

Over expression of the CMP-sialic acid transporter in Chinese hamster ovary cells leads to increased sialylation

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Abstract - Most glyco-engineering approaches used to improve quality of recombinant glycoproteins involve the manipulation of glycosyltransferase and/or glycosidase expression. We investigated whether the over expression of nucleotide sugar transporters, particularly the CMP-sialic acid transporter (CMP-SAT), would be a means to improve the sialylation process in CHO cells. We hypothesized that increasing the expression of the CMP-SAT in the cells would increase the transport of the CMP-sialic acid in the Golgi lumen, hence increasing the intra-luminal CMP-sialic acid pool, and resulting in a possible increase in sialylation extent of proteins being produced. We report the construction of a CMP-SAT expression vector which was used for transfection into CHO-IFN γ , a CHO cell line producing human IFN γ . This resulted in approximately 2 to 5 times increase in total CMP-SAT expression in some of the positive clones as compared to untransfected CHO-IFN γ , as determined using real-time PCR analysis. This in turn concurred with a 9.6% to 16.3% percent increase in site sialylation. This engineering approach has thus been identified as a novel means of improving sialylation in recombinant glycoprotein therapeutics. This strategy can be utilized feasibly on its own, or in combination with existing sialylation improvement strategies. It is believed that such multi-prong approaches are required to effectively manipulate the complex sialylation process, so as to bring us closer to the goal of producing recombinant glycoproteins of high and consistent sialylation from mammalian cells.

Index Terms— Chinese Hamster Ovary (CHO) cells, Over expression, Protein Quality, Sialylation, Transport.

I. INTRODUCTION

Glycosylation of recombinant proteins affect critical properties such as its solubility, thermal stability and bioactivity [1]. In particular, the presence of sialic acid, the terminal sugar for N-linked glycans, has been known to prevent recognition of the glycoprotein by asialoglycoprotein receptors [2], hence increasing its circulatory

life time in the blood. It is thus one of the goals of recombinant glycoprotein production to achieve maximum and consistent sialylation on these recombinant glycoproteins.

The sialylation process is a complex series of step-wise reactions carried out by a series of proteins, each having its own special function [3]. Glyco-engineering approaches have been used extensively to control this complex process (reviewed in [4] & [5]). In particular, previous work to improve sialylation involved the over expression of sialyltransferases [6]-[8]. This strategy has worked to varying extents though complete sialylation of the recombinant glycoproteins was never achieved.

In addition, it has also been known that nucleotide sugar availability and transport of the proteins through the various compartments of the ER and Golgi are important determinants of extent of protein glycosylation [9]. Various groups have attempted to control the earlier factor by manipulating intracellular sugar pools through addition of nucleotide sugar pre-cursors in the cell culture medium. Particularly, N-acetylmannosamine (ManNAc) has been known to be a specific precursor for increasing intracellular sialic acid pools [10]. It was reported that the addition of increasing amounts of up to 20mM ManNAc to CHO cell cultures producing IFN γ increased the intracellular sialic acid concentration correspondingly. This in turn led to an improvement in sialic acid content of recombinant IFN γ of up to 15%. However, addition of 40mM of ManNAc did not improve sialylation any further. More interestingly, when 20mM ManNAc was fed to NS0 cells producing a recombinant humanized IgG1 [11] and to both CHO and NS0 cells producing TIMP-1 [12], a sialylation increase in the recombinant proteins was not detected despite up to 12 fold increase in intracellular sialic acid concentration. These observations concur with the earlier results reported by Gu *et al.* [10], to indicate a limiting step in the sialylation process at some other point. Hills *et al.* [11] postulated that either the sialyltransferase activity and/or the CMP-sialic acid in the Golgi lumen was limiting. The sialyltransferase over expression strategy had been attempted but the issue of limiting CMP-sialic acid supply had not been addressed. This led us to hypothesize that increasing the expression of the CMP-sialic acid transporter (CMP-SAT) in the cells would increase the transport ability of the CMP-sialic acid in the Golgi lumen, hence increasing the intra-luminal CMP-sialic acid pool. This increased CMP-sialic acid pool

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would alleviate the limiting supply and bring about an increase in sialylation extent of glycoproteins produced in the cells, including the recombinant protein.

The CMP-SAT belongs to a large family of nucleotide sugar transporters, which are antiporters with their corresponding nucleoside monophosphates [13]. The hamster CMP-SAT cDNA was previously isolated through complementation cloning of Lec 2, a glycosylation mutant cell line that had a defect in the CMP-SAT [14]. Since this work was published, the reported cDNA and protein sequence has been deposited in the GenBank (Accession number Y12074) and Swiss Prot (Accession number CAA72794) respectively. The functional activity of the human [15] and murine [16] CMP-SAT was demonstrated, where the hamster homolog would have similar functionality. The membrane topology of this transmembrane protein has also been studied [17].

In this paper, we report the over expression of the hamster CMP-sialic acid transporter in CHO-IFN γ cells. It is our hypothesis that through the over expression of this transporter, an improvement in sialylation of the recombinant IFN γ can be achieved. As this approach is not known to be reported elsewhere, it represents a novel approach to improve sialylation. This strategy can be considered alone, or in combination with existing approaches, to result in better control of the sialylation process for maximal and consistent sialylation of recombinant proteins produced.

II. MATERIALS AND METHODS

A. Mammalian cell lines and media

A CHO cell line expressing human IFN- γ [18] was used for the cloning work. This cell line, referred to as CHO-IFN γ was grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT) and 0.25 μ M methothrexate. Cells were grown as monolayers in stationary T-flasks and incubated at 37°C under a 5% CO $_2$ atmosphere. Cells were detached from T-flasks by adding 0.05% (v/v) trypsin/EDTA solution (Sigma, St. Louis, MO) during regular sub-culturing.

Adherent clones obtained from the stable transfection procedure were adapted for growth in suspension. The initial suspension cultures were grown in protein free HyQ PF-CHO (HyClone), supplemented with 4mM L-glutamine, 0.25 μ M methothrexate, 20U/ml penicillin-20 μ g/ml streptomycin mix (Invitrogen), 0.1% (v/v) Pluronic[®] F-68 (Invitrogen) and 5% (v/v) FBS. These cultures were incubated at 37°C and under an 8% CO $_2$ atmosphere on shaker platforms set at 130rpm in a humidified incubator. The FBS in the suspension media was then gradually stepped down until the cells were able to grow healthily in serum free HyQ PF-CHO supplemented media.

B. Full length cDNA synthesis from CHO-K1

Total RNA was prepared from CHO-K1 by the SV Total RNA Isolation System (Promega, Madison, WI) according

to manufacturer's instructions. All reverse transcription reagents were from Promega. Full length cDNA was synthesized using Moloney Murine Leukaemia Virus Reverse transcriptase (M-MLV RT) for 1 h at 42°C in a reaction mix containing 5x M-MLV reaction buffer, 10mM of each dNTP and 25 units of recombinant RNasin[®] ribonuclease inhibitor.

C. Polymerase Chain Reaction (PCR) Amplification of CMP-SAT

The cDNA prepared from CHO-K1 total RNA was used as a template to amplify the coding region of the CMP-SAT cDNA, based on primers designed from the previously cloned hamster CMP-SAT [14]. BamHI and HindIII restriction sites were introduced upstream and downstream of the coding region for subsequent subcloning. The 5'-PCR primer used was 5'-ATAGGATCCTGCTCAGGCGA-GAGA-3' and the 3'-PCR primer used was 5'-GACAAGCTTTCACACACCAATGAC-3', where the introduced restriction sites are underlined, and the incorporated coding regions of the CMP-SAT are in bold.

All PCR reagents were from Promega. The reaction mix contained 2 μ l of DNA template, 1x Pfu buffer, 250 μ M of each dNTP, 1 μ M of each primer and a Taq-Pfu polymerase mix (approximately 5U). PCR conditions were: 94°C for 6min, followed by 35 cycles of 94°C for 1min, 56°C for 1min, and 72°C for 1min, and a final extension at 72°C for 8min.

D. Construction of CMP-SAT expression vector

The PCR product was first subcloned into pCR[®]-TOPO[®] (Invitrogen) to verify the sequence of the hamster CMP-SAT. The verified PCR product was then subcloned into pCMV-Tag vector (Stratagene, La Jolla, CA), an expression vector containing a FLAG[®] epitope at the N-terminus, and sequenced again. The final plasmid pCMV-FLAG-SAT, was purified using the Maxi Plasmid Purification Kit (Qiagen, Hilden, Germany) and its concentration quantified for transfection into CHO-IFN γ .

E. Transient and stable transfection of DNA into CHO-IFN γ

The transfection was carried out using Fugene 6 transfection reagent (Roche, Basel, Switzerland). Cells were grown overnight in 6-well plates with 0.5 million cells per well and transfected with approximately 1.5 μ g of circular plasmid per well the next day. The Fugene-DNA complex was prepared according to manufacturer's instructions in a 4:1 Fugene 6 transfection reagent (μ l) to DNA (μ g) ratio.

For transient transfections, cells were grown for 48h before they were harvested for FACS analysis. For generation of stable cell lines, the cells were grown for 48h before the media was changed to selection media containing 700 μ g/ml of Geneticin (Sigma), as determined in titrating experiments. The cells were maintained in the selection media for 3 weeks, where the untransfected cells in the selection media died within a week. After 3 weeks, Geneticin-resistant colonies were randomly picked and

subsequently expanded to stable adherent cell lines.

F. FACS analysis

FACS analysis was carried out by labeling cells intracellularly with anti-FLAG[®] M1 mouse monoclonal antibody (Sigma). Adherent CHO-IFN γ was transiently transfected with the following vectors: pcDNA3.1 (+) (Invitrogen), pCMV-FLAG[®]-Luc (Stratagene) and pCMV-FLAG[®]-SAT using Fugene 6 transfection reagent. pCMV-FLAG[®]-Luc is a positive control vector that results in expression of FLAG[®]-Luciferase protein. Approximately 1.5 million cells were used in each FACS preparation. This single cell suspension obtained from each sample was fixed and permeabilized using a Fix & Perm[®] Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). They were then labeled with 1:870 dilution of anti-FLAG[®] M1 mouse monoclonal antibody for 15min. Cells were subsequently washed in 1% BSA/PBS and incubated with 1:500 dilution of secondary anti-mouse IgG FITC (Dako, Copenhagen, Denmark) for 15min in the dark. The cells were then analyzed using the FACSCalibur[™] System (BD Biosciences, San Jose, CA), and the results were computed using the accompanying software's analysis tools.

G. Total RNA extraction and cDNA synthesis from CHO-IFN γ clones

Approximately 10 million cells were collected from stable adherent cell lines and total RNA was extracted using TRIzol[™] reagent (Invitrogen) as follows. Cells were resuspended in 1ml of Trizol[™] reagent and syringe-sheared 50 times with a 21 gauge needle. After a 10min incubation, 200 μ l of chloroform was added and sample was microfuged at 14,000rpm for 15min at 4°C. The upper aqueous layer was transferred to a new RNase-free tube and an equal volume of isopropanol was added. The tubes were then incubated at -20°C for 2h or more. Following that, a visible RNA pellet was seen after microfugation at 14,000rpm for 15min at 4°C. The pellet was washed with 75% (v/v) ethanol, air-dried and dissolved in 35 μ l of DEPC water. RNA quantification was carried out using the GeneQuant[™] Pro RNA/DNA Calculator (Amersham Biosciences, Piscataway, NJ). RNA quality was assessed using the absorbance ratio of 260nm to 280m, where a ratio of 1.9 and above was considered an indicator of RNA with sufficient purity.

Reverse transcription of respective 10 μ g RNA samples to first strand cDNA was carried out with 400U of Improm-II reverse transcriptase and 0.5 μ g of Random Primers (Promega) at 42°C for 60min according to manufacturer's instructions. The reaction was terminated at 70°C for 5min. cDNA was used for subsequent real-time PCR analysis.

H. Real-time PCR

Real-time PCR was carried out using the ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR conditions were: 95°C for 10min, followed by 40 cycles at 95°C for 15s and 60°C for 60s.

The reaction buffer of 25 μ l 1x SYBR[®] Green PCR Master Mix (Applied Biosystems) contained 2.5pmol forward and reverse primers and 2 μ l of cDNA from CHO-IFN γ samples as prepared above. Samples were run in duplicate during each run. Primers for detection of total CMP-SAT and recombinantly expressed CMP-SAT were designed as shown in Fig. 1. Primers for detection of CHO β -actin was 5'-AGCTGAGAGGGAAATTGTGCG-3' as the forward primer, and 5'-GCAACGGAACCGCTCATT-3' as the reverse primer. Standard curves were generated simultaneously for each real-time PCR reaction that was carried out, where serial dilutions of pCMV-FLAG[®]-SAT was used for detection of total and recombinant CMP-SAT and a CHO- β -actin plasmid was used for actin detection.

Using the accompanying software analysis tool, a threshold cycle, C_t is arbitrarily defined as the cycle which a given sample crosses a threshold fluorescence value, where C_t is proportional to the amount of starting DNA template. Thus, a linear plot of C_t versus the logarithm of concentration was interpolated to find the concentration of the unknown samples. Each total and recombinant CMP-SAT concentration was normalized with its respective β -actin concentration, and results from each of the samples from the over expressing clones were compared relative to normalized concentrations obtained from the untransfected CHO-IFN γ sample.

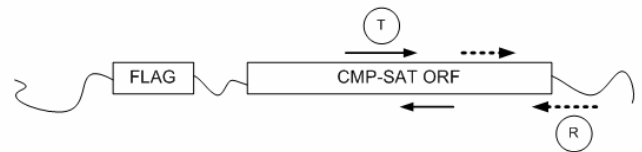


Fig. 1. Primer design for real time PCR. To detect total CMP-SAT expression, primer set T was designed internal to the CMP-SAT open reading frame (ORF) where 5'-primer was 5'-TGATAAGTG-TTGGACTTTTAGC-3' and 3'-primer was 5'-CTTCAGTTGAT-AGGTAACCTGG-3'. To detect recombinantly expressed CMP-SAT, primer set R was designed such that the 5'-primer was within the CMP-SAT ORF and the 3'-primer flanked the ORF and the pCMV-Tag plasmid sequence, as shown in the figure. 5'-primer was 5'-CTGCAGCC-ATTGTTCTTCTAC-3' and 3'-primer was 5'-GTATCGATAAGC-TTTCACACACC-3'.

I. SDS-PAGE and Western blot analysis

Approximately 10 million cells were collected from stable adherent cell lines and washed twice with ice-cold PBS. The cell pellet was lysed and reduced for electrophoresis under conditions described by Eckhardt [14]. Normalized protein lysate samples of 50 μ g were loaded onto a 13% polyacrylamide gel based on a standard Coomassie protein assay (Pierce, Rockford, IL). The fractionated proteins were then electro blotted onto a PVDF membrane (Biorad, Hercules, CA). Blocking was carried out overnight at 4°C using 5% (w/v) non-fat milk in PBS containing 0.1% (v/v) Tween 20. The membrane was then incubated for 1h at room temperature with purified anti-CMP-sialic acid transporter (anti-CMP-SAT). Purified anti-CMP-SAT was obtained through affinity chromatography of rabbit anti-serum against a peptide corresponding to a C-terminus portion of hamster CMP-SAT (Open Biosystems,

Huntsville, AL). The binding of the antibodies was detected using an ECL detection kit (Amersham Biosciences) following manufacturer's instructions. Additional normalization was carried out by immunoblotting for the expression of actin. Membranes were incubated in stripping buffer (100mM β -mercapethanol, 2% (w/v) SDS, 62.5mM Tris-HCl adjusted to pH 6.7) at 50°C for 30min to remove the first set of antibodies, before repeating the western blot analysis with anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

J. Purification and quantification of IFN γ

Supernatant from both the stable clones and untransfected parent CHO-IFN γ was collected from adherent and suspension cultures at the time of peak cell density in duplicate sets and filtered (0.22 μ m). IFN γ was purified through an immunoaffinity column made from purified mouse anti-human IFN γ clone B27 (BD Pharmigen, San Diego, CA), which was run on an AKTA Explorer 100 chromatographic system (Amersham Biosciences, Uppsala, Sweden). 50ml of prepared sample was loaded at 0.2 ml/min onto the anti-human IFN γ column which had been equilibrated with loading buffer (20mM sodium phosphate buffer and 150mM sodium chloride adjusted to pH 7.2). The column was then washed with loading buffer and the sample was eluted at 0.02 ml/min with elution buffer (150mM sodium chloride adjusted to pH 2.5 with HCl). The column was then regenerated with loading buffer for subsequent runs. Quantification of IFN γ was carried out using reverse phase HPLC, where standards of known IFN γ concentration had been run and compared with the actual samples. A Vydac C18 1mm by 250mm column was used (Grace Vydac, Hesperia, CA). The sample was eluted over a 30min linear gradient from 35% (v/v) to 65% (v/v) buffer B (Buffer A: HPLC grade water with 0.1% (v/v) trifluoroacetic acid; Buffer B: HPLC grade acetonitrile with 0.1% (v/v) TFA) at a flow rate of 0.05ml/min.

K. Sialylation analysis of IFN γ

Total sialic acid was measured using a modified version of the thiobarbituric acid assay (TAA) [19]. Three to 5 μ g of purified IFN γ was used for each assay sample, where sialic acid was cleaved from IFN γ using sialidase (0.0025U each) (Roche) treatment before the actual assay. Briefly, each digested sample was mixed with 250 μ l of periodic acid reagent (25mM periodic acid in 0.125N H₂SO₄) and incubated at 37°C for 30min. 200 μ l of arsenite solution (2% sodium arsenite in 0.5N HCl) was added to destroy the excess periodate, before 2ml of thiobarbituric acid reagent (0.1M 2-thiobarbituric acid, adjusted to pH 9 with NaOH) was added. The mixture was then heated at 98°C for 7.5min. Upon cooling on ice, the sample was mixed vigorously with 1.5ml of acid/butanol mixture (n-butanol containing 5% (v/v) 12N HCl) to extract the fluorometric substrate. The sample was then centrifuged at 3,000rpm for 3min. The clear organic phase was transferred to a 10mm cuvette and the fluorescence intensity (λ_{ex} = 550nm, λ_{em} = 570nm) was measured using a Cary Eclipse Fluorescent

Spectrophotometer (Varian Inc, Palo Alto, CA). Sialic acid content of each sample was then quantified based on a standard curve generated from pure sialic acid samples which were treated according to the same procedure simultaneously. The TAA was repeated 3 times, where each sample was run in duplicate. A total of 6 to 8 measurements were used for comparison in the 2-tailed Student's T-test.

L. Glycan site occupancy analysis of IFN γ

Site occupancy of the IFN γ was measured using a Beckman Coulter P/ACE™ MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) using micellar electrokinetic capillary chromatography (MEKC). A 50 μ m diameter x 52cm (40cm length to detector) unfused silica capillary (Beckman Coulter) was used for the separation. Before each separation run, the capillary was cleaned with 0.1M NaOH for 15min, flushed with filtered MQ water for 10min and subsequently equilibrated with running buffer (30mM sodium borate, 30mM boric acid and 100mM sodium dodecyl sulfate at pH 9) for another 15min. Samples were pressure injected at 3psi over 10sec and a 12kV voltage was applied to the capillary over 60 to 80min. The chromatograms were analyzed through peak integration and the percentages of 2-site, 1-site and nonglycosylated peaks were calculated.

III. RESULTS AND DISCUSSION

A. Establishment of CHO-IFN γ cell lines with over expressed CMP-sialic acid transporter

The PCR amplification of the full length CMP-sialic acid transporter (CMP-SAT) was carried out based on primers designed around the CMP-SAT cDNA as described earlier. The expression vector pCMV-FLAG®-SAT was then constructed with this PCR product and plasmids were purified to a concentration between 0.4 to 0.6 μ g/ml for transfection. Forty Geneticin-resistant colonies were picked from transfected cells containing pCMV-FLAG®-SAT. Four cell lines were randomly picked for subsequent analysis. The random selection was carried out since initial efforts to screen the transfected cells for recombinant CMP-SAT expression was not successful, as elaborated in the subsequent section. A control transfection with the null vector, pCMV-Tag was also performed. Fifteen colonies were picked and expanded to null vector cell lines, of which one was chosen arbitrarily to be the blank plasmid control. In addition, a set of untransfected CHO-IFN γ cells were grown together with these cell lines to maintain passage history.

B. FACS analysis of over expressed CMP-sialic acid transporter in transiently transfected cells

FACS analysis was carried out to detect FLAG®-CMP-SAT expression in transfected cells. As a negative control, cells transfected with pcDNA3.1(+) was used. The null vector pCMV-Tag was not used since the FLAG® epitope would still be expressed and detected by FACS. In addition, it was found that negative control cells which had gone

through the same process of transfection made a better control than untransfected cells. The results of the FACS analysis are shown in Fig. 2. The results obtained were as expected. To establish a basis of comparison, a marker region M1, was used to arbitrarily define 1 % of the cell population with higher fluorescence in the negative control. This same marker region defined an increase to 4.9 ± 0.7 % (n=2) of the cell population for the cells transfected with pCMV-FLAG[®]-SAT, indicating the expression of FLAG[®]-CMP-SAT. In addition, an increase to 4.8 ± 1.0 % (n=2) of the cell population in the positive control indicated the expression of FLAG[®]-Luciferase.

With the above results from transient transfection experiments, it was expected that stable cell lines would result in a more significant shift of the cell population to contain higher fluorescence. As such, this FACS procedure could potentially be used to screen the clones for relative expression of CMP-SAT. However, a distinct population shift could not be demonstrated in the FACS analysis of the stable clones (results not shown).

C. Detection of over expressed CMP-sialic acid transporter in stable cell lines

Over expression of CMP-SAT was detected at the transcript level using real-time PCR and at the protein level using Western blot analysis. Real-time PCR is considered a sensitive method to quantify low transcript levels [20]. It was thus found to be suitable for comparing CMP-SAT expression in the over expressing CHO-IFN γ clones versus the untransfected CHO-IFN γ . In addition, due to the specificity of the primers in amplifying selected gene regions, it allowed comparison of total and recombinant expression of CMP-SAT. Results of the real-time PCR are shown in Fig. 3.

The results obtained for the stable cell lines from real-time PCR were more conclusive compared to the FACS analysis. In this case, when the expression of CMP-SAT was compared amongst stable cell lines, a distinct difference could be seen. From (A) of Fig. 3, each of the samples from the 4 adherent clones had relatively similar threshold cycles, which on average indicated a 2 to 5 times increase in expression of total CMP-SAT compared to the untransfected CHO-IFN γ (Table I). The results were even more apparent when recombinant CMP-SAT expression was measured (B). Each of the samples from the 4 clones showed expression, whereas the untransfected parent CHO-IFN γ and blank plasmid control had threshold cycles similar to the water template, which served as a negative control. For these samples, the threshold cycle is reached due to fluorescence caused by primer-dimer formation. The apparent difference in transcript levels of approximately 3 orders of magnitude confirmed recombinant CMP-SAT expression in the clones. This in turn resulted in an overall increase in expression of total CMP-SAT in the positive clones.

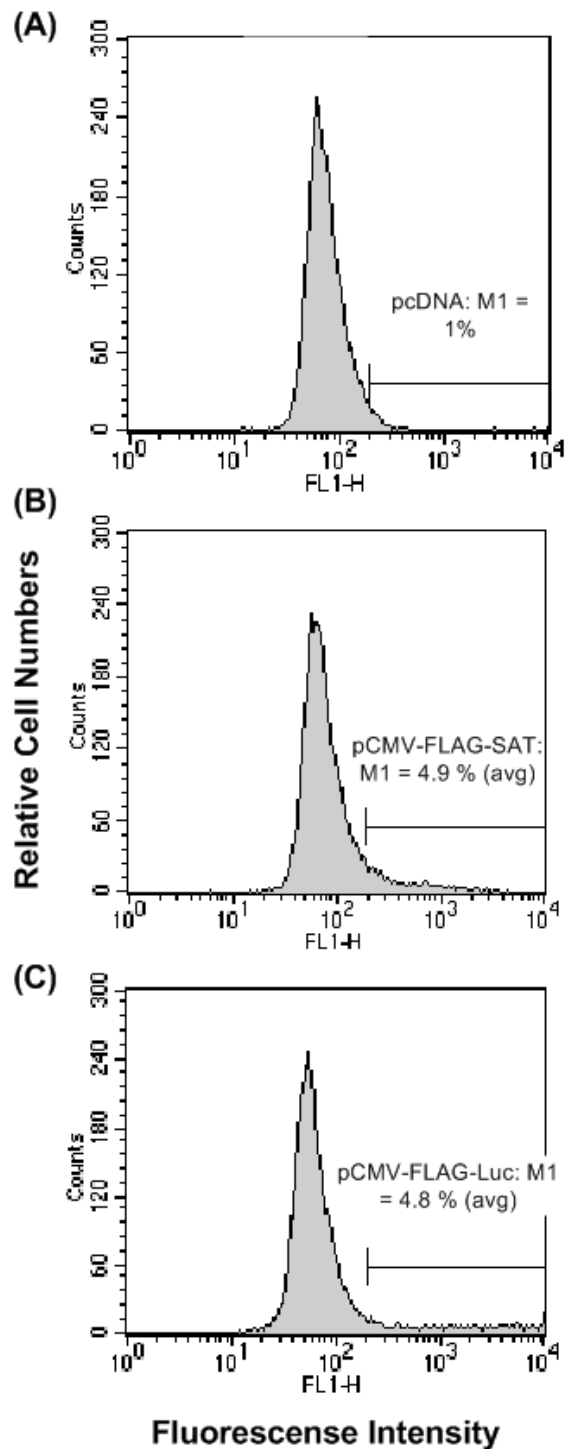


Fig. 2. FACS analysis of transiently transfected cells. Cells were transiently transfected with negative control pcDNA3.1 (+) (A), actual plasmid pCMV-FLAG[®]-SAT (B), and positive control pCMV-FLAG[®]-Luc (C). The comparison of the M1 marker regions showed the expression of the FLAG[®]-CMP-SAT and FLAG[®]-Luciferase in the respective samples.

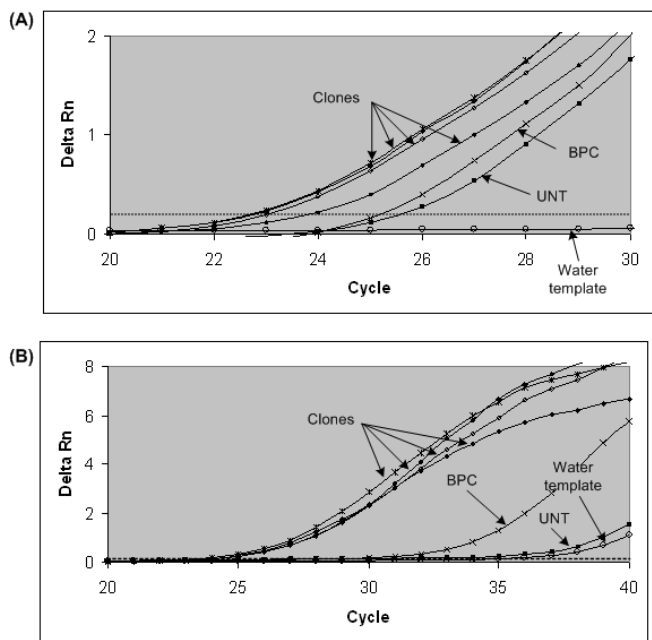


Fig 3. Real time PCR analysis of stable CMP-SAT clones versus untransfected CHO-IFN γ . Total CMP-SAT expression (A) was compared between the samples from the positive clones (clone 1 \blacklozenge , clone 2 \blacklozenge , clone 3 \blackstar and clone 4 \blackstar) and untransfected CHO-IFN γ (UNT \blacksquare). In (B), recombinant CMP-SAT expression was compared. The samples from the blank plasmid control (BPC \times) and water (\circ) served as the other negative controls for the samples and standards respectively. Standard curves were generated by running serial dilutions of known concentrations of plasmids, and hence CMP-SAT, as mentioned in the Materials and Methods. Slope values for plots of C_t versus $-\log_{10}$ mole of total and recombinant CMP-SAT were 3.48 ± 0.07 ($r^2=0.97$) and 3.37 ± 0.04 ($r^2=0.99$) respectively. Based on these standard curves, concentration of CMP-SAT could be calculated for each set of samples. This experiment was carried out at least 3 times with similar results.

CMP-SAT protein over expression in the various clones was detected through Western blot analysis, using a polyclonal antibody which recognizes the C-terminus of the CMP-SAT. The protein expression of CMP-SAT in the clones was compared relative to the untransfected CHO-IFN γ and blank plasmid control (Fig. 4). It was originally intended that the expression of FLAG[®]-CMP-SAT would allow detection of the recombinantly expressed CMP-SAT through commercially available FLAG[®] antibodies. Moreover, it was reported previously that the presence of the N-terminal FLAG[®] sequence would not affect its localization [14] and functional activity [15]. However, the high background caused by non-specific binding with the CHO cell lysate samples prevented the FLAG[®]-CMP-SAT from being detected specifically and conclusively. As a result, antibodies against CMP-SAT itself were used instead.

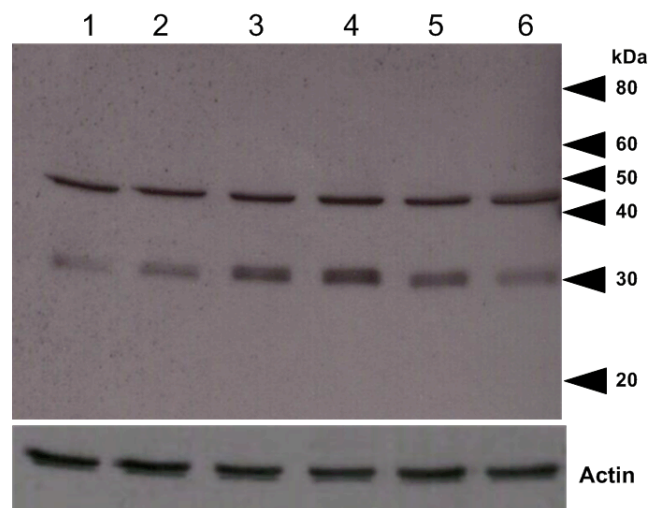


Fig 4. Western blot analysis of CMP-SAT stable clones versus untransfected CHO-IFN γ . FLAG[®]-CMP-SAT was detected using a polyclonal antibody against CMP-SAT as described in Materials and Methods. The bands at approximately 30kDa were deduced to be CMP-SAT with reference to the hamster [14], human [15] and murine CMP-SAT [16] that were previously reported. Samples were cell lysate from (1) untransfected CHO-IFN γ (UNT), (2) Clone 1, (3) Clone 2, (4) Clone 3, (5) Clone 4 and (6) blank plasmid control (BPC).

D. Sialylation analysis of recombinant IFN γ in stable cell lines

The effectiveness of this strategy was demonstrated when a corresponding increase in sialic acid content of the recombinant IFN γ produced by the clones over expressing CMP-SAT was measured. The IFN γ produced was purified from culture supernatant and the sialic acid was cleaved using sialidase treatment. This free sialic acid from IFN γ was measured using the TAA assay (Fig. 5). The average sialic acid content of IFN γ was then obtained by normalizing the sialic acid with the amount of IFN γ analyzed.

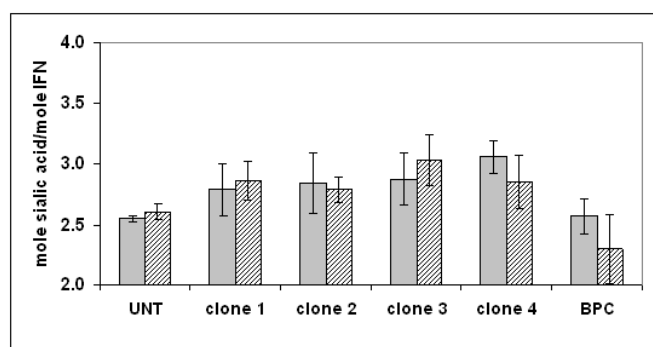


Fig. 5. Sialic acid analysis using a modified thiobarbituric acid assay [19]. The average sialic acid content of IFN γ in moles sialic acid per mole IFN γ for adherent clones (\square) and suspension clones (\square) are as shown. For each set of data, a 2-tailed Student's T test was performed. Average sialic acid content readings of IFN γ from each stable cell line were compared with values obtained from untransfected CHO-IFN γ (UNT) and the blank plasmid control (BPC). The p values obtained were less than 0.05 and measurements were considered statistically significant.

The increase in sialic acid content of IFN γ from the clones was measured relative to untransfected CHO-IFN γ and the blank plasmid control. This was demonstrated in both the IFN γ obtained from the adherent and suspension cell lines (Tables I and II). Moreover, the effect of sialic acid increase was found from randomly selected clones, where the sialic acid content of IFN γ was statistically different from the blank plasmid control and the untransfected CHO-IFN γ (Fig. 5). This reduced the possibility that the sialylation increase was due to clonal differences, rather than CMP-SAT over expression.

TABLE I
IFN γ SIALIC ACID CONTENT FOR ADHERENT CLONES

Cell line	Average sialic acid content (mole sialic acid/mole IFN γ)	Number of glycans per IFN γ ^a	Site sialylation	% inc. ^b	Fold increase in total CMP-SAT exp. ^d
UNT ^c	2.61±0.07	1.79	1.46		1.0
Clone 1	2.86±0.16	1.78	1.61	7.5	5.1
Clone 2	2.79±0.10	1.74	1.63	8.5	4.2
Clone 3	3.03±0.21	1.80	1.68	11.0	4.2
Clone 4	2.85±0.21	1.74	1.64	9.0	2.4
BPC ^c	2.30±0.28	1.77	1.30	(8.0)	1.1

^aNumber of glycans per IFN γ was calculated based on site occupancy data. Its formula is the denominator of (1).

^bPercentage increase (% inc.) was the increase in percent site sialylation as compared with the untransfected CHO-IFN γ

^cUNT represents the untransfected CHO-IFN γ and BPC represents the blank plasmid control

^dResults were calculated from real-time PCR experiments, where relative quantification of normalized total CMP-SAT levels was carried out with respect to untransfected CHO-IFN γ .

TABLE II
IFN γ SIALIC ACID CONTENT FOR SUSPENSION CLONES

Cell line	Average sialic acid content (mole sialic acid/mole IFN γ)	Number of glycans per IFN γ ^a	Site sialylation	% inc. ^b	Fold increase in total CMP-SAT exp. ^d
UNT ^c	2.55±0.05	1.75	1.46		1.0
Clone 1	2.81±0.20	1.70	1.65	9.6	3.3
Clone 2	2.88±0.11	1.68	1.72	12.8	2.2
Clone 3	2.88±0.13	1.74	1.66	10.0	2.6
Clone 4	3.05±0.09	1.71	1.78	16.3	3.1
BPC ^c	2.30±0.28	1.71	1.51	2.6	1.0

^aNumber of glycans per IFN γ was calculated based on site occupancy data. Its formula is the denominator of (1).

^bPercentage increase (% inc.) was the increase in percent site sialylation as compared with the untransfected CHO-IFN γ

^cUNT represents the untransfected CHO-IFN γ and BPC represents the blank plasmid control

^dResults were calculated from real-time PCR experiments, where relative quantification of normalized total CMP-SAT levels was carried out with respect to untransfected CHO-IFN γ .

The site occupancy of the IFN γ from adherent and stable clones was measured and results are shown in Fig. 6. As expected, the over expression of CMP-SAT did not significantly affect the site occupancy of IFN γ , since the over expression of CMP-SAT does not affect the transfer of the glycan to the protein, which is what influences site occupancy. However, the site occupancy data was used to normalize the average sialic acid content of IFN γ against the

number of available N-linked sites, to give a more accurate index of measurement known as site sialylation, as in (1). Site sialylation is an index that enables us to directly consider the ability of the cell to sialylate an available site when manipulated by various conditions (Fox, 2003).

$$\frac{\text{Molecules sialic acid}}{\text{Available N-linked site}} = \frac{\text{Mole sialic acid / mole IFN}\gamma}{0.01(2 \cdot \%2N + 1 \cdot \%1N + 0 \cdot \%0N)} \quad (1)$$

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

Tables I and II show the site sialylation data calculated from the IFN γ sialic acid content data for the adherent and suspension cell lines respectively. The adaptation of the adherent clones to suspension growth did not significantly affect the extent of sialylation increase. For the adherent clones, a sialylation increase of between 7.5 to 11.0% was measured. This was accompanied by a 2.4 to 5.1 fold increase in total CMP-SAT expression measured. For the suspension clones, a sialylation increase of between 9.6 to 16.3% was measured, which was accompanied by a 2.2 to 3.3 fold increase in total CMP-SAT expression.

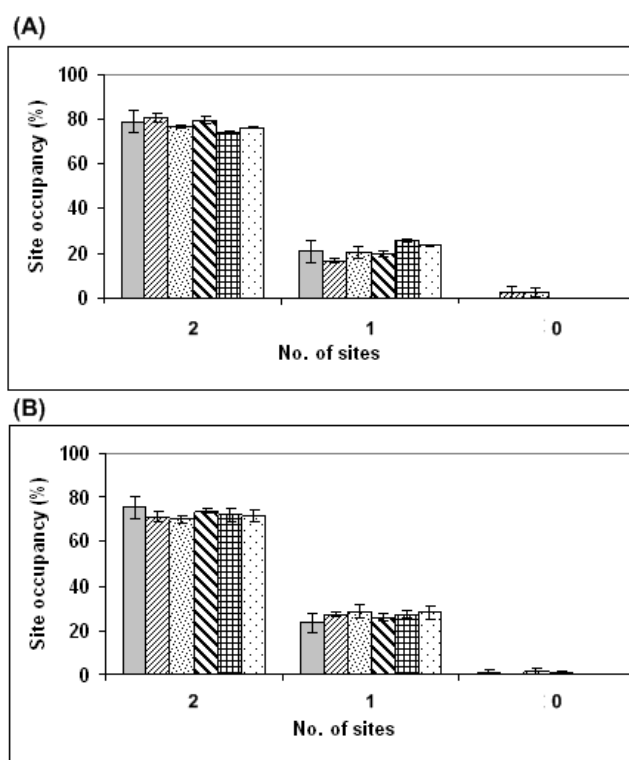


Fig. 6. Glycan site occupancy data of IFN γ from adherent clones (A) and suspension clones (B) using MEKC. There were no distinct differences in the site occupancy of both the over expressing CMP-SAT cell lines (clone 1 \square , clone 2 \square , clone 3 \square and clone 4 \square) and the blank plasmid control (\square), as well as the untransfected CHO-IFN γ (\square). 0-site glycosylated IFN γ could not be detected in the IFN γ obtained from some of the cell lines. The standard deviation was obtained from duplicate runs of each sample.

Our results demonstrate the effectiveness of over expressing CMP-SAT to improve sialylation of recombinant proteins in CHO cells. To our best knowledge, this approach has not been reported elsewhere.

However, similar glyco-engineering approaches have been reported extensively. Some groups have manipulated glycosylation patterns of existing glycoproteins, making mutations in their polypeptide chain to add oligosaccharides [22] or mutating the positions of oligosaccharides [23], to produce more efficacious proteins. A more common approach involves the genetic manipulation of the host glycosylation pathway to generate glycoform distributions that are more predictable and consistent. This can be through the introduction or over expression of glycosyltransferase genes [24]-[26] or antisense inhibition of endogenous glycosylation genes [27] into the host cells. Of these approaches, many groups have attempted to over express sialyltransferases to improve sialylation ([6]-[8] and [28]-[30]). Bragonzi *et al.* [6] created a universal CHO cell line which over expressed α 2,6-sialyltransferase (α 2,6-ST) that was used to modulate extent of α 2,3-linked sialic acids endogenously produced by CHO cells. Weikert *et al.* [8] reported an increase of average sialic acid content of TNFR-IgG by 30% upon over expression of α 2,3-sialyltransferase (α 2,3-ST). Fukuta *et al.* [7] measured a maximum sialic acid increase of 23% when α 2,3 and α 2,6-ST was over expressed in combination with the glycosylation enzyme involved with branching, β 1,6-N-acetylglucosaminyltransferase (GnT-V). The sialylation improvement obtained from the reported approach of 9.6 to 16.3% was comparable to these findings.

Through the results presented, we verified the hypothesis that a limiting supply of CMP-sialic acid in the Golgi lumen was caused by the lack of its transport into the Golgi carried out by the CMP-SAT. This strategy proved to be effective in increasing the extent of sialylation in recombinant IFN γ produced. However, the effectiveness of this strategy is subject to the cell-type variations in its glycosylation machinery as well as cellular demand for sialic acid. The endogenous expression of CMP-SAT may vary in different cells, and this strategy will prove more effective in cells with low amounts of CMP-SAT. Alternatively, if the supply of CMP-sialic acid is artificially increased, for example, through feeding of N-acetylmannosamine (ManNAc); improving the transport of CMP-sialic acid through CMP-SAT over expression may also be useful. In fact, the saturation effect observed by Gu *et al.* [10] on feeding 40mM ManNAc should be alleviated through CMP-SAT over expression if our hypothesis is correct. This is currently being tested in our laboratory. The demand for sialic acid depends on the amount of acceptor sites in the Golgi lumen [12], which can in turn depend on the type of recombinant glycoprotein being produced and the rate of its production or specific productivity. In other words, a highly glycosylated protein which is being produced at high specific productivities results in a situation where the endogenous glycosylation machinery is unable to cope and where sialylation improvement strategies such as CMP-SAT

over expression may prove more effective. Thus, we are currently employing our strategy on another CHO cell line which has some of the above characteristics as a further proof of concept. This will also demonstrate this strategy is generic as with sialyltransferase over expression.

In conclusion, we have established a novel means of improving sialylation of recombinant proteins. The use of CMP-sialic acid transporter over expression was demonstrated to improve sialylation of IFN γ produced by CHO cells. This established strategy can be considered alone or in combination with existing strategies for sialylation improvement. For example, N-acetylmannosamine feeding can be used to increase the CMP-sialic acid supply in the cells, with CMP-sialic acid transporter over expression used to improve its transport into the Golgi for sialylation. Such multi-prong approaches are required to effectively manipulate the complex sialylation process and help to address potential limiting factors more extensively. This will bring us closer to the goal of producing recombinant glycoproteins of high and consistent sialylation from mammalian cells.

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