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Mutations in the Human UDP-N-Acetylglucosamine 2-Epimerase Gene Define the Disease Sialuria and the Allosteric Site of the Enzyme

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Summary

Sialuria is a rare inborn error of metabolism characterized by cytoplasmic accumulation and increased urinary excretion of free N-acetylneuraminic acid (NeuAc, sialic acid). Overproduction of NeuAc is believed to result from loss of feedback inhibition of uridinediphosphate-N-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase) by cytidine monophosphate-N-acetylneuraminic acid (CMP-Neu5Ac). We report the cloning and characterization of human UDP-GlcNAc 2-epimerase cDNA, with mutation analysis of three patients with sialuria. Their heterozygote mutations, R266W, R266Q, and R263L, indicate that the allosteric site of the epimerase resides in the region of codons 263–266. The heterozygous nature of the mutant allele in all three patients reveals a dominant mechanism of inheritance for sialuria.

Introduction

Free N-acetylneuraminic acid (NeuAc) is a negatively charged carbohydrate present in most tissues and body fluids as the terminal acidic residue of N-linked glycoproteins (Schauer 1982). In the first committed step of NeuAc synthesis, UDP-N-acetylglucosamine (UDP-GlcNAc) is converted to N-acetylmannosamine (ManNAc) and UDP by UDP-GlcNAc-2-epimerase (fig. 1). This enzyme is feedback inhibited by the activated sialic acid donor, CMP-Neu5Ac (Kornfeld et al. 1964; Sommar and Ellis 1972), which also provides NeuAc for placement on glycoconjugates in the trans-Golgi (Beaudet and Thomas 1995). The glycoconjugates are eventually degraded in lysosomes, and one of the first steps is removal of a NeuAc residue by neuraminidase (Sommar and Ellis 1972). The resultant free NeuAc is transported out of lysosomes by a membrane carrier (Gahl et al. 1995; Renlund et al. 1986) that also recognizes glucuronic acid (Blom et al. 1990). NeuAc is filtered but not reabsorbed by the human kidney (Seppala et al. 1990).

Several human disorders of sialic acid metabolism have been recognized (Gahl et al. 1996). Sialidosis is due to deficiency of lysosomal neuraminidase activity (Beaudet and Thomas 1995), and galactosialidosis results from a defect in the protein that stabilizes galactosidase and neuraminidase (Galjart et al. 1988). In both of these disorders, conjugated or bound NeuAc is stored within lysosomes. In Salla disease and infantile free sialic acid storage disease (ISSD), unconjugated NeuAc accumulates in lysosomes because of defective transport across the lysosomal membrane (Mancini et al. 1986; Renlund et al. 1986; Tietze et al. 1989). ISSD, observed worldwide (Tondeur et al. 1982; Stevenson et al. 1983), appears to be a more severe form of Salla disease, which occurs primarily among Finnish people (Aula et al. 1979; Renlund et al. 1983). In a different NeuAc storage disease, sialuria (MIM 269921), free sialic acid accumulates in the cell cytoplasm, and gram quantities of NeuAc are excreted in the urine (Montreuil et al. 1968; Thomas et al. 1985; Wilcken et al. 1987). Using fibroblasts cultured from sialuria patients, we previously demonstrated that the metabolic defect involves lack of feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-Neu5Ac, causing constitutive overproduction of free NeuAc (Weiss et al. 1989; Seppala et al. 1991; Krasnewich et al. 1993). The four reported cases of sialuria (Montreuil et al. 1968; Wilcken et al. 1987; Seppala et al. 1991; Krasnewich et al. 1993) are characterized by variable degrees of developmental delay, coarse facial features, and hepatomegaly. Whether sialuria is a dominant or a recessive disorder has not been determined.

The rat UDP-GlcNAc 2-epimerase, a bifunctional enzyme possessing N-acetylmannosamine (ManNAc) kinase activity as well as epimerase activity (Hinderlich et al. 1997; Stasche et al. 1997), has been sequenced, and this provides a basis for investigating the human dis-

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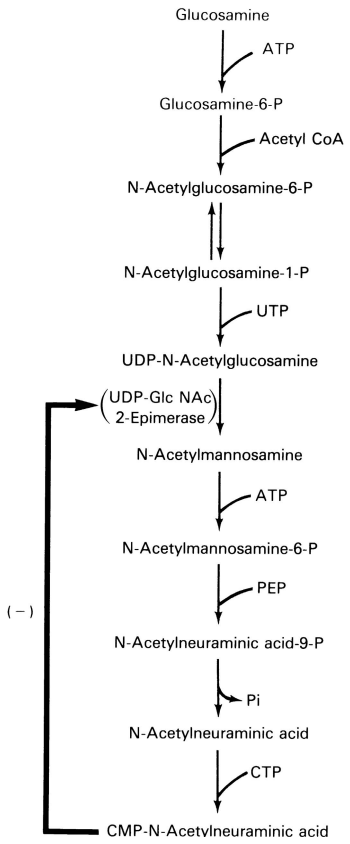

Figure 1 The N-acetylneuraminic acid synthetic pathway, showing feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-Neu5Ac.

order, sialuria. To elucidate the molecular mechanism for defective allosteric regulation of UDP-GlcNAc 2-epimerase in this disease, we cloned and sequenced the human cDNA for the enzyme and determined the mutations in the three available sialuria patients.

Subjects and Methods

Patients and Cells

Patient 1 was A.W., described by Wilcken et al. (1987) as a 2-year-old girl with moderate developmental delay, hepatosplenomegaly, slightly coarse facial features, a large tongue, macrocephaly, and massive urinary excretion of free sialic acid. At age 7 years, she had mild intellectual impairment, with fine-motor difficulty, but attended regular school (Don and Wilcken 1991). Her growth was at the 10th percentile, and the organomegaly persisted. Patient 2 was J.C., reported by Weiss et al. (1989). This 10-month-old boy had hepatomegaly and a coarse voice and facial features. His growth and development, however, remained normal at least through 5 years of age (Gahl et al. 1996). Patient 3 was T.W., extensively described by Krasnewich et al. (1993). This 4.5-year-old boy exhibited hepatomegaly, dysmorphic facies, and hirsutism, but normal growth. At age 6 years, his full-scale IQ was 68.

Fibroblasts from patient 1 were kindly provided by Drs. Bridget Wilcken (Sydney, Australia) and John Hopwood (Adelaide, Australia). Cells from patient 2 were sent by Drs. Greg Barsh and Seymour Packman (San Francisco), and cells from patient 3 by Drs. Wilma Krause (Atlanta) and David Wenger (Philadelphia). The fibroblasts were propagated in Dulbecco's modified Eagle's minimum essential medium fortified with 10% fetal calf serum (Gibco), 2 mM glutamine, nonessential amino acids, and antibiotics as described elsewhere (Renlund et al. 1986; Tietze et al. 1989).

Molecular Studies

We used the sequence of the recently cloned rat UDP-GlcNAc 2-epimerase (Hinderlich et al. 1997; Stasche et al. 1997) to search for homologous human sequences in the dbEST database with an NBI BLAST server. Three human sequences matched various regions of the rat sequence, on the basis of predicted amino acid sequences. Oligonucleotides designed from the human sequences served as primers to PCR amplify the human cDNA from a liver cDNA library (Marathon Ready, Clontech). We determined the 5′ flanking sequence by PCR amplification using Marathon Ready cDNA containing a universal AP adaptor. We identified the 3′ flanking sequence by PCR amplification using the SuperScript human liver cDNA library and a T7 universal primer.

Restriction enzyme digestion, ligation, and cloning procedures were performed as described elsewhere (Sambrook et al. 1989). PCR amplifications were done for 35 cycles (94°C for 30s, 65°C for 30s, and 72°C for 2 min), followed by a single incubation at 72°C for 10 min. DNA sequence analysis was performed by means of the T7 Quick Denature Plasmid sequencing kit (Amersham Life Sciences).

Mutation Analysis

We prepared fibroblast RNA, using Trizol Reagent (Life Technologies). It was converted to cDNA by the SuperScript Pre-amplification System (Life Technologies) and was used as a template for PCR amplification. Direct and clonal sequencing were performed with Amersham's Sequenase PCR Product and the Plasmid Sequencing Kits, respectively. Each patient's cDNA was subjected to analysis for sequence anomalies by using BESS-T Scan TM Mutation Detection and Localization Kit (Epicentre Technologies) according to the manufacturer's instructions.

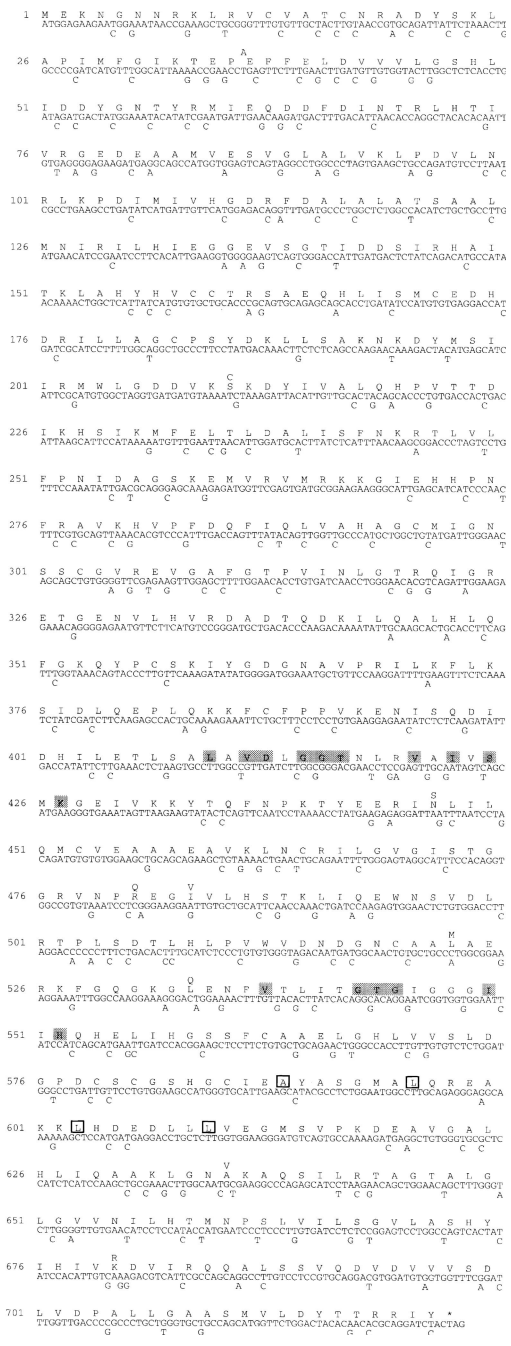

Figure 2 Nucleotide sequence and deduced amino acid sequence of human UDP-GlcNAc 2-epimerase/ManNAc kinase. Divergent amino acids in the rat sequence (SwissProt accession number Y07744) are shown above the human amino acid sequence. Divergent nucleotides in the rat sequence are shown below the human nucleotide sequence. Numbering of the amino acids starts with the putative initiation methionine and follows the rat sequence. The end of the coding region is indicated by an asterisk (*). Two clusters of amino acid residues that comprise putative ATP-binding regions are shaded, and the critical residues of a putative leucine zipper of the predicted kinase domain (Stasche et al. 1997) are boxed.

Northern Blot Analysis

A multiple-tissue northern blot was obtained from Clontech and hybridized by means of a probe containing nucleotides 777–1452 of the human UDP-GlcNAc 2-epimerase cDNA, as described elsewhere (Sambrook et al. 1989).

Expression and Epimerase Assay

Using the GST Gene Fusion System (Pharmacia Biotech), we accomplished expression of UDP-GlcNAc 2-epimerase by inserting the cDNA into vector pGEX-2T and transforming *Escherichia coli BL21* with this vector. After induction with isopropyl-β-D-thiogalactoside, the cells were harvested and extract supernatants assayed for epimerase activity. The conversion of UDP-[3H]GlcNAc to [3H]ManNAc was assayed as described elsewhere (Seppala et al. 1991), with detection of products by high-pH anion-exchange chromatography on a BioLC carbohydrate analyzer (Dionex). For thermostability studies, fibroblast extracts were incubated at 37°C for 0, 2, 10, and 30 min prior to assay of epimerase activity. Epimerase activity was expressed as picomoles of ManNAc produced per milligram of protein per minute.

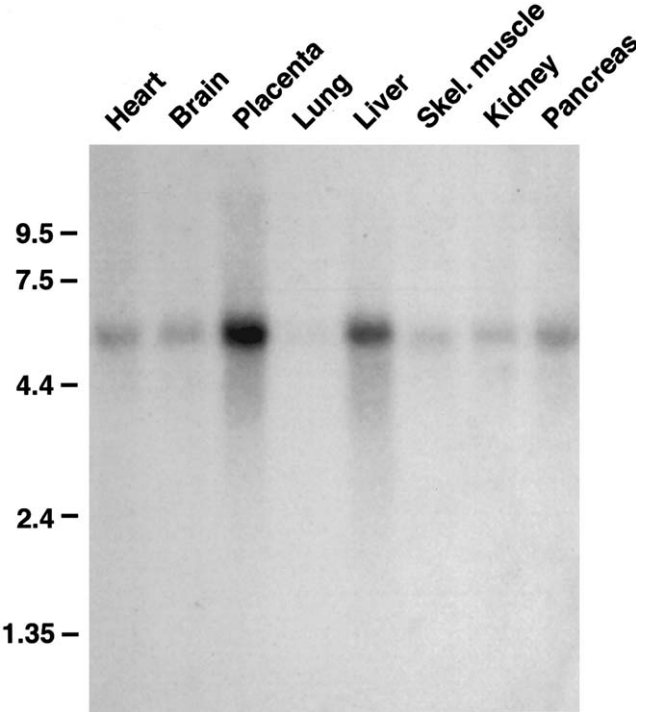

Figure 3 Northern blot of UDP-GlcNAc 2-epimerase gene expression in various human tissues.

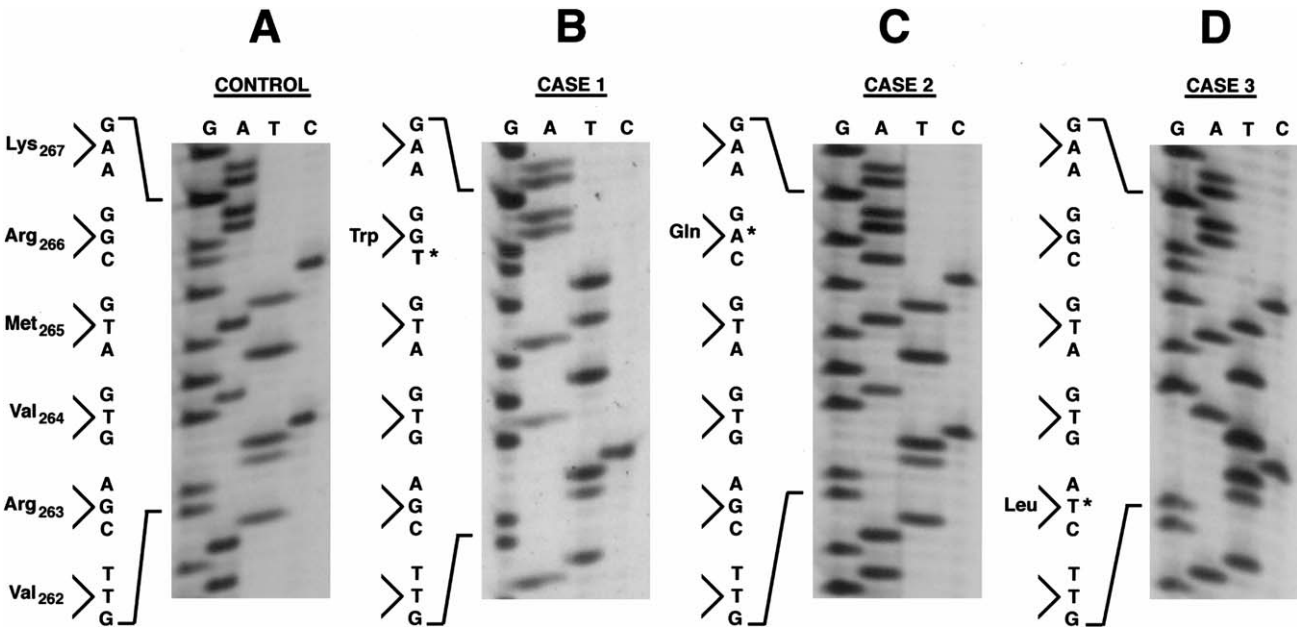

Figure 4 UDP-GlcNAc 2-epimerase mutations in three patients with sialuria. Sequences encompass codons 263–266. *A*, Normal control. *B*, Patient 1, showing missense change R266W due to a C→T transition in the third base of codon 266. *C*, Patient 2, showing missense change R266Q due to a G→A transition in the second base of codon 266. *D*, Patient 3, showing a missense change R263L due to a G→T transversion in the second base of codon 263.

Results

The full-length sequence of the human UDP-GlcNAc 2-epimerase, as judged by the presence of an open reading frame, exhibited 98.6% identity with the rat sequence at the amino acid level and 88% identity at the nucleotide level (fig. 2). Two putative ATP-binding sites were localized to the carboxy-terminal half of the sequence on the basis of a comparison with known ATP-binding domains of hexokinases (fig. 2). A separate comparison revealed 50% similarity of the amino terminal part of our human sequence with the UDP-GlcNAc 2-epimerases of *Bacillus subtilis* (SwissProt Protein Sequence Data Bank accession number P39131), *E. coli* (SwissProt accession number P27828) and *Salmonella borreze* (SwissProt accession number P52642), placing the epimerase activity in the amino-terminal portion of the protein.

Northern blot analysis using a multiple tissue filter revealed a single band of ~5.5 kb with a high level of expression of the human UDP-GlcNAc 2-epimerase in liver and placenta (fig. 3).

The cDNAs of three sialuria patients were subjected to BESS-T Scan analysis (Epicentre Technologies), which revealed sequence anomalies indicative of point mutations in the UDP-GlcNAc 2-epimerase/ManNAc kinase gene. Subsequent sequencing of wild-type and mutant subclones verified the point mutations in one allele of each patient (fig. 4). For patients 1 and 2, the anomaly was in codon 266; for patient 3, it was in codon 263. Consistent with pathologic mutations, each base change led to replacement of a positively charged arginine with an amino acid of different charge or solubility: hydrophobic tryptophan in patient 1, neutral glutamine in patient 2, and hydrophobic leucine in patient 3. For each patient, a cDNA clone that did not contain the base alteration of the mutant clone was completely sequenced; no amino acid–altering base changes were found. Northern blot analysis revealed normal size and amount of UDP-GlcNAc 2-epimerase RNA in each patient's fibroblasts (data not shown).

Full-length cDNA clones of a normal and mutant UDP-GlcNAc 2-epimerase gene were expressed in *E. coli* and assayed for epimerase activity. Bacteria transfected with vector alone gave virtually no background activity; there was no conversion of 3H-UDP-GlcNAc to ManNAc (data not shown). Bacteria transfected with the normal allele of patient 1 showed significant epimerase activity, with substantial production of ManNAc (fig. 5). This was inhibited 85% by 100 μM CMP-Neu5Ac, consistent with normal allosteric inhibition (Seppala et al. 1991). The extract of *E. coli* transfected with plasmid containing the mutant epimerase gene of patient 3 showed normal ManNAc production from

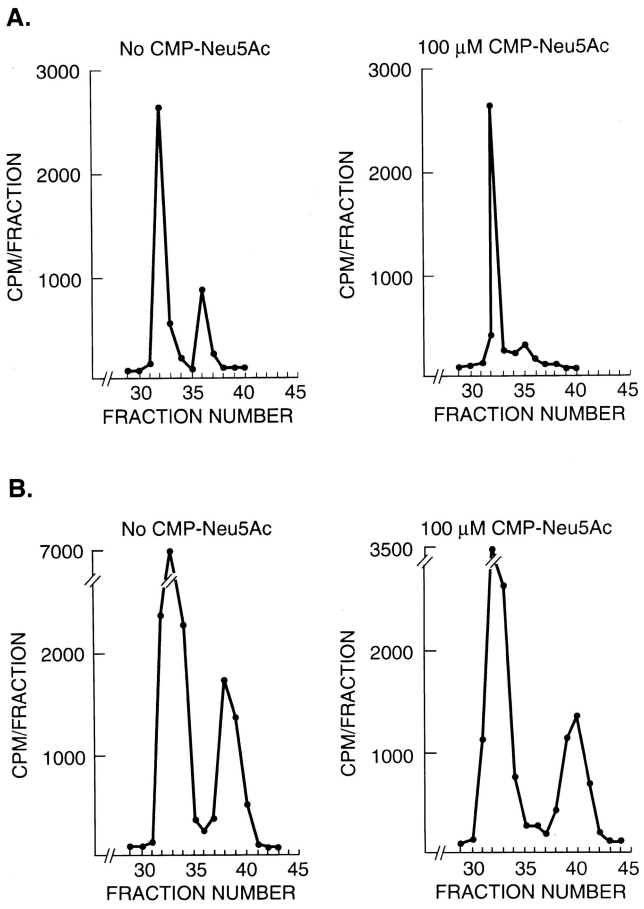

Figure 5 UDP-GlcNAc 2-epimerase enzymatic activity expressed in *E. coli*. The substrate, UDP-[3H]GlcNAc (7.5 nmol in a final volume of 112.5 μl), was incubated for 6 min at 37°C in the presence of 50 μl of *E. coli* extract with 100 μM CMP-Neu5Ac absent (*left panel*) or present (*right panel*). [3H]GlcNAc elutes first in fractions 30–35, whereas the product of the epimerase, [3H]ManNAc, elutes in later fractions. CMP-Neu5Ac inhibits the epimerase, reducing the amount of [3H]ManNAc produced. *A,* Epimerase activity of *E. coli* expressing the normal allele of patient 1. Epimerase activity was inhibited normally (i.e., 85%) by 100 μM CMP-Neu5Ac. *B,* Epimerase activity of *E. coli* expressing the mutant allele of patient 3. The epimerase activity is not decreased, but it is inhibited only 15% by 100 μM CMP-Neu5Ac.

UDP-GlcNAc, but this epimerase activity was inhibited only 15% by 100 μM CMP-Neu5Ac (fig. 5).

The thermostability of the normal and sialuria enzymes was investigated by measuring UDP-GlcNAc 2-epimerase activity in nontransfected fibroblast extracts that were incubated for various lengths of time at 37°C. Control fibroblasts exhibited a half-life of 12 min for the epimerase activity, compared with 13 min for the sialuria fibroblasts (patient 3). In duplicate experiments, the mean baseline epimerase activity of the control fibroblasts was 37 pmol of ManNAc/mg protein/min, compared with 70 pmol/mg protein/min for the sialuria cells.

Discussion

As shown by Stasche et al. (1997), the rat UDP-GlcNAc 2-epimerase is bifunctional, possessing both the 2-epimerase activity and ManNAc kinase activity. The rat enzyme self-associates as a fully active hexamer and as a dimer with only kinase activity. The human enzyme, with nearly 99% identity to the rat enzyme at the amino acid level, also appears bifunctional. The ATP-binding sites and the presence of a leucine zipper motif (a putative dimerization domain) in the carboxy terminus of the protein suggest that the kinase activity resides there (Stasche et al. 1997). This assignment is supported by the similarity of nucleotides 417–722 of our human sequence with those of the glucose kinases of *Staphylococcus xylosus, Streptomyces coelicolor,* and *B. subtilis* and with hexokinase A of *Saccharomyces cerevisiae*. Similarity of the human amino terminal sequence with other epimerases places the epimerase activity in the protein's amino-terminal portion. The clustering of patients' mutations in codons 263–266 strongly suggests that this is the allosteric site for CMP-Neu5Ac binding.

Since only one allele of each sialuria patient contained a mutation, and expression studies using a patient's normal allele resulted in normal, inhibitable epimerase activity (fig. 5), the disease appears dominant in inheritance. That is, heterozygosity for a mutated allosteric site of UDP-GlcNAc 2-epimerase is sufficient to cause sialuria. In this case, the mutant epimerase activity continues to produce free sialic acid and CMP-Neu5Ac, which inhibits the normal but not the mutant epimerase. With no brake on the rate-limiting step in sialic acid production, intracellular free sialic acid levels increase indefinitely, leading to the clinical and laboratory findings of sialuria. Dominant inheritance has also been reported in the hyperinsulinism and hyperammonemia syndrome, in which GTP fails to feedback-inhibit glutamate dehydrogenase because of mutations affecting the enzyme's allosteric site (Stanley et al. 1998). In sialuria, the absence of any symptoms in the parents of the affected children indicates that the base changes represent new mutations. Parental DNA was not available for direct analysis.

The exact mechanism by which the three epimerase mutations cause dysregulation of sialic acid synthesis remains unknown. The standard explanation is that CMP-Neu5Ac fails to feedback inhibit UDP-GlcNAc 2-epimerase. However, the two-fold increase in baseline epimerase activity of sialuria fibroblasts, reported in the present study and elsewhere (Weiss et al. 1989), suggests that greater stability of the enzyme may also contribute

to increased sialic acid production. This hypothesis was not supported by our normal in vitro thermostability measurements. Still, there may be factors that increase epimerase stability in vivo and were not measured in vitro. For example, the CMP-Neu5Ac of sialuria cells may bind to the allosteric site of the epimerase and stabilize the enzyme in vivo but may be released by the in vitro extraction, resulting in normal stability measurements. Alternatively, the extracted epimerase of the control cells may have retained CMP-Neu5Ac bound to it during the enzyme assay, whereas the mutant epimerase did not; this would account for increased epimerase activity in the sialuria extract. In the first case, the CMP-Neu5Ac would bind and stabilize the epimerase but would not inhibit its activity. In the second case, CMP-Neu5Ac would not bind to the allosteric site of the mutant epimerase. We consider this the simplest explanation, but we cannot rule out the possibility that the primary mutations also allow for increased in vivo stability of the epimerase, perhaps mediated by CMP-Neu5Ac binding.

In the rat, the bifunctional epimerase gene was reported to be expressed only in liver (Stasche et al. 1997), whereas we found expression in a variety of tissues (fig. 3). It is likely that any tissue that synthesizes glycoconjugates would require the sialic acid synthetic machinery.

Cloning, mutation analysis, and functional expression studies have revealed the molecular mechanisms of sialic acid accumulation in sialuria and have explained the disorder's inheritance pattern. Additional sialuria patients are required, to initiate genotype-phenotype correlations. However, identification of the allosteric site of UDP-GlcNAc 2-epimerase allows for immediate investigation into the interaction of CMP-Neu5Ac with the enzyme. In particular, the issue of whether CMP-Neu5Ac binds to the mutant epimerase can be addressed.

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Electronic-Database Information

Accession number and URLs for data in this article are as follows:

dbEST: Database of "Expressed Sequence Tags," http://www.ncbi.nlm.nih.gov/dbEST/index.html (for human sequences homologous to the recently cloned rat UDP-GlcNAc 2-epimerase)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for sialuria [MIM 269921])

SwissProt Protein Sequence Data Bank (SwissProt): Database of amino acid sequences (via Blast search tool), http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast (for comparison of the human UDP-GlcNac 2-epimerase with other species)

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