Expression of Glycosylated and Nonglycosylated Human Transferrin in Mammalian Cells. Characterization of the Recombinant Proteins with Comparison to Three Commercially Available Transferrins†

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ABSTRACT: The coding sequence for human serum transferrin was assembled from restriction fragments derived from a full-length cDNA clone isolated from a human liver cDNA library. The assembled clone was inserted into the expression vector pNUT and stably transfected into transformed baby hamster kidney (BHK) cells, leading to secretion of up to 125 mg/L recombinant protein into the tissue culture medium. As judged by mobility on $NaDodSO_4$–PAGE, immunoreactivity, spectral properties (indicative of correct folding and iron binding), and the ability to bind to receptors on a human cell line, initial studies showed that the recombinant transferrin, is identical to three commercial human serum transferrin samples. Electrospray mass spectrometry (ESMS), anion-exchange chromatography, and urea gel analysis showed that the recombinant protein has an extremely complex carbohydrate pattern with 16 separate masses ranging from 78 833 to 80 802 daltons. Mutation of the two asparagine carbohydrate linkage sites to aspartic acid residues led to the expression and secretion of up to 25 mg/L nonglycosylated transferrin. ESMS, anion-exchange chromatography, and urea gel analysis showed a single molecular species that was consistent with the expected theoretical mass of 75 143 daltons. In equilibrium binding experiments, the nonglycosylated mutant bound to HeLa S_3 cells with the same avidity and to the same extent as the glycosylated protein and the three commercial samples. These studies demonstrate conclusively that carbohydrate has no role in this function.

The transferrins are a group of glycosylated metal-binding proteins which function in the transport of iron to cells and as bacteriostatic agents in a variety of biological fluids (Brock, 1985; Huebers & Finch, 1987; Harris & Aisen, 1989; Aisen, 1989; Thorstensen & Romslo, 1990; Chasteen & Woodworth, 1990). Examination of the primary structures of various transferrins indicates that a gene duplication event occurred during evolution, resulting in the present day, 80-kDa protein which binds two metal ions in homologous lobes. X-ray crystallographic studies (Anderson et al., 1987, 1989, 1990; Baker et al., 1991; Bailey et al., 1988; Sarra et al., 1990) reveal the presence of two globular lobes each made up of two domains which define a deep cleft containing the binding site for each metal ion and a synergistic anion. In all transferrins for which X-ray data are available, the ferric ion is directly coordinated to two tyrosines, one histidine, one aspartic acid, and two oxygens from the synergistic carbonate anion. In spite of the similarity of the two sites, they differ with regard to thermodynamic, kinetic, and spectroscopic behaviors, all of which are affected by the salt concentration of the medium (see review articles cited above). A unified picture is slowly emerging as to the molecular basis of the observed differences and their relationship to the mechanisms of metal binding and release.

Recombinant technology may offer the key to unlocking the remaining mysteries related to exactly how transferrin binds and releases iron. The ability to change single amino acids, to incorporate isotopically substituted amino acids, and to produce homogeneous deglycosylated protein allows definitive inquiries to be made into the role of carbohydrate as well as to the role of individual residues involved in anion binding, the salt effect, receptor interaction, and modulation of metal binding. The highly homogeneous samples provided by recombinant technology are ideal for X-ray structure analysis, which is ultimately required to gain a complete understanding of the relationship of various residues at the molecular level. An additional rationale for producing human serum transferrin (hTf)[1] by recombinant technology is to provide a new and safer source of this protein for use in tissue culture. Human transferrin is one of several components found in virtually all serum supplements and serum replacements. There is an absolute requirement for Tf (iron) by many actively dividing cells (Barnes & Sato, 1980; Newman et al., 1982; Trowbridge et al., 1984; Chan et al., 1992).

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[1] Abbreviations: hTf, human serum transferrin; rhTf, recombinant human serum transferrin, hTf N413D/N611D, recombinant nonglycosylated human serum transferrin in which the asparagine residues at positions 413 and 611 are mutated to aspartic acid residues; BHK cells, baby hamster kidney cells; ESMS, electrospray mass spectrometry; DMEM–F-12, Dulbecco's modified Eagle's medium–Ham F-12 nutrient mixture; MTX, methotrexate; NTA, nitrilotriacetate.

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The recombinant human serum transferrin produced must be fully functional in reversible iron binding and in binding to the cellular receptor. Thus, although 2 recent publications report the production of this protein in *Escherichia coli* (Hershberger et al., 1991; Ikeda et al., 1992), it does not appear that the 19 disulfide linkages essential to the native conformation and to reversible metal binding are formed (see Discussion). By contrast, fully functional recombinant lactoferrin has been produced in baby hamster kidney cells (Stowell et al., 1991), and also by a filamentous fungus, *Aspergillus oryzae* (Ward et al., 1992). We report here the successful expression in baby hamster kidney cells of recombinant human serum transferrin with and without the glycosylation sites intact.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium–Ham F-12 nutrient mixture (DMEM–F-12) was obtained with or without phenol red from Sigma as was Joklik's minimum essential medium. Defined and supplemented newborn calf serum (catalog no. A-2151-D) was obtained from Hyclone and pretested to assure adequate growth of baby hamster kidney (BHK) cells. The serum replacement, Ultroser G, and penicillin/streptomycin sulfate solution were from Gibco. Corning expanded surface roller bottles, Wheaton Omnivials, and Dynatech Romovawells were obtained from a local distributer. $Na^{125}I$ was from Du Pont NEN. All of the chromatographic resins, DEAE-Sephacel, Sephacryl S-100 HR, and Polyanion SI were from Pharmacia. Methotrexate from Cetus was purchased at a local hospital pharmacy. Centricon 30 microconcentrators, YM-30 ultrafiltration membranes, and a spiral cartridge concentrator (CH2PRS) fitted with a S10Y10 cartridge were from Amicon. Rabbit anti-mouse immunoglobulin G was purchased from Organon Teknika. All chemicals and reagents were analytical grade or purer.

Human serum tranferrin was purchased both from Sigma and from Boehringer Mannheim. A generous gift of 20 mg of human transferrin was received from Scipac Limited (Broad Oak Rd, Sittingbourne, Kent ME9 8AQ, United Kingdom).

A monoclonal antibody designated $\alpha HT+N_1$ was prepared in our laboratory and found to be specific for the amino-terminal lobe of transferrin. Another antibody, designated E-8, specific to the C-terminal domain of transferrin was also used in these studies. A complete description of these antibodies is given elsewhere (Mason & Woodworth, 1991). Neither of these antibodies recognizes bovine transferrin.

Methods

Isolation of Human Serum Transferrin cDNA. The coding sequence for human serum transferrin was assembled from restriction enzyme digestion fragments derived from a full-length cDNA clone isolated from a human liver library that was kindly provided by Dr. Stuart Orkin of Harvard University (Funk et al., 1990). Since the parental plasmid (pKT-218) of the original clone had a limited number of unique restriction enzyme recognition sites, a series of cloning steps were required to introduce the coding sequence into a convenient vector. This process was initiated by cloning a *Hpa*II/*Bam*HI fragment from the 5′ end of the cDNA into the vector pUC 18 (Messing, 1983). The resulting plasmid was digested with *Bam*HI and *Hin*dIII, and a *Bam*HI/*Hin*dIII fragment from the human transferrin cDNA was cloned adjacent to the initial fragment. This plasmid was then digested with *Hin*dIII and *Pst*I, and a final *Hin*dIII/*Pst*I fragment from the 3′ end of the transferrin cDNA was cloned to complete the assembly of the full-length coding sequence. Digestion of the resulting plasmid with *Sac*I and *Sph*I released the full-length coding sequence as a single restriction fragment which was subsequently made blunt using T4 DNA polymerase and dNTPs and then cloned into the large *Sma*I fragment of pNUT (Palmiter et al., 1987). Plasmid DNA was prepared from *Escherichia coli* JM105 and purified by two successive centrifugation steps with cesium chloride gradients.

Removal of Glycosylation Sites from hTf. Human serum transferrin contains two N-linked oligosaccharides, at Asn-413 and Asn-611 (MacGillivray et al., 1982), corresponding to AAT and AAC codons in the cDNA sequence, respectively (Yang et al., 1984). These codons were converted to GAT and GAC by oligonucleotide-directed mutagenesis using the dut^- and ung^- method (Kunkel, 1985). The mutagenic oligonucleotides:

5′-GCAGAAAACTACGATAAGAGCGATAAT-3′

5′-CTATTTGGAAGCGACGTAACTGACTGC-3′

(the mutated codons are underlined) were synthesized on an Applied Biosystems 391 DNA synthesizer, and were purified by reverse-phase chromatography using a SEP-PAK (Waters) column (Atkinson & Smith, 1984).

The template for the mutagenesis was a plasmid containing the DNA coding sequence for the C-lobe of transferrin cloned into pUC named pUC2-3; as shown in Figure 1, this plasmid contains a *Not*I site in the interlobe bridge coding region and a *Sma*I site in both the 5′ and 3′ untranslated regions (K. Savage, unpublished results). Each of the two mutagenic oligonucleotides was used separately to introduce the desired mutations into pUC2-3; the resulting plasmids were Tf-N413D and Tf-N611D (see Figure 1). The presence of the mutated codons was confirmed by DNA sequence analysis (Sanger et al., 1977). Each plasmid was cut with *Acc*I and *Stu*I, the DNA fragments were separated by agarose gel electrophoresis, and the fragments containing the mutated residues were recovered from gel slices using GENECLEAN (Bio101, La Jolla, CA). The fragments were then ligated back into the *Acc*I site of the full-length transferrin cDNA clone in pUC19. The structure of the final construction, hTf(N/D), was confirmed by restriction mapping and DNA sequence analysis. The transferrin cDNA was then released with *Sac*I and *Sph*I; the ends were made blunt by treatment with the Klenow fragment of DNA polymerase I in the presence of dNTPS and ligated directly into pNUT restricted with *Sma*I (Palmiter et al., 1987). The correct orientation of the pNUT-hTf(N/D) clone was confirmed by restriction-endonuclease mapping.

Expression Vector and Cell Culture. Baby hamster kidney cells were grown in DMEM–F-12 medium with 10% fetal bovine serum to approximately 10^7 cells per 100-mm dish and then transfected with 10 μg of plasmid by the calcium phosphate coprecipitation technique described by Searle et al. (1985). After 24 h, the medium was changed to DMEM–F-12 containing 500 μM methotrexate to select the plasmid-containing cells. Once selected, the cells were passaged serially as described below. Frozen stocks of BHK cells transformed by the pNUT-hTf plasmid were stored in 95% fetal calf serum and 5% dimethyl sulfoxide (DMSO) in a liquid nitrogen cryostat. Frozen cells ($\sim 1 \times 10^6$) were initially grown in DMEM–F-12 containing 5% newborn calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Metho-

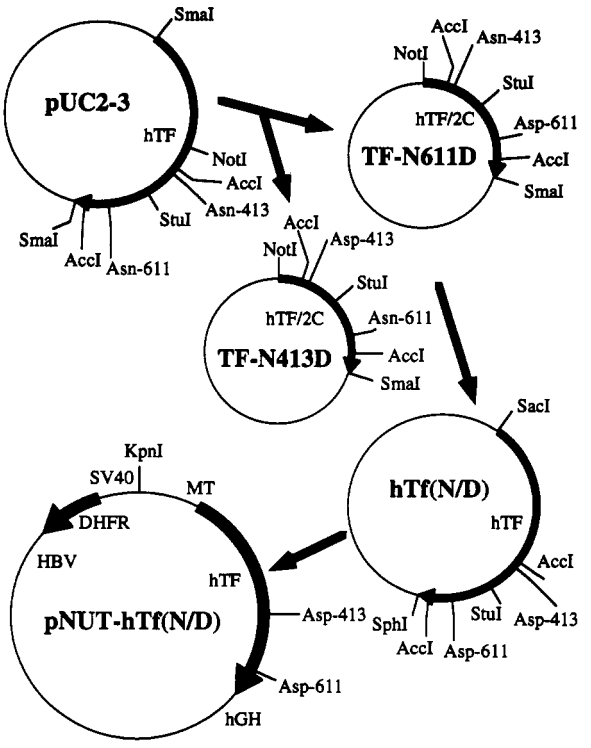

FIGURE 1: Construction of the hTf N413D/N611D expression vector in pNUT. Using a plasmid called pUC2-3 which contains the DNA coding region for the C-terminal lobe of hTf, each of the two mutagenic oligonucleotides described under Methods was used separately to introduce the desired mutations. The two resulting plasmids, Tf-N413D and Tf-N611D, were cut with *Acc*I and *Stu*I; the DNA fragments containing the mutated residues were removed from agarose gel slices and ligated into the *Acc*I site of a full-length Tf cDNA clone in pUC19 to give hTf(N/D). This plasmid was cleaved with *Sac*I and *Sph*I, the ends were made blunt, and the fragment was cloned into the *Sma*I site of pNUT to give pNUT-hTf(N/D). In this plasmid, the cDNA is under the control of the metallothionein promoter (MT) and the human growth hormone transcription termination signals (hGH). pNUT also contains the SV40 early promoter (SV40) driving expression of a mutated form of the dihydrofolate reductase (DHFR) cDNA using transcription termination signals from human hepatitis B virus (HBV).

trexate (500 μM) was used routinely in all cultures from which cells were harvested for frozen stocks, but methotrexate was absent from cells that were expanded for mass culture. In a typical experiment, cells were passed at ~80% confluency using trypsin/EDTA. Sequential passage of the original cells was to five 100-mm dishes (10-mL volume), then to five T-175 flasks (30-mL volume), and finally to five expanded-surface roller bottles (200-mL volume). At the T-175 passage, a serum substitute, Ultroser G, at a level of 1%, was used instead of 5% fetal calf serum in DMEM–F-12 lacking phenol red. Cells in the roller bottles were kept in a Bellco cell production roller apparatus set at a speed of 1 rpm. Details of cell culture and maintenance have been described previously (Mason et al., 1991).

Isolation and Characterization of Recombinant hTf. Once production levels were high, medium without Ultroser G could sustain production of recombinant protein for at least two more changes of medium. This greatly simplified the isolation of the expressed full-length recombinant human serum transferrin. To isolate the recombinant protein, harvested culture medium was made 0.01% with respect to phenylmethanesulfonyl fluoride and sodium azide to inhibit proteases and bacterial growth, respectively. Sufficient $Fe(NTA)_2$ was added to saturate the transferrin present. The medium was reduced in volume to <10 mL with a spiral cartridge, then a 400-mL stirred cell, a 50-mL stirred cell, and finally two Centricon 30 microconcentrators. Often a centrifugation step (10000*g* for 15 min) was necessary to clarify the medium. The transferrin was purified by passage over an anion-exchange column (Polyanion SI, 1 × 10 cm) as described for the recombinant amino-terminal human transferrin half-molecule (Funk et al., 1990; Mason et al., 1991).

$NaDodSO_4$–PAGE (12% separating gel with 4% stacker) was performed using the Laemmli buffer system (Laemmli, 1970) and the mini-Protean II slab cell apparatus from Bio-Rad. Gels were cast and run according to the manufacturer's instructions. The urea gel procedure was adapted and modified to allow use of the mini-gel apparatus. The separating gel was made exactly as described previously (Brown-Mason & Woodworth, 1984). Use of a discontinuous stacking gel of 13 mM Tris-HCl, pH 6.8, and 3.9% acrylamide/0.1% bis(acrylamide) improved resolution of the diferric species. Gels were run at 200 V for 1–1.5 h.

Visible absorption spectra were recorded on a Cary 219 spectrophotometer using 0.1 M KCl as the reference. The molecular weight of human serum Tf containing two biantennary glycans is 79 555 (see below). The absorbance at 280 nm of a solution containing 1 mg/mL protein was taken as 1.09 for apo-hTf and 1.40 for diferric hTf (Harris, 1977). Millimolar extinction coefficients at 280 nm are therefore 86.7 and 111.4 cm^{-1} mM^{-1} for these same proteins. The extinction coefficient calculated for apo-hTf by the method of Gill and von Hippel (1989) is within 4% of the value above.

Electrospray Mass Spectrometry Analysis. The samples were analyzed on a VG Quattro BQ mass spectrometer (VG Biotech Ltd., Altrincham, U.K.). This instrument consists of an electrostatic spray ion source operating at atmospheric pressure followed by a quadrupole mass analyzer with a mass range for singly charged ions of 4000. The hTf samples were dissolved in 50:50 (v/v) acetonitrile/water containing 0.5% formic acid at a concentration of 5 pmol/μL. Aliquots of 10 μL of each solution were injected into a stream of 50:50 (v/v) acetonitrile/water flowing at 5 μL/min into the spectrometer source. Data were acquired over the m/z range 1900–2700 at 10 s/scan with the data system operating as a multichannel analyzer. Data were summed from 5–10 injections of sample over a period of 10–20 min. Mass measurements were made from data transformed onto a true molecular mass scale using the software supplied with the instrument. Calibration employed the multiply charged ion series from a separate introduction of bovine trypsinogen (M_r = 23 980.9).

Radioimmunoassay for Recombinant HTf. A competitive solid-phase immunoassay was used to determine the concentration of recombinant hTf in the culture medium and at various stages of the purification (Mason & Woodworth, 1991). Briefly, 1 μg of rabbit anti-mouse immunoglobulin G in 100 μL of 14 mM Na_2CO_3/34 nM $NaHCO_3$ was added to each Removawell and incubated overnight at 4 °C. The wells were washed with 3 × 200 μL of assay buffer (50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl, 0.02% NaN_3, and 0.1% bovine serum albumin). An appropriate dilution (1:8300 in assay buffer) of the monoclonal antibody, αHT+N1, or E-8 in a volume of 200 μL was added to all wells except those used to determine background. After incubation for 1–1.5 h at 37 °C, each well was washed with 3 × 200 μL of assay buffer. Boehringer Mannheim diferric hTf radioiodinated with Iodogen (Mason & Woodworth, 1991) was added at a level of 5 ng/200 μL in the presence or absence of unlabeled standards and samples. A standard curve was generated by competition of diferric ^{125}I-hTf with 16–400 ng/well of

unlabeled hTf. After incubation for 1–2 h at 37 °C, the wells were washed as above, separated, placed into Omnivials, and assayed for radioactivity in a Packard Auto γ 500 scintillation counter.

Radioiodination of Human TF for Cell-Binding Studies. For cell-binding studies, 1 mg of each transferrin was iodinated using the McFarlane procedure (McFarlane, 1963). After incubation of 10 μL of ICl with 300 μCi of $Na^{125}I$ for 5 min at room temperature, the protein in <100 μL of alkaline glycine buffer was added and incubated for an additional 10 min. After being quenched with 5 μL of saturated tyrosine solution, the sample was quantitatively transferred to a 1-mL column (diposable syringe with a glass wool plug) of Bio-Rad P6DG desalting resin which had been centrifuged for 4 min in a clinical centrifuge. Another 4-min centrifugation resulted in complete separation of the radiolabeled sample from the free iodine. The iodinated protein was subjected to trichloroacetic acid precipitation to assure that the associated radioiodine was covalently bound (Goding, 1986). The protein was then exchanged into 20 mM Hepes, pH 7.4, containing 0.15 M NaCl and stored frozen. Typically ~80% incorporation of the initial radioiodine was obtained with greater than 98% covalently incorporated. The specific activity was ~20 000 cpm/pmol.

Cell-Binding Experiments. HeLa S_3 cells were the generous gift of Dr. Joan Moehring (Department of Microbiology, University of Vermont College of Medicine). Cells were routinely grown in DMEM–F-12 containing 10% newborn calf serum. Prior to beginning a binding experiment, the cells were harvested with Versene, and taken up in Joklik's minimum essential medium–20 mM Hepes–2% BSA (JMEM–BSA). Endogenous bovine transferrin was removed from the HeLa cells by incubation for 10 min at 37 °C at a 5-fold dilution with JMEM–BSA. After centrifugation of the cells and removal of the supernatant, this procedure was repeated twice. The cells were then incubated for an additional 10 min in the presence of 10 mM NH_4Cl to inhibit the removal of iron from transferrin (Morgan, 1981; Harding & Stahl, 1983; Rao et al, 1983; Klausner et al., 1983; Mason et al., 1987). Removal of the endogenous transferrin is somewhat superfluous since bovine transferrin has a very low affinity for human receptors and would not effectively compete with human transferrin in the binding studies (Penhallow et al., 1986). For each diferric hTf sample to be tested, cell suspensions (300 μL containing ~2.2 × 10^6 cells) were added to eight different Omnivials containing between 3 and 80 pmol of radiolabeled diferric transferrin. An identical set of vials was set up containing a 100-fold excess of unlabeled Boehringer Mannheim diferric transferrin to determine the amount of nonspecific binding. After 30 min of incubation at 37 °C with gentle shaking, portions of the cell suspension (3 × 100 μL) were pipetted into microfuge tubes containing 0.9 μL of ice-cold JMEM–BSA over 300 μL of dibutyl phthalate and centrifuged for 2 min in a Beckman microfuge. The aqueous and organic phases were aspirated to just above the cell pellet. The bottom of the tube containing the cell pellet was released by a hot wire into a plastic tube (12 × 75 mm) and assayed for radioactivity. A second approach involved competing six different amounts (4–120 pmol) of each of the different hTf samples against a constant amount (6.4 pmol) of radioiodinated Boehringer Mannheim hTf. The program Ligand was used to analyze the data from both types of experiment assuming a single class of binding sites in each case (Munson & Rodbard, 1980).

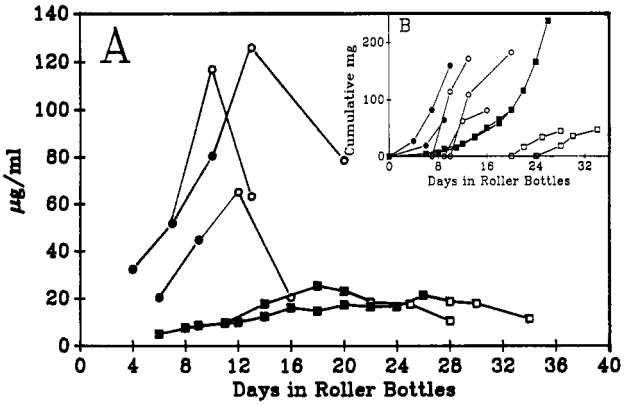

FIGURE 2: Production level, expressed as micrograms per milliliter (A) and as total cumulative milligrams collected (inset B), of rhTf (●) and rhTf N411D/N611D mutant (■) by BHK cells in continuous production in five expanded-surface roller bottles. On the days indicated, 1000 mL of DMEM–F-12–1% Ultroser G medium was collected and assayed for recombinant protein. The open symbols indicate the batches which were collected in the absence of Ultroser G.

RESULTS

Isolation of Human TF cDNA. As previously described (Funk et al., 1990), a human liver cDNA library was screened using a 24-base oligonucleotide to the 5′ sequence of the human TF cDNA as a probe. A single positive colony was found. Extensive restriction enzyme mapping of the plasmid isolated from the clone agreed with the patterns predicted from the human TF cDNA isolated from the same library by Yang et al. (1984). DNA sequence analysis of the 5′ and 3′ termini of the clone showed that it was identical to the full-length clone isolated by Yang et al.

Vector Construction and Expression. The expression vector pNUT has already been described thoroughly (Funk et al., 1990). As detailed under Methods, a *SacI*/*SphI* fragment which contained the full-length coding sequence as a single restriction fragment was first made blunt and then cloned into the *SmaI* site of pNUT. In addition, the full-length transferrin coding sequences in which the codons for asparagine residues at positions 413 and 611 were converted to those for aspartic acid residues were made as shown in Figure 1. Both plasmids were transfected separately into BHK cells, and the resulting transformants were selected using 500 μM MTX. The entire population of cells which survived the selection was taken. In previous attempts at clonal selection, it was found that cells which had higher levels of expression divided much more slowly than cells with lower levels of expression. Thus, there was no particular advantage to clonal selection in terms of overall production of protein in a given amount of time.

BHK cells containing the plasmids were each cultured in five expanded-surface roller bottles. Three different production runs for the recombinant glycosylated hTf (rhTf) and two for the deglycosylated hTf N413D/N611D mutant are shown in Figure 2. Levels of expression of the rhTf were as high as 126 μg/mL in the initial runs (two top curves), falling to approximately 65 μg/mL by the third run. Levels of expression of the N413D/N611D mutant were more modest, reaching a maximum of 25 μg/mL. The final yield of rhTf in the presence of Ultroser G was approximately 80, 60, and 64 mg for the three runs shown; rhTf in the absence of Ultroser G gave 170, 180, and 80 mg. The N413D/N611D mutant with Ultroser G yielded 235 and 80 mg for the two production runs shown and 45 mg for each of the collections without

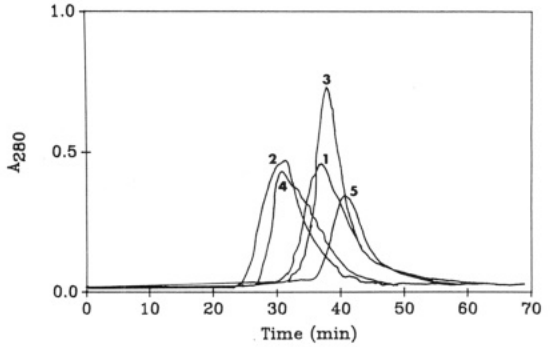

FIGURE 3: Chromatograms of the five human transferrin samples run on a Polyanion SI column. Samples are (1) Boehringer Mannheim Fe_2hTf, (2) Sigma Fe_2hTf, (3) Scipac Fe_2hTf, (4) recombinant Fe_2hTf, and (5) recombinant Fe_2hTf N413D/N611D mutant. Approximately 8 A_{280} units of each Tf was run.

Ultroser G. Both recombinant proteins were equally well recognized by the two monoclonal antibodies $\alpha HT+N_1$ and E-8 which are specific to the N- and C-terminal domains, respectively.

To date, we have been unable to separate reliably the rhTf from the human serum TF present in the serum replacement Ultroser G which, at a level of 1%, contains 2.8 μg/mL ($\pm$0.6, n = 10). This amount of hTf is insignificant when the expression level of rhTf is high, but is an important consideration when the levels are lower, as with the rhTf N413D/N611D mutant. To assure that there was no interference of the hTf in the medium with the rhTf, the cells were grown for two or three medium changes in the absence of Ultroser G (open symbols in Figure 2). Survival of the BHK cells for several days probably indicates that the rhTf being secreted into the medium is providing the critical function of iron delivery to the cells. Each batch (1 L) of harvested tissue culture medium was collected, reduced to a small volume as described under Methods, and exchanged into 50 mM Tris-HCl, pH 8.0. The sample was chromatographed on a Polyanion SI column developed with a 0–0.3 M NaCl gradient over 60 min. Both glycosylated rhTf and nonglycosylated rhTf are separated in pure form from one other major protein, which does not elute from the column until application of 1 M NaCl. Approximately 60% of the rhTf and rhTf(N413D/N611D) measured by radioimmunoassay in the original medium was recovered. To illustrate the rather striking differences in the elution profiles, both purified rhTf samples and three commercially available human serum transferrins were each run sequentially on the Polyanion SI column under identical conditions (Figure 3). Sigma hTf (peak 2) and the rhTf (peak 4) eluted at approximately 0.15 M NaCl, Scipac (peak 3) and Boehringer Mannheim hTf (peak 1) eluted at approximately 0.19 NaCl, and rhTf N413D/N611D (peak 5) eluted at approximately 0.21 M NaCl. The Sigma and rhTf samples were the most heterogeneous; the Scipac and N413D/N611D mutant gave the sharpest and most symmetrical elution profiles.

After anion-exchange chromatography, the samples were pooled, reduced in volume, and exchanged into 0.1 M KCl using Centricon 30 microconcentrators. Spectra were obtained for each sample; the spectral ratios are presented in Table I. These agree reasonably well with previously reported values (Harris, 1977) and indicate that all of the samples were correctly folded and able to bind two iron atoms. In addition, aliquots of each hTf were analyzed by SDS–PAGE, urea–PAGE, and electrospray mass spectrometry. SDS–PAGE (Figure 4A) shows that the samples are all reasonably

Table I: Spectral Ratios of the Various Human Serum Transferrins

Fe_2hTf source	A_{280}/A_{465}	A_{465}/A_{405a}
Boehringer Mannheim	21.4	1.38
Sigma	21.6	1.32
Scipac	21.4	1.37
recombinant glycosylated	20.1	1.40
recombinant nonglycosylated	20.4	1.35

[a] These ratios were obtained by using the apoprotein as a reference for the iron-containing protein as described previously (Woodworth et al., 1991).

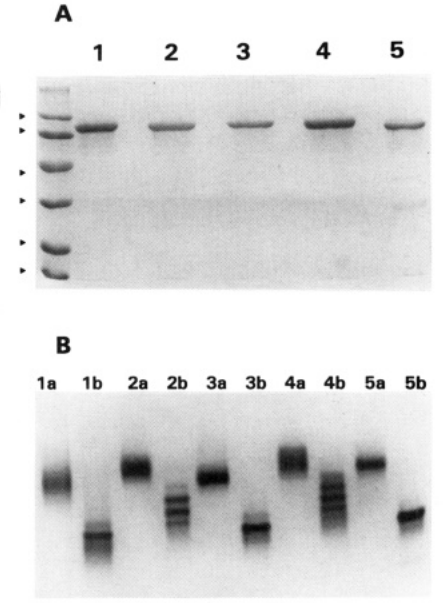

FIGURE 4: $NaDodSO_4$–PAGE of the five transferrin samples run under reducing conditions and visualized with Coomassie blue (A). Samples are (1) Boehringer Mannheim hTf, (2) Sigma hTf, (3) Scipac hTf, (4) recombinant hTf, and (5) recombinant hTf N413D/N611D mutant. Bio-Rad low molecular mass standards are shown in the lane to the left of the first sample. Molecular masses, top to bottom, are 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa. (B) Urea-PAGE of the five transferrin samples under nonreducing conditions and visualized with Coomassie blue. Samples are as above except that the apoprotein is shown in the a lanes and the diferric protein is shown in the b lanes. Approximately 5 μg of each sample was run.

monodisperse and of approximately the same molecular weight, except for the rhTf(N413D/N611D) mutant, which migrates slightly faster than the other hTf samples. This result is consistent with our previous work (Mason & Woodworth, 1991) in which hTf treated with *N*-glycanase migrated ahead of untreated hTf. Urea gels (Figure 4B) are traditionally used to determine the iron status of transferrins (Makey & Seal, 1976). Binding of iron results in both charge differences and an increase in resistance to denaturation in the presence of 6 M urea. Addition of a discontinuous stacker to the usual Makey–Seal gel resulted in added resolution, particularly of the fully iron-saturated proteins (Figure 4B). As with the anion-exchange chromatography, the largest amount of heterogeneity is observed with the glycosylated Fe_2rhTf (lane 4B) and the Sigma Fe_2HTf (lane 2b) samples. The least heterogeneity is observed with the recombinant nonglycosylated sample (lane 5b). Intermediate heterogeneity is observed with the Boehringer Mannheim (lane 1b) and Scipac Fe_2hTf samples (lane 3b). The urea gel results confirm that all of the hTf samples were fully iron-saturated.

A rational explanation for the urea gel results is provided by electrospray mass spectrometry analysis as shown in Table II. The theoretical mass derives from the calculated mass of 75 143.13 Da for the protein (Yang et al., 1984) and of 4412.16

Table II: Electrospray Mass Analysis of the Various Human Serum Transferrins

transferrin	theoretical mass[a] (Da)	experimental mass[b] (Da)
Boehringer Mannheim	79 555	79 619
Sigma	79 555	79 571
Scipac	79 555	79 559
single individual	79 555	79 552
recombinant glycosylated	79 555	78 833–80 802[c]
recombinant nonglycosylated	75 143	75 146

[a] The theoretical mass derives from a calculated mass of 75143.13 Da for the protein (Yang et al., 1984) and of 4412.16 Da for two biantennary glycans (Spik et al., 1988). [b] See Results for further details about these findings. [c] The recombinant glycosylated hTf gave a complex pattern of 16 peaks with masses in the range shown.

Da for the two biantennary glycans (Spik et al., 1988). The Boehringer Mannheim and Sigma samples had broad peaks with some evidence (no distinct peaks) of the presence of higher molecular mass species. The Scipac hTf and the hTf from a single individual had sharp molecular mass peaks with some evidence of the presence of higher molecular mass species. The recombinant glycosylated hTf gave a complex pattern of 16 individual peaks with masses in the range shown in Table II. The recombinant glycosylated sample was separated into five distinct peaks following chromatography on the Polyanion SI using a shallow salt gradient (0–0.15 M NaCl over 60 min) (data not shown). ESMS analysis revealed that each of these samples consisted of 3 or 4 species adding up to the 16 peaks observed in the original sample. In addition, a second sample gave a totally different but equally complex pattern. In contrast to these results, the nonglycosylated recombinant hTf gave a sharp peak at the molecular mass shown, followed by three further sharp peaks corresponding to the sulfated adduct moieties at mass + 98, mass + (2 × 98), and mass + (3 × 98).

Cell-Binding Studies. In order to test the functional integrity of the five different hTf samples, equilibrium binding studies were undertaken using two different approaches. First, each hTf sample was radioiodinated, and direct binding to HeLa S_3 cells was measured in the presence and absence of a 100-fold excess of unlabeled Boehringer Mannheim Fe_2hTf. In all instances, the amount of nonspecific binding was very low, less than 5% of the specific binding. The data from the equilibrium binding experiment were analyzed by the nonlinear curve-fitting program of Munson and Rodbard to determine the affinity and binding site number for each TF (Munson & Rodbard, 1980). A typical data set is presented in Table IIIA. The results show that all of the transferrins bound with approximately the same affinity and to the same extent.

The second approach involved competing different amounts of each of the transferrins (unlabeled) against a constant amount of radioiodinated Boehringer Mannheim diferric hTf. The results of a typical data set from this approach are presented in Table IIIB. The two experiments shown were done on different days which probably accounts for the difference in the number of binding sites per cell observed. In many experiments over a number of years (Penhallow et al., 1986), we have found between 0.8 and 2 × 10^6 sites/cell, which probably reflects the metabolic state of the cells. We conclude that binding of the recombinant Tf samples is indistinguishable from binding of the commercially available Tf samples.

Table III [a]

transferrin	K_d' [b] (nM)	TF/cell[c] × 10^{-6}	N[d]
(A) Results of Equilibrium Binding Experiments in which Binding of Radioiodinated Diferric Transferrin to HeLa S_3 Cells Was Directly Measured			
recombinant glycosylated	31.3 ± 3.6	2.09 ± 0.14	0.004 ± 0.003
recombinant nonglycosylated	23.4 ± 2.5	1.96 ± 0.13	0.013 ± 0.003
Boehringer Mannheim	17.8 ± 2.3	1.31 ± 0.08	0.019 ± 0.003
Sigma	19.9 ± 1.5	1.76 ± 0.14	0.009 ± 0.004
Scipac	22.5 ± 2.9	1.76 ± 0.09	0.008 ± 0.002
(B) Results of Equilibrium Binding Experiments in which Six Different Amounts of Unlabeled Transferrin Were Competed against a Constant Amount of Boehringer Mannheim Radioiodinated Transferrin			
recombinant glycosylated	22.6 ± 2.4	0.99 ± 0.03	0
recombinant nonglycosylated	19.8 ± 7.4	0.91 ± 0.07	0
Boehringer Mannheim	29.7 ± 1.6	1.00 ± 0.10	0.015 ± 0.006
Sigma	19.6 ± 9.7	0.79 ± 0.07	0.018 ± 0.005
Scipac	30.0 ± 1.8	1.04 ± 0.10	0.013 ± 0.006

[a] In (A), 7482 cpm bound (3.28 × 10^5 TF/cell) in the absence of competitor. A total of 44 cpm bound in the presence of 100-fold excess of unlabeled competitor. [b] In both experiments, K_d' denotes the apparent equilibrium binding constant. [c] TF/cell denotes the number of TF molecules bound per cell. [d] N denotes the ratio of nonspecifically bound to free transferrin.

DISCUSSION

Using a molecular biological approach, we have successfully produced high levels of recombinant human serum transferrin which appears to behave like hTf isolated from serum as judged by a number of independent criteria. These include size, immunoreactivity, spectral ratios (indicative of iron binding), and ability to bind to TF receptors on HeLa S_3 cells. We have also produced recombinant human serum transferrin in which the asparagine residues at positions 413 and 611 have been mutated to aspartic acid residues, thereby blocking carbohydrate attachment. The nonglycosylated mutant is slightly smaller in size, shows immunoreactivity similar to the unmutated Tf, has similar spectral ratios, and binds to HeLa cell receptors in a manner which is indistinguishable from the rhTf and three commercial hTf samples.

The amount of recombinant protein produced, 125 mg/L for the rhTf and 25 mg/L for the N413D/N611D mutant, compares favorably with amounts reported in the literature. Thus, lactoferrin is expressed at 20 mg/L by BHK cells (Stowell et al., 1991) and at 5–25 mg/L by the filamentous fungus *Aspergillus oryzae* (Ward et al., 1992). It is obvious that the mutant is produced at far lower levels than the recombinant glycosylated hTf. In our experience with expression of the amino-terminal half-molecule of human Tf, production levels of all mutants are always lower (Woodworth et al., 1991). Furthermore, the high level realized for rhTf in the first and second runs was not repeated in the third run. This trend has also been found in the production of hTf/2N. Expression levels are highest post-transfection and tend to fall with subsequent passages and especially freezing and thawing of the plasmid-containing BHK cells. Although all of the factors involved in the decrease are not well understood, we have recently found that at least part of the problem derives from releasing the adherent cells using only Versene, i.e., EDTA. This strategy results in cells which are increasingly less adherent. As noted previously (Mason et al., 1991), the main reason for the fall in expression level over time appeared to be a loss of cell mass from the roller bottle surface. After we switched to a dilute trypsin solution to harvest cells, expression levels have increased and remained higher for a long period of time.

Although there are two reports of human Tf being successfully expressed in *E. coli* (Hershberger et al., 1991; Ikeda et al., 1992), the ability of these proteins to reversibly bind iron was not demonstrated. In the study of Hershberger et al., the isolated recombinant Tf was refolded by air oxidation of the protein in pH 10.5 glycine buffer containing cysteine. A "competitive receptor binding assay" showed a 1.2% recovery of activity with the rhTf after folding. Quantitative data on recombinant protein production levels are not provided in either study.

The BHK cell line we used had been transformed by polyoma virus. Membrane proteins from this particular transformed cell line differ from those on untransformed BHK cells because they contain fucose residues, have more sialic acid residues, and have more highly branched carbohydrate chains (Ogata et al., 1976; Takasaki et al., 1980). The more complex carbohydrate has been attributed to increased levels of various enzymes involved in carbohydrate metabolism (Yamashita et al., 1985). A general feature of recombinant proteins expressed in mammalian cells seems to be a more complex carbohydrate pattern than is found in the naturally occurring proteins (Tsuda et al., 1988; Aeed et al., 1992). It has also been shown that there are growth-associated changes in the glycosylation of hTf secreted by HepG2 cells (Hahn & Goochee, 1992). It is thus not surprising that the expressed recombinant hTf has a complex carbohydrate pattern with more highly branched chains and that different batches of cells give rise to rhTf with different glycans.

Electrospray mass spectrometry showed that the serum transferrin from Scipac and that from a single individual consisted predominantly of a single molecular mass which corresponds, within experimental error, to the mass calculated from the published protein (Yang et al., 1984) and glycan structures (Spik et al., 1988). The heterogeneity observed in the Boehringer Mannheim and Sigma hTf samples (see Table II) may be traceable to the fact that the serum used was from a pool of individuals, each with his/her own characteristic glycan pattern. Alternatively, the methods used to purify the hTf could contribute to the differences. The urea gel with a discontinuous stacker gives a fast and simple way to assess the carbohydrate complexity. It is evident from these analytical studies that ESMS provides a faster and less tedious (though less detailed) approach to dissecting carbohydrate compositions than those methods conventionally used. For example, a recent study used a combination of serial lectin affinity chromatography, fast atom bombardment mass spectrometry, and ^{1}H nuclear magnetic resonance spectroscopy to map completely the N-glycosylation sites of hTf (Fu & van Halbeek, 1992). This approach yielded information about linkage patterns in addition to indicating the number and type of individual oligosaccharides. The study confirms earlier work (Marz et al., 1982) which indicated that the microheterogenity of hTf glycans is more extensive than previously realized. A renewed interest in the glycan composition of hTf derives from its possible use as a marker for alcohol abuse (Stibler, 1991) and hepatocellular carcinoma (Yamashita et al., 1989). ESMS may be adequate for these clinical determinations.

Spik et al. (1988) were unable to find a role for carbohydrate on hTf. They reported that complete deglycosylation had no effect on iron binding, recognition by reticulocytes, or iron transport into the cell, although no data were shown. This observation has also been made by another group (Hershberger et al., 1991), although again no data were shown. Our work quantitatively confirms this finding; we see no apparent differences in the avidity or extent of binding to receptor whether the hTf has (1) highly complex carbohydrate or (2) no carbohydrate at all. No appreciable differences are seen in the binding to HeLa cells of the three commerical Tf preparations which also show large variations in their glycosylation patterns. Furthermore, the deglycosylated hTf produced using a molecular biological approach is extremely pure and not subject to the usual concerns accompanying enzymatic removal of carbohydrate.

The detailed studies by Regoeczi and his co-workers on glycan variants of rat Tf showed that certain changes including deletion of the glycan alter its half-life in the circulation and affect iron transport in vivo (Marz et al., 1982; Regoeczi et al., 1987; Hu et al., 1992a,b). Future studies must include assessment of the in vivo behavior of the nonglycosylated hTf and use of radiolabeled iron to assess the ability of the recombinant transferrins to donate iron to cells.

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