Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 9140–9145, August 1998
Biochemistry

Mammalian cytidine 5′-monophosphate *N*-acetylneuraminic acid synthetase: A nuclear protein with evolutionarily conserved structural motifs

(nuclear localization/nucleotidyltransferases/sialic acid/polysialic acid)

ANJA-K. MÜNSTER*, MATTHIAS ECKHARDT*, BARRY POTVIN†, MARTINA MÜHLENHOFF*, PAMELA STANLEY†, AND RITA GERARDY-SCHAHN*‡

*Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany; and †Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461

Edited by Phillips W. Robbins, Massachusetts Institute of Technology, Cambridge, MA, and approved June 1, 1998 (received for review March 27, 1998)

ABSTRACT **Sialic acids of cell surface glycoproteins and glycolipids play a pivotal role in the structure and function of animal tissues. The pattern of cell surface sialylation is species- and tissue-specific, is highly regulated during embryonic development, and changes with stages of differentiation. A prerequisite for the synthesis of sialylated glycoconjugates is the activated sugar-nucleotide cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac), which provides a substrate for Golgi sialyltransferases. Although a mammalian enzymatic activity responsible for the synthesis of CMP-Neu5Ac has been described and the enzyme has been purified to near homogeneity, sequence information is restricted to bacterial CMP-Neu5Ac synthetases. In this paper, we describe the molecular characterization, functional expression, and subcellular localization of murine CMP-Neu5Ac synthetase. Cloning was achieved by complementation of the Chinese hamster ovary *lec32* mutation that causes a deficiency in CMP-Neu5Ac synthetase activity. A murine cDNA encoding a protein of 432 amino acids rescued the *lec32* mutation and also caused polysialic acid to be expressed in the capsule of the CMP-Neu5Ac synthetase negative *Escherichia coli* mutant EV5. Three potential nuclear localization signals were found in the murine synthetase, and immunofluorescence studies confirmed predominantly nuclear localization of an N-terminally Flag-tagged molecule. Four stretches of amino acids that occur in the N-terminal region are highly conserved in bacterial CMP-Neu5Ac synthetases, providing evidence for an ancestral relationship between the sialylation pathways of bacterial and animal cells.**

Sialic acids form terminal residues of cell surface glycans and affect the chemical properties of cell surfaces. Several of the >30 naturally occurring sialic acids are involved in the formation of cellular recognition structures and the regulation of cell–cell interactions during embryonic development (reviewed in refs. 1 and 2). Highly malignant tumors overexpress sialic acid and polysialic acid (PSA) (3, 4), and drugs inhibiting sialylation reduce tumor growth and metastasis (5, 6). In bacteria, sialylated and polysialylated capsular components provide important virulence factors mediating resistance to host defense mechanisms (reviewed in ref. 7).

The synthesis of sialic acids in pro- and eukaryotic cells begins with UDP-*N*-acetylglucosamine (UDP-GlcNAc), which becomes epimerized to UDP-*N*-acetylmannosamine (ManNAc) by the enzyme UDP-GlcNAc 2-epimerase (enzyme code 5.1.3.14). Although in bacteria, two pathways exist to obtain *N*-acetylneuraminic acid (Neu5Ac) directly from ManNAc (8), three consecutive steps are required to produce Neu5Ac in the eukaryotic cell (reviewed in ref. 9). Activation of Neu5Ac to the nucleotide sugar cytidine 5′-monophosphate (CMP)-Neu5Ac is required to provide the donor used by sialyltransferases of both pro- and eukaryotic cells (10, 11), and this reaction is catalyzed by CMP-Neu5Ac synthetases (enzyme code 2.7.7.43; 9).

In the mammalian cell, activation of free sialic acid to CMP-sialic acid is necessary for several reasons: (*i*) Sialyltransferases are Golgi residents, and only CMP-Neu5Ac, not free sialic acid, is transported specifically into the Golgi lumen (11); (*ii*) the sugar nucleotide and not free Neu5Ac is the substrate of sialic acid modifying enzymes (12, 13); and (*iii*) the cytosolic pool of CMP-Neu5Ac controls the activity of the upstream enzyme UDP-GlcNAc 2-epimerase in a feedback mechanism (14, 15). Therefore, the enzyme responsible for producing CMP-Neu5Ac, CMP-Neu5Ac synthetase, is an ideal target for exogenous regulation of the sialylation pathway in mammalian cells.

Partially purified CMP-Neu5Ac synthetases from different animal tissues have been characterized (reviewed in ref. 10). In the course of these experiments, the surprising observation was made that, in nucleated cells, >90% of the CMP-Neu5Ac synthetase activity associates with the nuclear fraction. This is in marked contrast to other nucleotide–sugar synthetases that are found in the cytoplasm (16). Furthermore, activation by CTP is restricted to sialic acids and 2-keto-3-deoxy-manno-octulosonic acid. These sugars react with CTP to give sugar nucleotide monophosphates (17, 18). All other nucleotide sugars are nucleotide diphosphates.

Although the biosynthetic pathway leading to CMP-Neu5Ac has been known for >30 years (19, 20), molecular information on the enzymes involved at each step is restricted to bacteria (8, 21). In mammals, only two enzymes of the pathway are characterized: UDP-GlcNAc 2-epimerase and ManNAc kinase are part of one bifunctional molecule (15, 22). In this study, we report the molecular characterization of the murine CMP-Neu5Ac synthetase.

EXPERIMENTAL PROCEDURES

Materials. Endoneuraminidase NE (endoNE) specifically degrades α-2,8-linked PSA. The enzyme was purified as described

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: CHO, Chinese hamster ovary; CMP-Neu5Ac, cytidine 5′-monophosphate *N*-acetylneuraminic acid; endoNE, endoneuraminidase NE; MAA, *Maackia amurensis*; PSA, polysialic acid; wt, wild-type.
Data deposition: The sequence data reported in this paper have been submitted to the European Molecular Biology Laboratory database (accession no. AJ006215).

‡To whom reprint requests should be addressed. e-mail: rgs@mikrobio.h.shuttle.de.

(23). mAb 735 that binds α-2,8-linked PSA chains (24) was used after purification on protein A-Sepharose (Pharmacia).

Cell Culture. Chinese hamster ovary (CHO)-K1 cells were maintained in DMEM Ham's medium F12 (Seromed, Berlin) supplemented with 1 mM sodium pyruvate and 5% fetal calf serum, LEC29 (25), and LEC29.Lec32 cells (26) in Alpha-MEM (GIBCO/BRL) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% fetal calf serum and NIH 3T3 cells (ATCC CRL 1685) in Eagle's minimal essential medium (GIBCO/BRL) supplemented with 10% fetal calf serum. Culture media usually contained penicillin and streptomycin at a concentration of 100 units per milliliter.

Expression Cloning. An oligo(dT)-primed cDNA library constructed from the murine pituitary cell line AtT20 in the eukaryotic expression vector pABE (27) was used for complementation cloning. After transformation into *Escherichia coli* MC1061/p3, the library was plated at a density of ≈2,000 cfu per plate, and plasmids were isolated with the Plasmid Mini Kit (Qiagen, Hilden, Germany) from each pool. For transfection, 2×10^5 LEC29.Lec32 cells were cotransfected with 500 ng of library pool DNA (or with the empty vector pABE for negative control) and 500 ng of plasmid pPSVE1-PyE, which encodes the polyoma large T antigen (28). Two days after transfection using Lipofectamine (GIBCO/BRL), PSA-positive transfectants were identified immunohistochemically with mAb 735 as described (27). One of 48 pools was positive. This pool was subdivided, and the transfection procedure was repeated. After three additional rounds of sibling selection, plasmid pAM15 was isolated.

A major difficulty in cloning was that LEC29.Lec32 cell populations contain PSA-positive pseudo and bona fide revertants. To keep the background low, daily removal of PSA-expressing cells was achieved by panning (29) on dishes coated with the mAb 735.

DNA sequencing on both strands was performed by the dideoxy chain termination method (30) using T7 DNA polymerase (Pharmacia) or Thermosequenase (United States Biochemical) and [α-^{35}S]-dATPs. Reactions were primed with vector-specific oligonucleotides and subsequently with primers derived from the known sequence of the cDNA.

Construction of Expression Vectors. The eukaryotic expression vector pFlagB1 was prepared by ligating the oligonucleotides FLAGs (5′-CGCCACCATGGACTACAAGGATGATGATG-ATAAGG-3′) and FLAGas (5′-*GATCCC*TTATCATCATCA-TCCTTGTAGTCCATGGTGGC*GGTAC*-3′), which encode the Flag M5 epitope (N-MDYKDDDDK-C) and carry the *Kpn*I and *Bam*HI restriction sites (italicized) into the *Kpn*I/*Bam*HI sites of the eukaryotic expression vector pCDNA3 (Invitrogen). For pAM16Flag, the entire coding region of the CMP-Neu5Ac synthetase was isolated from pAM15 by using *Bst*XI and *Not*I and was ligated into the corresponding sites of the vector pFlagBI (resulting in plasmid pCMP2). To obtain in-frame fusion of the Flag- and CMP-Neu5Ac synthetase sequences, the 5′-end of CMP-Neu5Ac synthetase was amplified by PCR by using the primers AM34 (5′-CG*GGATCC*GACGCGCTGGAGAAGG-GGGCC-3′) and AM27 (5′-CCCTGTAAGTAACCCATCTC-3′), which bind to nucleotides 4–24 and 670–689 of the coding sequence, respectively. The PCR product was digested with *Bam*HI and *Eco*RI (restriction site at 376 of the coding sequence) and was ligated into the *Bam*HI/*Eco*RI sites of the plasmid pCMP2. The resulting plasmid pAM16Flag contains an N-terminally Flag-tagged CMP-Neu5Ac synthetase.

To introduce the murine CMP-Neu5Ac synthetase cDNA into the prokaryotic expression vector pQE30 (Qiagen), pAM16Flag was digested with *Bam*HI and *Xho*I, and the restriction fragment was ligated into the corresponding sites of pQE30 to create pQEAM16.

Immunofluorescence. NIH 3T3 cells, cultured on glass coverslips, were transfected with varying amounts of either pAM16Flag or pFlagB1 by using SuperFect (Qiagen). After 24–30 hours, cells were fixed in 4% paraformaldehyde in PBS (10 mM sodium phosphate, pH 7.4/150 mM NaCl) for 22 min at room temperature and were permeabilized with 0.2% Triton X-100/PBS for 8 min at room temperature. The Flag epitope was detected by sequential incubation with anti-Flag mAb M5 (Kodak) (diluted 1:500) and fluorescein (DTAF)-conjugated goat anti-mouse Ig (Dianova, Hamburg, Germany) diluted 1:200 in PBS containing 20% horse serum. Antibody incubations were carried out for 30 min at 37°C. Control staining with the nuclear dye 2-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole (Hoechst 33258, Hoechst Pharmaceuticals) was performed at a concentration of 650 ng/ml for 4 min at room temperature. Fluorescence labeling was visualized by using a Zeiss Axioplan microscope.

Functional Expression of CMP-Neu5Ac Synthetase. PSA or sialic acid expression and enzyme activity of cell lysates were used as a measure of CMP-Neu5Ac synthetase activity in homologous and heterologous systems. For Western blot experiments, SDS/Page was performed on 8–10% polyacrylamide gels. Western blots were developed with the anti-Flag antibody M5, the PSA specific mAb 735, and digoxigenin-labeled *Maackia amurensis* (MAA) lectin (Boehringer Mannheim), which specifically binds to sialic acid α-2,3-linked to galactose. Immunoreactions were performed as described (27) or according to manufacturers instructions.

To rescue the *lec32* mutation, 3×10^6 transiently transfected LEC29.Lec32 cells were harvested 48 h after transfection, were lysed in 200 μl lysis buffer (50 mM Tris, pH 8.0/1% Nonidet P-40/2 mM EDTA/1 mM $MgCl_2$/1 mM PMSF/10 units/ml aprotinin) and were analyzed by Western blot analysis before and after endoNE digestion of the samples as described (31).

Heterologous expression of the murine CMP-Neu5Ac synthetase in *E. coli* strain EV5 (32) was examined by transforming either 10 ng of pQEAM16, the empty vector pQE30 (Qiagen), or plasmid pUE17 (33) encoding the CMP-Neu5Ac synthetase from *Neisseria meningitidis* serogroup B (kindly provided by M. Frosch, University Würzburg, Germany). Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactoside. Bacteria were harvested after 2 h, were sonicated, and were analyzed for PSA-capsule expression by Western blot analysis with mAb 735 before and after treatment with endoNE.

To assay CMP-Neu5Ac synthetase activity, LEC29.Lec32 cells were cotransfected with either 5 μg pAM15 or empty vector pABE mixed with 1 μg of the plasmid PSV2NEO by using Lipofectamine (GIBCO/BRL). Stable transfectants were selected with 1 mg/ml G418 (GIBCO/BRL). Colonies were isolated as a pool with 5 mM EDTA in PBS, and CMP-Neu5Ac synthetase activity was assayed by using ^{14}C-Neu5Ac as described (26).

To investigate the subcellular localization of CMP-Neu5Ac synthetase, a Flag-tagged protein was expressed in LEC29.Lec32 cells by using the vector pAM16Flag. Transfected cells were harvested after 48 h, and cytosolic and nuclear fractions were prepared (34) and analyzed by Western blot analysis with the anti-FLAG mAb M5.

Northern and Southern Blot Analyses. Total RNA from murine tissues and cell lines was isolated by guanidinium isothiocyanate extraction and centrifugation through CsCl gradients. Total RNA (15 μg) was fractionated on a 1% agarose gel (0.5 M formaldehyde) and was transferred to a nylon membrane. Blots were hybridized with a digoxigenin-labeled antisense RNA probe transcribed from the coding region of the murine CMP-Neu5Ac synthetase cDNA (nucleotides 376 to 1299) subcloned into pBluescript (Stratagene). Hybridization was performed for 16 h at 68°C in 50% formamide, 5× standard saline citrate (SSC), 50 mM sodium phosphate, 7% SDS, 0.1% N-lauroylsarcosine, and 2% blocking reagent (Boehringer Mannheim). Membranes were washed in 0.1× SSC and 0.1% SDS at 68°C for 30 min, and bound RNA probes were revealed as described (27).

Genomic DNA from wild-type (wt) LEC29.Lec32 and a revertant cell line was digested with *Bam*HI *Bgl*II, *Nco*I, *Nsi*I, *Pvu*I, *Sac*I, *Stu*I, or *Xba*I. After electrophoresis on 0.8% agarose, the gel was blotted to Hybond-N membrane (Amersham) and was hybridized to a 569-bp *Sac*I/*Blp*I fragment excised from pAM15 and labeled with ^{32}P-dCTP by random oligonucleotides (Prime It, Stratagene). Hybridization was at 42°C overnight, and blots finally were washed in 0.5× SSC and 0.1% SDS at room temperature.

DNA and Protein Sequence Analysis. Homology searches were performed at the National Center for Biotechnology Information by using the algorithm GAPPED BLAST and PSI-BLAST (35) and the databases SwissProt, the Protein Database, SPupdate, and Trembl. For multiple alignments ALIGN (36) was used. Motif searches were carried out with MEME (37), BLOCKMAKER (38), and MAST (37).

RESULTS

Isolation of a Murine CMP-Neu5Ac Synthetase cDNA. CHO cells with a defect in CMP-Neu5Ac synthetase activity have been isolated recently on the background of the dominant LEC29 CHO mutant (26). The *LEC29* mutation activates an α(1,3)fucosyltransferase normally not expressed in CHO cells (25) whereas the recessive mutation *lec32* essentially abolishes CMP-Neu5Ac synthetase activity (26). Sialylation of glycoproteins and glycolipids is reduced >95% by the *lec32* mutation (26) and leads to loss of PSA on the neural cell adhesion molecule (39) in LEC29.Lec32 cells (see Fig. 2). Reexpression of PSA would identify transformants in which the *lec32* mutation is complemented. This cloning strategy provides a rapid and highly sensitive way to isolate genes involved in sialylation pathways (27, 39, 40).

To obtain a cDNA that complements the *lec32* mutation, plasmid pools of 2,000 cfu from a cDNA library of the murine pituitary tumor cell line AtT20 were transfected together with the pPSVE1-PyE plasmid that contains the polyoma large T antigen (28) into LEC29.Lec32 cells. Before transfection, revertants expressing PSA were depleted by panning on the PSA-specific mAb 735. Pools with positive transfectants were identified by immunohistochemistry with mAb 735. After four rounds of sibling selection, a single cDNA clone named pAM15 was isolated.

Nucleotide Sequence and Deduced Amino Acid Sequence. The insert of 1,712 nucleotides contained in clone pAM15 predicts a protein of 432 amino acids with a calculated molecular mass of 48,058 Da (Fig. 1). Only seven nucleotides upstream of the translation initiation codon were present in the cDNA, but an A at position −3 and a G at position +4 of the start codon ATG form part of a consensus motif for eukaryotic translation initiation (41). Extension of the 5′-sequence by 5′-RACE yielded an additional 20 nucleotides and did not identify an additional in-frame ATG. The 3′-noncoding region contains a polyadenylation signal AATAAA starting 20 bp upstream of the Poly(A) tail. Two potential *N*-glycosylation sites were present at amino acids 212 and 427. Consistent with predictions that CMP-Neu5Ac synthetase activity is in the nucleus of eukaryotic cells (16, 42), the primary sequence shows three clusters of basic amino acids that could serve as nuclear localization signals (open boxes in Fig. 1).

pAM15 Rescues the *lec32* Mutation. Sialylation patterns of wt CHO cells and the mutants LEC29 and LEC29.Lec32 were compared by Western blot analysis with PSA-specific mAb 735 (ref. 24; Fig. 2 *Left*) and MAA lectin that specifically binds α-2,3-linked sialic acids (ref. 43; Fig. 2 *Right*). The heterogeneous smear at ≈250 kDa shows that wt and LEC29 cells express almost identical levels of PSA, which is bound to the neural cell adhesion molecule in CHO cells (39). Both cell lines also bind similar amounts of MAA. In contrast, signals from both reagents are reduced markedly in the double mutant LEC29.Lec32. The faint staining observed with MAA and stronger staining with the highly sensitive mAb 735 can be explained by revertants or leakiness of the *lec32* mutation (26). Binding of mAb 735 was abolished after digestion with the PSA-specific endoNE (23). LEC29.Lec32 transfectants expressing the pAM15 cDNA exhibited wt staining for both reagents, showing that pAM15 rescues the *lec32* mutation.

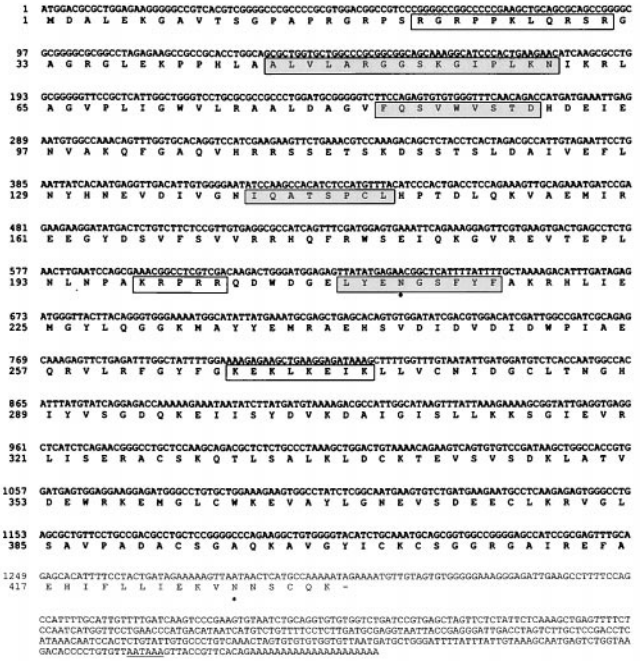

FIG. 1. Nucleotide and deduced amino acid sequence of murine CMP-Neu5Ac synthetase. Two potential *N*-glycosylation sites are indicated by asterisks, and a polyadenylation signal is underlined. Three potential nuclear localization signals are shown as open boxes. Stretches highly homologous to cloned bacterial CMP-Neu5Ac synthetases are shown as shaded boxes.

Nuclear Localization of Flag-tagged CMP-Neu5Ac Synthetase. To study the subcellular distribution of murine CMP-Neu5Ac synthetase, an N-terminally Flag-tagged CMP-Neu5Ac synthetase (pAM16Flag) was expressed transiently in LEC29.Lec32 cells. In Western blot analysis (Fig. 3), the anti-Flag mAb M5 detected a single band of ≈50 kDa only in pAM16Flag transfected cells, and no signal was detected in mock transfected cells. The signal was most prominent in nuclear extract, but a faint band also could be detected in the cytoplasmic fraction. The apparent molecular mass of 50 kDa is in good agreement with the calculated molecular mass of 48 kDa. Strong nuclear localization

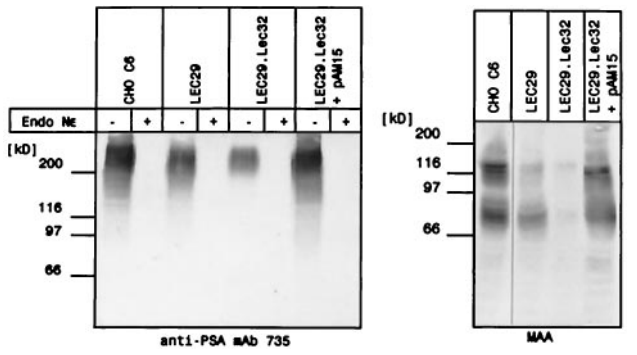

FIG. 2. (*Left*) Western blot analysis of PSA expressed by CHO wt C6, mutant LEC29, and the mutant LEC29.Lec32 before and after transfection with the plasmid pAM15, encoding the murine CMP-Neu5Ac synthetase. The signal in LEC29.Lec32 is caused by the presence of Lec32 revertants. To confirm the specificity of the signal, samples were treated with endoNE (+). (*Right*) A third aliquot of cell lysate was developed with digoxigenin-labeled lectin MAA specific for α-2,3-linked sialic acid.

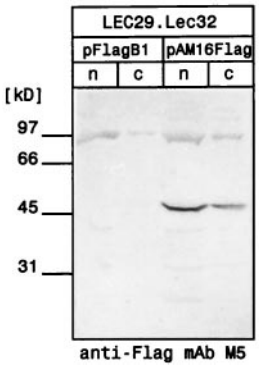

FIG. 3. Nuclear localization of CMP-Neu5Ac synthetase by Western blot analysis. LEC29.Lec32 cells were transfected transiently with pAM16Flag or with empty vector pFlagB1. After 48 hours, cytosolic (c) and nuclear (n) extracts were prepared and subjected to Western blot analysis with the anti-Flag mAb M5. A band at ≈50 kDa is prominent in the nuclear extract of cells transfected with pAM16Flag.

of the Flag-tagged protein was observed at the single cell level in transiently transfected NIH 3T3 cells (Fig. 4). The immunofluorescence signal obtained with mAb M5 in transfected cells exactly colocalized with nuclei stained by Hoechst 33258. Although strong nuclear staining was obtained at low levels of transgene expression, at high cDNA concentrations or transfection efficiency, a faint cytosolic staining was visible beside the strong nuclear signal (data not shown).

Enzymatic Activity of Recombinant CMP-Neu5Ac Synthetase. To show that pAM15 caused expression of CMP-Neu5Ac synthetase activity in transfectants, LEC29.Lec32 cells were cotransfected with pAM15 cDNA and pSV2neo, and stable transfectants selected with G418 were assayed for CMP-Neu5Ac synthetase activity. Compared with the vector control, synthetase activity was increased by a factor of ≈22 in cells transfected with pAM15 (Table 1).

To demonstrate complementation in a heterologous system, *E. coli* EV5, which is a capsule negative mutant of the neuroinvasive strain *E. coli* K1, was transfected with the CMP-Neu5Ac synthetase cDNA. The loss of the PSA capsule in EV5 was caused by a mutation in the CMP-Neu5Ac synthetase gene (32). For expression in *E. coli*, the coding region of pAM15 was cloned into the bacterial expression

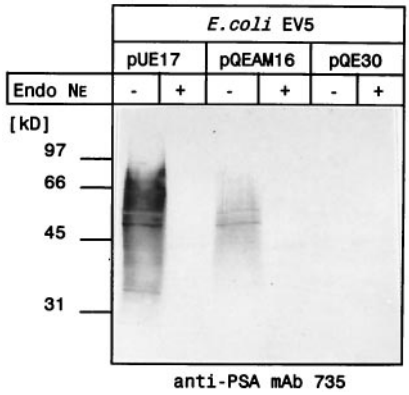

FIG. 5. Functional expression of murine CMP-Neu5Ac synthetase in *E. coli* mutant EV5. *E. coli* EV5 cells were transformed with pQEAM16, encoding the murine CMP-Neu5Ac synthetase, or empty vector pQE30 or pUE17, containing the CMP-Neu5Ac synthetase from *N. meningitidis*. Expression of the transformed DNA was induced with 0.2 mM isopropyl-β-D-thiogalactoside, and the presence of PSA capsule was determined by Western blot analysis by using anti-PSA mAb 735. PSA was removed specifically if samples were treated with endoNE (+).

vector pQE30 to give pQEAM16. Plasmid pUE17 (33) containing the CMP-Neu5Ac synthetase from *N. meningitidis* was transfected as a positive control. Transfer of either the *N. meningitidis* B gene (pUE17) or the mammalian CMP-Neu5Ac synthetase gene (pQEAM16) but not the empty vector QE30 induced expression of the endoNE-sensitive PSA capsule (Fig. 5).

Northern and Southern Blot Analyses. In Northern blots hybridized with a CMP-Neu5Ac synthetase probe, a band of ≈2 kb was obtained with RNA from CHO cells and mouse tissues but not with RNA from LEC29.Lec32 cells (Fig. 6). Equal loading of the gel was confirmed by ethidium bromide staining (data not shown). Therefore, the *lec32* mutation either reduces transcription of the CMP-Neu5Ac synthetase gene or dramatically decreases the stability of transcripts. The latter seems likely because low levels of sialylated glycoproteins are present in LEC29.Lec32 cells (26), and CMP Neu5Ac synthetase transcripts were detectable by reverse transcription–PCR. A revertant selected with ricin possessed wt levels of the 2-kb transcript (data not shown).

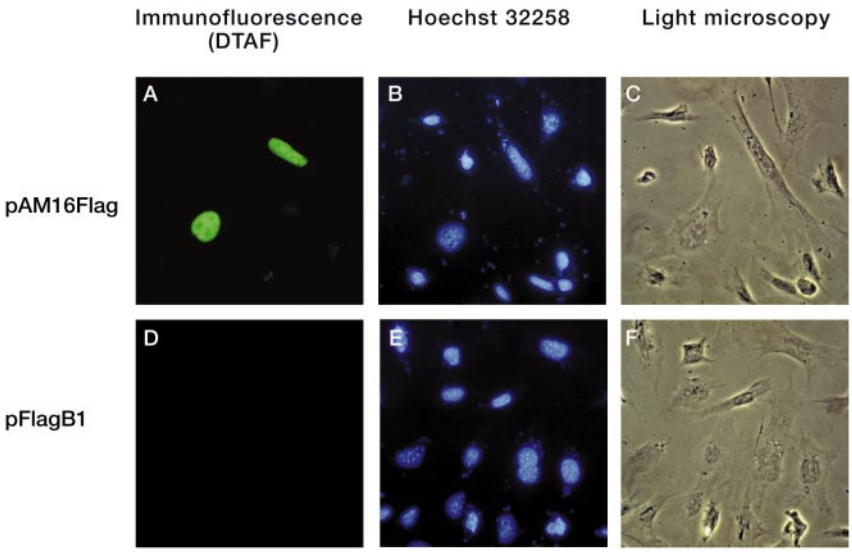

FIG. 4. Nuclear localization of CMP-Neu5Ac synthetase by immunofluorescence. NIH 3T3 cells were transfected transiently with pAM16Flag (*A–C*), and control samples were transfected with empty vector pFlagB1 (*D–F*). Immunofluorescence was carried out with the anti-Flag mAb M5 and fluorescein (DTAF)-conjugated goat anti-mouse Ig (*A* and *D*). Nuclear staining was performed with Hoechst 32258 on the same microscopic samples (*B* and *E*). In *C* and *F*, a light microscopic image of the cells is shown (400×).

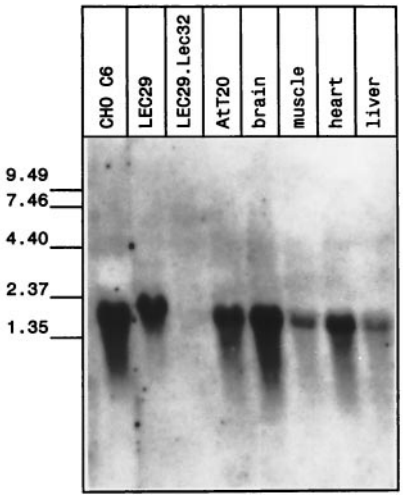

FIG. 6. Northern blot analysis of total RNA isolated from CHO wt cells, mutant LEC29 and LEC29.Lec32, and the mouse pituitary tumor cell line AtT20 and from murine brain, muscle, heart, and liver samples. mRNA (15 μg per lane) was resolved on a 1% agarose formaldehyde gel and, after transfer to a nylon membrane, was developed with a digoxigenin-labeled RNA-probe, transcribed from the coding region of the CMP-Neu5Ac synthetase cDNA.

Southern analysis was performed with genomic DNA from wt LEC29.Lec32 and revertant cells after digestion of genomic DNA with eight different restriction enzymes. All digests gave rise to the same pattern of two or more hybridizing bands with a 560-bp probe from the coding region (data not shown). Therefore, the *lec*32 mutation does not appear to have arisen from a major gene rearrangement.

DISCUSSION

The expression cloning of mammalian CMP-Neu5Ac synthetase brings to three the glycosylation genes that have been isolated by using reexpression of PSA detected by mAb 735 as the criterion for complementation of a glycosylation defect (27, 39, 40). The strategy may be applied to cloning any gene whose inactivation causes a severe reduction in sialic acid addition to cell surface glycans such that PSA is not synthesized. Once complementation of the defect occurs, mAb 735 will detect PSA with an unusual high sensitivity because a single PSA chain provides a multitude of epitopes for this mAb. Thus, leaky mutations and revertants present significant problems as noted herein.

The *lec32* mutation causes an asialo phenotype similar to the *lec2* CHO mutation, which inhibits the transport of CMP-Neu5Ac into the lumen of the Golgi apparatus (27, 40, 44). Although clones exhibiting the *lec2* defect have been isolated in different laboratories and from different cell lines (reviewed in ref. 45), the *lec32* mutation has so far appeared only once. Moreover, in contrast to Lec2 mutants, LEC29.Lec32 populations generate revertants at a rather high frequency, perhaps indicating a growth advantage for cells expressing an active CMP-Neu5Ac synthetase. However, revertants do not confound assays of synthetase activity (ref. 26 and Table 1) or Northern blot analysis (Fig. 6).

Table 1. Reconstitution of CMP–Neu5Ac synthetase activity in pAM15 transfected LEC29.LEC32 cells

Cell line	Plasmid	Activity, nmol/min/mg protein
wt CHO	—	1.98
LEC29.Lec32	—	0.006
LEC29.Lec32 (pool)	pABE	0.023
LEC29.Lec32 (pool)	pAM15	0.51

CMP-Neu5Ac synthetase activity was determined in wt CHO and LEC29.Lec32 cells before and after stable transfection with the murine CMP-Neu5Ac synthetase cDNA pAM15 or the empty vector pABE.

Evidence that the cDNA cloned by complementation of LEC29.Lec32 cells is indeed a mammalian CMP-Neu5Ac synthetase comes from several sources: (*i*) The sequence contains four motifs highly conserved in bacterial CMP-Neu5Ac synthetases (Table 2); (*ii*) the sequence contains three putative nuclear localization signal sequences, and cell fractionation (Fig. 3) and immunofluorescence (Fig. 4) show that it is localized predominantly to the nucleus, as predicted from previous studies (16, 42, 46); (*iii*) transfection of the cloned cDNA into LEC29.Lec32 cells corrects all aspects of the Lec32 phenotype (Fig. 2 and Table 1); and (*iv*) the cDNA rescues the *E. coli* K1 mutant EV5 that has a mutation in the CMP-Neu5Ac synthetase gene (47). The purification of CMP-Neu5Ac synthetase from bovine anterior pituitary glands has been reported recently (48), and its migration in SDS/PAGE is in good agreement with that of the murine CMP-Neu5Ac synthetase (Fig. 3). By contrast, no similarity exists between the murine synthetase and the protein reported as rat liver CMP-Neu5Ac synthetase (49). The N-terminal sequence and amino acid composition of the molecule purified from rat liver differs completely from the sequence we obtained, and, thus, it appears that the molecule from rat liver is not a CMP-Neu5Ac synthetase.

Studies aimed at defining the biosynthesis of bacterial capsules have identified six bacterial CMP-Neu5Ac synthetases (ref. 50 and references therein). Moreover, in *Campylobacter coli*, a gene involved in the sialylation of flagellin has been reported to express significant homology to CMP-Neu5Ac synthetases (51, 52), but whether this molecule functions as an enzyme is presently not known. Alignment of murine CMP-Neu5Ac synthetase to their bacterial orthologues reveals a degree of homology identical to that found within the group of bacterial enzymes. Four highly conserved motifs, which are expressed at almost identical positions in the bacterial enzymes, also were found in the murine sequence (Table 2). Although there is little data on the functional relevance of these motifs, motif I, which is most N-terminally located, fulfills essential catalytic functions (53). The motif shares homology to other nucleotidyltransferases and therefore is believed to be part of the CTP-binding site (50). It seems likely that the preservation of the four motifs is responsible for the catalytic activity of the mammalian enzyme in *E. coli*. CMP-Neu5Ac synthetase is the first enzyme involved in the metabolism of sialic acid that shows strong conservation from bacteria to mammals.

An intriguing feature of CMP-Neu5Ac synthetase is its predominant localization to the nucleus because other nucleotide–sugar synthetases localize to the cytoplasm. However, ≈10% of the enzyme activity was detected in the cytoplasmic fraction (42, 54), and a single report (55) identified 1% of the enzymatic activity in a Golgi fraction. Although we never saw Golgi localization, overexpression of the gene in the LEC29.Lec32 and NIH 3T3 cells gave a faint cytoplasmic signal in Western blots (Fig. 3) and immunofluorescence experiments. With the availability of recombinant enzyme and the potential to express cytoplasmic variants, it will be possible to determine whether nuclear localization is essential to fully complement the *lec32* mutation. It may be that nuclear localization of the synthetase functions to partially sequester CMP-Neu5Ac from the cytoplasm. CMP-Neu5Ac is an allosteric inhibitor of UDP-GlcNAc 2-epimerase, the enzyme that initiates sialic acid synthesis (14, 15) and thereby regulates its own production. Moreover, storage of free CMP-Neu5Ac in the nucleus may be necessary to protect the nucleotide-sugar against modifying activities located in the cytoplasm, such as the hydroxylase that converts Neu5Ac to *N*-glycolylneuraminic acid (NeuGc) by acting on CMP-Neu5Ac (12, 13). Finally, the localization of a large proportion of CMP-Neu5Ac synthetase to the nucleus potentially indicates a second cellular function for this enzyme or its product. Studies aimed at generating soluble forms

Table 2. Alignment of the four domains conserved in all CMP–Neu5Ac synthetases

Species	aa	motif I	aa	motif II	aa	motif III	aa	motif IV	Acc. no.
mouse	45	ALVLARGGSKGIPLKN	83	FQSVWVSTD	140	IQATSPCL	209	LYENGSFYF	AJ006215†
Haemophilus influenzae	9	AIIPARAGSKGIKDKN	47	FDQIVVTSD	105	LQPTSPLR	173	YRANGAIYI	U32807‡
N. meningitidis	7	AVILARQNSKGLPLKN	45	FDRIIVSTD	103	LQPTSPLR	172	FRPNGAIYI	X78068†
E. coli	7	AIIPARSGSKGLRNKN	45	FEKVIVTTD	102	LQPTSPFR	170	YHPNGAIFI	P13266*
Streptococcus agalactiae	5	CIIPARSGSKGLPDKN	45	KKDIFVSTD	102	LQVTSPLR	170	YYPNGAIFI	U19899†
Haemophilus ducreyi	5	AIIPARAGSKGIKDKN	43	FDHIIVSSD	100	IQPTSPLR	169	YRANGAIYI	U54496‡
Helicobacter pylori	5	AIVLARSSSKRIKNKN	43	FEKVFISSD			170	YHDAGLLYM	AE000550‡
Consensus sequence		AIIpAR-GSKGi--KN		F--i-VStD		LQPTSPLR		Y--NGAIYI	

The table aligns the four amino acid stretches of high conservation contained in the murine and bacterial enzymes. Numbers indicate the amino acid residue, with which the motifs start. In the consensus sequence, amino acid residues identical in at least five of seven sequences are shown as capital letters; those conserved in at least four sequences are shown in small letters. Boxed residues are identical for all species. Abbreviations: aa, amino acids; acc. no., accession number.
*SwissProt database.
†European Molecular Biology Laboratory database.
‡GenBank database.

of the enzyme should show whether nuclear localization is required to fully complement the *lec32* phenotype.

We are thankful to Dag Harmsen for automated sequencing of the CMP-Neu5Ac synthetase, to Annette Menkel for expert help with the immunofluorescence studies, and to Dieter Bitter-Suermann for continuous support. Thanks also are extended to Willie Vann for helpful discussions. Eric Vimr and Minoru Fukuda are kindly acknowledged for providing *E. coli* strain EV5 and the vector pPSVE1-PyE, respectively. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ge801/3-1) and Boehringer Mannheim (Penzberg) to R.G-S. and from the National Institutes of Health, National Cancer Institute Grant RO1 36434 to P.S. A Ph.D. grant from the Hans-Böckler-Stiftung supported A.M.

1. Kelm, S. & Schauer, R. (1997) *Int. Rev. Cytol.* **175,** 137–240.
2. Varki, A. (1993) *Glycobiology* **3,** 97–130.
3. Kern, W. F., Spier, C. M., Miller, T. P. & Grogan, T. M. (1993) *Leuk. Lymphoma.* **12,** 1–10.
4. Heitz, P. U., Komminoth, P., Lackie, P. M., Zuber, C. & Roth, J. (1990) *Verh. Dtsch. Ges. Pathol.* **74,** 376–377.
5. Harvey, B. E. & Thomas, P. (1993) *Biochem. Biophys. Res. Commun.* **190,** 571–575.
6. Takano, R., Muchmore, E. & Dennis, J. W. (1994) *Glycobiology* **4,** 665–674.
7. Jann, B. & Jann, K. (1990) *Curr. Top. Microbiol. Immunol.* **150,** 19–42.
8. Vann, W. F., Tavarez, J. J., Crowley, J., Vimr, E. & Silver, R. P. (1997) *Glycobiology* **7,** 697–701.
9. Corfield, A. P. & Schauer, R. (1982) in *Sialic Acids: Chemistry, Metabolism and Function*, ed. Schauer, R. (Springer, New York), Vol. 10, pp. 195–261.
10. Kean, E. L. (1991) *Glycobiology* **1,** 441–447.
11. Hirschberg, C. B. (1996) in *Organellar Ion Channels and Transporters,* eds. Clapham, D. E. & Ehrlich, B. E. (Rockefeller Univ. Press, New York), pp. 105–120.
12. Schlenzka, W., Shaw, L., Kelm, S., Schmidt, C. L., Bill, E., Trautwein, A. X., Lottspeich, F. & Schauer, R. (1996) *FEBS Lett.* **385,** 197–200.
13. Kawano, T., Kozutsumi, Y., Kawasaki, T. & Suzuki, A. (1994) *J. Biol. Chem.* **269,** 9024–9029.
14. Kikuchi, K. & Tsuiki, S. (1973) *Biochim. Biophys. Acta* **327,** 193–206.
15. Hinderlich, S., Stäsche, R., Zeitler, R. & Reutter, W. (1997) *J. Biol. Chem.* **272,** 24313–24318.
16. Coates, S. W., Gurney, T., Jr., Sommers, L. W., Yeh, M. & Hirschberg, C. B. (1980) *J. Biol. Chem.* **255,** 9225–9229.
17. Roseman, S. (1962) *Proc. Natl. Acad. Sci. USA* **48,** 437–441.
18. Ghalambor, M. A. & Heath, E. C. (1966) *J. Biol. Chem.* **241,** 3216–3221.
19. Warren, L. & Blacklow, R. S. (1962) *J. Biol. Chem.* **237,** 3527–3534.
20. Kean, E. L. & Roseman, S. (1966) *J. Biol. Chem.* **241,** 5643–5650.
21. Vimr, E. R., Steenbergen, S. & Cieslewicz, M. (1995) *J. Ind. Microbiol.* **15,** 352–360.
22. Stäsche, R., Hinderlich, S., Weise, C., Effertz, K., Lucka, L., Moormann, P. & Reutter, W. (1997) *J. Biol. Chem.* **272,** 24319–24324.
23. Gerardy-Schahn, R., Bethe, A., Brennecke, T., Mühlenhoff, M., Eckhardt, M., Ziesing, S., Lottspeich, F. & Frosch, M. (1995) *Mol. Microbiol.* **16,** 441–450.
24. Frosch, M., Görgen, I., Boulnois, G. J., Timmis, K. N. & Bitter-Suermann, D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1194–1198.
25. Potvin, B. & Stanley, P. (1991) *Cell Regul.* **2,** 989–1000.
26. Potvin, B., Raju, T. S. & Stanley, P. (1995) *J. Biol. Chem.* **270,** 30415–30421.
27. Eckhardt, M., Mühlenhoff, M., Bethe, A. & Gerardy-Schahn, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7572–7576.
28. Bierhuizen, M. F. & Fukuda, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9326–9330.
29. Seed, B. & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3365–3369.
30. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
31. Mühlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M. & Gerardy-Schahn, R. (1996) *EMBO J.* **15,** 6943–6950.
32. Vimr, E. R. & Troy, F. A. (1985) *J. Bacteriol.* **164,** 854–860.
33. Edwards, U., Müller, A., Hammerschmidt, S., Gerardy-Schahn, R. & Frosch, M. (1994) *Mol. Microbiol.* **14,** 141–149.
34. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
35. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402.
36. Depiereux, E., Baudoux, G., Briffeuil, P., Reginster, I., De Bolle, X., Vinals, C. & Feytmans, E. (1997) *Comput. Appl. Biosci.* **13,** 249–256.
37. Bailey, T. L. & Elkan, C. (1994) *Ismb.* **2,** 28–36.
38. Henikoff, S., Henikoff, J. G., Alford, W. J. & Pietrokovski, S. (1995) *Gene* **163**, GC17–GC26.
39. Eckhardt, M., Mühlenhoff, M., Bethe, A., Koopman, J., Frosch, M. & Gerardy-Schahn, R. (1995) *Nature (London)* **373,** 715–718.
40. Eckhardt, M. & Gerardy-Schahn, R. (1997) *Eur. J. Biochem.* **248,** 187–192.
41. Kozak, M. (1991) *J. Biol. Chem.* **266,** 19867–19870.
42. Van den Eijnden, D. H. (1973) *J. Neurochem.* **21,** 949–958.
43. Wang, W. C. & Cummings, R. D. (1988) *J. Biol. Chem.* **263,** 4576–4585.
44. Deutscher, S. L., Nuwayhid, N., Stanley, P., Briles, E. I. B. & Hirschberg, C. B. (1984) *Cell* **39,** 295–299.
45. Stanley, P. (1993) in *Cell Surface and Extracellular Glycoconjugates*, eds. Roberts, D. D. & Mecham, R. P. (Academic, San Diego), pp. 181–222.
46. Kean, E. L. & Bruner, W. E. (1971) *Exp. Cell Res.* **69,** 384–392.
47. Vimr, E. R., Aaronson, W., Silver, R. P. (1989) *J. Bacteriol.* **171,** 1106–1117.
48. Concepcion, N., Vionnet, J. & Vann, W. F. (1997) *Glycoconjugate J.* **14,** S25.
49. Rodriguez-Aparicio, L. B., Luengo, J. M., Gonzalez-Clemente, C. & Reglero, A. (1992) *J. Biol. Chem.* **267,** 9257–9263.
50. Tullius, M. V., Munson, R. S., Jr., Wang, J. & Gibson, B. W. (1996) *J. Biol. Chem.* **271,** 15373–15380.
51. Guerry, P., Doig, P., Alm, R. A., Burr, D. H., Kinsella, N. & Trust, T. J. (1996) *Mol. Microbiol.* **19,** 369–378.
52. Doig, P., Kinsella, N., Guerry, P. & Trust, T. J. (1996) *Mol. Microbiol.* **19,** 379–387.
53. Zapata, G., Picone, R. & Vann, F. W. (1995) *Glycoconjugate J.* **12,** 519.
54. Van Dijk, W., Maier, H. & Van den Eijnden, D. H. (1976) *Biochim. Biophys. Acta* **444,** 816–834.
55. Creek, K. E., Weisman, D. C. & Morre, D. J. (1980) *Eur. J. Cell Biol.* **23,** 157–165.