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Cloning, Expression, and Chromosomal Mapping of a Human Ganglioside Sialidase

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Here we report the cDNA sequence of a human ganglioside sialidase. The cDNA was isolated from a human brain cDNA library by screening with a 240 bp probe generated by polymerase chain reaction using primers based on the sequences of rat cytosolic and bovine membrane sialidases which we previously cloned. The 3.0 kb cDNA encodes an open reading frame of 436 amino acids containing a putative transmenbrane domain and an Arg-Ile-Pro and three Asp-box sequences characteristic of sialidases and showing overall 83% and 39% identities to the bovine and rat enzymes, respectively. Northern blot analysis revealed high expression in skeletal muscle and testis, but low level in kidney, placenta, lung, and digestive organs. Transient expression of the cDNA in COS-1 cells resulted in a 130-fold increase in sialidase activity compared to the control level, and the activity was found to be almost specific for gangliosides. Fluorescent *in situ* hybridization allowed the human sialidase gene localized to chromosome 11 at q 13.5. © 1999 Academic Press

Key Words: **sialidase; gangliosides; cDNA cloning; chromosomal mapping.**

Mammalian sialidases, catalyzing an initial step reaction for glycoconjugate degradation, have been suggested to participate in regulation of many cellular processes (1, 2), whereas their counterparts in microorganisms may be involved in nutrition and pathogenesis (3). We previously presented the evidence for four types of sialidase in rat tissues differing in subcellular localization and enzymatic properties: cytosolic (4), lysosomal (5) and two membrane sialidases (6). We then cloned the first manmalian sialidase cDNA for a cytosolic type (7) on the basis of the peptide sequence for the enzyme purified from rat skeletal muscle, and demonstrated that the mammalian sialidase also contained the motifs including Arg-Ile-Pro and Asp-box sequences, which had been reported to be conserved in sialidases of microbial origin (8). A cDNA encoding a soluble sialidase from Chinese hamster ovary cells (9) and recently the human homologue (10) were cloned by two other groups, showing high sequence identity with rat cytosolic sialidase. Lysosomal sialidase gene was identified in the major histocompatibility complex (MHC) region of man by analyzing the genomic DNA (11) or searching for the conserved sequences in the Expressed Sequence Tags data base (12, 13), and the mouse counterpart was cloned using the human cDNA probe (14–16). Recently we were able to obtain a plasma membrane-associated sialidase cDNA, the third type mammalian sialidase cDNA, using the amino acid sequence of the purified enzyme from bovine brain and characterized to encode an enzyme specific for gangliosides (17). These three types of mammalian sialidase share 19–38% sequence identity and seem to possess unique sequences according for their respective subcellular localizations. Transfection of these cDNAs has confirmed that they have distinct substrate specificities as characterized so far in enzyme fractions isolated from mammalian tissues (4, 18–20).

The bovine membrane sialidase is a 48 kDa protein with a type I membrane protein orientation lacking a signal sequence, and when transiently transfected into COS-7 cells it hydrolyzes almost specifically gangliosides other than GM1 and GM2 and hardly acts on glycoprotein and oligosaccharides. Northern blot analysis showed a 7.4 kb transcript, and the same size of transcript was observed in human tissues using the

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Abbreviations used: 4MU-NeuAc, 4-methylumbelliferyl-neuraminic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; 2-morpholinoethanesulfonic acid, MES.

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A

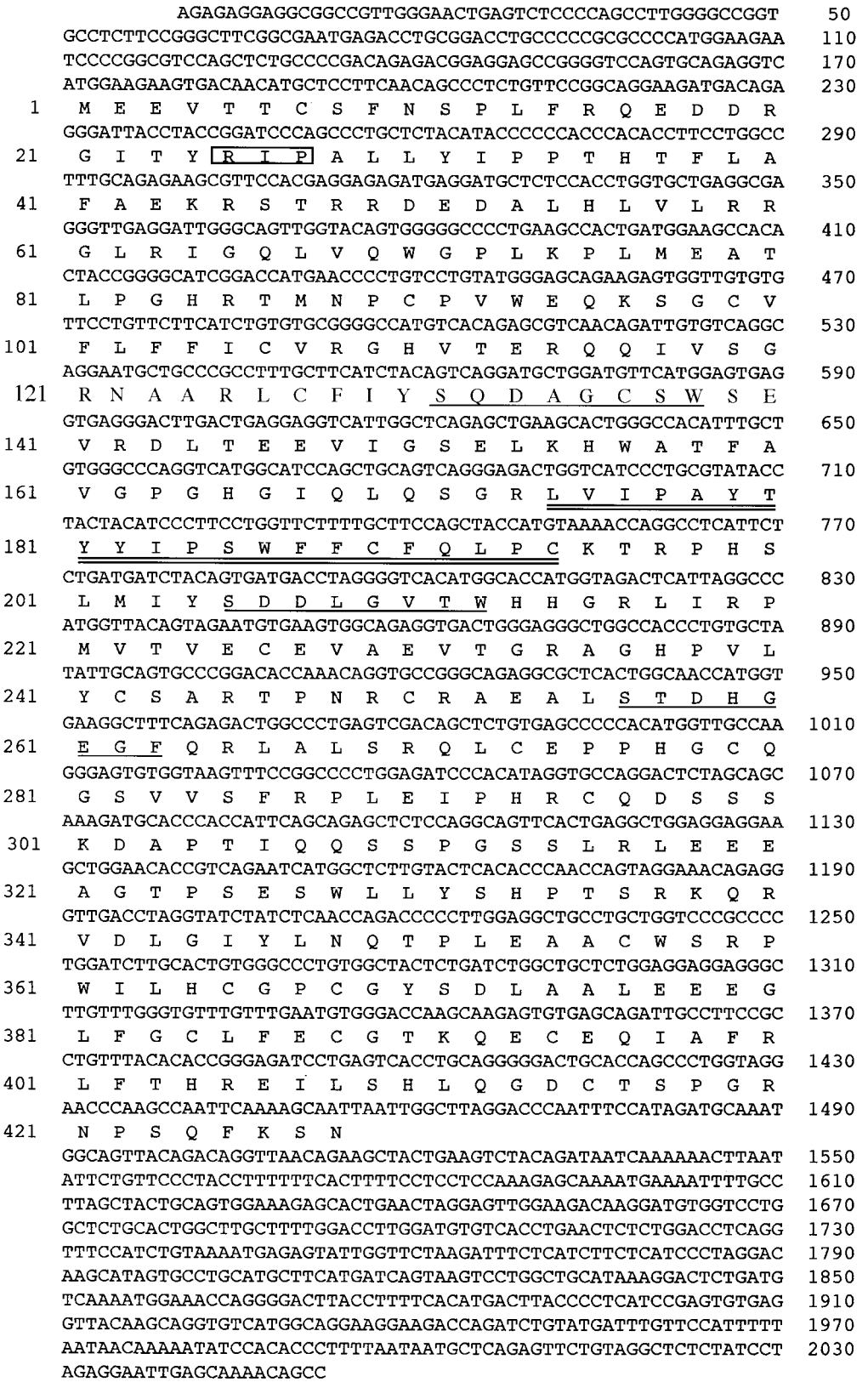

FIG. 1. Nucleotide and deduced amino acid sequences of the human ganglioside sialidase. (A) Single-letter notations are used for amino acids. The residues for the Arg-Ile-Pro sequence and for Asp-boxes, characteristic of sialidases, are boxed and are underlined with a single solid line, respectively. The putative transmembrane domain is underlined with a double line. (B) Alignment of the human and bovine ganglioside sialidase sequences according to the method of Lipman-Pearson. Identical residues are indicated by asterisks (*). The positions of active site residues predicted on the basis of the method described by Milner *et al.* (1997) are shown by closed diamonds (◆). β-Sheets by Chou-Fasman protein secondary prediction indicated by lines above the sequence.

B

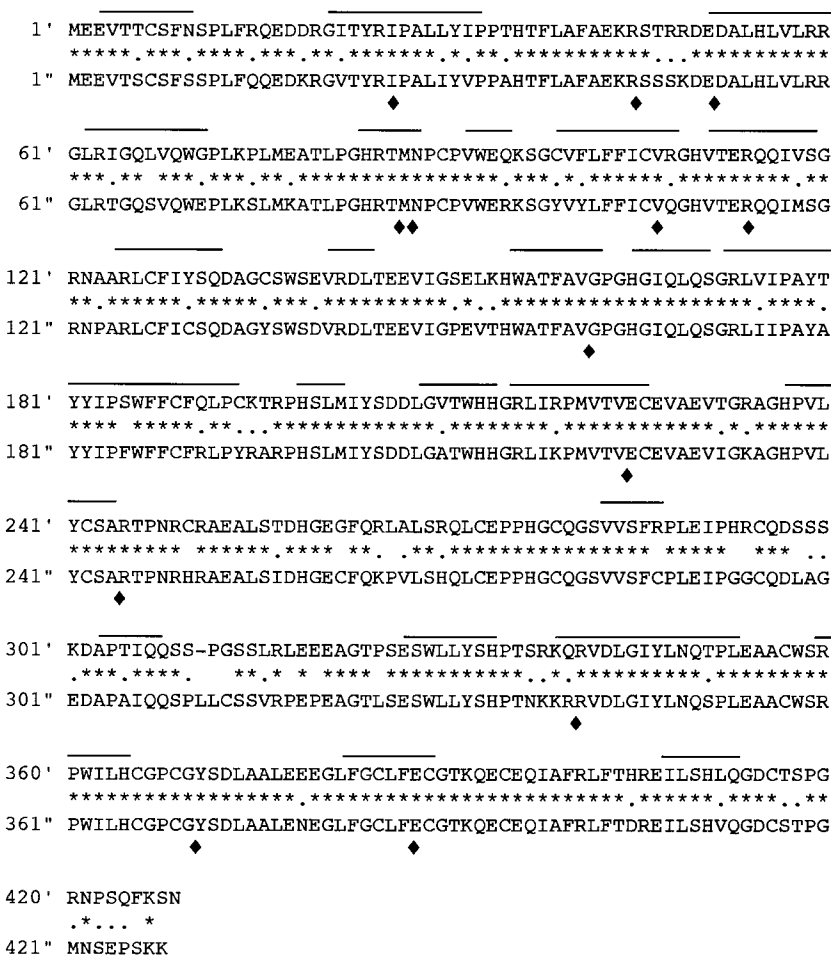

FIG. 1—*Continued*

bovine cDNA probe. Analysis of a cDNA fragment of the human gene obtained by polymerase chain reaction (PCR) with oligonucleotide primers for the bovine gene demonstrated high sequence identity between the two genes. We took advantage of this to clone the human gene for the purpose of obtaining a tool for understanding its function and expression mechanism, since the membrane sialidase specific for gangliosides is supposed to be involved in cell-cell interaction, cell differentiation, and transmembrane signalling by acting on gangliosides at cell surfaces (21, 22). In fact, it has been reported that this sialidase participates in human neuronal cell differentiation (23, 24). In addition, induction of anchorage-independent growth and tumorigenicity of mouse epidermal cells is accompanied by an increase of the membrane ganglioside sialidase (25).

We now describe the molecular cloning and characterization of a cDNA for a human ganglioside sialidase, and the chromosomal localization of the gene. The sequence data reported in this article have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB008185.

MATERIALS AND METHODS

Cloning of human sialidase cDNA. To obtain a cDNA probe, first strand cDNAs were synthesized from the poly $(A)^+$RNA of human brain (Clontech) using random primers and murine leukemia virus reverse transcriptase (Superscript RNase H^-, Gibco BRL), and applied as templates for the PCR. The cDNA fragment of human brain was amplified under the conditions described previously (17) with the primers [5′GGACACCGGACCA TGAACCCCT GTCCT-3′ (sense) and 5′CCTGGCCCCACA GCAAAAGTGGCCCA-3′ (antisense)], for a region in which the amino acid of the bovine sialidase sequence (amino acids 83–163) is identical to that of rat cytosolic sialidase. The 240bp fragment thus amplified, showing 87% identity to the corresponding region of the bovine gene, was used to screen a human brain λgt10 library (Clontec). Four positive clones were isolated from 6×10^5 recombinants under conditions of high stringency, subcloned into Bluescript and sequenced by the dideoxy chain termination method in both directions using an AutoRead Sequencing kit (Pharmacia).

TABLE I

Expression of the Human Ganglioside Sialidase in COS-1 Cells

Substrate	pME18S Specific activity[a] (units/mg protein)	pMEhmSD	
		Specific activity[a] (units/mg protein)	Hydrolysis relative[b] to GD3 (%)
GD3	5.3	691	100
GD1a	2.3	541	79
GD1b	1.7	356	52
GM3	2.6	572	84
GM2	2.5	14.1	2
Fetuin	0.7	5.2	1
Sialyllactose	13.5	49.3	5
4MU-NeuAc	22.9	96.4	11

[a] The values are means of three experiments.
[b] The values are expressed as the percentage of specific activity (the activity with pMEhmSD minus that with pME18S) relative to GD3 hydrolysis.

Expression in COS cells. Enzymatic studies were carried out in COS-1 cells, obtained from RIKEN Cell Bank, transiently transfected with the expression plasmid, because the cells showed low level of endogenous sialidase activity. The expression plasmid (pMEhmSD) was constructed using the pME 18S vector under control of the SRα promotor. The 1.3 kb fragment (nucleotides -3 to 1297) was amplified by PCR at an annealing temperature at 60°C using a clone covering the open reading frame as the template and ligated into the EcoRI sites of pME 18S. COS-1 cells grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO_2 were transfected with the expression plasmid by electroporation and after 48 h growth in culture, were harvested, sonicated in 9 volumes of phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF, and centrifuged at 1000 $\times$ g for 10 min. The supernatant (crude extract) was used for sialidase assays and the remaining supernatant was further centrifuged at 100,000 $\times$ g for 1 h. In certain cases the resulting pellet and the supernatant were used for sialidase assays as membrane and cytosolic fractions, respectively. The reaction mixture contained 50 nmol of substrate as bound sialic acid, 0.2 mg of bovine serum albumin, 10 μmol of sodium acetate (pH 4.6) and 0.2 mg of Triton X-100. After incubation at 37°C for 10–30 min, released sialic acid was determined by the thiobarbituric acid method as described elsewhere (4). Sialidase activity towards 4-methylumbelliferyl-neuraminic acid (4MU-Neu5Ac) was assayed by spectro-fluorometrical measurement of 4-methylumbelliferone (4MU) released (4). To observe pH profile for the activity, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), and citrate-phosphate buffers also were used. One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/h.

Northern blotting. Multiple tissue Northern blots of poly $(A)^+$ RNAs were purchased from Clonthec Laboratories, were hybridized with labeled 1.3 kb cDNA probe containing the entire openreading frame in a solution containing 5$\times$ SSPE, 5$\times$ Denhardt, 0.5% SDS, 50% (v/v)formamide and 50 μg/ml salmon sperm DNA, and then washed in 2$\times$ SSC, 0.1% SDS and finally in 0.2$\times$ SSC, 0.1% SDS at 42°C.

Chromosomal localization of the human sialidase gene. A cosmid clone was isolated from a human placenta genomic library in pWE 15 (Clontech) using the 1.3 kb cDNA probe containing the entire open-reading frame. The obtained positive cosmid clone was labeled with digoxigenin-dUTP (Boehringer-Mannheim, Germany) by nick translation, and hybridized to human metaphase chromosomes of a karyotypically normal male. Specific hybridization signals were detected by incubating the hybridized slides were tetramethylrhodamine isothiocyanate-conjugated anti-digoxigenine antibody, and chromosomes were identified by counterstaining with 4′,6-diamindino-2-phenyl-indole (Boehringer-Mannheim).

RESULTS

Isolation of human sialidase cDNA. In our previous study (17) Northern blot analysis of human tissue RNA using the bovine cDNA probe suggested the existence of human gene homologue. Using the bovine enzyme primers for a region identical to rat cytosolic sialidase, the PCR actually produced the same size of human cDNA fragment having an 87% amino acid identity to the corresponding region of the bovine sialidase. We therefore aimed to isolate a complete human cDNA by screening human cDNA library using the human cDNA fragment probe. As shown in Fig. 1A, the nucleotide sequence of the composite cDNA consists of 2052 nucleotides with a putative initiation codon at nucleotide 171 and a stop codon at 1455. There is an AT-rich sequence in 3′-noncoding region and no polyadenylation signal, which is similar to the bovine gene. Translation of this open reading frame generates a protein of 428 amino acids with a molecular mass of 48251 Da. The sequence contains a Arg-Ile-Pro sequence, three Asp-boxes, and a putative trans-membrane domain, showing an overall 83% amino acid identity to the bovine sialidase (Fig. 1B). Like the bovine gene, high contents of cystein residues and β-sheet structures were found. When compared with the sequence of *Salmonella typhimurium* sialidase (26), based on the mode of Milner *et al.* (11), putative active site amino acid residues were all identical to those of the bovine enzyme (Fig. 1B).

Expression of the human sialidase. The cDNA was demonstrated to encode a ganglioside-hydrolizing sialidase by transfection into COS-1 cells. The transfection increased sialidase activity toward gangliosides 120-fold in the presence of 0.1% Triton X-100. Over 89% of

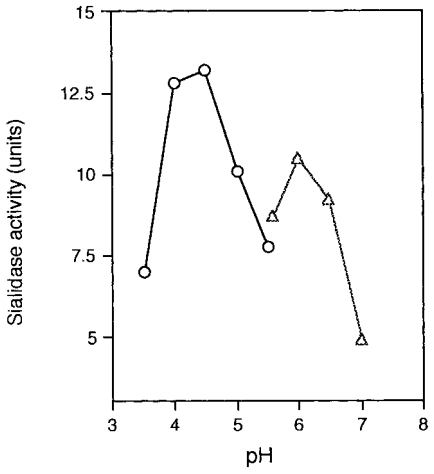

FIG. 2. Effect of pH on the activity of the ganglioside sialidase. Sialidase activities were in COS-1 cells transfected with human sialidase cDNA assayed using ganglioside substrate GD1a and sodium acetate buffer (○) for pH 3.5–5.5 and sodium phosphate buffer (Δ) for pH 5.5–7.0.

the activity of the expressed sialidase in the crude extract of the cells was recovered in the particulate fraction, and little activity remained in the cytosolic fraction, suggesting that it is membrane-bound. As shown in Table I, the sialidase hydrolized gangliosides preferentially, with hardly any activity against glycoproteins or oligosaccharides, similar to the bovine enzyme. Gangliosides GD3, GM3, GD1a and even GD1b were good substrats. Unlike the bovine enzyme with only one activity peak at pH 4.8, the human enzyme showed two peaks in its pH curve, at pH 4.5–4.8 and at pH 6.0–6.5 (Fig. 2).

Northern blot analysis. To compare the relative abundance of the message in human tissues,tissue mRNA blots were hybridized with radioactivity labeled human cDNA probe. As shown in Fig. 3, the human sialidase gene was expressed most abundantly in skeletal muscle and testis, at a relatively high level in pancreas, liver, heart, thymus and brain, and at a low level in kidney, lung, placenta and digestive organs. A 7.4-kb transcript was the major form expressed among these tissues, and an approximately 2.0 kb band was also found in several tissues including skeletal muscle.

Chromosomal localization. Using the sialidase cDNA probe, we isolated a genomic clone of 24 kb. Analysis of this clone revealed that it contained almost all the open reading frame downstream of its Bam HI site. The chromosomal assignment of the human sialidase gene was then determined by fluorescence in situ hybridization of the cosmid clone to normal human metaphase chromosomes. Doublet signals were only observed at a single location at 11q 13.5, as shown in Fig. 4. Although single back ground signals were observed at other chromosomal sites, none of them were labeled more than once.

DISCUSSION

We have isolated a cDNA encoding a sialidase from a human brain cDNA library using sequence information from the previously cloned rat (7) and bovine (17) sialidases. Our conclusion that the cDNA is for a ganglioside-hydrolyzing sialidase is supported by the following evidence: 1) transfection of the expression plasmid (pMEhmSD) into COS-1 cells resulted in an 130-fold increase in sialidase activity toward gangliosides in the presence of 0.1% Triton X-100, whereas no activity increase was observed with pME18S-transfected cells; and 2) the deduced amino accid sequence contained sialidase conserved sequences including Asp-boxes, Arg-Ileu-Pro and (Val)-Gly-Pro-Gly. Compared to other mammalian sialidases, the primary sequence exhibited 83% identity to bovine membrane sialidase (17), 39% to rat cytosolic (7), and 19% to human lysosomal sialidase (11–13), indicating that it is a human homologue of bovine membrane sialidase. A very high content of cystein residues (21 residues, 4.9% of all amino acids) was found in line with the bovine enzyme (21 residues, 4.9%), in contrast with human lysosomal sialidase (9 cystein residues, 2.1%) and rat cytosolic sialidase (10 residues, 2.6%).

The sialidase expressed in COS cells hydrolyzed GM3 and GD3 efficiently but GM1 and GM2 only poorly, the substrate specificity being consistent with

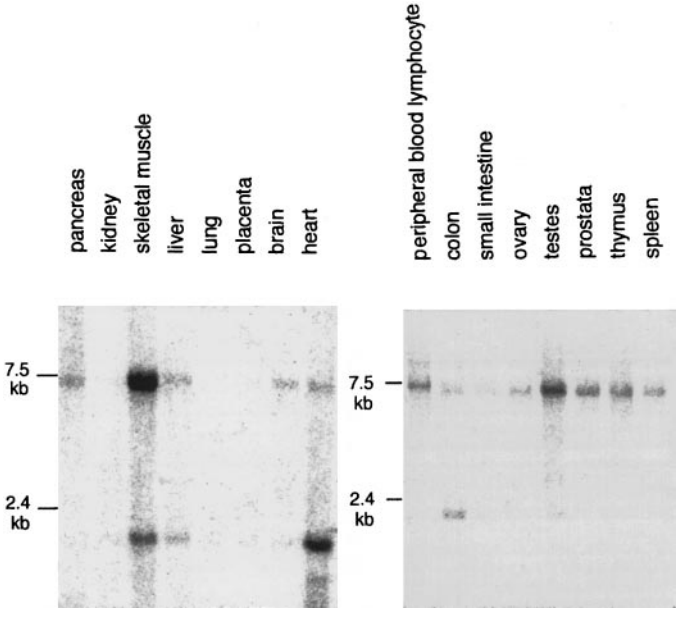

FIG. 3. Differential expression of the ganglioside sialidase in various human tissues. Northern blots of RNA from human tissues were hybridized with a cDNA for the human sialidase as described in the Materials and Methods.

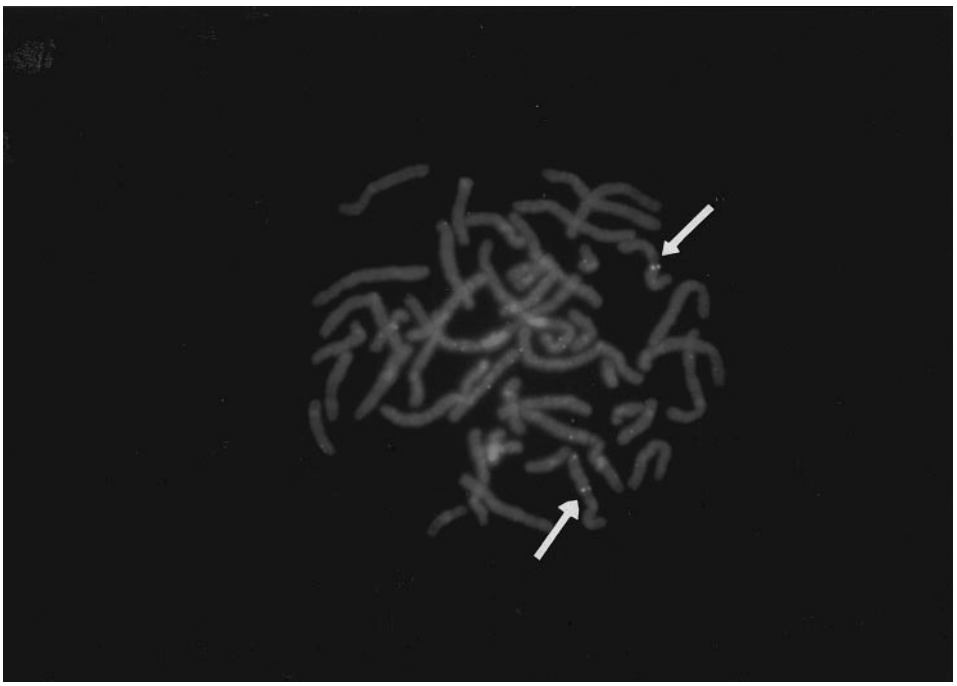

FIG. 4. Chromosomal localization of the human ganglioside sialidase gene determined by fluorescence in situ hybridization with a cosmid probe for the sialidase. Arrows indicate positive signals on 11q 13.5.

obsevations of Kopitz *et al.* using a partially purified sialidase from human brain (27). Interestingly, the pH profile proved to be different from that of the bovine enzyme, with considerable activity (60–70% of the maximum at pH 4.8) at near neutral pH in phosphate buffer, while the bovine enzyme shows a rapid decline of activity at that pH. Although the physiological significance of the neutral human sialidase activity is uncertain, it may act as a lectin-like molecule by binding gangliosides at cell surface.

The high expression levels in neuro-muscular tissues such as skeletal and cardiac muscle and brain, as demonstrated by Northern blot analysis, suggests some important roles of this human sialidase in neuromuscular functions including neuritogenesis and neurotransmission, as observed previously for the rat cytosolic sialidase that has essential participation in muscle cell differentiation (28). The report by Kopitz *et al.* (29) has also suggested an involvement of this sialidase in neural differentiation of human neuroblastoma cells, presenting evidence of galectin 1 as a major receptor for the sialidase product GM1. The sialidase-mediated carbohydrate interaction probably results not only in cell differentiation but also growth control, so that change in sialidase expression levels might disturb this regulation and facilitate malignant transformation (25, 30, 31). Only the limited evidence described above for the physiological function of mammalian ganglioside sialidases can be offered at present. However, it is plausible that the ganglioside sialidase cloned here is essentialy engaged in ganglioside metabolism, especially in modification of cell surface gangliosides, while the MHC-related lysosomal sialidase takes part mainly in glycoprotein catabolism by collaborating with endoglycosidases and proteases. In human cells these two sialidases appear to be the most important enzymes involved in regulation of glycoconjugate degradation and hence the biological function of sialo-glycoconjugates, because the expression of human cytosolic gene has been described only in skeletal muscle (10).

As a basis for examination of functional relationships with neuronal or malignant diseases, we have determined the chromosomal localization of this sialidase gene by FISH analysis using a cosmid clone as probe. The results indicates that the human gene is localized to chromosome 11, band q 13.5 which is different to the human lysosomal sialidase located on chromosome 17 (12, 13). The genes for myosin VIIA, pyruvate carboxylase, neurosensory deafness DFNB2 and DFNAII have been assigned to the region adjacent to 11q 13.5. Manipulation of this gene in eukaryotic cells and mice should facilitate understanding of the in vivo functions of this enzyme.

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