Posttranscriptional Regulation of Chimeric Human Transferrin Genes by Iron†

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ABSTRACT: Transferrin, the transferrin receptor, and ferritin are integral to the body's management of iron, an element required for life but highly toxic when present in excess. The transferrin receptor and ferritin are regulated posttranscriptionally by iron: the transferrin receptor by mRNA stability and ferritin by mRNA translation. Results described here indicate that transferrin, like ferritin, is regulated by iron at the level of translation. Chimeric genes introduced into the mouse genome were composed of the human transferrin 5′ regulatory region fused to the chloramphenicol acetyl transferase (CAT) reporter gene. Iron administration to transgenic mice resulted in a significant decrease of transferrin-directed CAT enzyme activity and CAT protein in liver, but no significant decrease in human transferrin-CAT mRNA levels. Binding of specific RNA iron regulatory elements by proteins in cytoplasmic extracts have been shown to regulate ferritin and transferrin receptor synthesis. Similar results have been obtained with transferrin mRNA. A decreased binding of human transferrin 5′-untranslated region RNA by factors in cytoplasmic extracts of livers from mice receiving iron was found when compared to extracts from control mice. A human transferrin RNA–protein complex migrated electrophoretically with the same mobility as a ferritin iron responsive element RNA–iron responsive element binding protein complex. The ferritin iron responsive element RNA also competed with the human transferrin 5′-untranslated region RNA–protein complexes formed and vice versa. Therefore, iron modulation of human transferrin may share a factor common or similar to that observed in ferritin and transferrin receptor iron modulation.

Transferrin (TF),[1] the major plasma protein which binds and transports iron, is regulated in humans and rats by iron levels. In rats, iron deficiency induced by diet stimulates liver TF synthesis at the level of transcription, yet chronic iron overload does not affect levels of serum TF (Idzerda et al., 1986). However, in cultured rat hepatocytes, levels of secreted TF decrease with iron treatment which appears to modulate TF posttranscriptionally (Lescoat et al., 1989). In humans, iron overload leads to a decrease in TF expression while iron deficiency leads to an increase in TF expression (Lane, 1966; Morgan, 1983). Consistent with a posttranscriptional mode of iron regulation, humans with the iron overloading disease hemachromatosis and normal individuals have similar TF mRNA levels in the liver. However, hemachromatosis patients have decreased serum TF levels (Pietrangelo, 1991).

Storage and distribution of iron are essential for cell metabolism. Transferrin serves at least two major roles: (1) TF in body fluids, such as serum, has an important role in bacteriostasis. Because of its strong affinity for iron (K_D = 10^{-22} mol^{-1}) and its low iron saturation (~25–30%), circulating TF limits the supply of free iron to many bacteria which require iron for proliferation, and (2) TF transports iron across cell membranes for storage and for use in the synthesis of iron containing proteins. The roles of ferritin, TF receptor, and TF are all intertwined for the purpose of iron management. The TF–iron complex is bound by transferrin receptors in cell membranes and endocytosed. Under acidic conditions, iron is released from the endosomes to ferritin for storage or, alternatively, is released for the synthesis of other iron-containing proteins (Huebers et al., 1983; Idzerda et al., 1986).

Previous studies have demonstrated that iron regulation of ferritin and transferrin receptor involves highly conserved RNA stem–loop structures called iron responsive elements (IRE). Ferritin and transferrin receptor are coordinately regulated in response to iron levels by a single protein, the iron responsive element binding protein (IRE-BP) which binds to the ferritin and TF receptor IRE's (Rouault et al., 1988; Brown et al., 1989; Mullner et al., 1988; Casey et al., 1989; Theil, 1990; Harrell et al., 1991). Iron responsive elements were first detected in the 5′-untranslated region (UTR) of ferritin mRNA and the 3′-UTR of the transferrin receptor mRNA (Hentze et al., 1987a; Aziz & Munro, 1987; Casey et al., 1988). Iron regulation of ferritin was the first example of translational regulation of eukaryotic mRNA's (Aziz & Munro, 1986; Hentze et al., 1987a,b). The single IRE in the 5′-UTR of ferritin is a translational cis-regulatory element. The transferrin receptor contains a series of five IRE's in its 3′-UTR which act as RNA stability cis-regulatory elements (Casey et al., 1988). The IRE is defined as a 28-nucleotide sequence which includes a 10 base pair stem structure with an unpaired midstem C and a six-nucleotide loop (Figure 1A). The six-nucleotide loop is highly conserved; five of these nucleotides, CAGUG, are important for binding by the IRE-BP (Casey et al., 1989; Hentze et al., 1988; Leibold et al., 1990; Rouault et al., 1988). In addition, the unpaired C in the stem and the length of the stem are highly conserved. Iron responsive elements which are bound by the IRE-BP have also been found in the 5′-UTR of porcine heart aconitase mRNA, murine aminolevulinic acid synthase mRNA, *Drosophila* toll mRNA, and human aminolevulinic acid synthase mRNA (Cox et al., 1991; Dandekar et al., 1991).

In this paper we present evidence that human transferrin (hTF) is regulated by iron at the level of translation. Transgenic mice which carry chimeric genes composed of the human TF 5′-flanking sequences fused to the chloramphenicol

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[1] Abbreviations: TF, transferrin; IRE, iron responsive element; IRE-BP, iron responsive element binding protein; UTR, untranslated region; hTF, human transferrin; hTF-CAT, human transferrin–chloramphenicol acetyl transferase transgene; DEPC, diethyl pyrocarbonate.

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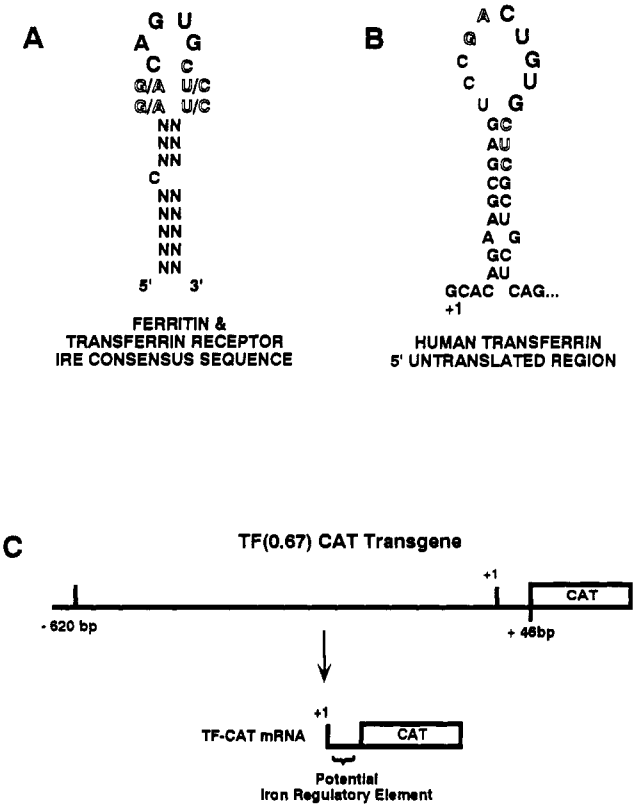

FIGURE 1: Comparison of the ferritin and transferrin receptor IRE consensus sequence with the human TF 5′-UTR sequence. (A). The ferritin and transferrin receptor IRE consensus sequence derived from the ferritin 5′-UTR stem–loop and the five 3′-UTR transferrin receptor stem–loops. The large boldface CAGUG is a pentanucleotide shown to be important for specific IRE-BP binding. The outlined letters represent additional conserved bases. The ΔG value for the ferritin IRE stem–loop structure is –0.3 kcal/mol. (B) Possible secondary structure in the 5′-UTR of hTF mRNA. A loop structure can be formed from two imperfect indirect repeats in the 5′-UTR (Adrian et al., 1986). The large boldface letters represent the conserved IRE pentanucleotide sequence. The outlined letters represent additional conserved bases. This structure, predicted using the RNA Fold program, has a ΔG value of –9.8 kcal/mol. (C) This diagram illustrates the TF(0.67)CAT transgene construct and the hTF-CAT mRNA transcribed from the transgene. The transcription start site, +1, has been verified by S1 nuclease analysis and primer extension experiments (Adrian et al., 1990). The hTF-CAT mRNA contains 46 of the 50 bases of the hTF 5′-flanking region fused to CAT RNA. hTF-CAT mRNA transcribed from the TF(1.2)CAT transgene also contains the 46 base hTF 5′-UTR.

acetyl transferase (hTF-CAT) reporter gene were used to demonstrate in vivo the response of hTF to iron. Liver expression of the protein product of hTF-CAT transgenes was suppressed following intraperitoneal injections of iron. However, liver hTF-CAT mRNA levels were unaffected by the iron treatment. The only human TF sequences in the hTF-CAT mRNA are 46 of the 50 bases of the 5′-UTR. The CAT sequences in the transgene have previously been shown not to be regulated by iron (Hentze et al., 1987a). Therefore, the 46 nucleotides of the hTF 5′-UTR have been identified as the region of hTF mRNA responding to iron modulation (Figure 1C). Analysis of the hTF 5′-UTR reveals a potential stem–loop structure which contains 9 of the 11 conserved IRE bases (Figure 1B). However, the overall conformation of the hTF 5′-UTR stem–loop predicted by computer modeling differs from the ferritin and transferrin receptor IRE's with a larger loop, lack of a bulging C, and different stem pairing. RNA–cytoplasmic protein binding assays have been used for studies of ferritin and TF receptor iron regulation (Aziz & Munro, 1986; Hentze et al., 1987a; Mullner et al., 1989; Barton et al., 1990; Leibold et al., 1990; Testa et al., 1991). Similar binding assays were used here to demonstrate specific binding of liver cytoplasmic proteins to the human TF 5′-UTR and differential binding of extracts from iron treated versus control mice. The ability of hTF 5′-UTR binding protein(s) to bind the ferritin IRE was also determined using competition binding assays.

MATERIALS AND METHODS

Transgenic Mice. Development and the descriptions of the transgenic mouse lines used in this study were reported by Adrian et al. (1990). Briefly, fertilized C57BL/6J mouse eggs were injected with DNA constructs containing –0.622 to +0.046 kb or –1.152 to +0.046 kb of the 5′-flanking region of the human transferrin (hTF) gene fused to the bacterial CAT gene protein coding region, TF(0.67)CAT and TF(1.2)CAT, respectively. Homozygous lines produced from these founder mice were used for this study. The mouse lines used, transgene construct, and number of transgene copies were as follows: A26X, TF(0.67)CAT, 5; A23IV, TF(0.67)-CAT, 1; A19III, TF(0.67)CAT, 5–10; A48VI, TF(1.2)CAT, 2; A46II, TF(1.2)CAT, 1. These transgenic lines showed no correlation between transgene copy number and CAT enzyme activity.

Extraction of Total RNA. Liver tissue was removed from anesthetized and exsanguinated mice, frozen in liquid nitrogen, and stored at –80 °C until used for RNA extractions. Extractions were performed according to Chomczynski and Sacchi (1987). The tissue (about 1 g) was dropped into 8 mL of incomplete solution D [4 M guanidium thiocyanate (Fluka)], 25 mM sodium citrate (pH 7), and 0.1 M 2-mercaptoethanol). The tissue was homogenized using a Brinkman homogenizer, and 0.05 vol of 10% sarcosyl was added. The samples were mixed, and 0.1 vol of 2 M sodium acetate (pH 4) was added. An equal volume of phenol and 0.1 vol of chloroform were added. Samples were mixed, incubated for 15 min on ice, and centrifuged at 12000*g* for 20 min at 4 °C. The aqueous phase was transferred to a new tube. An equal volume of isopropanol was added, and the samples were incubated for 60 min at –20 °C. Samples were centrifuged at 12000*g* for 20 min at 4 °C. The pellet was dried, resuspended in diethyl pyrocarbonate treated distilled H_2O, and stored in liquid nitrogen. RNA concentrations were determined by absorbance at 260 nm using a spectrophotometer.

DNA Probes for Northern Blot Analysis. The mouse TF probe was isolated from a commercial mouse cDNA library using PCR primers complementary to the 5′ end of exon 7 and the 3′ end of exon 9 (Chen & Bissell, 1987). No cross-hybridization of the mouse TF with human RNA was observed. Human genomic ferritin DNA was provided by ATCC clone pUCM11 (Hentze, 1986). A 465 bp *Sst*I fragment was isolated from this pUCM11 clone. Cloned rat albumin cDNA probe digested with *Pst*I restriction enzyme was used as a control probe (Chatterjee et al., 1987). The CAT DNA probe was isolated following a *Hin*dIII-*Bam*HI restriction digest of the pSVOCAT plasmid (Gorman et al., 1982). DNA fragments were isolated from NuSieve Low Melting GTG agarose gels and radioactively labeled according to Feinberg and Volgelstein (1983).

PCR primers used to isolate the mouse TF fragment are as follows: 5′ primer: 5′ (amino acid no. 287) GGC AAG GAG GAC TTG ATC TGG GAG 3′, 3′ primer: 5′ GAG ACC ACT GAG GAC TGC ATT GAA (amino acid no. 407) 3′.

Northern Blot. Northern analysis was performed using a modification of the protocol by Lehrach et al. (1977). RNA samples were denatured by heating for 15 min at 65 °C in loading buffer containing 48% deionized formamide, 1× MOPS, 17.3% formaldehyde, 5.3% glycerol, and 5.3% diethyl pyrocarbonate (DEPC) treated H_2O saturated with bromophenol blue. Following addition of ethidium bromide, each sample was fractionated by size on a 1.3% agarose, 2.2 M formaldehyde gel in 1× MOPS. RNA was transferred from the agarose gel to Nitro-2000 charged nylon membrane. The RNA was baked onto the filter for 2 h and hybridized to the appropriate ^{32}P-labeled DNA probe in 50% formamide, 5× Denhardt's, 5× SSPE, 0.1% SDS, 100 μg/mL denatured salmon sperm DNA, and 10% dextran sulfate overnight at 42 °C. Nonspecifically bound probe was washed off using two washes at room temperature with 2× SSC and 0.1% SDS followed by two 42 °C washes with 1× SSC and 0.1% SDS. The amount of bound probe was quantitated using a Betagen Betascope. Filters were stripped for reprobing as described previously (Adrian et al., 1990).

Analysis of Liver CAT Activity. An approximately 100-mg portion of the caudal edge of the left lobe of the liver was consistently utilized for CAT enzyme assays. The remainder of the liver was frozen in liquid nitrogen for subsequent mRNA analyses. Levels of CAT enzyme activity in liver tissue extracts from transgenic mice were determined by the procedure of Gorman et al. (1982). Briefly, the liver tissue was homogenized in 250 mM Tris (pH 7.8) using a Brinkman homogenizer. The homogenate was freeze-thawed using a dry ice–ethanol bath and 37 °C water bath. The homogenate was centrifuged for 10 min at 4 °C and the supernatant transferred to a new tube and heated for 5 min at 65 °C. The sample was centrifuged again for 5 min at 4 °C; the supernatant was transferred to a new tube and stored at −20 °C.

The protein concentration was determined by the Lowry assay (Lowry et al., 1951). To determine CAT activity, the protein extract was incubated with 250 mM Tris (pH 7.8), 0.2 μCi of [^{14}C] chloramphenicol, and 0.53 mM acetyl CoA. The reaction was incubated for 30 min or 1 h at 37 °C. The samples were then spotted on thin layer chromatography plates and chromatographed in 5% methanol and 95% chloroform. The CAT enzyme activity was quantitated using a Betagen Betascope. The CAT enzyme activity was shown to parallel CAT protein levels which were determined using a CAT ELISA kit from 5′–3′ (Boulder, CO).

Administration of Iron Compounds to Transgenic Mice. Adult transgenic mice matched by age, sex, and founder line were injected intraperitoneally with 10 mg of iron per kilogram of body weight as ferric ammonium citrate (17% iron), ferric chloride, ferric citrate, or ferric nitrate dissolved in 0.9% pyrogen-free saline in 100-μL volumes. Control injections of 23.2 mM ammonium citrate or 117 mM NaCl were also dissolved in 0.9% pyrogen-free saline (155 mM NaCl) and administered in 100-μL volumes. For the 48-h and 72-h studies, each mouse was injected at 24-h intervals, and tissues were dissected from the mouse 24 h after the final injection. Mice receiving the 48-h treatment received a total of 20 mg of Fe per kilogram of body weight, and the 72-h treated mice received a total of 30 mg of Fe per kilogram of body weight. For the 4-h and 24-h studies, each mouse was given a single injection of 25 mg of Fe per kilogram of body weight of ferric ammonium citrate or 23.2 mM ammonium citrate, and the tissues were dissected from these mice either 4 or 24 h after injection.

Templates for in Vitro Transcription. DNA templates were constructed by annealing T7 promoter DNA oligonucleotides with either hTF 5′-UTR or ferritin IRE DNA oligonucleotides (Mueller & Wold, 1989). Bold letters in the T7/hTF promoter oligonucleotide signify hTF sequences, while bold letters in the T7/ferritin IRE promoter oligonucleotide correspond to ferritin sequences. The bold letters indicate the region of oligonucleotide complementarity between the T7 promoter oligonucleotides and the hTF 5′-UTR or ferritin IRE oligonucleotides. Annealing of the oligonucleotides permits transcription from these templates to synthesize the desired RNA sequences.

T7/hTF promoter

5′ CCAAGCTTCTAATACGACTCACTATA**GCACAGAAGCGA** 3′
3′ GGTTCGAAGATTATGCTGAGTGATAT 5′

hTF 5′-UTR

5′ CTTCCGGGTG CGGCGCTGAG CAGCGAGCAC AGTCTTACTC GCTTCTGTGC 3′

T7/rat ferritin IRE promoter

5′ CCAAGCTTCTAATACGACTCACTATA**CCGCGGTTTCCT** 3′
3′ GGTTCGAAGATTATGCTGAGTGATAT 5′

Ferritin IRE

5′ GAGCACCGGG TTCCGTTCAA GCACTGTTGA AGCAGGAAAC CGCGG 3′

Synthesis and Purification of RNA Transcripts. The DNA templates were transcribed using an in vitro transcription kit (Promega). The reaction mixture contained 12 μM CTP, 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM UTP, ~0.5 μg of DNA template, 5 μCi of [α-^{32}P]CTP, 10 mM DTT, and 2 units/μL T7 polymerase in a 25-μL volume. The mixture was incubated for 2 h at 32 °C. RNA transcripts were resolved on a 12% denaturing polyacrylamide gel in 1× TBE, excised from the gel and eluted overnight using 500 mM ammonium acetate and 1 mM EDTA. The sample was incubated in 2.5 vol of absolute ethanol overnight at −20 °C, microcentrifuged for 15 min at 4 °C, dried, and resuspended to 10 000 cpm/μL in DEPC treated H_2O. For accurate quantitation of competing RNA, ^{3}H-labeled RNA transcripts were synthesized in the presence of 1.0 μCi of [5,6-^{3}H]UTP, 9.6 μM UTP, 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, ~0.5 μg of DNA template, 10 mM DTT, and 2 units/μL T7 polymerase in a 25-μL volume. Amounts of RNA transcripts synthesized were calculated on the basis of specific activity of the [5,6-^{3}H]UTP or [^{32}P]CTP (Barton et al., 1990). Because of the low energy β emission of ^{3}H, its signal was not detected by the autoradiographic or Betascope scanning procedures used.

Mouse Liver Cytoplasmic Protein Extracts. Nuclear proteins were extracted according to the procedure of Dignam et al. (1983). All buffers used were cold (4 °C), and all procedures were performed in a cold room. Liver tissues were dissected from mice treated with ferric ammonium citrate or ammonium citrate as described above. The tissue was placed in a small weigh boat and minced with a scalpel. The tissue was rinsed with 5 vol of saline and the liquid decanted off. Five volumes of buffer A [10 mM Hepes (pH 7.9 at 4 °C), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT] was added and the sample mixed on a rocker for 10 min. The buffer was

Table I: Comparison of CAT Activity in Iron-Treated Mice and Controls

			iron treatment			control			
expt no	line[a]	time[b]	dose[c]	salt[d]	CAT (cpm)[e]	salt[d]	CAT (cpm)[e]	%control[f]	p values[g]
1	A48VI (1.2)	72	0.5	$FeCl_3$	20600 ± 1680	NaCl	20200 ± 1980	102.1	0.889
			5	$FeCl_3$	15700 ± 1850			78.1	0.180
			10	$FeCl_3$	11500 ± 2010			56.9	0.025
2	A19III (0.67)	24	10	$FeCl_3$	2800 ± 280	NaCl	4100 ± 650	67.1	0.228
		48		$FeCl_3$	2800, 1600	saline	4000 ± 600	53.9	0.182
		72		$FeCl_3$	2900, 1400			52.5	0.183
3	A48VI (1.2)	72	10	$Fe(NO_3)_3$	2600 ± 190	saline	4400 ± 210	59.7	0.000
4	A48VI (1.2)	72	10	$Fe(NO_3)_3$	9600 ± 650	NaCl	14200 ± 530	67.6	0.007
				$FeCl_3$	8000 ± 830			56.3	0.000
5	A46II (1.2)	72	10	Fe cit	7700 ± 720	saline	11900 ± 1490	64.3	0.012
6	A23IV (0.67)	72	10	$FeCl_3$	6900 ± 740	NaCl	12100 ± 1090	57.2	0.009
7	A46II (1.2)	72	10	$FeCl_3$	6100 ± 330	saline	11800 ± 810	51.5	0.012
				Fe am cit	5800 ± 450	am cit	9000 ± 360	63.9	0.018
8a	A26X (0.67)	4	10	Fe am cit	53600 ± 5720	am cit	54200 ± 2100	98.9	0.951
						saline	44100 ± 5900	121.6	0.943
8b[h]	A26X (0.67)	24	10	Fe am cit	18400 ± 2670	am cit	21600 ± 1350	85.5	0.317
						saline	18700 ± 3380	98.6	0.952
8c	A26X (0.67)	48	10	Fe am cit	48300 ± 3590	am cit	53400 ± 5860	90.4	0.472
						saline	53600 ± 5830	90.1	0.432
8d	A23IV (0.67)	72	10	Fe am cit	6100 ± 760	am cit	8400 ± 510	73.1	0.052

[a] The "line" is the transgenic mouse founder line used for that experiment. The length of TF 5′-flanking region in kb present in the hTF-CAT transgene is in parentheses. [b] "Time" indicates the duration of the iron treatment in hours. A detailed description is presented under Materials and Methods. [c] Dose indicates mg of iron/kg of body weight/day of intraperitoneal injections. [d] Iron salts were dissolved in "normal" (155 mM) saline; control solutions were "saline", 155 mM NaCl; "NaCl", 117 mM NaCl in normal saline; or "am cit", 23.2 mM ammonium citrate in normal saline. [e] The cpm values in the table represent the means plus and minus the standard error of the mean. [f] The percent of control is the mean iron value divided by the mean control value times 100. [g] Probabilities, p values, were calculated using a one-way analysis of variance. [h] The assay was performed with a 30-min reaction, all other assays used a 60-min reaction. Five or more mice were included in each treatment group except experiment no. 2. In experiment no. 2, the number of mice receiving iron treatment in the 24-h group was four, the number of mice in the 48-h group was two and the number of mice in the 72-h group was two; both control groups contained four mice. All mice were homozygous for TF CAT transgenes except for expt 3 in which mice were heterozygous.

decanted, and an additional 2 vol of buffer A were added. The sample was then homogenized in a Dounce homogenizer with the B pestle using about 10 strokes. The sample was centrifuged for 10 min at 2000 rpm in a Beckman table-top (J-6) centrifuge in the cold room. The supernatant was transferred to a SW40 Beckman tube and 0.11 vol of buffer B (300 mM Hepes, 1.4 M KCl, and 30 mM $MgCl_2$) was added. The sample was centrifuged for 60 min, at 28 500 rpm (100000*g*), at 4 °C in a Beckman SW40Ti rotor. The supernatant (S100) was dialyzed against 20 vol of buffer D [20 mL of 20 mM Hepes (pH 7.9 at 4 °C), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT] and stored in aliquots at −80 °C. The protein concentration was determined using the Lowry assay (Lowry et al., 1951). Binding specificity was quantitated using a Betagen Betascope.

RNA-Protein Binding Assay. Binding reactions were performed as described by Aziz and Munro (1986). All reactions were performed on ice. The binding assay contained 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 200 mM KCl, 25% glycerol, 5 mM dithiothreitol, ~10 000 cpm of RNA transcript (0.04 ng), and 20 μg of cytoplasmic extracts in a 10-μL volume. DEPC treated water was used for making all buffers. The reactions were incubated for 30 min, and 1 μL of heparin (50 mg/mL) was added. The reactions were incubated for 10 min on ice and were electrophoresed in a 5% nondenaturing polyacrylamide gel or an 8% nondenaturing polyacrylamide gel (acrylamide/bis, 60:1) for ~3–4 h at 14 V/cm. The gel was submitted to autoradiography. All binding reactions were reproducible. Two or more preparations of cytoplasmic proteins and two or more preparations of each RNA probe and RNA competitors were examined.

Rocket Immunoelectrophoresis. Serum levels of mouse TF and serum amyloid protein were determined using the rocket immunoelectrophoresis procedure of Laurell (1966). Antibody preparations for mouse TF were obtained from Cappel-Organon Teknika (West Chester, PA), and antibody preparations for serum amyloid protein were obtained from Calbiochem Corporation (San Diego, CA). Samples were quantitated by measuring the peak height of the rocket immunoprecipitate of each sample.

RESULTS

Effect of Iron Treatment on hTF-CAT mRNA Levels and CAT Enzyme Activity in Livers of Transgenic Mice. In humans, serum TF levels are suppressed by iron overloading. To determine if expression of the human hTF-CAT transgene was also affected by iron, hTF-CAT transgenic mice received intraperitoneal injections of iron or control salts, and their livers were subsequently assayed for CAT enzyme activity and hTF-CAT mRNA levels. CAT enzyme activity was assayed in an approximately 100-mg portion of the caudal edge of the left liver lobe. The remainder of each liver was utilized for determination of RNA concentrations. Comprehensive data of CAT enzyme activities from 11 different iron treatment experiments are included in Table I. These experiments tested different transgenic lines, different hTF-CAT constructs, and different iron salts. In all studies, hematocrits, weights, coat conditions, and any obvious pathology were recorded for each mouse to monitor its general health. The mice treated with iron for 72 h show a 40–50% decrease in the CAT enzyme activity regardless of the form of iron treatment (Table I). Comparison of CAT enzyme activity and CAT ELISA assays showed that the enzyme activity accurately reflected the CAT protein levels. For example, in experiment 7 CAT enzyme activities were ammonium citrate = 9000 ± 360 and ferric ammonium citrate = 5800 ± 449 and CAT ELISA assays values were ammonium citrate = 103.25 ± 7.53 ng of CAT protein/mg of total protein and ferric ammonium citrate = 61.50 ± 4.19 ng of CAT protein/mg of total protein.

Table II: Comparison of RNA Levels in Iron-Treated Mice and Controls

			iron treatment			control			
RNA assayed[a]	line[b]	time[c]	dose[d]	salt[e]	mRNA (cpm)[f]	salt[e]	mRNA (cpm)[f]	%control[g]	*p* values[h]
mTF (6)	A23IV (0.67)	72	10	$FeCl_3$	1355 ± 54	NaCl	1350 ± 49	100.4	0.559
mTF[i]	A48VI (1.2)	72	10	$Fe(NO_3)_3$	15500, 15900	NaCl	14400	105.5	
mTF (7)	A46II (1.2)	72	10	$FeCl_3$	499 ± 21	saline	394 ± 23	126.6	0.315
				Fe am cit	421 ± 21	am cit	340 ± 8	123.8	0.150
mTF (8c)	A26X (0.67)	48	10	Fe am cit	424 ± 53	am cit	355 ± 39	119.5	0.317
						saline	403 ± 34	105.35	0.738
TF-CAT (7)	A46II (1.2)	72	10	$FeCl_3$	41 ± 4	saline	45 ± 4	90.4	0.553
				Fe am cit	35 ± 4	am cit	42 ± 3	84.6	0.316
TF-CAT (8c)	A26X (0.67)	48	10	Fe am cit	119 ± 5	am cit	135 ± 12	88	0.240
						saline	144 ± 17	82	0.103
ferritin (7)	A46II (1.2)	72	10	$FeCl_3$	121 ± 5	saline	136 ± 9	89.3	0.391
				Fe am cit	134 ± 11	am cit	122 ± 3	110.1	0.753
ferritin (8c)	A26X (0.67)	48	10	Fe am cit	35 ± 8	am cit	30 ± 11	115.7	0.738
						saline	36 ± 14	98.9	0.980

[a] mRNA levels were determined on mouse livers previously assayed for CAT enzyme activity. The experiment column includes the mRNA assayed and the corresponding CAT enzyme assay experiment number from Table I. [b] The line is the transgenic founder mouse line used for that experiment. The length of the TF 5′-flanking region in kb present in the hTF-CAT transgene is in parentheses. [c] "Time" indicates the duration of the iron treatment in hours. A detailed description is presented under Materials and Methods. [d] The dose in mg/kg indicates the amount of iron in each daily injection per kg of body weight. [e] Iron salts were dissolved in "normal" (155 mM) saline; control solutions were "saline", 155 mM NaCl; "NaCl", 117 mM NaCl in normal saline; or "am cit", 23.2 mM ammonium citrate in normal saline. [f] The cpm values in the table represent the means plus and minus the standard error of the mean. [g] The percent of control is the mean iron value divided by the mean control value times 100. [h] Probabilities, *p* values, were calculated using a one-way analysis of variance. [i] Five or more mice were included in each treatment group except this experiment. In this experiment, two mice received iron treatment and one received the control treatment. Thirty micrograms of total RNA were used for each analysis and albumin was used as a control for equal loading of the samples on the gel.

Levels of hTF-CAT, mouse TF, and albumin mRNA were determined by Northern blot analysis. Albumin mRNA was included as an internal control. RNA levels were quantified by scanning blots with a Betagen Betascope. Table II contains data from three different 72-h iron treatment experiments and one 48-h iron treatment experiment. Details of injection regimens are described under Materials and Methods. No significant changes in levels of any of the mRNA's were induced by iron treatment. The ferritin data from the northern blots of livers from iron treated mice were included as a control for the iron treatments. The lack of iron-induced change in ferritin mRNA levels is in agreement with data published by Aziz and Munro (1986) and Rouault et al. (1987).

Mice treated with iron for 72 h showed little hTF-CAT mRNA change with a 40–50% decrease in the CAT enzyme activity (Tables I and II). Similar results were obtained whether using $FeCl_3$ versus NaCl, ferric citrate versus NaCl, ferric nitrate versus NaCl, or ferric ammonium citrate versus ammonium citrate. Therefore, the effect on hTF-CAT expression is due to iron, not its anion. In addition, only small decreases were seen in CAT enzyme activity for the 24-h and 4-h treatment groups. The slight change in mRNA levels and the dramatic decrease in CAT enzyme activity suggest that iron regulation of TF synthesis is mainly posttranscriptional but may also have a small transcriptional component. Both transgenes, TF(0.67)CAT and TF(1.2)CAT, utilized in this study contain DNA corresponding to 46 of the 50 nucleotide human TF 5′-UTR (Figure 1C). Thus, the mRNA transcribed from these transgenes contains 46 bases of hTF 5′-UTR fused to CAT protein coding sequences [as shown in Adrian et al. (1990)]. Previous studies have shown that the CAT gene per se does not respond to iron levels (Hentze et al., 1987a). Therefore, any translational modulation is probably directed via the hTF 46 nucleotide 5′-UTR.

Effect of Iron Salts on Serum Protein Levels. Human TF is a negative acute phase reactant, whereas mouse TF is a positive acute phase reactant (Kushner, 1982). Rocket immunoelectrophoresis (Laurell, 1966) was performed on serum samples from each of the treated mice to screen for changes in protein levels and the induction of an acute phase response by the iron treatment. These assays demonstrated a moderate increase in serum amyloid protein, a sensitive acute phase reactant, in the $FeCl_3$ treated mice. Mouse TF decreased and serum albumin levels did not change. No serum amyloid protein was detected in the ferric ammonium citrate treated mouse serum. Because serum amyloid protein levels increased in response to ferric chloride, the subsequent studies were conducted using ferric ammonium citrate as the form of iron treatment with ammonium citrate as the control treatment. Ferric ammonium citrate solutions have a pH of 7 and have been used in studies of translational regulation of ferritin in rats (Leibold, & Munro, 1988).

RNA–Protein Binding Studies. The preceding studies suggest that human TF-CAT genes are regulated translationally via 46 bases of the 5′-UTR. Therefore, binding assays were performed using the hTF 5′-UTR RNA and the ferritin IRE RNA (see Materials and Methods) with the liver extracts prepared 4 h after the injection of ferric ammonium citrate or ammonium citrate (4-h extracts) or with the extracts prepared 24 h after the three injections given at 24-h intervals (72-h extracts). A sample gel shift assay resolved on a 5% nondenaturing polyacrylamide gel is shown in Figure 2. A single major iron-modulated RNA–protein complex is formed with the hTF 5′-UTR RNA that comigrates with the iron-modulated ferritin IRE RNA–protein complex (Figure 2). The lower ferritin IRE RNA–protein bands and the faint upper hTF 5′-UTR RNA bands do not show differential binding of iron-treated and control extracts. Similar results for the ferritin IRE RNA–protein binding have been reported by Leibold and Munro (1988). RNA–protein complex formation was quantitated by scanning the gel with a Betagen betascope. A comprehensive analysis with results from five mice for each iron and each control treatment group was performed. For comparison, ammonium citrate values were arbitrarily expressed as 100%. Using 4-h extracts, hTF 5′-UTR RNA-protein complex formation was decreased in extracts from iron-treated mice (72.50 ± 10.6%) versus the controls (100 ± 10.6%; $p = 0.040$). hTF 5′-UTR RNA–protein complex formation also decreased when using 72-h ferric ammonium citrate extracts (73.5 ± 5.4%) versus controls

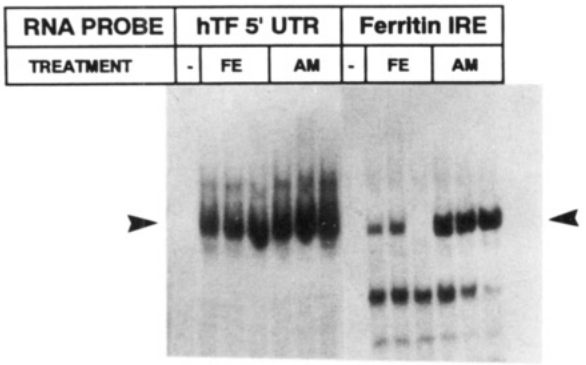

FIGURE 2: Comparison of RNA–protein complexes formed in binding reactions which contain ^{32}P-labeled hTF or ferritin RNAs and liver cytoplasmic extracts. Cytoplasmic extracts from livers of mice wihich had received injections of 25 mM ferric ammonium citrate (FE) or 23.2 mM ammonium citrate (AM) 4 h before sacrifice were incubated in binding reactions with either the ^{32}P-labeled hTF 5′-UTR or the ^{32}P-labeled ferritin IRE RNA. Complexes were fractionated by electrophoresis in a nondenaturing 5% polyacrylamide gel. For each RNA probe, each lane represents proteins from a different iron-treated or control mouse liver. Details of the binding reactions are presented under Materials and Methods. The arrow indicates RNA–protein complexes which are more abundant in extracts from control livers than extracts from iron treated livers, i.e., show differential binding.

(100 ± 11.64%; $p = 0.072$). A marked decrease in binding to the ferritin IRE sequences is only seen using the 4-h extracts (ferric ammonium citrate = 34.8 ± 10.7%; ammonium citrate = 100 ± 2.8%; $p = 0.002$). This agrees with studies by Leibold and Munro (1988) in which differential binding of iron-treated extracts to the ferritin IRE was demonstrated only with rat extracts prepared between 1 and 8 h after iron treatment with the maximum difference at 4 h. However, with hTF 5′-UTR RNA–protein binding assays, differential binding at 4 h and 72 h was seen, yet no change in the hTF-CAT enzyme activity was observed until 48–72 h.

To determine the binding specificity of proteins to the hTF 5′-UTR RNA, competition binding assays were performed. To obtain accurate quantitation, specific competing RNAs were synthesized in the presence of [^{3}H]UTP (see Materials and Methods). ^{3}H-labeled ferritin IRE RNA and ^{3}H-labeled hTF 5′-UTR RNA were used as competitors for ^{32}P-labeled hTF 5′-UTR RNA and ^{32}P-labeled ferritin IRE RNA probes (Figure 3A,B). In contrast to the gel shift patterns observed in the 5% native gel (Figure 2), two minor bands were observed in addition to the major hTF 5′-UTR RNA–protein bands when complexes were resolved on an 8% native gel (Figure 3A). These experiments indicate that the same protein(s) are binding to both the ferritin and hTF IRE RNA's and that their binding affinity is greater for the ferritin IRE RNA. Quantitation by the Betascope showed that binding was decreased 50% at a 20:1 ratio of ^{3}H-labeled hTF 5′-UTR RNA to ^{32}P-labeled hTF 5′-UTR RNA probe. However, a decrease of more than 75% was realized at a 20:1 ratio of ^{3}H-labeled ferritin IRE RNA to ^{32}P-labeled hTF 5′-UTR RNA probe. A 75% decrease in binding was observed at a 50:1 ratio of ^{3}H-labeled hTF 5′-UTR RNA to ^{32}P-labeled hTF 5′-UTR RNA probe. However, a 100% decrease in binding was achieved at a 50:1 ratio of ^{3}H-labeled ferritin IRE RNA to ^{32}P-labeled hTF 5′-UTR RNA probe.

Using the ^{32}P-labeled ferritin IRE RNA as a probe (Figure 3B), the ^{3}H-labeled ferritin IRE RNA decreased homologous binding by 50% at a 10:1 ^{3}H-labeled competition to probe RNA ratio and greater than 74% binding at a 20:1 ^{3}H-labeled competition to probe RNA ratio. The ^{3}H-labeled hTF 5′-

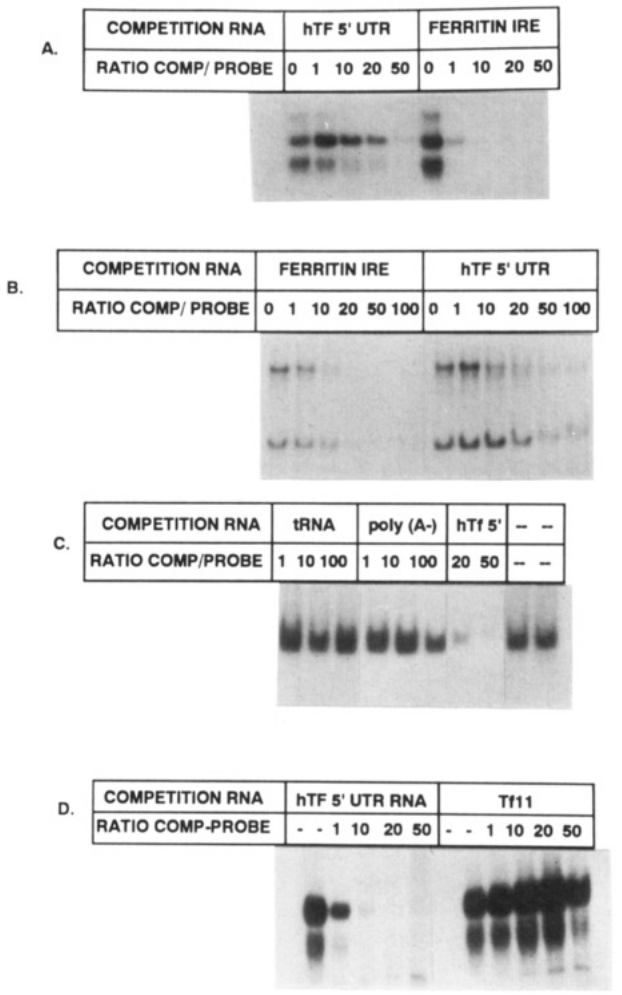

FIGURE 3: Determination of specificity of cytoplasmic protein(s) binding to the hTF 5′-UTR RNA. (A) Competition for ^{32}P-labeled hTF 5′-UTR RNA–cytoplasmic protein binding by ferritin IRE RNA and hTF 5′-UTR RNA. Liver cytoplasmic extracts were incubated with the ^{32}P-labeled hTF 5′-UTR RNA in the presence of heterologous (ferritin IRE) and homologous (hTF 5′-UTR) ^{3}H-labeled RNA. The "ratio of comp/probe" indicates molar ratios of competing RNA to probe RNA. These binding reactions were resolved on an 8% nondenaturing polyacrylamide gel. (B) Competition for ^{32}P-labeled ferritin IRE RNA–cytoplasmic protein binding by ferritin IRE RNA and hTF 5′-UTR RNA. Liver cytoplasmic extracts were incubated with the ^{32}P-labeled ferritin IRE RNA in the presence of heterologous (hTF 5′-UTR) and homologous (ferritin IRE) ^{3}H-labeled RNA. The "ratio of comp/probe" indicates molar ratios of competing RNA to probe RNA. These binding reactions were resolved on an 8% nondenaturing polyacrylamide gel. (C) Competition for ^{32}P-labeled hTF 5′-UTR RNA-cytoplasmic protein binding by nonspecific competitors. Cytoplasmic extracts were incubated with the ^{32}P-labeled hTF 5′-UTR in the presence of either poly(A$^-$) RNA or tRNA as nonspecific competitors. Competition (ratio of comp/probe) ranged from a 1- to 100-fold excess of cold competitor RNA. The hTF 5′-UTR RNA was used as a specific competitor for comparison. The last two lanes on the right are uncompeted binding assays using the hTF 5′-UTR RNA probe and cytoplasmic extracts. These binding reactions were resolved on a 5% nondenaturing polyacrylamide gel. (D) Competition for ^{32}P-labeled hTF 5′-UTR RNA–cytoplasmic protein binding by a mutated hTF (hTF11) RNA. Cytoplasmic extracts were incubated with the ^{32}P-labeled hTF 5′-UTR in the presence ^{3}H-labeled hTF 5′-UTR RNA (positive control) or in the presence of ^{3}H-labeled hTF11 RNA which is missing the upstream 19 nucleotides of the hTF RNA. The "ratio of comp/probe" indicates molar ratios of ^{3}H-labeled competing RNA to ^{32}P-labeled hTF 5′-UTR probe RNA. These binding reactions were resolved on an 8% nondenaturing polyacrylamide gel.

UTR RNA did not reduce binding as well as the ^{3}H-labeled ferritin IRE RNA against the ^{32}P-labeled ferritin IRE RNA probe: only a 5% decrease in binding was observed at a 20:1

cold competition to probe RNA ratio and about a 40% decrease in binding was achieved at a 50:1 cold competition to probe RNA ratio.

Nonspecific competitors, tRNA and poly(A⁻) RNA, did not reduce binding of cytoplasmic protein(s) to the ^{32}P-labeled hTF 5′-UTR RNA probe (Figure 3C). This indicates that the protein(s) binding the IRE's are specific. Also hTF11, a mutated hTF RNA in which the 5′ 19 bases of the putative hTF IRE have been deleted, did not interfere with cytoplasmic protein(s) binding to ^{32}P-labeled hTF 5′-UTR RNA (Figure 3D). This indicates that the 5′ portion of the UTR is required for binding and probably iron regulation.

DISCUSSION

Regulation of human TF by iron has long been recognized, but the mechanism unknown (Lane, 1966; Morgan, 1983). This study presents evidence that the regulation of human TF is posttranscriptional and probably translational. Studies using transgenic mice containing hTF-CAT chimeric genes detected only a small change of messenger RNA levels in response to 72-h iron treatments. However, CAT enzyme activity demonstrated decreases of 40–50% after 72-h iron treatment suggesting that hTF is modulated at the level of translation. Studies by Lescoat et al. (1989) using cultured rat hepatocytes support these data since they also showed iron regulation of TF with no change in TF mRNA levels. Possibilities considered for the iron regulation of hTF-CAT in transgenic mouse livers include suppression of translation directed by hTF mRNA sequences, regulation due to CAT RNA sequences, or CAT protein instability. Iron regulation via CAT mRNA or CAT protein stability is highly unlikely. Iron has been shown not to regulate CAT gene expression (Hentze et al., 1987), and CAT is a very stable enzyme; its activity withstands heating to 65 °C (Gorman et al., 1982). In addition, TF sequences are implicated in iron regulation because serum TF levels are known to be suppressed by iron overloading in humans (Lane, 1966; Morgan, 1983). The hTF-CAT transgene contains 46 of the 50 nucleotide 5′-UTR of hTF fused to the CAT protein-coding sequences; the iron cis-regulatory element for hTF is located within this 46 nucleotide TF region.

To further investigate the mechanism of iron regulation of hTF, binding assays were performed using RNA transcripts of the hTF 5′-UTR with cytoplasmic extracts from livers of iron-treated and control-salt treated mice. Iron-treated and control cytoplasmic protein extracts showed differential binding to the hTF 5′-UTR RNA with a significantly greater amount of binding in the control extracts. The hTF 5′-UTR RNA–protein complex which demonstrates differential binding in these assays comigrates with the ferritin IRE RNA–protein iron modulated band. The binding by protein(s) in the cytoplasmic extract to the hTF 5′-UTR RNA is specific. Nonspecific RNA's such as tRNA and poly(A⁻) RNA do not compete for protein binding. In addition, an RNA lacking the 5′ half of the hTF IRE, hTF11, neither competes with the radiolabeled hTF 5′-UTR RNA for cytoplasmic protein binding nor binds cytoplasmic extracts when it is radiolabeled.

In contrast, competition analysis between the hTF 5′-UTR and the ferritin IRE demonstrated that the ferritin IRE specifically eliminates binding of the hTF 5′-UTR RNA to proteins in the cytoplasmic extracts. Ferritin is known to be iron regulated via protein binding to the IRE in the 5′-UTR of the mRNA. A number of workers have shown that a single protein binds the ferritin IRE RNA (Rouault et al., 1988; Walden et al., 1988; Brown et al., 1989); this provides support for the hypothesis that the IRE-BP is also binding the hTF 5′-UTR RNA.

In the ferritin IRE model, there is an increase of mRNA translation following release of the IRE by the transacting factor IRE-BP. The binding of the IRE-BP to the ferritin IRE and the transferrin receptor IRE's is regulated in response to alterations in the cellular iron state (Goessling et al., 1992). Work by Harrell et al. (1991) using chemical and enzymatic cleavage of the ferritin IRE RNA bound by the IRE-BP indicates that the IRE-BP binds and protects the stem, loop, and bulging C of the IRE. They suggest that this IRE complex suppresses translation initiation by blocking RNA recruitment by ribosomes.

RNA sequence analysis of the hTF 5′-UTR using the RNA fold program from PC Gene predicts the formation of a stem–loop structure in the nucleotides from +5 to +32 of the 5′-UTR RNA. This version of the program does not consider G–U pairing. The predicted free energy of the structure (ΔG) is −9.8 kcal/ mol. The predicted free energy of the ferritin IRE is −0.3 kcal/mol (Barton et al., 1990). Therefore, the proposed hTF stem–loop structure is more stable than the ferritin stem–loop structure.

Modulation of translation of the TF mRNA is different from that observed in ferritin mRNA. Iron treatment in humans and in transgenic mice containing a chimeric hTF-CAT gene results in a 40–50% decrease in serum hTF levels (Lane, 1966; Morgan, 1983). This subtle regulation is quite different from ferritin which can increase 30–50-fold (3000–5000%) in response to iron level changes (Shull & Theil, 1982, 1983; Aziz & Munro, 1986; Didsbury et al., 1986). Transferrin mRNA contains a putative IRE-like stem–loop structure in its 5′-UTR. The binding assays presented in this paper suggest that an increase in binding of cytoplasmic factors to the TF 50 nucleotide 5′-UTR results in an increase in TF mRNA translation; this is the opposite of ferritin regulation which with increased iron levels shows a decrease in binding to the mRNA and an increase in ferritin synthesis (Aziz & Munro, 1986; Hentze et al., 1987a,b). One example of the hTF type of modulation is seen with the bacteriophage Com protein (Wulczyn et al., 1991). The Com protein binds specifically to the side of the stem–loop structure of the Mom RNA. The action of binding by the Com protein up-regulates Mom mRNA translation. Such studies suggest that binding of the IRE-BP could up-regulate hTF translationally like Mom. It has been proposed that the IRE-BP in a high-affinity state binds the regulatory sequences in one manner and the IRE-BP in a low-affinity state binds in a different manner (Rouault et al., 1990; Hentze et al., 1989). Perhaps, in the hTF 5′-UTR, the larger loop requires high-affinity binding to open the loop, therefore promoting recruitment of hTF RNA by the ribosomes for translation. With this type of mechanism, the IRE-BP binding may be weak enough that, after opening the hTF stem–loop, the IRE-BP falls off the hTF 5′-UTR and allows the translational machinery to proceed. This model is supported by the greater affinity of the binding protein(s) for ferritin than the hTF sequences which are shown in this study.

As discussed above, there may be similarities in iron regulation of ferritin and hTF due to IRE-BP binding, but there must also be important differences since ferritin is up-regulated by iron while hTF is down regulated by iron. That differences exist is demonstrated by the significantly different binding patterns of proteins in cyptoplasmic extracts to the ferritin IRE RNA versus the hTF 5′-UTR RNA. Variations in RNA sequences and secondary structures in the IRE's probably mediate their divergent regulation.

The regulation of ferritin and transferrin receptor by iron involves the RNA's and has been extensively characterized. This work indicates that the third major protein involved with iron metabolism, TF, is also regulated at the RNA level. Future studies will include characterization of the hTF stem–loop structure and functional assays of this translational regulatory element.

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