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Expression Cloning of a cDNA Encoding a Sulfotransferase Involved in the Biosynthesis of the HNK-1 Carbohydrate Epitope*

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The HNK-1 carbohydrate epitope is expressed on several neural adhesion glycoproteins and as a glycolipid, and is involved in cell interactions. The structural element of the epitope common to glycoproteins and glycolipids has been determined to be sulfate-3-GlcAβ1→3Galβ1→4GlcNAc. The glucuronyltransferase and sulfotransferase are considered to be the key enzymes in the biosynthesis of this epitope because the rest of the structure occurs often in glycoconjugates. Here we describe the isolation of the rat sulfotransferase cDNA *via* an expression cloning strategy. The clone finally isolated predicts a protein of 356 amino acids, with characteristics of a type II transmembrane protein and with no sequence similarity to other known sulfotransferases. Both the enzyme expressed as a soluble fusion protein and homogenates of cells transfected with the full-length cDNA could transfer sulfate from a sulfate donor to acceptor substrates containing terminal glucuronic acid.

The carbohydrate antigen recognized by the monoclonal antibody HNK-1 was originally described as a marker for human natural killer cells (1). Later it was shown to be expressed predominantly on glycolipids and glycoproteins from nervous tissue (2–5). The expression pattern of the HNK-1 carbohydrate in both the central and peripheral nervous system is spatially and developmentally regulated (6–11). The HNK-1 carbohydrate epitope is carried by many, but not all, neural recognition glycoproteins and is involved in homo- and heterophilic binding of these proteins (for a review, see Ref. 12). Of special interest is the association of the epitope with Schwann cells myelinating motor but not sensory axons (10), where it may be involved in the preferential reinervation of muscle nerves by motor axons after lesion (13, 14).

Determination of the structure of the glycolipid (15) and glycoprotein (16) forms has shown that both carry sulfate-3-GlcAβ1→3Galβ1→4GlcNAc at the nonreducing end. The minimal requirement for recognition by HNK-1 is unknown, but the antibody only binds to the sulfated form(17). Several other monoclonal antibodies have been isolated that recognize identical or similar structures(4, 18); of these, L2–412 is important for this study, because it also recognizes the non-sulfated form of the carbohydrate(19).

The key enzymes in the biosynthesis of HNK-1 carbohydrates are a glucuronyltransferase (20, 21), transferring GlcA in β1→3 linkage to a terminal galactose, and a sulfotransferase (22), responsible for coupling sulfate to the C-3 position of this GlcA residue. A cDNA encoding the glucuronyltransferase involved in the biosynthesis of at least the HNK-1 glycoprotein epitope has recently been cloned (23). We describe here the cloning of a cDNA coding for a sulfotransferase active on terminal glucuronic acid residues and whose expression can render cells immunoreactive with HNK-1 antibody when cotransfected with a glucuronyltransferase cDNA.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Plasmids—CHOP2 cells (24) were grown in minimal essential medium α supplemented with 10% fetal calf serum, penicillin/streptomycin, and 200 μg/ml G418 (all from Life Technologies, Basel, Switzerland). For transfections, G418 was omitted. Hybridoma supernatants containing antibodies HNK-1 (mouse, Ref. 1) and L2–412 (rat, Ref. 4) were produced as described(18) and used without further purification. The glucuronyltransferase cDNA was in the mammalian expression vector pEF-BOS (23).

Poly(A)$^+$ RNA from cerebral cortex of newborn rats was converted to double-stranded cDNA using a kit from Stratagene, and a 1–3 kb fraction prepared by gel electrophoresis was cloned into the vector pXMD1 (25). Plates with transformed colonies were replicated to nitrocellulose filters, which were then further processed for storage as filter "sandwiches" at −80 °C (26). Bacteria remaining on the plates were regrown overnight and collected for preparation of plasmid DNA. Subpools were prepared by cutting replica sandwiches into 10–12 pieces and regrowing the bacteria from one side of the sandwich.

For production of a fusion protein, the sulfotransferase cDNA downstream from the *Msc*I site at nucleotide 319 (see below) was subcloned into the filled *Eco*RI site of plasmid pPROTA (27).

Transfection and Immunostaining of CHOP2 Cells—In cotransfection experiments, 2 parts of test plasmids were supplemented with 1 part plasmid coding for glucuronyltransferase (23). CHOP2 cells were transfected using DEAE-dextran (28) but without chloroquine treatment.

Glutaraldehyde-fixed monolayers of transfected cells were stained with HNK-1 or L2–412 antibody, followed by HRP-conjugated secondary antibody (goat anti-mouse for HNK-1 and goat anti-rat for L2–412, both from Jackson ImmunoResearch) and color development with 3-amino-9-ethylcarbazole.

Sulfotransferase Assays—CHOP2 cells were harvested by scraping in phosphate-buffered saline 2 days after transfection with the sulfotransferase cDNA plasmid. After centrifugation, the cells from an 80-cm^2 flask were taken up in 250 μl of 100 mM Bis-TRIS, pH 6.6, containing mixed protease inhibitors (chymostatin/pepstatin A/leupeptin/antipain/aprotinin, all at 10 μg/ml). Aliquots were stored at −20 °C and only

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF022729.

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thawed once. Cerebral cortex of 7-day-old rats was homogenized in the same buffer (25% w/v) and similarly aliquoted.

The sulfotransferase assays were done in 100 mM Bis-TRIS (pH 6.6), 10 mM $MnCl_2$, 2.5 mM ATP, and 0.1% Triton X-100, in a final volume of 20 μl including 10 μl of the transfected cell homogenate (50 μg protein) or 5 μl of 25% brain homogenate (30 μg protein), 100 pmol of [^{35}S]PAPS[1] (900 Bq; from New England Nuclear, diluted with unlabeled PAPS from Sigma), and 10 nmol of acceptor substrate. The acceptors used were 4-nitrophenyl-β-D-galactose (Galβ-pNP), 4-nitrophenyl-β-D-glucuronic acid (GlcAβ-pNP) (both Fluka) or 2-heptanoylamidoethyl-(3-O-β-D-glucuronyl)-β-D-galactose (GlcAβ1→3Galβ-R).[2] The mixture was incubated for 2 h at 37 °C with mild shaking. Then 100 μl of 4:6, water:methanol was added to the samples and centrifuged, and the residue was re-extracted with 100 μl of 1:1 water:methanol. Combined supernatants were dried, redissolved in 10 μl of 1:1, water:methanol, and run on aluminum-supported HPTLC plates (Silica Gel 60, Merck) in 5:4:1, chloroform:methanol:0.25% KCl/water. Activity was assessed using a Phosphorimager (Molecular Dynamics) or by autoradiography.

For the protein A fusion protein, the medium of transfected cells was replaced 1 day after transfection by medium with 5% low immunoglobulin calf serum (Life Technologies, Basel, Switzerland) and incubated for 2 more days. The medium (20 ml) was filtered (5 μM) and incubated overnight with 100 μl of human IgG-agarose beads (Sigma) after addition of 0.05% sodium azide and mixed protease inhibitors (see above). The beads were washed 3 times with 1 ml of 100 mM Bis-TRIS, pH 6.6, containing 0.05% sodium azide, 1 mM $MnCl_2$, and 1 mg/ml bovine serum albumin and then stored in this buffer at 4 °C in the same volume as the cell pellets. Assays were carried out directly with the beads as with cell homogenates, except that Triton X-100 was omitted.

RESULTS

Expression Cloning of the HNK-1 Sulfotransferase cDNA—For expression screening, we used the cell line CHOP2 (24), a derivative of the Lec2 cell line (29) that lacks the CMP-sialic acid transporter. This mutation results in an increase in glycoproteins and glycolipids terminating in β4-galactose, potentially increasing the amount of substrate available to the glucuronyl and subsequently acting sulfotransferase. Transfection of CHOP2 cells with the recently cloned glucuronyltransferase (23) indeed led to very clear surface staining of the cells with antibody L2–412, which recognizes the nonsulfated form of the carbohydrate (Fig. 1*E*).

Plasmid DNA from 40 pools of 5,000–10,000 cerebral cortex cDNA clones was cotransfected with the glucuronyltransferase cDNA. Two days after transfection, the monolayers were stained with HNK-1, and the number of colored cells in each plate was scored. In one pool, about 20 positive cells were seen, indicating the sulfotransferase was expressed in these cells (Fig. 1*A*). In two rounds of subdividing positive pools, the number of positive cells increased first to several hundred (Fig. 1*B*) and then to several percent of all cells (Fig. 1*C*). A single clone isolated from the last pool gave, upon cotransfection, HNK-1-positive cells at about the same frequency as seen when cells were transfected with glucuronyltransferase cDNA alone and stained with antibody L2–412 (Fig. 1, *D* and *E*). This isolated cDNA clone, therefore, is likely to encode the HNK-1 sulfotransferase.

A GlcA-dependent Sulfotransferase Activity Is Found in Transfected Cells—The HNK-1 reactivity after transfection with the sulfotransferase cDNA is dependent on the presence of the glucuronyltransferase. Only a very faint HNK-1 reactivity, but clearly higher than in mock transfected cells, is seen after transfection with the sulfotransferase alone (Fig. 1*F*). Western blotting of proteins isolated from transfected cells (Fig. 2) confirms these results. Mock transfected cells gave no signal with L2–412 or HNK-1, cells transfected with glucuronyltransferase cDNA alone showed only L2–412 reactive proteins, and cells transfected with both glucuronyltransferase and sulfotransferase cDNAs were positive with both antibodies. Faint staining of probably a single protein is seen with both L2–412 and HNK-1 in blots of proteins from cells transfected with only the

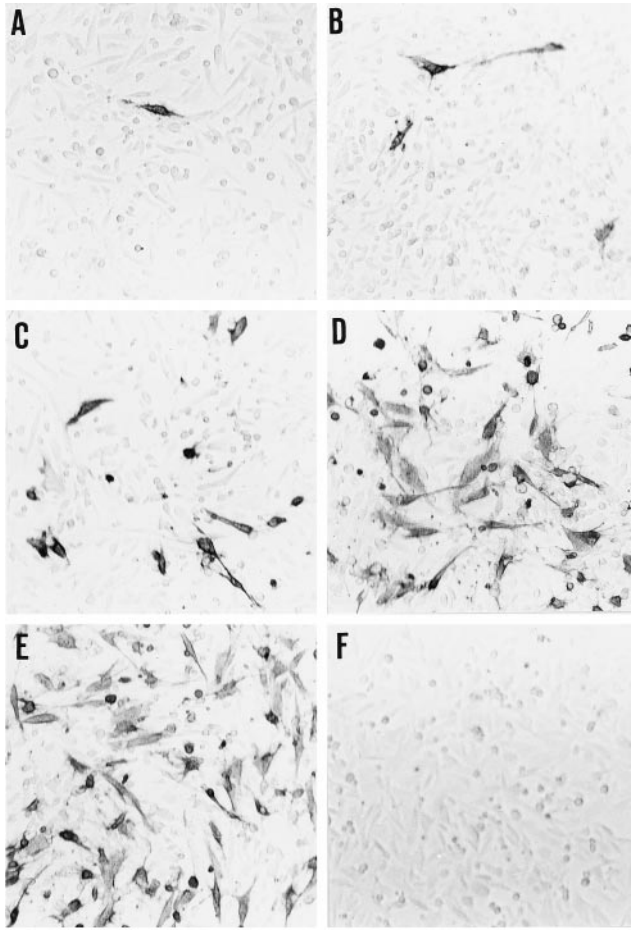

FIG. 1. **Immunostaining of transiently transfected CHOP2 cells.** Panels *A-D* and *F* show staining with antibody HNK-1, panel *E* shows staining with L2–412. Cotransfection with the glucuronyltransferase cDNA and pools of 5,000–10,000 clones from the primary library gave a few immunopositive cells with one pool (*A*). Progressively higher frequencies of positive cells were found upon two rounds of subdividing positive pools (*B* and *C*), reaching a maximum with the single sulfotransferase cDNA clone (*D*). *E*. Cells transfected only with glucuronyltransferase cDNA. *F*, cells transfected with the sulfotransferase but not glucuronyltransferase cDNA clone.

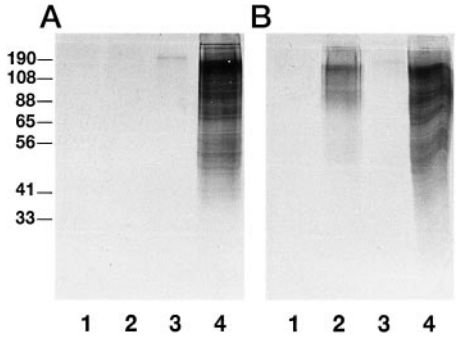

FIG. 2. **Western blot analysis of proteins from transfected CHOP2 cells.** Blots were stained with antibody HNK-1 (*A*) or L2–412 (*B*). Cells were transfected with no DNA (*lanes 1*), with the glucuronyltransferase cDNA alone (*lanes 2*), with sulfotransferase cDNA alone (*lanes 3*), or with both transferase cDNAs (*lanes 4*).

[1] The abbreviations used are: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; Galβ-pNP, 4-nitrophenyl-β-D-galactose; GlcAβ1→3Galβ-R, 2-heptanoylamidoethyl-(3-*O*-β-D-glucuronyl)-β-D-galactose; GlcAβ-pNP, 4-nitrophenyl-β-D-glucuronic acid; HPTLC, high performance-thin layer chromatography; EST, expressed sequence tag.

[2] A. V. Kornilov, L. O. Kononov, A. A. Sherman, and N. E. Nifant'ev, unpublished data.

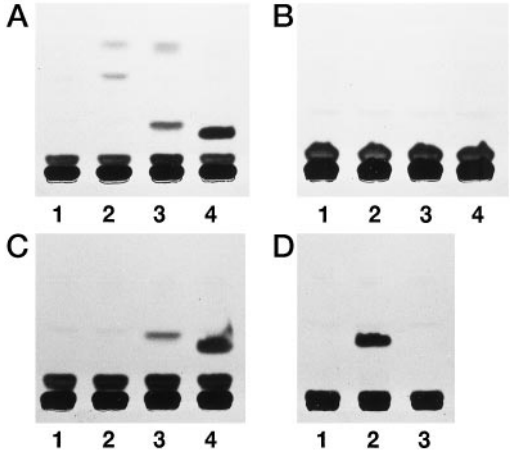

FIG. 3. **In vitro sulfotransferase assays.** Homogenates were incubated with [^{35}S]PAPS and different potential acceptor substrates. The reaction products were analyzed by HPTLC. *A*, cerebral cortex homogenate; *B*, mock transfected CHOP2 cells; *C*, CHOP2 cells transfected with the sulfotransferase cDNA clone. *Lanes 1* (for *A*, *B*, and *C*), no acceptor; *lanes 2*, Galβ-pNP; *lanes 3*, GlcAβ-pNP *lanes 4*, GlcAβ1→3Galβ-R. The bands closest to the origin comigrate with [^{35}S]PAPS. Other bands observed in all lanes may represent either degradation products or transfer of sulfate to endogenous acceptors. The labeled material running with high mobility in lanes *2* and *3* of *panel A* may arise from transfer of sulfate to released pNP, as it is always observed when using pNP substrates. *D*, sulfotransferase activity of the protein A-sulfotransferase fusion protein captured on IgG-agarose beads, assayed using GlcAβ1→3Galβ-R as acceptor substrate. IgG beads were incubated with medium from cells expressing either the nonsecreted form of the sulfotransferase, without protein A (*lane 1*), the protein A fusion protein (*lane 2*), or the translation product from a pPROTA vector containing the sulfotransferase in antisense orientation (*lane 3*).

sulfotransferase cDNA (Fig. 2, *A* and *B, lanes 3*), suggesting that Chinese hamster ovary cells already expose a low level of acceptor that can be used by the transfected sulfotransferase. This protein is presumably responsible for the faint HNK-1 immunostaining seen on whole cells transfected only with sulfotransferase cDNA.

The presence of sulfotransferase activity was confirmed by enzyme assays *in vitro*. Sulfotransferase activity was determined with Galβ-pNP, GlcAβ-pNP, and GlcAβ1→3Galβ-R as acceptors (Fig. 3). Homogenates of cells transfected with the isolated sulfotransferase clone showed activity toward GlcAβ-pNP and GlcAβ1→3Galβ1-R, but not to Galβ-pNP. Homogenates of mock-transfected cells gave no sulfotransferase activity; cerebral cortex homogenate could use both GlcAβ-pNP and Galβ-pNP as acceptor, suggesting the presence of more than one sulfotransferase in this tissue. The sulfotransferase activity toward the disaccharide acceptor GlcAβ1→3Galβ-R in brain as measured from *panel A*, *lane 4*, is 154 pmol/mg-h, very close to the maximal activity measured by Chou and Jungalwala (22) using a glycolipid acceptor. Transfected CHOP2 cells show an activity of 80 pmol/mg-h. There was no increase in activity with longer oligosaccharides (data not shown), and activity toward GlcAβ-pNP was about 5 times lower in both rat brain and CHOP2 cells. The enzyme encoded by the cloned cDNA is therefore able to perform the same transfer of sulfate to terminal β-linked GlcA residues as measured in rat cerebral cortex.

The Cloned cDNA Encodes the Sulfotransferase Itself—To more conclusively demonstrate that the isolated cDNA clone encodes the sulfotransferase itself, we produced a fusion protein that could be readily separated from other cellular components. The part of the cDNA encoding the putative cytoplasmic and transmembrane domains was replaced by DNA encoding protein A preceded by a signal sequence. Fusion protein secreted into the medium was captured on human IgG-agarose beads, and sulfotransferase activity was determined (Fig. 3*D*). Activity was found with the bound protein A fusion protein, but not when the sulfotransferase cDNA was cloned in the reverse orientation or when the protein A moiety was absent. The measured enzyme activity produced per cell is about twice as high for the secreted protein A fusion as for the membrane-bound sulfotransferase measured in cell homogenates.

The Sulfotransferase Has No Sequence Similarity to Known Proteins—The cloned 2649-base pair cDNA contains an open reading frame encoding a protein of 356 amino acids (Fig. 4). The protein probably is a type II transmembrane protein, as a potential transmembrane region is observed close to the N terminus, and the enzymatic activity is expected to be located in the endoplasmic reticulum or Golgi lumen. No significant sequence similarity was observed between the translation product of the cloned cDNA and any known protein. More than ten overlapping human ESTs were found that potentially encode the human homolog of the cloned rat sulfotransferase. Several other ESTs showed a much lower similarity with the rat cDNA clone and may encode two different homologs of the human gene, indicating that there probably is a human gene family of at least three members.

DISCUSSION

Several cDNAs encoding enzymes involved in glycosylation have been isolated by expression cloning (30). The most often used technique is panning and plasmid recovery from transfected mammalian cells. However, although the panning procedure will enrich the desired plasmid, after one or several rounds of panning, recovered plasmids are still divided into pools and tested for expression of the sugar epitope (sibling selection) (30–32). We found it much more efficient to directly start a sibling selection procedure.

The cloned sulfotransferase cDNA was shown to induce HNK-1 reactivity in CHOP2 cells only in combination with a glucuronyltransferase, indicating that these two enzymes, together with common enzymes already present in CHOP2 cells, are required and sufficient for the biosynthesis of the HNK-1 epitope on glycoproteins. It is, however, not known if these two enzymes are responsible for the synthesis of all HNK-1 carbohydrate epitopes observed in nervous tissue. While the HNK-1 carbohydrate epitope in nervous tissues is only observed on a limited number of proteins (11, 12), very many proteins seem to carry the epitope after expression of the enzymes in CHOP2 cells. The situation in CHOP2 cells may be abnormal, owing to lack of competition for acceptor by other enzymes, such as sialyltransferases and fucosyltransferases. *In vitro* sulfotransferase assays showed that the cloned cDNA encodes an enzyme capable of transferring sulfate from PAPS to acceptor substrates containing terminal GlcA. The disaccharide GlcAβ1→3Galβ-R is as good an acceptor as the complete glycolipid used previously to characterize the natural enzyme(22), and the acceptor preferences of the enzyme encoded by the cloned cDNA parallel those seen with brain homogenate. The cloned enzyme therefore seems potentially capable of synthesizing the known HNK-1 structures on glycolipids and glycoproteins (15, 16).

Surprisingly, the cloned sulfotransferase showed no significant sequence similarity to other sulfotransferases, not even to the recently cloned sulfatide sulfotransferase (33). The latter enzyme transfers sulfate from PAPS to the C-3 of galactose residues, a reaction very similar to that of the HNK-1 sulfotransferase, expected to transfer the sulfate to C-3 of GlcA residues. As cytoplasmic sulfotransferases that use various substrates all have some sequence similarity (34), it might be expected that sulfotransferases acting on carbohydrate struc-

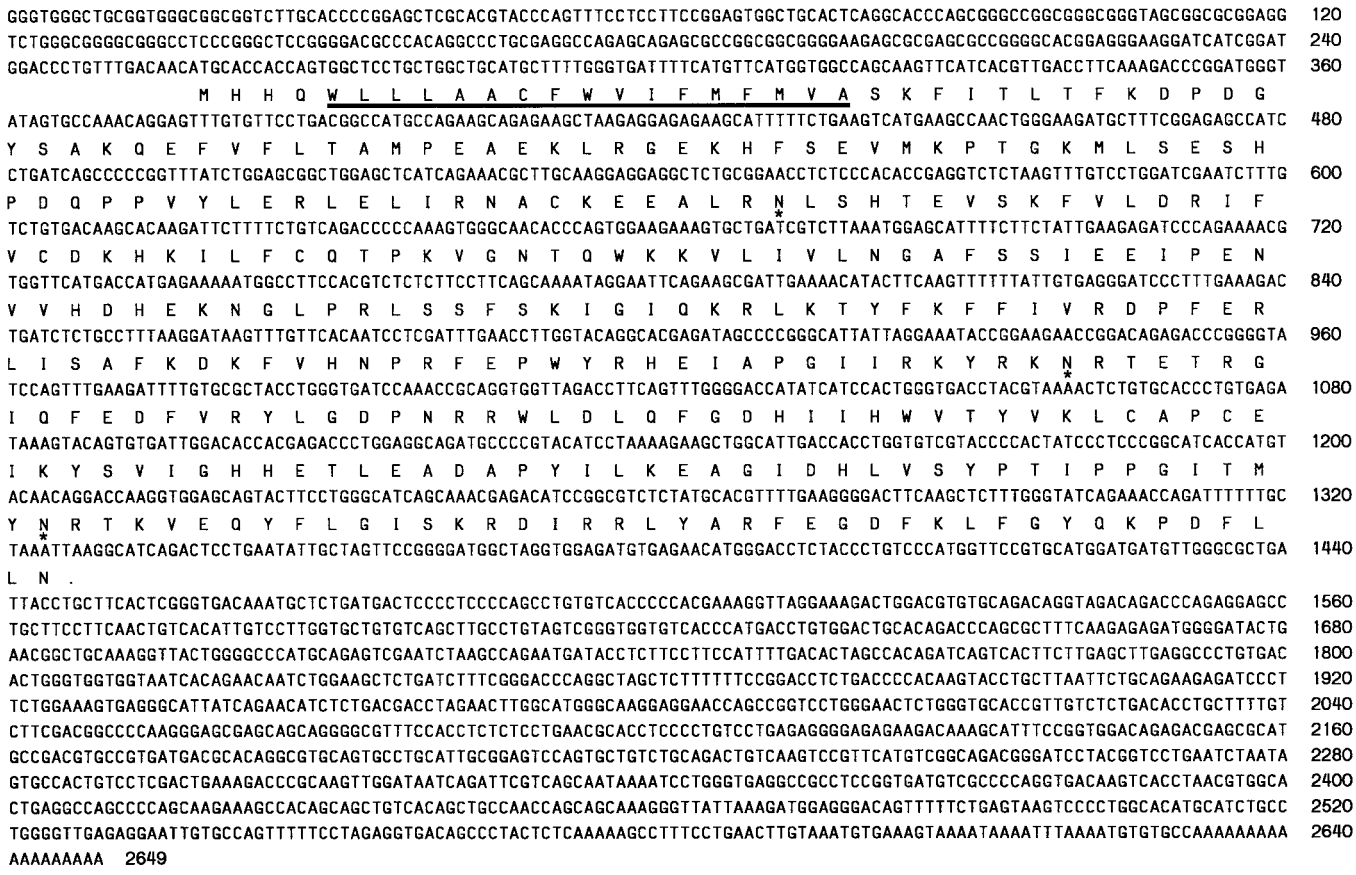

FIG. 4. **Complete nucleotide and deduced amino acid sequence of the HNK-1-sulfotransferase cDNA clone.** The putative transmembrane region in the translation product is *underlined*, and potential *N*-linked glycosylation sites are indicated by *asterisks*.

tures would also be structurally related. This is, however, not found among the enzymes whose cDNAs have been cloned so far. These comprise two different *N*-heparan sulfate sulfotransferases (35, 36) showing 70% sequence identity with each other, chondroitin 6-sulfotransferase (37), sulfatide sulfotransferase (33), and the HNK-1 sulfotransferase. However, all these enzymes show the same membrane topology and are predicted to be type II membrane proteins with a short N-terminal cytoplasmic domain and a larger luminal catalytic domain. This structure is typical of Golgi glycosyltransferases(38).

Several human ESTs are very similar to the cloned rat HNK-1 sulfotransferase cDNA, and probably encode the same enzyme in humans. Surprisingly, no such mouse ESTs are present in the data bases. Further ESTs, both human and mouse, are found that may encode more distant relatives of the enzyme. Although these transcripts can of course encode completely different enzymes, it is interesting that for both the HNK-1 glucuronyltransferase (23) and the HNK-1 sulfotransferase a family of related genes seems to exist. A similar situation may occur as for the fucosyltransferase gene family (39), wherein the enzymes differ only slightly in acceptor specificity. Such variations might be responsible for at least some of the marked differences observed in developmental and spatial patterns of HNK-1 immunostaining when different species are compared (7, 11).

The availability of clones for both a glucuronyltransferase and a sulfotransferase responsible for the biosynthesis of the HNK-1 epitope will greatly enhance studies on the regulation of the expression of these carbohydrate structures. The enzymes can be used to produce substantial amounts of HNK-1 carbohydrate, allowing more detailed testing than heretofore possible of its role in the nervous system.

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