

## Primary Structure of $\beta$ -Galactoside $\alpha$ 2,6-Sialyltransferase

CONVERSION OF MEMBRANE-BOUND ENZYME TO SOLUBLE FORMS BY CLEAVAGE OF THE NH<sub>2</sub>-TERMINAL SIGNAL ANCHOR\*

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This report describes the primary structure of a rat liver  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase (EC 2.4.99.1), a Golgi apparatus enzyme involved in the terminal sialylation of N-linked carbohydrate groups of glycoproteins. The complete amino acid sequence was deduced from the nucleotide sequence of cDNA clones of the enzyme. The primary structure suggests that the topology of the enzyme in the Golgi apparatus consists of a short NH<sub>2</sub>-terminal cytoplasmic domain, a 17-residue hydrophobic sequence which serves as the membrane anchor and signal sequence, and a large luminal, catalytic domain. NH<sub>2</sub>-terminal sequence analysis of a truncated form of the enzyme, obtained by purification from tissue homogenates, reveals that it is missing a 63-residue NH<sub>2</sub>-terminal peptide which includes the membrane binding domain. These and supporting results show that soluble forms of the sialyltransferase can be generated by proteolytic cleavage between the NH<sub>2</sub>-terminal signal-anchor and the catalytic domain.

Sialyltransferases are a family of glycosyltransferases which catalyze the transfer of sialic acid (SA) to terminal positions on the carbohydrate groups of glycoproteins and glycolipids in the general reaction: CMP-SA + HO-acceptor  $\rightarrow$  CMP + SA-O-acceptor (1, 2). Like many other glycosyltransferases, sialyltransferases are found primarily in the Golgi apparatus of cells where they participate in post-translational glycosylation pathways (3, 4). They are also found in body fluids such as breast milk, colostrum, and blood (5, 6). At least 10-12 different sialyltransferases are required to synthesize all the sialyloligosaccharide sequences known. Each of these can be distinguished enzymatically by their specificity for the sequence of the acceptor oligosaccharide and the anomeric linkage formed between the sialic acid and the sugar to which it is attached (1). To date, four of these enzymes have been purified to homogeneity (1, 7-9). Although their enzymatic properties have been extensively studied, there is only fragmentary information about their structures and the basis of their intra- and extracellular localizations (1, 7-11).

This report describes the cDNA cloning and primary structure of a rat  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase which forms the SA $\alpha$ 2,6Gal $\beta$ 1,4GlcNAc-sequence common to many N-

linked carbohydrate groups. This enzyme has been purified from rat liver (7, 8), and the analogous enzyme has been purified from bovine colostrum where it exists in soluble form (12, 13). The rat enzyme has been localized to the *trans*-Golgi cisternae and *trans*-Golgi network of hepatocytes and intestinal goblet cells by immunogold electron microscopy (14, 15). A similar localization has been inferred from immunofluorescence microscopy for the bovine enzyme in fibroblasts (16). These observations are in keeping with the current concept of the Golgi apparatus being compartmentized into *cis*-, *medial*-, and *trans*-regions, corresponding to early, middle, and late functions, respectively (17). In intestinal absorptive cells, however, this sialyltransferase appears to be localized throughout the *cis*-, *medial*-, and *trans*-Golgi regions (15). The molecular basis for the subcellular localization of the sialyltransferase, or of any other glycosyltransferase, is presently unknown.

The physical relationship between intracellular sialyltransferase and the same soluble enzyme found in blood and colostrum is also incompletely understood. However, it has been demonstrated that cultured hepatocytes secrete the enzyme (18) and that inflammation (19, 20) or tumor burden (21-23) causes an increase both in the amount of liver sialyltransferase and in the levels of the enzyme found in blood. Although immun-blot analysis of SDS gels of rat liver Golgi preparations shows that the membrane-bound enzyme has a molecular weight of 47,000 (14), the purified, soluble form of the enzyme has an *M<sub>r</sub>* of 37,000-43,000 (7, 8). Thus, the purified sialyltransferase appears to be a catalytically active, proteolytic fragment of the membrane-bound form in the Golgi apparatus (14).

The amino acid sequence and additional biochemical evidence described in this report clarifies the relationship between membrane-bound and purified, soluble forms of the sialyltransferase. This information further implies a domain structure and topology of the enzyme in the Golgi apparatus and suggests that limited proteolysis is the factor responsible for the release of soluble sialyltransferase into body fluids. Evidence which indicates that other glycosyltransferases may share similar topology to that described for the sialyltransferase is discussed.

### EXPERIMENTAL PROCEDURES

**Materials**—The Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase (7), EC 2.4.99.1, and affinity purified anti-sialyltransferase antibodies (14) were purified as previously described. *Escherichia coli* strains BNN97 (ATCC 37194) and Y1089 (ATCC 37196) were purchased from American Type Culture Collection. Restriction enzymes were purchased from New England Biolabs, Pharmacia LKB Biotechnology Inc., and Bethesda Research Laboratories. Nitrocellulose was manufactured by Schleicher & Schuell (type BA85, 0.45  $\mu$ m). High pressure liquid

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chromatography (HPLC)<sup>1</sup>-grade acetonitrile, water, and isopropanol were purchased from Malinkrodt. Unless otherwise specified, all other biochemicals were of the highest quality commercially available, and organic and inorganic chemicals were of reagent grade or higher.

**Buffers**—The composition of the commonly used buffers are: SSPE (sodium chloride:sodium phosphate:EDTA buffer), 150 mM NaCl, 10 mM sodium phosphate, 1 mM disodium EDTA, pH 7.4; SSC (sodium chloride:sodium citrate buffer), 150 mM NaCl, 15 mM sodium citrate, pH 7; Denhardt's reagent, 0.02% Ficoll type 400 (Sigma), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin fraction V (Sigma); TBS (Tris-buffered saline), 50 mM Tris, pH 8.0, 0.15 M NaCl; gel sample buffer, 20% glycerol (v/v), 2%  $\beta$ -mercaptoethanol (v/v), 3% SDS (w/v; ultrapur, International Biotechnologies), 0.001% bromophenol blue, 130 mM Tris, pH 6.8; Tris/NaCl buffer, 150 mM NaCl, 0.02% sodium azide, 10 mM Tris, pH 7.5; wash buffer, 600 mM NaCl, 0.1% SDS, 0.05% Nonidet P-40 (v/v), 10 mM Tris, pH 8.6.

**Isolation of RNA**—Total rat liver RNA was prepared as described by Chirgwin *et al.* (24), as was poly(A)<sup>+</sup> mRNA which was selected by two cycles of binding to oligo(dT)-cellulose type 2 (Collaborative Research).

**Rat Liver cDNA Expression Library**—A rat liver cDNA library was constructed in the expression vector  $\lambda$ gt11 essentially as described by Huynh *et al.* (25). After preparation of cDNA from rat liver mRNA, ligation of *EcoRI* linkers, and digestion with *EcoRI* the excess *EcoRI* linkers were removed by gel filtration on a 1-ml column of Bio-Gel A-15 (Bio-Rad) prepared in a 1.0-ml disposable pipet plugged with siliconized glass wool. cDNA was ligated into the single *EcoRI* site in the lac Z gene of  $\lambda$ gt11, and the recombinant DNA was packaged into phage using an *in vitro* packaging kit supplied by Amersham Corp. The library contained  $2.3 \times 10^6$  recombinant plaques as 19% of the viable phage with an average insert size of 700–1000 bp. A second rat liver cDNA library containing  $6.8 \times 10^6$  independent clones with an average insert size of 1 kb was purchased from Clontech Laboratories.

**Antibody Screening of Library**—Approximately  $1.5 \times 10^6$  phage were screened for production of  $\beta$ -galactosidase sialyltransferase fusion protein at a density of 60,000 plaque-forming units/150-mm plate as described by Huynh *et al.* (25) with some modifications. Nitrocellulose replicas of each plate were blocked for 1 h in Tris-buffered saline containing 3% gelatin and then incubated in a solution containing affinity purified sialyltransferase antibody diluted 300-fold in Tris-buffered saline with 1% gelatin. All washes were performed three to four times in the absence of detergent to increase sensitivity. Positive clones were visualized with <sup>125</sup>I-protein A (ICN Radiochemicals). Agar plugs from the region of the positive signal were removed, and the eluted phage were replated and rescreened until all the plaques on the plate produced a signal. One repeatedly positive clone,  $\lambda$ ST1, was obtained.

**Preparation of Sialyltransferase cDNA Inserts from  $\lambda$ gt11 Recombinants**—A  $\lambda$ ST1 lysogen was generated in *E. coli* Y1089 cells (25) which was then the source of  $\lambda$ ST1 phage and sialyltransferase fusion protein preparations. Insert cDNA from  $\lambda$ ST1 (719 bp) was subcloned (26) into pBR322 (pST1). Insert cDNA (ST1) was prepared from pST1 by excision with *EcoRI*, electrophoresis on 1% agarose gels, electroelution of the separated fragment, and purification by phenol:chloroform:isoamylalcohol (25:24:1) extraction (26). The cDNA from  $\lambda$ gt11 clones  $\lambda$ ST2– $\lambda$ ST5 was similarly prepared following excision from  $\lambda$  phage prepared from plate lysates (26).

**Nucleotide Screening of cDNA Library**—A total of  $7.8 \times 10^6$  recombinants from a rat liver cDNA library in  $\lambda$ gt11 (Clontech Laboratories) were plated at a density of 200 plaque-forming units/cm<sup>2</sup>. Phage DNA was transferred to nitrocellulose filters and screened as described by Davis *et al.* (27) with [<sup>32</sup>P]dCTP nick-translated ST1 with a specific activity of  $10^6$  cpm/ $\mu$ g (26). Filters were prehybridized and hybridized as described by Wahl *et al.* (31) using 1 ml solution/100 cm<sup>2</sup> at each step. Filters were rinsed twice briefly at 20 °C and then 15 min at 50 °C in 2  $\times$  SSC, 0.1% SDS, and then for 1 h at 50 °C in 0.1  $\times$  SSC, 0.1% SDS. Dried filters were exposed to film (Kodak

XAR-5) with an intensifying screen (Cronex lightning plus) for 14 h at –80 °C. Positive clones were plaque-purified and amplified as plate stocks (27). Insert cDNAs with 3' or 5' extensions of ST1 were identified by restriction mapping (26) and by differential hybridization to 3' or 5' nick-translated probes generated from a single *PstI* restriction site in ST1 at 557 bp. Other sialyltransferase cDNAs were similarly mapped.

**SDS-Gel Electrophoresis and Immun-blotting**—SDS-polyacrylamide (10%) gel electrophoresis was performed as described by Laemmli (28). Samples were kept in a boiling water bath for 5 min prior to loading on the gel. Protein bands were visualized by Coomassie Blue. For immun-blotting, proteins were transferred electrophoretically to nitrocellulose, which was then blocked with gelatin and incubated with diluted antisera or affinity purified sialyltransferase antibodies (1:200). Bound antibodies were visualized as described previously (14) using the Immun-blot reagents supplied by Bio-Rad.

**Preparation of Epitope-selected Antibody**—Lysates of induced  $\lambda$ ST1 and  $\lambda$ gt11 lysogens in *E. coli* Y1089 cells were used as a source of expressed proteins. Crude lysates were obtained by growing induced lysogens for 1 h after temperature shift and addition of 1 M isopropyl- $\beta$ -D-thiogalactoside. Cells harvested from 100-ml cultures were suspended in 1/20 of the original volume in Tris-buffered saline and frozen and thawed twice. This was followed by addition of Triton X-100 to 0.6%, and the suspension was kept stirred in an ice bath for 1 h. The supernatant (lysate), obtained after centrifugation at 10,000  $\times$  g for 30 min, was adsorbed to 84-mm diameter nitrocellulose filters. Filters were then incubated with polyclonal anti-sialyltransferase antibody, and bound antibody was eluted essentially as described by Weinberger *et al.* (29). Antibody prepared in this way becomes diluted 100- to 200-fold and thus was used for immun-blot analysis without further dilution.

**Northern Hybridization**—Samples of total or poly(A)<sup>+</sup> RNA were denatured in 50% formamide at 65 °C and electrophoresed in 1% agarose gels containing formaldehyde (30). RNA was transferred to nitrocellulose, and the filters were prehybridized and hybridized as described by Wahl *et al.* (31) with 0.5% SDS added to the prehybridization and hybridization solutions. Nick-translated ST1 was used as a probe at a specific activity of approximately  $10^6$  cpm/ $\mu$ g. Filters were washed twice at room temperature in 2  $\times$  SSC, 0.1% SDS, and twice at 65 °C in 0.1  $\times$  SSC, 0.1% SDS followed by two 45-min washes at 65 °C in 0.1  $\times$  SSC, 0.5% SDS. Filters were then dried prior to autoradiography.

**Preparation of Peptides by Cyanogen Bromide Cleavage**—Purified sialyltransferase (2.5 mg) was reduced and S-carboxymethylated as described by Crestfield *et al.* (32). The S-alkylated protein was extensively dialyzed against water, taken to dryness under vacuum, and subjected to cleavage by cyanogen bromide (33). Dried peptides were solubilized in 20% formic acid and 24% acetic acid and were separated by HPLC using a Waters liquid chromatograph with Model 45 pumps, Model 480 detector, Model 680 automated gradient controller, and a Z-Module System with a Radial-PAK 10- $\mu$ m C18 Bondapak cartridge. Initial separation was obtained using a gradient of 5 mM trifluoroacetic acid and acetonitrile, with a program increasing acetonitrile from 0 to 40% over 20 min at 1 ml/min, from 40 to 60% over 40 min, and 60 to 100% over 10 min. Peptides were detected at a wavelength of 220 nm and pooled. Each of eight peaks were rechromatographed on the same column using a linear gradient starting with 5 mM trifluoroacetic acid and increasing the concentration of 100% isopropyl alcohol from 0 to 20% over the first 10 min, 20 to 40% over 30 min, and 40 to 100% over 20 min at a flow rate of 1 ml/min. Each major peak was subjected to NH<sub>2</sub>-terminal amino acid analysis (34) and amino acid composition by the University of California, Los Angeles, Microsequencing Facility. A single peptide, eluting in the first gradient at 40–41% acetonitrile and in the second gradient at 28–29% isopropyl alcohol, was judged to be pure enough for sequence analysis.

**Amino Acid Sequence Determination**—Automated sequence analysis (35, 36) was performed in a gas-phase sequencer (Applied Biosystems) using a standard protein program or a new program designated MHNAC supplied by M. Hunkapiller of Applied Biosystems. The use of a trifluoroacetic acid-activated glass fiber disc was adapted from the procedure of S. Kent as previously described (37). The PTH-derivative obtained from each sequencer cycle was identified by reverse-phase HPLC (38). For NH<sub>2</sub>-terminal sequence analysis of the purified sialyltransferase (50  $\mu$ g) the protein was precipitated from 50% glycerol, 0.05 M sodium cacodylate, pH 6.5, 300 mM NaCl with 4  $\times$  volume of cold ethanol at –20 °C for 2 h. The precipitate was collected by centrifugation and washed once with cold ethanol. The

<sup>1</sup> The abbreviations used are: HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; kb, kilobase pairs; bp, base pairs.  $\lambda$ ST1– $\lambda$ ST5 refer to  $\lambda$ gt11 bacteriophage with cDNA inserts corresponding to sialyltransferase mRNA. ST1–ST5 refer to the cDNA inserts carried by the  $\lambda$  clones  $\lambda$ ST1– $\lambda$ ST5. Immun-blot refers to the procedure for detection of proteins transferred to nitrocellulose filters with specific antibodies visualized by the Immun-blot reagents (Bio-Rad).

pure peptide obtained by cyanogen bromide cleavage and HPLC chromatography was sequenced by the University of California, Los Angeles, Microsequencing Facility.

**DNA Sequencing of Sialyltransferase cDNAs**—The cDNA inserts obtained by *Eco*RI digests of  $\lambda$ ST1,  $\lambda$ ST2, and  $\lambda$ ST4 clones were shotgun-cloned (39) into the polylinker of M13mp18 or M13mp19 (40). Both strands were sequenced using the dideoxy chain termination method (41) with  $^{35}$ S-dATP (Du Pont-New England Nuclear). Most of the sequence was determined by synthesizing successive oligonucleotide primers (Vega/DuPont Coder 300 Oligodeoxynucleotide Synthesizer) every 200–250 base pairs along the DNA (39), whereas the remainder was deduced using the M13 (–20) universal primer (New England Biolabs) after shotgun subcloning restriction enzyme digests of the appropriate cDNA clones into M13mp18 or M13mp19. Sequence data were aligned and analyzed by computer using DNA Inspector II (Textco).

**Solubilization of Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-Sialyltransferase from Rat Liver Golgi**—Rat liver Golgi were prepared by the method of Morré (42) and stored at  $-80^{\circ}\text{C}$  in 50 mM sodium cacodylate, pH 6.5, 0.1 M NaCl, 50% glycerol. To a freshly thawed vial, 2 volumes of 0.1 M NaCl, 50 mM sodium cacodylate, pH 6.5, were added. The suspension was centrifuged for 20 min at  $20,000 \times g$  and the pellet resuspended in the same buffer at twice the original volume. This suspension was divided into six equal parts and Triton CF-54 added to give a range of detergent concentrations (0–1.5%). Incubations were performed for 3 h at  $37^{\circ}\text{C}$  or  $4^{\circ}\text{C}$  with occasional shaking. Aliquots were removed for enzyme activity assays and the remaining suspensions centrifuged for 20 min at  $20,000 \times g$ . The supernatants were carefully removed, and the pellets were washed once in the same buffer prior to solubilization in sample treatment buffer for SDS-polyacrylamide gel electrophoresis (28).

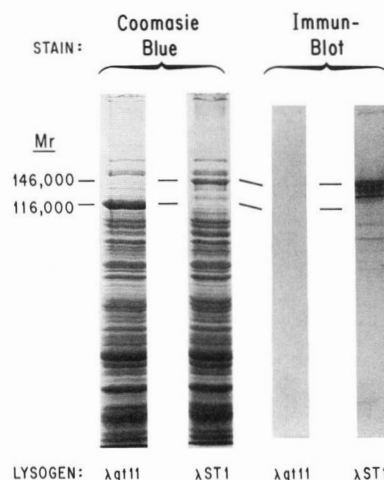
**Immunoprecipitation of Sialyltransferase from  $^{35}$ S-Labeled Hepatocytes**—Rat liver hepatocytes ( $1 \times 10^6$  cells/ml) were labeled for 2 h with [ $^{35}$ S]methionine to a specific activity of  $3.5 \times 10^6$  cpm/ml and solubilized with a detergent mixture (SDS/Triton X-100/deoxycholate) as described by Edwards *et al.* (43). Immunoprecipitin (*Staphylococcus aureus* bearing protein A; Bethesda Research Laboratories) was treated according to the manufacturer's instructions and resuspended in a 10% (w/v) solution of 10 mM Tris, pH 7.4, 150 mM NaCl, 0.02% sodium azide. For each reaction, 600  $\mu$ l of solubilized hepatocyte extract was precleared 5 times with 60  $\mu$ l of Immunoprecipitin at  $4^{\circ}\text{C}$  for 15 min. The Immunoprecipitin pellets were collected by centrifugation at  $16,000 \times g$  for 30 s at  $20^{\circ}\text{C}$ . Affinity purified anti-sialyltransferase antibodies (8  $\mu$ l) were preincubated for 1 h at  $37^{\circ}\text{C}$  alone or with 4  $\mu$ g of purified sialyltransferase in 50  $\mu$ l of Tris/NaCl buffer prior to addition to the hepatocyte membranes. After 16 h at  $4^{\circ}\text{C}$ , 60  $\mu$ l of Immunoprecipitin was added and incubation was continued for 15 min at  $4^{\circ}\text{C}$ . The pellet was washed with 4 aliquots (1 ml) of wash buffer. To dissociate bound protein, pellets were heated at  $100^{\circ}\text{C}$  for 5 min in 40  $\mu$ l of gel sample buffer prior to SDS-polyacrylamide (10%) gel electrophoresis. The dried gel was exposed to film (Kodak XAR-5) with an intensifying screen (Cronex lightning plus) for 9 days at  $-80^{\circ}\text{C}$ .

## RESULTS

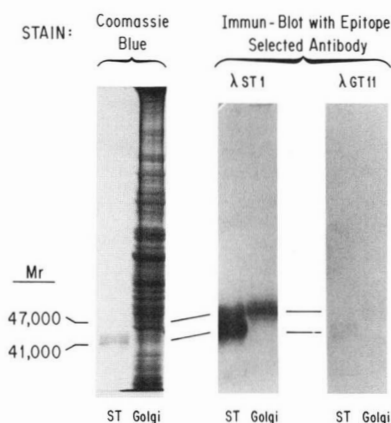
**Isolation of the Initial cDNA Clone of  $\beta$ -Galactoside  $\alpha$ 2,6-Sialyltransferase**—Because polyclonal antibodies to the sialyltransferase had previously been raised in rabbits, affinity purified, and characterized (7, 14), antibody screening of an expression library was chosen to obtain an initial cDNA clone. Accordingly, a  $\lambda$ gt11 expression library was prepared from rat liver mRNA with cDNAs inserted at the single *Eco*RI restriction site in the  $\beta$ -galactosidase gene as described by Huynh *et al.* (25). Using the affinity purified sialyltransferase antibody, the library was screened for recombinants producing a  $\beta$ -galactosidase sialyltransferase fusion protein. From a total of  $2 \times 10^5$  recombinants screened, a single positive clone,  $\lambda$ ST1, was identified and plaque-purified. A lysogen of  $\lambda$ ST1 was prepared in *E. coli* Y1089 cells which was subsequently used for production of  $\lambda$ ST1 and extracts containing the fusion protein (25). Comparison of the induced extracts from lysogens of  $\lambda$ gt11 and  $\lambda$ ST1 are shown in Fig. 1. The  $\beta$ -galactosidase ( $M_r$  116,000), seen as a predominant band in the Coomassie Blue-stained gel of the  $\lambda$ gt11 lysogen extract, is absent

from the  $\lambda$ ST1 lysogen extract and is replaced by a new band at  $M_r$  146,000. This band is intensely stained by the sialyltransferase antibody, as seen in the adjacent lanes.

Sialyltransferase antibodies were also epitope-purified as described by Weinberger *et al.* (29) by adsorption to the fusion protein in  $\lambda$ ST1 lysogen extracts immobilized on nitrocellulose filters. These antibodies inhibited sialyltransferase activity (not shown) and, in immun-blots of SDS gels (Fig. 2), stained both purified ( $M_r$  = 41,000) and Golgi forms ( $M_r$  =



**FIG. 1. Identification of  $\beta$ -galactosidase sialyltransferase fusion protein in  $\lambda$ ST1 lysogen lysate.** Lysates from  $\lambda$ ST1 and  $\lambda$ gt11 lysogens were subjected to SDS-gel electrophoresis as described under "Experimental Procedures." Duplicate lanes of each sample were either stained with Coomassie Blue (left) or transferred to nitrocellulose filters and processed for immun-blot staining using anti-sialyltransferase antibody (right). The  $\beta$ -galactosidase ( $M_r$  = 116,000) in the  $\lambda$ gt11 lysogen extract is replaced in the  $\lambda$ ST1 extract by an  $M_r$  = 146,000 fusion protein visualized with the anti-sialyltransferase antibody in the immun-blot.



**FIG. 2. Epitope-purified antibody recognizes purified and mature Golgi forms of the sialyltransferase.** Polyclonal sialyltransferase antibody was epitope-purified (29) by adsorption to and elution from nitrocellulose filters coated with lysates from  $\lambda$ ST1 and  $\lambda$ gt11 lysogens as described under "Experimental Procedures." Triplicate samples of purified sialyltransferase (ST; 1  $\mu$ g) or Golgi apparatus proteins (Golgi; 140  $\mu$ g) were subjected to SDS-gel electrophoresis. Lanes containing each sample were then stained with Coomassie Blue (left) or transferred to nitrocellulose and immun-blot stained with the epitope-selected antibody preparations. Staining of the purified and Golgi forms of the sialyltransferase with antibody adsorbed to  $\lambda$ ST1 lysogen proteins (middle) but not control antibody adsorbed to  $\lambda$ gt11 lysogen proteins (right) indicates that the  $\lambda$ ST1 fusion protein carries epitopes in common with the purified sialyltransferase.

47,000) of the sialyltransferase (14). In contrast, a control antibody preparation, produced by "mock purification" of sialyltransferase antibodies on nitrocellulose filters coated with a  $\lambda$ gt11 lysogen extract, gave only trace staining of the sialyltransferase bands (Fig. 2). Thus,  $\lambda$ ST1 was tentatively concluded to contain a cDNA insert that had a portion of the coding sequence of the sialyltransferase.

**Northern Analysis of Rat Liver mRNA**—To estimate the size of the sialyltransferase mRNA, the  $\lambda$ ST1 insert (717 bp) was used as a probe for Northern analysis of total RNA and poly A-selected mRNA. As seen in Fig. 3, a single mRNA band of approximately 4.7 kb was identified.

**Complete Coding Sequence of the  $\beta$ -Galactoside  $\alpha$ 2,6-Sialyltransferase**—Using the  $\lambda$ ST1 insert (ST1) as a probe, a rat liver cDNA library in  $\lambda$ gt11 (Clontech Laboratories) was screened by nucleotide hybridization. Clones comprising both 5' and 3' extensions were obtained, and rescreening with 3' fragments of the largest clones yielded several clones with additional 3' extension. Fig. 4 shows a homology map and sequencing strategy of several overlapping cDNA clones ( $\lambda$ ST1- $\lambda$ ST5) which comprise 4.6–4.7 kb, accounting for most of the sialyltransferase mRNA. ST1, ST3, ST4, and portions of ST2 have been sequenced using the Sanger dideoxy method (41) yielding the sequence corresponding to 1700 bp of the 5' end of the sialyltransferase mRNA encompassing the entire coding sequence of the enzyme.

The DNA sequence of the ST3 coding strand and the inferred amino acid sequence of the largest open reading frame are shown in Fig. 5. Although the entire sialyltransferase cDNA has not been completely characterized, the position of the sialyltransferase coding sequence indicates a 5'-untrans-

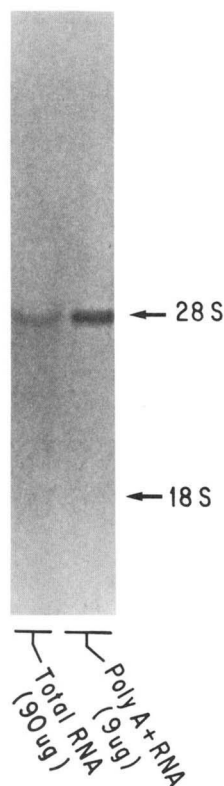


FIG. 3. The  $\lambda$ ST1 cDNA insert hybridizes to a single rat liver mRNA. Northern blot analysis of total and poly(A)<sup>+</sup> selected rat liver RNA probed with nick-translated  $\lambda$ ST1 insert (717 bp) reveals a single band of hybridization corresponding to an mRNA of approximately 4.7 kb (see "Experimental Procedures"). Arrows indicate the position of the 18 and 28 S ribosomal RNAs.

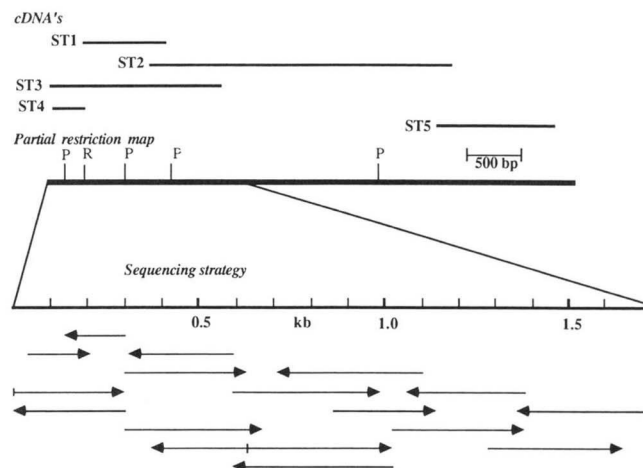


FIG. 4. Homology map and sequencing strategy of sialyltransferase cDNA clones. Restriction sites are indicated for *EcoRI* (R) and *PstI* (P).

lated sequence of at least 180 bp and a 3'-untranslated sequence of approximately 3.2 kb, both of which are unusually long (44). It is of interest that there were two additional AUGs in the 5'-untranslated region at -158 and -116, with 3' in frame stop codons at -131, -134, -95, and +2. The AUG at -116 has been confirmed in two different clones (ST3 and ST4). Upstream AUGs are relatively rare (44–77) and have been associated with translational control (46–49).

The largest open reading frame codes for a polypeptide of 403 residues with a predicted  $M_r$  of 46,700. Peptide overlaps with gas-phase sequencing of the intact purified sialyltransferase (arrow) and a sialyltransferase peptide produced by cyanogen bromide cleavage are shown in the black boxes, confirming that the cDNA was indeed that of the sialyltransferase. The NH<sub>2</sub>-terminal sequence of the intact protein gave two overlapping sequences beginning at residues 64 and 66, indicative of proteolysis. These could be readily distinguished at each cycle since the yield from the primary sequence, beginning at residue 66, was twice that of the minor sequence. At residue 67, the two sequences revealed both Asn and Asp (1:3). Normally this would be called as Asn because the sequencing chemistry is consistent with loss of the amino group from Asn, but Asp was confirmed from the cDNA sequence in two different clones. It may be that the sequence analysis at this position reflects natural polymorphism in the purified sialyltransferase prepared from pooled rat livers.

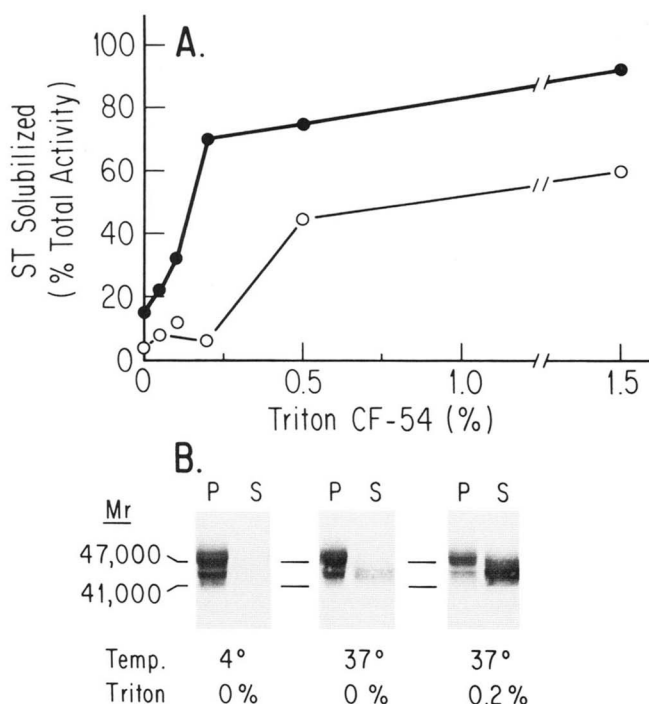
Overlapping clones were identical in sequence with ST3 with two exceptions. A single nucleotide difference between ST3 and ST1 at nucleotide 368 was G in ST1 instead of A, changing amino acid 123 from Tyr to Cys. The second exception was a 50-bp sequence at the 5' end of ST2 which was completely nonhomologous with ST3. In this case, the nonhomologous sequence is presumed to be a cloning artifact originating from the synthesis of the cDNAs, as observed by others (50).

A Hopp-Woods hydrophilicity plot (51) revealed one potential membrane spanning region located ten residues from the NH<sub>2</sub>-terminus (Fig. 5). It consists of 17 hydrophobic amino acids bordered on either end by 2 or 3 lysines. Since the sialyltransferase has its catalytic site in the lumen of the Golgi apparatus and is a membrane-bound enzyme (4), it requires both a signal sequence to target the enzyme to the endoplasmic reticulum (52) and a membrane-spanning region. Because the enzyme has a single hydrophobic sequence, it appears to be a member of a group of proteins with noncleavable NH<sub>2</sub>-terminal signal-anchor sequences (52).



FIG. 5. Complete amino acid sequence of sialyltransferase inferred from the nucleotide sequence of the sialyltransferase cDNA. Peptide sequence overlaps (black boxes) include the NH<sub>2</sub>-terminal sequence of the purified sialyltransferase (arrow). Stippled areas indicate residues that were not identified. Potential glycosylation sites with the sequence Asn-X-Thr/Ser are boxed. The proposed signal-anchor sequence is underscored with the cross-hatched box bordered at either end by open boxes highlighting charged lysine residues.

<sup>2</sup> K. Riggs, J. Weinstein, and J. Paulson, unpublished results.



**FIG. 6. Conversion of membrane-bound Golgi sialyltransferase to soluble forms.** Aliquots of rat liver Golgi membranes were incubated for 3 h at 4 (○) or 37 °C (●) with increasing concentrations of Triton CF-54, and membranes were then collected by centrifugation as described under "Experimental Procedures." Solubilized sialyltransferase activity found in the membrane-free supernatant is shown in A. Immunoblot analysis of the molecular weight forms present in the pellet (P) and supernatant fractions (S) for selected conditions are shown in B, demonstrating that the solubilized enzyme is enriched in lower molecular weight forms of the enzyme.

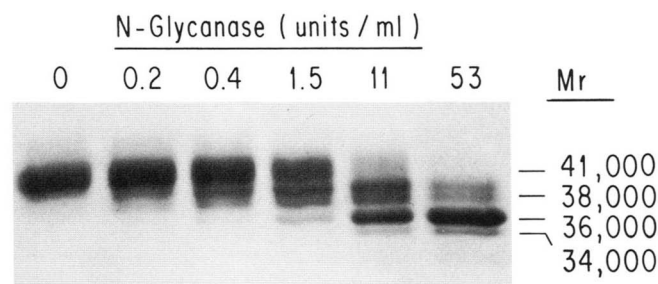
predominant bands at an  $M_r$  47,000. The lower molecular weight forms in the pellet fractions appear to be generated during work-up of the samples. Indeed, they appear in freshly prepared Golgi fractions unless samples are boiled immediately after adding SDS-gel sample buffer or unless inhibitors of thiol proteinases and serine proteinases are added.<sup>2</sup>

The results demonstrate that proteases present in purified Golgi preparations can degrade the sialyltransferase to lower molecular weight forms which are catalytically active and provide direct evidence that the lower molecular weight forms found in the supernatant have lost their membrane binding domain.

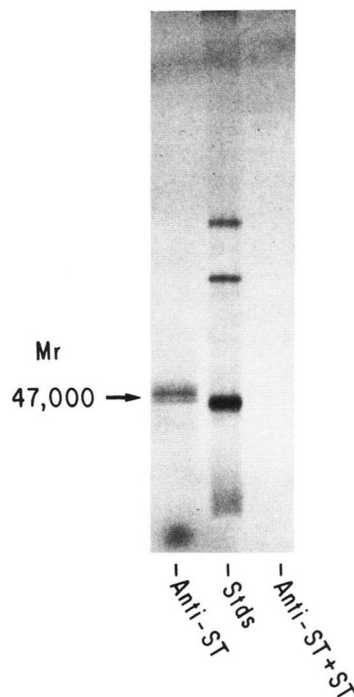
**The Sialyltransferase Is a Glycoprotein**—Three potential sites of *N*-linked glycosylation are found in the predicted amino acid sequence of the sialyltransferase, shown in Fig. 4 as the boxed recognition sequences Asn-X-Thr/Ser (2). To see if these sites were utilized, the purified sialyltransferase ( $M_r$  41,000) was subjected to incubation with increasing amounts of *N*-glycosidase F, which cleaves *N*-linked oligosaccharides *en bloc* at the GlcNAc $\beta$ Asn linkage. The resulting products were analyzed by SDS gels as shown in Fig. 7. The purified sialyltransferase migrates as a diffuse band centered at  $M_r$  = 41,000 (7), which often appears as a doublet (not shown). Increasing amounts of *N*-glycosidase F resulted in the production of doublets centered at  $M_r$  38,000 and  $M_r$  35,000. Increased amounts of *N*-glycosidase F or longer incubation times did not appreciably change the limit digest shown in Fig. 6. The results are consistent with the removal of two *N*-linked oligosaccharides, each having the effect of changing the apparent mass of the enzyme on SDS-gel electrophoresis by 3,000 daltons. If only two of the three potential

sites are utilized, the site with the sequence Asn-Pro-Ser is most likely the one not glycosylated (2).

**Immunoprecipitation of Sialyltransferase from <sup>35</sup>S-Labeled Hepatocytes**—Adding the contribution of the *N*-linked carbohydrate groups of 6000 daltons to the predicted mass of the sialyltransferase polypeptide of 46,700 daltons gives a total expected mass of 52,700 daltons. This is considerably larger than the observed molecular weight of the mature form of the sialyltransferase in the Golgi apparatus ( $M_r$  = 47,000). To see if higher molecular weight precursors could be detected in newly synthesized sialyltransferase, hepatocytes were labeled with [<sup>35</sup>S]methionine for 2 h, solubilized, and subjected to immunoprecipitation. As shown in Fig. 8, the labeled sialyl-



**FIG. 7. N-Glycosidase F digestion of the sialyltransferase.** Sialyltransferase (5  $\mu$ g) in 5  $\mu$ l of 1% SDS was heated for 3 min at 100 °C and then digested with *N*-glycosidase F (Boehringer Mannheim) in 40  $\mu$ l of 10 mM  $\beta$ -mercaptoethanol, 200 mM sodium phosphate, pH 8.6, 0.75% Nonidet P-40 for 1 h at 37 °C. Electrophoresis on a SDS-polyacrylamide (10%) gel was followed by Coomassie Blue staining to visualize proteins.



**FIG. 8. Immunoprecipitation of sialyltransferase (ST) from <sup>35</sup>S-labeled hepatocytes.** Affinity purified sialyltransferase antibody, preincubated with (right) or without (left) purified sialyltransferase (4  $\mu$ g), was added to separate aliquots of solubilized <sup>35</sup>S-labeled rat liver hepatocytes and incubated overnight at 4 °C. Antibodies and immune complexes were adsorbed to *S. aureus* cells (Immunoprecipitin), collected by centrifugation, solubilized with gel sample buffer, and subjected to SDS-gel electrophoresis. The <sup>35</sup>S-labeled proteins were detected by autoradiography. Additional details are given under "Experimental Procedures."

transferase was found to be approximately 47,000 daltons as observed for the sialyltransferase in purified Golgi preparations. No higher molecular weight species were seen.

#### DISCUSSION

The proposed topology of the mature  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase in the Golgi apparatus membranes and its relationship to a catalytically active soluble form are depicted in Fig. 9. The membrane-bound species has a short cytoplasmic domain, a transmembrane domain of 17 residues, and a luminal domain or catalytic domain made up of the rest of the protein. The proposed orientation of the sialyltransferase is based on fact that there is a single  $\text{NH}_2$ -terminal hydrophobic domain at the amino terminus of the peptide, suggesting that it serves both as the signal sequence and the membrane anchor. The strong positive charges introduced by the 2 lysines at the COOH-terminal end of the membrane-spanning domain are not compatible with cleavage by signal peptidase, supporting the concept that this sequence is a true signal anchor (53). Current knowledge of the mechanism of signal peptide-mediated translocation of the protein across the endoplasmic reticulum dictates that the amino-terminal end would face the cytoplasm and the COOH terminus would be translocated into the lumen (52). This leaves the bulk of the protein forming the catalytic domain in the lumen, the expected site of action of the enzyme in sialylation of glycoproteins. Indeed, the luminal localization of the rat liver sialyltransferase catalytic domain in the Golgi apparatus has been amply demonstrated (4, 54). Thus, the sialyltransferase appears to have a topology similar to that of other membrane proteins which have  $\text{NH}_2$ -terminal signal-anchors including the transferrin receptor, the asialoglycoprotein receptor, and the influenza virus sialidase (55–57).

$\text{NH}_2$ -terminal sequence analysis of the purified sialyltransferase ( $M_r = 41,000$ ) compared to the sequence of the mature form of the enzyme in the Golgi apparatus suggests that it results from proteolytic removal of an  $\text{NH}_2$ -terminal peptide of about 63 residues including the membrane binding domain. Conversion of the membrane-bound form of the sialyltransferase to soluble forms is also observed following incubation of Golgi apparatus preparations at 37 °C, with the primary soluble species generated having a molecular weight of 43,000 (Fig. 6). These results demonstrate that the membrane binding domain is not required for enzymic activity and suggests a "stem" structure or exposed region extending from the

luminal side of the transmembrane domain which is susceptible to proteolytic cleavage, as diagrammed in Fig. 9.

Several observations suggest that conversion of membrane-bound sialyltransferases to soluble forms also occur *in vivo*. Soluble sialyltransferases are found in body fluids such as blood, lymph, breast milk, colostrum, and semen (5, 6, 58, 59). A soluble  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase from bovine colostrum has been purified to homogeneity as two molecular weight forms,  $M_r = 45,000$  and 56,000 (12, 13). Neither of these could be incorporated into phospholipid vesicles under conditions which allowed incorporation of other membrane proteins such as cytochrome  $b_5$  and glycophorin A, suggesting that they had lost their membrane binding domains.<sup>3</sup> For intestinal goblet cells the  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase is found throughout the mucin droplets and in the mucin layer at the cell surface (15, 60). This finding strongly suggests that the sialyltransferase in these cells is released from the membrane in the *trans*-region of the Golgi apparatus. Thus, it can be distributed evenly in the mucin storage droplets, where it is then subject to exocytosis like any other secretory glycoprotein. Further study is needed to generalize from these observations. However, it appears likely that the presence of sialyltransferases in various body fluids results from the proteolytic release of the catalytic domain of the enzyme from the  $\text{NH}_2$ -terminal membrane anchor. Presumably, this requires exposure of the sialyltransferase to intracellular proteases, co-localized with the enzyme, in the *trans*-Golgi cisternae and *trans*-Golgi network or exposure to extracellular proteases acting on the enzyme bound to membrane fragments of disrupted cells.

To what extent can the domain structure of the sialyltransferase described here be compared with that of other glycosyltransferases? Numerous other glycosyltransferases have been reported as soluble enzymes in serum, milk, or colostrum including sialyl-, fucosyl-, galactosyl-, *N*-acetylglucosaminyl-, and *N*-acetylgalactosaminyltransferases (1). Size heterogeneity of purified glycosyltransferases is also typically observed (1, 61–64), and this has been proposed to be a consequence of the removal of portions of the polypeptide not essential for activity, such as those which interact with Golgi membranes (1, 61, 65, 66). In one well-studied case, two forms of a  $\beta$ -galactoside  $\alpha$ 2,3-sialyltransferase were purified from porcine submaxillary glands with apparant masses of 49,000 and 44,000 daltons (11, 67). Only the larger form could be incorporated into lipid vesicles, suggesting that the smaller form had lost a membrane binding domain. Metabolic labeling ( $^{35}\text{S}$ ) of a HeLa cell galactosyltransferase followed by immunoprecipitation at various times showed that the enzyme has a half-life of 19 h inside the cell with  $M_r = 54,000$  and is then secreted into the medium as a soluble as a soluble enzyme of  $M_r = 52,000$  (68). Taken together, these observations suggest that many glycosyltransferases will share a general topology which allows a catalytically active domain to be proteolytically cleaved from a membrane domain that anchors it to the membranes of the endoplasmic reticulum or Golgi apparatus. Such topology, however, would be consistent with either an  $\text{NH}_2$ -terminal or COOH-terminal membrane binding domain.

The partial sequence of only one other glycosyltransferase has been reported to date, that of the bovine and human  $\beta$ -*N*-acetylglucosaminide  $\beta$ 1,4-galactosyltransferase, providing the most direct comparison of primary structure with the sialyltransferase (69–72). Narimatsu *et al.* (69) and Shaper *et al.* (70) have obtained cDNAs accounting for 3.7 kb of the 3' end of a 4.8-kb mRNA. As seen for the sialyltransferase, there is a long 3'-untranslated sequence of about 2.7 kb. The

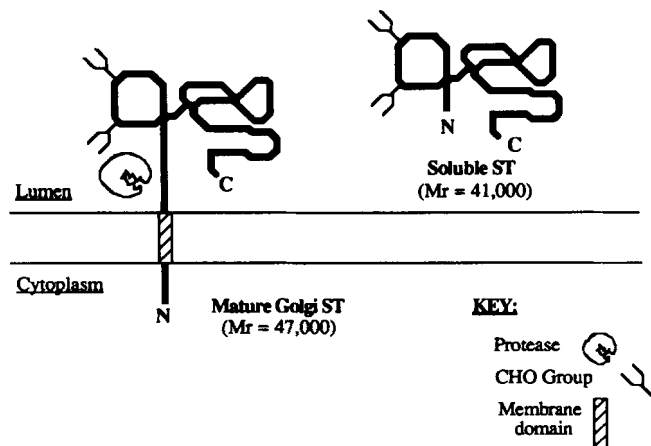


FIG. 9. Proposed topology of sialyltransferase (ST) and relationship of membrane-bound and soluble forms of the enzyme. CHO, carbohydrate.

<sup>3</sup> J. Paulson and R. L. Hill, unpublished results.

remaining 5' sequence codes for an  $M_r = 37,500$  peptide which includes the COOH-terminal sequence of the purified galactosyltransferase followed by a stop codon. This peptide accounts for most if not all of the smallest catalytically active soluble form of the enzyme ( $M_r = 40,000$ ) purified from milk (69, 70). The sequence contains no hydrophobic stretch which could serve as a membrane spanning domain. It follows then that the membrane binding domain must be toward the NH<sub>2</sub> terminus, coded for in the missing 5' end of the mRNA. Thus, it appears that the domain structure of the galactosyltransferase has striking similarities to that of the sialyltransferase reported here.

Although the sialyltransferase and galactosyltransferase carry out quite different glycosylation reactions, both have been localized to the *trans*-cisternae of the Golgi apparatus stack (14, 15, 73), and they appear to have similar domain structures. Yet, a computer-generated homology matrix of the nucleotide sequences of the coding regions of the two enzymes using a 6-base window of comparison showed no regions of homology.<sup>4</sup> Visual inspection of the amino acid sequences revealed a short stretch of Gly-Pro-Gly-Pro-Gly in the sialyltransferase (residues 125–129) that was analogous to a stretch, Pro-Gly-Pro-Gly-Pro-Gly-Pro-Gly (residues 40–47) in the galactosyltransferase, but the significance of this similarity is not clear at present. In view of the current interest of targeting and localization of proteins in the secretory pathway, it will be of considerable interest to compare the NH<sub>2</sub>-terminal sequences of the two enzymes when that of the galactosyltransferase becomes available.

The availability of cDNAs of glycosyltransferases will permit initial investigations into the molecular biology of glycosylation. Particularly pertinent will be to understand the extent to which developmental and tissue-specific expression of glycosyltransferases determine the carbohydrate structures produced by cells. The availability of a cDNA clone containing the complete coding sequence of the sialyltransferase will also allow the use of molecular approaches to identify the biochemical basis for the unique intracellular localization of the enzyme in the *trans*-Golgi cisternae and *trans*-Golgi network.

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<sup>4</sup> E. Ujita Lee and J. Paulson, unpublished results.



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