Engineering Intracellular CMP-Sialic Acid Metabolism into Insect Cells and Methods to Enhance Its Generation†

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ABSTRACT: Previous studies have reported that insect cell lines lack the capacity to generate endogenously the nucleotide sugar, CMP-Neu5Ac, required for sialylation of glycoconjugates. In this study, the biosynthesis of this activated form of sialic acid completely from endogenous metabolites is demonstrated for the first time in insect cells by expressing the mammalian genes required for the multistep conversion of endogenous UDP-GlcNAc to CMP-Neu5Ac. The genes for UDP-GlcNAc-2-epimerase/ManNAc kinase (EK), sialic acid 9-phosphate synthase (SAS), and CMP-sialic acid synthetase (CSAS) were coexpressed in insect cells using baculovirus expression vectors, but the CMP-Neu5Ac and precursor Neu5Ac levels synthesized were found to be lower than those achieved with ManNAc supplementation due to feedback inhibition of the EK enzyme by CMP-Neu5Ac. When sialuria-like mutant EK genes, in which the site for feedback regulation has been mutated, were used, CMP-Neu5Ac was synthesized at levels more than 4 times higher than that achieved with the wild-type EK and 2.5 times higher than that achieved with ManNAc feeding. Addition of *N*-acetylglucosamine (GlcNAc), a precursor for UDP-GlcNAc, to the media increased the levels of CMP-Neu5Ac even more to a level 7.5 times higher than that achieved with ManNAc supplementation, creating a bottleneck in the conversion of Neu5Ac to CMP-Neu5Ac at higher levels of UDP-GlcNAc. The present study provides a useful biochemical strategy to synthesize and enhance the levels of the sialylation donor molecule, CMP-Neu5Ac, a critical limiting substrate for the generation of complex glycoproteins in insect cells and other cell culture systems.

Many of the high-value therapeutic proteins in the market and in clinical development today are glycoproteins. The specific glycan structures present on these glycoproteins are essential for their structure, stability, and functionality (*1*, *2*) and affect a number of physiological properties including in vivo half-life, bioavailability, and tissue targeting. The glycan structures attached to glycoproteins can also vary with species, tissue, and developmental stage of the organism. Mammalian cells typically produce proteins with complex glycans terminating in sialic acid, which are a family of nine carbon 2-keto-3-deoxy sugars (*3*). The most abundant of over 50 different sialic acids identified in nature is *N*-acetylneuraminic acid (Neu5Ac)[1]. By virtue of its negative charge and terminal position on many glycans, sialic acid has a unique and significant effect on the stabilization of the structure (*4*, *5*), antigenicity, and in vivo circulatory half-life of the proteins to which it is attached (*2*). Sialic acids present on cell surface glycans have been implicated in cell−cell signaling and cell-molecular interactions in processes such as inflammation and the immune responses (*1*). Changes in sialylation have also been implicated in oncogenic transformations and tumorogenesis in vertebrates (*3*, *6*, *7*).

In contrast to mammalian cells, insect cells typically produce nonsialylated glycans which contain paucimannosidic (low mannose), high mannose, or occasionally hybrid structures (*8*). Such simple glycan structures greatly reduce the bioavailability and functional activity of glycoproteins due to their rapid in vivo clearance, rendering the glycoproteins of insect cell origin less suitable for therapeutic use. To make insect cells synthesize mammalian type complex sialylated glycans, the cells require the expression of all the requisite glycosyltransferases combined with the presence

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[1] Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; SAS, sialic acid 9-phosphate synthase; ManNAc, *N*-acetylmannosamine; Sf9, *Spodoptera frugiperda*; UDP-GlcNAc, uridine diphosphate GlcNAc; EK, UDP-GlcNAc-2-epimerase/ManNAc kinase; GlcNAc, *N*-acetylglucosamine; KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; Man, Mannose; CMP-Neu5Ac, cytidine monophosphate Neu5Ac; ManNAc-6-P, ManNAc-6-phosphate; Man-6-P, Mannose-6-phosphate; CSAS, cytidine monophosphate sialic acid 9-phosphate synthase; CMP-KDN, cytidine monophosphate 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; AcSAS, *Autographa californica* baculovirus containing sialic acid-9-phosphate synthase gene; DMB, 1,2-diamino-4,5-methylene dioxybenzene dihydrochloride; HPLC, high-performance liquid chromatography; PEP, phosphoenolpyruvate; Neu5Ac-9-P, *N*-acetylneuraminic acid 9-phosphate; AcEK, *Autographa californica* baculovirus containing UDP-GlcNAc-2-epimerase/ManNAc kinase gene; AcEKR263L, *Autographa californica* baculovirus containing R263L mutation UDP-GlcNAc-2-epimerase/ManNAc kinase gene.

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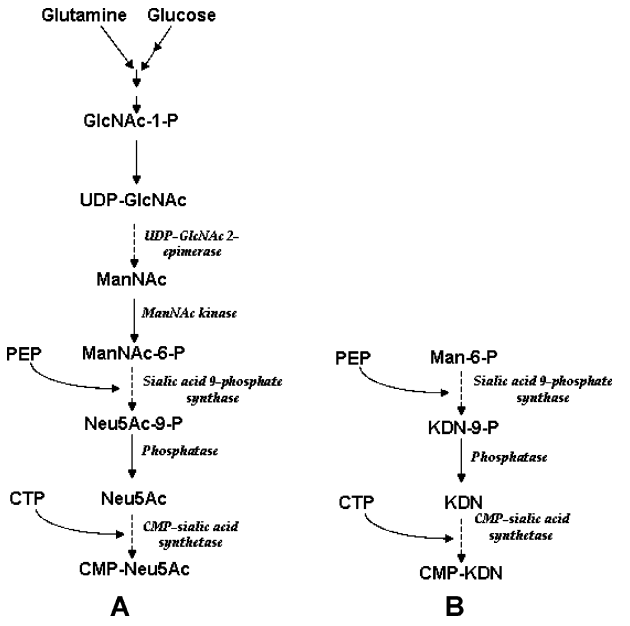

FIGURE 1: (A) CMP-Neu5Ac and (B) CMP-KDN synthesis pathway in mammalian cells. Dotted arrows indicate metabolic activities which are insufficient in insect cells.

of the various donor sugar nucleotides. Previously, the glycosyltransferase activities in insect cells have been studied, and the deficiciency in activities such as *N*-acetylglucosaminyltransferase-I, *N*-acetylglucosaminyltransferase-II, β(1,4)-galactosyltransferase, and α(2,6)-sialyltransferases have been overcome by expressing mammalian glycosyltransferases (*8*−*10*). The expression of these glycosyltransferase activities must be complemented with the presence of the required sugar nucleotides in order for cells to synthesize mammalian-type complex glycans. The presence of various sugar nucleotides also have been investigated in several insect cell lines (*11*, *12*). While a number of sugar nucleotides involved in glycosylation are present in substantial quantities in insect cells (*12*), levels of cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), the donor sugar nucleotide for sialylation, is negligible in insect cells grown in serum-free medium (*11*−*13*). The precursor to CMP-Neu5Ac, *N*-acetylneuraminic (Neu5Ac), is also found to be absent or present in insignificant quantities in insect cells (*14*). Since CMP-Neu5Ac and Neu5Ac are not easily incorporated into cells from the medium (*15*), metabolic engineering strategies have been considered in order to achieve sufficient intracellular levels of Neu5Ac and CMP-Neu5Ac in vivo. This approach involves identifying the requirements for CMP-Neu5Ac synthesis in insect cells and complementing activities that are missing with genetic engineering and substrate supplementation.

The pathway steps dedicated to the biosynthesis of CMP-Neu5Ac in mammalian cells are shown in Figure 1A. UDP-GlcNAc, typically generated intracellularly from sugars, amino acids, amino sugars, and UTP, is first epimerized to *N*-acetylmannosamine (ManNAc). This reaction is catalyzed by UDP-*N*-acetylglucosamine-2-epimerase (UDP-GlcNAc-2-epimerase). ManNAc is subsequently phosphorylated by ManNAc kinase activity to yield ManNAc-6-phosphate (ManNAc-6-P). In mammalian cells, these two activities are present on a single bifunctional enzyme UDP-GlcNAc-2-epimerase/ManNAc kinase (EK), in which the N-terminal domain contains the 2-epimerase activity and the C-terminal domain includes the kinase activity (*16*, *17*). Next, Neu5Ac-9-phosphate is synthesized in the presence of sialic acid 9-phosphate synthase (SAS) by the condensation of ManNAc-6-phosphate and phosphoenolpyruvate (PEP) (*18*). This SAS enzyme is also capable of synthesizing an alternative sialic acid, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid-9-phosphate (KDN-9-P), from mannose-6-phosphate (Man-6-P) (*14*). The Neu5Ac-9-phosphate and KDN-9-P are then dephosphorylated by unknown dephosphorylases to yield Neu5Ac and KDN, respectively. Finally, the synthesized sialic acid is activated by CTP in order to synthesize CMP-Neu5Ac or CMP-KDN in the presence of CMP-sialic acid synthetase (CSAS) (*13*). In mammalian cells, this activation occurs in the nucleus where the CSAS is localized (*13*, *19*). This sugar nucleotide diffuses into the cytoplasm where it is subsequently transported into the Golgi by CMP-sialic acid transporter and utilized by sialyltransferases in the generation of sialylated glycans attached to proteins and lipids (*20*).

Most insect cells lack desired expression levels of a number of enzymes in the CMP-Neu5Ac synthesis pathways and, as a result, are unable to synthesize significant quantities of CMP-Neu5Ac or the precursors Neu5Ac and ManNAc (*13*). The UDP-GlcNAc-2-epimerase activity, responsible for generating ManNAc, has been reported to be under the detection limit in Sf9 cells (*17*). While insect cells do not express UDP-GlcNAc-2-epimerase/ManNAc kinase, they do have a ManNAc kinase activity derived from endogenous GlcNAc kinase (*21*). Insect cells also have insufficient levels of sialic acid 9-phosphate synthase and CMP-sialic acid synthetase activities (*13*, *14*).

Initial efforts to engineer CMP-Neu5Ac synthesis pathway in Sf9 cells involved infecting these cells with a baculovirus, AcSAS, containing the human sialic acid 9-phosphate synthase gene (*sas*) (*14*). Sf9 cells, when cultured in ManNAc supplemented medium and infected with AcSAS, were able to synthesize Neu5Ac and an alternate sialic acid KDN (*14*). While the primary product of the SAS enzyme in insect cells is Neu5Ac-9-P, the cells were capable of dephosphorylating the sialic acid product using endogenous phosphatase activity. Subsequently, synthesis of CMP-Neu5Ac was achieved when cells were co-infected with AcSAS and another baculovirus containing the human CMP-sialic acid 9-phosphate synthetase (CSAS) gene in the presence of ManNAc supplementation (*13*). In the absence of ManNAc supplementation, Sf9 cells generated significant intracellular CMP-KDN levels from endogenous Man-6-P levels in the cells. Aumiller et al. (*22*) followed up on this research by producing N-glycans in a recombinant Sf9 cell line stably expressing sialic acid synthase and CMP-sialic acid synthetase along with five glycosyltransferases. Once again, these cells required ManNAc supplementation for the synthesis of CMP-Neu5Ac and effective sialylation of glycans. While Sf9 cells lack sufficient intracellular pools of the sialylation precursor, ManNAc, analytical studies have indicated the presence of significant pools of the UDP-GlcNAc-2-epimerase/ManNAc kinase substrate, UDP-GlcNAc, in insect cells (*11*, *12*). Recently, Sf9 cells were co-infected with two baculoviruses containing the genes for sialic acid 9-phosphate synthase and UDP-GlcNAc-2-epimerase/ManNAc kinase (*23*). With this co-infection, Sf9 cells were able

to synthesize high levels of Neu5Ac from their endogenous pools of UDP-GlcNAc. The addition of GlcNAc to the medium resulted in the generation of even higher levels of Neu5Ac as a result of higher levels of substrate available for generation of Neu5Ac.

In this study, we have evaluated the ability to achieve a complete synthesis of CMP-Neu5Ac in Sf9 cells using metabolic engineering of the relevant pathways. However, unlike the synthesis of Neu5Ac, CMP-Neu5Ac production must also consider the allosteric feedback inhibition of UDP-GlcNAc epimerase activity by the nucleotide sugar product, CMP-Neu5Ac (*24*, *25*). To address this feedback regulatory loop, a mutant UDP-GlcNAc-2-epimerase/ManNAc kinase, which lacks the site for feedback inhibition by CMP-Neu5Ac, was used, and production levels were compared to those achieved with the wild-type epimerase/kinase enzyme. The effect of substrate supplementation on CMP-Neu5Ac production was also evaluated by considering the addition of GlcNAc to the system. These studies will provide significant insight into the development of biochemical strategies that can be used to generate high levels of donor sugar nucleotides in insect cells, mammalian cells, and other cell culture systems. Since the levels of CMP-Neu5Ac can often limit the sialylation process in cells, such an approach may be beneficial for generating glycan structures with improved sialylation characteristics in various cell culture systems.

MATERIALS AND METHODS

Creation of Point Mutants of UDP-GlcNAc-2-epimerase/ManNAc Kinase. Point mutations in UDP-GlcNAc-2-epimerase/ManNAc kinase were introduced using the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the rat cDNA of UDP-GlcNAc-2-epimerase/ManNAc kinase cloned into the pFastBacHTA vector (*17*) as the template. The primers used were 5'-GCAAGGAGATGGTTCTAGTGATGCGGAAGAAGG-3' for the generation of the R263L mutant and 5'-GGTTCGAGTGATGTGGAAGAAGGGCATCGAGC-3' for the generation of the R266W mutant. Temperature-cycling amplification was performed with *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA). Parental template DNA was then digested with *Dpn*I. The synthesized plasmids containing the desired mutation were transformed into supercompetent InvαF′ *E. coli* cells (Invitrogen, Carlsbad, CA). The sequences of all the constructs were verified before use.

These mutants were expressed in insect cells using the Bac-to-Bac system (Invitrogen, Carlsbad, CA) as described in Blume et al. (*26*). In brief, constructs of the point mutants were transformed into DH10Bac *E. coli* cells for transposition into bacmid-DNA. Recombinant bacmid-DNA was isolated and transfected into Sf9 insect cells, which produce recombinant baculoviruses containing the modified UDP-GlcNAc-2-epimerase/ManNAc kinase genes. Baculoviruses were then used for protein overexpression in Sf9 cells; the proteins were purified by Ni−NTA affinity chromatography and analyzed as previously described (*26*).

Preparation of Sugar Solutions. Sugar solutions (1 M) of ManNAc and GlcNAc (Sigma chemicals, St. Louis, MO) were prepared by dissolving them in water followed by filter sterilization.

Cell Culture. Sf9 (Invitrogen, Carlsbad, CA) cells were grown in serum-free Sf900 medium in shaker flasks. Approximately 2.4×10^6 Sf9 cells taken from cell cultures at densities between 1.5 and 2.0×10^6 cells/mL were plated in each well of a 6-well plate. After the cells adhered, the medium was removed and 2 mL of fresh medium was added to each well. Cells in each well were either infected with 20 μL of each baculovirus or left uninfected. Baculovirus infections were performed as described in O'Reilly et al. (*27*). The medium was supplemented by the appropriate sugar solution at the time of infection.

Cells were harvested 96 h postinfection by removing the cell culture medium and washing the cells twice with phosphate buffered saline (Invitrogen, Carlsbad, CA). The cells were resuspended in either deionized water (for sialic acid and total protein measurement) or 75% ice-cold ethanol (for CMP-sialic acid measurements), vortexed, and then sonicated with a Tekmar sonic disruptor (Cincinnati, OH) for 30 s at 50% cycle at a power setting of 5. Samples were analyzed for total protein content using a BCA assay kit (Pierce, Rockford, IL) and a 96-well plate reader (Molecular Devices, Sunnyvale, CA) and analyzed for sialic acids and CMP-sialic acids as described below.

Neu5Ac/KDN Detection. Previous studies have indicated the absence of sialic acid on cellular proteins for Sf9 cells lacking the expression of both heterologous galactosyltransferase and sialyltransferase genes (*28*, *29*). Consequently, any incorporation of Neu5Ac or KDN into cell proteins was ignored. The free sialic acid content was measured by the procedure as described by Hara et al. (*30*). Ninety-five microliters of 1, 2-diamino-4, 5-methylene dioxybenzene dihydrochloride (DMB; Sigma, St. Louis, MO) reducing solution (7.0 mM DMB in 1.4 M acetic acid, 0.75 M β-mercaptoethanol, and 18 mM sodium hydrosulfite) was added to 5 μL of sample and incubated at 50 °C for 2.5 h from which 3−10 μL was used for HPLC analysis on a Shimadzu (Columbia, MD) VP series HPLC using a Waters (Milford, MA) Spherisorb 5-μm ODS2 column. A Shimadzu RF-10AXL fluorescence detector with 448-nm emission and 373-nm excitation wavelengths was used for detecting peaks. An acetonitrile, methanol, and water mixture (9:7:84, v/v/v) with a flow rate of 0.7 mL/min was the mobile phase. Response factors of Neu5Ac and KDN were established with authentic standards based on peak areas for quantifying sample sialic acid levels. Sialic acid content was normalized based on the total protein content measured with the BCA assay kit (Pierce, Rockford, IL) and a Molecular Devices (Sunnyvale, CA) microplate reader.

CMP-Sialic Acid Measurement. Cells harvested 96 h postinfection by removing cell culture medium and washing cells twice with phosphate buffered saline (Invitrogen, CA) were split into two fractions. One fraction was resuspended in water and lysed using a Tekmar Sonic Disruptor (Cincinnati, OH). This was used for quantifying total protein. The other fraction was resuspended in 300 μL of ice-cold 75% ethanol and lysed by sonication. Cells resuspended in ice-cold 75% ethanol were centrifuged at 14 000 rmp for 10 min, and the soluble fraction obtained was lyophilized. The lyophilized samples were then resuspended in 120 μL of 40 mM phosphate buffer, pH 9.2, filtered, and analyzed by HPAEC as described previously (*12*). The CMP-Neu5Ac levels were normalized with respect to total protein deter-

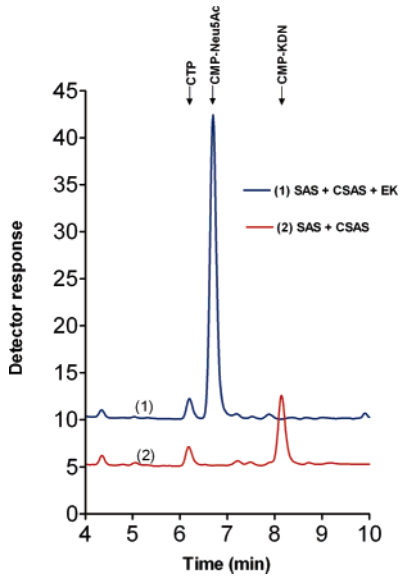

FIGURE 2: Comparison of CMP-Neu5Ac and CMP-KDN production levels in Sf9 cells cultured in serum-free medium and co-infected with AcSAS, AcCSAS, and AcEK with the levels obtained following co-infection with AcSAS and AcCSAS only. The cells were harvested 4 days postinfection, and the sugar nucleotides extracted were separated by HPAEC with UV detector measuring A_{260}. Detector responses on the chromatograms have been normalized using the total protein concentration in lysates for each sample.

mined in the supernatant of PBS-washed cells and lysed in water by sonication, using a BCA protein assay kit (Pierce, Rockford, IL).

RESULTS

CMP-Neu5Ac Can Be Synthesized in Sf9 Cells from Endogenous in Vivo Metabolites. Previously, Lawrence et al. (*14*) showed that Neu5Ac could be generated in Sf9 cells by infecting them with a baculovirus containing the human sialic acid 9-phosphate synthase gene (AcSAS) and supplementing the cell culture medium with Neu5Ac precursor ManNAc. Also, Sf9 cells were able to synthesize CMP-Neu5Ac when the medium was supplemented with ManNAc and the cells were co-infected with AcSAS and a baculovirus containing CMP sialic acid synthase gene (AcCSAS) (*13*). In an effort to generate CMP-Neu5Ac completely from insect cells' endogenous intracellular metabolites, insect cells were infected with baculoviruses AcSAS and AcCSAS in concert with a baculovirus containing the gene of rat UDP-GlcNAc-2-epimerase/ManNAc kinase (AcEK).

From the cell lysates of the triply infected cells, intracellular sugar nucleotides were extracted and examined by HPAEC. Following the simultaneous infection of these three baculoviruses, Sf9 cells were able to synthesize CMP-Neu5Ac, even when grown in serum-free medium without any medium supplementation (Figure 2). The synthesis of CMP-Neu5Ac with this triple infection confirms the presence of sufficient levels of the intracellular precursor, UDP-GlcNAc. In contrast, cells grown in the same medium and infected with AcSAS and AcCSAS in the absence of AcEK infection did not generate detectable levels of CMP-Neu5Ac. Clearly, the levels of intracellular ManNAc present are insufficient for generating CMP-Neu5Ac without the inclusion of a heterologous EK enzyme. However, Sf9 cells co-infected with only AcSAS and AcCSAS did synthesize CMP-KDN as alternative nucleotide sugar in agreement with results previously reported by Lawrence et al. (*13*) (Figure 2). The levels of CMP-Neu5Ac obtained were 16.94 nmol/mg of protein for cells expressing all three sialic acid pathway genes, while the levels of CMP-KDN were found to be less than 3 nmol/mg of protein from cells expressing SAS and CSAS alone.

As a comparison, Sf9 cells cultured in medium containing 10 mM ManNAc were infected with AcSAS and AcCSAS and the CMP-Neu5Ac levels were measured and compared to the levels obtained with a triple infection (AcSAS + AcCSAS + AcEK). As observed in Figure 3A, CMP-Neu5Ac levels obtained with ManNAc feeding were 1.7 times higher than the levels obtained from endogenous substrates in concert with the heterologous EK protein. The intracellular precursor Neu5Ac levels measured in this experiment were also higher in the cells fed with ManNAc as compared to the cells expressing EK (Figure 3B). In contrast, when Sf9 cells were infected with AcSAS and fed ManNAc in the absence of AcCSAS, the levels of Neu5Ac synthesized (18.7 nmol/mg) were 3-fold lower than the Neu5Ac levels obtained with AcSAS and AcEK co-infection (54.7 nmol/mg). Indeed, for the cells co-infected with AcSAS and AcEK, Neu5Ac production was lowered considerably when the cells were also co-infected with AcCSAS virus. This decline in Neu5Ac production when expressing CSAS is likely due to the feedback inhibition of the UDP-GlcNAc-2-epimerase enzyme by the CMP-Neu5Ac product. This feedback inhibition of EK activity may also be the cause of the lower CMP-Neu5Ac levels observed in Figure 3A when this metabolite was generated from endogenous UDP-GlcNAc using heterologous EK as compared to obtaining CMP-Neu5Ac from fed ManNAc without any endogenous source. While ManNAc obtained from EK was limited due to feedback regulation by CMP-Neu5Ac, levels of intracellular ManNAc incorporated from the medium were not subject to feedback inhibition by CMP−Neu5Ac.

Generation of Sialuria Mutants of Rat UDP-GlcNAc-2-epimerase/ManNAc Kinase. The decreased synthesis of CMP-Neu5Ac in the presence of endogenous ManNAc synthesis may be attributed to feedback inhibition of UDP-GlcNAc-2-epimerase activity by the product CMP-Neu5Ac. To determine whether this bottleneck to CMP-Neu5Ac synthesis could be overcome, baculovirus constructs were generated containing rat UDP-GlcNAc-2-epimerase/ManNAc kinase mutants that included either an arginine to leucine mutation at amino acid position 263 (R263L) or an arginine to tryptophan mutation at amino acid position 266 (R266W). The two mutants were previously found in the human UDP-GlcNAc epimerase/ManNAc kinase genes of patients of sialuria (*31*), a hereditary metabolic disease in humans characterized by the intracellular accumulation of undesirably high levels of Neu5Ac. The mutations in UDP-GlcNAc-2-epimerase prevent the binding of CMP-Neu5Ac to UDP-GlcNAc-2-epimerase, and thus, the cells are unable to regulate the levels of CMP-Neu5Ac synthesized, causing the metabolite to accumulate to toxic levels. The accumulation results in excessive excretion of free sialic acid accompanied by the inability to coordinate voluntary muscular movements and severe psychomotor deterioration (*32*).

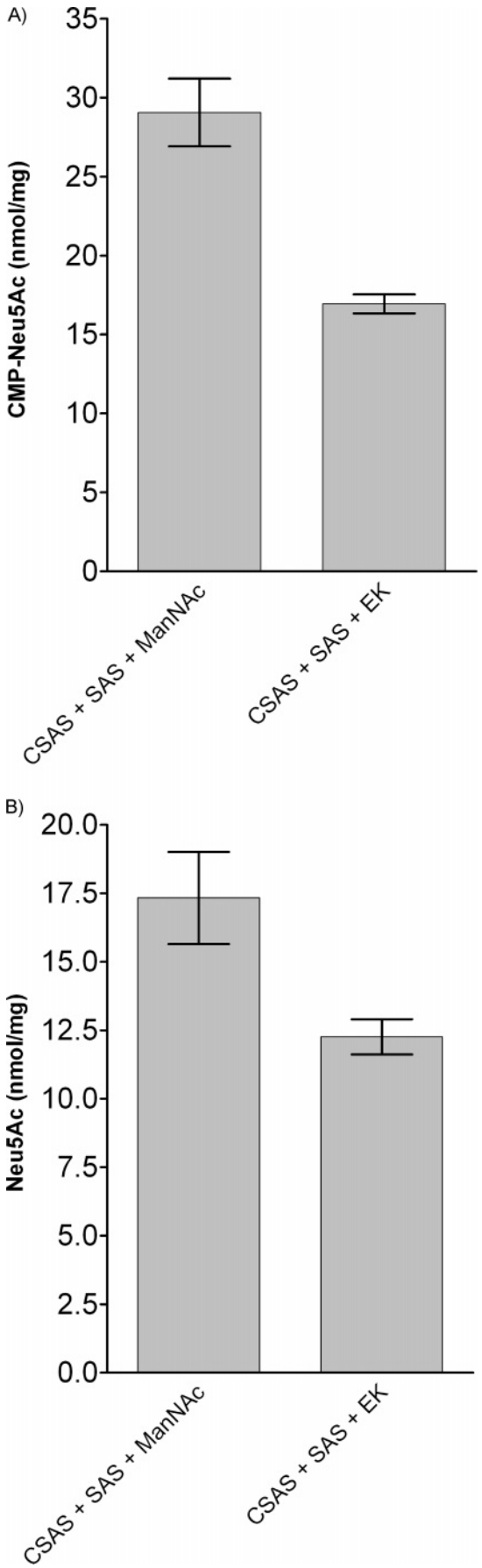

FIGURE 3: (A) CMP-Neu5Ac levels and (B) Neu5Ac levels in Sf9 cells cultured in serum-free medium co-infected with AcCSAS and AcSAS along with 10 mM ManNAc feeding compared to levels obtained following co-infection with AcCSAS, AcSAS, and AcEK.

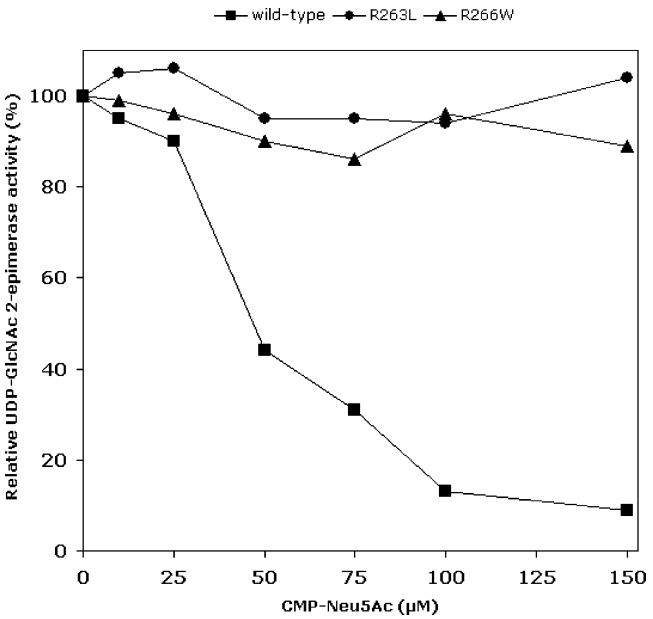

FIGURE 4: Inhibition of wild-type and mutated UDP-GlcNAc-2-epimerase/ManNAc kinase by CMP-Neu5Ac. Enzymes were expressed in Sf9 cells, purified by affinity chromatography, and assayed for UDP-GlcNAc-2-epimerase activity in the presence of CMP-Neu5Ac. Values are means of duplicate experiments.

In this study, rat UDP-GlcNAc-2-epimerase/ManNAc kinase was used, and therefore, the analogous amino acids of the rat enzyme, which are conserved, were first changed by site-directed mutagenesis, and then baculoviruses were generated in the Bac-to-Bac system. To verify that these mutant rat enzymes displayed the same phenotype as the human sialiuria mutant UDP-GlcNAc-2-epimerase/ManNAc kinase, the His-tagged recombinant proteins were expressed in Sf9 cells and purified by affinity chromatography on Ni−NTA agarose column. The purified proteins were assayed for UDP-GlcNAc-2-epimerase activity in the presence of CMP-Neu5Ac (Figure 4). While the wild-type enzyme was found to be inhibited in a concentration-dependent manner, no significant inhibition was observed in either of the two mutant UDP-GlcNAc-2-epimerase/ManNAc kinase (EKR263L, EKR266W) enzymes. Thus, similar to previously characterized human sialuria mutants, R263L and R266W mutants were not feedback inhibited.

Increased CMP-Neu5Ac Synthesis with R263L Mutant UDP-GlcNAc-2-epimerase/ManNAc Kinase (EKR263L). The decreased synthesis of CMP-Neu5Ac in the presence of endogenous ManNAc synthesis is attributed to feedback inhibition of UDP-GlcNAc-2-epimerase activity by the CMP-Neu5Ac product. To determine whether this bottleneck to CMP-Neu5Ac synthesis could be overcome, the EKR263L and EKR266W mutants were expressed in insect cells. When Sf9 cells were co-infected with AcEKR263L and AcSAS, the levels of Neu5Ac generated intracellularly (48.2 nmol/mg) were not significantly lower than the levels obtained with AcEK and AcSAS co-infection (54.7 nmol/mg). Thus, the replacement of the Arg residue at 263 with Leu amino acid appears to have a minimal effect on the capacity of the UDP-GlcNAc-2-epimerase/ManNAc kinase enzyme to generate sufficient ManNAc substrate in insect cells for subsequent Neu5Ac production. Similar initial results were also obtained with the EKR266W mutant.

Next, Sf9 cells were co-infected with AcEKR263L, AcSAS, and AcCSAS, and the levels of Neu5Ac and CMP-Neu5Ac were compared with the levels obtained by co-infecting Sf9 cells with wild-type AcEK, AcSAS, and AcCSAS. As shown in Figure 5A, the Neu5Ac levels obtained using the mutant EKR263L were approximately 4.5 times higher than the levels obtained with the wild-type EK. A similar increase was detected in the CMP-Neu5Ac levels with the use of the EKR263L as compared to levels obtained by using wild-type EK (Figure 5B). Previously, the CMP-Neu5Ac levels generated were 1.7 times lower when using the wild-type EK to generate ManNAc as compared to adding ManNAc directly to the culture medium as a result of feedback inhibition of the 2-epimerase activity. However, when the heterologous EKR263L mutant was expressed in combination with SAS and CSAS, the CMP-Neu5Ac levels were 2.5 times higher than the levels obtained with 10 mM ManNAc feeding and AcSAS + AcCSAS co-infection. These results indicate that the synthesis of CMP-Neu5Ac in triply infected Sf9 cells is limited primarily by the feedback regulation of UDP-GlcNAc-2-epimerase activity and not by the absence of sufficient endogenous UDP-GlcNAc substrates. Furthermore, by using the EKR263L mutant we can minimize the feedback inhibition effects of CMP-Neu5Ac and synthesize higher levels of CMP-Neu5Ac than levels obtained with other available strategies, including feeding precursor substrates such as ManNAc directly to the cells. We have previously shown that incorporation of ManNAc into the Neu5Ac pathway can be limited by a transport bottleneck into the cell and a phosphorylation limitation by the GlcNAc kinase (*23*).

Effect of Substrate Supplementation on CMP-Neu5Ac Synthesis in Insect Cells. While the addition of ManNAc was not as effective in generating CMP-Neu5Ac as using endogenous UDP-GlcNAc and the EK mutant, the effect of other substrate feeding strategies on CMP-Neu5Ac production was also considered. Previously, Sf9 cells have been observed to generate very high levels of intracellular Neu5Ac when supplemented with the substrate *N*-acetylglucosamine (GlcNAc) in combination with the AcEK and AcSAS co-infection (*22*). GlcNAc in the medium is taken up by the cells and converted to UDP-GlcNAc by a series of enzymatic steps. The supplementation of the cell culture medium with 10 mM GlcNAc feeding increases the intracellular UDP-GlcNAc levels in Sf9 cells up to 10-fold (Viswanathan and Betenbaugh, unpublished result). The UDP-GlcNAc may then be metabolized to CMP-Neu5Ac by the action of EK, SAS, and CSAS. To investigate if GlcNAc supplementation could affect CMP-Neu5Ac synthesis, the Sf9 cell culture medium was supplemented with 10 mM GlcNAc. The effect of GlcNAc supplementation on Neu5Ac production levels for Sf9 cells co-infected with AcEKR263L and AcSAS is similar to that obtained with AcEK and AcSAS co-infection. In GlcNAc-supplemented cells, the level of Neu5Ac generated with AcEKR263L and AcSAS co-infection was 337 nmol/mg and the level obtained with AcEK and AcSAS co-infection was 343 nmol/mg. With either the wild-type or mutant EK, insect cells supplemented with GlcNAc were observed to generate more than 6-fold higher Neu5Ac levels as compared to levels obtained without GlcNAc supplementation to indicate that the increased UDP-GlcNAc levels are being utilized efficiently by the EKR263L enzyme to

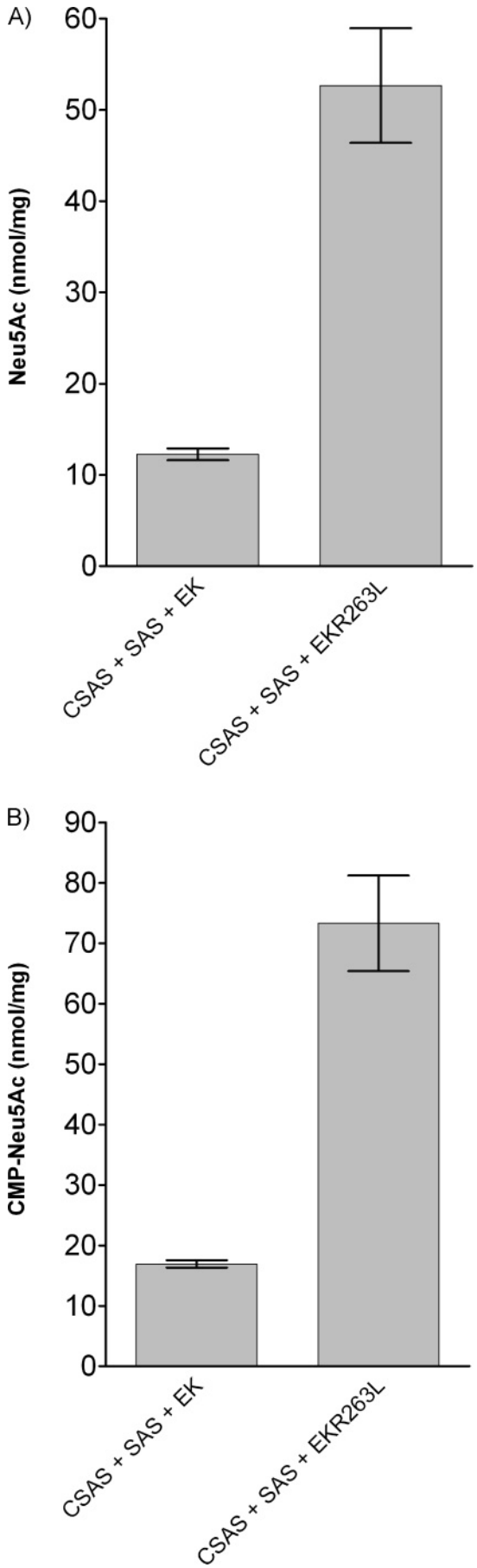

FIGURE 5: (A) Neu5Ac and (B) CMP-Neu5Ac levels in Sf9 cells co-infected with AcSAS, AcCSAS, and AcEK compared to levels in cells co-infected with AcSAS, AcCSAS, and AcEKR263L.

generate Neu5Ac. When the cells were co-infected with AcCSAS along with AcEKR263L and AcSAS, Sf9 cells generated CMP-Neu5Ac levels that were more than 3-fold higher than the levels obtained without GlcNAc supplementation (Figure 6A) and more than 7-fold higher than the levels obtained using ManNAc supplementation rather than the AcEKR263L infection. This increase in CMP-Neu5Ac level with GlcNAc feeding indicates that the availability of UDP-GlcNAc is one of the principal limitations in the synthesis of CMP-Neu5Ac for insect cells expressing heterologous EKR263L, SAS, and CSAS. However, when the levels of precursor Neu5Ac were examined in these Sf9 cells infected with the same three baculoviruses in the presence or absence of 10 mM GlcNAc, the Neu5Ac levels in the GlcNAc-supplemented cells were more than 7.5-fold higher than the levels obtained in the absence of any GlcNAc supplementation (Figure 6B). Thus, the relative increase in Neu5Ac levels with GlcNAc feeding (Figure 6B) was significantly higher than the increase in CMP-Neu5Ac levels (Figure 6A) for the same infection and feeding conditions. The smaller increase in CMP-Neu5Ac levels obtained with GlcNAc feeding relative to the increase in Neu5Ac levels implies a second downstream bottleneck in the production of CMP-Neu5Ac that occurs in the presence of high levels of Neu5Ac accompanying GlcNAc feeding. The limitation may exist in the available levels or enzymatic activity of the CSAS enzyme, or alternatively, the bottleneck may be due to limitations in the availability of CTP for the activation of Neu5Ac.

DISCUSSION

Previous studies in our laboratory have focused on the metabolic synthesis of sialic acid in insect cells. Heterologous SAS expression has been combined with ManNAc feeding in insect cells to synthesize significant levels of Neu5Ac (*14*). The additional coexpression of CSAS has resulted in high levels of CMP-Neu5Ac product in these MaNAc-fed cells (*13*). In addition, coexpression of SAS and EK in Sf9 cells resulted in synthesis of Neu5Ac from endogenous substrates (*23*). These previous studies have also suggested potential bottlenecks in the synthesis pathways. The observation that Neu5Ac levels were increased in the presence of endogenous EK activity suggested that a bottleneck exists in the uptake of ManNAc from the medium of cells. Indeed, subsequent experiments demonstrated a limitation in the transport of ManNAc into cells as well as a bottleneck in the phosphorylation of ManNAc at higher levels of ManNAc supplementation. The ManNAc transport bottleneck could be overcome by peracetylation of ManNAc residues (*33*), but the peracetylated substrates were also found to be toxic to the cells (*23*). As a result, these studies suggested that endogenous synthesis routes for generating metabolites in the sialic acid metabolic pathway may be preferred over ManNAc supplementation in insect cells as these routes avoid the problems associated with the cellular uptake of ManNAc. The elimination of ManNAc supplementation would also eliminate the need to modify the medium to include this substrate.

In this study, we have demonstrated the capacity to synthesize the activated form of Neu5Ac, CMP-Neu5Ac, in insect cells completely from endogenous metabolites. Previous studies in our laboratories and others have shown that

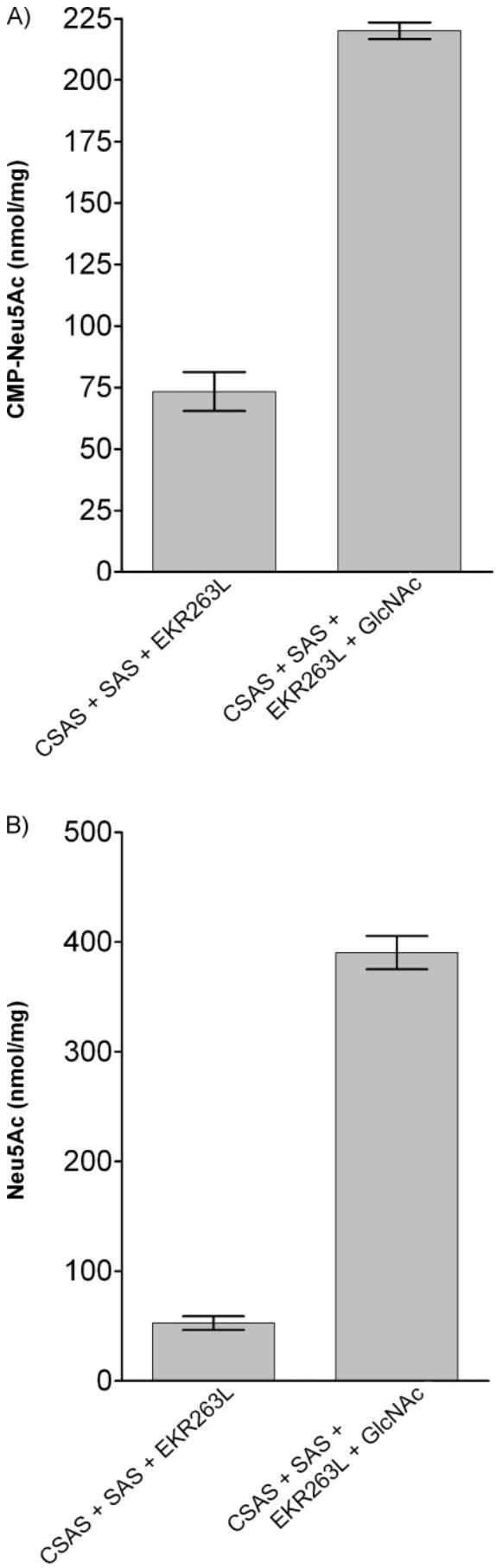

FIGURE 6: Effect of 10 mM GlcNAc feeding on levels of (A) CMP-Neu5Ac and (B) Neu5Ac in Sf9 cells co-infected with AcSAS, AcCSAS, and AcEKR263L.

insect cells include significant intracellular levels of the sialylation precursor, UDP-GlcNAc (*11*, *12*). In this study, CMP-Neu5Ac, the nucleotide sugar substrate for sialylation, was generated from endogenous substrates by combining the EK and SAS expression with the expression of CSAS, required for generating CMP-Neu5Ac. This is the first report of insect cells being able to synthesize CMP-Neu5Ac without any additional precursor supplementation and demonstrates that mammalian metabolic pathways can be inserted successfully into insect cells or other nonmammalian host systems.

However, a comparison of the intracellular CMP-Neu5Ac and Neu5Ac levels in cells coexpressing all three genes to those levels obtained with ManNAc feeding suggested that feedback inhibition of the UDP-GlcNAc-2-epimerase enzyme was limiting the production of CMP-Neu5Ac in insect cells. Indeed, UDP-GlcNAc-2-epimerase responsible for the synthesis of ManNAc is known to be a critical regulator of synthesis of CMP-Neu5Ac and cell surface sialylation in mammalian cells (*34*), and UDP-GlcNAc-2-epimerase activity is feedback-inhibited by CMP-Neu5Ac (*24*, *25*). This allosteric inhibition of the epimerase activity by CMP-Neu5Ac provides the cells with an efficient mechanism for regulating the intracellular levels of CMP-Neu5Ac. While the unrestrained synthesis of Neu5Ac in sialuria patients has undesirable effects on the health, we hypothesized such a mutant cell line may provide potential benefits in synthesis of high levels of CMP-Neu5Ac for biotechnology applications. For this purpose, two baculoviruses were generated containing two different sialuria-like mutant rat UDP-GlcNAc-2-epimerase/ManNAc kinases (EKR263L and EKR266W). The epimerase activity of these mutants was not inhibited by CMP-Neu5Ac and displayed the same phenotype that is described for the human enzyme of the sialuria patients. By expressing the rat EK containing the Arg to Leu mutation at residue 263 to prevent feedback inhibition by CMP-Neu5Ac, we were able to generate CMP-Neu5Ac levels that were more than 4 times higher than those obtained in cells expressing the wild-type EK. Even more significant is that the levels of CMP-Neu5Ac were more than 2.5 times higher than those obtained by using ManNAc supplementation to indicate that endogenous production of the activated sugar nucleotide is more efficient than adding this precursor substrate. The increased CMP-Neu5Ac levels obtained from endogenous metabolites as compared to ManNAc feeding suggest that the same bottlenecks associated with ManNAc supplementation exist for CMP-Neu5Ac synthesis as observed previously for Neu5Ac synthesis. These findings also indicate that current strategies that seek to improve CMP-Neu5Ac production and sialylation by adding ManNAc because it avoids the feedback regulation step may be problematic due to bottlenecks in the incorporation of ManNAc into cells.

To determine if there was a second bottleneck in the CMP-Neu5Ac production once the CMP-Neu5Ac feedback inhibition was avoided by the use of mutant EK, the medium was supplemented with GlcNAc, a precursor of the epimerase substrate, UDP-GlcNAc. Our findings that both the intracellular Neu5Ac and CMP-Neu5Ac levels increased with fed GlcNAc suggest that the levels of endogenous UDP-GlcNAc were limiting the production of CMP-Neu5Ac in insect cells engineered to express SAS, CSAS, and the mutant EK. Interestingly, the addition of GlcNAc to the medium increased the Neu5Ac levels by more than 7-fold, while the levels of CMP-Neu5Ac improved only by a factor of 3. Since the increase in CMP-Neu5Ac levels did not parallel the increases in Neu5Ac following GlcNAc feeding, another bottleneck to CMP-Neu5Ac synthesis was manifested. This bottleneck, which is present at the step involving the conversion of Neu5Ac to CMP-Neu5Ac, may be due to inadequate intracellular supplies of the CSAS enzyme, a limitation in its enzymatic activity at high Neu5Ac levels, or inadequate levels of the CTP cosubstrate involved in the reaction. Subsequent studies will examine if the limitation is in the availability of CTP by providing CTP precursors such as cytidine or in the supply of enzyme by increasing the expression of CSAS relative to the other enzymes.

This is the first time that insect cell metabolism has been manipulated to enable the complete synthesis of all the principal nucleotide sugars involved in N-linked glycosylation without the addition of any dedicated precursors to the medium. When these finding are considered in conjunction with the efforts of Aumiller et al. (*22*) to express heterologous glycosyltransferases in insect cells, we envision the capability to complete the synthesis of complex humanized glycans in insect cells without the addition of any exogenous substrates. Such a strategy may also be used to enhance the sialylation of glycoproteins generated in other culture systems, including mammalian cells. In fact, a number of recombinant proteins expressed in mammalian cells have exhibited deficiency in levels of sialylation, and this limitation has often been attributed to the availability of the donor CMP-Neu5Ac (*15*). When recombinant proteins are expressed under strong promoters, the sialic acid synthesis machinery may not able to meet the demands of the corresponding glycoprotein synthesis machinery. Indeed, Gu and Wang (*15*) demonstrated that supplementing mammalian cell culture medium with ManNAc could increase the levels of CMP-Neu5Ac and sialylation present on interferon-gamma. Using the strategy developed in this study of combining GlcNAc feeding with expression of a mutant EK, we could further increase the levels of CMP-Neu5Ac beyond those obtained by simple feeding of ManNAc. Also, the higher cost of ManNAc compared to GlcNAc and limitations in the uptake of ManNAc from the medium may make this approach more economical for generating high levels of the essential donor CMP-Neu5Ac molecules.

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