The Effect of Culture Temperature on Recombinant IFN-γ Production and Quality

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ABSTRACT-The goal of this research project is to analyze the effect of culture temperature on the production and quality of IFN-γ produced and secreted by suspension culture CHO cells.The effect of low temperature on IFN-γ glycosylation, which is under the control of a battery of enzymes whose activities will be influenced by temperature, is unknown. Work is focused on implementing a system for accurately monitoring the glycosylation of IFN-γ and then using the system for quantifying the effect of culture temperature on glycosylation. The system consists of immunoaffinity purification of IFN-γ, followed by capillary electrophoresis for determining glycosylation macroheterogeneity and MALDI-TOF MS and HPLC for determining glycosylation microheterogeneity. Initial results suggest that glycosylation macroheterogeneity is slightly decreased (~5%) at low temperature, thereby identifying a potential quality "bottleneck" for the use of low temperature to increase IFN-γ production.

Low temperature (32^{o}C) shifts the cells towards the non-growth, G1 portion of the cell cycle. In batch culture, if cells are shifted to low temperature once a reasonably high cell density is reached, an approximately 4-fold improvement in total IFN-γ production compared to 37^{o}C culture is achieved. Pseudo-continuous culture was used to show that IFN-γ production is statistically significantly higher at 32^{o}C compared to 37^{o}C even when nutrient depletion is not a concern ($p < 0.5$). In fed-batch bioreactor culture, cells grown at low temperature display a short period of growth followed by a prolonged stationary phase of high specific IFN-γ productivity (~4-fold higher than compared to 37^{o}C) whereas cells at 37^{o}C grow rapidly, reach a peak cell density and then begin to die immediately. The net result is a 2-fold increase in total IFN-γ production at low

temperature. Real-time RT-PCR was used to show that the amount of IFN-γ mRNA present during the 32°C stationary production phase is approximately 4-fold higher than the amount present during the exponential growth phase of the 37°C culture.

To further explore the effect of low temperature on cell RNA levels, total RNA per cell was quantified during the course of batch cultures at 32°C and 37°C. Total RNA levels were found to be approximately 50% higher at 32°C than 37°C. The kinetics of the low temperature RNA concentration profile was modeled to obtain transcription (Ks) and degradation (Kd) rate constants and these were found to be consistent with literature values. This finding suggests that temperature shift may offer a novel approach for measuring RNA kinetic parameters in any cell system that can tolerate mild temperature changes.

Index Terms – Animal Cell Cultivation, Low Temperature Growth, mRNA Stability, Protein Quality

I. INTRODUCTION

IFN-γ contains two potential N-linked glycosylation sites at Asparagine 25 (Asn^{25}) and Asn^{97}. IFN-γ exhibits variable site occupancy (macroheterogeneity), with 0, 1 or 2 sites occupied. IFN-γ also exhibits variable oligosaccharide structures at the occupied sites (microheterogeneity). For therapeutic purposes, the desirable outcome is to have sites occupied and maximally sialylated. The scheme being implemented for measuring IFN-γ glycosylation was adapted from Gu (Gu, 1997) and is shown in Figure 1. To date, the immunoaffinity purification, capillary electrophoresis, trypsin digest and first RP-HPLC separation have been established. Currently, MALDI-TOF MS is being optimized for identifying glycopeptides amongst the peptide fractions. We have been able to identify several Asn^{25} and Asn^{97} glycopeptides in separate HPLC fractions, as expected from Gu's work (Gu, 1997)

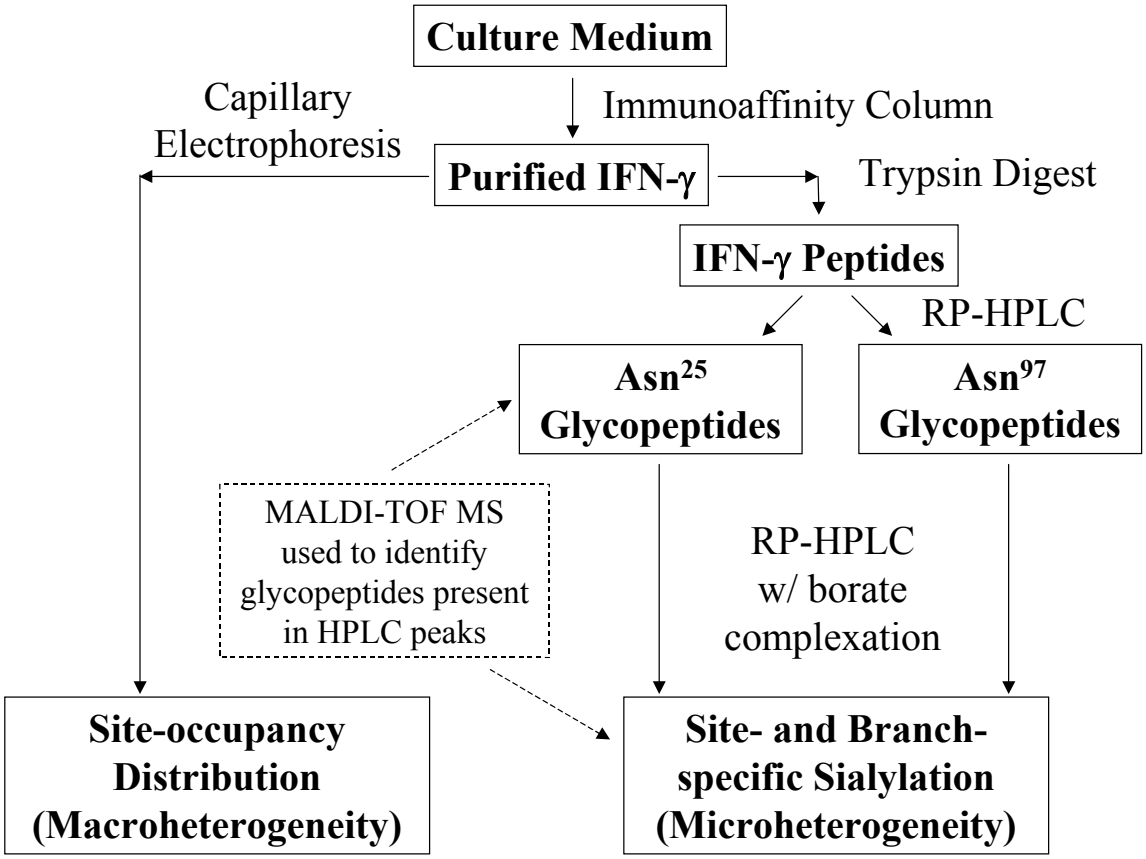

Figure 1: IFN-γ Glycosylation Analysis Scheme

II. MATERIALS AND METHOD AND RESULTS AND DISCUSSION

The remaining work is to identify additional glycopeptides from the initial HPLC step. Then, the site-specific glycopeptides will be separated using HPLC again, the glycopeptides will be identified using MALDI-TOF MS and the site- and branch-specific sialylation can be quantified. Then, the system will be ready for quantifying glycosylation differences between IFN-γ produced at 37°C and 32°C. Initial results suggest that IFN-γ site-occupancy is reduced by about 5% at 32°C compared to 37°C, as shown in Figure 2. The figure presents site-occupancy results for two samples from 37°C and 32°C fed-batch cultures. These results must be confirmed, but they would be consistent with reduced glycosylation enzyme activity at the lower temperature and would identify a potential glycosylation "quality bottleneck" for low temperature production.

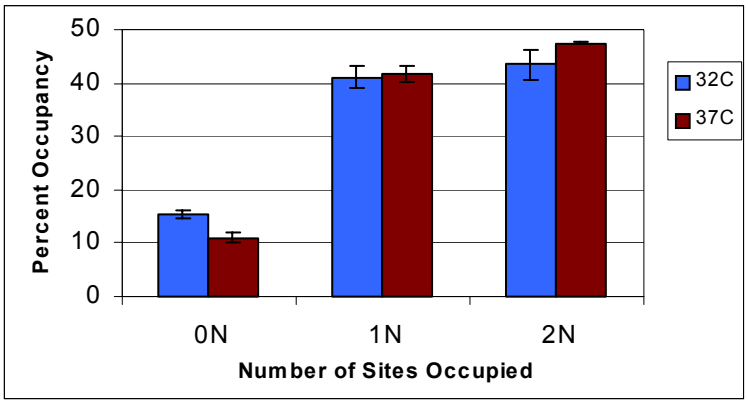

Figure 2: IFN-γ Site-Occupancy at Different Culture Temperatures (n=2)

A. BATCH CULTURES

The batch culture results presented during the previous reporting period have been repeated and expanded upon to include additional temperature shift profiles. Two seeding densities were tested, namely 2.5 x 10^5 cells/mL and 2 x 10^6 cells/mL. For both cases, cultures were conducted in duplicate. The temperature strategies tested were as follows:

- Constant temperatures: 32°C, 37°C and 40°C
- Temperature Shift #1: Cells shifted during exponential growth (TS1)
- Temperature Shift #2: Cells shifted once peak density reached (TS2)
- Temperature Cycling: Cells grown 8 hours at 37°C and 16 hours at 32°C (C)

After one day of culture, flow cytometry was used to verify that low temperature shifts cells towards the G1 portion of the cell cycle as shown in Figure 3.

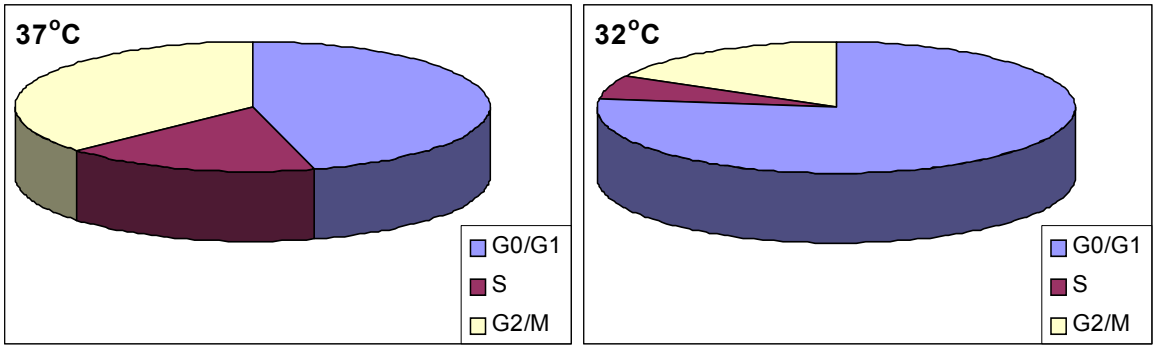

Figure 3: Cell Cycle Distribution One Day Post-Seeding for Low Density Batch Culture

The cultures were ended once cell viability dropped below 50% and the total IFN-γ production was measured (Figure 4)

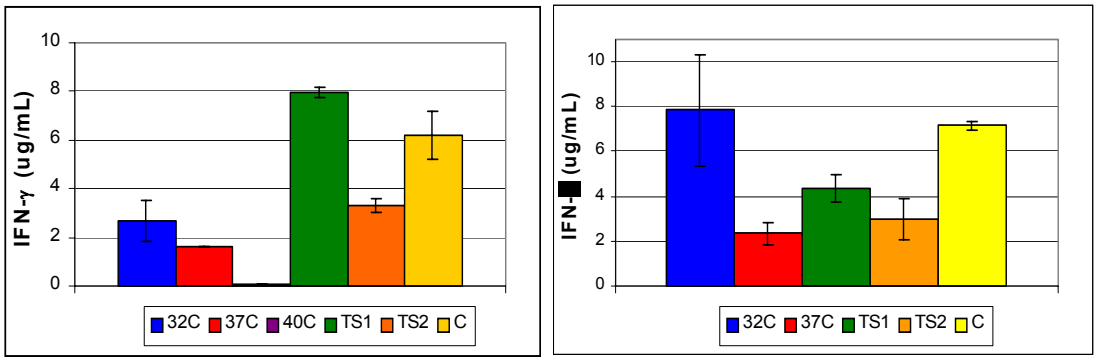

Figure 4: Total IFN-γ Production in (a) Low Density and (b) High Density Batch Culture

One observation is that growing cells at elevated temperature (40°C) results in practically no IFN-γ production. The remaining data is consistent with what was presented during the last reporting period, namely that to maximize production, both specific productivity and integrated viable cell density (IVCD) should be maximized. In batch culture, this is accomplished by growing cells to a relatively high density (or seeding at a high cell density) to achieve a high IVCD and then shifting to low temperature, where specific productivity is highest. Thus, temperature shift strategy works well for low density seeding and constant 32°C culture works well for high density seeding.

B. PSEUDO-CONTINUOUS CULTURES

Pseudo-continuous cultures were used to verify whether low temperature and temperature cycling gave statistically significantly higher IFN-γ production than 37°C culture. In batch culture, nutrient depletion occurs and in batch and fed-batch culture, metabolic byproducts will accumulate to levels that may be harmful to the cells. To remove these detrimental effects from an analysis of IFN-γ production, pseudo-continuous cultures were used. Cultures were seeded at 1 x 10^6 cells/mL. Every 24 hours, the cells were counted, fluid was removed from the culture and concentrated and normal feed media was added. The amounts added and removed were calculated to return the cell concentration to 10^6 cells/mL, return the glucose and glutamine concentrations to 20mM and 4mM, respectively, and return the culture volume to 25mL (in 125-mL shaker flasks). Four different temperature strategies were tested, namely 37°C continuously, 32°C continuously, a cycling strategy consisting of 6 hours at 32°C and 18 hours at 37°C (Cycle 1), and a cycling strategy consisting of 12 hours at 32°C and 12 hours at 37°C (Cycle 2). Each condition was conducted in triplicate and the experiment was conducted for 7 days.

As expected, it was found that cell growth rates decreased with increasing time spent at low temperature, as shown in Figure 5a. In addition, whereas the 37°C growth rate remained relatively high over the course of the seven-day experiment, the 32°C growth rate decreased to practically zero by day 4 of the experiment, demonstrating that true growth arrest takes place (Figure 5b).

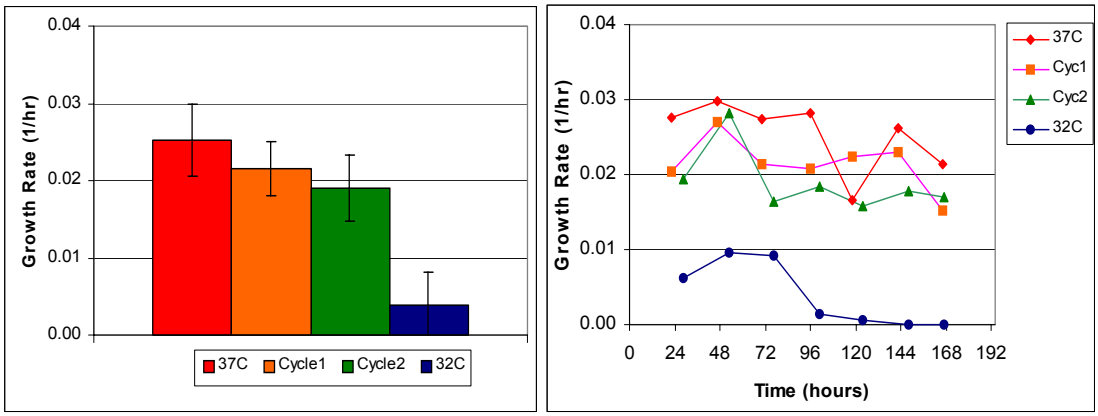

Figure 5: (a) Seven-Day Average Growth Rate and (b) Daily Average Growth Rate

After allowing 48 hours for the cultures to adjust to the new conditions, the total IFN-γ production was measured over the course of the remaining five days. The results for *total* IFN-γ production are presented in Figure 6. It appears that IFN-γ production increases as time spent at low temperature increases. However, only the 32°C culture produces IFN-γ at statistically significantly higher levels than the 37°C culture ($p < 0.05$ using t-test), with a 60% increase in total production over the course of 5 days. These results show that even when nutrient depletion is not a concern, low temperature results in higher IFN-γ production than standard 37°C culture. The results also show that temperature cycling does not enhance production above what is seen for 32°C alone. Thus, temperature cycling will no longer be investigated as a new means for enhancing IFN-γ production.

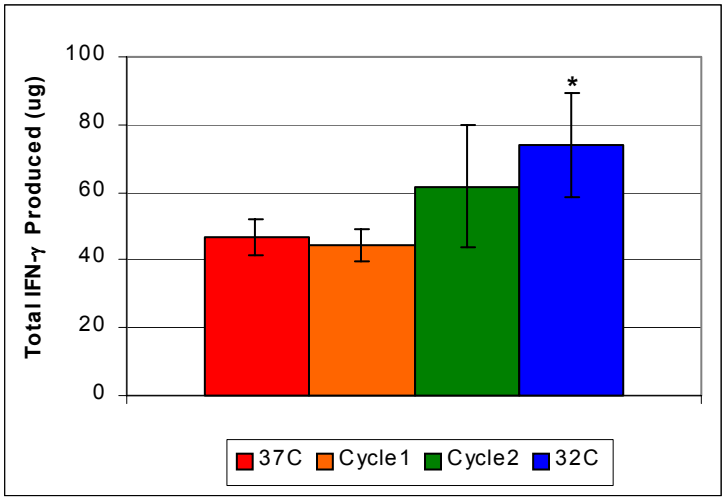

Figure 6: Total IFN-γ Produced During Day 2-7 of Pseudo-continuous Culture

C. FED-BATCH BIOREACTOR CULTURES

During the previous reporting period, it was reported that low temperature did not appear to give beneficial results for fed-batch cultures. Based on the results from the pseudo-continuous cultures showing the benefits of low temperature production, the fed-batch work was revisited using bioreactors. The advantage of the bioreactor is that the

temperature, pH, and dissolved oxygen can be accurately controlled, which is not the case for shaker flasks. Two bioreactor runs, using 37°C and a 32°C, have been conducted. The runs were terminated once cell viability dropped below 90%. The growth curves from the two runs are shown in Figure 7.

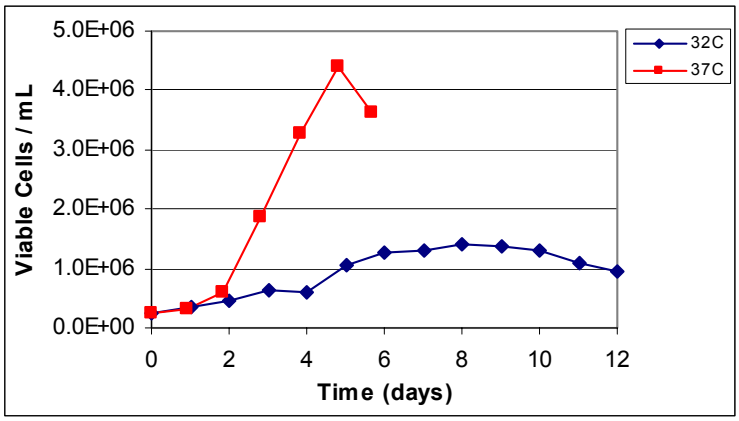

Figure 7: Fed-Batch Bioreactor Growth Curves

The 37°C cultures grew very rapidly, reached a high cell density and then began to die. In contrast, the low temperature culture grew slowly and reached a stationary phase that was maintained for about 5 days. IFN-γ production data for the two runs is given in Figure 8.

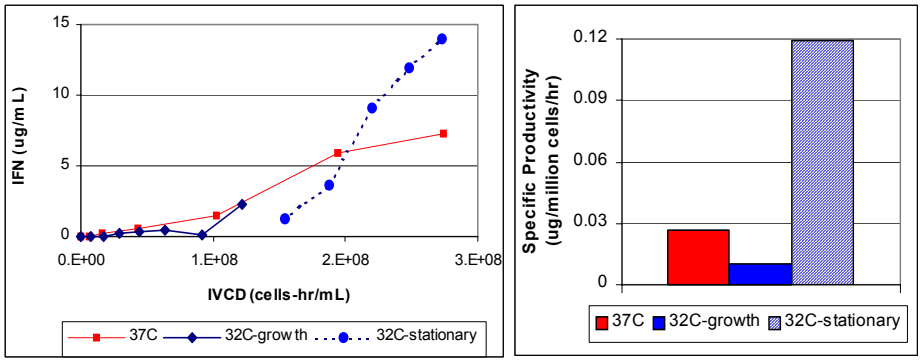

Figure 8: Fed-batch Bioreactor (a) Total Production and (b) Specific Production

It appears that IFN-γ specific production is a relatively constant value of approximately 0.03ug/million cells/hr at 37°C, as demonstrated by the approximately linear increase versus IVCD (Figure 8a). In contrast, the 32°C culture appears to consist of biphasic production, in that the specific production is low during the growth phase (0.01ug/million cells/hr; approximately 1/3 of the value at 37°C) but high during the stationary phase (0.12ug/million cells/hr; approximately 4 times the value at 37°C). The net result is that total IFN-γ production is doubled for the low temperature culture. The molecular mechanisms behind the increase in production are discussed next.

D. RNA ANALYSIS

1) Real Time RT-PCR for IFN-γ mRNA Quantification

The results from the pseudo-continuous and the fed-batch bioreactor cultures show that specific productivity of IFN-γ is increased at low temperature. One possible mechanism for the increase is that the amount of IFN-γ mRNA per cell is increased at low temperature.

In order to accurately quantify IFN-γ mRNA, a real-time, reverse transcriptase (RT) PCR protocol has been developed. Real-time PCR allows one to monitor the progress of the PCR following each cycle. The PCR mix includes the dye SYBR Green, which fluoresces when bound to double stranded DNA. The level of fluorescence is directly proportional to the concentration of double stranded DNA, which is the product of PCR. Based on the cycle number at which a particular sample crosses a threshold fluorescence value, termed the threshold cycle (C_t), the concentration of the mRNA of interest can be determined by comparison with a standard curve, which is generated by running simultaneous reactions on mRNA standards of known concentrations. An IFN-γ standard curve generated during one of the RT-PCR runs is presented in Figure 9.

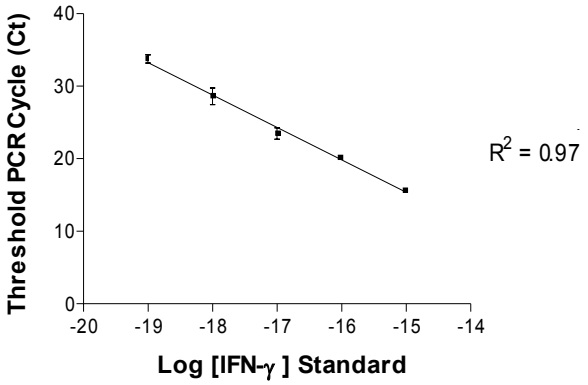

Figure 9: Real Time RT-PCR IFN-γ Standard Curve

In addition, one must have an approach for normalizing the results. Typically, RT-PCR data is normalized versus a housekeeping gene, usually GAPDH or β-actin. Other methods include normalizing versus one of the abundant ribosomal RNA molecules or versus total RNA.

Real time RT-PCR has been used to investigate the mechanism of increased IFN-γ observed in the fed-batch bioreactors. The samples chosen were day 10 of the 32°C and day 3 of the 37°C fed-batch bioreactor runs (please see Figure 7). The 32°C sample was chosen to correspond with the middle of the high productivity stationary phase and the 37°C sample was chosen to correspond with the middle of the exponential growth phase. As discussed previously, the specific productivity during the 32°C stationary phase was four times higher than that during 37°C culture, which did not exhibit a stationary phase. The RT-PCR result for one of the runs on these two samples is given in Figure 10. The GAPDH housekeeping gene was tested simultaneously with the IFN-γ gene. Following normalization of the data from two RT-PCR runs, the 32°C contains 4±0.2 times as much IFN-γ mRNA as the 37°C sample. This result is consistent with the 4-fold increase in

specific IFN-γ protein production seen between the samples. Thus, the initial RT-PCR results suggest that the increase in IFN-γ production at low temperature is a result of an increased level of IFN-γ mRNA.

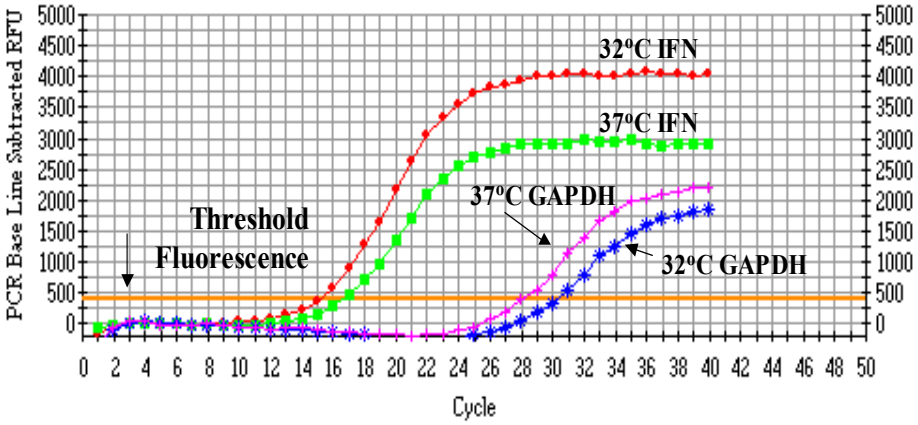

Figure 10: RT-PCR Results in Real Time for Fed-Batch Bioreactor Cells

2) Analysis of Total RNA Kinetics

The issue of normalizing the RT-PCR results warrants further discussion and investigation. There are several instances in which the "housekeeping" genes copy number per cell can vary significantly (Bustin, 2000) and a temperature change might be one such instance. Another option for normalizing the IFN-γ mRNA levels between samples would be by using a "per cell" or "per total RNA" basis. However, the effect of temperature on total RNA per cell is not known. To investigate the implications of using different normalization approaches, a batch experiment was conducted to determine whether *total* RNA levels *per cell* vary during the course of culture or vary at different culture temperatures. Cultures were seeded at $5x10^5$ cells/mL and grown at either 32°C or 37°C. Each condition was run in triplicate. Every 24 hours, one million cells were collected from each flask and the total RNA was isolated and quantified using spectrophotometry. The cultures were terminated once cell viability dropped below 90%. The growth and viability curves are presented in Figure 11 and the total RNA profiles are presented in Figure 12.

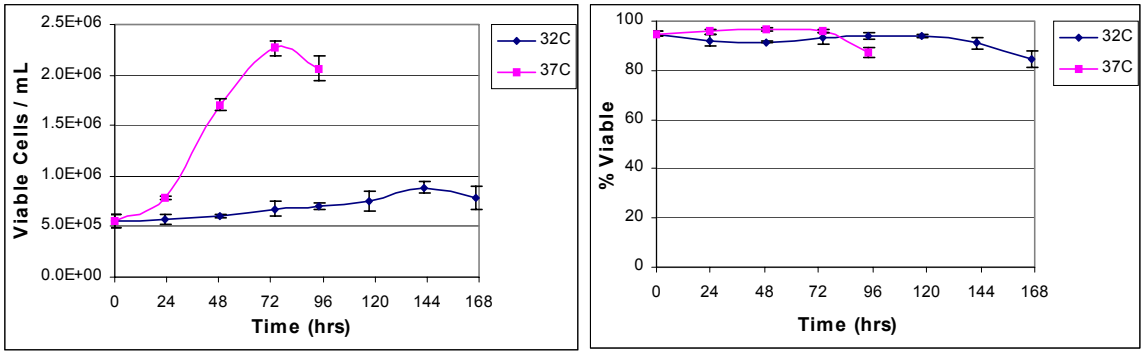

Figure 11: Batch Culture (a) Growth and (b) Viability Curves

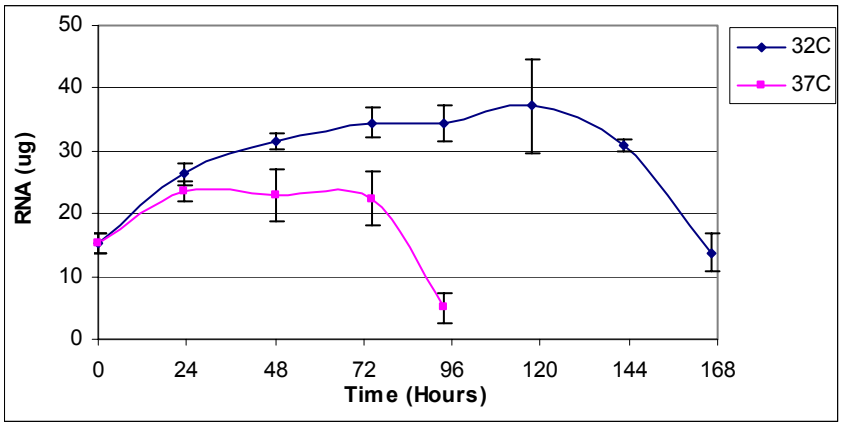

Figure 12: Total RNA Isolated from One Million Cells During Batch Culture

Several interesting results were observed from the total RNA data. First, as indicated by the relatively small error bars for most points, RNA isolation per cell is quite reproducible, which is a positive finding given the number of steps required in RNA isolation and quantification. Second, it is observed that total RNA content per cell is higher at low temperature, reaching a level about 50% higher than the peak level for 37°C cells. Third, the total RNA content per cell plummets at the onset of cell death! The implications of these three observations are discussed next.

The fact that RNA isolation per cell is reproducible suggests that normalizing RNA data on a per cell basis is possible. Thus, copy number of IFN-γ on a per cell basis can be compared at different temperatures and used for comparing against the results obtained by normalizing versus a housekeeping gene.

The observation that total RNA per cell is higher at low temperatures has several implications. First, it shows that normalizing IFN-γ mRNA on a "per total RNA" basis will significantly skew the data on a per cell basis. Second, there may be a generic mechanism that increases the amount of all the RNA in the cell at low temperature, meaning that the copy number of most RNA molecules on a per cell basis increases. Third, the fact that greater than 80% of total RNA present in a cell is ribosomal RNA (rRNA) (Lodish *et al*, 2000) suggests that rRNA levels are significantly elevated at low temperature. Since rRNA is associated with ribosomes, the level of ribosomes, and thus the cell's ability to produce protein, is potentially increased at low temperature, too.

To examine the molecular mechanism leading to increased total RNA levels at low temperature, the RNA concentration temporal profile was modeled. The following RNA mass balance equation was applied (adapted from Bibila and Flickinger, 1992):

$$dC/dt = Ks - (Kd + \mu)C$$

where C is the *total* RNA concentration (ug/million cells), t is time (hours), Ks is the rate of RNA synthesis (ug/million cells/hr), Kd is the rate of RNA degradation (1/hr), and μ is the cell growth rate (1/hr). In reality, each RNA species will have its own unique synthesis and degradation rate, but for the purpose of this model, namely to explain total RNA concentration, all RNA is being lumped together and single synthesis and degradation rates considered. Growth rates were determined from the cell growth data (see Figure 11a - 0.019/hr at 37°C and 0.0034/hr at 32°C). Only 32°C data was modeled because there was insufficient 37°C data to obtain a reliable non-linear regression. For the 32°C data, one of the three flasks was found to

vary significantly from the other two in terms of RNA content for the 120-hour data point (this explains the large error bar on Figure 12 for this particular time point). This flask was not included in the modeling. The results are shown in Figure 13, which compares the model prediction to experimental data, and Table 1, which lists the kinetic parameters obtained from the non-linear regression.

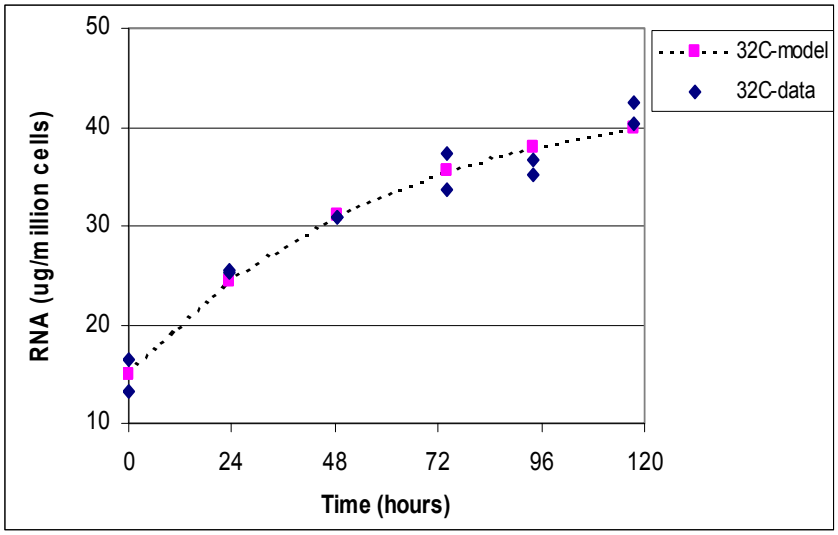

Figure 13: Total RNA Kinetics at 32°C: Model versus Experimental Data

Table 1: Total RNA Kinetics – Model Parameters

Parameter	32°C Value
Ks (ug/million cells/hr)	0.74
Kd (1/hr)	0.013

A "reasonability test" can be applied to the model parameters presented in Table 1 by comparing the estimated values to literature values. For mammalian rRNA, typical half-lives are <3 days to 9 days (Yi *et al*, 1999). RNA half-life is given by the following formula (Ross, 1995):

$$\ln(0.5) = -Kd*t_{1/2}$$

By applying this formula with the model predictions of Kd given in Table 1, total RNA average half-life is estimated as 2.2 days for 32°C cultures, which is of the correct order of magnitude for literature values of rRNA half-lives. Considering that mRNA half-lives are much less than rRNA half-lives, the actual rRNA half-life at 32°C will be even higher than 2.2 days, and thus closer still to literature values.

For synthesis rates (Kd), it is estimated that a mammalian cell must produce approximately 7 million pre-rRNA molecules per cell per cell cycle to provide enough rRNA for ribosomes (Lodish, 2000). Taking into account pre-rRNA molecular weight and 32°C CHO cell doubling time, this corresponds to ~0.3ug/million cells/hr of rRNA synthesis. Total RNA synthesis will be somewhat higher because of tRNA and mRNA synthesis. Thus, the RNA synthesis rate of 0.74ug/million cells/hr estimated from the data (Table 1) is of the correct order of magnitude. Thus, both model parameters (Kd and Ks) appear to be physically reasonable.

This finding has exciting implications for measuring half-lives of any RNA molecule. The difficulty with measuring RNA half-lives is that cells often exist in a dynamic steady state, in which there is constant transcription and degradation, but where the RNA concentration does not change with time and thus no difference can be measured directly. Currently, the way half-lives are measured is to either arrest transcription using a chemical agent, such as actinomycin-D, and monitor the disappearance of the RNA of interest with time or add radioactive nucleosides to the culture in a pulse or continuous fashion, and then monitor radioactivity of the RNA of interest with time (Ross, 1995). These approaches have complicating issues, such as the effect of chemical agents on the cells and the use of radioactive materials. Using temperature shift, it is likely that the steady-state levels of many, if not all, RNA molecules will be perturbed. Shifting cells to low temperature to obtain a new steady-state concentration and then monitoring the return to the original (or physiological) steady-state following a shift back to 37^{o}C will result in the accumulation of an RNA profile that can then be regressed to obtain Ks and Kd values.

The last item to be addressed regarding the total RNA profiles presented in Figure 12 is the rapid drop in RNA concentration seen on the final day of each culture. Although this doesn't have much bearing on my project directly, I find it quite interesting and would like to say a word or two about it. The final points correspond to 87% and 85% cell viability for the 37^{o}C and 32^{o}C cultures, respectively, which are reasonably high viabilities. Yet, the RNA content per cell at these points has dropped to about 20% and 40% of the peak values for the 37^{o}C and 32^{o}C cultures, respectively. Recently, researchers have found that rRNA degradation appears to be involved in the inhibition of protein synthesis during the early stages of apoptosis (Nadano and Sato, 2000). Using gel electrophoresis, these researchers identified two rRNA bands resulting from a single cleavage of the 28S rRNA. The data presented in Figure 12 adds to Nadano and Sato's work by showing the rapid and thorough nature of the degradation that occurs well before cell death is apparent by traditional cell counting methods. The implications of this finding is that total RNA concentration may be used as an early indicator of apoptosis onset and that apoptosis inhibition in recombinant protein production needs to be directed at very early events in apoptosis in order to prevent the protein synthetic machinery from being destroyed

IV. CONCLUSIONS

The use of lower temperature for the growth of recombinant CHO cells has show to be an effective way to increase product concentration. Reducing the growth temperature from 37^{o} C to 32^{o} C has resulted in the increase of the production of Interferon γ. There is, however, a draw back in the reduction of the growth temperature which is the accompanied cell's specific growth rate. However, the nearly four-fold increase in the recombinant Interferon γ off-sets the reduced growth of the cell. The increase in the specific productivity of Interferon γ was found to be from the increase in the mRNA. Conceptually, it is the believed that this generic method to increase the production of recombinant proteins by animal cells can be employed.

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