A FERRITIN-RESPONSIVE INTERNAL RIBOSOME ENTRY SITE REGULATES FOLATE METABOLISM
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Running Title: Ferritin-Responsive IRES
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Cytoplasmic serine hydroxymethyltransferase (cSHMT) enzyme levels are elevated by the expression of the heavy chain ferritin (FTH) cDNA in cultured cells without corresponding changes in mRNA levels, resulting in enhanced folate-dependent de novo thymidylate biosynthesis and impaired homocysteine remethylation. In this study, the mechanism whereby FTH regulates cSHMT expression was determined. cSHMT translation is shown to be regulated by a FTH-responsive internal ribosome entry site (IRES) located within the cSHMT mRNA 5'-untranslated region (5'UTR). The cSHMT 5'UTR exhibited IRES activity during in vitro translation of bicistronic mRNA templates, and in MCF-7 and HeLa cells transfected with bicistronic mRNAs. IRES activity was depressed in FTH-deficient mouse embryonic fibroblasts and elevated in cells expressing the FTH cDNA. FTH was shown to interact with the mRNA binding protein CUGBP1, a protein known to interact with the α and β subunits of eukaryotic initiation factor eIF2. siRNA-mediated depletion of CUGBP1 decreased IRES activity from bicistronic templates that included the cSHMT 3'UTR in the bicistronic construct. The identification of this FTH-responsive IRES represents a mechanism that accounts for previous observations that FTH regulates folate metabolism.

The term folates refers to a family of enzyme cofactors that carry and chemically activate single carbons at three different oxidation states and function in a metabolic network known as folate-mediated one-carbon metabolism (1). Folate is required for the synthesis of purines and thymidylate and for the remethylation of homocysteine to methionine (Figure 1). Methionine can be adenosylated to form S-adenosylmethionine (AdoMet), which is a cofactor and primary methyl donor for numerous methylation reactions including DNA, RNA, protein, lipid and neurotransmitter methylation (2). Disruptions within this metabolic network are common and result from nutrient deficiencies and/or penetrant single nucleotide polymorphisms (3). Impairments in one-carbon metabolism alter gene expression and genome stability and are associated with numerous pathologies and developmental anomalies including cancer, cardiovascular disease and neural tube defects (3). Biomarkers of impaired folate-dependent one-carbon metabolism include elevated uracil content in DNA, hypomethylated DNA and elevated S-adenosylhomocysteine (AdoHcy) and homocysteine.

Cytoplasmic serine hydroxymethyltransferase (cSHMT) is a folate-dependent enzyme that preferentially partitions activated carbons toward the thymidylate biosynthesis pathway and impairs the homocysteine remethylation pathway (4) (Figure 1). Previous studies have demonstrated that cSHMT enzyme levels respond to changes in heavy chain ferritin (FTH) expression but not light chain ferritin (FTL) expression (5). Transfection of the FTH cDNA in cultured cells markedly elevates cSHMT enzyme levels without affecting mRNA levels, leading to more efficient de novo thymidylate biosynthesis and impaired homocysteine remethylation (4,5). FTH is a subunit of the iron storage protein ferritin, which is a spherical and multimeric protein that is comprised of 24 heavy chain and light chain subunits. The relative ratio of heavy chain and light chain polypeptides within a ferritin heteropolymer varies by tissue type (6). FTH possesses ferroxidase activity; the active site binds and oxidizes Fe2+ to Fe3+ which is subsequently stored in the core of the ferritin molecule. In this manner, FTH prevents the formation of reactive oxygen species (ROS) resulting from Fe2+ oxidation through the Fenton
reaction. FTL does not exhibit ferroxidase activity but is thought to aid in iron mineralization (7). FTH is an essential gene in mice; FTH-null embryos do not survive past past embryonic day 9.5 (8).

The human cSHMT mRNA has two alternatively spliced forms of its 5’UTR that are encoded by exons 1-3 (9). Exon 2, which encodes an Alu J SINE insertion in reverse orientation (10), is alternatively spliced in a cell-specific manner (11). The cSHMT hnRNA is spliced to yield a full-length 332 nucleotide 5’UTR (AluUTR) which has ~62% GC content and includes exon 2, and a shorter 193 nucleotide 5’UTR (UTR) that lacks exon 2 and has ~71% GC content (11). The high GC content and extensive secondary structure of the 5’UTR indicates the potential for translational regulation of cSHMT expression.

Translation can be regulated by the sequence and structure of the 5’UTR of mRNAs (12). Eukaryotic mRNAs contain 7-methyl-guanosine 5’ cap structure (m7Gp) that serves as the docking site for the initiation factor eIF4F and the 43S ribosomal subunit. The 43S ribosomal subunit scans through the 5’UTR of the mRNA and unwinds secondary structure using the helicase activity of eIF4A until it reaches an AUG start codon where the 60S subunit joins the complex to initiate translation (13). Depending on the length and secondary structure of the 5’UTR, scanning may impede cap-mediated translation (14). External stress such as nutrient deprivation and heat-shock can impair cap-initiated translation (15). Translation initiation can occur in a cap-independent manner by recruitment of the 43S ribosomal subunit to internal sequences within the 5’UTR. This process is mediated by IRES Trans-Acting Factors (ITAFs) that bind to internal ribosome entry sites (IRES) (16). Here, we present evidence that indicates cSHMT translation is regulated by a FTH-responsive IRES.

Materials and Methods

Materials. All chemicals were purchased from SIGMA unless stated otherwise. Restriction/modification enzymes were from New England Biolabs, Stratagene, Ambion or Promega. Sheep anti-human cSHMT and sheep anti-human FTH antibodies were described previously (5). Mouse anti-human CUGBP1 and mouse anti-HA antibodies were purchased from Santa Cruz Biotechnology, Inc.

Cell Culture and Reagents. Human mammary adenocarcinoma cells (MCF-7) and HeLa cells were obtained from ATCC. Cells were cultured with α-minimal essential medium (α-MEM; Hyclone Laboratories) supplemented with 11% fetal bovine serum (Hyclone Laboratories) and maintained at 37°C in a 5% CO2 atmosphere.

Generation of Stable Cell Lines. The human FTH cDNA lacking the 5’ and 3’UTRs was generated by PCR using reverse transcribed MCF-7 cell total RNA as a template, and cloned into eukaryotic expression vector pcDNA3.1(+/−) (Invitrogen) using standard procedures as described previously (5). Briefly, the forward primer was 5’ TA GGATCC ATG ACG ACC GCG TCC the underlined sequence is a Bam HI site to aid in cloning into the vector. The reverse primer was 5’ TA CGGCCG TTA GCT TTC ATT ATC with the underlined sequence a Not I site. The FTH/pcDNA3.1 linear DNA approximately 4μg, was transfected in 70-80% confluent MCF-7 cells using Fugene transfectamine from Roche, following the manufacturer’s suggested protocol. The transfected cells were grown in DMEM for 24 hours, then one 100mm plate of transfected cells were trypsinized and passaged into ten, 100mm, plates and placed in DMEM with 1mg/ml of Geneticin, following Invitrogen’s suggested protocol for selection of FTH/pcDNA3.1 expressing cells. The resulting individual clones were then selected and passage into 6 well cell culture cluster plates and maintained in DMEM with 800μg/ml of Geneticin. A DMSO stock and cell pellet for each clone was collected for western analysis for the expression of FTH, cSHMT and GAPDH.

Isolation of mouse embryonic fibroblasts (MEFs). Embryos were generated by crossing fth+/− mice (gift from C. Beaumont) on a Balb/c background (8) and genotyped by PCR at embryonic day 14 (E14) (8,17). Fth+/− and fth−/− fibroblasts were isolated from the embryos and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) following the protocol described elsewhere (18).
**Immunoblotting.** Cells were harvested by trypsinization and washed with phosphate-buffered saline, then lysed at 100°C for 10 min in buffer containing 2% SDS, 100 mM dithiothreitol, and 60 mM Tris (pH 6.8). Cellular proteins (40-120 µg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10-12% polyacrylamide gels. Proteins were transferred at 4°C to a polyvinylidene fluoride microporous membrane (Millipore Corp.) using a Transblot apparatus (Bio-Rad). Following transfer, membranes were incubated with primary antiserum for 15 h at 4°C, washed with phosphate-buffered saline and 0.1% Tween 20 and then incubated for 1-3 h with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the horseradish peroxidase SuperSignal chemiluminescent substrate system (Pierce). For cSHMT detection, affinity-purified sheep anti-human cSHMT antibody was diluted 1:20,000, and rabbit anti-sheep IgG-HRP (Pierce) was diluted 1:5000. For FTH detection, affinity-purified sheep anti-human FTH antibody was diluted 1:500, and rabbit anti-sheep IgG-HRP (Pierce) was diluted 1:5000. For CUGBP1 detection, mouse monoclonal anti-human CUGBP1 antibody was diluted 1:500, and goat anti-mouse IgG-HRP (Pierce) was diluted 1:5000. Mouse anti-human glyceraldehyde-3-phosphate dehydrogenase antibody (Novus) was used at a 1:100,000 dilution. The protein bands were quantified using ChemiImager 4400 from Alpha Innotech Corp. (San Leandro, CA).

**Yeast-two hybrid assay.** The human FTH cDNA was amplified and cloned into the pGBK plasmid using the following primers: 5'-TAATACGACTCAGATATG-3' and 5'-AAGCTTAGATCCATTAGGAGAAAGCTTG-3'. The BamHI and SalI sites are shown in bold. The pGBK-FTH vector was transformed into yeast strain AH109 and stable clones were maintained in Trp- dropout medium Y187 yeast cells with a pretransformed HeLa cDNA library (Clonetech) were mated to the pGBK-FTH-AH109 cells following the Clonetech Matchmaker protocol. After a 24 h mating, cells were plated on His-, Ade-, Leu-, Trp- dropout medium containing X-α-gal, and positive colonies were isolated after a 4 day incubation at 30°C. DNA from positive clones was isolated and sequenced at the Cornell BioResource Center. Clones were validated against negative controls according to the Matchmaker protocol.

**Immunoprecipitations.** Cells were lysed in M-PER buffer containing protease inhibitor cocktail. Extracts (150 µg) were incubated for 30 min at 4°C with 30µl of protein A/G conjugated agarose beads to remove nonspecific matrix-binding proteins. The precleared extracts were incubated with 5 µg of either mouse anti-human CUGBP1 or mouse anti-human HA antibodies overnight at 4°C with 30µl of protein A/G agarose beads. The beads were collected and washed 3 times with 1X phosphate-buffered saline. SDS-PAGE sample buffer containing 2% SDS was added to the beads and the sample was heated at 100°C to release bound proteins from the beads. The samples were analyzed by immunoblotting as described above.

**Generation of bicistronic constructs.** Bicistronic constructs were generated in the pSP64 poly A vector (Promega). The base construct contained (in the 5' to 3' direction) the Renilla luciferase cDNA (containing three tandem stop codons 3' of the open reading frame) cloned into the HindIII site, the cSHMT 5'UTR cloned into the HindIII and NcoI sites and Firefly luciferase cDNA cloned into the NcoI and XbaI sites of the vector. The Renilla luciferase cDNA containing the three tandem stop codons was amplified using the following primers: 5'-TATAATCGACTCAGATATGAGAAAGCTTG-3' and 5'-AAGCTTAGATCCATTAGGAGAAAGCTTG-3'. The HindIII sites are in shown in bold, the T7 promoter is in italics and the three stop codons are underlined (reverse primer). The human cSHMT 5'UTR, AluUTR (332 nt) and the alternatively spliced variant UTR lacking exon 2 (193bp) have been described previously (9). The BiP IRES (a gift from Peter Sarnow) was subcloned into this vector replacing the cSHMT 5'UTR cloned into the NcoI and HindIII sites. The reverse complement of the cSHMT 5'UTR (rcUTR) was cloned into the base bicistronic construct using the HindIII and NcoI sites such that the reverse complement of the UTR was located between the two reporter genes. The mouse cSHMT 5'UTR was subcloned into the bicistronic construct, using the primers
5’TAAGCTTGCGATCCACTTGC-3’ and
5’-TACCATGGTGCACTGGTCCAGAG-3’.
For the construction of a truncated UTR that
lacked 30nt from the 3’ end, the reverse primer
used was 5’- CCTGTCCTAAGCCGCG -3’ (NcoI site in bold (the forward primer was
the same as for the full length UTR)). The
tUTR was subcloned into the bicistronic
construct, replacing the UTR. For constructs
containing the eSHMT UTR at the 5’ end of the
Renilla open reading frame, the eSHMT 5’UTR
was cloned using the primers 5’-
TAGCTAGCGCCTGGCGCGCAG and 5’-
TAAAGCTTCATTGCACTGGTTCGAAG-3’
which contain NheI and HindIII sites in bold,
respectively. The eSHMT 3’UTR (635 nt) was
amplified from reverse
transcribed total RNA isolated from MCF-7
cells (iScript kit, BIORAD) by PCR using the
following primers: 5’-
TCGAGGGAGGAGCCGGCCACTCTG-3’
and
5’-TACCGGGCTGGTGTCTCAACC-3’.
The SmaI sites are in bold. All constructs
were sequenced verified.

Generation and purification of bicistronic
mRNA. DNA templates (2 μg) were linearized
with EcoRI and purified using the Roche PCR
clean-up column. The template was transcribed
using either the T7 or SP6 Megascript (Ambion)
kit for use in in vitro translation assays (5mM
cap analog was added to reactions according to
the manufacturer’s instructions), or the T7 or
SP6 mMessage mMACHINE (Ambion) kit for use
in mRNA transfections (which includes 4mM
cap analog in the reacton mix). The crude
mRNA was treated with DNase I for 15 min at
37°C and precipitated overnight in 2 M LiCl at
-80°C. All RNA procedures were conducted
under RNase-free conditions and all mRNA was
stored with RNase inhibitor (Promega). The
mRNA was further purified with oligo-dT beads
(MicrofastTrac 2.0, Invitrogen) before use to
ensure that only full length PolyA RNA served
as templates. For preparation of radiolabeled
mRNA for in vitro translation experiments, 50μCi of [α-32P]-labeled rUTP (800Ci/mM,
Perkin Elmer) was included in in vitro
transcription assays. The assays were then
carried out as described above. The mRNA purity
was verified by electrophoresis.

In vitro translation assays. All experiments were
performed at a final mRNA concentration of 2 nM
(about 5 ng/μl) to ensure that the translation system
was below saturation. The mRNA templates were
heated to 65°C for 10 min and then cooled to room
temperature prior to their addition to the reaction
mixture to allow uniform secondary structures to
form. Translation reaction mixtures (25 μl)
containing 12.5 μl of rabbit reticulocyte lysate,
amino acid mixture (1mM each),1 mM MgOAc,
2 mM dithiothreitol, 100 ng yeast tRNA and 35 mM
KCl (Flexi rabbit reticulocyte lysate system kit,
Promega) were incubated at 30°C for 15 min with
the bicistronic mRNA templates. All
enzyme/mRNA incubations contained RNase
inhibitor (Promega). The translation reaction
mixtures were quenched on ice and luciferase
activity was determined using the Dual-Glo kit from
Promega. The activities were read on a Veritas
microplate luminometer (Turner Biosystems).

mRNA transfections. Bicistronic mRNAs were
transfected into HeLa, MCF-7 or Mouse Embryonic
Fibroblasts (MEF) cells grown to 90-95%
confluence in 6-well plates. Transfections were
carried out using 2.5 μg capped mRNA, 500ul of
OptiMEM I (Invitrogen) and 5μl of DMRIE-C
transfection reagent (Invitrogen) per well. After a 4
h incubation at 37°C, the transfection solution was
removed, α-MEM (1 ml) was added to each well
and the cells incubated for an additional 16 h under
standard culture conditions. Translation from the
bicistronic constructs was quantified by measuring
luciferase activity using the Dual-Glo kit from
Promega following the manufacturer’s instructions.
Firefly and Renilla luciferase activities were
recorded using a Veritas luminometer (Turner
Biosystems). To inhibit cap-mediated translation,
rampamycin dissolved in ethanol was added to the
cells 4 h before transfection to a final concentration
of 20ng/ml. An equal volume of carrier (ethanol)
was added to control samples.

siRNA transfection. The CUGBP1 siRNA was
purchased from Qiagen’s pre-designed HP siRNA
library (Hs_CUGBP1_2 HP siRNA (sense: r(GGA
ACU CUU CGA ACA GUA U)dTdT; antisense:

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r(AUA CUG UUC GAA GAG UUC C)dCdG)). Ambion silencer negative control siRNA (cat no: 4611) served as a control for all siRNA transfections. The transfections were performed using HiPerFect siRNA transfection reagent (Qiagen). HeLa cells were plated to 20% confluence in 6 well plates the day of transfection. 5nM of either CUGBP1 or control siRNA was added to each well with the HiPerFect reagent following the manufacturer’s instructions. The cells were incubated with siRNA for 48 h under normal culture conditions (37°C, 5% CO2). After incubation, cells were either lysed and subjected to SDS-PAGE/immunoblotting analysis to determine knockdown efficiency or used in mRNA transfection experiments as described above.

Results

The human cSHMT 5’UTR exhibits IRES activity. The contribution of the cSHMT 5’UTR and 3’UTR to the regulation of cSHMT translation was investigated by generating a series of bicistronic mRNAs that served as templates for in vitro translation reactions (Table 1). The base bicistronic template contained a 5’ capped (7-methyl-guanosine analog) Renilla luciferase (Rluc) open reading frame (ORF) that terminated with three tandem, in-frame stop codons (TAA). The cSHMT 5’UTR (UTR or AluUTR) was placed at the 3’ end of the Rluc ORF and 5’ of the Firefly luciferase (FFluc) ORF, which was followed by a thirty nucleotide polyA tail (Table 1, template 1). From this mRNA template, translation initiation of the Rluc ORF occurs through cap-mediated binding of the eIF4F complex and recruitment of the ribosome. Translation of the FFluc ORF could potentially occur through three independent mechanisms: 1) mRNA degradation resulting in the formation of a monocistronic FFluc template, 2) failure of the ribosome to dissociate from the template at one of the three tandem termination codons located 3’ of the Rluc ORF, otherwise known as translation re-initiation; or 3) cap-independent recruitment of the ribosome by the cSHMT 5’UTR serving as an IRES. To negate the role of mRNA degradation in FFluc translation, all bicistronic mRNAs were labeled with [α-32P]-rUTP and the integrity of the transcript was verified by gel electrophoresis prior to and following all in vitro translation reactions; no evidence for mRNA splicing or degradation was observed for any of the templates (Supplementary Figure 1).

The ability of the cSHMT 5’UTR to function as an IRES was investigated during in vitro translation reactions and in transiently transfected MCF-7 cells using bicistronic mRNA templates. The base bicistronic template (Table 1, template 1) yielded both Rluc and FFluc activity in in vitro translation reactions and in transfected cells, thereby providing evidence that the cSHMT 5’UTR exhibits IRES activity, which is expressed in Table 1 as the FFluc/Rluc ratio. Because others have shown that IRES function is orientation dependent (19), a template containing the reverse complement of the cSHMT 5’UTR (rcUTR) was generated (Table 1, template 3). Upon transfection in MCF-7 cells, rcUTR (template 3) yielded 3% of the IRES activity observed from the UTR (template 1), providing evidence that the FFluc activity generated from template 1 did not result from re-initiation of translation and that the cSHMT UTR is an IRES. In in vitro translation reactions, rcUTR IRES activity was 2% of that generated from template 1. Neither the UTR nor the rcUTR contained AUG initiation codons, ruling out the possibility of premature translation initiation within the 5’UTR. These initial findings provide evidence that the cSHMT 5’UTR contains IRES activity that cannot be explained by mRNA degradation of the bicistronic template (Supplementary Data, Figure 1) or ribosomal re-initiation (Table 1, template 3).

The alternatively spliced cSHMT UTR lacking exon 2 (template 1) exhibited 2-fold greater activity than the full length AluUTR (template 2) in both in vitro translation reactions and in transfected cells. Deletion of 30 nucleotides from the 3’ end of the cSHMT 5’UTR (trUTR) (Table 1, template 4) immediately proximal to the AUG codon decreased IRES activity by about 75% in in vitro reactions; similar results were observed upon deletion of sequences from the 5’end of the cSHMT 5’UTR, indicating that the entire UTR is required for maximal IRES activity (data not shown).
The mouse cSHMT 5'UTR (mUTR) was placed within the bicistronic construct to determine if the IRES activity associated with the human cSHMT UTR is species specific (Table 1, template 5). The mUTR exhibited only 27% of the IRES activity observed for the human 5'UTR in in vitro translation reactions, indicating that the mUTR is not as efficient as the human cSHMT IRES. The mUTR is 210 nt long with 54% GC-content; the human 5'UTR has 71% GC content. The mouse and human 5'UTRs share only 42% sequence identity and the mouse UTR does not contain a SINE element, nor is it alternatively spliced like the human cSHMT 5'UTR.

The cSHMT mRNA contains a 3'UTR of 635 nucleotides. IRES activity has been shown to be influenced by 3'UTR sequences (20) (21), which can destabilize RNAs (22) or provide favorable contacts with the translation initiation machinery to enhance translation initiation (23). Modification of template 1 by placement of the cSHMT 3'UTR at the 3' of the FFLuc ORF (Table 1, template 6) increased the IRES activity by at least 2-fold compared to IRES activity generated from the UTR alone (template 1) in both in vitro reactions and in transiently transfected MCF-7 cells, indicating that the cSHMT 3'UTR stimulates IRES activity. Inclusion of the cSHMT 3'UTR in the template containing the rcUTR (Table 1, template 7) did not stimulate IRES activity.

**Strength of the cSHMT IRES.** The efficiency of cSHMT IRES-mediated translation initiation relative to cap-mediated translation initiation was investigated. Cap-mediated translation of the endogenous cSHMT mRNA requires ribosomal scanning through the cSHMT 5'UTR, which is known to decrease translation rates. IRES-mediated translation initiation can occur either independent of ribosomal scanning or through a "land and scan" mechanism that involves both internal entry and limited scanning (24). Additional mRNA templates were synthesized to include the cSHMT UTR (Table 1, template 8) at the 5' end of the Rluc ORF. Translation from template 8, which enables both cap-mediated and IRES-mediated translation of Rluc, increased the FFLuc/Rluc activity ratio 3.7 fold compared to translation from the base template 1 in in vitro translation reactions. The data from template 8 indicates that cSHMT IRES-mediated translation alone, indicated by FFLuc activity, is about half as efficient as translation that involves potential for both cSHMT IRES-mediated and cap-mediated translation initiation (Rluc activity).

The IRES activity of the cSHMT 5'UTR was also compared to the activity of the immunoglobulin heavy-chain binding protein (BiP) 5'UTR (25,26). The BiP IRES is one of the most robust and well-characterized mammalian IRESs identified to date. A bicistronic mRNA template containing the BiP IRES (Table 1, template 9) yielded similar activity as observed for the cSHMT 5'UTR (Table 1, template 1), but less than 50% of the activity as observed for template 6 (which contains the cSHMT 3'UTR) during in vitro translation reactions. In transient transfections, the cSHMT 5'UTR exhibited only 50% of BiP IRES activity, but template 6 yielded similar activity to the BiP IRES. The cSHMT 3'UTR also stimulated BiP IRES activity 1.6 fold in in vitro translation reactions when placed 3'of the FFLuc ORF (template 10). However, unlike the cSHMT IRES, no stimulation of the BiP IRES by the cSHMT 3'UTR was observed in transfected MCF-7 cells (Table 1).

Under normal cellular conditions, 5'-cap structures and IRES elements compete for limiting concentrations of initiation factors required for translation. Like most cellular IRESs identified to date, the cSHMT 5'UTR does not compete well with cap-dependent translation, as evidenced by the reduced FFLuc/Rluc ratio in transfected cells compared to nuclease-treated in vitro translation extracts. However, under conditions of cellular stress, quiescence, and apoptosis, the binding of the translation initiation machinery to the 5'-cap is physiologically impaired, enabling more efficient IRES-mediated translation initiation. In order to determine the strength of the cSHMT IRES in a cellular environment that is more favorable to cap-independent translation, we reduced the availability of 5'-cap structures by preincubating the cells with the macrolide antibiotic, rapamycin for 4 h prior to transfection (27). Rapamycin inhibits the mTOR kinase pathway and inhibits cap binding by eIF4E (28). Under these conditions, IRES activity increased 5-fold in rapamycin-treated MCF-7 cells.
transfected with the base template 1 compared to nontreated cells (Table 1); the activity ratio was increased three-fold for the bicistronic construct containing the BiP IRES (Table 1, template 9). Under these conditions, the FFluc activity generated by cSHMT- and BiP-IRES-mediated translation was 15% and 18% of Rluc activity generated by cap-mediated translation, respectively, from the bicistronic construct.

Heavy chain ferritin modifies IRES activity. The role of FTH in IRES-mediated translation initiation of cSHMT was investigated by transfecting the base bicistronic template 1 (Table 1) containing the cSHMT 5’UTR in FTH-deficient cells. Mouse embryonic fibroblasts (MEFs) isolated from fth<sup>−/−</sup> embryos exhibited reduced FTH protein levels (~50%) compared to MEFs isolated from their wild-type fth<sup>+/+</sup> littermates (data not shown) (8). IRES activity was decreased 30% in MEFs isolated from fth<sup>−/−</sup> compared to MEFs isolated from fth<sup>+/+</sup> embryos (Figure 2). The IRES activity of the BiP bicistronic construct was not affected by fth genotype, indicating that only the cSHMT IRES is ferritin responsive. IRES activity was also investigated in 3 independent stable MCF-7 cell lines expressing the FTH cDNA (Table 2). IRES activity was elevated in all three cell lines, although the results did not reach statistical significance for clone 3 (Table 2). These results provide a mechanism to account for the increase in cSHMT protein resulting from FTH expression in cultured cells (5).

Heavy Chain Ferritin Interacts with CUGBP1. FTH could stimulate IRES activity either indirectly through signalling pathways (29) or directly by acting as an Internal ribosome entry site Trans-Acting Factor (ITAF). To differentiate between these two potential mechanisms, the role of FTH in IRES-mediated cSHMT translation initiation was explored by screening for FTH binding partners. Previous studies have demonstrated that ferritin is a high-affinity but non-specific RNA-binding protein in rat liver (30), but its interaction with components of the translational machinery has not been reported. The FTH cDNA was fused to the Gal4 DNA binding domain and the fusion protein was screened against a HeLa cDNA library by yeast two-hybrid assay. Of the 42 clones isolated, 85% were identified were FTL or FTH (Table 3). Three clones were identified as CUGBP1. The CUGBP1-FTH interaction was confirmed through replicate plating and by co-immunoprecipitation of FTH with mouse anti-CUGBP1 antibodies (Figure 3). CUGBP1 is a mRNA-binding protein that has been implicated in translational regulation of C/EBP beta (31) by interacting with the alpha and beta subunits of initiation factor eIF2 (32). This interaction of eIF2 with CUGBP1 enhances C/EBP beta translation (33). CUGBP1 binds several RNA motifs including the bruno response element (BRE)(34). The cSHMT 3’UTR, but not the 5’UTR contains this cis element at nucleotide positions 501-508 (UGUAUGUU).

The contribution of CUGBP1 to IRES-mediated translation of cSHMT was investigated by siRNA-mediated knockdown of CUGBP1 in HeLa cells (Figure 4). HeLa cells transfected with CUGBP1 siRNA were depleted of CUGBP1 protein compared to cells treated with scrambled siRNA (Figure 4A). CUGBP1 depletion decreased cSHMT protein levels approximately 25% in HeLa cells (Figure 4A). Transfection of the base bicistronic template 1 containing the cSHMT UTR resulted in a 20% decrease in IRES activity in CUGBP1-depleted cells compared to cells transfected with scrambled siRNA, however this was not statistically significant (p>0.05) (Figure 4B). When the cSHMT 3’UTR was included in the bicistronic construct (Table 1, template 7) CUGBP1 depletion resulted in a 40% decrease in IRES activity (Figure 4C). Ongoing studies indicate that CUGBP1 levels in the cell greatly exceed the concentration of cSHMT mRNA, accounting for the modest decrease in IRES activity with CUGBP1 depletion (unpublished data). Collectively, these data indicate that the 3’UTR-CUGBP1 interaction stimulates IRES activity, and that FTH exerts its stimulatory effect by interacting with CUGBP1.

Discussion

This study provides evidence that the iron storage protein ferritin regulates the expression of cSHMT by enhancing the activity of an IRES element within the cSHMT 5’UTR. Several IRES elements have been demonstrated to enable adaptive responses to environmental stresses including heat shock and hypoxia (35), but few have been shown to confer
nutrient regulation, and to date none enable cross talk among metabolic pathways (36,37). The role of FTH in modulating intracellular free iron concentrations, and its ability to enhance the expression of a cSHMT may enable coordinate regulation of folate and iron metabolism, and potentially enable the regulation of one-carbon metabolism by iron.

The cSHMT 5’UTR displays comparable IRES activity to the BiP 5’UTR (26) in both in vitro translation assays and in mRNA transfection studies when the cSHMT 3’UTR is included in the template. Characterization of mammalian IRES elements has been controversial because of the lack of adequate controls that: 1) validate RNA stability, 2) monitor splicing of bicistronic RNAs and 3) monitor for cryptic promoter elements in 5’UTRs (38,39). In this study, we avoided these potential limitations by ensuring the integrity of the mRNA prior to and following in vitro translation assays. We also avoided potential cryptic promoter activity in the 5’UTR of cSHMT by conducting mRNA as opposed to DNA transfections in cells.

The addition of the cSHMT 3’UTR to the 3’ end of the bicistronic construct (Table 1, template 6) increased IRES activity 2-3 fold in MCF-7 cells and in in vitro translation assays. Elements in the 3’UTR and the poly(A) tail of many genes have been shown to influence translation either positively(13,40,41) or negatively (42-45) (46,47). 3’UTRs from other genes have been shown to enhance and/or stimulate both viral (20,48) and mammalian IRES activity (49). Although 3’UTR elements can potentially affect both cap- and IRES-mediated translation, the cSHMT 3’UTR did not influence the BiP IRES activity (as defined as the FFluc/Rluc ratio) in cell transfection studies, indicating that the cSHMT 3’UTR does not preferentially stimulate the BiP IRES-mediated translation relative to cap-mediated translation.

The relative strength of many IRES elements in RNA transfection experiments has been raised as a concern (38). In this study, we demonstrate that cSHMT IRES-mediated translation likely occurs with near equal or greater efficiency as cap-mediated translation in in vitro translation reactions. Studies of template 8 (Table 1) demonstrate that the IRES activity of the cSHMT 5’UTR was about 50% as efficient as translation from the cSHMT 5’UTR when both cap- and IRES-mediated translation was enabled. Furthermore, inclusion of the cSHMT 3’UTR further stimulated cSHMT IRES activity by 2.6 fold (Table 1, template 6), indicating that IRES-mediated translation may be more robust than cap-mediated translation. In transfected cells, the cSHMT IRES activity was only 6% of that observed for cap-mediated translation initiation when the cSHMT 3’UTR was included in the template (template 6). We were unable to compare cSHMT IRES activity to cap-mediated translation with ribosomal scanning (template 8) because neither FFluc nor Rluc activity was generated from this construct upon transfection in cells for reasons unknown. However, we expect that inclusion of ribosomal scanning in the template should increase substantially the relative activity of IRES-mediated translation relative to cap-mediated translation in cells.

This study suggests that FTH and CUGBP1 are ITAFs that interact during cSHMT IRES-mediated translation, and that CUGBP1 functions through the 3’UTR (Figure 4) whereas FTH functions through the 5’UTR (Figure 2). siRNA-mediated depletion of CUGBP1 lowered IRES activity only in constructs containing the cSHMT 3’UTR, indicating the interaction of CUGBP1 and the 3’UTR. The cSHMT 3’UTR has a CUGBP1 response element at nucleotide positions 501-508 (UGUAUGUU) that may be a binding site for CUGBP1. FTH depletion lowered IRES activity in MEFs, whereas expression of the human FTH cDNA in MCF-7 cells increased IRES activity. The effect of FTH on IRES activity was independent of the presence of the cSHMT 3’UTR in the bicistronic construct for both cell models. Based on our data we can propose a model for the involvement of FTH and CUGBP1 in cSHMT IRES activation (Figure 5). CUGBP1 binds to its consensus sequence on the 3’UTR of cSHMT and ferritin, which has previously been shown to bind mRNA (30) binds at the 5’UTR. The physical interaction between CUGBP1 and FTH recruits the translation initiation machinery to the cSHMT IRES. Ongoing studies will provide additional evidence to develop and differentiate between these models.
This study also provides a mechanism for cross talk between folate and iron metabolism (50). Increased FTH expression occurs independently of iron concentrations in cancer (51) and pregnancy (52), and FTH is a component of the TNFα-induced inflammatory response (29). FTH sequesters intracellular free iron and creates a “functional” iron deficiency in the cell when its expression is increased independent of increased intracellular iron uptake. By sequestering iron, increased FTH expression helps prevent the formation of iron-induced reactive oxygen species (ROS) (53), which during inflammation increase DNA damage and the need for DNA repair (54,55). Folate metabolism is also altered during these physiological states (51), and FTH-induced increases in cSHMT expression during inflammation likely provide thymidylate nucleotides for DNA replication and repair. Previously, we have shown that FTH-mediated increases in cSHMT expression alters the flux of folate-dependent one-carbon units to increase de novo thymidylate biosynthesis while impairing homocysteine remethylation (4,5). Whereas induction of FTH levels by expression of the FTH cDNA elevated cSHMT in cultured cells, induction of FTH expression by the addition of ferric citrate to culture medium did not induce cSHMT levels (5), indicating that it is the FTH protein, and not the FTH-induced changes in the cellular free iron pool, that regulates cSHMT activity. Identifying the physiological states that induce cSHMT IRES activity will be key to further characterization of the mechanism and physiological function of this FTH-inducible IRES element, and its potential role in folate-related pathologies.

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Table 1: Detection of IRES activity using bicistronic constructs. Each bicistronic construct contained renilla luciferase cDNA (Rluc) with a cap analog at the 5’ end and three sequential in-frame stop codons at the 3’ end. The cSHMT 5’UTR (or modifications thereof) or a known IRES (BiP) was inserted 3’ of the Rluc gene. The firefly luciferase gene (FFluc) is the second cistron in the RNA and was placed 3’ of the IRES elements. Some constructs contained the cSHMT 3’UTR located 3’ of the FFluc ORF. IRES activity is expressed as the ratio of total firefly luciferase activity over total renilla luciferase activity (FFluc/Rluc). All mRNA transfections were performed in MCF-7 cells. All values are the average of at least 5 measurements and variation is expressed as standard deviation of the mean.
| Template Number | Bicistronic mRNA | Potential IRES | Activity in vitro translation (FFluc/Rluc) | Activity mRNA transfection (FFluc/Rluc) | Activity mRNA transfection (10 ng/ml Rapamycin) (FFluc/Rluc) |
|----------------|-----------------|----------------|-------------------------------------------|----------------------------------------|---------------------------------------------------------------|
| 1              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT 5'UTR | 0.15 ± 0.01 (100%) | 0.03 ± 0.002 (100%) | 0.15 ± 0.04 |
| 2              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT 5’-AluUTR | 0.07 ± 0.004 (47%) | 0.016 ± 0.002 (53%) |
| 3              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT Reverse Complement 5'UTR | 0.002 ± 0.001 (2%) | 0.001 ± 0.0005 (3%) |
| 4              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT 5’UTR truncated | 0.04 ± 0.01 (27%) | |
| 5              | cap→ Rluc→ FFluc→ AAAAAA | Murine cSHMT 5'UTR | 0.04 ± 0.01 (27%) | |
| 6              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT 5'UTR | 0.4 ± 0.05 (266%) | 0.06 ± 0.005 (200%) |
| 7              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT Reverse Complement 5'UTR | 0.002 ± 0.001 (2%) | 0.002 ± 0.001 (7%) |
| 8              | cap→ Rluc→ FFluc→ AAAAAA | cSHMT 5'UTR | 0.56 ± 0.05 (370%) | |
| 9              | cap→ Rluc→ FFluc→ AAAAAA | BiP 5'UTR | 0.16 ± 0.01 (107%) | 0.06 ± 0.003 (200%) | 0.18 ± 0.05 |
| 10             | cap→ Rluc→ FFluc→ AAAAAA | BiP 5'UTR | 0.25 ± 0.04 (160%) | 0.07 ± 0.003 (233%) |
Table 2: IRES activity in MCF-7 cells expressing the human FTH cDNA. FTH protein levels were determined by western blots and quantified by densitometry. IRES activity is expressed as a relative ratio of FFluc/Rluc activity following mRNA transfection of bicistronic constructs; activity in MCF-7 cells is given a relative value of 1.0. Data represent an average of at least 5 independent experiments and variation is expressed as standard variation of the mean. Significance was calculated using Student’s t-test.

| MCF-7 cell Stable Clone | FTH Protein | IRES Activity Template 1 | IRES Activity Template 6 |
|-------------------------|-------------|--------------------------|--------------------------|
| Empty Vector            | 1.00        | 1.0                      | 1.0                      |
| FTH clone 1             | 3.42        | 3.4 ± 1.4 (p< 0.05)      | 3.7 ± 1.2 (p < 0.02)     |
| FTH clone 2             | 3.66        | 1.8 ± 0.3 (p< 0.003)     | 1.7 ± 0.4 (p< 0.02)      |
| FTH clone 3             | 3.99        | 1.1 ± 0.2 (p> 0.1)       | 1.3 ± 0.3 (p > 0.1)      |
Table 3: Identification of FTH binding partners using a yeast two-hybrid screen. All positive clones were validated to ensure the proteins were not auto-activating $\beta$-galactosidase ($\beta$-gal) expression.

| Gene name   | #of colonies | Description                        |
|-------------|--------------|------------------------------------|
| FTL         | 23           | Light chain ferritin               |
| FTH         | 13           | Heavy chain ferritin               |
| CUGBP1      | 3            | CUG-repeat binding protein         |
| ALPI        | 2            | Alkaline phosphatase               |
| Golgin-95   | 1            | Golgi protein, 95 kDa              |
Folate-mediated one-carbon metabolism

Tetrahydrofolate (THF)-mediated one-carbon metabolism is required for the synthesis of purines and thymidylate and the remethylation of homocysteine to methionine. The hydroxymethyl group of serine is the major source of one-carbon units which are generated in the mitochondria in the form of formate, or in the cytoplasm through the activity of cytoplasmic serine hydroxymethyltransferase. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for cytoplasmic folate metabolism. The one carbon is labeled in “bold”. cSHMT, cytoplasmic serine hydroxymethyltransferase; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

The cSHMT IRES is FTH-responsive.

MEFs were isolated from *fth*/*+* and *fth*/*−/−* embryos and cultured in DMEM medium. Cells were transfected with bicistronic reporter constructs containing either the cSHMT 5'UTR (A) or the BiP 5'UTR (B). cSHMT IRES activity was decreased by 30% in *fth*/*−/−* MEFs as compared to *fth*/*+/+* MEFs (p < 0.01). BiP IRES activity decreased slightly in *fth*/*−/−* MEFs but was not statistically significant (p > 0.05). All values are the average of 10 independently-derived primary MEF cell lines, and variation is expressed as SEM.

FTH interacts with CUGBP1.

The FTH protein immunoprecipitated from MCF-7 cell extracts that were incubated with an antibody generated against CUGBP1, but not with a control HA antibody. The immunoprecipitates were subjected to SDS-PAGE (60 μg/lane) and probed with an anti FTH antibody. Lanes: 1) MCF-7 cell extract; 2) anti-HA immunoprecipitate (neg. control); 3) anti-human CUGBP1 immunoprecipitate. The lower band in lane 3 is consistent with the size of a proteolytic fragment of FTH found in haemosiderin (56).

CUGBP1 depletion lowers IRES activity in bicistronic constructs containing the cSHMT 3'UTR.

HeLa cells were transfected with no siRNA (mock), non-specific siRNA (scrambled siRNA) or CUGBP1 siRNA. After a 48 h incubation with the siRNAs, cells were either: harvested and CUGBP1, cSHMT and GAPDH protein levels determined by immunoblotting (Panel A) or transfected with bicistronic RNA constructs containing the cSHMT 5'UTR (Table 1, template 1) (panel B) or transfected with the bicistronic construct containing the cSHMT 5'UTR and 3'UTR (Table 1, template 6) (panel C). Levels of expression of GAPDH and cSHMT were quantified from immunoblots, and cSHMT protein levels were decreased by 25% in CUGBP1 depleted cells. All values are the average of at least 5 transfections, and variation is expressed as SEM.

Model for the role of FTH and CUGBP1 in cSHMT IRES activation.

In this model, CUGBP1 binds the cSHMT 3'UTR and FTH binds to the 5'UTR. The physical interaction of CUGBP1 and FTH recruits the translation initiation machinery to the cSHMT IRES.

Stability of the bicistronic mRNAs prior to and after *in vitro* translation assays.

RNA constructs were transcribed *in vitro* with α-[32P]-rUTP to generate labeled RNA transcripts. *In vitro* translation assays were performed with the labeled RNA as described in materials and methods. The RNAs were resolved on an agarose gel and transferred to a positively charged nylon membrane. The lane numbers correspond to template numbers listed in Table 1. For each construct, the right lane represents poly (A) purified RNA before *in vitro* translation and the left lane is the template after *in vitro* translation reactions.
Figure 3
Figure 4

A.

| Treatment            | CUGBP1 | cSHMT | GAPDH |
|----------------------|--------|-------|-------|
| Mock Transfection    |        |       |       |
| Scrambled siRNA      |        |       |       |
| CUGBP1 siRNA         |        |       |       |

B.

| siRNA Type           | FFuc/Rluc |
|----------------------|-----------|
| siRNA Scrambled      | 1.2       |
| siRNA CUGBP1         | 0.8       |

C.

| siRNA Type           | FFuc/Rluc |
|----------------------|-----------|
| siRNA Scrambled      | 1.2       |
| siRNA CUGBP1         | 0.6       |
Figure 5

Supp Fig. 1
A Ferritin-responsive internal ribosome entry site regulates folate metabolism
Collynn F. Woeller, Jennifer T. Fox, Cheryll Perry and Patrick J. Stover

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