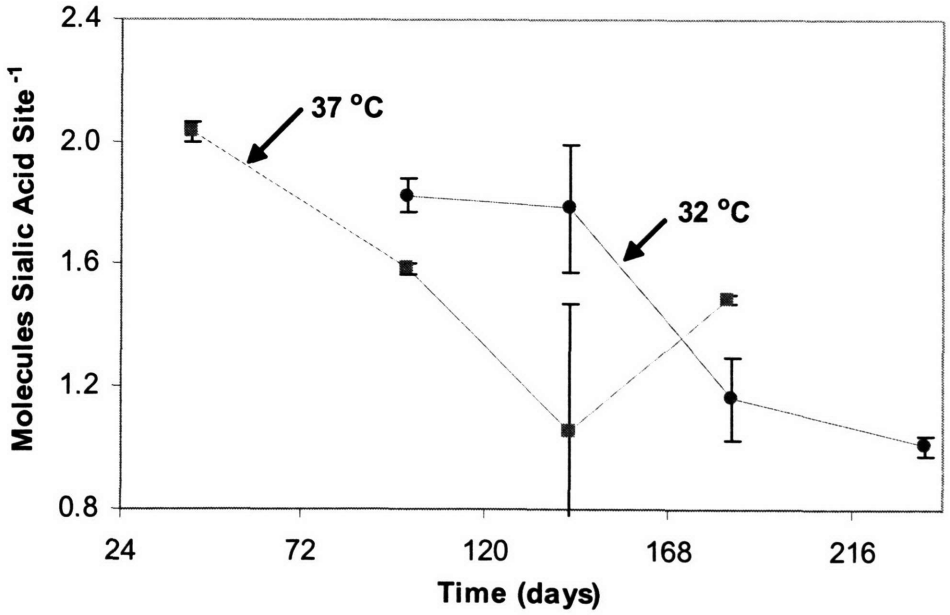

Figure 9-4. Site sialylation in adherent batch cultures. The site sialylation at various points during culture is shown for 37 °C (■) and 32 °C (●) culture. Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

9.3 IFN-γ GLYCOSYLATION DURING PSEUDO PERFUSION CULTURE

The pseudo perfusion system used for this study has been discussed in detail in Chapter 6. During the culture, samples were collected periodically for glycosylation analysis. The percentage of 2N glycoforms obtained at various points in culture for the three different conditions, 32 °C, 37 °C and TS, are shown in Figure 9-5. There is no significant difference in glycoform composition produced in any of the cultures, with the percentage of 2N glycoform fluctuating around 75% for all three conditions.

The site sialylation was determined for the pseudo perfusion batch cultures and the values are shown in Figure 9-6. There is no obvious difference between the sialylation for any of the three conditions considered. All three conditions yield about 1.8 molecules of sialic acid per available site. However, there does appear to be a slight decrease in sialylation with time.

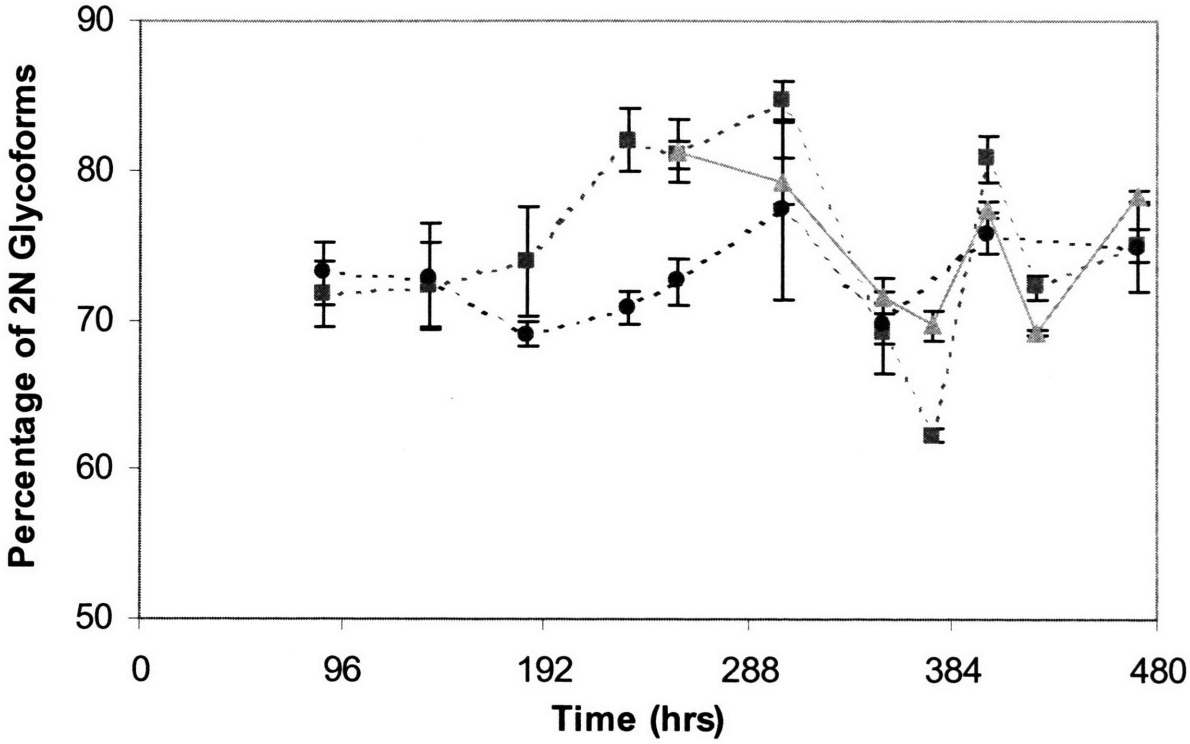

Figure 9-5. Percentage of 2N IFN-γ glycoform produced during pseudo perfusion culture. The percentage of 2N glycoform at various points during culture is shown for 37 °C (--■--), 32 °C (--●--) and TS (▲). Figure Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

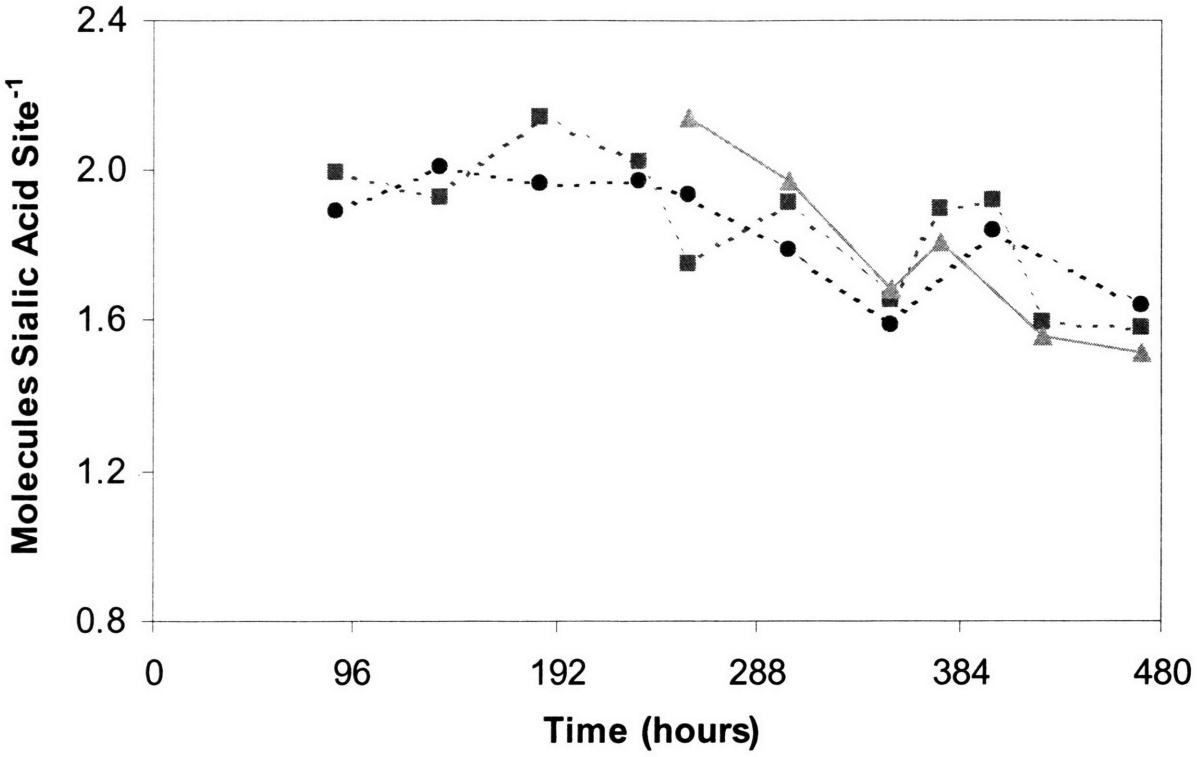

Figure 9-6. Site sialylation in pseudo perfusion cultures. The site sialylation at various points during culture is shown for 37 °C (--■--), 32 °C (--●--) and TS (▲). Y-axis scale was chosen for comparison with figures throughout this chapter, which will all use the same Y-axis scales.

9.4 DISCUSSION AND CONCLUSIONS

The effect of temperature on glycosylation under various culture conditions is summarized in Table 9-1. Several trends regarding hypothermic culture emerge when considering all the data. First, in batch culture hypothermia has a statistically significant ($p<0.02$) negative effect on IFN-γ site occupancy. This trend was seen with both the bioreactor and adherent cell batch systems, with the average percent of 2N glycoforms at 32 °C decreasing by 16% and 7%, respectively, when compared to 37 °C. Further, in the bioreactor culture it was noted that the TS sample percent 2N dropped by about 10% following the change from 37 °C to 32 °C, providing further evidence that hypothermia does indeed decrease site occupancy in batch culture. This is the first report of hypothermia having a detrimental impact on glycosylation, with all other reports showing positive or no change in glycosylation (see Section 2.4.4). In batch culture, the endpoint is the most crucial measurement, as this reflects the quality of the product at the point of harvest. In this regard, the trend of decreased site occupancy under hypothermic conditions holds, with endpoint percent of 2N glycoform being 5-6% lower than at 37 °C for both batch culture conditions tested (see Figure 9-1 and Figure 9-3).

Regarding microheterogeneity, the average value for batch culture suggests that 32 °C results in a few percent higher site sialylation than 37 °C (0-7% increase). However, the 32 °C cultures tended to exhibit sharper decreases in sialylation towards the end of the batch than the 37 °C cultures. As noted above, for batch culture the endpoint is most crucial and the site sialylation at 32 °C was about 10-30% lower than that at 37 °C (see Figure 9-2 and Figure 9-4). In summary, in the batch culture system both macro- and microheterogeneity appear to suffer adverse effects during hypothermic culture, but at this time only the macroheterogeneity decrease can be stated as being statistically significant.

When cells are grown under perfusion conditions, the detrimental effects of hypothermia on glycosylation are eliminated. Site occupancy becomes indistinguishable at the two culture temperatures and site sialylation is not adversely affected and may in fact be improved by a few percent. It has been previously shown that nutrient depletion can cause the site occupancy of IFN-γ produced by our CHO-γ cell line to decrease (Xie et al, 1997). This is likely the reason site occupancy of IFN-γ decreases with time in batch cultures, as seen before (Castro, 1995; Curling, 1990; Goldman, 1998; Xie et al, 1997) and also observed here (see Figure 9-1 and Figure 9-3). In one case, fed-batch culture was able to alleviate the decrease in site occupancy seen in batch culture (Xie et al, 1997) and in another case perfusion culture was also able to alleviate the batch decrease (Goldman et al, 1998). It appears that perfusion is successful in alleviating the site occupancy decrease here too, which implies that the detrimental effect of temperature on glycosylation is due to nutrient limitations and that providing fresh feed can alleviate the problem. It is possible that one of the numerous enzymes responsible for transporting, converting or attaching substrate to proteins during glycosylation is hindered by lower temperature and this effect is exacerbated in the presence of low nutrient concentrations.

In conclusion, glycosylation appears to be slightly decreased at low temperature in batch culture but does not appear to be significantly positively or negatively affected by hypothermic cultivation in the case of nutrient replenishment, and thus product quality should not pose a major hurdle for implementing hypothermic cultivation in a biopharmaceutical manufacturing process. This is particularly apparent when one notes that the trend in industry is to move away from batch culture towards fed-batch and perfusion, owing to higher cell densities and extended culture duration under these conditions.

Table 9-1. Effect of culture temperature on IFN-γ glycosylation

	Batch Bioreactor Culture		Batch Adherent Culture		Pseudo Perfusion Culture	
	%2N	# Sialic Acid / Site	%2N	# Sialic Acid / Site	%2N	# Sialic Acid / Site
32 °C [1]	*56 ± 2	1.5 ± 0.3	*66 ± 2	1.6 ± 0.4	74 ± 4	1.8 ± 0.2
37 °C	66 ± 6	1.5 ± 0.1	71 ± 2	1.5 ± 0.4	75 ± 7	1.7 ± 0.1
32 °C : 37 °C	0.84	1.00	0.93	1.05	0.99	1.07

[1]The 32 °C data combines the 32 °C and TS results for the bioreactor and pseudo perfusion systems
*Significantly different from 37 °C by unpaired Student's t test ($p < 0.02$)

10. CONCLUSIONS AND RECOMMENDATIONS

10.1 THESIS CONCLUSIONS

It has been known for many years that mammalian cells become arrested in the G_0/G_1 phase of the cell cycle when grown under mild hypothermic conditions. More recently, several research groups have reported CHO cells having enhanced specific productivity of recombinant glycoproteins when grown under these mild hypothermic conditions. Given the success of G_0/G_1 phase controlled proliferation processes in general for enhancing specific productivity, it was assumed prior to this thesis that the improvement exhibited at low temperature was due to cell arrest in the G_0/G_1 phase of the cell cycle.

The CHO-γ cell line used in this thesis was shown to be a cell line exhibiting enhanced specific productivity during hypothermic cultivation. The enhanced specific productivity was maintained in the well-controlled bioreactor environment under both batch and fed-batch conditions, indicating that it was the direct effect of temperature, as opposed to an indirect effect such as nutrient depletion or different oxygen concentration, that was responsible for the improvement.

Similar to all controlled proliferation methods, one drawback of hypothermic cultivation is that cell growth is impaired, meaning that cell densities are much lower and consequently the significantly higher specific productivity does not always translate into higher total production. Worse still, when one considers volumetric productivity, hypothermic cultivation becomes even less appealing. This is because the slower growth rate means batches are run for a longer period of time and thus volumetric productivity (product concentration / batch time) is diminished.

To address this issue of reduced volumetric productivity, one approach that has been proposed in the literature is to grow the cells at 37 ^{o}C for the first phase of the culture to obtain

high cell density and then reduce the temperature to obtain high specific productivity. The decision on when to reduce the temperature is an optimization problem, trading off the high cell density and shorter batch times of 37 °C against the high specific productivity and longer batch times of hypothermia. Herein, a model for choosing the optimal time during a batch culture at which to reduce the temperature from 37 °C to 32 °C in order to maximize volumetric productivity was proposed and validated. The model predicted that day 3 was optimal and this was validated by the data, giving a 90% improvement in volumetric productivity over 37 °C culture and a 40% improvement over 32 °C culture.

The optimization model offers one option to alleviate the slow growth and low cell densities that plague hypothermic culture. However, using this model is still a tradeoff situation, wherein one must compromise on one desirable trait, high productivity, to obtain another desirable trait, high growth rate. A better option is to simultaneously obtain both positive traits. This would be achieved by promoting active growth under hypothermic conditions. However, the general consensus was that the positive effect of low temperature on productivity is due to growth arrest, and that it would thus be infeasible to obtain both active growth and high specific productivity. Therefore, it was essential to confirm whether the positive effect of low temperature was due to growth arrest, since if it were, active hypothermic growth would be a pointless endeavor.

The pseudo perfusion system was used to separate the effect of growth rate and temperature on specific productivity. This system allowed for a fairly wide range of cell cycle distribution at both 32 °C and 37 °C and thus it was possible to simultaneously observe the effect of temperature and cell cycle on productivity. It was found that CHO-γ is a growth-associated producer at both temperatures, with productivity increasing as the percentage of S phase cells

increased. The positive effect of temperature was a separate effect, meaning for the same S phase distribution at 32 °C and 37 °C, the lower temperature gave a significantly higher productivity. In the CHO-γ cell line, it was shown that specific productivity is proportional to IFN-γ mRNA levels and that *both* active growth and hypothermia were found to increase IFN-γ mRNA levels.

Based on the independence of hypothermia from growth state and the knowledge that CHO-γ is a growth-associated producer, it was hypothesized that active hypothermic growth would be the optimal platform for maximizing total IFN-γ production, due to the high cell density of active growth and the high specific productivity of hypothermia. This hypothesis was validated using two independent methods. In the first method, mitogens were used to successfully stimulate cell growth at low temperature, causing improved growth rates and increasing the duration during which the culture remained in exponential growth. The high productivity of hypothermia was maintained or enhanced by the active growth, and consequently total production was increased by several-fold over the 37 °C and 32 °C controls.

The second method used to validate active hypothermic growth was by screening for CHO-γ cells capable of improved growth at low temperature. Several such clones were isolated and found to grow markedly better than the control at low temperature whilst growing about the same or lower at 37 °C. As was the case with growth factor supplementation under hypothermic conditions, these clones had both higher growth rate and also remained in exponential growth for a longer duration than the control cells, consequently achieving much higher peak cell density. These clones maintained high productivity during hypothermic growth and therefore produced much higher total IFN-γ concentrations than the control at either 37 °C or 32 °C.

The isolation of hypothermic growth clones was extended to the non-recombinant CHO-K1 cell line. The screen was again successful at isolating a group of clones capable of enhanced hypothermic growth. However, in this case the growth characteristics were far more impressive than with the CHO-γ hypothermic clones, with peak cell density at 32 °C more than 50% higher than what was achieved at 37 °C. The cultures lasted a longer duration too, and consequently the increase in IVCD was 2.8-fold higher than 37 °C controls. These impressive increases in growth characteristics coupled with the findings in this thesis on the positive effect of hypothermia on productivity suggest that the CHO-K1 hypothermic cell lines will be ideal production platforms for recombinant glycoproteins.

10.2 RECOMMENDATIONS FOR FUTURE RESEARCH

Given the potential of hypothermia to greatly enhance production of valuable recombinant glycoproteins, it is worth considering research directions that could allow for a better understanding of the hypothermic effect on mammalian cells and also how to best apply hypothermic culture for improving biomanufacturing.

10.2.1 Mechanistic insight into the mammalian cold shock response

Not all CHO cell lines have shown improved productivity at low temperature (Ryll et al, 2000). There may be many more such CHO lines that have not been reported in the literature. Obtaining a line that *does not* exhibit enhanced productivity during hypothermic culture and then subjecting the cell line to the pseudo perfusion analysis (cell cycle, recombinant mRNA levels and specific productivity) would be particularly insightful for understanding the cold shock better. This would provide an idea as to how generic some of the findings from the CHO-γ line

are for hypothermia in general, such as the higher recombinant mRNA levels, increased mRNA stability and growth-associated productivity at both temperatures. For example, finding that recombinant mRNA levels are still higher at low temperature despite specific productivity being lower would indicate that the production "bottleneck" has been shifted to a downstream process, such as translation, glycosylation or secretion.

At the other extreme, it would be fruitful to conduct a pseudo perfusion analysis on a CHO line that has enhanced productivity at lower temperature but produces much higher total product than CHO-γ. The CHO-γ gives batch culture total product concentration of 2-15 $\mu g\ ml^{-1}$ in the experiments considered in this thesis, with the lower end representing 37 °C control and the upper end representing active hypothermic growth. In contrast, the CHO-EPO line used by Yoon and colleagues (2003) produced 40 $\mu g\ ml^{-1}$ at 37 °C and 110 $\mu g\ ml^{-1}$ under hypothermia during standard batch cultures, about 20-fold higher than CHO-γ under similar conditions. This is encouraging as it shows that hypothermic culture is still relevant for high producing cell lines. Consequently, it would be very informative to subject this CHO-EPO cell line, or a similar high producer still showing higher hypothermic productivity, to the pseudo perfusion analysis. This would determine whether enhanced mRNA levels are still the differentiator explaining enhanced hypothermic productivity. If not, then further work would need to be done to determine the cause of the enhancement. Either outcome would be exciting and useful for hypothermic culture and could lead to a whole range of new ideas for optimizing cell culture.

There is still surprisingly little known about the proteins involved in the mammalian cold shock. The proteins CIRP and p53 were identified as two key players connecting mild hypothermia and G_0/G_1 growth arrest. However, there are almost certainly others involved and identifying these proteins would be interesting in general and may help future efforts to optimize

CHO cells for hypothermic culture. Genomics and proteomics are increasingly popular tools for cell culture applications (Korke et al, 2002) and could be applied to determine more of the genes involved in the cold shock response. Gene expression profiles can be measured using hybridization microarray technology and protein profiling is typically done using two-dimensional gel electrophoresis coupled with mass spectrometry protein identification (Korke et al, 2002). One of the major issues when undertaking these genomic and proteomic analyses is devising suitable control conditions. In a batch culture, the gene expression is likely changing with time to reflect the changing cell densities and the medium environment. Thus, directly surveying 32 °C and 37 °C cells in batch culture may not be very useful, given that the growth dynamics are very different at these two temperatures, and consequently so are the cell cycle distribution, medium composition and so on. The pseudo perfusion system described herein offers a way to separate growth state effects from temperature effects. All cells are growth-arrested in a viable state and the medium replacement prevents large differences in nutrient or metabolite concentrations from developing. The system reaches a pseudo-steady state, as can be seen from the near constant IFN-γ production during the day 15-18 period. In addition, the fact that productivity is still higher under hypothermic conditions indicates that the proteins involved in causing the increased productivity are still being expressed. Thus, the pseudo perfusion culture appears to be a good system for conducting profiling analyses to specifically determine expression differences resulting from the temperature difference.

The proteomics analysis can be taken a step further by considering post-translational modifications. Stress signals are often propagated via kinase or phosphatase enzymes (Paul et al, 1997), which change the phosphorylation status of various target proteins. Changes in phosphorylation can be determined by staining protein gels with antibodies that recognize

phosphate conjugated to amino acids. Kaufmann and colleagues (Kaufmann et al, 1999) have already applied this technique to CHO hypothermic culture to show that low temperature does cause phosphorylation changes in at least two proteins. However, the proteins undergoing modification were not identified. Continuing this line of work and identifying the proteins would help to unravel the cold shock response and may identify the proteins functioning early in the cold shock signaling cascade. These proteins could then be targeted in cell line engineering projects to obtain desirable hypothermic mutants.

10.2.2 Optimizing hypothermic culture

The results presented herein showed that adding serum to commercial, protein-free medium had contrasting effects, depending on the culture temperature. At 37 °C, serum addition did not change total production at all. At 32 °C, however, adding serum caused production to increase 4- to 5-fold over the 32 °C control. This serves as an extreme example that although commercial media may be optimal for 37 °C culture, which is likely the temperature condition at which media are developed and optimized, they are far from optimum for hypothermic culture. The performance of hypothermic culture may improve significantly if the concentration of sugars, salts and amino acids were optimized for low temperature. A well-planned factorial design could quickly determine whether there is room for significant improvement in total production by developing and optimizing a medium specifically for hypothermic applications.

Hypothermic culture could also be optimized at a much earlier step in the process, namely clonal selection. The most popular CHO expression system for pharmaceutical production is the DHFR- CHO line, which allows for the amplification of recombinant DNA by cotransfecting with the DHFR gene and then growing in the presence of methotrexate, a DHFR

inhibitor (Meents et al, 2002). Gene amplification occurs by gradually subjecting the cells to increasing levels of methotrexate and then measuring productivity of the recombinant product to find high-producing clones. To the best of my knowledge, this selection process is carried out at 37 °C. In this case, if one were left with a clone that happens to produce even higher under hypothermic conditions, this would be a fortunate but not a predictable outcome. The recommendation here is to conduct parallel screenings, where all parameters are identical except for the screening temperature and to select the high producers from each of the parallel tracks. Two interesting questions will be addressed: (1) are the clones at low temperature generally higher producers than those at 37 °C? (2) Which selection process gives the best overall clone? The answers will demonstrate how generic the hypothermic effect is and also how applicable it will be for high producing cell lines.

A third strategy for optimizing hypothermic culture would be by designing better expression vectors specifically for hypothermic culture. For example, the research group that discovered CIRP has also found some of the transcription factors involved in the mammalian hypothermic response (Fujita, 2003). By engineering elements recognized by such factors into expression vectors, one could promote high-level transcription during hypothermic culture. Coupled with the enhanced stability seen at low temperature, which appears to be generic, such a strategy could lead to much higher recombinant mRNA levels and subsequent protein expression during hypothermic culture. Unfortunately, the information on the transcription factors remains unpublished currently (Fujita, 2003).

Another option for expression vector engineering would be to include upstream elements that promote translation under hypothermic conditions. As discussed in Section 2.2.2, the cold-inducible protein RBM3 contains an IRES in the 5' leader region of the gene that causes up to 5-

fold higher translation rates at 33 °C compared to 37 °C (Chappell et al, 2000). This element is therefore an obvious candidate for being placed upstream of the recombinant gene to promote high-level translation of recombinant protein during hypothermic culture.

10.2.3 Promoting and evaluating active hypothermic growth

This thesis has shown that promoting active growth at low temperature, achieved by growth factors or isolating hypothermic clones, is a worthwhile endeavor that should be considered further. These two approaches should be tested at the same time (i.e. mutants treated with growth factors) to see if the beneficial effects on cell growth are additive or even synergistic.

In addition, one can test whether the mutants maintain their positive effect on hypothermic growth when adapted to and grown in suspension culture, which is the scenario of most interest for industrial application. Also, it would be interesting to determine how the mutants perform during fed-batch culture, a culture regime of much interest for industrial production. Finally, various recombinant proteins can be cloned into the CHO-K1 mutants developed during this thesis to see if the mutants consistently give higher total production when grown at hypothermic conditions than the three controls (37 °C control cells; 32 °C control cells; 37 °C mutant cells).

Also, CHO cells can be rationally engineered to obtain active hypothermic growth. In the first approach, genes known to cause G_0/G_1 growth arrest can be targeted for transient, inducible or stable knockout. Example candidate targets include CIRP or the CKIs (p21, p27 etc.; refer to Section 2.1).

In a second genetic engineering approach, proteins known to promote progression from G_0/G_1 to S phase could be over-expressed. This approach is particularly appealing because there is some precedence that such a strategy can alleviate growth arrest caused by growth factor deprivation. For example, Renner and colleagues over-expressed cyclin E in CHO cells and found that this cell line was capable of proliferation in serum-free medium in the absence of bFGF, which was not the case for the control CHO cell (Bailey et al, 2002; Renner et al, 1995).

Another gene worth considering over-expressing for obtaining active hypothermic growth is the transcription factor E2F-1, discussed in Section 2.1, which is responsible for activating various S phase genes. Lee et al over-expressed E2F-1 in CHO cells and discovered that this in turn resulted in high expression of cyclins A and E and active proliferation in serum-free medium (Lee et al, 1996). Interestingly, a proteomics analysis showed that the effects of E2F-1 over-expression on the level of cell cycle protein expression was distinct from the effect of bFGF, although both proteins caused active proliferation. Thus, a combination of E2F-1 and bFGF may yield very good growth.

Finally, this thesis started with a reference to hibernation and ends with one, too: a particularly interesting gene to consider over-expressing in CHO cells to obtain active hypothermic growth is c-fos, discussed previously in Section 2.3.1, which together with c-jun forms the transcriptional activator AP-1, promoting several genes involved in cell cycle progression. Levels of c-fos mRNA in the hypothalamus of the golden-mantled ground squirrel have been shown to rise constantly during hibernation, peaking during the arousal from hibernation (Bitting et al, 1994), suggesting that this factor may play a major role in alleviating aspects of the cold shock response during hibernation arousal, and thus may be capable of alleviating hypothermic growth arrest.

NOMENCLATURE

E	Enzyme activation energy (J $mole^{-1}$)
f_{G1}	Fraction of cells in the G_1 phase (unitless)
f_{G2M}	Fraction of cells in the G_2/M phase (unitless)
f_S,	Fraction of cells in the S phase (unitless)
K	Monod growth constant (mg ml^{-1})
K_s	Monod glucose consumption constant (mg ml^{-1})
k_D	mRNA degradation rate constant (hr^{-1})
k_D^{actin}	mRNA degradation rate constant for β-actin (hr^{-1})
k_D^{IFN}	mRNA degradation rate constant for IFN-γ (hr^{-1})
k_T	mRNA transcription rate (molecules $cell^{-1}$ hr^{-1})
k_T^{actin}	mRNA transcription rate for β-actin (molecules $cell^{-1}$ hr^{-1})
k_T^{IFN}	mRNA transcription rate for IFN-γ (molecules $cell^{-1}$ hr^{-1})
N	Adherent cell density (viable cell $well^{-1}$)
N_0	Adherent cell seeding density (viable cell $well^{-1}$)
N_f	Fast-growing cells - adherent cell density (viable cell $well^{-1}$)
$N_{f,0}$	Fast-growing cells - adherent cell seeding density (viable cell $well^{-1}$)
N_n	Normal-growing cells - adherent cell seeding density (viable cell $well^{-1}$)
$N_{n,0}$	Normal-growing cells - adherent cell seeding density (viable cell $well^{-1}$)
P	IFN-γ concentration (μg ml^{-1})
q_{IFN}	Specific IFN-γ production rate (μg $cell^{-1}$ hr^{-1})
q_{max}	Maximum specific glucose consumption rate (mg $cell^{-1}$ hr^{-1})
q_s	Specific glucose consumption rate (mg $cell^{-1}$ hr^{-1})
R	Ideal gas constant (8.314 J $mole^{-1}$ K^{-1})
$R(t)$	mRNA cellular concentration at time t (molecules $cell^{-1}$)
R_{ss}^{actin}	mRNA steady-state cellular concentration for β-actin (molecules $cell^{-1}$)
R_{ss}^{IFN}	mRNA steady-state cellular concentration for IFN-γ (molecules $cell^{-1}$)
R_{ss}	mRNA steady-state cellular concentration (molecules $cell^{-1}$)
S	Glucose concentration (mg ml^{-1})

t	Time (hr)
$t_{1/2}$	mRNA half-life (hr)
T	Temperature (K)
T_d	Cell doubling time (hr)
T_{G1}	Time spent in the G_1 phase during one cell doubling (hr)
T_{G2M}	Time spent in the G_2/M phase during one cell doubling (hr)
T_S	Time spent in the G_2/M phase during one cell doubling (hr)
X	Viable cell concentration (viable cell ml^{-1})
$v(T)$	Enzyme activity at temperature, T (mole g $enzyme^{-1}$ hr^{-1})
v_0	Maximum enzyme activity (mole g $enzyme^{-1}$ hr^{-1})

Greek Letters

λ_{em}	Emission wavelength (nm)
λ_{ex}	Excitation wavelength (nm)
μ	Specific growth rate (hr^{-1})
μ_f	Specific growth rate of fast growers (hr^{-1})
μ_{max}	Maximum specific growth rate (hr^{-1})
μ_n	Specific growth rate of normal growers (hr^{-1})

Abbreviations

AML	Adenovirus major late
AMP	Adenosine 5'-monophosphate
Asn	Asparagine
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BHK	Baby hamster kidney
cDNA	Complimentary DNA
C/EBPα	CCAAT/enhancer-binding protein α
CDK	Cyclin-dependent kinase
CHO	Chinese hamster ovary

CHO-γ	Chinese hamster ovary cells expressing recombinant human interferon-gamma
CHO-K1	Non-recombinant Chinese hamster ovary cells
CIRP	Cold-inducible RNA-binding protein
CKI	Cyclin-dependent kinase inhibitors
CMV	Cytomegalovirus
CS-RBD	Consensus sequence RNA-binding domain
CSF-1	Colony stimulating factor-1
CSP	Cold shock proteins
C_t	Real time PCR fluorescence threshold cycle
cyc	Cyclin
dNTP	Deoxynucleoside Triphosphates
DB	Downstream box (ribosome binding site) protein motif
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EF1	Elongation factor 1
ELISA	Enzyme-linked Immunosorbent Assay
EPO	Erythropoietin
ER	Estrogen receptor
ER	Endoplasmic reticulum
F-MEL	Friend murine erythroleukemia cell
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FSH	Follicle stimulating factor
G_0	Quiescent cell state indistinguishable from G_1
G_1	First gap phase of the cell cycle
G_2	Second gap phase of the cell cycle

G_2/M	Combined G_2 and M phases of the cell cycle
Gal	Galactose
GlcNAc	N-acetylglucosamine
GRP	Glycine-rich RNA-binding protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPAEC-PAD	High performance anion exchange chromatography and pulsed amperometric detection
HPLC	High performance liquid chromatography
HSP	Heat shock proteins
IFN-γ	Recombinant human interferon-gamma
IGF-I	Insulin-like growth factor-I
IL-2	Interleukin-2
IRES	Internal ribosome entry site
IRF-1	Interferon-responsive factor 1
IVCD	Integrated viable cell density
kDa	Kilo-Dalton
LDH	Lactate dehydrogenase
M	Mitosis phase of the cell cycle
Man	Mannose
MECC	Micellar electrokinetic capillary chromatography
mRNA	Messenger RNA
N-linked	Asparagine-linked
NS0	Non-secreting myeloma
OCR	Oxygen consumption rate
ODN	Oligonucleotides
Oligo(dT)	Oligodeoxythymidine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
poly(U)	poly(uracil)
pRb	Retinoblastoma protein

R	Restriction point in the cell cycle
RNA	Ribonucleic acid
RNase	RNA nuclease enzyme
RP	Reverse phase
rRNA	Ribosomal RNA
RT	Reverse transcriptase
S	DNA synthesis phase of the cell cycle
SDS	Sodium dodecyl sulphate
SEAP	Secreted alkaline phosphatase
Ser	Serine
SSII	Superscript reverse transcriptase
SV40	Simian virus 40
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor β
Thr	Threonine
t-PA	Tissue-type plasminogen activator
TPO	Thrombopoietin
TS	Temperature shift
UV	Ultraviolet

0N	Zero of two potential IFN-γ N-linked glycosylation sites occupied
1N	One of two potential IFN-γ N-linked glycosylation sites occupied
2N	two of two potential IFN-γ N-linked glycosylation sites occupied

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Building a Biotechnology Franchise inside Big Pharma:

Operations and Manufacturing Strategy

PhDCEP Capstone Project

Executive Summary

The market for recombinant proteins and antibodies (henceforth "biologics") has grown at a remarkable rate over the last twenty years, with over $33 billion in sales in 2003 and expected sales in excess of $100 billion by 2010. Because of this tremendous growth and size, the biologics market is expected to become an area of increasing interest to the world's largest pharmaceutical companies, or big pharma. This has been the case for some of the top companies, with big pharma entering into an increasing number of partnerships with biotechnology firms, with the intent of deriving some of their revenues from biologics.

The Operations group of a company is responsible for bringing a company's products to market. This study develops an operations strategy framework for a big pharma company that is trying to build a biologics franchise, and then seeks to test whether the recommended strategy is being followed by big pharma, in practice. The first portion of the paper studies general operations frameworks developed by operations academics and practitioners, and then applies these frameworks to the specific case of biologics operations. Based on these frameworks, the recommended operations strategy for a big pharmaceutical company, *committed to being a successful player in the biologics market*, is as follows:

- Build up the capacity and skills necessary to effectively produce the biologics in-house;
- Build capacity in excess of projected internal production requirements, since the opportunity cost of having insufficient capacity far exceeds the carrying costs of excess capacity and also because the excess capacity could be used strategically for becoming a "partner of choice" for small biotechnology firms;
- Outsource the fill-and-finish portion of production, if desired;
- Design a vertical (process stage), centrally-controlled network of operations facilities, with facilities located in areas with a large population of the professional employees required for biologics production; and
- Integrate product and process development into the operations, through such practices as early operations representation on discovery projects, as this could be a major competitive advantage in rapidly and effectively bringing drugs to market.

The second portion of the paper studies the top biotechnology firms (Amgen, Genentech, Serono, Biogen IDEC, Genzyme and others) as case studies of how biologics operations in successful firms are carried out in practice. These top firms were found to view manufacturing and operations as a strategic asset and were found to follow the recommended practice of manufacturing in-house, using contractors only for short-term capacity needs. Thus, these companies have a strong focus on both product and process (operations) development, and effectively follow the operations strategy advocated in the first section.

In the third and final section, the Top 20 pharmaceutical companies ("big pharma") were studied to determine the operations strategy these companies are pursuing in regards to biologics. Four distinct models emerged. The first model, "minimal biologics activity", is an approach with a low or unclear commitment to either biologics product or operations development. Somewhat surprisingly, this model was used by 5 of the 6 largest drug companies (Pfizer, GSK, Sanofi-Aventis, Merck and AstraZeneca). The second model, "product-focused", is an approach with a fairly high commitment to having biologics in the product portfolio, through either internal R&D or external collaborations, but with a low commitment to biologics operations, preferring to outsource manufacturing. This model is used by BMS, Lilly, Takeda, Schering-Plough and Merck KgA. The third model, "process-focused", is an approach with a fairly high commitment to building biologics operations capabilities, such as in-house manufacturing, but with a limited amount of biologics product development evident. This model is used by Novartis, Boehringer-Ingelheim and Bayer. The fourth model, "simultaneous process-product development", is an approach that has a high level of both biologics product development and biologics operations expertise. In other words, it is the model advocated in the first part of this study and the approach used by top biotechnology companies. This model is used by J&J, Roche, Abbott and Wyeth. These four companies are predicted to make significant gains in the biologics market, relative to their Top 20 peers, unless the other companies adapt their operations models.

Introduction

During my PhD thesis, I focused on using hypothermic culture for optimizing the total production of a particular biological product, interferon-γ, by mammalian cell culture. These types of research studies are important to the healthcare industry, because they allow drug companies to produce their products at a lower cost. Reducing costs through manufacturing optimization is part of a much broader area of corporate management, namely operations. "Operations" is the group within a company responsible for producing the products and delivering them to the customers.

In this study, the capstone paper that is meant to tie together my technical education from the Chemical Engineering Department with my business education from the Sloan School of Management, I will discuss a framework for developing a suitable operations strategy for producing biologics within a large pharmaceutical company. This is an interesting and challenging problem, because biologics are not the traditional domain of big pharma. These companies are accustomed to producing small molecule drugs through chemical processes, which requires a different set of organizational skills, all the way from the bench scientists through to the sales people. However, the biologics market has had a stretch of dizzying growth and has now reached a point where it is too large for big pharma to ignore, even if big pharma may not necessarily be in the best position to sell biologics. For example: from 1990 to 2002, the biotech industry enjoyed 16% average annual revenue growth. Biotech revenues were $33 billion in 2002 and are expected to eclipse $100 billion by 2010. The biologics industry is growing at a rate twice that of total pharmaceuticals. As an example of this growth, 6% of marketed products are biologics whereas 40% of Phase II or III products are biologics, with over 600 biotech products in phase II or III (all statistics from Bende, 2003).

Operations strategy is a particularly challenging management area in biologics for a host of reasons, most of which relate to the large upfront investment costs and uncertainty of commercial success of the drugs being developed. First, the capital outlay required to produce a biologic product is significant, with a commercial-scale mammalian cell culture facility costing $150-500 million (Business Insights, 2003). Second, this capital outlay must be made 4-5 years ahead of the expected start-up time (Business Insights, 2003). Third, these capital expenditures must be made at a point in time at which there is no assurance that the biologic in the clinic will ever receive regulatory approval. Fourth,

assuming the product is marketed, there is a great deal of uncertainty related to the product yield of the manufacturing process, the amount of product that will be required per patient and the eventual success of the product in the market.

Finally, a word on terminology and scope: Most of the Top 20 pharmaceutical companies have been involved in producing "biologics" for many years. For example, Aventis, Merck and GSK have been, and continue to be, prolific producers of vaccines. Many big pharmaceutical companies also have recombinant protein products, produced through the use of bacterial or yeast cell culture. **However, for the purposes of this study, a "biologic" is being defined as a complex, recombinant protein (such as a glycoprotein) or an antibody**, both of which currently rely on mammalian cell culture for production, and also have additional complex production issues, such as product quality and glycosylation. Producing these types of products requires different equipment, organizational skills and people than the biologics that have been produced by big pharmaceutical companies for years.

Study Goal and Approach

The goal of this work is to establish an intelligent business model for building and operating a successful biologics business within the confines of a major pharmaceutical company, and then to study the Top 20 Pharmaceutical Companies ("big pharma") to see whether they are following this model. To address this goal, I will use the following approach. First, I will study the frameworks and ideas that have been put forth by academics and practitioners of operations strategy, and try to apply these frameworks to the particular issues encountered in the biologics business. In particular, I will draw strongly from the work of Robert Hayes, Gary Pisano, David Upton and Steven Wheelwright from the Harvard Business School and from Charles Fine at the MIT Sloan School of Management. Second, I will study the current practices of top biotechnology companies to try and understand the operations strategy of these successful firms. Third, I will take the lessons learnt from studying theory and case studies, and synthesize the information to put forth a sound business model for building and operating a successful biologics business inside the confines of big pharma. Fourth, I will apply this framework to evaluate big pharma's excursion into biologics, to see which, if any, of the companies are following the recommended approach.

Frameworks from Operations Theory

In this section, I will draw on the work of academics in the operations field as I attempt to develop a recommended operations framework for biologics. There is a rich set of structural and infrastructural issues to be considered when formulating an operations strategy (Hayes et al, Chapter 2). On the structural side, one must address the issues of capacity, materials sourcing, facilities and information technology. On the infrastructural side, one must address resource allocation, human resources systems, work planning and control, quality systems, measurements and rewards system, a product and process development system, and organizational issues. Here, I will discuss some of these structural and infrastructural issues, and how they apply in the case of biologics. I will organize the discussion into five sections, focusing on the following key topics, which I feel are particularly relevant and should be in the forefront when considering the operations strategy for a biologics "start up" inside big pharma: (1) Materials sourcing, (2) capacity, (3) network design (facilities), (4) product and process co-development and (5) driving an operations edge.

Materials Sourcing: To Make or Not to Make? That is the Question

Very few business issues have received as much attention in recent years as outsourcing. As in many other manufacturing industries, numerous contract manufacturing organizations (CMOs) exist for producing biologics (e.g. Boehringer Ingelheim, DSM and Lonza). For many companies, in a variety of different industries, outsourcing makes sense. It allows an organization forego the significant capital expenditures required to build capacity and lets the organization focus on more profitable "core competencies". However, outsourcing is also fraught with risks, including coordination problems, a loss of manufacturing knowledge and a lack of control over your own products. The ultimate decision as to whether to outsource or to produce in-house must weigh the costs and benefits of these two approaches.

A hot debate in the biologics field today is the availability of manufacturing capacity. The industry has experienced significant capacity shortages in the past. Today, the general impression seems to be that capacity in the mid-term will probably be well-utilized, but adequate, for the "most probable" drug approval case (Chovav et al, 2003; Levine, 2004). If a more optimistic drug approval scenario prevails,

there will once again be a capacity shortage. However, I argue that the total industry capacity should not be the major metric that a company looks at when considering whether to outsource or not. Even if the industry is awash with excess capacity, this does not mean a company will have effective access to this capacity nor does it mean a company should relinquish the production of its goods to a contract manufacturer. Rather, one should consider the strategic implications of the "make versus buy" decision, and choose the path with the most strategic merit. In this section, I will apply frameworks developed by operations theorists for making this decision.

The framework of Hayes and his colleagues at HBS is very useful for considering the outsourcing issue. The framework weighs the outsourcing decision along three dimensions, namely "coordination", "strategic control" and "intellectual property". This framework is duplicated in **Exhibit 1**, and I have bolded points from the framework which I feel are particularly relevant for biologics.

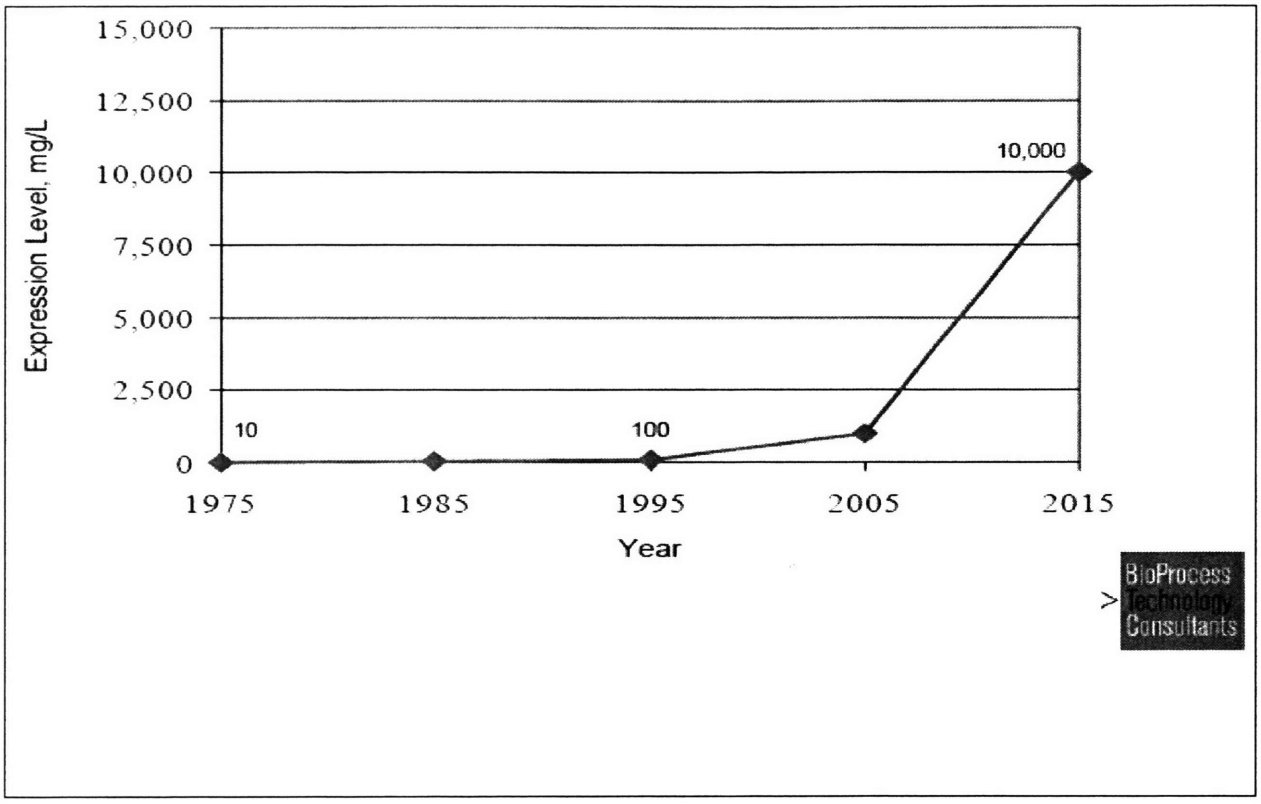
Exhibit 1: A Framework for Organizational Boundaries (Source: Hayes et al, 2005)

	Vertical Integration	**Arms-length relationships**
Coordination	**"Messy" interfaces; adjacent tasks involve a high degree of mutual adaptation, exchange of "tacit" knowledge, and learning-by-doing. Requisite information is highly idiosyncratic**	Standardized interfaces between adjacent tasks; requisite information is highly codified and standardized (prices, quantities, delivery schedule, etc.)
Strategic Control	Very high: significant investments in highly durable **relationship-specific assets** needed for optimal execution of tasks. **Investments cannot be recovered if relationship terminates.** • Co-location of specialized facilities • Investments in brand equity • **Large proprietary learning curves** • **Long-term investments in idiosyncratic R&D programs**	Very low: assets applicable to businesses with a large number of other potential customers or suppliers
Intellectual Property	**Unclear or weak IP protection** Easy-to-imitate technology **"Messy" interfaces between different technological components**	Strong IP protection Difficult-to-imitate technology "Clean" boundaries between different technological components

In regards to the issue of "coordination", this is particularly challenging in biologics process development. The process requires a high degree of tacit knowledge, and developing a process that works effectively at the commercial scale requires a good deal of iteration. Thus, coordination issues suggest that vertical integration is appropriate for biologics.

Next, considering issues of "strategic control" in biologics, there are at least two points that stand out. First, there is a significant, sunk investment in a relationship-specific asset in the form of regulatory approval for a facility. If the relationship is ended, the customer will need to obtain approval again with the new contractor, which requires both time and money. Second, there are large, potentially proprietary learning curves involved in biologics production. One needs only look at the tremendous strides that have been made over the past 30 years in the productivity of mammalian cell culture, as well as the predicted future productivity, to realize that a large amount of learning is still taking place in the industry (**Exhibit 2**). In 1975, typical productivity was 10 mg/L. Productivity is expected to reach levels of 10,000 mg/L by 2015, a 1,000 fold increase over the 1975 performance! If all work is outsourced, the contractors will reach a point of productivity that will be increasingly difficult for a client company to match, if they ever decided they needed to take capacity in-house again. This will give the top CMOs increasing power in the biologics value chain, increasing their bargaining power and ability to command rents. This "knowledge and capacity dependency" is a very dangerous position for a company to find itself in, as will discussed later in this section when drawing on the work of Charles Fine. Thus strategic control issues suggest that vertical integration is appropriate for biologics.

Exhibit 2: Historical and Projected Animal Cell Culture Production Levels (Source: Levine, 2004)

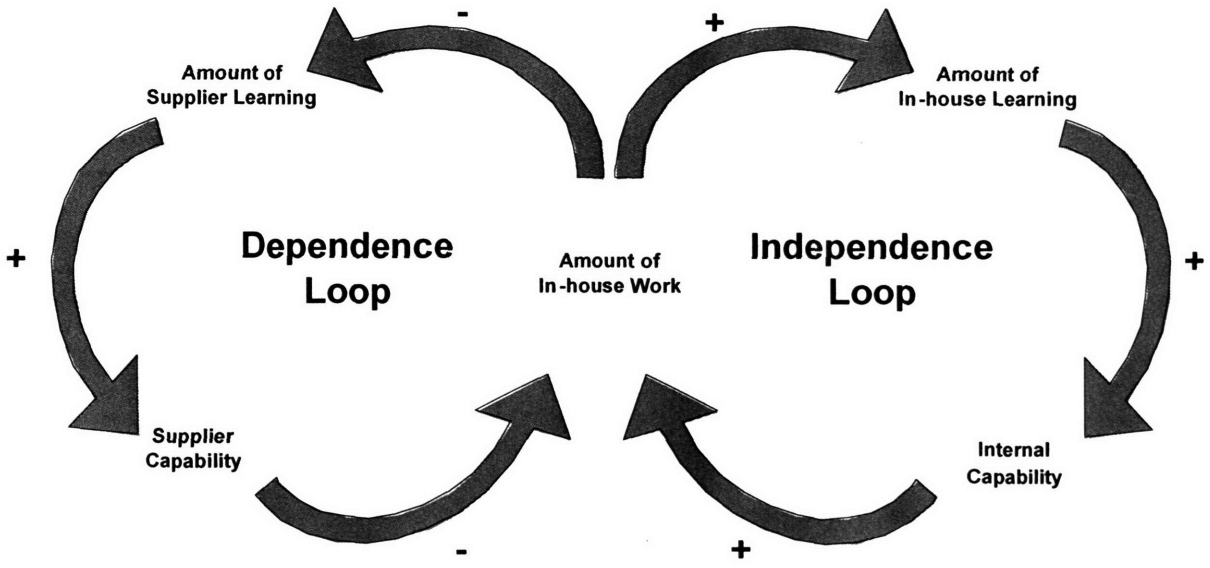


Finally, regarding "intellectual property", there are clearly issues related to IP in producing biologics. Since the product is partially the process for biologics, there is significant risk involved when entrusting your process IP to a CMO. For example, although the actual protein sequence of the biologic product might be well known, the specific process conditions used to maximize productivity or control product quality are potentially valuable trade secrets. These same CMOs will also have partnerships with a company's competitors, and even though secrecy clauses will be signed, it is next to impossible to prevent tacit knowledge from diffusing to other projects, within the same CMO organization. Further, with the unclear issue of biogenerics looming on the horizon, and the unknown role CMOs will play in the biogenerics market, some of a company's critical process information could eventually end up in the hands of a biogeneric competitor, making the biogeneric competitors process development, ramp-up, production efficiency and product quality initiatives much faster, resulting in faster erosion of an innovator's end-of-patent-life sales. In the biologics market of the future, protecting these types of trade

secrets will be of paramount importance. Thus, IP issues suggest that vertical integration is appropriate for biologics.

A different framework that can be used for considering the outsourcing decision has been developed by Charles Fine at MIT Sloan. The framework is partially based on the notion that by outsourcing, an organization will make its suppliers capabilities stronger and will make its own manufacturing capabilities weaker. This relationship is summarized in **Exhibit 3**. In the "Independence Loop", as a company increases the amount of in-house work, in-house learning increases, which increases in-house capability. This makes the in-house operations group more appealing for additional work, and thus the amount of in-house work increases further. In the "Dependence Loop", as a company decreases the amount of in-house work, supplier learning increases, which increases supplier capabilities. This makes the supplier more appealing for additional work, and thus the amount of in-house work decreases further.

Exhibit 3: Dynamics of Capability Sourcing and Capability Development (Adapted from Fine 1998**)**



Because of these dynamics, Fine asserts that a company should only depend on a supplier for capacity and not for both capacity and knowledge, especially if the component being outsourced is highly

integrated and complex. The outsourcing decision can be framed as a 2 x 2 matrix, as depicted in **Exhibit 4**.

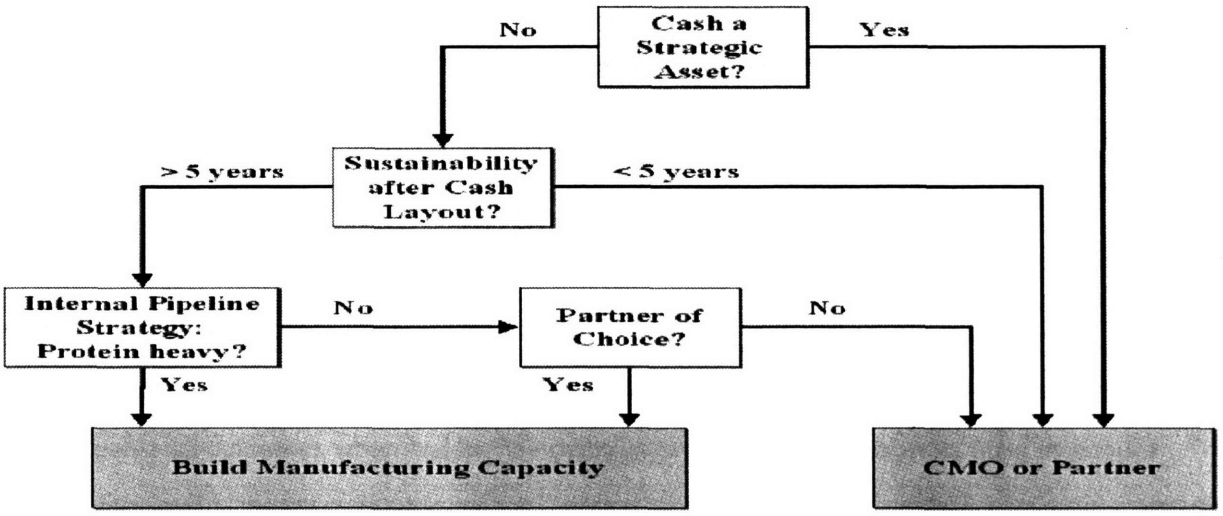
Exhibit 4: Organizational Dependency and Product Decomposability (Source: Fine 1998)

	Dependent for Knowledge and Capacity	**Dependent for Capacity Only**
Item is Modular (Decomposable)	**A POTENTIAL OUTSOURCING TRAP** Your partners could supplant you. They have as much or more knowledge and can obtain the same elements as you.	**BEST OUTSOURCING OPPORTUNITY** You understand it, you can plug it into your process or product, and it probably can be sourced from several sources. It probably does not represent competitive advantage in and of itself. Buying it means you save attention to put into areas where you have competitive advantage, such as integrating other things.
Item is Integral (Not decomposable)	**WORSE OUTSOURCING SITUATION** You don't understand what you are buying or how to integrate it. The result could be failure since you will spend so much time on rework and rethinking.	**CAN LIVE WITH OUTSOURCING** You know how to integrate the item so you may retain competitive advantage even if others have access to the same item.

It is debatable as to whether a biologic is "modular" or "integral", but I feel it is closer to being integral, mainly due to the integrated nature of the product and process in biologics, as discussed earlier. In regards to knowledge dependency, it is unclear whether an organization will be able to retain the knowledge required to *efficiently* and *effectively* produce biologics while at the same time outsourcing the actual production. This is due to the highly tacit level of the knowledge accumulated in biologics production. Thus, it is likely that the organization will rely on the CMO for both capacity and knowledge, particularly in order to produce at a high productivity. Therefore, there is good reason to believe that outsourcing biologics belongs in the "worst outsourcing situation" depicted in **Exhibit 4**.

These considerations have lead analysts to conclude that "for most profitable biotech companies, building capacity makes strategic sense" (Chovav et al, 2003). Analysts at UBS have developed a simple approach for making the sourcing decision, which takes into account a company's ability to fund capacity development, the company's long-term biologics pipeline and also whether the company aims to be a "partner of choice" for small biologics companies (Chovav et al, 2003). The decision tree is summarized in **Exhibit 5**.

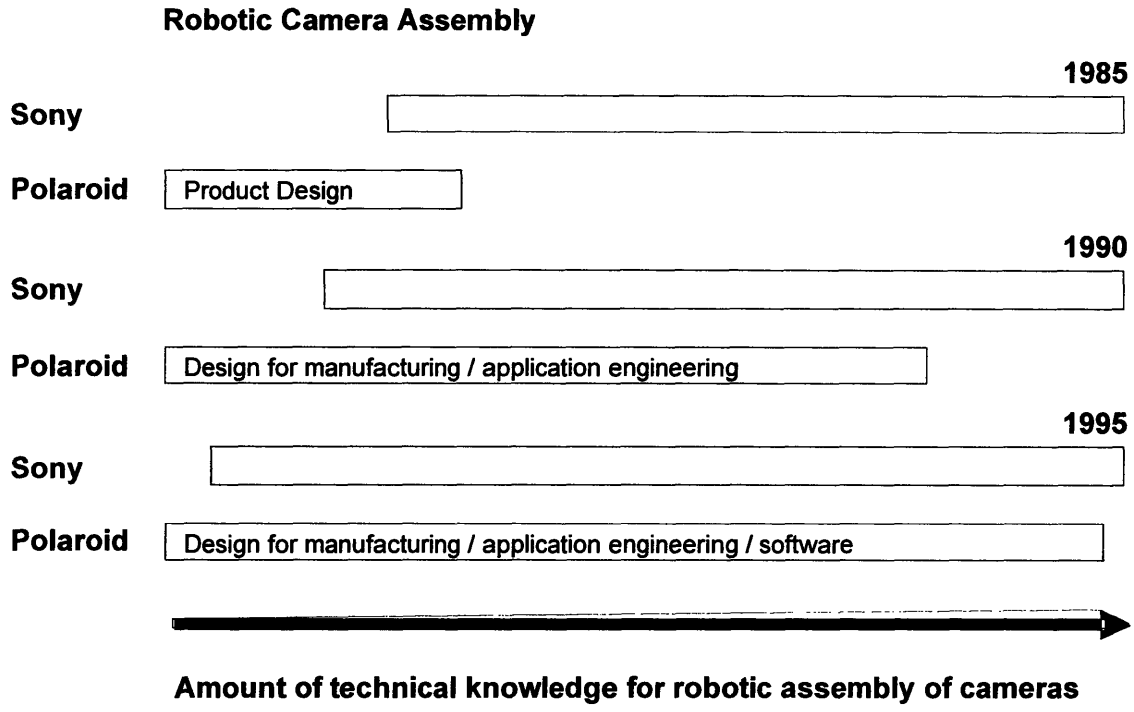
Exhibit 5: Build-or-Buy Decision Tree (Source: Chovav et al, 2003)



For a Big Pharma company with an internal pipeline strategy that is focused on biologics, or with a strategy of being a "partner of choice" for small biologics companies, the decision tree will lead to the conclusion to build in-house manufacturing capacity. The "partner of choice" issue is a particularly important one, given the increasingly competitive nature of making licensing deals with biotechnology companies. Given the choice between a partner with a robust manufacturing operation and experience and a partner without any experience or capacity for manufacturing, the small biotechnology firms will logically choose the partner with more capabilities and experience, all other things being equal, since these skills will translate into more effective product-process codevelopment and hence faster time to market. From this perspective, manufacturing skills can be seen as a strategic asset for a big pharma company interested in pursuing alliances with small biotechnology firms.

Finally, just because a company makes a decision to build in-house capacity and skills, does not mean that the company should not also work with CMOs. On the contrary, it would be wise to work with CMOs, especially in the short- to medium-term, as the organization builds its own capabilities. Consider, for example, the case of Polaroid in the early 1980s, as they worked to develop their own internal, automated production skills (Fine, 1998). Initially, Polaroid had little internal knowledge about robotics and automated assembly of consumer products, and decided to partner with Sony, in order to develop the required skills. The two companies worked together very closely, and with time, Polaroid's internal skills and scope greatly improved. The evolution of the two companies skills are shown in **Exhibit 6**. If well managed and structured, a rich, collaborative relationship with a knowledgeable CMO can be an excellent tool for developing a company's internal skills.

Exhibit 6: Polaroid's Evolving Capability Insourcing and Internal Development (Source: Fine 1998)



Manufacturing Capacity: Balancing the "too little" versus "too much" Risks

Firms need to continuously evaluate their capacity for producing goods today and into the future. This decision is particularly difficult in the biologics industry, given the long lead times required to build manufacturing capacity and the uncertainty surrounding clinical trial outcomes for multiple indications, drug dosage amounts, manufacturing productivity and market uptake of the product. The previous section has stressed the belief that for a company committed to building a biologics business, manufacturing should be brought in-house as soon as possible. This section will focus on the amount of capacity that should be built or made available.

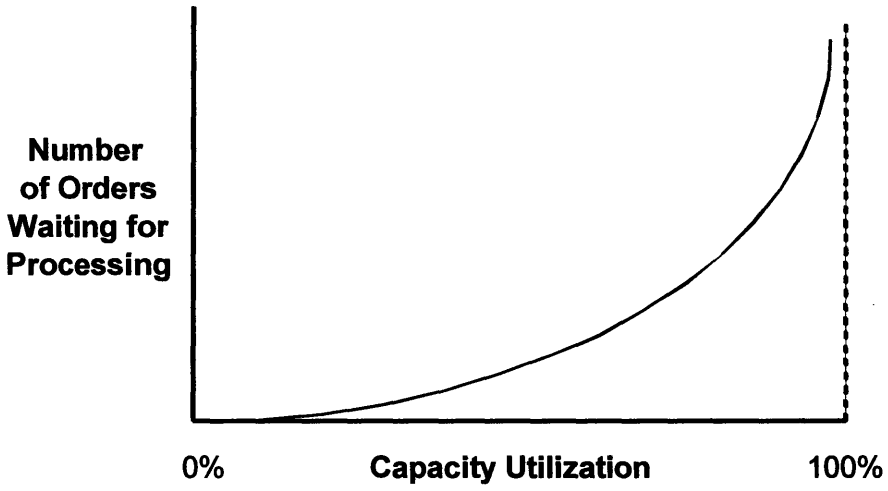
In framing the capacity decision, I will draw on several theoretical and practical considerations that can serve as guidelines when making the decision on how much capacity to build or acquire. The first consideration is "queuing theory", which provides a simple model for thinking about the effect of running a process in which there is inherent variability very close to maximum capacity. Queuing theory is based on the assumption that order arrival time and order processing time are both governed by exponential probability distributions. In other words, if the arrival rate of orders at a facility is "α", the

probability that no orders will arrive during some longer period of time (0 to T) is equal to $e^{-\alpha T}$. If the processing rate, "p', follows the same distribution, it can be shown that the average number of orders waiting to be processed is given by:

$$N = \alpha / (p-\alpha) \qquad \textbf{(Equation 1)}$$

As a numerical example, if orders arrive at the manufacturing facility at the average rate of 4 per month and the plant can process orders at an average rate of 5 per month, the average numbers of orders waiting to be processed at any time is equal to 4 (4/(5-4)). So, even though the example system on average has a 25% excess of processing capacity ((5-4)/4), there are still going to be delays in order processing, due to the variability in order arrival and processing. The qualitative nature of the relationship between waiting and capacity utilization is shown in **Exhibit 7**. The punch line is that in the face of variability in demand and processing time, as an operation approaches its capacity limits, congestion and its associated organizational problems can escalate rapidly. The lesson here is that a manufacturing system with variability in demand and processing rates should be designed for a rated capacity that is well above the expected process requirements.

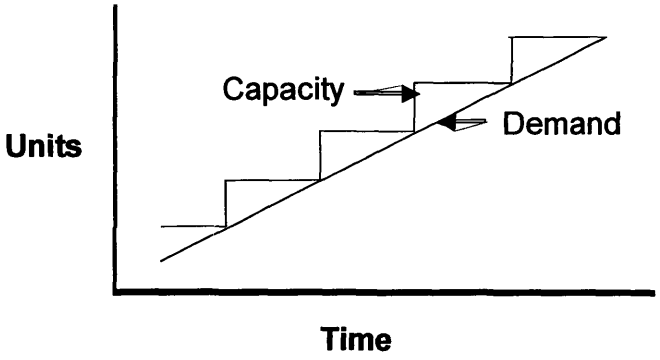
Exhibit 7: Number Waiting as a function of Capacity Utilization (Adapted from Hayes et al, 2005)



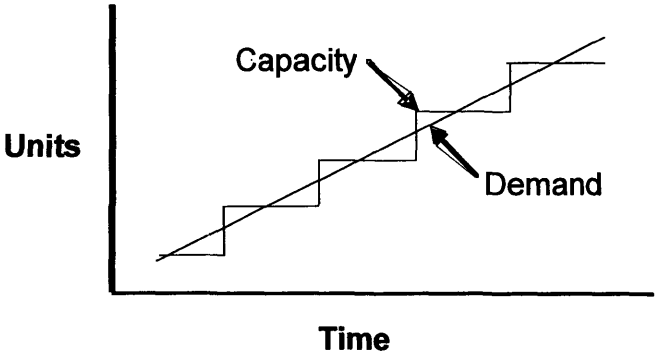
A second consideration when making capacity decisions is the timing of capacity increments and the size of the capacity cushion. Hayes and colleagues lay out three different capacity expansion strategies, as shown in **Exhibit 8**. Policy A represents one extreme, wherein the company always strives to have capacity in excess of expected demand. Policy C represents the other extreme, wherein the company strives to have capacity trail expected demand.

Exhibit 8: Alternative Capacity Expansion Strategies (Source: Hayes et al, 2005)

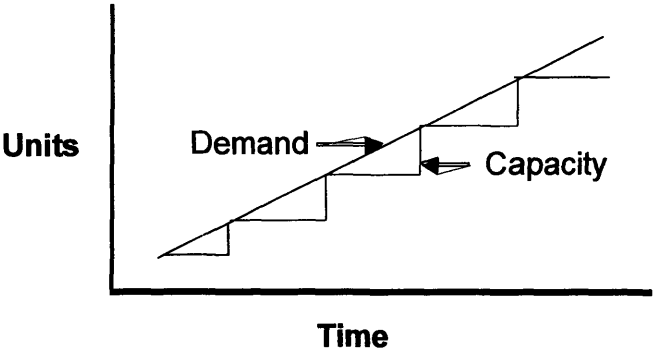
Policy A: Capacity leads demand



Policy B: Capacity in approximate equilibrium with demand



Policy C: Capacity lags demand



One way to choose between these policy alternatives is to consider the opportunity cost of not having enough capacity (C_s) versus the cost of having unneeded capacity (C_x), a problem often referred to as the "newsboy problem", in honor of the tough decision a cash-strapped newsboy must make every

morning when choosing how many non-refundable papers to buy from the newspaper company. The newsboy model implies that the capacity cushion should be related to the ratio (Hayes et al, 2005):

$$(C_s - C_x) / C_s \qquad \textbf{(Equation 2)}$$

In general, if this ratio is greater than 0.5, more capacity than the median expected requirement should be provided (i.e. **Exhibit 8** Policy A). If the ratio is less than 0.5, a negative cushion (i.e. **Exhibit 8** Policy C) makes economic sense. Although this model relies on certain simplifications, and the values of C_s and C_x are difficult to estimate, the model nonetheless is a useful framework for thinking about the tradeoffs that exist in the capacity decision. Using some rough numbers, one can estimate values of C_s and C_x for biologics. For example, the estimated carrying cost, C_x, of a facility operating at 50% capacity (e.g. Synergen's Antril facility) is \$2 – 3 million / month (Levine, 2004). The estimated opportunity cost, C_s, of a 50% shortage of a blockbuster biologic (e.g. Immunex's Enbrel) is \$40 - \$45 million / month (Levine, 2004). Using these rough values in Equation 2, one obtains a newsboy ratio of 0.93 to 0.96. These numbers can be interpreted as saying that for a blockbuster biologic, enough production capacity should be provided so that there would be less than a 4 - 7% probability that demand for the new drug would exceed capacity in the first year. Hayes and colleagues found a newsboy ratio of 0.83 for a pharmaceutical company (Hayes et al, 2005), suggesting that this high ratio is an inherent characteristic of the pharmaceutical industry. As further evidence of this fact, a recent report by McKinsey and Company estimates that a biologic can be a profitable product even if the capacity utilization is as low as 25% (Lohmeyer et al, 2002). Together, these findings suggest that a biologics producer should provide capacity well in excess of demand, and strive to follow the "capacity leads demand" policy outlined in **Exhibit 8**.

There are qualitative reasons that the "capacity leads demand" approach is the best choice for biologics. First, a large capacity cushion allows a company to respond to unexpected surges in demand. With drugs, it is often hard to accurately predict demand *a priori*, so the surge cushion is useful in this industry. Second, a large cushion can provide a competitive advantage, relative to similar products, if customers are not getting the required level of service from the competitor. For example, one of the

prime beneficiaries of Amgen's (formerly Immunex's) shortage of Enbrel is Johnson & Johnson's competing anti-TNF monoclonal antibody, Remicade. Third, since the biologics market is expected to continue to experience significant growth in the years to come, the risk of building too much capacity is lessened in such a growth environment. Fourth, there are ethical reasons that a drug company would strive to make sure they have sufficient capacity to deliver their life saving drugs at all times. In an industry where products save lives, running short of capacity is a matter of life and death.

A third consideration when making capacity decisions regards strategic externalities that can be obtained by having capacity. One such strategic externality is the deterrent role that capacity can play in a capital intensive industry, such as biologics production. By building capacity, a company in such an industry is often able to deter competitors from building capacity. This is a common strategy employed by a master of operations, Walmart, whose willingness to build stores in small, underserved markets many years ago has deterred competitors from entering these markets and having to share a small "pie" with Walmart, a most unappetizing proposition (Hayes et al, 2005). As I will discuss later in this paper, one of the big pharma companies that is competing very effectively in biologics is Roche. With the deterrent power of capacity in mind, it may come as no surprise that Roche makes its capacity expansion plans public, as this may work quite well to deter competitors expanding, and allow Roche to become a master of biologics operations, at the expense of competitors' learning, since the competitors may be tempted to outsource their production, if they sense that the industry is awash with capacity. A second strategic externality is the bargaining power that excess capacity will give a company when entering into licensing negotiations with smaller biotechnology companies. This was touched on in the section on outsourcing, but the general idea is that by being able to offer a potential licensor or partner manufacturing capacity, a big pharma company becomes a much more attractive partner for the small company. Given the ever expanding importance and role of in-licensing as part of big pharma's strategy, such a strategic advantage could pay big dividends.

A fourth consideration when making capacity decisions is the role of concurrent product and process development. As will be covered in a later section, in an industry such as biologics where there is a significant amount of product and process innovation, and rapid time to market is so crucial, it can be a significant advantage to have a highly efficient concurrent process and product development operation.

By having excess capacity, it is envisioned that a biologics producer would be able to move the developing process into a commercial facility at an early stage during development, which would speed up the development cycle.

Taken together, the four considerations outlined in this section all point towards the idea that biologics operations should plan for capacity to lead demand, given the important reasons and strategic advantages of having, on average, excess production capacity. Unlike, for example, in other capital intensive industries such as the commodity chemicals or steel businesses, where cost is king and excess capacity can be a deadly drag on an organization, production costs are not the primary driver of competitive advantage in the biologics business. Consequently, capacity decisions must be considered from these strategic perspectives, too.

Designing the Operations Network

Once a company has chosen to keep operations in-house and has made a decision on the amount of capacity to develop, another significant decision to be made regards the basic design of the operations network. The network design consists of decisions regarding the number, location, specialty and control of the different facilities in the operations network.

The choice of the number of facilities to have involves a trade-off between the economies of scope and scale (e.g. shared overhead, human resources etc.) and the diseconomies of scope and scale (cost of increasing complexity). On this point, the big pharma companies are probably quite adept at making this tradeoff, owing to their tremendous skill and history in traditional pharmaceutical production.

The choice of the location of the facilities in a network are driven by a set of trade-offs regarding such issues as labor cost, labor skills, tax incentives and so on. In the pharmaceutical business, tax incentives seem to be a major driver of location choices, with Puerto Rico and recently Ireland and Singapore as favorite choices, given the tax incentives offered by the governments of these regions to pharmaceutical manufacturers. In regards to biologics, it would be wise to add that the manufacturing process requires a higher level of skilled scientists and engineers than traditional small molecule drug manufacturing. For this reason, the skills of the local labor pool should be a major factor in the decision on where to locate a biologics facility. Most of the biotechnology companies have their USA

manufacturing either in California or the Northeast (Massachusetts, Rhode Island, Maryland), where there is a better than average pool of well trained scientists and engineers.

In regards to choosing the specialty of the facilities in the network, a company can choose either a horizontal or a vertical design. A horizontal, or product line, system means that a single facility handles the entire business needs (raw materials through to sales and marketing) of a single product. A vertical, or process stage, system means that a single facility handles an entire process stage, such as manufacturing, for multiple products. The choice between these different systems depends on the particular structure of the industry and the needs of the organization. **Exhibit 9** summarizes the conditions under which each system is most appropriate, with those points that seem to make sense to biologics operations shown in bold.

Exhibit 9: Choosing a horizontal or vertical operations network (Adapted from Hayes et al, 1998)

Horizontal (product focused) when:	**Vertical (process stage) when:**
• Process technology is not too complex or output can be purchased from contractors	• **Process technology is so complex and fast changing that it requires the attention of a large number of skilled employees**
• Competitive strategy requires close interaction with a fast moving market	• Low cost is a higher priority than fast delivery or flexible response • **Large economies of scale exist**

Based on the criteria in **Exhibit 9**, it would seem that vertical network designs make the most sense for biologics. The process technology of biologics production is complex enough that it requires a sizeable staff of scientists and engineers. Further, there are substantial economies of scale in locating these skilled employees in a small number of process-stage plants, since it would be costly to duplicate their talents at each plant in a network of product-focused plants. Also, there are economies of scale in the equipment for biologics production. In regards to the tradeoff between low cost and fast delivery or flexible response, in biologics production there is probably no need for a highly flexible response, since

once regulatory approval is obtained for a facility, the company would like to minimize changes in the process. So, if flexibility is not a major issue or requirement, low cost naturally becomes desirable.

The final point regarding the broad network structure of biologics is whether to exercise centralized or decentralized control and policies over the network. In today's business environment, there is plenty of talk about worker empowerment and the merits of decentralized networks, but that is not to say that the "old school" centralized networks of yesteryear don't have merit. **Exhibit 10** summarizes the conditions under which each system of control and policy making is most appropriate, with those points that seem to make sense to biologics operations shown in bold.

Exhibit 10: When is Centralization or Decentralization appropriate? (Adapted from Hayes et al, 1998)

Centralized / Standardized when facilities:	**Decentralized / Autonomous when facilities:**
• **Produce similar products**	• Produce different products
• **Serve similar customers who value uniformity**	• Serve customers with different needs
• **Operate in environments with similar constraints and/or resources**	• Operate in very different local environments

In my opinion, the arguments in **Exhibit 10** all support a centralized and standardized network for biologics operations. As I have defined biologics in this paper, the products are similar enough that the same equipment, processes and labor skills are generally applicable to the entire product portfolio. Further, customers in this market definitely value uniformity and consistency. In fact, the FDA outright demands it! Lastly, the environments that the companies operate in should have similar constraints and resources, with local nuances playing small roles in the production of life-saving medicines.

In summary, the ideas presented in this section support the following approach to biologics operations:

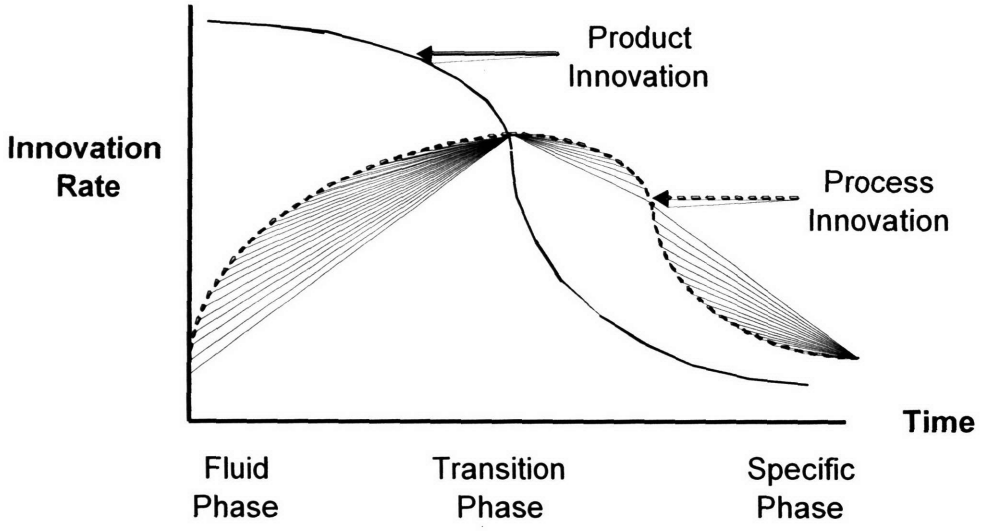
- Locate in areas that are tax advantaged and have an abundance of highly skilled employees.
- Design each facility to focus on a particular process stage, such as protein manufacturing or fill-and-finish, as this allows pooling of the required, specialized talent for biologics operations.

- Exercise central control over the network, since the products produced are similar and there is an extremely high value to product uniformity.

The Power of Concurrent Product and Process Development

The role of process development in product development has historically been characterized by the "product life cycle" model, the features of which are reproduced in **Exhibit 11**.

Exhibit 11: The (Outdated) Product Life Cycle Model (Adapted from Hayes et al, 2005)



Briefly, the product life cycle model states that during the first phase of product development, the fluid phase, the rate of product innovation is high and process innovation or development is relatively low. At some point, the industry or system undergoes a transition, where now the key to success is process innovation. Finally, one reaches the specific phase, wherein the rate of any type of innovation is low.

This model is useful for thinking about some industries, but not for all, and particularly for our case, it neglects to give users a clear picture of the dynamics in biologics product development. A more appropriate model or framework has been put forth by Hayes and colleagues and is summarized in **Exhibit 12**.

Exhibit 12: The Relationship between Product and Process Innovation (Source: Hayes et al, 2005)

Rate of Process Innovation	Rate of Product Innovation: Low	Rate of Product Innovation: High
High	**Process Focused** • Commodity Chemicals • Steel • Shipbuilding *Process Development focuses on Cost Reduction*	**Process Enabling** • **Pharmaceuticals/Biotechnology** • Specialty chemicals • Semiconductors • Advanced materials • High precision, miniature electronics • Services *Process Development focuses on solving complex technical problems, fast time to market, and fast ramp-up*
Low	**Mature** • Apparel • Agriculture • Cement • Paper • Process *Process development focuses on cost reduction*	**Product Focused** • Software • Entertainment • Assembled products *Either little process development or a focus on design for manufacturability*

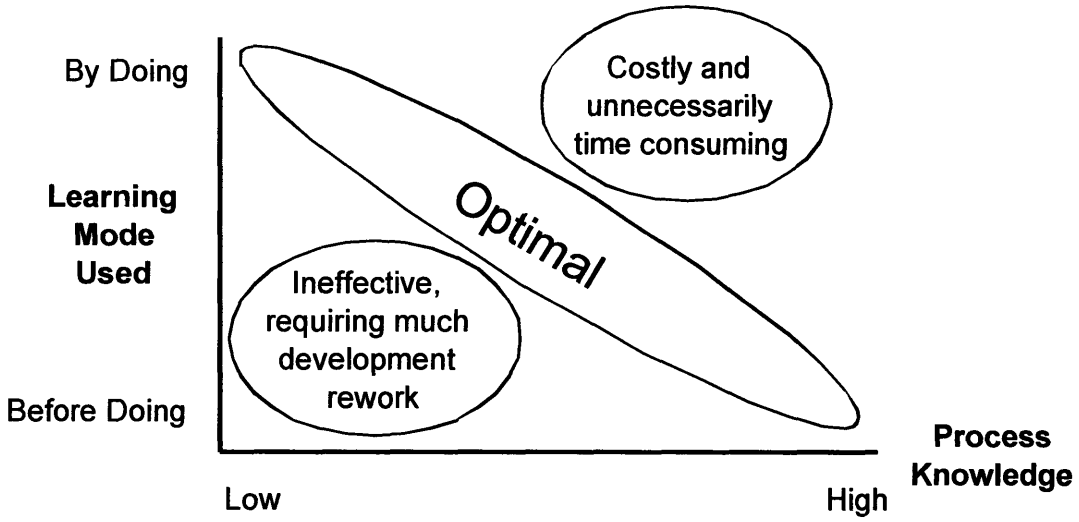
Comparing **Exhibit 12** to **Exhibit 11**, it is clear that the two frameworks share ideas, but that the 2 x 2 matrix adds one additional "phase" to the product life cycle. The "fluid", "transition" and "specific" phases of **Exhibit 11** are the same as the "product-focused", "process focused" and "mature" quadrants, respectively, of **Exhibit 12**. The additional concept captured by **Exhibit 12** and neglected by the traditional product life cycle approach is the "Process Enabling" quadrant, a region where "process development focuses on solving complex technical problems, fast time to market, and fast ramp-up". In this quadrant, process and product innovation must be carefully synchronized, since the capability for fast, efficient and high quality process development has a direct effect on new product introductions. It should come as no surprise that pharmaceuticals and biotechnology are grouped by Hayes et al into the

"process enabling" quadrant, given the fact that delaying the introduction of a blockbuster drug by a single day is worth several million dollars in unrecoverable revenues.

To go about optimizing and synchronizing product and process development, Hayes and colleagues note that a company must carefully address three drivers of performance. The first driver is integrating product and process development. This can be accomplished by having integrated teams, by using design-for-manufacturing (DFM) concepts, and by using prototyping early and often. In mapping these concepts to biologics, the integrated teams can be addressed by having operations personnel involved in early stages of new products. Projects can probably be started with a minimal amount of resources early on to try and optimize cell lines and production conditions, even before it is clear that the biologic will become a pre-clinical compound. Likewise, DFM concepts mean that early efforts should be employed to make sure the cell line and purification needs of the product are being considered by operations at an early stage. Prototyping can come into play by starting to produce the products at an early stage in reaction vessels with the same geometry and conditions as the commercial scale vessels. This will be easier to accomplish if an organization has excess commercial production capacity.

The second driver is the timing of technology transfer from the laboratory to the manufacturing floor. This involves designing a framework for process development experiments. In particular, an organization needs to balance the costs and benefits of "learning before doing" versus "learning by doing", as summarized in **Exhibit 13**.

Exhibit 13: Learning by doing versus learning before doing (Adapted from Hayes et al, 2005)



If process knowledge is high, it is wasteful to spend resources on learning by doing, since it would be much more efficient to learn before doing, by using, for example, laboratory experiments, simulations and models. On the other hand, if process knowledge is low, it is better to engage in learning by doing, since there is not that much to be gained by trying to optimize the process at the laboratory level or by engaging in simulations and modeling. It is better to get out on the floor and "get your hands dirty" in accumulating tacit knowledge and solving problems at the commercial level.

Biologics production process knowledge probably falls somewhere in the middle of the two extremes. On the one hand, scientists and engineers have made strides in understanding these processes and in developing useful laboratory assays, models and simulations for predicting process behavior. On the other hand, biologics production involves the use of a platform (animal cells) that we do not yet fully understand, and there are still problems in scaling these processes up to a commercial scale. So, for the foreseeable future, a biologics producer should still plan on a good deal of "learning by doing", in order to develop a robust process. This should be planned for in product-process development.

The third driver is the decision on whether to keep process development and technology choices centralized or decentralized at each site. In making this decision, Hayes et al have laid out three questions that a company should address:

1. *How important are "local" differences in markets or operating conditions?* In the case of biologics production, the answer is probably "not very important", although there could be issues regarding local utility supplies and so on. This response suggests central process development is best.
2. *How fully can the process be optimized prior to transfer to operations?* In the case of biologics, as discussed previously, the answer is probably "somewhat", but there is still going to be a good deal of "learning by doing" at the local site. This response suggests a balance between a centralized and decentralized development approach.
3. *Do the major improvements in performance generally occur by incrementally improving a particular generation of process technology or by developing completely new generations of process technology?* In the case of biologics, since regulatory approval is required for process changes, there is probably limited incremental improvements after the process is up and running. This response suggests central process development is best, with process breakthroughs (e.g. a new cell line platform) being rolled out by the central organization, and applied to the next generation of products.

In summary, the analysis in this section has lead to the conclusion that biologics product and process co-development should involve highly integrated teams and integration concepts (e.g. DFM, prototyping). Further, the process should involve a good deal of centralized development work, but that there must be a willingness to get the process into the local site well before the end of the development cycle, since there is a good deal of "learning by doing" as well as local issues that will need to be addressed before the unit starts producing.

Driving an Operations Edge

This last section draws further on the work of Hayes and colleagues in considering how big pharma should go about implementing an operations edge when starting up or improving a biologics franchise within their organization. Starting up a biologics franchise inside the walls of a large pharmaceutical company is classified as a breakthrough or structural improvement effort (as apposed to

an incremental change), since it involves the organization learning to do some very different types of tasks than it is accustomed to. For example, most big pharma companies have a well developed process for generating pre-clinical compounds, involving a pipeline of work flowing from biologists to medicinal chemists, who might then spend years optimizing the chemical structure of these compounds. In the biologics world, there may not be a strong need for medicinal chemists, and their organizational clout and stature could be threatened by a company's growing emphasis on biologics. This is just one example of why the implementation of a biologics franchise is by no means a small task for big pharma. Further, given the significant changes required, such an effort cannot be driven fully from within the organization, as this sort of effort will require a fair amount of explicit knowledge. The organization must be willing to go out and acquire this knowledge.

The framework developed by Hayes involves five points for a company to address when undergoing an improvement effort. First, a company should clearly state the nature of the improvement strategy. For our specific case, it could be a simple sentence acknowledging the importance to the company of pursuing a biologics business (why are we doing this?): "We, Big Pharma, aim to improve our pipeline and long-term prospects by actively engaging in biologics, a field with significantly higher mid-term growth than small molecules".

Second, the company should establish the expectations and goals of the improvement effort. These goals should be as explicit as possible, as they will serve to determine whether the improvement initiative worked or not. For the case at hand, namely developing a biologics business, the goals could be the following:

- Obtain 35% of our revenues from biologics by 2015; or
- Market 6 biologics by 2010; or
- Obtain 3 new licensing deals, which can be attributed to our focus on biologics operations excellence, with small biotechnology companies by 2010

Third, the company needs to organize for implementing the strategy. In the case of a breakthrough improvement effort, it will be critical for the strategy to have top management support and a top management champion. Further, the actual activities that will be carried out to achieve the strategy should be noted at this stage. For the biologics case, this could be something along the lines of:

1. Bring licensed, phase II compounds X and Y to market by working with Lonza (a CMO).
2. Assign a team of scientists and engineers to Lonza, to retain knowledge in-house.
3. At the same time, begin building 30,000 liters of capacity at our Berkeley CA location.
4. Transfer the manufacturing know how for compound X to the new facility.
5. Etc.

Fourth, the organization must be sure to provide the appropriate resources for achieving success. In the case of developing a biologics franchise, these resources are significant. Besides funding, the strategy will require a new set of workers (biochemical engineers from MIT, protein scientists, glycosylation scientists, regulatory experts etc.). It will also pay to use external consultants and expertise at this point, since the budding organization will probably be significantly behind the industry leaders at this point. Also, the organization will need resources for obtaining licenses to particularly useful technologies, such as Lonza's glutamine synthetase (GS) system for optimizing cell culture productivity.

Fifth, the organization should develop a rich set of contingency plans to make sure the strategy is flexible enough to withstand the risks and uncertainty inherent in the biologics field. Some potential events and their contingency plans could be as follows:

- Internal products do not receive FDA approval:
    - Pursue new biologics licensing deals in the short-term to shore up the pipeline, using our manufacturing capacity as a negotiating asset
    - Partner with another company and offer them capacity for a discount (we will learn a great deal from the experience, even if we don't make a profit).
    - Consider entering biogenerics
    - Sell the plant and exit biologics
- Insufficient production capacity:
    - Maintain strong ties with Lonza as we build up our own capacity, always keeping an option on a portion of their capacity.
- Excess capacity:

- Pursue licensing deals – we are a more appealing partner, since we have capacity and expertise ready to go.
- Partner with another company in need of short-term capacity.
- Use a portion of the excess capacity for product / process development and improvement work (i.e. incorporate earlier "learning by doing" in our process development cycle)
- Consider entering biogenerics

By following these five steps, as well as by applying some of the ideas and frameworks presented in earlier sections, big pharma can improve the likelihood of any endeavor into biologics being successful, and also maximize the edge that operations will bring to the new biologics business.

Operations in the Biotechnology Industry

In the previous section, I have tried to develop an "ideal" biologics operations strategy. In this section, I will look at what the top biotechnology companies in the world are doing, in practice. **Exhibit 14** lists eleven companies engaged in producing biologics, seven of which were ranked in the top 10 in terms of biotechnology sales in 2003 (Contract Pharma, 2004). Three of the top 10 companies have been excluded from this analysis because their products do not qualify as complex biologics, using the definition given in the introduction to this paper. In their place, I have added four smaller biotechnology companies with interesting business models and extensive collaborations with big pharma.

Exhibit 14: Top 10 Biotechnology Companies (and others)

Company	Headquarters	2003 Rank	2003 Revenues ($B)
Amgen	Thousand Oaks, CA	1	7.9
Genentech	San Francisco, CA	2	2.6
Serono	Geneva, Switzerland	3	1.9
Biogen IDEC	San Diego, CA	4	1.9
Genzyme	Cambridge, MA	5	1.1
MedImmune	Gaithersburg, MD	7	0.99
Millennium	Cambridge, MA	9	0.24
ImClone	New York, NY	NR	0.081
Protein Design Labs (PDL)	Fremont, CA	NR	0.067
Abgenix	Fremont, CA	NR	0.017
Human Genome Sciences (HGSI)	Rockville, MD	NR	0.0082

To consider the importance of operations to these top biotechnology companies, I have analyzed the companies' annual reports, 10-K forms (available at www.sec.gov), the comprehensive list of FDA-approved biological products (www.biopharma.com) and web sites to gain a better understanding of their operations and manufacturing networks and strategy. The results clearly indicate that these top players see in-house manufacturing and operations as critical for their business success, and treat them as core

competencies, continually striving to improve them. A sampling of some of the company testimonials and observations regarding the importance of operations are summarized in **Exhibit 15**. Text in quotation marks is taken from the attributed source. The unquoted, bolded text are my observations and commentary.

Exhibit 15: Biotechnology Testimonials on the Importance of Operations

Company	Testimonials / Observations / Author's Commentary	Source
Amgen	**Comment on the importance of process improvement:** "In connection with our ongoing process improvement activities associated with products we manufacture, we continually invest in our various manufacturing practices and related processes with the objective of increasing production yields and success rates to gain increased cost efficiencies and capacity utilization."	2003 Form 10-K
Genentech	**Comment on the importance of process development:** (1) "However, we believe our competitive position is enhanced by our commitment to research leading to the discovery and development of new products and manufacturing methods". **Comment on the importance of capacity expansion:** (2) "On the operations front, we continue to plan for manufacturing needs in both the short- and long-term. We have increased manufacturing efforts in both our South San Francisco and Vacaville facilities in an effort to meet growing product demand"	2003 Form 10-K
Serono	**Quantitative data on their continuing operations improvements:** (1)"We have integrated operations that allow us to manufacture and market the products we derive from our R&D efforts. The use of biotechnology techniques has allowed us to improve our manufacturing efficiency and helped us to increase our product gross margin to 85.0% in 2003 from 67.7% in 1995 and to increase our net margin to 19.3% of revenues in 2003 from 4.2% in 1995." **Details on the quality difficulties facing biologics, and the ability of biologics companies to tackle these issues via operations:** (2) "In order to control product variability, we have developed a highly controlled manufacturing process for Gonal-f. This manufacturing process allows us to produce recombinant human FSH with a highly consistent isoform profile. Furthermore, we have now identified a new more precise physico-chemical method to determine the potency of the product. As a result, Gonal-f is now filled-by-mass (i.e., protein weight). By doing so, we eliminate the intrinsic variability of the rat bioassay and ensure high batch-to-batch and vial-to-vial consistency of r-hFSH content." **Operations contingency planning:** (3) "For certain key products, we have two production facilities available to ensure a continuity of supply in the event of contamination, catastrophe or other unforeseen event at one of our facilities."	2003 Form 20-F
Biogen IDEC	**A clear sign that outsourcing is not part of their long-term plans:** (1) "We manufacture and expect to continue to manufacture our own commercial requirements of bulk [drug]."	2003 Form 10-K

	Capacity as a strategic asset for licensing and partnering deals: (2) "Additionally, we believe our manufacturing capacity will make us an attractive partner for companies seeking to partner on promising biologic products in development."	
Genzyme	**Recognizes manufacturing issues as 1 of 30 main risks facing the company:** (1) "our ability to manufacture sufficient amounts of our products for development and commercialization activities and to do so in a timely and cost-effective manner" **Manufacturing scale-up difficulties recognized for at least 1 product:** (2) "We are making progress in increasing our supply of Myozyme and scaling up our manufacturing capacity."	2003 Form 10-K
MedImmune	**Interestingly, MedImmune outsources their small molecule product (Ethyol) but produces their biologic product (Synagis) in-house, which might be a useful case study for small-molecule-heavy big pharma.**	2003 Form 10-K
ImClone	**Internal manufacturing has apparently resulted in a significant decrease in COGS for Erbitux:** "During 2004, we sold all inventory produced by Lonza and we anticipate that all production of ERBITUX in 2005 will be performed at our BB36 manufacturing facility. Therefore, the sales price to our corporate partners in 2005 is expected to be significantly lower (i.e. roughly one-half) than the costs we charged our partner in 2004 since our unit cost to manufacture ERBITUX is significantly lower than the cost paid to Lonza for manufacturing ERBITUX." **Further, ImClone is dramatically increasing manufacturing capacity, adding to their existing 30,000 liters with an additional 110,000 liters.**	2004 Form 10-K
PDL	**PDL started out as a technology company, offering monoclonal antibody (MAb) humanizing technology. They are now moving to become a fully-integrated biotechnology company, and see manufacturing as a major advantage. Consequently, they are building significant capacity:** "We believe our knowledge and capabilities in this area provide a significant degree of competitive advantage over those companies that currently lack such fully integrated operations. In particular, we have over a decade of manufacturing experience based upon a serum-free and protein-free production process, and we believe that this approach is a significant competitive advantage."	Company web site (pdl.com)
Abgenix	**Abgenix sees their manufacturing skills as part of their "partner of choice" offering:** Abgenix's manufacturing facility is designed for multiple antibody products with a focus on mammalian processes. Our experienced staff offers significant expertise in all phases of antibody manufacturing from preclinical to worldwide market supply and is committed to being the partner of choice.	Company web site (abgenix.com)
HGSI	**Even a cash-strapped Biotech sees manufacturing as a worthy investment:** "A worldwide shortage of protein drug manufacturing facilities and expertise exists. We believe our in-house skills, facilities and experience in this area are a vital strategic asset."	Company web site (hgsi.com)

From the testimonials and data presented in **Exhibit 15**, it is clear that even biologics companies with revenues of well below $1 billion per year see manufacturing as a strategic necessity, giving them control over their own operational excellence initiatives and improving their strategic position in the market. To better understand the manufacturing strategy pursued by these companies, one can develop a square matrix depicting the manufacturing relationships that exist amongst the companies listed in **Exhibits 14 and 15**. This matrix is shown in **Exhibit 16**. The specific details of each of the relationships depicted in **Exhibit 16** are given in **Appendix A**.

Exhibit 16: Manufacturing Relationships amongst Top Biotechnology Firms

	CUSTOMER ORGANIZATION										
	Amgen	Genen.	Serono	Biogen	Genz.	MedIm.	ImClone	PDL	Abgenix	HGSL	3rd Parties
Amgen	S, D										M
Genentech	C	S, D		C							M
Serono			S, D								M
Biogen				S							M
Genzyme					S						
MedImmune						S					M, C
ImClone							S, D				
PDL								S			
Abgenix	P								L		P
HGSI										S, D	
3rd Parties	C	M, C				C	C				

Legend: C = contract manufacturer; D = additional capacity under development; L = limited internal manufacturing (pilot plants, clinical trial material); M = Customer markets the product manufactured by a partner; P = Partner manufactures clinical trial material for the customer; S = Internal manufacturing capacity

The matrix in **Exhibit 16** is sparse, almost diagonal, with very few relationships other than along the diagonal (i.e. in-house manufacturing). This means that every one of the top biologics producers, as

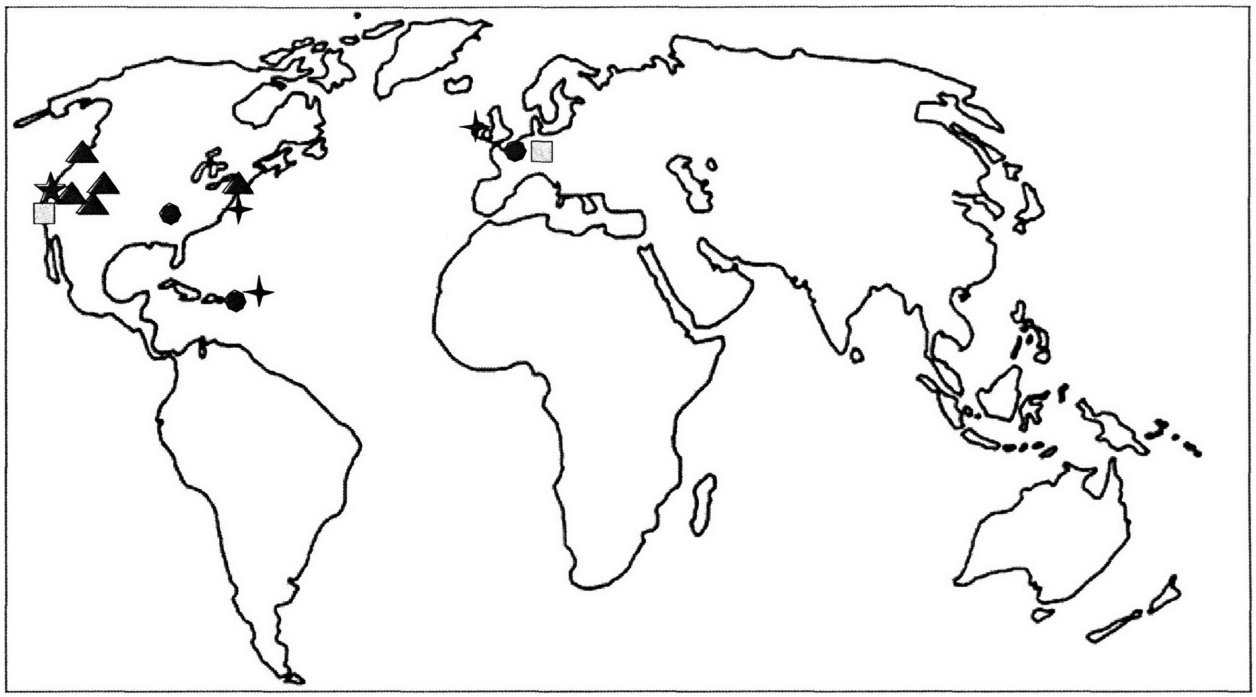
well as several very small companies, has a strategy of using in-house manufacturing. By examining the details of the few 3rd party relationships (see Appendix A), it is clear that these biotechnology companies only rely on 3rd party manufacturing when faced with a capacity crunch. For example, Amgen currently has a manufacturing relationship with Lonza for relieving the short-term Enbrel supply shortage, while Amgen builds up its own capacity. Also, the fact that the majority of these companies provide 3rd party manufacturing services (last column in the matrix) suggests that at some point in their histories, these companies have had excess capacity, which is expected in an industry where a "capacity leads demand" approach seems appropriate, as discussed earlier. Further, note that at least half of these companies are developing additional capacity ("D").

The relationships depicted in **Exhibit 16** are only related to the production of active pharmaceutical ingredient (API; i.e. the protein itself). Most, if not all, of the companies analyzed here seem to outsource the "fill-and-finish" portion of production to 3rd parties, which likely means that these companies consider this step non-strategic and suitable for outsourcing.

Finally, a word on the network design of the top biologics producers. As shown in **Exhibit 14**, these companies are generally headquartered on the East or West coast of the USA, in cities that are rich with top universities and the highly trained employees required to run a biologics operation. Further, these companies seem to adhere to the ideas discussed in the previous section on operations theory by having fairly centralized operations. For example, the major facilities in the worldwide network of Amgen, the world's largest biotechnology company, with revenues greater than the rest of the top 5 combined, are shown in **Exhibit 17**. The majority of the manufacturing appears to be conducted near the company's California headquarters. Only now is the company starting to expand significantly overseas, probably due to the tax advantages of certain geographies.

Exhibit 17: Amgen Worldwide Manufacturing and Packaging Network

(Source: Data compiled from 2003 10-K)



Legend:
- Company Headquarters
- Inhouse Biologics Bulk Drug Manufacturing
- Inhouse Fill and Finish / Packaging / Distribution
- Outsourced Biologics Manufacturing
- Outsourced Packaging / Distribution
- Current Expansion Projects

Synthesis: The Recommended Operations Strategy

Based on operations theory and the operations practices of the top biotechnology companies, both of which have been discussed in some detail up to this point, the recommended operations strategy for a big pharmaceutical company, *committed to being a successful player in the biologics market*, is as follows:

- Build up the capacity and skills necessary to effectively produce the biologics in-house;
- Build capacity in excess of projected internal production requirements, since the opportunity cost of having insufficient capacity far exceeds the carrying costs of excess capacity and also because the excess capacity could be used strategically for becoming a "partner of choice" for small biotechnology firms;
- Outsource the fill-and-finish portion of production, if desired;
- Design a vertical (process stage), centrally-controlled network of operations facilities, with facilities located in areas with a large population of the professional employees required for biologics production; and
- Integrate product and process development into the operations, through such means as early operations representation on discovery projects, as this could be a major competitive advantage in rapidly and effectively bringing drugs to market.

This operations strategy would need to be well synchronized with a product, or business development, strategy, to ensure that the pipeline is well stocked with biologics. The product development strategy would focus on in-house biologics R&D, external collaborations and licensing agreements, line extension work, and post-patent activities, such as improving the physiological properties of the biologic (e.g. Amgen's evolution from EPO to Aranesp) or by engaging in areas such as biogenerics production. This synchronization between operations (process development) and product development, a major advantage in "process enabling" industries such as drug development (**Exhibit 12**), will be discussed further in the next section.

The Report Card: How is Big Pharma Doing?

In this final section, I pass judgment on big pharma's performance to date in the biologics business. Specifically, I have looked at the biologics franchise of the Top 20 pharmaceutical companies, based on 2003 revenues (Contract Pharma, 2004). These companies are listed in **Exhibit 18**.

Exhibit 18: Top 20 Pharmaceutical Companies

Company	2003 Rank	2003 Revenues ($B)
Pfizer	1	39.7
GlaxoSmithKline (GSK)	2	29.7
Merck	3	21.0
Johnson & Johnson (J&J)	4	19.5
Aventis[1]	5	19.0
AstraZeneca	6	18.8
Novartis	7	16.0
Bristol-Myers Squibb (BMS)	8	14.9
Roche	9	12.2
Lilly	10	11.9
Wyeth	11	11.9
Abbott	12	9.3
Sanofi-Synthelabo[1]	13	9.1
Takeda	14	7.6
Schering-Plough (S-P)	15	6.7
Boehringer-Ingelheim (B-I)	16	6.3
Bayer	17	5.4
Schering AG	18	3.6
Sankyo	19	3.1
Merck KgA	20	2.0

[1]Aventis and Sanofi have completed a merger that retroactively would give them the 3rd rank, based on 2003 revenues

Although I have tried to obtain a complete and accurate set of data regarding the activity of each of the Top 20 pharmaceutical companies, there can be no assurances that I have learnt anymore than what each of these companies wants me to learn and is willing to disclose publicly, or that has entered the public domain by other means. Thus, if a company has a "secret" biologics program, I would probably not have found any evidence of this program, and the company would receive a low grade from me, even though they may, in fact, have a very robust and emerging program. However, in certain areas, my data is complete. For example, the list of FDA-approved biologics (available at www.biopharma.com) was used to generate the list of products that each company has on the market. If a company did not register on this list, then it can be said with a high level of certainty that the company does not have any approved or marketed biologics.

Throughout this paper, I have tried to stress the belief that operational excellence is important for success in the biologics business. Obviously the other factor for succeeding in the biologics business is by having excellent biologics *product* development. In terms of evaluating the performance of these Top 20 companies, I will use a framework that tries to measure how each company performs on these two dimensions, namely product and operations, or process, performance. Being able to simultaneously excel at both product and process development is expected to be a major advantage in a "process enabling" industry, such as biotechnology.

To measure the company performance along these two dimensions, namely product and process development, I will use the metrics and scoring presented in **Exhibit 19**. For each metric, I will score a company on a scale of 0 to 2, using the evaluation criteria shown in the exhibit for assigning a score.

Exhibit 19: Criteria for Scoring Biologics Product and Process Development Performance

Metric	Score = 0	Score = 1	Score = 2
	***Product* Development Performance**		
In-house biologics R&D	None known	At least 1 biologic developed in-house	Devoted biologics R&D facility
Biologics pipeline	None known	At least 1-2 biologics in the pipeline	More than 2 biologics in the pipeline
Marketed biologics	None known	At least 1-2 biologics on the market	More than 2 biologics on the market and at least 1 blockbuster ($1B+ in 2003)
Biologics collaborations (*non-manufacturing*)	None known	At least 1-2 biologics-related collaborations	More than 2 biologics-related collaborations
Post-launch or Post-patent activities	None known	Minor activities (e.g. new indications)	Major activities (e.g. biogenerics, Aranesp developed from EPO)
	***Process* Development Performance**		
In-house manufacturing	None known; all outsourced	Under development	Have commercial capacity
Manufacturing capacity	None known; all outsourced	Adequate; occasional outsourcing	Significant; excess capacity or expansion
Process contractor	None known	Manufacture biologic for at least 1 other company; Licensor of process technology[1]	Serve as CMO to multiple organizations
Concurrent product/process engineering	None known	Pilot plant	A "pledge" to concurrent engineering was found in company documents
Distribution network	No biologics; no biologics distribution	Biologics distribution in at least 1 market	Biologics distribution worldwide

[1]A good licensing example is Lonza's glutamine synthetase (GS) system for improving cell line productivity (See Appendix B)

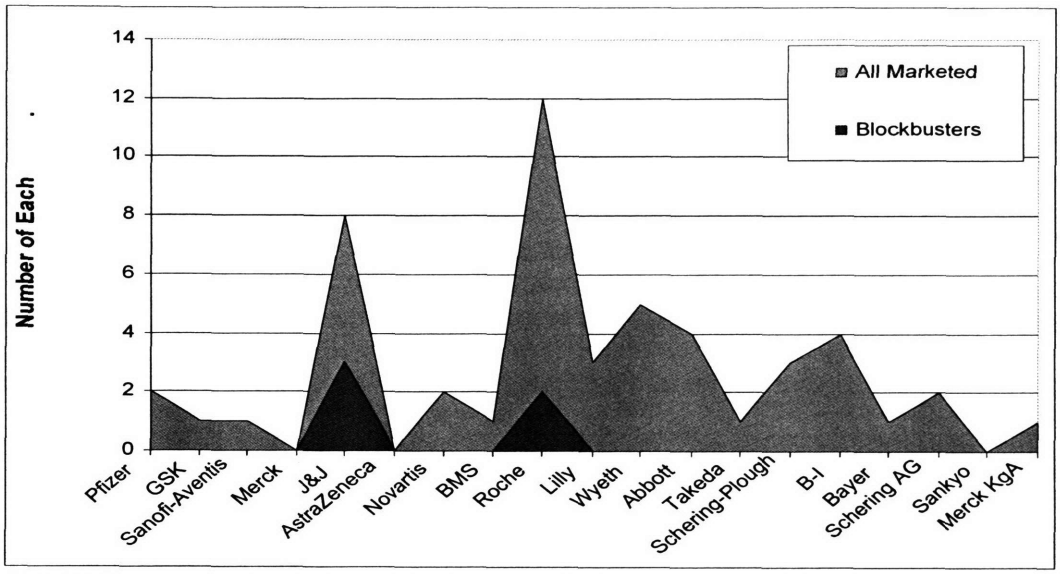
The data required for assigning a score to each company for each of the above 10 metrics came from a variety of sources, as now described:

1. *In-house biologics R&D*: Company websites, partner websites, press releases and annual reports

2. *Biologics pipeline*: Company websites, partner websites, press releases and annual reports
3. *Marketed biologics*: List of all FDA-approved biologics (www.biopharma.com); company websites, partner websites, press releases and annual reports (Compiled in **Appendix B**)
4. *Biologics collaborations*: Company websites, partner websites, press releases and annual reports (Compiled in **Appendix C**)
5. *Post-launch or post-patent activities*: Company websites, partner websites, press releases, annual reports and Contract Pharma
6. *In-house manufacturing*: List of all FDA-approved biologics (www.biopharma.com) shows each drug's manufacturer; company websites, press releases and annual reports (Manufacturing network is summarized in **Appendix A**)
7. *Manufacturing capacity*: See "In-house manufacturing above"
8. *Process contractor*: See "In-house manufacturing above"
9. *Concurrent product / process engineering*: Company websites (testimonials compiled in **Appendix D**)
10. *Distribution network*: List of all FDA-approved biologics (www.biopharma.com) shows each drug's marketer, for major geographic regions.

When compiling the data necessary for calculating the product and process development score for each company, several interesting observations emerged. First, there is a great deal of variance in the level of activity of the Top 20 companies in terms of currently marketed biologics. As shown in **Exhibit 20**, there are significant peaks and valleys, with some companies very actively involved in biologic products and with other companies completely absent. Further, there is surprisingly little activity from the Top 5 companies, with only J&J having a significant presence in the biologics market today. Activity also drops off at the lower end of the rankings. The most activity is taking place in the middle of the pack, with Roche, Lilly, Wyeth and Abbott all performing well.

Exhibit 20: The Number of Biologics Currently Marketed by Big Pharma (see Appendix B)



A second interesting observation is the level of variance in biologics manufacturing strategy. Whereas the top biotechnology companies all engaged in in-house manufacturing (see **Exhibit 16**), big pharma is much more likely to use contract manufacturing, as shown in **Appendix A**. Comparing the matrix for pharma companies presented in **Appendix A** to that for biotechnology companies (**Exhibit 16**), the differences are quite stark. The pharma matrix is not nearly as sparse, with lots of contract manufacturing being used. Further, the diagonal in the pharma matrix is less than half non-zero terms, showing that many of these companies neither have nor are developing manufacturing capabilities.

Using the scoring system described in **Exhibit 19**, in conjunction with the data obtained for each of the Top 20 pharmaceutical companies as described above, a product and process development score was calculated for each company. The raw scores are shown in **Appendix E** and are summarized in **Exhibit 21**.

Exhibit 21: Big Pharma Product and Process Focus Scores (see **Appendix E**)

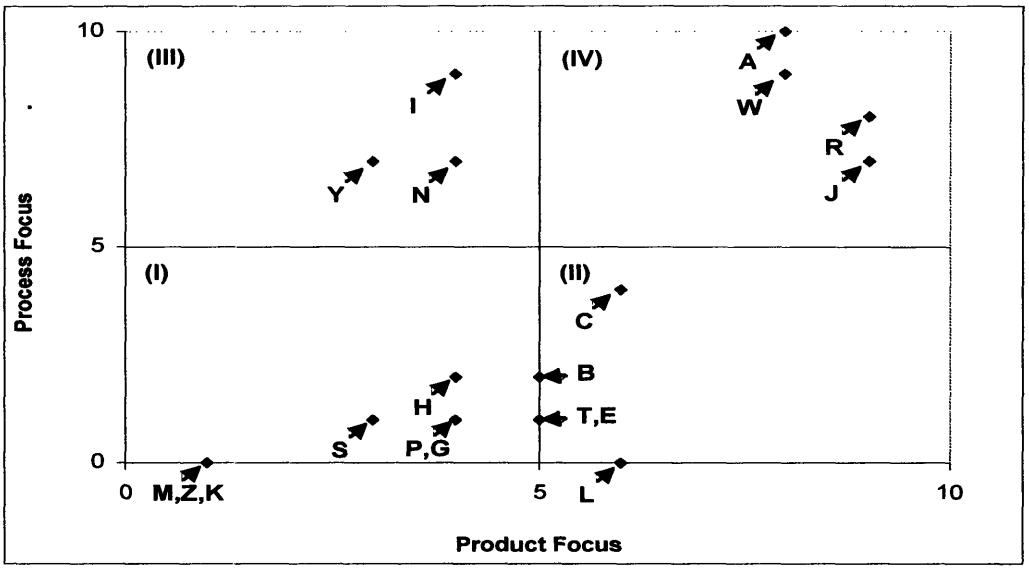


EXHIBIT LEGEND:

Company	Symbol	Company	Symbol	Company	Symbol	Company	Symbol
Pfizer	P	AstraZeneca	Z	Wyeth	W	Bayer	Y
GSK	G	Novartis	N	Abbott	A	Schering AG	H
Sanofi-Aventis	S	BMS	B	Takeda	T	Sankyo	K
Merck	M	Roche	R	S-P	C	Merck KgA	E
J&J	J	Lilly	L	B-I	I		

Exhibit 21 can be divided into four different quadrants:

(I) ***Minimal biologics activity***: Limited success (or effort) in developing or maintaining a biologics product portfolio or a biologics operation;

(II) ***Product-Focused approach***: substantial focus on developing or maintaining a biologics product portfolio, but a limited focus on operations;

(III) ***Process-focused approach***: substantial focus on developing biologics operations, but limited success (or effort) in developing or maintaining a biologics portfolio; and

(IV) ***Simultaneous Process-Product approach***: substantial focus on both developing and maintaining a biologics portfolio as well as developing an operations network for producing the products

Exhibit 22 summarizes where each of the Top 20 companies fall. As was the case with marketed biologics (**Exhibit 20**), **Exhibit 22** shows that the top 5 companies do not appear to have a strong focus on biologics, with 5 of the top 6 companies falling into the "minimal biologics activity" quadrant. These companies have either chosen not to participate in the biologics market and focus on small molecules instead, or have been very slow in entering the market, to date.

Exhibit 22: Top 20 Pharma Performance or Strategy in Biologics

Quadrant	Companies (rank)
Minimal biologics activity (I)	Pfizer (1); GSK (2); Sanofi-Aventis (3); Merck (4); AstraZeneca (6); Schering AG (17); Sankyo (18)
Product-focused approach (II)	BMS (8); Lilly (10); Takeda (13); S-P (14); Merck KgA (19)
Process-focused approach (III)	Novartis (7); B-I (15); Bayer (16);
Simultaneous process-product approach (IV)	J&J (5); Roche (9); Wyeth (11); Abbott (12);

Of the companies that are seriously focused on at least some portion of the biologics value chain (Quadrants II –IV), which quadrant has the greatest chance of success? Let us now explore the expected fates of companies in each of these three quadrants.

The companies in the product-focused quadrant (II) are developing strong product portfolios, but do not yet have the operational expertise to effectively bring the products to market on their own. These companies will be forced to out-license their products or partner with a CMO. These companies will have at least two weaknesses, limiting the potential of their biologics franchises: (1) their lack of operational expertise will make concurrent product-process development more difficult, relative to process-focused organizations and (2) they will need to share some of their product value with operating partners.

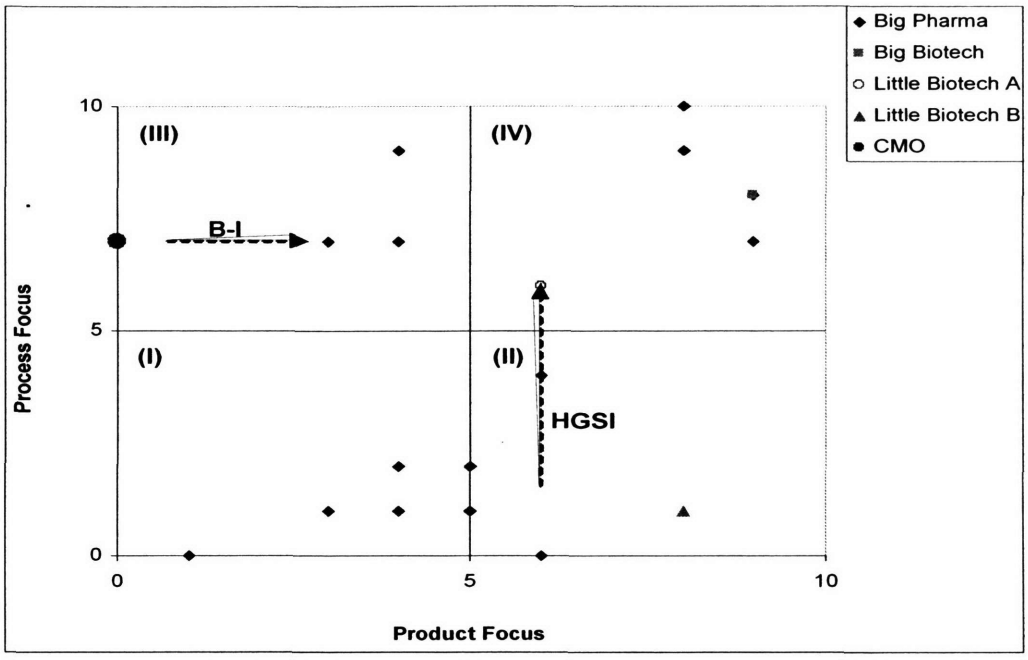
The companies in the process-focused quadrant (III) are developing strong operations, but may not have any products to bring to market. If there is a shortage of operations know-how and capacity in the future, these companies could benefit by using their operations expertise as leverage in licensing negotiations with product-focused companies. However, if there is not a shortage of operations expertise or capacity, these companies will find themselves in a difficult position, since they depend on others for products. The extreme example of companies in this quadrant are the pure-play CMOs.

I would argue that Quadrant IV is the best strategic approach. The simultaneous focus on products and operations, as recommended throughout this study as being highly appropriate for biologics, will allow Quadrant IV companies to continually improve their concurrent engineering practices, which will let them bring products to market faster, a major advantage in the drug development industry. These companies will be the "partners of choice" for many biotechnology companies, owing to their all-around skills in the biologics business.

Further, the companies in Quadrant IV are following the same strategy for biologics that has worked so well for the top 5 biotechnology companies (Amgen, Genentech, Serono, Biogen IDEC and Genzyme). The dual focus on product and process development and improvement has served these companies very well. In fact, even biotechnology firms with revenues well below $1 billion per year (e.g. Abgenix, HGSI, ImClone, PDL) have realized the merits of this model, and are now trying to develop both product portfolios and operations skills, as was discussed in the previous section (e.g. see **Exhibit 15**). These are examples of companies that were initially product-focused, but are now moving from Quadrant II into Quadrant IV.

Similarly, B-I is an example of a biologics process-focused company moving from Quadrant III into Quadrant IV. B-I has long been a CMO of choice. They have used these skills in working with biotechnology companies (e.g. Genentech) and have been able to benefit from these relationships by obtaining co-marketing deals. Now, B-I is moving into early stage biologics R&D, with antibody discovery and development work with, for example, MorphoSys and Micromet AG. **Exhibit 23** overlays the product-process performance of the Top 20 pharma companies with the top biotechnology companies and some of the smaller companies, illustrating the transition that B-I and small biotech firms such as HGSI are trying to make from their current positions into the desirable Quadrant IV.

Exhibit 23: Transition to the Simultaneous Product-Process Focused Quadrant



Finally, which of the Quadrant IV companies (J&J, Roche, Wyeth and Abbott) is in the best position to succeed in the biologics business, based on operations excellence? Without diving into the specifics of each company's portfolio, there seems to be another dimension along which these company's operations differ, and that is the level of centralized control. As discussed in the earlier section on operations theory, the complexity of biologics operations and the need for product consistency across all locations, suggests that a centralized control structure should be more appropriate for biologics, than a network with a high degree of local autonomy. Wyeth and Abbott both appear to be using a much more centralized approach than J&J, which appears to act more as a holding company, leaving its two biologics subsidiaries, Centocor and Ortho Biotech, to function semi-independently. On the basis of this point, I will give the long-term biologics advantage to Wyeth and Abbott over J&J, at least in terms of developing operational excellence.

What about the final member of Quadrant IV, Roche? They appear to be hedging their bet, and have adopted a blended approach, owning a majority equity stake in Genentech, a fully-integrated biotechnology company, while also developing their own, internal, fully-integrated biologics franchise. With over 40% of the world's biologics production capacity, a focus on simultaneous product and process development, and an aim "to be a world leader in biopharmaceutical R&D, production and marketing" (2004 Annual Report), I like their odds.

I would like to conclude with a "dark horse" prediction of a company that I envision becoming a leader in biologics, on the basis of following the operations model outlined in this report, namely Novartis. What Novartis is doing that sets them apart from the any other Top 20 pharmaceutical company is that they are aggressively pursuing a fully-integrated biogenerics business through their subsidiary, Sandoz. The following quote was taken from the Novartis 2003 Form 20-F, and gives some background on the Sandoz push into biogenerics:

In developing our new Biopharmaceuticals Business, we are seeking to leverage our technology and expertise to develop, manufacture and market high-quality biopharmaceutical products, such as protein hormones and other human proteins, to be sold as substitutes for branded biopharmaceutical products after their patents have expired. Sandoz is also an important manufacturer of biopharmaceuticals for a number of third parties.

On the face of it, biogenerics might not seem to be a compelling business for a big pharma, since the margins are likely to be lower than what the companies are accustomed to for branded drugs. However, the advantage that a biogenerics business will bring to Novartis is an outstanding training grounds, or sandbox, for developing biologics operational excellence. In biogenerics, the ability to compete on cost will be a critical driver of success, which is not true for patented biologics. Further, if FDA approval of biogenerics follows the same rules as approval of small-molecule generics, the ability to get to market first will be a major driver of success too, since the first-to-market company will enjoy the 6-month competition-free period currently granted to the first manufacturer of an off-patent drug. In this **intensely** competitive environment, Sandoz will learn to produce biologics in the most efficient, low cost and rapid manner, or they will need to close their doors. The generics business, in the long-term, will be merciless to inefficient producers. This will be the ultimate training regimen for developing a powerful

biologics operation, and if Novartis is able to leverage this learning for their patented biologics business, they will become a powerful competitor in this market.

Acknowledgements

In addition to all of the people who made my PhD experience a success, I would also like to thank the business school professors who were instrumental in helping me think about the issues I have tried to address in this capstone paper. From MIT, I would like to thank Professor David Simchi-Levi and from the Harvard Business School, I would like to thank Professors Kent Bowen and Robert Huckman.

APPENDIX A: Manufacturing Alliances in the Biologics Business (Legend and Notes on the following pages)

BIG PHARMA CUSTOMER

	Pfizer	GSK	Merck	J&J	Aventis	Astra Zeneca	Novartis	BMS	Roche	Lilly	Wyeth	Abbott	Sanofi	Takeda	S-P	B-I	Bayer	Schering AG	Sankyo	Merck KgA	Third Parties
Pfizer																					
GSK																					
Merck																					
J&J										M (3)					M (1)						
Aventis																					
AstraZeneca																					
Novartis							S (2,12,31)														M (2)
BMS								L													
Roche									S, D (14)												
Lilly																					
Wyeth				A (6)							S,D (20,30)			M (20)							C (16)
Abbott												S,D (34)									C (4)
Sanofi-Synthelabo																					
Takeda																					
S-P															S (1)						
B-I	C (26)							C (27)			C (8)	C (21)		C (8)		S, D (22,32)		C (25, 26)		C (27)	C (8,21,25,26)
Bayer					C (5)												S (23)				C (5)
Schering AG																					
Sankyo																					
Merck KgA																					
Amgen				M (11)							M (19)										
Genentech							M (2)		S						C (32)						
Serono	M (9)																				
Biogen-IDEC												M (33)			M (33)			M (24)			
Genzyme												M (35)									
MedImmune												M (21)									
Abgenix	P (10)																				
Centocor				S						M (3)					M (1)						
Charles River Labs	M (28)									M (28)											
Corixa		M (29)																			
Chugai									S												
ImClone								D (27)												M (27)	
Lonza	C				C (36)			C (13,27)	C(17)	C (18)										C (27)	
Ortho Biotech				S																	
Unknown Third Parties																					

BIOTECHNOLOGY CUSTOMER

	Amgen	Genentech	Serono	Biogen-IDEC	Genzyme	MedImmune	Abgenix	HGSI	ImClone	Protein Design Labs	Third Parties
Amgen	S, D										M (11,19)
Genentech	C (15)	S, D		C							M (2)
Serono			S, D								M (9)
Biogen-IDEC				S							M (24)
Genzyme					S						
MedImmune						S (21)					M (21), C
Abgenix	P (7)						L, S				P (10)
HGSI								S			
ImClone									S (27)		
Protein Design Labs										S,D	
Novartis		M (2)									
Wyeth		C (16)									
Lonza		C (17)							C (27)		C (13,18)
BI	C (8)	C (32)				C (21)			C (27)		
Third Parties											

APPENDIX A: Manufacturing Alliances in the Biologics Business (Continued)

Legend:

A	Acquired manufacturing facility
C	Contract manufacturer
S	Internal commercial manufacturing
D	Manufacturing capabilty being developed
L	Limited internal manufacturing (pilot plants, clinical trial material)
M	Customer markets / promotes the product manufactured by the partner
P	Partner manufactures clinical trial material

Notes:

(1) Centocor (a J&J subsidiary) manufactures Remicade for S-P, but S-P has also built in-house capacity in Singapore

(2) Genentech currently manufactures Xolair, but Novartis is expected to take over full responsibility at the end of 2005 (Genentech 10-K)

(3) Centocor (a J&J subsidiary) manufactures ReoPro (MAb) for Lilly

(4) Abbott has performed contract mfg for several companies in the past (e.g. Serono, Novartis, Connetics, IBEX)

(5) Bayer manufactures generic Factor VIII (Helixate FS) for the EU market for Aventis Behring, the generics wing of Aventis

(6) Centocor (J&J subsidiary) acquired Wyeth's St. Louis biologics manufacturing facility in 2003

(7) Abgenix will manufacture clinical and early commercial supplies of panitumumab, with Amgen's support and assistance.

(8) For additional quantities of Enbrel

(9) Serono produces all Rebif

(10) Abgenix produces clinical materials for broad antibody collaboration

(11) Amgen produces all of J&J's US EPO (Procrit); J&J produces its own overseas EPO (Eprex)

(12) Via Sandoz, their generic subsidiary (Sandoz is very aggressively pushing into biogenerics)

(13) BMS has contracted with Lonza to produce commercial quantities of antibodies, should their two late stage compounds obtain FDA approval

(14) Roche and its subsidiaries, Genentech and Chugai own a 1/3 of the world's biologics manufacturing capacity

(15) Genentech manufactures some Enbrel for Amgen (based on a deal with Immunex)

(16) Wyeth produces some Herceptin for Genentech

(17) Lonza produces some Rituxan for Genentech

(18) Lonza produces all of Lilly's commercial Xigris

(19) Amgen produces the US Enbrel supply

(20) Developing Enbrel production capacity at their Ireland facility, with an expected online date in 2005

(21) MedImmune produces commercial quantities of Synagis, which it provides to Abbott for distribution, but also gets additional capacity from BI

(22) Significant, ongoing commitment to capacity, for internal products and contract manufacturing

(23) Manufacture Kogenate FS in Berkeley

(24) Biogen IDEC apparently produces all of the Zevalin distributed by Schering AG

(25) Campath production is outsourced to B-I

(26) These companies are known to use B-I's CMO services

(27) Lonza currently supplies commercial quantities of Erbitux for BMS and Merck KgA distribution. However, ImClone is developing significant in-house manufacturing. Also. B-I is listed as having played a role in manufacturing (www.biopharma.com), although there is no record of this in recent BMS or ImClone 10-K forms.

(28) Arcitumomab, a radiolabeled MAb for diagnostics; the MAb is manufactured by Charles River Labs; Pfizer owns the rights and Lilly has European mkt rights.

(29) Bexxar (tositumomab), a MAb used for diagnostic and treatment purposes of cancers

(30) Wyeth produces its own ReFacto (Factor VIII) at one of the USA's largest facilities, in Andover, MA; also produces bmp-2 glycoprotein, mylotarg (MAb), BeneFIX,

(31) Manufacture Simulect (organ transplant Ab) in-house

(32) Genentech manufactures the USA market needs of Activase and TNKase, which are co-marketed with S-P; B-I handles EU manufacturing and marketing

(33) Biogen IDEC produces Avonex for Abbott and S-P.

(34) In-house Humira production

(35) Genzyme produces the thyrotropin co-marketed by Abbott.

(36) Lonza is manufacturing Aventis and TKT developmental EPO (human cell line based).

APPENDIX B: Big Pharma Marketed Biologics Products

Company	Marketed Products	Blockbusters[1]
Pfizer	Rebif, Arcitumomab	-
GlaxoSmithKline (GSK)	Bexxar	-
Sanofi-Aventis	Generic Factor VIII (Helixate FS)	-
Merck	-	-
Johnson & Johnson (J&J)	Eprex, Procrit, Remicade, Reopro, Orthoclone	Eprex, Procrit, Remicade
AstraZeneca	-	-
Novartis	Xolair, Simulect	-
Bristol-Myers Squibb (BMS)	Erbitux	-
Roche	Activase, Avastin, DNase, Herceptin, Neorecormon, Pulmozyme, Rituxan, Tarceva, TNKase, Zenapax	Herceptin, Neorecormon (EPO)
Lilly	Arcitumomab, ReoPro, Xigris	-
Wyeth	Benefix, BMP-2, Enbrel, Mylotarg, ReFacto	-
Abbott	Avonex, Humira, Synagis, Thyrotropin	-
Takeda	Enbrel	-
Schering-Plough (S-P)	Activase, Remicade, TNKase	-
Boehringer-Ingelheim (B-I)	Actilyse, Beromun, Imukin, Metalyse	-
Bayer	Kogenate FS	-
Schering AG	Campath, Zevalin	-
Sankyo	-	-
Merck KgA	Erbitux	-

[1]Big pharma company share of the 2003 revenues was in excess of $1 billion

APPENDIX C: Collaborations in Biologics (Legend and notes are on following pages)

	Pfizer	GSK	Merck	J&J	Aventis	Astra Zeneca	Novartis	BMS	Roche	Lilly
Pfizer										M (46)
GSK										
Merck										
J&J										M (1)
Aventis										
AstraZeneca										
Novartis										
BMS										
Roche										
Lilly	M (46)			M (1)						
Wyeth									X,M (44)	
Abbott										
Sanofi-Synthelabo										
Takeda										
S-P				M (1)						
B-I										
Bayer										
Schering AG										
Sankyo										
Merck KgA								M (14)		
Amgen				L (7)					V (18)	
Genentech				L (3)			E,M,X (13,16)		E, L, M (15)	
Serono	M (5)									
Biogen-IDEC									X (19)	
Genzyme										
Chiron							B (4)			
MedImmune										
Millennium										
Intermune										
ImClone								M (14)		
Lonza				L (37)			L (37)		L (37)	L (37)
Abgenix	B (6)					B (12)				
Applied Molecular Evolution										A (20)
Berlex Labs										
CAT						D (12)				
Centocor				A (8)						M (1)
Chugai									E (17)	
Diversa			D (12)							
EMD Pharmaceuticals										
Fujisawa				M (10)						
Genetics Institute									X,M (44)	
HGSI										
ILEX Oncology										
Medarex										
Micromet AG										
MorphoSys								D (22)	D (22)	
Knoll Pharmaceuticals										
Ortho Biotech				A (9)						
Protein Design Labs									L (39)	
Tanabe Seiyaku				M (11)						
Tanox							X (13)			
Transkaryotic Therapies (TKT)					X (43)					
Xoma									X (21)	

Appendix C: Collaborations in Biologics (Continued)

	Wyeth	Abbott	Sanofi-Synthelabo	Takeda	S-P	B-I	Bayer	Schering AG	Sankyo	Merck KgA
Pfizer										
GSK										
Merck										
J&J					M (1)					
Aventis										
AstraZeneca										
Novartis										
BMS										M (14)
Roche	X,M (44)									
Lilly										
Wyeth				M (23)						
Abbott										
Sanofi-Synthelabo										
Takeda	M (23)									
S-P										
B-I										
Bayer										
Schering AG										
Sankyo										
Merck KgA										
Amgen	M (23)			M (23)						
Genentech		L (24)				L,X,M (31)	L (33)			
Serono										
Biogen-IDEC		M (40)			M (41)			D,X (34)		
Genzyme		M (42)						X,M (2)		
Chiron										
MedImmune		M (27)								
Millennium										
Intermune										
ImClone										M (14)
Lonza	L (37)	L (37)			L (37)			L (37)		
Abgenix										
Applied Molecular Evolution										
Berlex Labs								A (36)		
CAT		D (26)								
Centocor					M (1)					
Chugai										
Diversa										
EMD Pharmaceuticals										A (45)
Fujisawa										
Genetics Institute	A (38)									
HGSI				D (28)	D,L (29)					
ILEX Oncology								X,M (2)		
Medarex								D (35)		
Micromet AG						X (32)				
MorphoSys						D, X (30)	D (22)	D (22)		
Knoll Pharmaceuticals		A (25)								
Ortho Biotech										
Protein Design Labs										
Tanabe Seiyaku										
Tanox										
Transkaryotic Therapies (TKT)										
Xoma										

Appendix C: Collaborations in Biologics (Continued)

Legend:

A	Acquisition
B	Broad alliance
D	Discovery collaboration
E	Majority equity stake
L	Licensing agreement
M	Co-marketing agreement
X	Development collaboration
P	A partnership or collaboration of an unknown nature
V	Divestiture

Notes:

(1) Lilly has ex-Japan marketing rights to ReoPro (Fujisawa has the Japanese rights), per alliance with Centocor
(2) WW marketing rights of Campath. Alliance due to the 12/2004 Genzyme acquisition of ILEX Oncology
(3) Licensing rights to Remicade via Genentech agreement with Celltech
(4) Described as cooperating in R&D, mfg and marketing on an arm's-length basis
(5) Pfizer has US marketing rights to Rebif
(6) Broad antibody discovery / development agreement; at least one product (CP-675) in the clinic
(7) Amgen licensed US non-dialysis rights and all worldwide rights for EPO to J&J
(8) Acquired in 1999
(9) Acquired in 1990
(10) Marketing rights to ReoPro in Japan
(11) Distribution rights to Remicade in Japan and co-exclusive rights in parts of the Far East
(12) Broad antibody discovery / development programs
(13) Novartis, Genentech and Tanox are developing Xolair; Novartis and Genentech will co-market
(14) Erbitux co-marketing agreement
(15) Roche hold an approximate 60% equity stake in Genentech; Roche has also licensed at least 5 Genentech products (Rituxan, Herceptin, Pulmozyme, Activase, TNKase), where Roche holds ex-US marketing rights
(16) Novartis holds a 33% equity stake in Roche, and consequently, holds a 20% stake in Genentech
(17) Roche holds a majority stake in Chugai
(18) Roche divested its European Neupogen® and Granulokine® business to Amgen in 2002
(19) Roche, Genentech and Biogen IDEC were co-developing MABTHERA (Rituxan)
(20) 2004 acquisition of Applied Molecular Evolution
(21) Genentech developed Raptiva with Xoma
(22) Morphosys has an antibody development platform
(23) Co-markets Enbrel with Amgen (US) and Takeda (Japan)
(24) Abbott received a worldwide license to Humira
(25) 2001 acquisition of Knoll provides Abbott with Humira rights
(26) Very strong discovery and development collaboration with CAT (4 MAb in Phase III and 2 MAb in Phase II); CAT played a role in the development of Humira (biopharma.com)
(27) A co-marketing / ex-US distribution agreement for Synagis
(28) Per license with Human Genome Sciences, Takeda has selected ~100 novel gene and protein targets of interest for its small molecule and antibody development programs
(29) S-P opted to license an interferon from HGSI and is responsible for clinical dev. and commercialization.
(30) Exercised an option in 2003 to begin developing a MorphoSys antibody
(31) Have developed and marketed several drugs with Genentech (e.g. Actilyse, Metalyse, Imukin, Beromun), with BI typically holding the EU manufacturing and marketing rights
(32) BI to collaborate on process development and manufacturing of Micromet's anti-cancer antibody MT201
(33) Bayer has licensed worldwide rights to Kogenate FS (Factor VIII) from Genentech
(34) Schering AG has ex-US marketing rights to Zevalin
(35) Schering AG has access to Medarex's fully human monoclonal antibody technology
(36) Berlex Labs is a wholly-owned subsidiary of Schering AG and handles the US biologics distribution.
(37) Lonza Glutamine Synthesase expression system is licensed for at least one biologics production operations
(38) The Genetics Institute (GI) was acquired by American Home Products (AHP) in 1996
In 2002, AHP changed its name to Wyeth. GI is now part of Wyeth BioPharma
(39) Roche has licensed non-orgna transplant Zenapax rights to PDL, as of September 2003.
(40) Abbott has the Latin American marketing rights to Avonex.
(41) S-P has the European marketing rights to Avonex.
(42) Abbott has co-USA marketing rights to Thyrotrophin
(43) Trying to develop and market an EPO produced by human cells; rejected in US; approved in Europe, but not marketed, due to IP issues.
(44) Roche manufactures and sells Neorecormon (EPO) in non-US markets, based on a Genetics Institute patent
(45) Merck KgA has acquired or created EMD Pharma (Boston, Raleigh), which is focused on biologics.
(46) Lilly (Europe) and Pfizer co-market Arcitumomab

APPENDIX D: Commitment to Concurrent Product/Process Development

General: These comments are meant to show a company's commitment to integrating product development and operations (process development). As emphasized in this paper, such a commitment should improve overall product development, time to market and ramp-up. The company statements are given in quotation marks and the author's comments are shown in bold, preceding the company quotes.

Co.	Testimonials / Observations / Author's Commentary	Source
Abbott	**Cell line process development in conjunction with product development:** "Founded in 1989, the *Abbott Bioresearch Center* in Worcester, Massachusetts, is a world-class discovery and basic research facility committed to finding new treatments for autoimmune diseases. *Abbott Bioresearch Center* employs leading-edge technologies, discovery and manufacturing processes, including proprietary phage antibody display technology, and mammalian cell expression systems to produce human monoclonal antibodies." **They offer a fairly comprehensive slate of product-process development expertise:** "Abbott offers partners an unparalleled set of capabilities to optimize the value of antibody targets. These include ABC's unique technology platform comprising both proprietary and licensed–technologies, a ten–year track record pioneering the discovery and development of fully human antibody therapeutics, and a state–of–the–art facility for cGMP manufacture of protein therapeutics. The focus of ABC's advanced technology platform is on immunology, oncology, and transplant rejection as well as supporting antibody projects across the Abbott discovery effort. In 2001, Abbott named ABC its Center of Excellence in Immunoscience Discovery."	Company press release Company web site
B-I	**Very detailed commentary on the role of operations in optimizing product development work – very much in line with the recommended operations strategy presented in this paper:** "Boehringer Ingelheim's goal is to complete product development within seven years, ensuring R&D's internal customers products of the highest quality for minimum cost with maximized supply security, according to Dr Joachim Wenzel, Head of the spiriva® Launch Department. Managing the interface between R&D and Operations is a specific challenge during development: the main burden lies with the development team (the core team) and various subteams. Minimizing "time to market" is the target, but a production process should provide the balance between "minimum cost, necessary quality and sufficient supply security". Operations normally had to live with manufacturing processes for the life of products, as reformulation is expensive, and a change in registration often hard. Today, Boehringer Ingelheim's strategy is to fully integrate Operations into the development process. The interface between Operations and R&D is not a "one-off" handover, where R&D simply halts its activities, with Operations taking over from one day to the next. Today, the interface is a smooth transition, where production increasingly takes over responsibility. In the early phases of development, Operations' input is also extremely important, as here decisions are made that determine product costs. If the final formulation has been fixed, every additional test of alternate exipients or material for packaging has a direct impact on the development timeline. The seven-year target will not be met, if Operations later wants to use a less costly or more easily available compound. A major task for Operations representatives in development teams is to make sure that all cost avoidance measures are built into a product. The R&D subteam, which includes Operations, must also make sure, that the appropriate quality can be produced, when scaled up. Process	2004 Annual Report

	robustness needs to be evaluated at this phase: what impact does varying compressing speed have on the tablet, can the shipping carton for the capsules be exposed to sunlight, or how long can an intermediate be stored. Basic decisions on supply security are also made in a very early state of development, when the final formulation is being developed. A most important question is the long-term availability of components used for product development, packaging, or device development. In this development phase, Operations must ensure that long-term supply security is built into the product. After the Production Reallocation Projects, the research sites lost their production capabilities on site. Managing the interface between R&D and Production is therefore also a question of managing communication and cultural differences. Personal contact, and even more so, common manufacture of batches and analysis are essential for transfer processes. In future, due to the higher complexity of new chemical substances, more non-standard technologies will potentially be required to bring compounds to market, requiring even closer interaction between R&D and Operations. Moreover, after seven years of development a product has not necessarily achieved 100 per cent maturity for ordinary production. Both aspects are much easier to take care of, if product launches are always made from the same plant. A launch site concept could further optimize the interface between R&D and Operations by easing communication, resulting in the closest cooperation to overcome technical hurdles. There is a fine balance which Operations must seek to establish in the production process from the earliest stage of product development."	
Roche	**A commitment to the entire biologics value chain, with a current emphasis on manufacturing capacity:** "Roche, together with Genentech and Chugai, is a world leader in biotechnology. And this is an area where we intend to become even stronger, both in research and development and in production. Over the next several years, for example, we will invest some 2 billion Swiss francs in expanding the Group's biotech production capacity in Basel (Switzerland), Penzberg (Germany),Vacaville (USA) and Utsunomiya (Japan)."	2004 Annual Report
Wyeth	**A very strong commitment to integrating product development with operations:** "Integrating process development and manufacturing technologies is essential to maintaining leadership in the biopharmaceutical industry. Our primary objectives are: o To continue to effectively integrate development, manufacturing, quality assurance, and regulatory affairs and create a center of excellence for recombinant, protein-based development and manufacturing o To focus on biopharmaceutical activities such as integrated planning and decision-making, yield management, product innovation, and speed to market o To utilize process development in a pivotal role in supply management in order to increase productivity Wyeth BioPharma brings together the state-of-the-art biopharmaceutical science, technology, and production expertise necessary to produce recombinant protein biopharmaceuticals. The organization is built upon a foundation of people, facilities, technologies, and products that have contributed to the integrated organization. With current expansion in the United States and Ireland, Wyeth BioPharma is positioned to propel Wyeth's exciting biopharmaceutical pipeline and portfolio to industry leadership. To establish itself as a major player in the industry, Wyeth BioPharma is drawing on the total corporate strength of Wyeth and leveraging its own substantial pioneering spirit, unique skills, and biopharmaceutical expertise. The vision of Wyeth BioPharma is clear - to integrate innovative development with unparalleled excellence in manufacturing, quality, and commercialization, and thereby deliver first-in-class biopharmaceutical products that meet global clinical and market demand."	Company web site

APPENDIX E: Big Pharma Biologics Product and Process Development Scores

Company	Product Development[1]					Process Development[1]				
	P1	P2	P3	P4	P5	O1	O2	O3	O4	O5
Pfizer	0	1	1	2	0	0	0	0	0	1
GlaxoSmithKline (GSK)	2	1	1	0	0	0	0	0	0	1
Sanofi-Aventis	0	0	1	1	1	0	0	0	0	1
Merck	0	0	0	1	0	0	0	0	0	0
Johnson & Johnson (J&J)	2	2	2	2	1	2	1	1	1	2
AstraZeneca	0	0	0	1	0	0	0	0	0	0
Novartis	0	0	1	1	2	2	1	1	1	2
Bristol-Myers Squibb (BMS)	1	1	1	1	1	0	0	0	1	1
Roche	2	2	2	2	1	2	2	0	2	2
Lilly	2	0	1	2	1	0	0	0	0	0
Wyeth	2	2	1	2	1	2	2	1	2	2
Abbott	2	2	1	2	1	2	2	2	2	2
Takeda	1	0	1	2	1	0	0	0	0	1
Schering-Plough (S-P)	2	0	1	2	1	1	1	0	1	1
Boehringer-Ingelheim (B-I)	0	1	1	2	0	2	2	2	2	1
Bayer	0	0	1	1	1	2	1	1	1	2
Schering AG	1	0	1	2	0	0	0	0	0	2
Sankyo	0	0	0	1	0	0	0	0	0	1
Merck KgA	1	1	1	1	1	0	0	0	0	1

[1]**Category Index:**

Product Assessment Categories:

P1 = In-house biologics R&D

P2 = Biologics pipeline

P3 = Marketed biologics

P4 = Biologics collaborations

P5 = Post-launch or post-patent activities

Process Assessment Categories:

O1 = In-house manufacturing

O2 = Manufacturing capacity

O3 = Process contractor

O4 = Concurrent product / process engineering

O5 = Distribution network

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