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Expression of the Amino-Terminal Half-Molecule of Human Serum Transferrin in Cultured Cells and Characterization of the Recombinant Protein†

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ABSTRACT: A human liver cDNA library was screened with a synthetic oligonucleotide, complementary to the 5′ region of human transferrin mRNA, as a hybridization probe. The full-length human cDNA clone isolated from this screen contained part of the 5′ untranslated region, the complete coding region for the signal peptide and the two lobes of transferrin, the 3′ untranslated region, and a poly(A) tail. By use of oligonucleotide-directed mutagenesis in vitro, two translational stop codons and a *Hin*dIII site were introduced after the codon for Asp-337. This fragment was inserted into two different expression vectors that were then introduced into *Escherichia coli*. As judged by NaDodSO$_4$–polyacrylamide gel electrophoresis and Western blot analysis, however, recombinant hTF/2N was undetectable in bacteria transformed by these plasmids. Concurrently, we developed a plasmid vector for the expression of recombinant hTF/2N in eukaryotic cells. In this case, a DNA fragment coding for the natural signal sequence, the hTF/2N lobe, and the two stop codons was cloned into the expression vector pNUT, such that the expression of hTF/2N was controlled by the mouse metallothionein promoter and the human growth hormone termination sequences. Baby hamster kidney cells containing this hTF/2N-pNUT plasmid secreted up to 20 mg of recombinant hTF/2N per liter of tissue culture medium. Recombinant hTF/2N was purified from the medium by successive chromatography steps on DEAE-Sephacel, Sephadex G-75, and FPLC on Polyanion SI. The purified protein was characterized by NaDodSO$_4$–PAGE, urea–PAGE, amino-terminal sequence analysis, UV–visible spectroscopy, iron-binding titration, and proton NMR. By these criteria, the recombinant hTF/2N appeared to behave identically with the proteolytically derived half-molecule, but to show a higher degree of monodispersity than the latter protein. Addition of *m*-fluorotyrosine to the culture medium resulted in random incorporation of this amino acid into cellular protein in lieu of tyrosine. Purified recombinant ^{19}F-Tyr hTF/2N gave four well-resolved ^{19}F NMR resonances of 20–40 Hz line width, two with suggestions of shoulders.

The iron-binding pseudoglobulins collectively called transferrins or siderophilins comprise a class of proteins with strikingly similar features. X-ray crystallographic analyses of human lactoferrin (Anderson et al., 1987) and rabbit serum transferrin (Bailey et al., 1988) reveal that these proteins consist of two similar lobes connected by a short bridging peptide and that each lobe contains two domains defining a deep cleft containing the binding site for a metal ion and a synergistic anion. To date, the amino acid sequences for five transferrins have been reported (Jeltsch & Chambon, 1982; MacGillivray et al., 1983; Metz-Boutigue et al., 1984; Rose et al., 1986; Baldwin & Weinstock, 1988), and comparisons reveal 35–75% sequence identity among the proteins and between the two lobes of a given transferrin. Highly conserved residues have been implicated previously in the binding of metal ions and synergistic anions. Most previous physicochemical studies are consistent with the structures determined by X-ray crystallography; however, some aspects of the proposed iron-binding sites require further clarification. For instance, nuclear magnetic resonance (NMR)[1] titration studies

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[1] Abbreviations: hTF, human serum transferrin; hTF/2N, amino-terminal half-molecule of hTF; hGH, human growth hormone; BHK, cultured baby hamster kidney cells; DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified essential medium; FPLC, fast protein liquid chromatography; MTX, methotrexate; NMR, nuclear magnetic resonance; NTA, nitrilotriacetate; PAGE, polyacrylamide gel electrophoresis.

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of apo and Ga(III) complexes of ovotransferrin and its two half-molecules (Woodworth et al., 1987) and of the amino-terminal half-molecule of human serum transferrin (Valcour & Woodworth, 1987) implicated two histidyl side chains as ligands to the bound metal, whereas the X-ray crystal structures show a single histidyl side chain, two tyrosyl side chains, and an aspartyl side chain as ligands to bound Fe(III) (Anderson et al., 1987; Bailey et al., 1988).

Partial chemical dissection of the aromatic region of the proton NMR spectrum of Japanese quail lysozyme has been achieved by feeding a defined diet containing side chain perdeuterated histidine, phenylalanine, and tryptophan and [3,5-$^2H_2$]tyrosine. Incorporation of deuterated amino acids into egg white protein reached 90% within 5 days (Brown-Mason et al., 1981). Similar experiments with laying hens resulted in a maximum of 50% incorporation, rendering the ovotransferrin NMR spectra relatively difficult to interpret. To render this biological substitution with isotope-substituted amino acids feasible and more economical of isotope-enriched compounds and to obviate the proteolytic cleavage of holotransferrin to its half-molecules for study by NMR and physiological methodologies, we sought to express the cDNA for the amino-terminal half-molecule of human serum transferrin in transformed cells in culture. This report describes the construction of a vector for this purpose, transformation of baby hamster kidney cells, production in cell cultures of sufficient amounts of recombinant protein for the proposed work, characterization of the recombinant protein, and an initial trial at incorporating *m*-fluorotyrosine into recombinant transferrin. This approach will also allow site-directed mutagenesis experiments aimed at testing current assumptions about the functions of selected amino acid residues at or near the iron-binding site of the protein.

Materials and Methods

Materials

T_4 DNA ligase, DNA polymerase I (Klenow fragment), and T4 polynucleotide kinase were purchased from Pharmacia–PL Biochemicals. Restriction endonucleases were from Pharmacia–PL Biochemicals and Bethesda Research Laboratories. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. Nitrocellulose filters were from Schleicher & Schuell, ^{32}P-labeled nucleotides were from New England Nuclear, goat anti-human transferrin antiserum was from Sigma Chemical Co., formalin-fixed *Staphylococcus aureus* cells were from Bethesda Research Laboratories, the Protoblot immunoscreening detection system was from Promega, the oligonucleotide-directed mutagenesis kit was from Amersham, Dulbecco's modified essential medium and fetal bovine serum were from Gibco, and antihuman transferrin monoclonal antibody HTF-14 was from the Czechoslovakian Academy of Sciences. All other reagents were analytical grade or purer.

Methods

Isolation of Human Serum Transferrin cDNA. A human liver cDNA library constructed in the *Escherichia coli* expression vector pKT-218 (Prochownik et al., 1983) was kindly provided by Dr. Stuart Orkin (Harvard University). The cDNA library was screened by using a synthetic oligonucleotide coding for the amino-terminal eight amino acids of serum hTF as a hybridization probe. The oligonucleotide corresponded to nucleotides 88–111 of the human TF cDNA sequence reported by Yang et al. (1984). The oligonucleotide was end-labeled with T4 polynucleotide kinase and [^{32}P]ATP (Chaconas & van de Sande, 1980) and used to screen approximately 10^5 colonies. Restriction endonuclease mapping of positive clones and DNA sequence analysis were performed by using standard procedures with pUC19 and M13mp19 vectors, respectively (Maniatis et al., 1982; Messing, 1983; Sanger et al., 1977).

Expression Vector and Cell Culture. The eukaryotic expression vector pNUT (Palmiter et al., 1987) and baby hamster kidney (BHK) cells were generously provided by Dr. Richard D. Palmiter (Howard Hughes Medical Institute, University of Washington). After synthesis, oligonucleotides were purified on C_{18} reverse-phase columns (Sep-Pak, Waters Associates; Atkinson & Smith, 1984). Site-directed mutagenesis was performed by using the method of Taylor et al. (1985). Plasmid DNA was prepared from *E. coli* JM105 and purified by two successive centrifugation steps with cesium chloride density gradients. BHK cells were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum to approximately 10^7 cells per 10-cm dish and were subsequently 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 containing 100 μM methotrexate (MTX), and surviving cells were serially selected to 500 μM MTX. Large-scale roller bottle cultures were initiated by seeding approximately 5×10^7 cells into each 850 cm^2 roller bottle containing 100 mL of DMEM–MTX. Cultures were induced at 80% confluency by the addition of $ZnSO_4$ to the medium to a final concentration of 0.08 mM. The medium was harvested 40 h later.

Immunoprecipitation and Western Blotting. Immunoprecipitation of cell culture medium and cell lysates was performed by the method of Van Oost et al. (1986). Precipitates were analyzed by electrophoresis on 12% polyacrylamide gels in the presence of $NaDodSO_4$ (Laemmli, 1970), followed by blotting onto a nitrocellulose membrane. The blot was incubated in PBS containing 0.1 mg/mL gelatin, then treated with goat anti-hTF antiserum (250-fold dilution in PBS), and finally developed with an alkaline phophatase conjugated, rabbit anti-goat IgG antibody according to the supplier's instructions.

Amino Acid Substitution. To incorporate 3-fluorotyrosine into the recombinant hTF/2N as a ^{19}F NMR probe, the culture medium was supplemented with DL-*m*-fluorotyrosine (Sigma) at 16% of the concentration of L-tyrosine in the medium. The cells grew as well on this medium as on the medium lacking DL-*m*-fluorotyrosine.

Isolation of Recombinant hTF/2N. Harvested culture medium was made 0.01% in phenylmethanesulfonyl fluoride to inhibit proteases, and sufficient $Fe^{III}(NTA)_2$ was added to saturate all transferrin in the medium. After being stirred at room temperature, the solution was dialyzed for 24 h versus cold running tap water, and then for a few hours versus Milli-Q purified water. Concentrated Tris-HCl buffer, pH 8.4, was added to a final concentration of 5 mM; the preparation was centrifuged to remove any debris and was loaded onto a column (2.5 × 80 cm) of DEAE-Sephacel (Pharmacia) equilibrated with 10 mM Tris-HCl buffer, pH 8.4. The column was eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. Fractions showing a pink color were analyzed by $NaDodSO_4$–PAGE, and fractions containing the recombinant protein (M_r 37 000) were pooled. Such fractions also contained bovine transferrin and albumin resulting from the fetal calf serum in the tissue culture medium. After concentration of the pooled fractions to 5 mL on an Amicon PM-10 membrane, the protein was chromatographed on a column (2.5 × 90 cm) of Sephadex G-75 Superfine (Phar-

macia) equilibrated with 100 mM ammonium bicarbonate. Sometimes, a second chromatographic step through this column was necessary to resolve completely the hTF/2N from the bovine proteins. At this stage, the A_{465}/A_{410} was usually <1.0, indicating the presence of a contaminating heme protein (possibly hemopexin). The hTF/2N was finally purified to homogeneity by FPLC on a column (1 × 10 cm) of Polyanion SI (Pharmacia) using a linear gradient of NaCl (0–0.3 M) in 50 mM Tris-HCl, pH 8.0, over a period of an hour at a flow rate of 1 mL/min. Fractions of 1 mL were collected. Two to four protein bands emerged from the column, depending on the iron-binding status of the protein.

$NaDodSO_4$–PAGE was performed with 5–12% gradient gels, and urea–PAGE was performed according to a modification (Brown-Mason & Woodworth, 1984) of the Makey and Seal (1976) procedure. Electrofocusing was performed on a 0–50% sucrose gradient in a 110-mL glass column (LKB) with 0.8% Pharmalyte, pH 5–8 (Pharmacia). The column was prefocused overnight to a final current of 2 mA at 1000 V. The protein sample in 0.2 mL was diluted with 5 mL of solution withdrawn from the middle of the gradient. The sample was then reinjected into the isodense region of the column, and focusing was continued for 24 h. The gradient was collected from the bottom of the column in 1.5-mL fractions. Individual fractions were analyzed for A_{280} and for pH. Fractions with maximum A_{280} were selected as representing the p*I*'s of the apo- and iron-saturated proteins.

Iron was readily removed from the iron–protein by incubation in a buffer containing 1 mM NTA, 1 mM EDTA, and 0.5 M sodium acetate, pH 4.9. The apoprotein was concentrated to a minimum volume on a Centricon 10 (Amicon) column and then diluted and reconcentrated twice with water and twice with 0.1 N KCl. The apoprotein had a tendency to precipitate in pure water, but redissolved readily in 0.1 M KCl. The apoprotein was made 10 mM in $NaHCO_3$ and titrated with a suitable concentration of $Fe(NTA)_2$ while monitoring the absorbance at 465 nm.

Quantitative Immunoassay of Recombinant hTF/2N. A competitive solid-state immunoassay was used to assess the concentration of recombinant hTF/2N in the culture fluid and at various stages of the purification (Foster et al., 1982). Proteolytically derived Fe-hTF/2N (Lineback-Zins & Brew, 1980) was radioiodinated (Fraker & Speck, 1978) with Iodogen (Pierce Chemical Co.) and used as the standard. The monoclonal anti-hTF antibody HTF·14 was used as the probe (Bartek et al., 1984). This antibody recognizes only the amino-terminal lobe of hTF (Mason et al., 1988) and does not recognize bovine transferrin (Penhallow et al., 1986).

Amino-Terminal Sequence Analysis. The amino-terminal sequences of both the minor and major forms of recombinant hTF/2N were determined on an Applied Biosystems 470A protein sequencer in the Given Analytical Facility at the University of Vermont.

Periodic Acid–Schiff Stain. The presence of oligosaccharides in the recombinant hTF/2N was determined by staining the protein with periodic acid–Schiff reagent (Fairbanks et al., 1971).

Nuclear Magnetic Resonance Spectroscopy. Proton and fluorine NMR spectra were obtained on the 5.872-T Bruker WM NMR spectrometer in the Camille and Henry Dreyfus NMR Laboratory, Department of Chemistry, University of Vermont, operating in the Fourier-transform mode with quadrature detection. Dr. Christopher W. Allen of that department kindly allowed us to use his ^{19}F probe. For proton spectra, spectrometer settings were as described previously (Valcour & Woodworth, 1987). For ^{19}F spectra, the sweep width was 30 000 Hz, the acquisition time was 0.279 s, a receiver delay of 2.0 s intervened between acquisition and pulse of 15.0 μs (90°), and the sample was at 303 K. ^{19}F chemical shifts are relative to 0.1 M trifluoroacetic acid in ^2H_2O. Protein samples were 6–8 mg in 0.1 mL of 99.8 atom % ^2H_2O, and spectra were run on these samples in 0.1-mL capsules inserted into standard 5-mm NMR tubes containing ^2H_2O. Free induction decays of ^{19}F spectra were subjected to a line broadening of 10 Hz prior to Fourier transformation.

Results

Isolation of Human TF cDNA. Approximately 100 000 colonies of a human liver cDNA library (Prochownik et al., 1983) were screened by using a 24-base oligonucleotide to the 5′ sequence of the human TF cDNA as a hybridization probe. A single positive colony was obtained. Extensive restriction enzyme mapping of the plasmid isolated from this clone agreed completely 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 this clone confirmed that it was identical with the full-length clone isolated by Yang et al. All subsequent sequence analyses performed during the mutagenesis and subcloning of this cDNA conformed exactly to the sequence reported previously.

Vector Construction and Expression. Two translational stop codons and a unique *Hin*dIII recognition site were introduced into the linker region between the amino- and carboxy-terminal domains of the hTF cDNA sequence by oligonucleotide-directed mutagenesis. The predicted translation sequence from this construct ends at Asp-337, according to the serum hTF numbering sequence (MacGillivray et al., 1983). Initially, we attempted to express the cDNA coding for the amino-terminal domain in *E. coli*. To this end, we engineered a *Kpn*I restriction site into the sequence encoding the amino terminus of serum hTF protein, thus allowing the isolation of a *Kpn*I/*Hin*dIII restriction fragment that encoded the amino-terminal domain of hTF lacking the signal sequence. This fragment was then spliced onto a synthetic *E. coli* alkaline phosphatase signal sequence (Gray et al., 1985), such that the resulting recombinant protein would be exported into the periplasmic space of the bacterium. We had hoped that the transport of the nascent chain to the periplasmic space would aid the proper folding of the protein as had been described for bovine pancreatic trypsin inhibitor (Marks et al., 1986); however, the recombinant protein was undetectable by $NaDodSO_4$–PAGE or Western blotting, even though two separate expression vectors (pKT-223 and pUC19) were utilized in these experiments (data not shown).

The expression vector pNUT (Palmiter et al., 1987) contains a mouse metallothionein 1/human growth hormone gene fusion that has been shown to direct high levels of human growth hormone in transgenic mice (Palmiter et al., 1983). Important functional features of this vector include a mouse metallothionein 1 promoter to induce cDNA transcription in the presence of heavy metals, pUC18 sequences to allow replication and selection in *E. coli*, and a dihydrofolate reductase (DHFR) cDNA driven by the SV40 early promoter to allow selection in cell culture. The DHFR cDNA encodes a mutant form of the enzyme which has a 270-fold lower affinity for the competitive inhibitor methotrexate (MTX) (Simonsen & Levinson, 1983). This allows for the immediate selection of transfected cells in very high concentrations (0.5 mM) of MTX and abrogates the need for a recipient cell line that is deficient in DHFR.

To construct the expression vector pNUT-hTF/2N, the

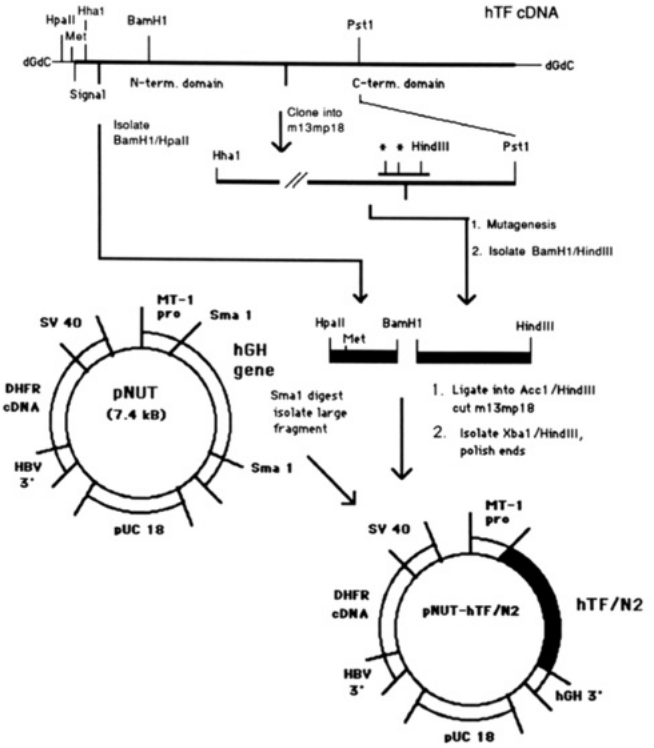

FIGURE 1: Construction of the hTF/2N expression vector in pNUT. A 2.3-kb cDNA encoding human serum TF was isolated from a human liver cDNA library, and a 1.5-kb *Pst*I/*Hal* fragment containing the complete amino-terminal domain coding sequence was cloned into M13mp18. Double translational stop codons and a *Hin*dIII recognition sequence were introduced by site-directed mutagenesis, allowing the isolation of a *Bam*HI/*Hin*dIII fragment which, when joined to a *Bam*HI/*Hpa*II fragment, encodes the amino-terminal domain and signal sequence. This fragment was cloned into the eukaryotic expression vector pNUT, giving the vector pNUT-hTF/N2. In this plasmid, the TF cDNA is under the control of the metallothionein promoter (MT-1 pro) and the human growth hormone transcription termination signals (hGH3'). pNUT also contains the SV40 early promoter (SV40) driving expression of a resistant DHFR cDNA (DHFR cDNA) using transcription termination signals from human hepatitis B virus (HBV). See text for details.

*Bam*HI–*Hin*dIII fragment from the bacterial expression vector was isolated (Figure 1). A *Hpa*II–*Bam*HI fragment from the original transferrin cDNA clone was also isolated (Figure 1). These two fragments were then ligated into M13mp18 replicative-form DNA that had been cut with *Acc*I and *Hin*dIII. Replicative-form DNA from the resulting M13 phage was isolated, the insert was released by cleavage with *Xba*I and *Hin*dIII, and the ends were made blunt-ended. These steps ensured that the fragment included the translational stop signals, retained the natural signal sequence for the protein, and was free of the dG/dC tail found in the original vector (Figure 1). This fragment was inserted into *Sma*I-cut pNUT, thus replacing the human growth hormone gene with a hTF/2N encoding cDNA, but leaving the transcriptional termination signal from the growth hormone gene intact. This plasmid was transfected into BHK cells, and the resulting transformants were selected in the presence of MTX.

To analyze the mRNA transcripts produced by the transfected BHK cells, total RNA was electrophoresed on an agarose gel in the presence of formaldehyde (Maniatis et al., 1982). After transfer to nitrocellulose, the blot was analyzed by using an oligonucleotide to the 3' untranslated region of the hGH gene as a hybridization probe. An inducible mRNA of approximately 1.4 kb was detected in the transfected cell line but not in mock-infected BHK cells (data not shown). This agreed with the predicted size of the hTF/2N mRNA, including the expected hGH 3' untranslated sequence and poly(A) tail.

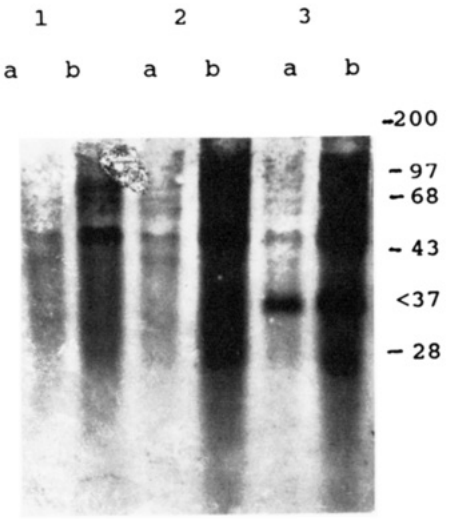

FIGURE 2: Western blot of immunoprecipitates from various baby hamster kidney cell lines. Samples of cell lysates (a) and medium (b) from Zn-induced cell cultures were precipitated with anti-hTF antiserum. Samples of the resuspended pellets were analyzed by $NaDodSO_4$–PAGE, transferred to nitrocellulose, and developed with anti-hTF antiserum followed by alkaline phosphatase conjugated anti-IgG. The hGH-pNUT and hTF/N2-pNUT cell lines were selected in 500 μ M MTX, and all cell culture was performed in DMEM/10% fetal calf serum. Lane 1, BHK cells; lane 2, hGH-pNUT transfected BHK cells; lane 3, hTF/N2-pNUT transfected BHK cells. The positions of molecular weight markers ($\times 10^{-3}$) are indicated to the right of the blot; the position of the additional protein band of M_r 37 000 is also indicated (<37) to the right of the blot.

To analyze the polypeptides produced by the transformed BHK cells, Western blot analysis was performed both on cell lysates and in the medium of various cell lines (Figure 2). Samples of BHK cells, BHK cells containing the hGH-pNUT plasmid, and BHK cells containing the hTF/2N-pNUT plasmid were grown in DMEM (BHK cells) or DMEM–MTX (BHK cells containing pNUT vectors). When the cells were reaching confluence, samples of medium were taken, and cell lysates were prepared. These samples were incubated successively with goat anti-hTF antiserum and formalin-fixed *S. aureus* cells (Van Oost et al., 1986). Bound proteins were eluted by incubation with $NaDodSO_4$, electrophoresed on a polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was then incubated with goat anti-hTF antiserum and rabbit anti-goat immunoglobulin conjugated to alkaline phosphatase. When cell lysates or medium from BHK cells (Figure 2, lanes 1a and 1b) or BHK cells with hGH-pNUT plasmid (Figure 2, lanes 2a and 2b) were analyzed, only the expected goat immunoglobulin bands (M_r 25 000 and 50 000) from the original goat anti-hTF antibodies and a small amount of cross-reacting material were observed. However, an additional band of M_r 37 000 was observed in cell lysates (Figure 2, lane 3a) or medium (Figure 2, lane 3b) of the BHK cells containing the hTF/2N-pNUT plasmid. The molecular weight of this polypeptide chain is in excellent agreement with the molecular weight of the hTF/2N molecule (37 833) calculated from the amino acid sequence. The homogeneity of the hTF/2N product indicates the successful removal of the signal sequence as cell lysate and secreted samples comigrate on SDS–PAGE. The antiserum appears to be highly specific for human TF species, since little bovine TF is apparent in the precipitates.

In large-scale cultures of the hTF/2N cell line grown in roller bottles, the concentration of hTF/2N in the medium was

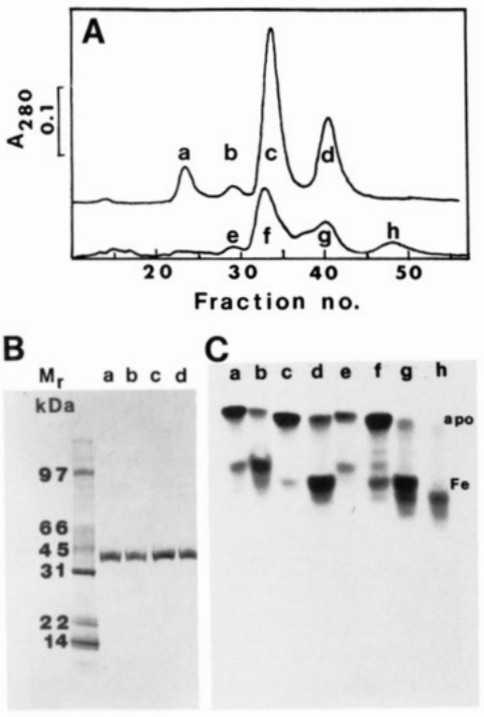

FIGURE 3: Isolation and PAGE analysis of hTF/2N. (Panel A) FPLC isolations on a column of Polyanion SI of recombinant hTF/2N (upper trace) and proteolytically derived hTF/2N (lower trace). (Panel B) $NaDodSO_4$–PAGE (5–12% gradient of acrylamide) of molecular weight standards (lane M_r) and 3 μg of each of peaks a–d from panel A. (Panel C) Urea–PAGE under nonreducing conditions of the FPLC peaks a–d (recombinant hTF/2N species) and peaks e–h (proteolytically derived hTF/2N species) from panel A. The positions of the apoprotein (apo) and iron-bound protein (Fe) are indicated. The conditions used for FPLC are given under Materials and Methods. FPLC fractions were pooled as follows: peak a (fractions 23–27), peak b (28–31), peak c (32–38), peak d (39–45), peak e (28–31), peak f (32–36), peak g (38–44), and peak h (46–51).

approximately 10–15 μg/mL as detected by radioimmunoassay.

Isolation and Characterization of Recombinant hTF/2N. Recombinant hTF/2N was purified by a three-step procedure that led routinely to an 80% yield of the major form of the protein, based on radioimmunoassay. The final purification on Polyanion SI led to quantitative resolution of the apo- and iron-saturated forms of both the minor (<5%) and major constituents of the protein (Figure 3, panel A), as corroborated by urea–PAGE (Figure 3, panel C). (Note that on urea–PAGE the slowest moving bands are apo-hTF/2N and the faster moving bands are Fe-hTF/2N.) SDS-PAGE gels (Figure 3, panel B) showed the major and minor forms of recombinant hTF/2N to be monodisperse and of equal molecular weight and the major component to be free of carbohydrate by PAS stain (data not shown). In general, these preparations appear to have better monodispersity than proteolytically derived hTF/2N (Lineback-Zins & Brew, 1980) (Figure 3). For example, the chromatographic peaks are more regular for the former, and the number of bands on urea–PAGE is greater for the latter. Spectral ratios for the iron-saturated recombinant protein are typically $A_{280}/A_{465} = 21$ and $A_{465}/A_{410} = 1.38$, which compare favorably with values for pure diferric transferrin isolated from human plasma. Titration of 3.68 A_{280} units of the apoprotein with $Fe(NTA)_2$ yields a slope corresponding to an $E^{mM}_{465} = 2.1$ and gives for the apoprotein $E^{mM}_{280} = 38.8$ (Figure 4), both reasonable values for a half-transferrin molecule (Lineback-Zins & Brew, 1980; Zak et al., 1983). The p*I*'s for the apo- and Fe-hTF/2N were 6.5 and 5.4, respectively.

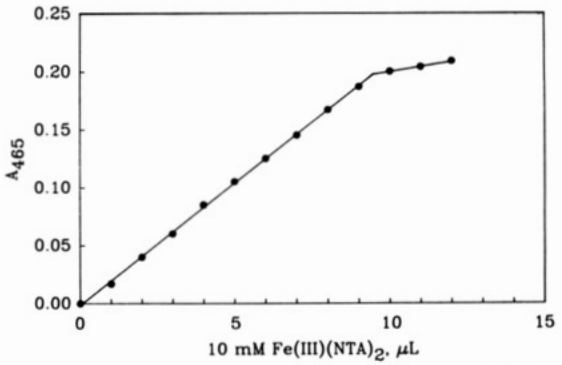

FIGURE 4: Titration of the major form recombinant hTF/2N with 10 mM $Fe^{III}(NTA)_2$. The amount of protein was 3.68 A_{280} units in 1.00 mL of 10 mM $NaHCO_3$. Visible spectra were run 5–10 min after each addition of iron to the magnetically stirred cuvette.

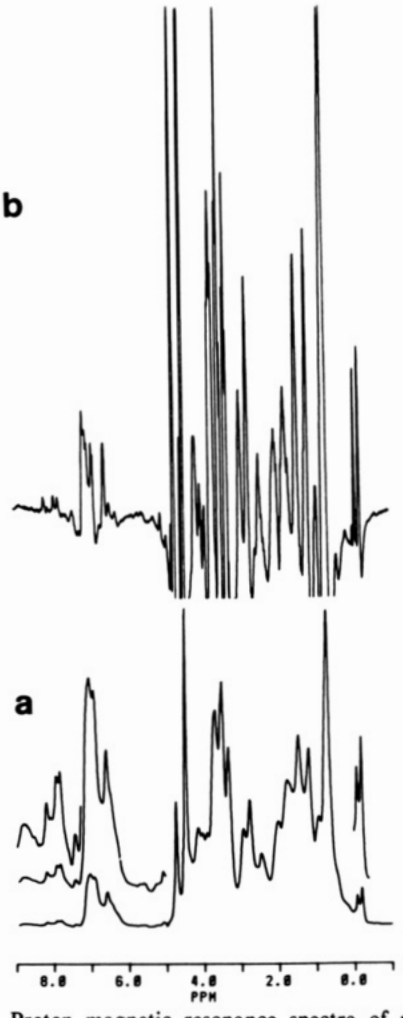

FIGURE 5: Proton magnetic resonance spectra of recombinant hTF/2N. (a) Fourier transform spectrum with a line broadening of 2 Hz. (b) Convolution difference spectrum with a line broadening of 4 Hz and DC = 4.0, NS = 68 500. The protein sample was 8 mg in 0.1 mL of 0.1 M KCl in ^2H_2O.

Amino-terminal sequence analysis of both the minor and major forms of recombinant hTF/2N gave results identical with those found (MacGillivray et al., 1983) for holo-hTF from serum (Table I).

The proton NMR spectrum of the recombinant protein (Figure 5) is very similar to that for the proteolytically derived hTF/2N (Valcour & Woodworth, 1987), but the resonance lines are sharper for the recombinant protein. The ^{19}F NMR spectrum of the protein derived from a cell culture grown on

Table I: Amino-Terminal Sequence of Human Transferrin and of the Recombinant Human Transferrin Amino-Terminal Half-Molecule[a]

protein	amino acid sequence	ref
human serum transferrin	V-P-D-K-T-V-R-W-C-A-V-S-	MacGillivray et al. (1983)
recombinant hTF/2N (major)[b]	V-P-D-K-T-V-R-W-X[c]-A-V-S-	this report
recombinant hTF/2N (minor)[d]	V-P-D-K-T-V-	this report

[a] The recombinant hTF/2N sequences were determined on an Applied Biosystems 470A protein sequencer. Approximately 200 pmol of each sample was analyzed. [b] Twelve sequencer cycles were analyzed. [c] No residue was identified at cycle 9; however, cysteine residues were not modified prior to the analysis. [d] Six sequencer cycles were analyzed.

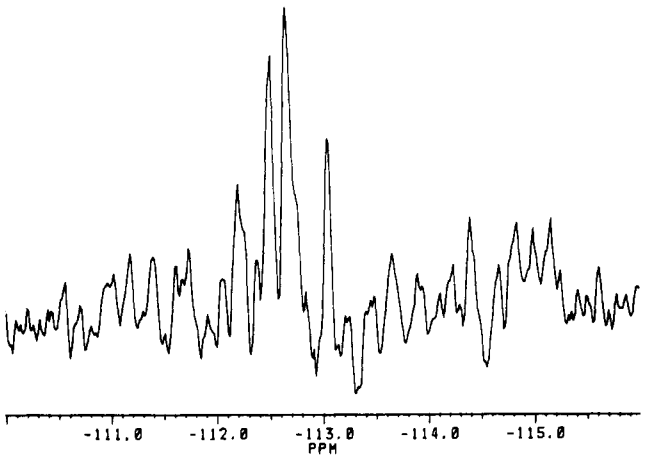

FIGURE 6: ^{19}F nuclear magnetic resonance spectrum of *m*-F-Tyr recombinant hTF/2N. The figure shows a Fourier transformation with a line broadening of 10 Hz, NS = 30000. The protein sample was 6 mg in 0.1 mL of 0.1 M KCl in ^2H_2O; the reference was 0.1 M trifluoroacetic acid in ^2H_2O.

medium supplemented with *m*-F-tyrosine (Figure 6) shows four well-resolved resonances, two possibly having an unresolved shoulder.

Discussion

By using recombinant DNA technology, we have successfully produced a hTF/2N molecule that functions identically with the proteolytically derived species as judged by several independent criteria. This represents the first reported expression in a stable cell culture system of a functionally active form of this important iron transport protein. Previously, the chicken ovotransferrin gene has been expressed in transgenic mice (McKnight et al., 1983), and a fusion protein of part of rat transferrin with β-galactosidase has been expressed in *E. coli* (Aldred et al., 1984). Except as this fusion protein, attempts to express TF or portions of the molecule in prokaryotic systems have been unsuccessful to date (Aldred et al., 1984; this report). The highly convoluted structure of the protein and the larger number of disulfide bridges in the molecule are probably the major impediments to expression in bacterial hosts. Our attempts to mimic partially the natural protein folding environment by targeting the protein for bacterial membrane transport via an attached alkaline phosphatase signal sequence were unsuccessful, forcing us to explore eukaryotic expression systems.

The pNUT-based hTF/2N construction described here produces high levels of recombinant protein without the need for a DHFR-deficient cell line or tedious resistance amplification procedures. BHK cells are well-suited for economical, large-scale growth, and we are currently examining their growth characteristics on micro-carrier supports in bioreactor vessels. By using either roller bottles or a fermentor with a capacity of several liters, we can easily produce sufficient recombinant protein even for techniques such as NMR that traditionally have required a high concentration of protein.

The minor form of recombinant hTF/2N isolated on Polyanion SI migrates more slowly than the major form on urea–PAGE (Figure 3, panel C), but at the same rate on SDS–PAGE (Figure 3, panel B). Thus, the apparent molecular weights are the same, but the relative degrees of unfolding in 6 M urea differ. [Note that the proteolytically derived apo-hTF/2N shows even faster migrating species in 6 M urea (Figure 3, panel C, fractions g and h). Contamination of apo-hTF/2N with Fe-hTF/2N and vice versa on these gels arises from the method of pooling FPLC fractions, from some loss of bound iron on the urea gel, and from binding of contaminating iron during workup of the FPLC samples.] Identical N-terminal sequences (Table I) show that the signal peptide has been removed from both minor and major forms of the recombinant protein. As in hTF/2N from human serum (Lineback-Zins & Brew, 1980), the recombinant hTF/2N is nonglycosylated. The cause of the difference between major and minor forms of hTF/2N is unknown at present. The minor form has never represented more than 5% of the total recombinant protein and is usually less than 1%. Thus, the goal of isolating a monodisperse recombinant hTF/2N (the major form) has been achieved.

The iron-binding behavior, p*I*'s, migration on NaDodSO$_4$–PAGE and urea–PAGE, and proton NMR spectra of the recombinant hTF/2N match reasonably well those of the hTF/2N derived from amino-terminal monoferric hTF by proteolysis with thermolysin (Lineback-Zins & Brew, 1980; Valcour & Woodworth, 1987), except as noted above. The major form of the recombinant protein shows a higher degree of monodispersity (Figure 3), and its proton NMR spectrum shows sharper resonance lines than does the proteolytically derived hTF/2N. There has been insufficient minor form for analysis by NMR.

Previous studies of the incorporation of *m*-fluorotyrosine into alkaline phosphatase from *E. coli* have established the efficacy of ^{19}F NMR for specifically probing the tyrosyl residues in a protein (Sykes et al., 1974; Hull & Sykes, 1974). Incorporation of *m*-F-tyrosine into the recombinant hTF/2N proves that selective amino acid substitution is possible in this cell culture system and gives us access to a specific NMR probe of tyrosyl side chains. This preparation behaves in all respects like the nonmodified protein as described above for the nonsubstituted recombinant. When we have optimized the cell culture conditions to achieve higher levels of incorporation, changes in the ^{19}F NMR spectrum on addition of paramagnetic and diamagnetic metals and on changes in pH will be useful in studying the tyrosyl residues specifically involved in metal binding. Incorporation of selectively deuterated aromatic amino acids will allow us to dissect the aromatic region of the proton NMR spectrum of the protein in similar fashion to the studies on lysozyme from Japanese quail (Brown-Mason et al., 1981).

Finally, we are currently in the process of expressing hTF/2C by similar methodologies to those described in this paper. In addition to NMR studies, expression of the carboxy-terminal domain will allow us to undertake physiological studies of binding and iron donation by the half-molecules to cells in culture (Brown-Mason & Woodworth, 1984; Mason et al., 1987; Penhallow et al., 1986).

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