THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 39, Issue of September 26, pp. 24319–24324, 1997
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Printed in U.S.A.

A Bifunctional Enzyme Catalyzes the First Two Steps in *N*-Acetylneuraminic Acid Biosynthesis of Rat Liver

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF UDP-*N*-ACETYL-GLUCOSAMINE 2-EPIMERASE/*N*-ACETYLMANNOSAMINE KINASE*

(Received for publication, June 3, 1997)

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***N*-Acetylneuraminic acid (Neu5Ac) is the precursor of sialic acids, a group of important molecules in biological recognition systems. Biosynthesis of Neu5Ac is initiated and regulated by its key enzyme, UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, EC 5.1.3.14)/*N*-acetylmannosamine kinase (ManNAc kinase, EC 2.7.1.60) in rat liver (Hinderlich, S., Stäsche, R., Zeitler, R., and Reutter, W. (1997) *J. Biol. Chem.* 272, 24313–24318). In the present paper we report the isolation and characterization of a cDNA clone encoding this bifunctional enzyme. An open reading frame of 2166 base pairs encodes 722 amino acids with a predicted molecular mass of 79 kDa. The deduced amino acid sequence contains exact matches of the sequences of five peptides derived from tryptic cleavage of the enzyme. The recombinant bifunctional enzyme was expressed in COS7 cells, where it displayed both epimerase and kinase activity.**

Distribution of UDP-GlcNAc 2-epimerase/ManNAc kinase in the cytosol of several rat tissues was investigated by determining both specific enzyme activities. Secreting organs (liver, salivary glands, and intestinal mucosa) showed high specific activities of UDP-GlcNAc 2-epimerase/ManNAc kinase, whereas significant levels of these activities were absent from other organs (lung, kidney, spleen, brain, heart, skeletal muscle, and testis). Northern blot analysis revealed no UDP-GlcNAc 2-epimerase/ManNAc kinase mRNA in the non-secreting tissues.

N-Acetylneuraminic acid (Neu5Ac)[1] is the most common naturally occurring sialic acid, as well as being the biosynthetic precursor of this group of compounds. The elementary 9-carbon carboxylated skeleton can be modified by various substitutions leading to more than 30 naturally occurring derivatives of Neu5Ac (1, 2). The expression of specific sialic acids depends on the species, the tissue, and the developmental stage of the organism (3). By virtue of their widespread distribution, their structural versatility, and their peripheral position in glycoconjugates, sialic acids are well suited as molecular determinants of specific biological functions (4) such as cellular adhesion (5, 6), formation or masking of recognition determinants (1, 2, 7), and stabilization of the structure of glycoproteins (8, 9). Furthermore, sialic acids are overexpressed in many tumor cells (10, 11), especially in Wilms tumor where they are present as polysialic acid (12). The metastatic and invasive potential of tumor cells is often correlated with the amount of expressed sialic acids (13, 14).

To study the functional role of sialic acids in normal and diseased tissues many efforts have been made to influence the amount of expressed sialic acids by inhibition of sialyltransferases (15), and to modulate the structure of sialic acids by the use of synthetic precursor analogs (16, 17). The results of the present study provide a possible new means for determining the role of sialic acids. Neu5Ac and CMP-Neu5Ac, its activated form, are biosynthesized in rat liver by five consecutive reactions (18). Recently we found that the two enzymes initiating the biosynthesis of Neu5Ac, UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, EC 5.1.3.14) and *N*-acetylmannosamine kinase (ManNAc kinase, EC 2.7.1.60) are parts of one bifunctional enzyme (19). This bifunctional enzyme, the key enzyme of Neu5Ac biosynthesis, was cloned and its cDNA was functionally expressed.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Life Technologies, Inc. (Gaithersburg, MD). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Nylon filters for screening, [α-^{32}P]dATP, and [α-^{35}S]dATP were from Amersham (Braunschweig, Germany). *N*-Acetyl-D-[1-^{14}C]mannosamine and UDP-*N*-acetyl-D-[U-^{14}C]glucosamine were from ICN (Eschwege, Germany). Peroxidase-conjugated swine anti-rabbit antibody was from Dako (Hamburg, Germany). Fluorescein isothiocyanate-conjugated swine anti-rabbit antibody was purchased from Dianova (Hamburg, Germany). All other chemicals were of the highest available quality and purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany).

Preparation of Peptides and Amino Acid Sequencing—UDP-GlcNAc 2-epimerase/ManNAc kinase was purified from rat liver as described (19) and subjected to NH_2-terminal sequencing by automated Edman degradation on an Applied Biosystems protein sequencer (model 473A). Peptides were generated by digestion of 20 μg of UDP-GlcNAc 2-epimerase/ManNAc kinase with 2 μg of trypsin in 100 mM Tris-HCl, pH 8.5, at 37 °C for 24 h. Peptides were separated on a reversed phase HPLC column (C_{18} column LiChro CART CHR D, Merck) with a discontinous gradient of 2 to 50% acetonitrile in 0.1% trifluoroacetic acid over 85 min at 0.4 ml/min, followed by 50 to 95% acetonitrile in 0.1%

* This work was supported by the Bundesministerium für Bildung und Forschung, Bonn, and the Fonds der Chemischen Industrie, Frankfurt/Main. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y07744.

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; UDP-GlcNAc 2-epimerase, UDP-*N*-acetylglucosamine 2-epimerase; ManNAc kinase, *N*-acetylmannosamine kinase; GlcNAc, *N*-acetylglucosamine; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; bp, base pair(s).

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TABLE I
Design of PCR primers

Sense (d and c) and antisense (a-d and a-e) primers for PCR were designed from sequences of peptides D and E, taking into consideration the rat codon usage frequencies.

Peptide	Primer	Amino acid sequence	DNA sequence "rat codon usage"
D		LIQEWNSVDL	CTGATCCAGGAGTGGAACWSYGTGGAYCTG
	d		CTGATCCAGGAGTGGAA
	a–d		GTTCCACTCCTGGATCA
E		VGAFGTPVINGGGKGCGCYTTCGGCACHCCHGTGATCAACCTGGGCACHMGV	
	e		GTGATCAACCTGGGCAC
	a–e		GTGCCCAGGTTGATCAC

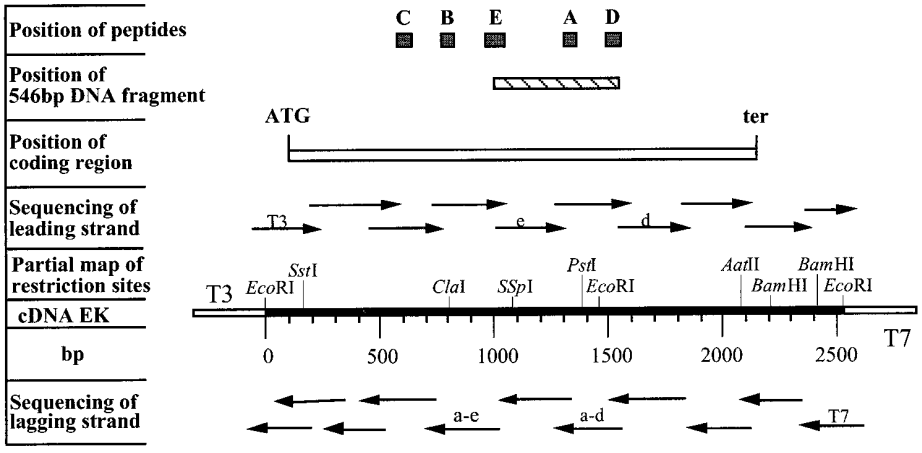

FIG. 1. **Physical map and sequencing strategy for cDNA of UDP-GlcNAc 2-epimerase/ManNAc kinase (cDNA EK).** Named arrows (*T3, T7, d, e, a-d,* and *a-e*) indicate different origins of sequencing. The restriction sites were determined both from the sequence and by digestion with appropriate restriction enzymes, and are indicated along cDNA EK. The coding region (*open bar* above the partial restriction map of cDNA EK) and the position of the PCR-generated 546-bp DNA fragment (*striped bar*), which was used to screen a cDNA library of rat liver, are indicated. The beginning of the coding region is indicated by *ATG* and the end is indicated by *ter*. The positions of peptides (*A, B, C, D*, and *E*) derived by tryptic digestion and reversed phase HPLC separation are also shown in relation to the cDNA sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase.

trifluoroacetic acid over 25 min at 0.4 ml/min. Effluent samples showing peaks of absorbance at 215 nm were collected and concentrated to 20 μl before peptide sequencing.

cDNA Library and Nucleotide Sequencing—Total RNA was extracted from rat liver tissue in an acidic guanidinium thiocyanate/phenol/chloroform mixture (20). Reverse transcription of poly(A)$^+$ RNA with oligo(dT)$_{15}$ primers was done using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The resulting reverse transcripts were used directly for PCR. Primers for PCR were designed from peptide sequences, taking into consideration the rat codon usage frequencies. PCR was performed using sense and antisense primers (125 pmol each) and 1.3 units of *Taq* DNA polymerase in a reaction mixture (50 μl) containing 1 μg of cDNA. Amplification was carried out by 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min using a TwinCycler II thermal cycler (AGS, Heidelberg, Germany). The PCR products were analyzed by agarose gel electrophoresis, purified, cloned in pCR II (Invitrogen, San Diego, CA), and sequenced by the dideoxy chain termination method (21) using a T7 sequencing kit from Pharmacia (Freiburg, Germany). The obtained 546-bp PCR product was radiolabeled with [α-^{32}P]dATP by the random priming method (22) using a Random Primed DNA Labeling System Kit from Life Technologies, Inc.; 1.2 $\times$ 10^6 clones of a rat liver λ ZAP II cDNA library (Stratagene, Heidelberg, Germany) were screened. Hybridization was performed in buffer of 5 $\times$ SSC (1 $\times$ SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 0.1% SDS, 200 μg/ml herring sperm DNA at 42 °C. Washes were at the same temperature with 2.0–0.3 $\times$ SSC, 0.1% SDS. Positive clones were converted to phagemids, consisting of pBluescript II SK$^-$ and cDNA insert, by *in vivo* excision in *Escherichia coli* XL1-Blue host with R408 helper phage. Plasmids were amplified and the inserted cDNA sequenced. T3, T7, and internal primers generated from the sequence were used for sequencing both strands. Sequence homology analysis was done with GenBank/EMBL and SWISS-PROT data bases using MacMolly software (Soft Gene, Berlin, Germany).

Expression of UDP-GlcNAc 2-Epimerase/ManNAc Kinase—UDP-GlcNAc 2-epimerase/ManNAc kinase coding cDNA from pBluescript II was subcloned into the eucaryotic expression vector pBK-CMV (Stratagene). COS7 cells were then transiently transfected with this construct using the calcium phosphate method (23). After 20 h, the calcium phosphate precipitate was removed and cells were further cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 24 h and then selected with 600 μg/ml G418. Expression of UDP-GlcNAc 2-epimerase/ManNAc kinase was monitored by immunofluorescence, immunoblotting, and determination of enzyme activity in cell lysates.

Immunofluorescence—For immunofluorescence, cells were grown on coverslips, fixed in solution containing 3% formaldehyde and permealized with 0.1% Triton X-100. Cells were blocked with 3% bovine serum albumin and then incubated with polyclonal antibody at room temperature. The polyclonal antiserum directed against UDP-GlcNAc 2-epimerase/ManNAc kinase was obtained by the method of Boulard and Lecroisey (24). Cells were then incubated with fluorescein isothiocyanate-conjugated anti-rabbit antibody and imaged by fluorescence microscopy.

Immunoblot Analysis—Cell lysates of COS7 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (25) using 10% acrylamide gels. Separated proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked for 12 h in 5% skim milk in buffer A (0.1% Tween 20, 150 mM NaCl, 3 mM KCl in 9 mM NaH_2PO_4, pH 7.2) and then incubated for 1 h in a 1/2000 dilution of polyclonal anti-UDP-GlcNAc 2-epimerase/ManNAc kinase antiserum in buffer A. Detection was performed using a peroxidase-conjugated swine anti-rabbit secondary antibody and an enhanced chemiluminescence (ECL) detection kit from Amersham.

Preparation of Tissue Cytosols—Male Wistar rats were perfused with 20 ml of 150 mM NaCl while the animals were under light ether anesthesia. Organs were removed and transferred to 2 volumes of homogenizing buffer containing 10 mM NaH_2PO_4, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM ManNAc, 0.1 mM UDP, and 1 mM phenylmethylsulfonyl fluoride. The tissues were homogenized with an Ultraturrax for 1 min at 10,000 rpm. The homogenates were ultracentrifuged at 100,000 $\times$ *g* for 60 min.

Enzyme Assays—UDP-GlcNAc 2-epimerase and ManNAc kinase were assayed as described (19). Tissue distribution was determined by

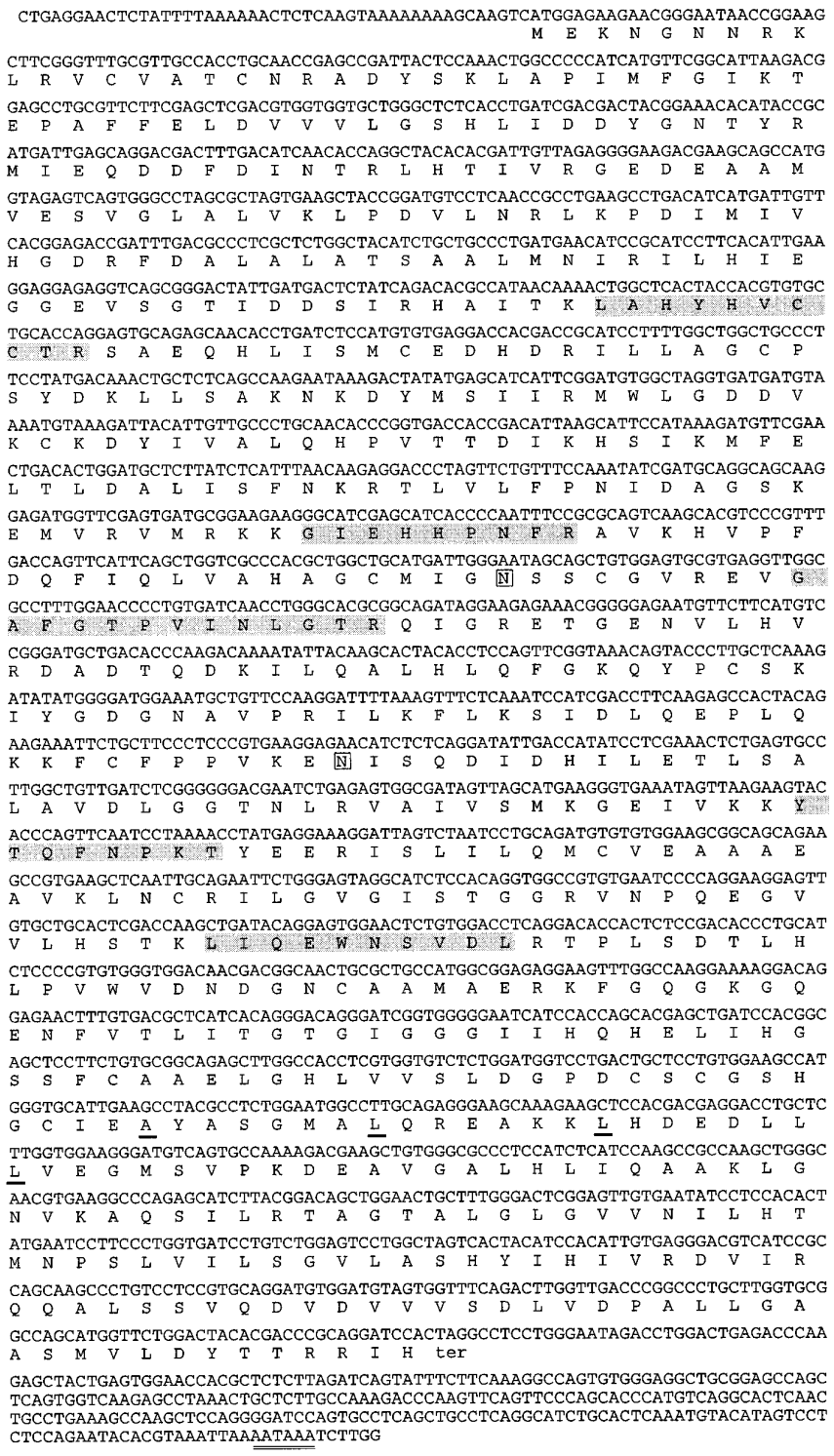

FIG. 2. **Nucleotide sequence and deduced amino acid sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase.** Nucleotides are numbered beginning with the first nucleotide of the cDNA insert following the *Eco* linker of the cDNA library at the *Eco*RI site. The predicted amino acid sequence is indicated *below* and is *numbered in bold* beginning with the first amino acid of the NH_2 terminus. *Shadowed* amino acid residues were determined by amino acid sequencing of purified rat UDP-GlcNAc 2-epimerase/ManNAc kinase prior to cloning. The end of the coding region is indicated by *ter* and the polyadenylation signal is *double underlined*. Potential asparagine-linked glycosylation sites are *boxed* and the leucine residues and one alanine residue of a leucine zipper-like motive (34, 35) are *underlined*. The sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under accession number Y07744.

assaying ManNAc kinase in the presence of 5 mM *N*-acetylglucosamine (GlcNAc) to inhibit the ManNAc kinase activity of *N*-acetylglucosamine kinase (GlcNAc kinase) (26). Protein concentration was determined according to the method of Bradford (27), using bovine serum albumin as standard.

Northern Blot Analysis—A rat multiple tissue Northern blot (CLONTECH, Palo Alto, CA) was hybridized with the [α-^{32}P]dATP radiolabeled 546-bp DNA fragment generated by PCR as described above. Hybridization was performed overnight at 42 °C in 50% formamide, 10 × Denhardt's solution, 5 × SSPE (1 × SSPE, 150 mM NaCl, 20 mM EDTA, 10 mM NaH_2PO_4, pH 7.4), 0.5% SDS, and 100 μg/ml denatured herring sperm DNA. The blot was washed four times at 42 °C in 2 × SSC and 0.1% SDS for 10 min and then twice at 50 °C in 0.1 × SSC and 0.1% SDS for 20 min. The bands were visualized by autoradiography.

RESULTS

Protein Sequencing and Amplification of cDNA Fragments by PCR—Amino acid sequences of five selected peptides were obtained after tryptic digestion of purified UDP-GlcNAc 2-epimerase/ManNAc kinase and fractionation of the peptides by reversed phase HPLC (Fig. 2). Sequencing of the NH_2 terminus of the 75-kDa polypeptide was without result, suggesting that the NH_2 terminus was blocked. The sequences of peptide D and peptide E were used for designing oligonucleotide primers (d, e, a-d, and a-e, where a-d and a-e are the antisense sequences of primers d and e, respectively) for PCR, taking into consider-

		PHOSPHATE 1		PHOSPHATE 2
2EPI_RAT	410	L A V D L G G T N L R V A I V S M K	537	F V T L I T G T G I G G G I I H
HXK1_RAT	529	L A L D L G G T N F R V L L V K I R	673	E I G L I V G T G T N A C Y M E
HXK2_RAT	81	L A L D L G G T N F R V L R V R V T	225	E I G L I V G T G S N A C Y M E
HXK3_RAT	539	L A L D L G G T N F R V L L V R V A	680	E M G L I V G T G T N A C Y M E
HXKH_RAT	75	L S L D L G G T N F R V M L V K V G	221	E V G M I V G T G C N A C Y M E
HXKB_YEAST	83	L A I D L G G T N L R V V L V K L G	227	K M G V I F G T G V N G A Y Y D
PBD		f x f D x G s s x x h h x h x p x p		t h h x x x G T s t x x h h x p

FIG. 3. **Mapping of phosphate-binding subdomains in UDP-GlcNAc 2-epimerase/ManNAc kinase.** The diagram aligns putative phosphate-binding subdomains of the ATPase domain in UDP-GlcNAc 2-epimerase/ManNAc kinase and several hexokinases. Abbreviations are: 2EPI_RAT, UDP-GlcNAc 2-epimerase/ManNAc kinase; HXK1_RAT, rat hexokinase 1; HXK2_RAT, rat hexokinase 2; HXK3_RAT, rat hexokinase 3; HXKH_RAT, rat glucokinase; HXKB_Yeast, yeast hexokinase B; PBD, phosphate-binding subdomain. *Letters* at PBD represent the amino acid nomenclature of Bork *et al.* (32) derived from sequence similarities of the ATP-binding domains of sugar kinases, actin, and hsp 70: *h*, purely hydrophobic (VLIFWY); *f*, partially hydrophobic (VLIFWYMCGATKHR); *t*, tiny (GSAT); *s*, small (GSATNDVCP); *p*, tiny plus polar (GSATNDQEKHR). *Underlined* amino acids match with PBD amino acids. *Shadowed* amino acids match of UDP-GlcNAc 2-epimerase/ManNAc kinase with other hexokinases.

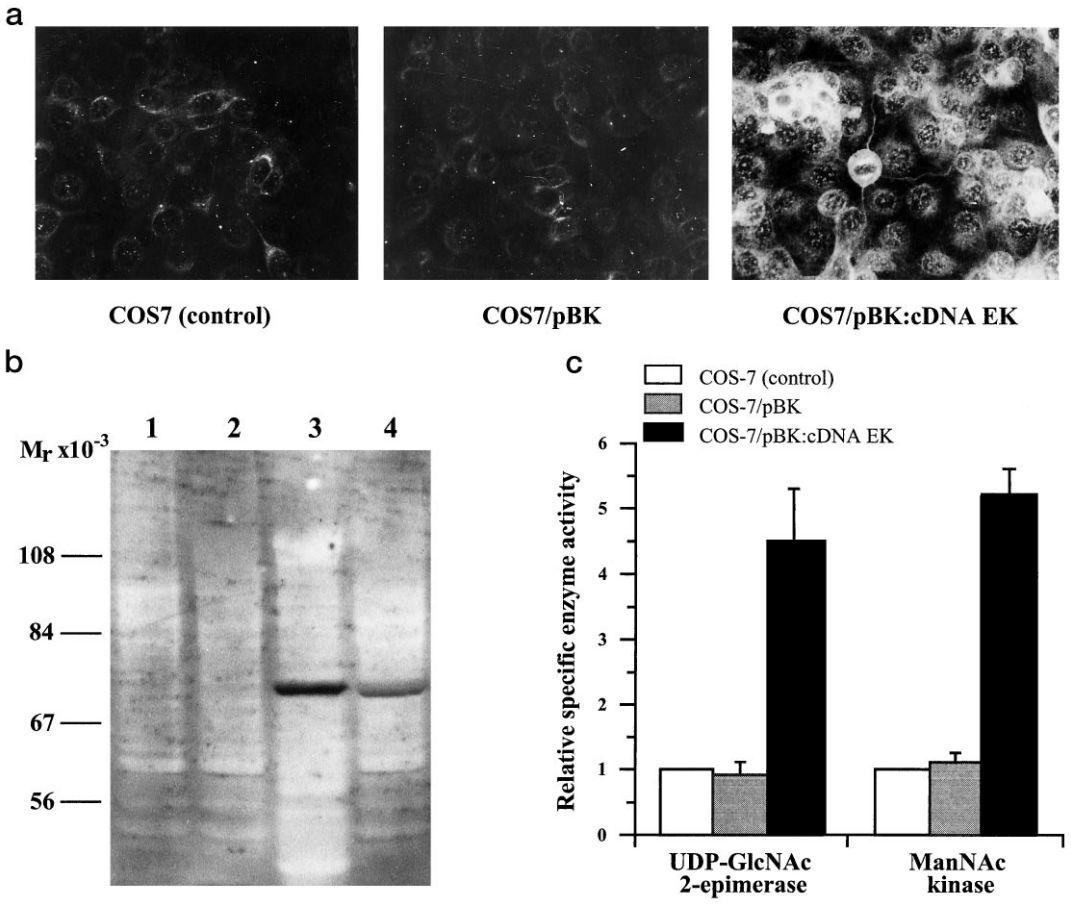

FIG. 4. **Transient expression of UDP-GlcNAc 2-epimerase/ManNAc kinase in COS7 cells.** COS7 cells were transfected with either expression plasmid pBK-CMV (*pBK*) or cDNA EK in expression plasmid pBK-CMV (*pBK:cDNA EK*). Non-transfected COS7 cells (*COS7 (control)*) served as the negative control. *a*, immunofluorescence; *b*, immunoblotting of cell lysates: *lane 1*, non-transfected COS7 cells; *lane 2,* COS7 cells transfected with pBK; *lane 3*, rat liver cytosol; *lane 4,* COS7 cells transfected with pBK:cDNA EK; molecular mass markers are indicated on the *left. c*, determination of catalytic activity of UDP-GlcNAc 2-epimerase and ManNAc kinase in cell lysates.

ation the rat codon usage (Table I). First, a DNA fragment of 546 bp was amplified from rat liver cDNA with primer e and primer a-d. The 546-bp DNA fragment was cloned in pCR II and then sequenced. The deduced amino acid sequence of the amplified fragment contained a complete match of peptide A.

Nucleotide Sequence and Deduced Amino Acid Sequence—The 546-bp DNA fragment was used to screen a cDNA library of rat liver. Eight hybridization positive clones were identified. After plaque purification and rescreening, DNA was prepared from each clone and characterized by restriction analysis. The partial restriction map of the longest cDNA clone, which was designated cDNA EK, is shown in Fig. 1. The additional seven clones had overlapping restriction maps. cDNA EK was chosen for further characterization. PCR primers d, e, a–d, and a–e were used as sequencing primers. Additional primers were constructed from the newly obtained sequence, and the complete cDNA sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase was determined in both directions. The strategy used for

deducing the nucleotide sequence is illustrated in Fig. 1. The determined DNA sequence of 2508 bp included the 546-bp long PCR-generated DNA fragment from bp 990 to bp 1536. The sequence of the leading strand is shown in Fig. 2. Examination of the nucleotide sequence showed an open reading frame starting at position 48 and ending at position 2213. The reading frame of 2166 nucleotides encoded 722 amino acids (Fig. 2). The deduced amino acid sequence contained complete matches for all five peptides of the digested UDP-GlcNAc 2-epimerase/ManNAc kinase. The position of these peptides with respect to the DNA sequence is shown in Fig. 1 and the sequences of these peptides within the 75-kDa polypeptide are shadowed in Fig. 2.

The initiation codon ATG is part of a complete consensus motive for eucaryotic translation (28). The 3′-terminal noncoding region includes the polyadenylation signal (29) from nucleotide 2497 to nucleotide 2502 (Fig. 2). Two potential asparagine-linked glycosylation sites with -Asn-Xaa-Ser- as consensus sequence were identified at amino acid positions 300 and 395, but no glycosyl residues were detected in the purified UDP-GlcNAc 2-epimerase/ManNAc kinase when assayed by peptide *N*-glycosidase F digestion and separation of the products by ion exchange chromatography by the method of Nuck *et al.* (30) (data not shown). In addition, no potential transmembrane spanning sequences or signal sequences for glycosylphosphatidylinositol anchoring were detectable, suggesting that UDP-GlcNAc 2-epimerase/ManNAc kinase is translated on free ribosomes as a cytosolic protein, and therefore has no opportunity to undergo *N*-glycosylation.

Determination of Sequence Similarity—Searches of EMBL/GenBank and SWISS-PROT sequence data showed that no identical sequence has been reported so far. In addition, we compared the amino acid sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase with the known sequences of 13 UDP-GlcNAc-binding enzymes, 15 UDP-glucose 4-epimerases, and 12 other epimerases, including the sequence of porcine GlcNAc 2-epimerase (31). The comparison revealed no similarities which may indicate the localization of the UDP-GlcNAc 2-epimerase domain within the bifunctional enzyme. Comparison with the sequences of several hexokinases (Fig. 3) revealed good matches at positions which are typical for phosphate-binding subdomains in ATPase domains of sugar kinases (Fig. 3) (32).

Expression of UDP-GlcNAc 2-Epimerase/ManNAc Kinase—To confirm that UDP-GlcNAc 2-epimerase/ManNAc kinase is the product of, and can be directly functionally expressed by, the cloned cDNA, cDNA EK was expressed in COS7 cells which were low in detectable UDP-GlcNAc 2-epimerase/ManNAc kinase activity. The recombinant plasmid pBK:cDNA EK contained the 2.5-kilobase pair cDNA EK fragment between *Xba*I and *Xho*I sites of the vector pBK-CMV. COS7 cells were then transfected with pBK:cDNA EK. Transfected cells were found to show an increased intensity of immunofluorescence in the cytosol (Fig. 4*a*). Immunoblot analysis of the cell lysates of transfected cells with a polyclonal antibody directed against rat liver UDP-GlcNAc 2-epimerase/ManNAc kinase revealed a polypeptide showing the same mobility as native UDP-GlcNAc 2-epimerase/ManNAc kinase on SDS-polyacrylamide gel electrophoresis (Fig. 4*b*). In cell lysates expressing the 75-kDa polypeptide the activities of UDP-GlcNAc 2-epimerase and ManNAc kinase were five times greater than those in cell lysates of either pBK-CMV-containing strains or nontransfected strains of COS7 cells (Fig. 4*c*).

Tissue Distribution of UDP-GlcNAc 2-Epimerase/ManNAc Kinase—The enzyme activities of UDP-GlcNAc 2-epimerase/ManNAc kinase were determined in the cytosols of 10 different rat tissues. Enzyme activities were only found in liver, salivary glands, and intestinal mucosa, whereas activities of UDP-GlcNAc 2-epimerase/ManNAc kinase in the cytosols of brain, heart, kidney, lung, skeletal muscle, spleen, and testis were below the detectable level (Table II). mRNA analysis was used to determine whether the tissues showing no UDP-GlcNAc 2-epimerase/ManNAc kinase activity contained even small amounts of the enzyme. Rat mRNA of liver, brain, heart, kidney, lung, skeletal muscle, spleen, and testis were probed at high stringency with the radiolabeled 546-bp DNA fragment. Liver showed a dominant signal at 2500 nucleotides corresponding to the cDNA of UDP-GlcNAc 2-epimerase/ManNAc kinase, but no positive hybridization signal was obtained with the mRNA of the other investigated tissues (Fig. 5).

TABLE II
UDP-GlcNAc 2-epimerase and ManNAc kinase activities of the cytosolic fraction of various tissues

For details see "Experimental Procedures." Enzyme activities are given as milliunits/mg of protein and are expressed as means ± S.D. for three different experiments.

Tissues	UDP-GlcNAc 2-epimerase	ManNAc kinase
Liver	3.1 ± 1.1	6.5 ± 1.7
Salivary glands	0.5 ± 0.2	1.7 ± 0.4
Intestinal mucosa	0.3 ± 0.1	1.0 ± 0.2
Lung	<0.1	<0.1
Spleen	<0.1	<0.1
Kidney	<0.1	<0.1
Testis	<0.1	<0.1
Brain	<0.1	<0.1
Heart	<0.1	<0.1
Skeletal muscle	<0.1	<0.1

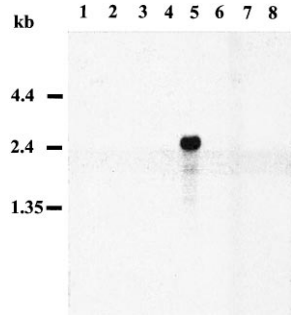

FIG. 5. **Northern blot analysis of several tissues of rat liver.** The Northern blot with mRNA of heart (*lane 1*), brain (*lane 2*), spleen (*lane 3*), lung (*lane 4*), liver (*lane 5*), skeletal muscle (*lane 6*), kidney (*lane 7*), and testis (*lane 8*) was hybridized as described under "Experimental Procedures." A kilobase marker is indicated to the *left*.

DISCUSSION

Recently we found that the two enzymes initiating the biosynthesis of Neu5Ac (UDP-GlcNAc 2-epimerase and ManNAc kinase) are parts of one bifunctional enzyme (19). Here we present the complete sequence of the cDNA encoding the bifunctional enzyme. The 722 amino acids derived from the open reading frame of the cDNA have a predicted molecular mass in agreement with that of the native enzyme, and contain exact matches of five peptides obtained from tryptic digestion of UDP-GlcNAc 2-epimerase/ManNAc kinase. Expression of the cDNA in COS7 cells resulted in a 75-kDa polypeptide, as well as increased activities of UDP-GlcNAc 2-epimerase and ManNAc kinase. We therefore conclude that the cDNA of the 75-kDa polypeptide encodes the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase.

A bifunctional enzyme normally consists of two distinct domains containing separate catalytic sites. As demonstrated for mouse ATP sulfurylase/adenosine 5′-phosphosulfate kinase (33) it is possible to determine these domains from their similarity to known sequences encoding separated enzymes with

equal or similar properties. Although we compared the amino acid sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase with several sequences of enzymes using UDP-GlcNAc as a substrate or catalyzing an epimerase reaction, we were not able to determine the localization of the UDP-GlcNAc 2-epimerase domain. Comparison with the ATP-binding domains of hexokinases revealed good matches for two phosphate-binding sites in the COOH-terminal half of the sequence (Fig. 3). The same area also contains a leucine zipper-like motif which participates in dimerization of DNA-binding proteins and membrane proteins (33, 34). This motif may be responsible for the higher stability of the dimeric structure that displays ManNAc kinase activity, although no cytosolic protein has yet been reported in which the leucine zipper motif plays a functional role. We therefore assume that the COOH-terminal part of the UDP-GlcNAc 2-epimerase/ManNAc kinase sequence contains the kinase domain.

Sialic acids are widely distributed in mammalian tissues and one might assume that sialic acid synthesizing enzymes occur in all tissues containing sialic acids. This assumption is not supported by Northern blot analysis, which failed to detect mRNA for UDP-GlcNAc 2-epimerase/ManNAc kinase in the examined tissues, in particular in brain, lung, and kidney, which definitely express sialic acids (36). Tissues with a low requirement for sialic acids may replenish their sialic acid pool with Neu5Ac from oligosaccharide turnover (2, 37). Maru *et al.* (31) reported an alternative pathway for the biosynthesis of Neu5Ac, in which ManNAc is directly synthesized from GlcNAc by the action of *N*-acetylglucosamine 2-epimerase, which occurs in most rat tissues (38). But the activity of ManNAc kinase is directly linked to that of UDP-GlcNAc 2-epimerase by the bifunctionality of UDP-GlcNAc 2-epimerase/ManNAc kinase. In this case GlcNAc kinase, occurring in several rat tissues (39), may phosphorylate ManNAc (26). A very appropriate method for testing for an alternative pathway of Neu5Ac biosynthesis is to construct a gene knock-out of UDP-GlcNAc 2-epimerase/ManNAc kinase in mouse. The prerequisite for this experiment is given with the results presented here.

Acknowledgments—We thank Eva Guhl for technical assistance and Dr. T. A. Scott for improving the English of this manuscript.

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