Structure of Human Transferrin Receptor Oligosaccharides: Conservation of Site-Specific Processing†

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ABSTRACT: The human transferrin receptor (TfR) has three N-linked oligosaccharides. A combination of site-directed mutagenesis and carbohydrate and protein chemistry was used to characterize the structures of the N-linked oligosaccharides and to map their locations. We find that the type of oligosaccharide at each position was unique for that particular site. Human TfR isolated from placentae was used to characterize the structure of the oligosaccharides found in the native TfR. Following digestion of purified TfR with trypsin, individual peptides were obtained via RP-HPLC and were assayed for monosaccharides by strong acid hydrolysis and HPAE-PAD. Peptides containing carbohydrate were subjected to amino acid sequencing to identify the specific Asn residue. The oligosaccharides at Asn 251 are of the complex type. HPAE-PAD and FACE analysis suggests that they are triantennary and trisialylated with core fucosylation. The glycopeptide containing the site at Asn 317 was obtained after limited tryptic digestion and RP-HPLC. FACE analysis reveals predominantly a family of sialylated hybrid oligosaccharides. The consensus sequences for each N-linked site were mutated in various combinations and the resultant TfRs expressed in mouse 3T3 cells. Endoglycosidase H digestion of the mutated TfRs indicates that the pattern of oligosaccharides is consistent with the type of oligosaccharides found at each position in human tissue and the glycosylation of one site does not directly affect the glycosylation of other sites. Previous studies indicated that the oliosaccharide at Asn 727 was high-mannose type [Hayes, G. R., et al. (1995) *Glycobiology 5*, 227−232]. These results indicate that the type of oligosaccharide found at each site is most dependent on the environment surrounding it.

The TfR[1] mediates the uptake of iron into the cell. It is a transmembrane glycoprotein which binds transferrin at the cell surface and transports transferrin into a low-pH environment within the cell, where iron is released. Both the TfR and apotransferrin recycle to the cell surface, and apotransferrin is released, allowing the TfR to bind more diferric transferrin (Dautry-Varsat et al., 1983; Klausner et al., 1983). The TfR undergoes many co- and post-translational modifications during its biosynthesis and targeting to the plasma membrane-endosomal system. The modifications include dimer formation (Seligman et al., 1979; Enns & Sussman, 1981), intersubunit disulfide bond formation (Goding & Burns, 1981; Sutherland et al., 1981; Trowbridge & Omary, 1981), acylation with palmitate (Omary & Trowbridge, 1981), phosphorylation of a serine residue (Schneider et al., 1982), N-linked glycosylation (Omary et al., 1981; Schneider et al., 1982), and O-linked glycosylation (Do et al., 1990; Do & Cummings, 1992; Hayes et al., 1992). Site-directed mutagenesis has been used extensively to determine the function of these modifications.

The most important modification for the correct folding and transport of the protein to the cell surface is N-linked glycosylation (Reckhow & Enns, 1988; Root et al., 1988; Hunt et al., 1989; Enns et al., 1991; Williams & Enns, 1991; Hoe & Hunt, 1992). Early studies indicated that the human TfR contains three N-linked glycosylation sites, consisting of two high-mannose oligosaccharides and one complex oligosaccharide (Schneider et al., 1982). Sequencing of the cDNA encoding the TfR showed that these glycosylation sites are at Asn residues 251, 317, and 727 (McClelland et al., 1984; Schneider et al., 1984). Glycosylation of the last N-linked site (Asn 727) is the most critical to the structure of the receptor (Williams & Enns, 1993). This glycosylation site is predominantly high-mannose in composition (Hayes et al., 1995).

In the present study, the oligosaccharide structures present at the other two N-linked glycosylation sites are reported. In this set of experiments, a combination of peptide sequencing, HPAE-PAD, and FACE analysis of native human TfR and expression of TfRs lacking various combinations of glycosylation sites in 3T3 cells was used to determine that Asn 251 is the predominant site of the complex oligosaccharide; Asn 317 appears to contain a mixture of hybrid

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[1] Abbreviations: TfR, transferrin receptor; N-linked, asparagine-linked; O-linked, serine/threonine-linked; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; RP-HPLC, reverse-phase high-pressure liquid chromatography; HPAE-PAD, high-pH anion exchange chromatography with pulsed-amperometric detection; *N*-glycanase, peptide-*N*-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (EC 3.5.1.52); endo H, endo-*N*-acetylglucosaminidase H (EC 3.2.1.96); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; endo-GluC, endoprotease GluC (EC 3.4.21.19); BiP, binding immunoglobulin protein; FACE, fluorophore-assisted carbohydrate electrophoresis; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; BCA, bicinchoninic acid; TFA, trifluoroacetic acid.

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oligosaccharides. Our findings suggest that the oligosaccharide distribution of the TfR obtained from human tissue is similar to that observed in the mouse 3T3 cells producing human TfR. These results imply that the environment at each site determines the carbohydrate composition of the oligosaccharide, and this is an intrinsic property of the protein.

MATERIALS AND METHODS

Materials. NIH-3T3 cells were obtained from the American Tissue Culture Collection. Human transferrin was purchased from Calbiochem. BioGel P-2 (fine), Affigel-15, and SDS−PAGE reagents were from BioRad Laboratories. Transferrin−Affigel was prepared as recommended by the manufacturer. Sequencing grade modified trypsin and endo-GluC (V8 protease) were from Promega, and *N*-glycanase (peptide-*N*-glycohydrolase, EC 3.5.1.52) and endo H were from either Boehringer-Mannheim or Genzyme Corp. The following complex type N-linked oligosaccharide standards were obtained from Oxford Glycosystems: A1, monosialylated, galactosylated biantennary (from human fibrinogen); A2, disialylated, galactosylated biantennary (from human fibrinogen); A3, trisialylated, galactosylated triantennary (from bovine fetuin); NA2, asialo, galactosylated biantennary (from human fibrinogen); NA3, asialo, galactosylated triantennary (from bovine fetuin); and NA4, asialo, galactosylated tetraantennary (from human acid glycoprotein). Bicinchoninic acid (BCA) protein assay reagent was from Pierce Chemical Co. Reagents and standards for FACE analysis were obtained from Glyko, Inc. All other chemicals were reagent grade or better and were obtained from commercial sources.

Trypsin Digestion and Peptide Purification. Human placental TfR was prepared by affinity chromatography on transferrin−Affigel essentially as described by Turkewitz et al. (1988), with the minor modifications described by Hayes et al. (1992). Purified human TfR (2 mg) was concentrated to 0.4 mL using Centricon-30 microconcentrators (Amicon) and precipitated with $CH_3OH/CHCl_3/H_2O$ (4:1:1) as described by Wessel and Flügge (1984). The precipitate was dissolved in 0.7 mL of 0.5 M Tris-HCl (pH 8.6) and 2% SDS, reduced, carboximidomethylated as described by Swiedler et al. (1983). The carboximidomethylated TfR was precipitated with $CH_3OH/CHCl_3/H_2O$ (4:1:1) and suspended by sonication in 8 M urea at a concentration of 10 mg/mL. Trypsin digestion (1 h at 37 °C) was performed in 0.5 mL of 50 mM NH_4HCO_3 containing 1.67 M urea, 1 mg of TfR, and trypsin at a 1:100 ratio of trypsin to TfR. Trypsin digestion was terminated by boiling for 5 min. Two additional incubations with trypsin (1:200 trypsin/TfR) were performed, each terminated by boiling for 5 min.

The tryptic digest was reduced to 0.25 mL in a Speed-Vac and was applied to a BioGel P-2 column (0.9 × 89 cm) equilibrated in 0.1% TFA. Peptides were eluted with 0.1% TFA at a flow rate of 0.4 mL/min, and 1 min fractions were collected. Aliquots (50 μL) were added to BCA in a microtiter plate, and the initial peak, corresponding to the void volume of the column, was determined by visual inspection of the plate after 1 h at 37 °C. Fractions containing the void volume peak were pooled, and the volume was reduced to <2 mL in a Speed-Vac. This fraction was applied to a C4 reverse-phase column (4.6 × 250 mm, Vydac) equilibrated in 0.1% TFA and separated using a linear gradient of 0 to 40% CH_3CN, containing 0.08% TFA, over 60 min at a flow rate of 1 mL/min. Peptides were detected by absorbance at 214 nm. An aliquot of each peak obtained from RP-HPLC was analyzed for carbohydrate content following strong acid hydrolysis in 4 M TFA at 125 °C for 1 h (Neeser, 1985).

The glycopeptide containing Asn 317 could not be obtained under the above conditions. This site was isolated following a single short tryptic digestion (30 min at 37 °C with a 1:100 ratio of trypsin to TfR). The entire digest was subjected to HPLC using a linear gradient of 0 to 80% CH_3CN, containing 0.08% TFA, over 120 min at a flow rate of 1 mL/min. The carbohydrate-containing peak eluting at about 52 min was repurified on the same gradient, and the peptide was partially sequenced. On the basis of the sequence data, the peptide was further digested with endo-GluC at 37 °C for 18 h to confirm its identity. The digest was subjected to RP-HPLC on the same gradient, and the peak eluting at 42 min was shown by peptide sequencing to contain the site at Asn 317. For carbohydrate analysis, the tryptic peptide was used without further digestion.

Amino Acid Sequence Analysis. Glycopeptides were subjected to amino acid sequence analysis (performed by T. Thannhauser, Cornell University) by automated Edman degradation on either a Porton 2090 peptide sequencer or an Applied Biosystems 477A protein sequencer. The GCG Sequence Analysis Software Package (Genetics Computer Group, 1991) was used to align peptide sequences with the sequence of TfR in the GenBank data base (McClelland et al., 1984; accession no. M11507; Schneider et al., 1984).

N-Glycanase Digestion and Oligosaccharide Analysis. Glycopeptides containing mannose were subjected to *N*-glycanase digestion and oligosaccharide analysis. Glycopeptide fractions were evaporated to dryness in the presence of sufficient SDS to give a final concentration of 0.1% in the digestion reactions (typically 10−50 μg) and resuspended in H_2O. The pH was raised by addition of 1 M Tris-HCl at pH 8.0 or an equal volume of 100 mM phosphate buffer at pH 8.3. Glycopeptides were incubated for 18 h at 37 °C with 0.5 unit of recombinant *N*-glycanase. The incubation was terminated by addition of TFA to 1%, and peptide was removed by solid-phase extraction on Sep Pak C18 (Millipore) cartridges. The deproteinized sample was then desalted using Sephadex G-10 packed in a tuberculin syringe and dried. Alternatively, the incubation was terminated by boiling for 5 min, and the samples were dried and labeled with ANTS for FACE analysis as described by the supplier.

HPAE-PAD Analysis. The carbohydrate content was determined by HPAE-PAD using a Dionex BioLC (Hayes et al., 1992). Monosaccharides were separated isocratically on a CarboPac PA-1 column using 15 mM NaOH as the eluant (1 mL/min) with postcolumn addition of 300 mM NaOH (1 mL/min).

Oligosaccharides were analyzed by HPAE-PAD as described by Hermentin et al. (1992) except that a CarboPak PA-1 column was used. Sialylated oligosaccharides were separated using a gradient of sodium acetate in 0.1 M NaOH (Hermentin et al., 1992; Table 1 gradient S). Asialo oligosaccharides were separated using a gradient of sodium acetate in 0.2 M NaOH (Hermentin et al., 1992; Table 1 gradient A). Sialylated and asialo oligosaccharide standards from Oxford Glycosystems were employed for comparison (it

should be noted that the trisialo, triantennary standard contains two oligosaccharides which can be separated by this procedure).

Analysis of Oligosaccharides by FACE. ANTS-labeled oligosaccharides were deproteinized as described above and desalted on a 1 mL column of BioGel P-2 packed in a 1 mL tuberculin syringe. Fractions of 0.1 mL were collected and fluorescence detected with a hand-held UV light. We found that all labeled oligosaccharides, ranging from trisaccharide to trisialylated triantennary, eluted in the void volume and were well separated from unreacted dye. The pooled fractions were dried and resuspended in 20 μL of H_2O and aliquots subjected to electrophoresis as described by the supplier. Additional aliquots were subjected to sequence analysis using the FACE N-linked oligosaccharide sequencing kit supplied by Glyko, Inc. Electrophoresis was carried out at 4 °C as described by the manufacturer. Gels were analyzed using the FACE Imaging System and software (Glyko, Inc.). In some cases, individual bands were isolated for oligosaccharide sequencing by briefly washing excised gel pieces in ethanol, followed by soaking overnight in H_2O.

Endoglycosidase H Analysis of Mutant TfRs. Site-directed mutagenesis of the TfR and transfection of the plasmids into 3T3 cells were described previously (Williams & Enns, 1991). Oligonucleotides were designed so that each glycosylation site had an alteration in the consensus sequence. Elimination of the first glycosylation site at Asn 251 was achieved by mutating Ser 253 to Ala (S253A). The second glycosylation site was eliminated by mutation of Asn 317 to Asp (N317D), and the third glycosylation site was eliminated by mutation of Asn 727 to Lys (N727K).

NIH-3T3 cells expressing mutant constructs were grown in 35 mm dishes in 2 mL of DMEM (GIBCO-BRL) and 10% FCS under a 5% CO_2 atmosphere at 37 °C. Extracts of 5×10^5 cells containing wild type (WT) TfR, S253A TfR, or N317D TfR or 1×10^6 cells containing N727K TfR, double-mutant TfRs, or TRPL TfR were solubilized in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 at pH 7.4 and immunoprecipitated as described previously (Williams & Enns, 1991). The immunoprecipitates were resuspended in 100 μL of 50 mM sodium citrate and 0.1% SDS at pH 6.0 and divided into two aliquots, with one of the aliquots being treated with 2.5 mu of endoglycosidase H for 16 h at 37 °C and the other aliquot mock treated with buffer alone. The samples were mixed with an equal amount of Laemmli buffer and separated by 8% SDS−PAGE (Laemmli, 1970). After transfer to NitroPlus 2000 (nylon-backed nitrocellulose, MSI), the samples were probed using 1:10000 sheep anti-human TfR followed by 1:10000 swine anti-goat IgG-conjugated horseradish peroxidase (Boehringer Mannheim) and visualized by enhanced chemiluminescence (Amersham) (Williams & Enns, 1993).

RESULTS

Early studies of the human TfR in cultured cells using a combination of tunicamycin, endo H, and SDS−PAGE analysis suggested that it possesses two high-mannose oligosaccharides and one complex oligosaccharide (Omary et al., 1981; Trowbridge et al., 1981; Schneider et al., 1983). We have analyzed and mapped the oligosaccharides found at the individual glycosylation sites in the human placental TfR. The oligosaccharides of the TfR expressed in transfected cells were also analyzed to determine the role of glycosylation in the transport of the TfR to the cell surface (Williams & Enns, 1993), to determine the degree of heterogeneity of the oligosaccharides at each site, and to assess whether the state of glycosylation of one site affects the glycosylation of the other sites.

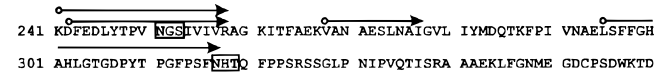

FIGURE 1: Amino acid sequence of the human TfR containing the N-glycosylation sites at Asn 251 and Asn 317. Amino acid sequence of the human TfR from Lys 241 through Asp 360. The consensus sequences for N-glycosylation are enclosed in boxes. Arrows indicate peptides sequenced as described in Materials and Methods. The entire peptide from Val 268 through Asn 317 was obtained as described in Figure 2, and the smaller peptide starting at Leu 295 was obtained and sequenced following endo-GluC treatment as described.

Identification of TfR Glycopeptides. We have previously described the isolation of two TfR glycopeptides, one containing the N-linked site at Asn 727 (Hayes et al., 1995) and the other containing the O-linked site at Thr 104 (Hayes et al., 1992). Using these conditions, two other glycopeptides, eluting from the RP-HPLC column at approximately 30% CH_3CN, can be isolated. The carbohydrate composition of both of these peptides was identical and contained GlcNAc/Man/Gal/NANA (1.8:1.00:1.06:0.22), suggesting the presence of complex oligosaccharides. Amino acid sequence analysis revealed that both peptides contain the glycosylation site at Asn 251 and differ only in the presence of an amino terminal Lys in one of the peptides. Complete sequence analysis of these peptides, summarized in Figure 1, showed that they coincided with Lys 241−Arg 258 and Asp 242 −Arg 258. These two peptides were combined for analysis of oligosaccharide structure.

The glycopeptide containing the site at Asn 317 was particularly refractory to analysis and required a modification of the proteolytic digestion protocol. As described in Materials and Methods and shown in Figure 2A, a brief tryptic digestion yielded a previously unobserved glycopeptide. A partial sequence of this glycopeptide coincided with Val 268−Ile 277 of human TfR (Figure 1). In order to verify that the complete peptide overlapped the site at Asn 317, it was digested with endo-GluC under conditions which should hydrolyze peptide bonds following glutamyl residues but not aspartyl residues. Following proteolysis and repurification on RP-HPLC (Figure 2B), one of the resultant peptides contained carbohydrate. This glycopeptide was sequenced and shown to overlap with amino acids Leu 295−Asn 317 (Figure 1). Thus, once the fact that the initial tryptic peptide contained the Asn 317 site was established, the peptide was used for further carbohydrate analysis without additional proteolysis or purification. Initial screening of the oligosaccharide at Asn 317 showed a monosaccharide composition of GlcNAc/Man/Gal in a ratio of 2.4:2.2:1, suggesting either a hybrid oligosaccharide or a mixture of high-mannose and complex−hybrid moieties.

Oligosaccharides released from the glycopeptides (Asn 251 and Asn 317) were analyzed by HPAE-PAD or FACE (Hu, 1995). Figure 3 shows the ANTS-labeled oligosaccharides released from Asn 251 and Asn 317 separated by electrophoresis. The oligosaccharides released from Asn 251 (Figure 3, lane 2) migrate as a triplet of bands running at 6.19−7.30 glucose units. The major band (6.56 glucose

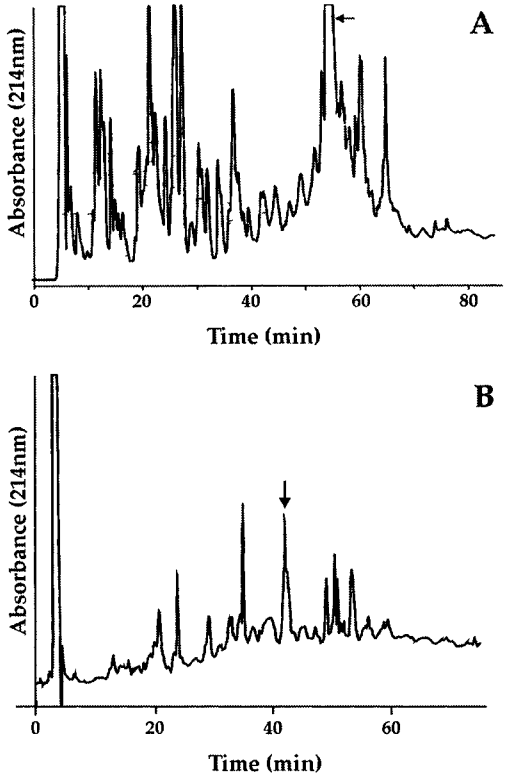

FIGURE 2: Purification of the glycopeptide containing Asn 317. The glycosylation site at Asn 317 was obtained as described in Materials and Methods. (A) The RP-HPLC elution profile of the whole 30 min tryptic digest. The peak containing the glycopeptide is indicated. The peak was repurified and treated with endo-GluC. (B) The elution profile of the endo-GluC digest. The glycopeptide containing Asn 317 is indicated.

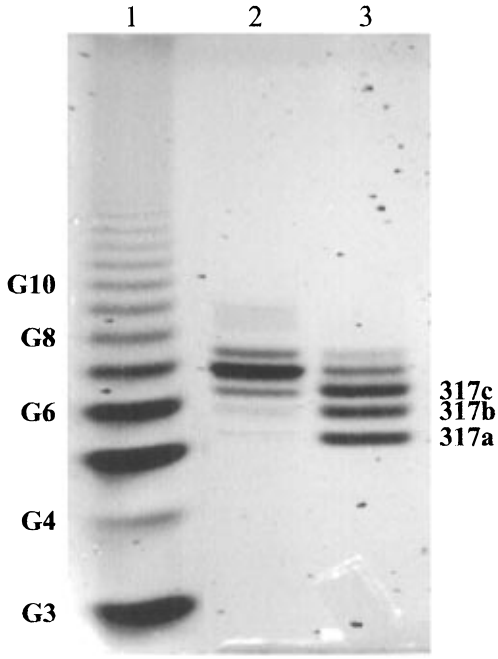

FIGURE 3: FACE analysis of oligosaccharides released from glycopeptides containing Asn 251 and Asn 317. Oligosaccharides released by *N*-glycanase were ANTS-derivatized for FACE analysis as described in Materials and Methods and separated by gel electrophoresis. Fluorescent oligosaccharides were analyzed using the FACE Imager and software: lane 1, maltooligosaccharide standards; lane 2, oligosaccharides released from Asn 251; and lane 3, oligosaccharides released from Asn 317. G3−G10 indicate the degree of polymerization; 317a−c indicate the three major bands from Asn 317.

units) accounts for 75% of the oligosaccharide. The pattern of oligosaccharides released from Asn 317 (Figure 3, lane 3) is more complex, having three major bands and several minor bands ranging from 5.24 to 8.42 glucose units. The three major bands (317a−c) account for 85% of the labeled oligosaccharide.

Analysis of Oligosaccharides Present at Asn 251. The compositional analysis of the oligosaccharide family at Asn 251 suggested it is a complex oligosaccharide. Therefore, the oligosaccharides released from Asn 251 by *N*-glycanase were analyzed by column chromatography using the method of Hermentin et al. (1992) as described in Materials and Methods. As shown in Figure 4A, when the oligosaccharides are desialylated by mild acid hydrolysis and subjected to HPAE-PAD analysis, two major peaks are observed, one coeluting with sialic acid and the second near the asialo, triantennary standard. A minor peak elutes near the asialo, biantennary standard. These results are confirmed by analysis of the intact oligosaccharides shown in Figure 4B. Thus, the major peak elutes near the two trisialo, triantennary standards, and a smaller peak elutes near the disialo, biantennary standard. The HPAE-PAD and compositional analysis suggest that the oligosaccharide family present at Asn 251, although not identical to the standard oligosaccharides, is composed primarily of trisialo, triantennary complex moieties, with a smaller fraction of sialylated, biantennary structures. These results are confirmed by the FACE analysis described below.

Since most of the oligosaccharide at the Asn 251 site appeared as a single band in FACE analysis (see Figure 3 above), enzymatic sequencing was performed without further purification. ANTS-labeled oligosaccharide was digested with a combination of glycosidases and analyzed by FACE (Figure 5A). Neuraminidase digestion resulted in a decrease in mobility of all three bands with the major band migrating with the G10 standard and minor bands migrating at 9.24 and 8.11 glucose units (Figure 5A, lane 3), consistent with the removal of three sialic acid residues from the bands migrating at 6.56 and 6.19 glucose units and one sialic acid from the band migrating at 7.3 glucose units. Concomitant treatment with neuraminidase and β-galactosidase (lane 4) resulted in a triplet migrating at 6.8, 6.41, and 6.1 glucose units, consistent with the removal of three galactose residues from the upper two bands of neuraminidase-treated oligosaccharides and two residues from the lower band. Treatment with neuraminidase, β-galactosidase, and β-*N*-acetylhexosaminidase resulted in a doublet migrating at 4.53 and 3.97 glucose units (lane 5), while addition of α-mannosidase to this mixture (lane 6) resulted in a doublet comigrating with the core trisaccharide and fucosylated core trisaccharide standards (lane 1). We were surprised to find that the majority of the oligosaccharide appeared to be fucosylated (approximately 76%), as very little fucose was seen in the carbohydrate composition studies used to screen for glycopeptides, probably due to the hydrolytic conditions employed. To confirm this result, ANTS-labeled oligosaccharide was treated with all four of the above enzymes followed by incubation in the absence or presence of α-fucosidase. As can be seen in Figure 5B, treatment with fucosidase resulted in the complete digestion of the upper band. These results are consistent with the interpretation that the majority of the oligosaccharides present at Asn 251 are core-fucosylated,

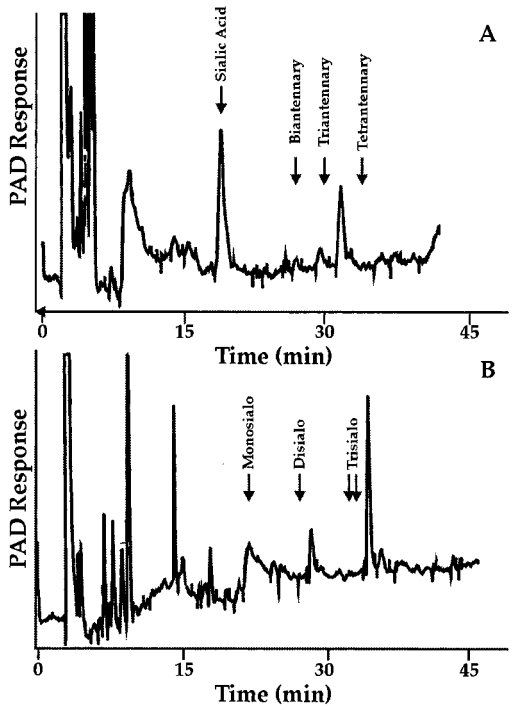

FIGURE 4: HPAE chromatography of Asn 251 oligosaccharides. Oligosaccharides were released from the purified glycopeptide by *N*-glycanase and analyzed by HPAE-PAD using the technique of Hermentin et al. (1992), as described in Materials and Methods. (A) Desialylated oligosaccharides were eluted using gradient A. Elution times of complex galactosylated asialo bi-, tri-, and tetraantennary standards are indicated by arrows. (B) Intact sialylated oligosaccharides were analyzed using gradient S. Elution times of complex galactosylated mono-, di-, and trisialylated standards are indicated by arrows. Isomers of the single trisialylated, triantennary standard are resolved by this technique.

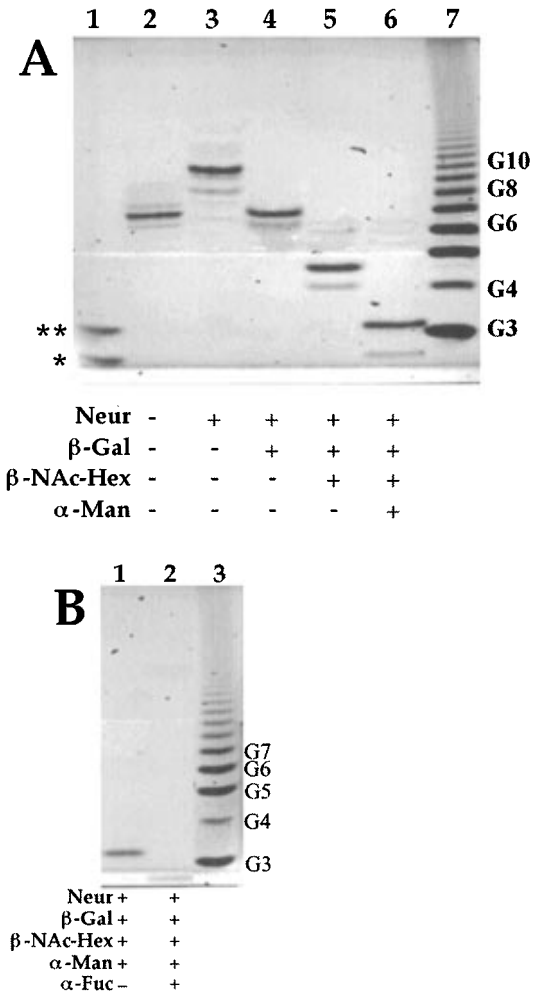

FIGURE 5: Sequence analysis of oligosaccharides released from Asn 251. ANTS-derivatized oligosaccharides were sequenced as described in Materials and Methods. (A) Lane 1, core trisaccharide and fucosylated core trisaccharide standards, (*) ManGlcNAcGlcNAc and (**) ManGlcNAc[Fuc]GlcNAc; lane 2, intact oligosaccharides; lanes 3−6, oligosaccharides treated with the combination of glycosidases, Neur (neuraminidase), β-Gal (β-galactosidase), β-NAc-Hex (β-NAc-hexosaminidase), and α-Man (α-mannosidase) as indicated; and lane 7, maltooligosaccharide standards (G3-G10). (B) Lane 1, oligosaccharides from Asn 251 treated with the combination of enzymes shown above (panel A, lane 6); lane 2, material from lane 1 treated with α-fucosidase; and lane 3, maltooligosaccharide standards (G3−G7).

trisialylated, triantennary structures. There are also smaller amounts of nonfucosylated trisialylated, triantennary and monosialylated, biantennary structures.

Analysis of the Carbohydrate Composition at Asn 317. Due to the difficulty in isolating the glycopeptide containing Asn 317, HPAE-PAD analysis was not performed on the oligosaccharides released from this site. We initially digested the mixture of ANTS-labeled oligosaccharides with α-mannosidase alone. This enzyme had only small effects on the distribution of oligosaccharides in the various bands (data not shown), indicating that little of the oligosaccharide present at Asn 317 consists of high-mannose structures. Preliminary enzymatic digestion with other glycosidases indicated that the mixture was too complex to analyze directly. Therefore, the three major bands (see Figure 3) were individually isolated by elution from the gel, and further studies were performed on the isolated oligosaccharides. Digestion of these oligosaccharides with β-galactosidase or β-*N*-acetylhexosaminidase had no effect, while treatment of each of the bands with α-mannosidase resulted in partial digestion, indicating the presence of exposed mannose residues but no exposed galactose or GlcNAc residues (data not shown).

Figure 6 shows the results of oligosaccharide sequencing of the three bands. The three major bands (317a−c) are composed primarily of a family of sialylated hybrid structures containing two, three, or four α-mannose residues, respectively. Of the three, only band 317a (panel A) appeared to be homogeneous. Treatment of this oligosaccharide with neuraminidase resulted in a decrease in mobility from 5.19 to 5.79 glucose units, indicating the release of a single sialyl residue. Treatment with neuraminidase and β-galactosidase increased the mobility (4.64 glucose units), while the addition of β-*N*-acetylhexosaminidase further increased the mobility, consistent with the presence of one galactose and one GlcNAc residue. Treatment with all four glycosidases resulted in a band comigrating with the core trisaccharide standard. The mobility shift (3.96 to 1.78 glucose units) suggests the release of three mannose residues. Therefore, this oligosaccharide appears to be a sialylated hybrid structure with a single accessible α-mannose residue.

Sequencing of bands 317b and 317c is shown in Figure 6 (panels B and C, respectively). Enzymatic digestion indicates that the major components present in each of these bands are sialylated hybrid structures having three (317b, panel B) and four (317c, panel C) α-mannosyl residues. Thus, they are closely related to the 317a oligosaccharide.

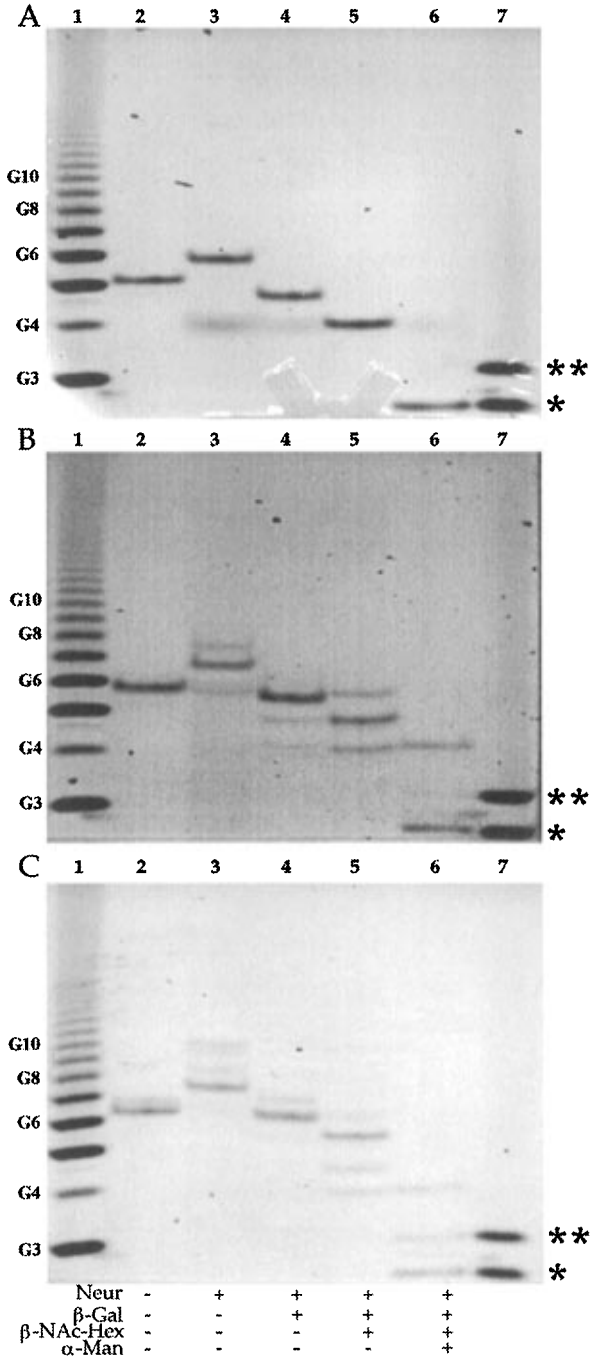

FIGURE 6: Sequence analysis of oligosaccharides released from Asn 317. ANTS-derivatized oligosaccharides were purified from the gel shown in Figure 3 by elution in water as described in Materials and Methods: (A) oligosaccharide 317a, (B) oligosaccharide 317b, and (C) oligosaccharide 317c. Each oligosaccharide was sequenced as described in Materials and Methods employing the enzymes indicated: Neur (neuraminidase), β-Gal (β-galactosidase), β-NAc-Hex (β-NAc-hexosaminidase), and α-Man (α-mannosidase). Lane 1, maltooligosaccharide standards (G3−G10); lane 2, intact oligosaccharides; lanes 3−6, oligosaccharides treated with the indicated glycosidases; and lane 7, core trisaccharide and fucosylated core trisaccharide standards. The standards include (*) ManGlcNAcGlcNAc and (**) ManGlcNAc[Fuc]GlcNAc.

Both of these bands also contain other oligosaccharides in small amounts. Digestion of 317b with neuraminidase (panel B, lane 3) results in the appearance of three bands, suggesting the release of zero, one, and two sialic acids (the major band corresponds to the monosialylated species described above). These three bands collapse to a single band upon treatment with β-galactosidase, while further treatment with β-hexosaminidase (lane 4) again produces three bands, one comigrating with the original band. This band disappears upon treatment with α-mannosidase, suggesting that the material resistant to neuraminidase is a high-mannose structure. The material containing two sialyl residues is probably a disialylated, biantennary structure, since an asialo, agalacto biantennary structure would be expected to comigrate with the asialo, agalacto hybrid structure in this system (Hu, 1995). A small portion remains undigested by α-mannosidase.

Upon neuraminidase digestion, the major component of band 317c loses one sialic acid and behaves like a hybrid oligosaccharide, similar to the pattern for bands 317a and 317b. A minor component (approximately 10%) loses three sialic acids as indicated by a decrease in mobility from 7.32 to 10.75 glucose units. Treatment with β-galactosidase and β-*N*-acetylhexosaminidase releases three galactose and three GlcNAc residues from this component. Further α-mannosidase digestion results in comigration with the fucosylated core trisaccharide standard. Thus, this component seems to be the same structure as that found at Asn 251.

Analysis of Human TfR Mutants Expressed in Mouse Cells. The analysis of the oligosaccharides on the placental TfR indicated that the complex, hybrid, and high-mannose modifications are not randomly distributed between sites but are distinctive for each site. The oligosaccharides of the human TfR expressed in mouse 3T3 cells were analyzed to determine whether the homogeneity of glycosylation at each site is a universal phenomenon for the TfR or a species/cell type phenomenon. In addition, we used mutant TfRs which lack various combinations of the three N-glycosylation sites to determine whether the state of glycosylation at one site influenced the composition of the oligosaccharides at the other sites. Endo H is an endoglycosidase that cleaves between the proximal GlcNAc residues of high-mannose and hybrid but not complex, N-linked oligosaccharides. It was used with the mutant TfRs lacking each individual glycosylation site or lacking a combination of two glycosylation sites to determine which of the individual glycosylation sites contained complex or high-mannose/hybrid type oligosaccharides.

The endo H sensitivities of the mutated TfRs lacking a single N-linked glycosylation site at Asn 251, Asn 317, or Asn 727 are visualized by the Western blot in Figure 7A. Both the wild type and the single mutants show partial sensitivity to endo H, indicating that at least one of the two remaining oligosaccharides contains a high-mannose/hybrid type oligosaccharide. As expected, the TRPL TfR, lacking all three N-linked oligosaccharides, is not sensitive to endo H. A fraction of the human TfR appears to be incompletely processed, as they are totally sensitive to endo H and possess all high-mannose/hybrid type oligosaccharides (Figure 7A, WT; Figure 7B, WT). The mobility of this lower-molecular weight band of the WT TfR is not completely reduced to that of the TRPL TfR. This result is most likely due to endo H cleavage between the two proximal GlcNAcs, leaving one GlcNAc residue attached to each Asn or other covalent post-translational modifications. The mutant TfR lacking the first glycosylation site (S253A TfR) is almost completely digested to the mobility of the TRPL TfR, indicating that Asn 251 is the major site of the complex carbohydrate. The slight heterogeneity observed suggests that one of the two other

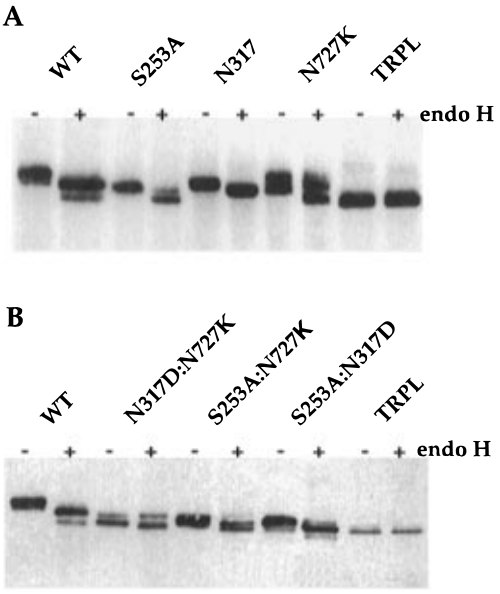

FIGURE 7: Endo H digestion of human TfR in 3T3 cells. Endo H digestion of human TfR mutants lacking single N-linked glycosylation sites (A) expressed in mouse NIH-3T3 cells. Wild type human TfR (WT) or human TfR lacking the first (S253A), second (N317D), or third (N727K) N-linked glycosylation sites, or all three sites (TRPL), was expressed in NIH-3T3 cells. Endo H digestion of human TfR mutants lacking two N-linked glycosylation sites expressed in mouse NIH-3T3 cells (B). Wild type human TfR (WT) and human TfR possessing only the first (N317D/N727K), the second (S253A/N727K), or the third N-glycosylation site (S253A/N317D), or lacking all three glycosylation sites (TRPL), were expressed in NIH-3T3 cells. Endo H digestion, gel electrophoresis, transfer, and detection were performed as described in Materials and Methods.

glycosylation sites may also contain a small fraction of complex oligosaccharide and/or endo H resistant hybrid structures (Kobata, 1979). Such endo H resistant hybrid oligosaccharides are observed in placental TfR preparations. The intermediate mobility of the mutant TfR missing the second glycosylation site (N317D TfR) indicates that one of the two sites is a high-mannose/hybrid type and the other is a complex type oligosaccharide. Although these results are consistent with the oligosaccharides at Asn 251 being complex and those at Asn 727 being high-mannose, the N727K TfR, lacking the C terminal site, is heterogeneous. Endo H digestion of this mutant TfR suggests half of the TfR contain one high-mannose and one complex site as expected while the other half of the receptors contain two high-mannose/hybrid sites. This reduced processing is due to the previously reported increased retention of N727K TfR in the endoplasmic reticulum (Williams & Enns, 1993). Thus, the results of the studies employing single-mutant TfRs indicate that Asn 251 is modified by a complex oligosaccharide and the other two sites are predominantly high-mannose/hybrid type oligosaccharides.

Double-mutant TfRs, possessing only single glycosylation sites at Asn 251, Asn 317, or Asn 727, were also analyzed by endo H digestion, SDS−PAGE, and Western immunodetection (Figure 7B). As expected for TfRs with single glycosylation sites, extracts of mouse cells transfected with these mutants showed TfRs having molecular weights lower than those of the WT and single mutant TfRs, but molecular weights higher than that of the TRPL TfR (Figure 7B).

Mutants lacking the Asn 727 site have been shown to be strongly retained in the endoplasmic reticulum, and only a small fraction are processed in the Golgi apparatus (Williams & Enns, 1993). The TfR containing only the first glycosylation site (N317D/N727K TfR) appears as a doublet. When it is subjected to endo H treatment (Figure 7B), the lower band undergoes a shift in molecular weight to a lower value, while the upper band is unaltered in mobility. This result is again consistent with Asn 251 being the site of complex oligosaccharides, but since it is inefficiently transported out of the endoplasmic reticulum, many of the oligosaccharides are incompletely processed. The TfR containing only the second glycosylation site at Asn 317 (S253A/N727K TfR) also migrates as a doublet on SDS−PAGE gels, but both bands shift upon endo H digestion, indicating that the oligosaccharide is hybrid/high-mannose in character. Since there is only one N-linked glycosylation site and both bands are equally sensitive to endo H digestion, the results suggest that the doublet is due to some other modification(s), such as acylation, O-glycosylation, and/or phosphorylation. The mutant possessing only the Asn 727 glycosylation site (S253A/N317D TfR) yields a single band which is sensitive to endo H digestion, entirely consistent with the interpretation that this site is high-mannose in composition. This result is in keeping with our previous finding that Asn 727 is modified with high-mannose oligosaccharides (Hayes et al., 1995). Therefore, the endo H data from the TfRs containing only one glycosylation site are consistent with the data from the TfRs containing two glycosylation sites. Since the identification of which sites contain high-mannose/hybrid sites and which site contains complex oligosaccharide is internally consistent among the combinations of single- and double-mutant TfRs, we can also conclude that the glycosylation of one site does not directly affect the other sites.

DISCUSSION

The human TfR was first identified as a membrane glycoprotein when it was characterized in preparations derived from placentae and cultured cells (Hamilton et al., 1979a,b; Seligman et al., 1979; Wada et al., 1979a,b). More detailed analysis using pulse-chase studies, susceptibility to endo H, and treatment of cells with increasing concentrations of tunicamycin, an inhibitor of N-linked glycosylation, indicated that the TfR possessed three N-linked glycosylation sites (Omary et al., 1981; Schneider et al., 1982, 1983). Mobility shifts upon SDS−PAGE revealed two endo H sensitive sites and one endo H resistant site, suggesting two high-mannose/hybrid type oligosaccharides and one complex type oligosaccharide. The endo H digestion patterns of the TfR showed distinct shifts in molecular weights, indicating a homogeneous distribution of oligosaccharide types rather than a random distribution of endo H resistant and sensitive sites. Cloning of the TfR 2 years later revealed three consensus sequences in the extracellular domain for N-linked glycosylation sites (McClelland et al., 1984; Schneider et al., 1984). Thus, all the potential N-linked sites are utilized in the human TfR.

The N-linked glycosylation sites of the TfR appear to be highly conserved in evolution. All the TfRs cloned to date, including human, mouse, rat, Chinese hamster, and chicken, have three regions of N-linked glycosylation. The first two N-linked glycosylation sites are equivalent to the human N-linked glycosylation sites at Asn 251 and 317 (McClelland et al., 1984; Schneider et al., 1984; Trowbridge et al., 1988; Roberts & Griswold, 1990; Gerhardt et al., 1991; Collawn

et al., 1993). The third region is in the C terminal portion. The human TfR has one glycosylation site at Asn 727. Rodents have two glycosylation sites in this region with the additional consensus sequence three amino acids N terminal to the equivalent of the human Asn 727 glycosylation site (Trowbridge et al., 1988; Roberts et al., 1990; Collawn et al., 1993). The last glycosylation site in the chicken TfR is in the same C terminal region and is equivalent to the third glycosylation site in rodents (Gerhardt et al., 1991).

N-Linked glycosylation is critical for the proper function of the TfR. Treatment of cells with tunicamycin, which inhibits N-linked glycosylation, results in diminished cell surface expression of the TfR (Reckhow et al., 1988; Hunt et al., 1989) and retention of the receptor in the endoplasmic reticulum. These results were confirmed by site-directed mutagenesis of the TfR N-linked sites (Williams & Enns, 1991, 1993; Hoe and Hunt, 1992). Although all three sites contribute to a certain extent to the proper folding and functioning of the TfR, the region around Asn 727 appears to be the most critical in the folding and transport of the TfR to the cell surface (Williams & Enns, 1993).

Because of the great importance of glycosylation in TfR folding and function, we undertook a series of studies to identify the specific oligosaccharides associated with each N-linked glycosylation site. Through a combination of oligosaccharide analysis and site-directed mutagenesis, the oligosaccharide composition of each N-linked glycosylation site was determined. Our studies indicate that the type of oligosaccharide is not randomly distributed at each glycosylation site, but rather, each site has its own distinctive oligosaccharide array. The Asn 251 site contains complex oligosaccharides, and Asn 317 contains hybrid oligosaccharides. As previously demonstrated (Hayes et al., 1994), the site at Asn 727 is entirely high mannose in character.

A survey of TfR oligosaccharide structures was reported by Orberger et al. (1992). Oligosaccharides of the TfR derived from two human placentae and HepG2 cells, a human hepatoma cell line, were analyzed. In their study, TfR was trypsinized and the oligosaccharides were released by endo H and *N*-glycanase digestion. Thus, the oligosaccharides were not mapped to specific sites. However, using mass spectral and methylation analysis, they identified a number of structures that we report here. The trisialylated, core-fucosylated triantennary oligosaccharide is a major component, as is the nonfucosylated counterpart. We have mapped these structures to Asn 251. In addition, hybrid structures having one, two, and three mannosyl residues on the α1,6 branch are major components. We find that these structures are isolated only from the Asn 317 site. In addition, minor components such as biantennary complex structures were observed in both studies. Combining our results with those of Orberger et al. (1992), we now find it possible to assign anomeric and linkage positions to the major TfR oligosaccharide species (e.g., see Figure 8) and to map their attachment to the polypeptide.

All of the structures identified in the present study and by Orberger et al. can be ascribed to either the Asn 251 or Asn 317 sites. However, they failed to detect high-mannose oligosaccharides from either of the two placentae they studied. The reason for this is unclear. In numerous placental preparations, we observed that the site at Asn 727 contained only polymannose moieties (Hayes et al., 1995). Perhaps, there is some genetic variability, and the two

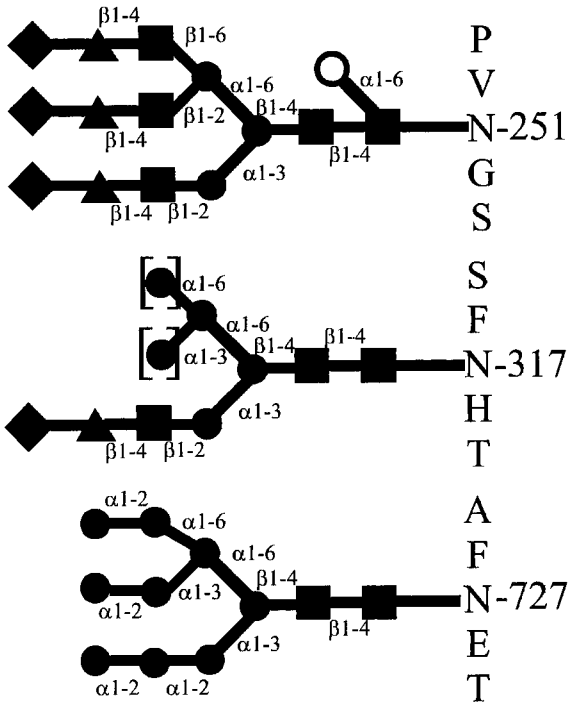

FIGURE 8: Proposed oligosaccharide structures at each N-glycosylation site on the human TfR. Proposed structures of the oligosaccharides found at each glycosylation site based on the data presented here and those of Hayes et al. (1995) and Orberger et al. (1992): open circles, fucose; closed circles, mannose; closed squares, *N*-acetylglucosamine; closed triangles, galactose; and closed diamonds, sialic acid. Square brackets indicate the heterogeneity (either presence or absence) of α-mannose in the family of hybrid structures.

subjects they studied were highly unusual. Such variability is consistent with the differences in distribution observed in the complex and hybrid oligosaccharides. It should be noted, however, that they reported the presence of high-mannose oligosaccharides in TfR from HepG2 cells, in agreement with earlier studies employing a variety of other cell lines and with our studies.

Interestingly, we also found that the class of oligosaccharide at each site of human TfR does not vary when expressed in a cell line of another species. Thus, we observed the same distribution of oligosaccharides at each site in human placentae and two rodent cell lines transfected with the human TfR (mouse and Chinese hamster, not shown). The only differences detected between the rodent and human cell lines were that the human TfR is not as efficiently processed in rodent cell lines and that some TfRs remain completely endo H sensitive. We have previously shown that incompletely processed TfRs are not transported to the cell surface (Williams & Enns, 1993). Incompletely processed TfR is not seen in any of the human cell lines we have examined (unpublished observations).

The results of our present study suggest that the type of oligosaccharide at each site is an intrinsic property of the TfR. Although it is well known that protein glycosylation is dependent on both the polypeptide and the cell type in which it is expressed, the human TfR exhibits the same general glycosylation patterns, whether produced in the placenta or in cell cultures. Furthermore, the mutations at each site do not appear to influence the glycosylation pattern found at the other sites. These results are generally similar to those observed for rat Thy-1, rat CD4, human cortico-

steroid binding globulin, and tissue plasminogen activator [see a review by Dwek (1996); Avvakumov & Hammond, 1994]. Rat Thy-1 from brain and thymocytes possesses three N-linked glycosylation sites. The Asn 23 site contains only high-mannose oligosaccharides; the Asn 74 site is mostly complex with some hybrid oligosaccharides, and the Asn 98 site contains all three types of oligosaccharides. Site-directed mutagenesis of each site individually reveals that processing at each site is independent of the others. In the case of the two oligosaccharides of rat CD4, glycosylation at each site is specific and independent, similar to what we see with the TfR. Processing of the six oligosaccharides of the human corticosteroid binding globulin is also site-specific, and mutagenesis studies revealed that processing of one site is independent of the other (Avvakumov & Hammond, 1994). In contrast, deletion of domains 3 and 4 of the rat CD4, including the glycosylation site at Asn 270, affects the processing of the oligosaccharide at Asn 159 (Dwek et al., 1993). In the case of tissue plasminogen activator, the occupancy of one glycosylation site can influence another site. The fine structure of the oligosaccharide at Asn 448 is affected by the occupancy of site 184 (Dwek, 1996). Thus, on the proteins studied to date, oligosaccharide processing tends to be site-specific, but processing of the oligosaccharide at one site may be influenced by glycosylation at other sites. In the TfR, they appear to be fully independent.

The observations we present suggest that the folded peptide domain structure of the completed polypeptide directs the cells' oligosaccharide-processing machinery to modify the moieties present at each locus in a site-specific manner. The sites that are less critical for the folding and transport of the TfR to the cell surface (i.e., Asn 251 and Asn 317) contain more highly processed oligosaccharides. The carbohydrate site that plays the smallest role in transport of the TfR to the cell surface (Williams & Enns, 1993), Asn 251, is almost entirely complex in nature and is presumably completely accessible to Golgi processing enzymes. It is tempting to speculate that the high-mannose carbohydrate at Asn 727 (Hayes et al., 1995), which is involved in the proper folding of the TfR during its biosynthesis, is sterically hindered from further processing by the protein backbone, or perhaps by accessory proteins.

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