0273-2289/05/125/0159/$30.00

Development of a Method to Quantify Gene Expression Levels for Glycosylation Pathway Genes in Chinese Hamster Ovary Cell Cultures

Kevin J.-R. Clark,[1] Sarah W. Harcum,*[,2] Jennifer Griffiths,[3] and Kevin M. Bailey[3]

Departments of [1]Chemical Engineering and [2]Bioengineering, Clemson University, 112 Biosystems Research Complex, 51 New Cherry Street, Clemson, SC 29634-0905, E-mail: harcum@clemson.edu; and [3]Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591

Received July 21, 2004; Revised December 23, 2004; Accepted January 18, 2005

Abstract

Changes in protein glycosylation owing to changes in environmental conditions are not well understood. To better understand these relationships, methods to quantify controlling factors are needed. Because enzymes are translated from genes, the ability to quantify gene expression levels for glycosylation-related enzymes would be advantageous. We developed quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays to monitor gene expression in Chinese hamster ovary (CHO) cells for five terminal glycosylation genes. The five enzymes were sialidase, a putative α2,3-sialyltransferase, β1,4-galactosyltransferase, cytosine monophosphate–sialic acid transporter, and uracil diphosphate–galactosyl transporter. Four of these CHO cell genes were publicly available from GenBank; however, the α2,3-sialyltransferase gene for *Cricetulus griseus* (CHO cell species) was not available and, therefore, was sequenced as a part of this work. The qRT-PCR primers and probes (based on the TaqMan™ chemistry) were designed and validated for these five genes. The gene expression profiles were obtained for CHO cells producing the recombinant interleukin-4/13 cytokine trap molecule in batch reactors.

Index Entries: Quantitative real-time polymerase chain reaction; mRNA; glycosylation; interleukin-4/13 cytokine trap; α2,3-sialyltransferase; sequencing.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

The glycosylation profile of a recombinant therapeutic can be an important process parameter. Although much study has been conducted on the environmental factors that affect glycosylation *(1,2)* and research has been conducted for mouse *(3–8)*, rat *(9)*, and human *(10–13)* glycosylation-related gene expression, little work has been published about the gene expression levels for glycosylation pathway genes in Chinese hamster ovary (CHO) cells *(14,15)*. Additionally, native gene expression changes have not been correlated with changes in recombinant protein glycosylation profiles for CHO cells cultured in batch reactors.

Quantitative real-time polymerase chain reaction (qRT-PCR) is a method that can be used to determine changes in gene expression levels. The throughput of qRT-PCR is limited compared to DNA microarrays. However, if only a small number of genes are to be analyzed, qRT-PCR is much less expensive than DNA microarrays. qRT-PCR assays are also sensitive to changes in gene expression and are robust enough to measure changes in gene expression over seven to eight orders of magnitude, if conditions require *(16–18)*. The lower detection limit of a qRT-PCR assay is generally considered to be one to five transcript copies per cell *(18,19)*. The high sensitivity and large dynamic range of qRT-PCR have allowed this technique to be used to validate viral clearance *(20)*, identify species *(21–23)*, and detect diseases *(18,24,25)*. However, to ensure high sensitivity and large dynamic ranges, each primer/probe set must be optimized and validated *(17,18,23,26)*. Assay parameters that typically need to be optimized include primer/probe sequences, melting temperature (T_M), probe length, distance between reporter and quencher, distance between primer and probe, and primer concentration *(23,27)*. Additionally, when possible, the probes should span an intron, so as to mitigate signal from DNA carryover contamination.

The objective of the present study was to develop and validate qRT-PCR primers and probes, based on the TaqMan™ chemistry, to measure gene expression levels for five key glycosylation-related genes in CHO cells. The five validated qRT-PCR assays were then used to determine the gene expression profiles for sialidase, a putative α2,3-sialyltransferase, β1,4-galactosyltransferase, cytosine monophosphate (CMP)–sialic acid transporter, and uracil diphosphate (UDP)–galactosyl transporter. CHO cells expressing the recombinant cytokine trap molecule interleukin-4/13 (IL-4/13) were used as the model system, and gene expression levels were monitored during the course of a batch culture. Additionally, it was necessary to sequence the α2,3-sialyltransferase gene for *Cricetulus griseus* (CHO cell species), because this sequence information was not publicly available.

Materials and Methods

Cell Culture

CHO cells expressing the recombinant IL-4/13 cytokine trap molecule were maintained in 1-L spinners at 5% CO_2, 60 rpm, and 37°C. The medium

was a proprietary defined medium (Regeneron, Tarrytown, NY) supplemented with 2 m*M* L-glutamine (Gibco, Carlsbad, CA). Cells were initially seeded in duplicate spinners at 0.2 ± 0.06 million cells/mL. Cell counts were determined using the trypan blue exclusion method. Measurements are the average of duplicate parallel 1-L spinners. Glucose and lactate concentrations were determined using a BioProfile 400 Analyzer (NOVA, Waltham, MA) per the manufacturer's instructions.

Sialidase Activity Assay

Cells were harvested and centrifuged for 10 min at 800 rpm and 4°C. The cell pellet was resuspended in ice-cold phosphate-buffered saline (PBS) (Gibco) and then centrifuged at 750*g* for 10 min at 4°C. The cell pellet was resuspended in 500 µL of ice-cold PBS and disrupted by passage through a 27 1/2-gage needle. The disrupted cells were centrifuged at 12,000*g* for 30 min at 4°C. The cell lysate was aliquoted and frozen at –20°C for later analysis. Sialidase activity was assayed using an adaptation of a method described by Berg et al. *(28)* and has been used to characterize and quantify CHO cell sialidases *(29–31)*. Briefly, each 20-µL sample was placed in a 1-mL centrifuge tube with 150 µL of substrate buffer and gently mixed. The substrate buffer used was 0.2 or 0.5 *M* sodium acetate buffer, pH 5.5, with 500 m*M* $CaCl_2$. Samples were centrifuged at 14,000*g* for 5 min to remove any precipitates. One hundred fifty microliters of sample was transferred to a 96-well plate. Thirty microliters of 0.125 m*M* artificial substrate 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (Sigma, St. Louis, MO) in substrate buffer was added to the reaction tube and gently aspirated. The samples were then incubated at 37°C for 45 min in the dark. The fluorescent signal (λ_{ex} = 366 nm and λ_{em} = 460 nm) was recorded using a GENios fluorometer (Tecan, Research Triangle Park, NC). A serial dilution of lysed CHO cells provided a standard to determine the linear range and detection limits of this assay. Sialidase activity was normalized relative to the first sample of the cultures.

Purification of Recombinant IL-4/13 Cytokine Trap and Analysis of Sialic Acid Content

Cell culture broth was harvested from the spinners, and cells were removed by centrifuging at 750*g* for 10 min and 4°C. The supernatant was loaded onto a 1-mL Hi-Trap™ Protein A affinity column (Amersham Pharmacia, Piscataway, NJ). The column was washed with 3 column vol of PBS, pH 7.0, with 350 m*M* NaCl. The column was washed a second time with 2 column vol of PBS, pH 7.0. The protein was eluted with 3 column vol of 40 m*M* acetic acid. Fractions were collected, and the protein concentration was determined by measuring the absorbance at 280 nm. Columns were subsequently stripped and reequilibrated with 2 column vol of 0.5 m*M* acetic acid and PBS, pH 7.0, respectively. Loading, washing, eluting, stripping and column equilibration were completed at a flow rate of 1 mL/min.

The sialic acid content of the IL-4/13 cytokine trap fusion protein was determined by a high-performance liquid chromatography (HPLC) method that used *o*-phenylenediamine (OPD) derivatization *(32,33)*. The sialic acid content is reported per potential N-glycosylation site on the protein. Acid-hydrolyzed sialic acids from purified IL-4/13 cytokine trap fusion were derivatized with OPD (Aldrich, Milwaukee, WI) and analyzed by reverse-phase HPLC (HP1090) on a C18 column (Ultrasphere ODS, 250 × 4.5 mm; Beckman, Fullerton, CA). Fluorescently labeled monosaccharide derivatives were detected by fluorescence spectroscopy (HP1046-A: λ_{ex} = 230 nm and λ_{em} = 425 nm). Sialic acid residues released from fetuin were used as standards.

Sequencing of CHO Cell Sialyltransferase Gene

Total RNA was isolated from CHO cells using an RNAqueous-4PCR kit (Ambion, Austin, TX) per the manufacturer's instructions, except the DNase treatment was increased to 2 µL of DNase for 1 h. Reverse transcription reactions were completed using 200 ng of total RNA. Oligo dT or GeneRacer™ reverse transcription primer with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions for first-strand synthesis. RNase H was used to degrade the RNA following first-strand synthesis. PCR reaction mixtures consisted of 2 m*M* dNTPs, 900 n*M* forward and reverse primers, 5 µL of 10X PCR buffer, 5 µL of 25 m*M* $MgSO_4$, 37 µL of nuclease-free water, and 0.5 µL of cDNA from reverse transcription reactions. PCR cycle parameters were 94°C for one 2-min cycle, then 35 cycles of 94°C for 30 s, 57°C for 30 s, and 70°C for 1 min. A final extension step of 70°C for 15 min was used. Sequencing was performed using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA) per the manufacturer's instructions.

Oligonucleotide primers were designed for the amplification of an internal fragment of the putative CHO cell α2,3-sialyltransferase from the published *Mesocricetus auratus* (AJ245700.1) sequence. Sense primer 5'-CAA-CTC-AGA-GAA-GAA-AGA-GCC-ATG-3' and antisense primer 5'-GCC-ACA-GGA-GCA-TTG-TTC-AA-3' were used to amplify a 280-bp fragment of the CHO cell sequence. To capture the stop codon of the CHO cell sequence, a GeneRacer reverse transcription primer was used to generate cDNA with a known 3' sequence. The sense primer from *M. auratus* and the GeneRacer antisense primer generated a fragment containing the CHO cell sialyltransferase stop codon. The 5' sequence of the CHO cell sialyltransferase was determined by using a sense primer based on the homology in the upstream region between *Homo sapiens* (BC010645), *Mus musculus* (BC050773.1), and *Oryctolagus cuniculus* (AF121967) α2,3-sialyltransferases. The sense primer used was 5'-GAG-GCA-GCC-GGG-ATG-ACA-3' and the antisense primer was the *M. auratus* primer. The full-length CHO cell sialyltransferase sequence was generated using the upstream sense primer and the antisense primer 5'-ATC-CCA-ACG-CTC-ATA-GAC-AGG-3'.

Table 1
Primer and Probe Sequences for TaqMan Assays

Gene	Primer direction	Primer sequence (5'–3')
α2,3-Sialyltransferase	FP	ACGCTCCTGTGGCTGGTTAT
	RP	GGCCGACTCAGGGTAGAAGAG
	PRB	ATGTGGGCTCCAAGACCACCATACGTC
β1,4-Galactosyltransferase	FP	GGTGGCCATCATTATCCCATT
	RP	CGGATGCAAATAATACAGCCAA
	PRB	CGCAACCGGCAGGAGCACCT
CMP–sialic acid transporter	FP	AGCCCCAAGGAACTGATGAA
	RP	ACCTGGTATACTGCTGCATCCA
	PRB	CTGAACAGCATACACCAGTGATGGCACA
UDP-galactose transporter	FP	TGCCACCTCCCTGTCTATTGT
	RP	AATAATGGGTCCAGGTGAAAGC
	PRB	TCCACTGTTGCCTCCATTCGCCTCT
Sialidase	FP	CCCTACAGACTCCCGGAAGAG
	RP	AGTCCGAGTAGGCACAGATGC
	PRB	ACCTGGTCAGCTCCCACCCTGTTG

Full-length CHO cell α2,3-sialyltransferase sequence information was deposited in GenBank (AY266675).

Quantitative Real-Time Polymerase Chain Reaction

CHO cell gene expression for the terminal portion of the glycosylation pathway was quantified using TaqMan chemistry. Primers and probes were designed using Primer Express (Applied Biosystems) for the following five genes: sialidase (U06143), α2,3-sialyltransferase (AY266675), β1,4-galactosyltransferase (AF318896), CMP–sialic acid transporter (Y12074), and UDP-galactose transporter (AF299335) (*see* Table 1 for primer and probe sequences). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probes were purchased as a predeveloped assay from Applied Biosystems (TaqMan Rodent GAPDH Control Reagents). Since only mRNA sequence information was available from GenBank, the amplicons used did not span introns. Reverse transcription negative controls were used to confirm the absence of significant genomic DNA contamination. TaqMan universal master mix and 6-carboxy-fluorescein/6-carboxy-tetramethyl-rhodamine-labeled probes were purchased from Applied Biosystems. Total RNA was quantified using RiboGreen (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. Five nanograms of total RNA was used for the TaqMan assays. Random hexamers and SuperScript reverse transcriptase (Invitrogen) were used per the manufacturer's instructions to synthesize the first strand. TaqMan reactions were carried out using an ABI 9700 thermal cycler in 96-well format. Reaction volumes were 50 μL. Cycle parameters were 50°C for 2 min and 94°C for 2 min,

followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. Each gene was normalized relative to its corresponding GAPDH control sample *(34,35)*. Actin and 18S rRNA have been used in parallel with GAPDH as the normalizers in our laboratory with insignificant differences in the experimental gene expression profiles. Additionally, analysis of variance (ANOVA) of the GAPDH expression across the experiment indicated no significant trends in the data ($p \geq 0.05$). Each biologic sample was analyzed in triplicate for each experimental gene and the normalizer gene. For better visualization of the gene expression profiles, the normalized gene expression for each gene was also normalized to the average of the first time point.

Results

qRT-PCR primers and probes, based on the TaqMan chemistry, were developed to measure gene expression levels for five key glycosylation-related genes in CHO cells. The five validated qRT-PCR assays were used to determine the gene expression levels of sialidase, α2,3-sialyltransferase, β1,4-galactosyltransferase, CMP–sialic acid transporter, and UDP-galactosyl transporter in CHO cells expressing the recombinant cytokine trap IL-4/13 during the course of a batch culture. However, first the α2,3-sialyltransferase gene needed to be isolated and sequenced for *C. griseus* (CHO cell species).

CHO Cell Sialyltransferase Sequence

The α2,3-sialyltransferase enzyme catalyzes the transfer of sialic acid from CMP–sialic acid to a juvenile glycan. Several sialyltransferases have been sequenced and studied; however, none of these reported sialyltransferases are *C. griseus* sialyltransferases. We sequenced a putative *C. griseus* α2,3-sialyltransferase IV. The sequencing was facilitated by the high homology of α2,3-sialyltransferase IV among other known mammalian organisms. The putative *C. griseus* sialyltransferase nucleotide sequence was found to be 96, 93, 92, 87, and 85% homologous with the coding regions of α2,3-sialyltransferase IV from *M. auratus* (AJ245700.1), *Rattus norvegicus* (XM-343368.1), *M. musculus* (BC050773.1), *H. sapiens* (BC010645.1), and *O. cuniculus* (AF121967), respectively. The full length for *C. griseus* α2,3-sialyltransferase sequence was deposited in GenBank (AY266675).

Optimization and Validation of qRT-PCR

qRT-PCR primers and probes were designed and validated to quantify the CHO cell gene expression levels for five key glycosylation pathway genes. Forward and reverse primer concentrations were optimized in order to determine the reaction conditions that resulted in the greatest fluorescent signal (ΔRn) and the lowest cycle threshold (C_T) *(36)*. Primer concentrations investigated were from 50 to 900 n*M* for each forward and reverse primer. For the CMP–sialic acid transporter gene, as the primer concentra-

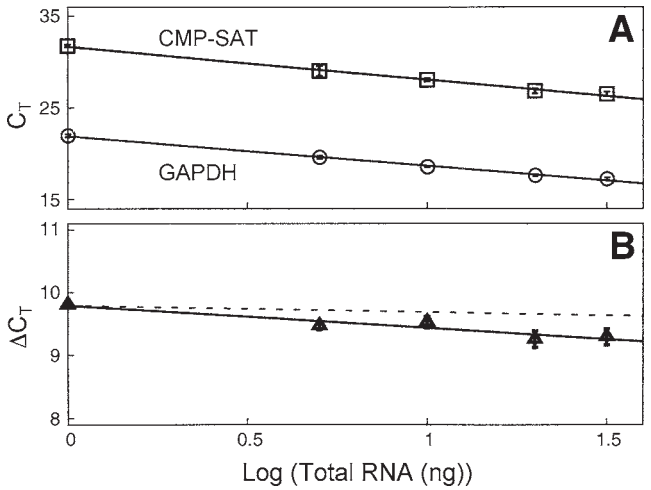

Fig. 1. TaqMan assay validation: **(A)** standard curves for (□) CMP–sialic acid transporter (SAT) and (○) GAPDH; **(B)** ΔC_T for CMP-SAT normalized to GAPDH (▲). The solid lines in (A) and (B) are lines fitted to the data. The dashed line in (B) represents the lower allowable boundary for the slope for the $\Delta\Delta C_T$ method.

tions increased the C_T decreased to a minimum of 26 cycles. In addition, as the primer concentrations increased the change in ΔRn reached a maximum value of 2.3 relative fluorescence units. At a primer concentration of 900 n*M* for both the forward and reverse primers, the C_T and ΔRn values were relatively constant at peak values (data not shown). The primer sets for the other four CHO cell glycosylation genes (sialidase, α2,3-sialyltransferase, β1,4-galactosyltransferase, and UDP-galactose transporter) were optimized similarly. For all five genes, the best primer concentration was determined to be 900 n*M* for both the forward and reverse primers.

To determine whether the primers and probes were suitable for quantification, samples were serially diluted and qRT-PCR was performed. Each of the five primer probe sets, as well as the predeveloped assay GAPDH, produced linear standard curves over the range of 1–30 ng of total RNA on log scale. Figure 1 shows a representative plot of the standard curve for CMP–sialic acid transporter and GAPDH. Each of the five assays was determined to be suitable for quantifying the relative changes in gene expression levels. The five primers and probes with GAPDH were also evaluated for their compatibility with the standard curveless method of relative gene quantification, i.e., the $\Delta\Delta C_T$ method, in which ΔC_T is defined as the difference between the C_T of the gene of interest and the C_T of the endogenous control, and $\Delta\Delta C_T$ is defined as the difference between the ΔC_T of the sample of interest and the ΔC_T of the normalizing sample *(37)*. The $\Delta\Delta C_T$ method requires equal amplification efficiency for the gene of interest and the endogenous control *(37)*. Equal amplification efficiency is demonstrated by a linear relationship from the ΔC_T vs the log of the total RNA curve with a slope ±0.1 C_T per log mass. For CMP–sialic acid trans-

Table 2
qRT-PCR Efficiency (μ) of Target Amplification, and Slope of ΔC_T vs log Mass of Total RNA With Respective 95% CIs for Each Target Gene of Interest

Gene	μ ± CI	Slope ± CI[a]
α2,3-Sialyltransferase	1.9 ± 0.02	–0.32 ± 0.07
β1,4-Galactosyltransferase	1.84 ± 0.01	–0.5 ± 0.08
CMP–sialic acid transporter	1.89 ± 0.03	–0.35 ± 0.06
UDP-galactose transporter	1.98 ± 0.03	–0.1 ± 0.09
Sialidase	1.93 ± 0.03	–0.25 ± 0.07
GAPDH	2.02 ± 0.03	N/A

[a]N/A, not applicable.

porter and GAPDH, the slope of the ΔC_T curve was found to be –0.35 ± 0.06 C_T per log mass (95% confidence interval [CI]), as shown in Fig. 1. This slope is outside the allowable range of ±0.1. The other four genes were evaluated similarly, and none of the five primer/probe sets were found to be suitable for the $\Delta\Delta C_T$ method. Therefore, standard curves were used to quantify changes in gene expression for the primers and probes used in this study. Table 2 provides the efficiencies of amplification for each of the six assays.

Expression Studies

To demonstrate that significant gene expression profiles could be obtained for CHO cell cultures, the effects of growth on the gene expression of five key glycosylation genes for duplicate CHO cell cultures expressing recombinant IL-4/13 cytokine trap were evaluated. Figure 2 shows the average cell densities of the parallel cultures. The gene expression levels for all five CHO cell glycosylation genes were each normalized to the first samples obtained from the cultures. Figure 2 shows the gene expression levels for the five genes under investigation. The gene expression levels of sialidase, α2,3-sialyltransferase, and CMP–sialic acid transporter were found to have a negative slope for at least the first 55 h ($p \leq 0.05$; two-tailed *t*-test) of culture time (Fig. 2). The slopes of the gene expression profiles for the three genes sialidase, α2,3-sialyltransferase, and CMP–sialic acid transporter were found to be the same ($p \geq 0.05$; ANOVA) during the first 55 h of culture. The gene expression levels for the final two sample points of the culture were found to be greater than the gene expression levels at 55 h for α2,3-sialyltransferase and CMP–sialic acid transporter ($p \leq 0.05$; one-tailed *t*-test). However, sialidase gene expression decreased continuously for 90 h, and then it increased between 90 and 115 h ($p \leq 0.05$; one-tailed *t*-test).

The gene expression levels of β1,4-galactosyltransferase and UDP-galactose transporter were also found to have a slope not significantly different from zero for at least the first 55 h of the cultures ($p \geq 0.05$; two-tailed *t*-test). The gene expression levels for β1,4-galactosyltransferase and

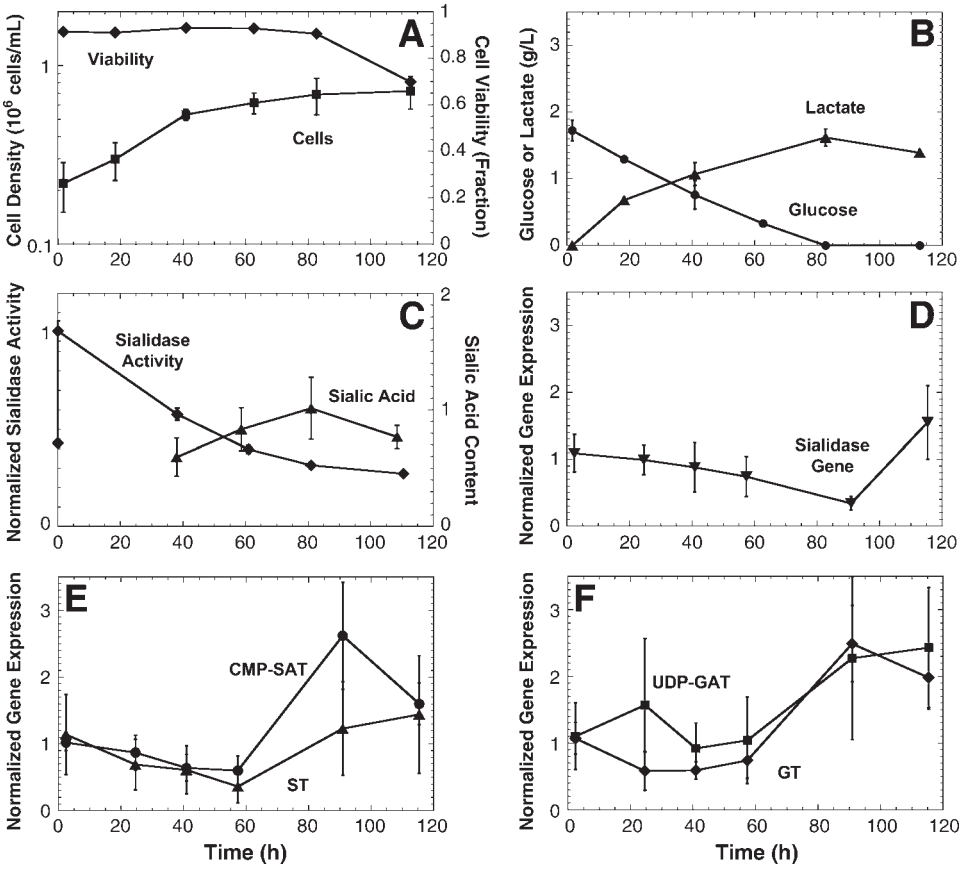

Fig. 2. Recombinant CHO cell growth characteristics and gene expression levels for duplicate spinner flasks: **(A)** cell density (■) and viability (◆); **(B)** glucose (●) and lactate (▲); **(C)** sialic acid content (▲) and normalized sialidase activity (◆); **(D)** sialidase gene expression (▼); **(E)** α2,3-sialyltransferase (ST) (▲) and CMP–sialic acid transporter (SAT) (●) gene expression levels; **(F)** β1,4-galactosyltransferase (GT) (◆) and UDP-galactose transporter (GAT) (■). Error bars represent 95% CIs.

UDP-galactose transporter were found to be the same ($p \geq 0.05$; two-tailed *t*-test) for the first 55 h. The gene expression levels for the final two sample points of the culture were found to be greater than the gene expression levels at 55 h for β1,4-galactosyltransferase ($p \leq 0.05$; one-tailed *t*-test). Although UDP-galactose transporter gene expression levels were found to be numerically greater for the final two time points of the culture relative to the 55 h time point, UDP-galactose transporter gene expression levels were not statistically different at the end of the culture compared to the 55-h time point.

Glucose concentration was determined to have reached zero by 77 ± 6 h ($p \leq 0.05$). The genes α2,3-sialyltransferase and CMP–sialic acid transporter were observed to have decreasing gene expression prior to glucose

depletion and then increased expression after glucose depletion. The genes β1,4-galactosyltransferase and UDP-galactose transporter were observed to have relatively stable expression prior to glucose depletion and increased expression after glucose depletion. Sialidase gene expression was observed to have a continuously decreasing expression even after glucose depletion ($p \leq 0.05$; one-tailed *t*-test).

Discussion

TaqMan primers and probes were designed and validated to measure the expression levels of five key glycosylation genes for CHO cells expressing recombinant IL-4/13 cytokine trap. The gene expression profiles for α2,3-sialyltransferase and CMP–sialic acid transporter were determined to be similar, and the gene expression profiles for β1,4-galactosyltransferase and UDP-galactose transporter were also determined to be similar. The sialidase gene expression profiles were determined to be different from the other four genes investigated.

Primers and Probes

The five primer and probe sets were observed to be suitable for measuring the change in gene expression levels relative to the predeveloped GAPDH assay. The amplification efficiency for the five assays was found to be different from that of GAPDH. Therefore, the standard curveless method ($\Delta\Delta C_T$) was not compatible with these primers and probes. Proudnikov et al. *(27)* demonstrated that the probe cleavage efficiency was, in part, dependent on the position of the fluorescent quencher, where probe cleavage contributes significantly to amplification efficiency. They demonstrated that placement of the quencher at an internal nucleotide on the probe, instead of the traditional 3' end of the probe, increased the sensitivity of the qRT-PCR assay. Because the present study was relatively small, the additional cost to reoptimize the efficiency was not warranted. However, for larger studies in which the designed primers and probes are used continuously, economics would favor development of the standard curveless method, because this would halve the number of reactions required for quantification of gene expression. The standard curve method was sufficient to evaluate the relative gene expression level profiles examined in our study.

Expression Studies

The relative gene expression levels of five key glycosylation-related genes were determined for CHO cells cultured in spinners. The CHO cells expressed a recombinant IL-4/13 cytokine trap molecule. The gene expression profiles of sialidase, sialyltransferase, CMP–sialic acid transporter, β1,4-galactosyltransferase, and UDP-galactose transporter are briefly discussed next in the context of protein function in the cell.

Sialidase

Sialidase plays an important role in protein glycosylation. Sialidases have been demonstrated to be active and capable of degrading glycoproteins in CHO cell culture supernatants *(30,38)*. The presence of sialidase in CHO cell supernatants has been attributed primarily to apoptosis, the subsequent cell lysis, and the release of cytosolic sialidases into the supernatant *(38–40)*. Although the enzymatic characteristics of the CHO cell sialidase have been studied *(38,39,41)*, not until recently have the gene expression levels for the sialidase gene in connection with the sialidase enzyme activity been studied. Hasegawa et al. *(42)* reported that different tissues had different gene expression profiles. Additionally, gene expression levels and enzyme activities have not always been well correlated. Hasegawa et al.'s *(42)* results indicated that sialidase enzyme activity was possibly not transcriptionally controlled. For example, the gene expression profiles for cytosolic sialidase in rat brain and liver were observed to be relatively constant over the development of the rat; however, the sialidase enzyme activity in both rat brain and liver increased during development of the rat. Additionally, the rate of sialidase activity accumulation was clearly different between the two tissues *(42)*.

In the present study, the CHO cell sialidase gene expression profile was observed to decrease throughout the culture until substrate depletion. Because the sialidase enzyme was translated from mRNA, decreased gene expression may have indicated a decreased need for the enzyme. The decreased need for sialidase activity might have also been the result of a decreased need for glycan degradation. CHO cells placed in fresh medium with low or no waste products may have signaled the need for increased sialidase activity, which required sialidase transcription rates to increase.

Sialyltransferase and CMP–Sialic Acid Transporter

α2,3-Sialyltransferase catalyzes the transfer of sialic acid from CMP–sialic acid to a subterminal galactose on a glycan. Several investigators have purified sialyltransferases and assayed the function and specificity of these sialyltransferases *(43–46)*. The gene expression profile of the α2,3-sialyltransferase IV in CHO cells has not been well studied. The α2,3-sialyltransferase enzyme is hypothesized to be transcriptionally controlled in other species *(10,11,13,47,48)*. Numerous isoforms of sialyltransferase have been identified in various species *(11,13,48)*. These various sialyltransferase isoforms are expressed via multiple promoters and alternative splicing *(5–7,11,13,48–50)*. Currently, not much has been reported about CMP–sialic acid transporter gene expression. Eckhardt et al. *(51)* sequenced CHO CMP–sialic acid transporter and concluded that CMP–sialic acid transporter had only one open reading frame. The CMP–sialic acid transporter has 10 transmembrane regions *(51,52)*. Eckhardt et al. *(51)* also measured the expression levels of CMP–sialic acid transporter in various mouse tissues, and CHO K1, 6B2, and Lec2 cells. Mouse liver and skeletal muscle tissues appeared to have the highest expression of CMP–sialic acid transporter vs

spleen tissues. The three CHO lines had comparable levels of CMP–sialic acid transporter gene expression.

In our study the CMP–sialic acid transporter and α2,3-sialyltransferase gene expression levels decreased until glucose depletion and then increased sharply. This information and the fact that the two enzymes are located close to one another on the metabolic pathway could indicate that α2,3-sialyltransferase and CMP–sialic acid transporter have similar control and expression characteristics in CHO cells. However, the similar control mechanism might not be universal, because the sialyltransferase enzyme has numerous isoforms generated by multiple promoters and alternative splicing, whereas for CMP–sialic acid transporter only one isoform has been identified *(51,52)*.

Galactosyltransferase and UDP-Galactose Transporter

The β1,4-galactosyltransferase enzyme catalyzes the transfer of galactose from UDP-galactose to either the terminal GlcNAc on a glycan or a glucose molecule in the lactating mammary gland to make lactose *(53)*. Thus far, β1,4-galactosyltransferase enzyme is thought to be transcriptionally controlled *(3–8)*, with tissue-specific gene expression levels *(5–7)*. Tissue-specific expression of galactosyltransferases has been reported to be controlled by several promoters, one of which was demonstrated to be occupied by the ubiquitous factor Sp1 *(6,7)*. As a result, β1,4-galactosyltransferase has two distinct isoforms: 4.1 and 3.9 kb. The Sp1 ubiquitous factor was observed to participate in the control of both isoforms. There was evidence that a putative repressor also controlled the expression of the 3.9-kb isoform *(5–7)*. The ubiquitous control may be responsible for the constant levels of β1,4-galactosyltransferase gene expression.

The UDP-galactose transporter enzyme catalyzes the transport of the sugar nucleotide UDP-galactose across the Golgi membrane and transports UDP back out of the Golgi *(54)*. To date, most of the gene expression data for UDP-galactose transporter have been mainly for cancer cells. Kumamoto et al. *(55)* reported that for colon cancer cells the gene expression levels of UDP-galactose transporter were significantly higher than in normal colon cells. They also demonstrated that the stable transfection of UDP-galactose transporter into various cultured human cancer cell lines significantly changed the glycosylation profile; specifically, the presence of more cancer-like glycans resulted.

In our study, β1,4-galactosyltransferase and UDP-galactose transporter gene expression were relatively similar to each other and stable until glucose depletion. The control of β1,4-galactosyltransferase expression is thought to be at the transcriptional level *(3–8)*, whereas the control of UDP-galactose transporter has not been determined. The observed similarities in gene expression suggest similar control. As with α2,3-sialyltransferase and CMP–sialic acid transporter, each gene probably has a certain amount of control independent of the other.

Our preliminary studies quantified the gene expression profiles for five glycosylation genes in CHO cells. In mouse, human, and rat, each of these genes was known to have numerous nucleotide sequences that were attributed to enzymes with slightly different substrate specificities and that catalyzed different sugar linkages *(7,13,56)*. However, in CHO cells, few nucleotide sequences corresponding to the genes investigated in our study have been identified *(57)*. Because it is likely that CHO cells have roughly the same number of glycosylation genes as mouse, the probes and primers used in our study most likely hybridized with glycosylation genes similar to the target genes, in addition to the target genes. From the Swiss-Prot Database, for mouse there are 17 identified sialyltransferase genes *(58)*. For CHO cells, only one sialyltransferase gene was available at the time of our study; therefore, it was not possible to determine the overlap of the probes and primers to other CHO cell sialyltransferase gene sequences. Further work is necessary to increase the number of genes available for analysis.

The objectives of our study were to develop gene expression assays for five key glycosylation enzymes and determine whether these assays could be used to monitor the gene expression profiles of CHO cell cultures. Owing to the limited number of CHO cell sequences publicly available, it was also necessary to sequence the putative α2,3-sialyltransferase gene for CHO cells. For quantification, the primers and probes used were based on the TaqMan chemistry. The primers and probes were validated to be suitable for standard curve quantification, which reports relative gene expression levels. These probes make it possible to detect and quantify changes in gene expression levels for a variety of experimental stimuli. In our study, it was observed that the gene expression levels of sialyltransferase and CMP–sialic acid transporter were decreasing during the industrially relevant portion of the cultures. Therefore, because α2,3-sialyltransferase and possibly CMP–sialic acid transporter are transcriptionally controlled, the amount of α2,3-sialyltransferase and CMP–sialic acid transporter enzyme also decreased. This change in enzyme levels has the potential to affect the sialylation of glycoproteins in general and overexpressed recombinant glycoproteins specifically. In the future, the genome of the CHO cell should be sequenced, and the transcriptome and proteome of the whole organism should be considered, in order to better understand CHO cell glycosylation.

Nomenclature

C_T = cycle threshold
ΔC_T = change in cycle threshold
$\Delta\Delta C_T$ = change in change in cycle threshold
ΔRn = fluorescent signal

Acknowledgment

This work was supported by the National Science Foundation under grant no. 0303782.

References

1. Hooker, A. D., Goldman, M. H., Markham, N. H., et al. (1995), *Biotechnol. Bioeng.* **48,** 639–648.
2. Hayter, P. M., Curling, E. M., Gould, M. L., et al. (1993), *Biotechnol. Bioeng.* **42,** 1077–1085.
3. Hu, W. S., Zhou, W. C., and Europa, L. F. (1998), *J. Microbiol. Biotechnol.* **8,** 8–13.
4. Kotani, N., Asano, M., Iwakura, Y., and Takasaki, S. (2001), *Biochem. J.* **357,** 827–834.
5. Hinton, D. A., Evans, S. C., and Shur, B. D. (1995), *Exp. Cell. Res.* **219,** 640–649.
6. Rajput, B., Shaper, N. L., and Shaper, J. H. (1996), *J. Biol. Chem.* **271,** 5131–5142.
7. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M. A., et al. (2001), *Biochimie* **83,** 727–737.
8. Masibay, A. S., Damewood, G. P., Boeggeman, E., and Qasba, P. K. (1991), *Biochim. Biophys. Acta* **1090,** 230–234.
9. Wen, D. X., Svensson, E. C., and Paulson, J. C. (1992), *J. Biol. Chem.* **267,** 2512–2518.
10. Baum, L. G., Derbin, K., Perillo, N. L., et al. (1996), *J. Biol. Chem.* **271,** 10,793–10,799.
11. Taniguchi, A. and Matsumoto, K. (1999), *Biochem. Biophys. Res. Commun.* **257,** 516–522.
12. Chung, M. I., Lim, M. H., Lee, Y. J., et al. (2003), *J. Microbiol. Biotechnol.* **13,** 217–224.
13. Grahn, A. and Larson, G. (2001), *Glycoconjugate J.* **18,** 759–767.
14. Wang, H., Tachibana, K., Zhang, Y., et al. (2003), *Biochem. Biophys. Res. Commun.* **300,** 738–744.
15. Ferrari, J., Gunson, J., Lofgren, J., Krummen, L., and Warner, T. G. (1998), *Biotechnol. Bioeng.* **60,** 589–595.
16. Wang, M. D., Yang, M., Huzel, N., and Butler, M. (2002), *Biotechnol. Bioeng.* **77,** 194–203.
17. Puig-Kroger, A., Sanz-Rodriguez, F., Longo, N., et al. (2000), *J. Immunol.* **165,** 4338–4345.
18. Klein, J. M. and McCarthy, T. A. (2002), *Pediatr. Res.* **51,** 62A.
19. Yang, M. and Butler, M. (2000), *Cytotechnology* **34,** 83–99.
20. Sears, H. J., Sawers, G., Berks, B. C., Ferguson, S. J., and Richardson, D. J. (2000), *Microbiology* **146,** 2977–2985.
21. Yang, M. and Butler, M. (2002), *Biotechnol. Prog.* **18,** 129–138.
22. Jeong, Y., Jinwoo, K., Suhyun, K., et al. (2003), *Plant Dis.* **87,** 890–895.
23. Lunge, V. R., Miller, B. J., Livak, K. J., and Batt, C. A. (2002), *J. Microbiol. Methods* **51,** 361–368.
24. Puig, S. and Perez-Ortin, J. E. (2000), *Syst. Appl. Microbiol.* **23,** 300–303.
25. Steinbach, D., Lengemann, J., Voight, A., et al. (2003), *Clin. Cancer Res.* **9,** 1083–1086.
26. Proudnikov, D., Yuferov, V., Laforge, K. S., Ho, A., and Kreek, M. J. (2003), *Mol. Brain Res.* **112,** 182–185.
27. Proudnikov, D., Yuferov, V., Zhou, Y., et al. (2003), *J. Neurosci. Methods* **123,** 31–45.
28. Berg, W., Gutschkergdaniec, G., and Schauer, R. (1985), *Anal. Biochem.* **145,** 339–342.
29. Warner, T. G., Chang, J., Ferrai, J., et al. (1993), *Glycobiology* **3,** 455–463.
30. Gramer, M. J., Goochee, C., Chock, V. Y., Brousseau, D. T., and Sllwkowski, M. B. (1995), *Biotechnology* **13,** 692–698.
31. Ferrari, J., Harris, R., and Warner, T. G. (1994), *Glycobiology* **4,** 367–373.
32. Anumula, K. R. (1997), in *Techniques in Glycobiology*, Townsend, R. R., Arland, J., and Hotchkiss, T., eds., Marcel Dekker, New York, pp. 349–357.
33. Gawlitzek, M., Ryll, T., Lofgren, J., and Sliwkowski, M. B. (2000), *Biotechnol. Bioeng.* **68,** 637–646.
34. Suzuki, T., Higgins, P., and Crawford, D. (2000), *BioTechniques* **29,** 332–337.
35. Freeman, W. M., Walker, S. J., and Vrana, K. E. (1999), *BioTechniques* **26,** 112–125.
36. Mackay, I. M. (2004), *Clin. Microbiol. Infect.* **10,** 190–212.
37. Livak, K. J. and Schmittgen, T. D. (2001), *Methods* **25,** 402–408.
38. Gramer, M. J. and Goochee, C. F. (1993), *Biotechnol. Prog.* **9,** 366–373.
39. Munzert, E., Muthing, J., Buntemeyer, H., and Lehmann, J. (1996), *Biotechnol. Prog.* **12,** 559–563.

40. Goochee, C. F. and Gramer, M. J. (1994), *Biotechnol. Bioeng.* **43,** 423–428.
41. Goochee, C. F., Gramer, M. J., Schaffer, D. V., and Sliwkowski, M. B. (1994), *J. Cell. Biochem.* **(S18D),** 263.
42. Hasegawa, T., Carnero, C. F., Wada, T., Itoyama, Y., and Miyagi, T. (2001), *Biochem. Biophys. Res. Commun.* **280,** 726–732.
43. Sasaki, K., Watanabe, E., Kawashima, K., et al. (1993), *J. Biol. Chem.* **268,** 22,782–22,787.
44. Kono, M., Ohyama, Y., Lee, Y. C., et al. (1997), *Glycobiology* **7,** 469–479.
45. Kitagawa, H. and Paulson, J. C. (1994), *J. Biol. Chem.* **269,** 17,872–17,878.
46. Endo, T., Koizumi, S., Tabata, K., and Ozaki, A. (2000), *Appl. Microbiol. Biotechnol.* **53,** 257–261.
47. Taniguchi, A., Morishima, T., Tsujita, Y., Matsumoto, Y., and Matsumoto, K. (2003), *Biochem. Biophys. Res. Commun.* **300,** 570–576.
48. Hirooka, T., Suganuma, N., Furuhashi, M., et al. (1996), *Endocr. J.* **43,** 423–428.
49. Paulson, J. C., Colley, K., Lee, E. U., and Roth, J. (1988), *Glycoconjugate J.* **5,** 330.
50. Taniguchi, A., Hioki, M., and Matsumoto, K. (2003), *Biochem. Biophys. Res. Commun.* **301,** 764–768.
51. Eckhardt, M., Muhlenhoff, V., Bethe, A., and Gerardyschahn, R. (1996), *Proc. Natl. Acad. Sci. USA* **93,** 7572–7576.
52. Eckhardt, M., Gotza, B., and Gerardy-Schahn, R. (1999), *J. Biol. Chem.* **274,** 8779–8787.
53. Bill, R. M., Revers, L., and Wilson, I. B. H. (1998), *Protein Glycosylation*, Kluwer Academic, Boston.
54. Berninsone, P. M. and Hirschberg, C. B. (2000), *Curr. Opin. Struct. Biol.* **10,** 542–547.
55. Kumamoto, K., Goto, Y., Sekikawa, K., et al. (2001), *Cancer Res.* **61,** 4620–4627.
56. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M. A., et al. (2000), *Biochem. J.* **352,** 37–48.
57. Lee, J. H., Sundaram, S., Shaper, N. L., Raju, T. S., and Stanley, P. (2001), *J. Biol. Chem.* **276,** 13,924–13,934.
58. Gasteiger, E., Gattiker, A., Hoogland, C., et al. (2003), *Nucleic Acids Res.* **31,** 3784–3788.