Heavy Chain Ferritin Enhances Serine Hydroxymethyltransferase Expression and \textit{de Novo} Thymidine Biosynthesis*

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We have elucidated a biochemical mechanism whereby changes in iron metabolism cause changes in folate-dependent one-carbon metabolism. Although animal and clinical studies have demonstrated that perturbations in iron status and metabolism alter folate metabolism, the biochemical mechanisms underlying these associations have yet to be identified. The effect of altered ferritin expression on folate metabolism was determined in human MCF-7 cells and SH-SY5Y neuroblastosoma. Cells expressing rat heavy chain ferritin (HCF) exhibited markedly increased expression of the folate-dependent enzyme cytoplasmic serine hydroxymethyltransferase (cSHMT). These effects were not seen when rat light chain ferritin was expressed. Additionally, cSHMT expression was not altered when HCF expression was induced in MCF-7 cells cultured with supplemental ferric citrate. This indicates that cSHMT expression is increased by elevated HCF concentrations, independent of increased iron availability, suggesting that cSHMT expression may respond to HCF-induced chelation of the regulatory iron pool. Increased HCF expression did not alter cSHMT mRNA levels, but did increase translation rates of cSHMT mRNA. The increase in translation was mediated, at least in part, through the cSHMT 5′-untranslated region of the transcript. MCF-7 cells with increased expression of cSHMT displayed increased efficiency of \textit{de novo} thymidylate biosynthesis, indicating that thymidylate synthesis is normally limited by cSHMT activity in MCF-7 cells. Our data suggest that the iron regulatory pool may play an important role in regulating folate metabolism and thereby thymidine biosynthesis.

The influence of iron deficiency, both induced and naturally occurring, on folate metabolism has been well documented in cell culture models, animal models, and humans. Iron deficiency can influence folate status, modify folate metabolism, and result in hematological pathologies similar to those normally attributed to folate deficiency (1). It is not certain if all observed effects of iron deficiency on folate status reflect actual changes in intracellular folate concentrations or are due to alterations in folate-dependent metabolism. Studies of iron-deficient rats have demonstrated a 90% decrease in serum folate levels compared with their iron-sufficient controls (2). The decreased serum folate is not secondary to intestinal malabsorption, changes in intestinal flora, isolated folate malabsorption, or changes in vitamin B\textsubscript{12} status. In addition, maternal iron deficiency in rats decreases the secretion of folate into milk (3–6), sacrificing folate metabolism in the pups while maintaining maternal serum and red blood cell folate levels. Many of the findings in rats have been replicated in other mammals and in pregnant and non-pregnant humans (2, 7, 8). In humans, iron deficiency impairs folate utilization in certain tissues, decreases polyglutamation levels of folate, decreases the secretion of folate into breast milk without decreasing maternal folate stores, and can cause granulocyte abnormalities characteristic of folate deficiencies (1, 4, 9–12). Despite the numerous clinical associations, the biochemical mechanisms underlying the influence of iron deficiency on folate metabolism have not been established (3–6, 11, 13).

The regulation of iron status and intracellular iron concentrations is complex. Cellular iron can be categorized into three pools: the functional, storage, and regulatory pools (14). The functional iron pool refers to the iron incorporated into proteins that require the mineral for enzymatic function or structural integrity, including hemoglobin and ribonucleotide reductase. The storage iron pool refers to iron that is associated with the iron storage protein ferritin (14). Finally, the regulatory iron pool is the iron that is in equilibrium with the iron regulatory proteins (IRPs).1 It is this pool of iron that is therefore critical for the translational regulation of mRNA species that contain iron regulatory elements, including ferritin and transferrin receptor transcripts (15). When the regulatory iron pool is decreased, IRPs bind to these mRNA elements and inhibit specific mRNA translation or degradation (15).

In the cell, iron is an effective and deleterious oxidizing agent. The cell prevents iron-mediated oxidative damage by chelating and storing intracellular iron within the protein complex that is ferritin. Ferritin is a heterogeneous protein composed of heavy chain (HCF) and light chain (LCF) subunits (16). In mammals, HCF and LCF share 54% sequence identity and assemble in varying proportions to make up a complex of 24 subunits (14). The ratio of heavy to light isomers varies depending on cell type and function (17). The heavy chain isomer has the capacity to sequester iron through its ferroxidase active sites, whereas LCF is believed to function in mineralizing iron at the core of the ferritin polymer (18). Increased HCF expression can be induced in cell culture models, resulting in chelation and subsequent lowering of the regulatory iron pool (19), whereas elevations in LCF levels \textit{in vivo} have no effect on the regulatory iron pool (20). Current literature indicates that changes in the ferritin subunit ratio generally result from transcriptional regulation of the gene encoding HCF. Hor-

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1 The abbreviations used are: IRPs, iron regulatory proteins; HCF, heavy chain ferritin; LCF, light chain ferritin; cSHMT, cytoplasmic serine hydroxymethyltransferase; THF, tetrahydrofolate; α-MEM, α-minimal essential medium; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region.
serves as the primary source of folate-activated one-carbon units for both purine biosynthesis, which requires the 10-formyl-THF cofactor; thymidine biosynthesis, which requires 5-methyl-THF; and methionine synthesis (homocysteine remethylation), which requires 5,10-methylene-THF; and methionine synthesis (Scheme 1). Chinese hamster ovary cells lacking mitochondrial SHMT activity are auxotrophic for glycine (27), suggesting that cSHMT activity cannot replace mitochondrial SHMT function. The inability of cSHMT to compensate for the loss of mitochondrial SHMT function indicates that the mitochondrial isozyme is not a primary source of glycine and perhaps one-carbon units. The SHMT enzyme catalyzes the tetrahydrofolate (THF)-dependent interconversion of glycine and serine (Scheme 1). This reaction is the primary source of folate-activated one-carbon units. There are three biosynthetic pathways that are associated with cytoplasmic folate metabolism and that require folate-activated one-carbon units: purine biosynthesis, which requires the 10-formyl-THF cofactor; thymidine biosynthesis, which requires 5,10-methylene-THF; and methionine synthesis (homocysteine remethylation), which requires 5-methyl-THF (26).

There are two intracellular isoforms of SHMT, one located in the mitochondria and the other in the cytoplasm. Mutagenesis studies have demonstrated that the mitochondrial isozyme is essential for the generation of glycine and presumably formate (Scheme 1). Chinese hamster ovary cells lacking mitochondrial SHMT activity are auxotrophic for glycine (27), suggesting that cSHMT activity cannot replace mitochondrial SHMT function. The inability of cSHMT to compensate for the loss of mitochondrial SHMT function indicates that the cSHMT isozyme is not a primary source of glycine and perhaps one-carbon units. The SHMT-catalyzed reaction is freely reversible in vitro using physiological concentrations of amino acid and folate substrates (28, 29). Therefore, the enzyme, when catalyzing serine degradation, can supply 5,10-methylene-THF for biosynthetic reactions; and when catalyzing serine synthesis, it can deplete 5,10-methylene-THF pools and regenerate THF for purine biosynthesis. Consequently, cSHMT is poised to regulate the supply of folate-activated one-carbon units for both DNA synthesis and methionine regeneration.

The ability of iron chelators to influence cSHMT expression may begin to provide a mechanistic rationale for the known associations between iron and folate metabolism. The cSHMT enzyme is potentially critical for regulating folate metabolism because of its capacity to regulate the flux of folate substrates, and cSHMT is known to be sensitive to extreme alterations in intracellular iron concentrations as those induced by chemical chelators (25). However, the use of chemical iron chelators to mimic iron deficiency is not an adequate model to test the physiological relevance of cSHMT regulation by iron. Therefore, in this study, we examined the ability of the endogenous iron chelator HCF to influence cSHMT expression and activity in cell culture models.

**Experimental Procedures**

**Cell Lines and Media—**Human MCF-7 mammary adenocarcinoma cells (ATCC HTB22) and SH-SY5Y neuroblastoma, a subline of the SK-N-SH neuroblastoma, have been previously described (28). Cells were cultured with α-minimal essential medium (α-MEM; HyClone Laboratories) supplemented with 11% fetal calf serum (HyClone Laboratories) and maintained at 37 °C in a 5% CO2 atmosphere.

**Generation of Rat HCF- and LCF-Expressing Cell Lines—**MCF-7 cells were cultured in 100-mm culture dishes with α-MEM and grown to 80% confluency. The cells were harvested by trypsination and washed with phosphate-buffered saline, and 1 × 10^6 cells were incubated on ice for 10 min before 10 μl of plasmid DNA was added. The cells were electroporated at 0.22 kV and 950 microfarads (using a Bio-Rad GenePulser). Cells were cultured with α-MEM for 48 h and then cultured with G418 sulfate (500 μg/ml)-supplemented α-MEM to select for stable integrants. The plasmid (pCDNA3 vector, Invitrogen) contained the open reading frame cDNA for either rat HCF or LCF (30). Over 10 colonies were isolated and passaged until stable lines were generated.

**Generation of Antisense cSHMT-Expressing Cell Lines—**The antisense cSHMT construct was generated by transcriptase-polymerase chain reaction (RT-PCR). Total mRNA was isolated from MCF-7 cells (Purgene™ RNA isolation kit) and converted to cDNA using Tth polynucleosome (Promega) and random hexamer primers. The first 405 base pairs (bp) of the human cSHMT cDNA were amplified using forward primer 5'-TTCACTGATATGGCCGCTGCTTCGACTGTGTTGG-3' and reverse primer 5'-ACTAGATATCAGCAATAGCAGCTGTATTGG-3'. The forward primer contains an EcoRI restriction site at the 5'-end, whereas the reverse primer contains a BamHI restriction site. The cycling conditions were 94 °C for 45 s, 55 °C for 45 s, and 70 °C for 90 s for a total of 30 cycles. The PCR product was digested with EcoRI and BamHI and subcloned into the pcDNA3 vector described above. The sequence was verified by nucleotide sequencing. Once verified, the construct was transfected into MCF-7 cells. Stable cell lines expressing the antisense construct were also generated by the procedure described above.

**Western Blot Analyses—**Cells were harvested by trypsination and washed with phosphate-buffered saline. Cell pellets were incubated at 100 °C for 10 min in buffer containing 2% SDS, 100 ms dithiothreitol, and 250 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 4–15 h with the horseradish peroxidase-conjugated secondary antibody. Rat ferritin (Sigma) was used as a standard for all ferritin Western blot analyses, and recombinant human cSHMT was used as a standard for cSHMT Western blot analyses. The membranes were visualized using the horseradish peroxidase–SuperSignal chemiluminescent substrate system (Pierce).

**For detection of HCF, sheep anti-human HCF antisera (30) was diluted 1:8000, and the secondary antibody (rabbit anti-sheep IgG, Pierce) was diluted 1:6500. For LCF detection, rabbit anti-rat LCF antiserum (30) was diluted 1:8000, and the secondary antibody (anti-rabbit IgG, Pierce) was diluted 1:5000. For thymidylate synthase detection, monoclonal mouse anti-human thymidylate synthase antiserum (Neomarkers) was diluted 1:1000, and the anti-mouse goat IgG secondary antibody (Pierce) was diluted 1:3500. For cSHMT detection, sheep anti-human cSHMT antisera (31) was diluted 1:7500, and rabbit anti-sheep IgG antisera was diluted 1:6500.

**Ferric Citrate Supplementation—**Cells were grown for 24 h in medium enriched with 0, 0.1, 1, or 10 μM ferric citrate. Two replicate plates were grown at each supplementation concentration. Cells were harvested using trypsine/EDTA, washed three times with phosphate-buffered saline, and stored at -80 °C until Western blot analyses were conducted.

**Detection of cSHMT mRNA Levels by RT-PCR—**Total cellular RNA was isolated from cells using RNAzol™ (Teltest, Inc.) and converted to cDNA using a first strand synthesis kit (CLONTECH). A competitive RT-PCR method based on the MIMIC strategy (CLONTECH) was used to measure cSHMT mRNA levels. A competitive
internal standard with the same primer-binding sites used to amplify the 300-bp segment of the cSHMT cDNA was generated by amplifying 500 bp of v-erbB using two composite primers (40-mers), 5'-GAACAGCTGCACTGGAACCAAGTGAAATTCCCTCGG-3' and 5'-CTCATACACACAACTCGACTAGTCGGAGGACCTTT-3'. The first 20 nucleotides of the 40-mer are complementary to cSHMT, whereas the following 20 nucleotides are complementary to v-erbB. The 540-bp internal standard was synthesized and purified following protocols described by the manufacturer. The primers used to amplify cSHMT cDNA and the internal standard were 5'-GAACAGCTGCACTGGAACCAAGTGAAATTCCCTCGG-3' and 5'-ACCTACACACACAACTCGACTAGTCGGAGGACCTTT-3', and the primers used to amplify glyceraldehyde-3-phosphate dehydrogenase standard were 5'-ACCA-AGTCGTCACCAGCAGCAAGTGAAATTCCCTCGG-3' and 5'-TCCACACCATGTTGCTGATTGTCGGAGGACCTTT-3'. The cycling parameters were 94 °C for 45 s, 65 °C for 45 s, and 72 °C for 60 s for both primer sets. Internal standards and target DNA fragments were then separated on a 1.8% agarose gel and stained with ethidium bromide solutions. DNA concentrations and cSHMT message copy number were quantified using a ChemiImager 4400 with AlphaEase Version 4.0 (Alpha Innotech Corp.).

Luciferase Reporter Gene Assays—As described previously, both the full-length and shorter alternatively spliced form of the cSHMT 5'-untranslated region (UTR) were cloned into the pGL3-luciferase reporter vector (Promega) such that the cSHMT 5'-UTR was fused to the luciferase open reading frame just as it is normally fused to the cSHMT open reading frame (31, 32). The LUTR-luc (where LUTR is the long 332-bp UTR and luc is luciferase) construct encodes the full-length cSHMT UTR fused to the luciferase open reading frame, whereas the SUTR-luc (where SUTR is the short 193-bp UTR) construct encodes the shorter alternatively spliced cSHMT 5'-UTR fused to the luciferase open reading frame. The constructs were transfected with a control construct for transfection efficiency (pRL-CMV vector, Promega) into MCF-7 and rat HCF-expressing MCF-7 cells. The transfection method has been described previously (25). 48 h following transfection, the cells were assayed for luciferase activity. The activity generated from the constructs was normalized to that generated from the co-reporter pRL-CMV-Renilla luciferase. The luciferase activities generated from the LUTR-luc and SUTR-luc constructs were normalized to luciferase activity generated from the same construct lacking the cSHMT 5'-UTR in the same cell lines to control for differences in basal translation rates among cell lines. All reported luciferase activity values represent the mean of at least five measurements, and the error bars represent the S.E. values.

In Vitro Translation Assays—RNAs corresponding to luciferase mRNA, luciferase mRNA with the cSHMT 5'-UTR fusion (Lluc-mRNA), and luciferase mRNA with the shortened alternatively spliced cSHMT 5'-UTR fusion (Sluc-mRNA), and Sluc-mRNA templates. All experiments were performed at a final mRNA concentration of 0.2 μg/ml to ensure that the catalytic system was below saturation. The mRNA templates were heated to 65 °C for 10 min and then cooled to room temperature before addition to the reaction mixture to allow for a more uniform secondary structure to form. All enzyme/mRNA incubations contained RNase inhibitor (Ambion Inc.). To determine the effects of rat ferritin and ferric citrate on cSHMT translation, various concentrations of rat ferritin or ferric ammonium citrate were added to the reaction mixture. Following a 35-min incubation at 30 °C, the translation reaction mixtures were quenched on ice, and the luciferase activity was determined.

Modified Deoxuryridine Suppression Assays—Cells were plated to a confluence of 1 × 10^4 cells/24-mm well and cultured overnight with α-MEM. The medium was changed to 3 ml of defined medium, which lacked ribonucleosides, deoxyribonucleosides, thymidine, hypoxanthine, serine, and glycine. After 48 h, the medium was refreshed, and deoxuryridine was added to a final concentration of 50 μM in experimental samples, but was excluded from control wells. After a 90-min incubation, 5 μl of [3H]thymidine (1 mCi/ml, PerkinElmer Life Sciences) was added to all wells. After a 24-h incubation, cells were harvested, and DNA was isolated (Purgene™ DNA purification kit). Once isolated and rehydrated, DNA concentrations were determined at A260. DNA solutions were transferred to scintillation tubes, and [3H]thymidine incorporation into DNA was determined using a scintillation counter to determine cpm/μg of DNA. Data are expressed as cpm/μg of DNA in control samples (lacking deoxuryridine) divided by cpm/μg of DNA in experimental samples (supplemented with deoxuryridine).

RESULTS

Effects of Ferritin on Intracellular cSHMT Concentrations—Iron chelators including mimosine (33, 34) and deferoxamine inhibit cSHMT transcription in MCF-7 cells (25), and the cSHMT protein is not detectable in cell extracts following 24 h of exposure to these chelators. The effect of iron chelation on cSHMT expression is cell-specific; cSHMT protein levels are not affected by iron chelators in human SH-SY5Y neuroblastoma. To determine if cSHMT expression was responsive to changes in the cellular iron regulatory pool, the effects of rat HCF and LCF expression on cellular cSHMT protein concentrations were determined in MCF-7 and SH-SY5Y cells. Others have shown that increased expression of HCF, but not LCF, in mammalian cells decreases the cellular concentration of free non-ferritin bound iron (19, 35). In our study, both MCF-7 and SH-SY5Y cells were transfected with a plasmid that expresses either rat HCF or LCF cDNA, and stable clones were isolated. Clonal lines were screened for changes in HCF, LCF, and cSHMT protein levels (Figs. 1 and 2).

Fig. 1 shows the effects of expressing rat HCF cDNA on cSHMT protein levels in SH-SY5Y neuroblastoma (A) and MCF-7 cells (B) as determined by Western blotting. The upper panels verify that HCF protein levels were increased in both G418-resistant MCF-7 and SH-SY5Y cell lines. Surprisingly, we found that cSHMT protein levels were also elevated 5–10-fold in all HCF-expressing MCF-7 cell lines, indicating that increased rat HCF expression has the opposite effect of chemical chelators on cSHMT protein concentrations. This effect on cSHMT protein concentrations was also seen in all SH-SY5Y cell lines. This indicates that, unlike the effects of iron chelators on cSHMT expression (25), the effects of HCF are not cell-specific for MCF-7 cells since both cell lines had increased cSHMT protein levels when rat HCF was expressed. As a control experiment, stable MCF-7 cell lines that express rat LCF were generated (Fig. 2A). The MCF-LCF5 clonal line displayed the highest level of LCF expression, although increased LCF expression was seen in all G418-resistant cell lines upon longer exposure of the blots. These cells would not be expected to display changes in the iron regulatory pool, as the
LCF subunit lacks the ferroxidase activity necessary for significant iron uptake by ferritin. As expected, MCF-7 cell lines expressing rat LCF did not display alterations in cSHMT concentrations (Fig. 2B). Collectively, these data indicate that cSHMT protein levels are increased either directly by increased HCF protein or indirectly by responding to HCF-induced changes in the iron regulatory pool.

**Effect of Exogenous Iron on cSHMT Expression**—The regulatory iron pool is sensitive to and typically reflective of exogenous iron concentrations. In cell culture models, it has been demonstrated that increasing iron in the culture medium results in increased ferritin synthesis and decreased synthesis of transferrin receptors (36). IRPs sense and respond to changes in the iron regulatory pool by altering the translation of ferritin mRNA and increasing the stability of the transferrin receptor mRNA (36). The ultimate role of IRPs is to maintain iron homeostasis irrespective of alterations in exogenous iron concentrations. The effects of increasing the iron regulatory pool on cSHMT expression were determined in MCF-7 cells (Fig. 3). Increasing the concentration of ferric citrate in the culture medium increased HCF protein levels as expected (Fig. 3, lower panel). However, the increase in HCF protein did not influence cSHMT protein levels. This indicates that the mechanisms that are utilized to increase ferritin expression as a function of iron do not influence cSHMT expression, which does not respond to increases in the iron regulatory pool or to iron overload. These data suggest that cSHMT protein expression, if responsive to changes in the iron regulatory pool, is influenced only by decreases in the iron regulatory pool under our culture conditions.

**Effect of HCF Expression on cSHMT mRNA**—Competitive RT-PCR assays were conducted to determine the molecular mechanisms whereby HCF alters cSHMT expression. The cellular cSHMT mRNA content was determined by co-amplification of the cSHMT cDNA and an internal control cDNA containing primer-binding sites identical to those of cSHMT. The cSHMT transcript levels were quantified in both wild-type MCF-7 and SH-SY5Y cells and compared with cells lines expressing rat HCF. No significant differences in cSHMT transcript levels were found between wild-type and HCF-expressing cell lines. The average concentration of the cSHMT transcript in both MCF-7 and SH-SY5Y cells was 0.0036 amol/µg of total RNA. These data indicate that increased HCF levels do not elevate cSHMT protein levels by increasing cSHMT mRNA concentrations.

**Effect of HCF on cSHMT Translation**—Expression of rat HCF in human cell cultures did not increase cSHMT mRNA as determined by quantitative RT-PCR, nor did it increase rates of cSHMT promoter activity as determined by gene reporter assays (data not shown). This indicates that ferritin increases either cSHMT translation rates or cSHMT protein stability.

Table I shows evidence that ferritin increased the translation rate of the cSHMT message and that this effect was mediated through the cSHMT 5’-UTR. As a result of alternative splicing, cSHMT transcripts contained either a long 332-bp UTR or a short 193-bp UTR. 5’-UTR sequences are often binding sites for proteins that regulate rates of translation (37–40). To determine if HCF-induced increases in cSHMT protein are mediated through the cSHMT 5’-UTR, luciferase reporter gene assays were performed. Two cDNA constructs (Lluc and Sluc) that contain the cSHMT 5’-UTRs fused to the 5’-end of the luciferase cDNA (32) were transiently transfected into MCF-7 cells. The addition of the cSHMT 5’-UTR to the luciferase cDNA increased luciferase activity by 2–3-fold relative to activity generated from the luciferase cDNA alone (data not shown) when transfected into MCF-7 cells. Interestingly, this effect was even more pronounced in cells expressing the HCF cDNA. HCF-expressing cells have enhanced luciferase activity relative to control cells resulting from the presence of the 5’-UTR (41). These data demonstrate that increased HCF expression increases translation of a reporter gene containing the cSHMT 5’-UTR. It is not clear if HCF is interacting directly with the cSHMT 5’-UTR, or if it is influencing the activity of other mRNA-binding proteins that may be sensitive to decreased intracellular iron concentrations.

Ferritin is a promiscuous mRNA-binding protein and has been demonstrated either to activate or to inhibit in vitro translation of a variety of unrelated mRNA species (42). To determine if ferritin directly interacts with the cSHMT 5’-UTR and enhances translation in vitro, the effect of ferritin protein on the translation efficiency of Lluc-mRNA templates was determined. The addition of commercial rat ferritin (50–150 pmol) to an in vitro translation reaction decreased translation of the Lluc template by >90% at all ferritin concentrations relative to reactions that lacked ferritin. The addition of ferric citrate at concentrations ranging from 0.1 to 100 µM had no effect on the in vitro translation reaction. Therefore, it is unlikely that the HCF protein acts independently or directly on cSHMT mRNA in vivo to increase cSHMT translation. It is more probable that the HCF-induced increases in cSHMT expression are a consequence of alterations in the iron regulatory pool.

**Effects of Increased cSHMT Expression on Folate Metabolism**—The induction of cSHMT protein concentrations by HCF suggests a mechanism whereby iron metabolism influences folate metabolism. However, changes in cSHMT protein levels may not necessarily result in increased cSHMT specific activity in the cell. It has been demonstrated, for example, that the
TABLE I

**Effect of the cSHMT 5'-UTR on luciferase expression**

| Cell line | Normalized ratio of UTR-luc/luc activity | p value* |
|-----------|----------------------------------------|----------|
| Luc activity |                                           |          |
| MCF-7      | 1                                       |          |
| MCF-HCF2   | 1.14                                    | <0.0003  |
| MCF-HCF5   | 1.68                                    | <0.0002  |
| MCF-HCF6   | 1.22                                    | <0.05    |
| Sluc activity |                                          |          |
| MCF-7      | 1                                       |          |
| MCF-HCF2   | 1.02                                    | NS       |
| MCF-HCF5   | 2.16                                    | <0.0001  |
| MCF-HCF6   | 1.42                                    | <0.002   |

* Student’s t test assuming unequal variances. NS, not significant.

The cSHMT enzyme is capable of synthesizing its own slow, tight-binding inhibitor, 5-formyl-THF. Studies in human neuro blasts indicate that cSHMT enzyme activity is inhibited by 5-formyl-THF in these cells (28). Therefore, the effects of altered cSHMT protein levels on cSHMT specific activity were determined using an *in vivo* deoxouridine suppression assay. This competition assay measures the efficiency of de novo thymidylate biosynthesis (Scheme 2). The effects of altered cSHMT activity on thymidine biosynthesis have never been determined. The cSHMT reaction is freely reversible *in vitro* at physiological concentrations of substrates and products, indicating that cSHMT, when catalyzing serine catabolism, is capable of providing one-carbon units in the form of methylene-THF for thymidylate biosynthesis. Alternatively, when catalyzing serine synthesis, cSHMT may deplete methylene-THF levels and thereby inhibit thymidylate biosynthesis. To validate that the dUrd suppression assay is sensitive to changes in the specific activity of cSHMT, MCF-7 cells expressing high levels of the luciferase cDNA or with the luciferase cDNA containing the long cSHMT 5'-UTR (Luc) or the alternatively spliced cSHMT 5'-UTR (Sluc) cloned into the expression vector pGL3. A control Renilla luciferase vector was cotransfected with all pGL3 vectors. After 48 h, luciferase activity was determined. All luciferase activities generated from pGL3-basic were normalized to luciferase activity generated from pRL-CMV with Renilla luciferase activity. Data are presented as a ratio of normalized luciferase activity generated from the UTR-luciferase (Lluc) construct to activity generated from the luciferase construct.

**Effects of cSHMT Expression on Genome-wide Protein Expression in MCF-7 Cells**—In the course of generating cell lines with altered cSHMT expression, it was observed that increases in cSHMT enzyme levels are associated with alterations in the SDS-PAGE profile generated from total MCF-7 cellular protein extracts. As shown in Fig. 5, Coomassie Blue-stained SDS-polyacrylamide gels of crude cell extracts demonstrated that MCF-7 cells expressing the sense cSHMT construct or the rat HCF construct have similar protein profiles, but differ from wild-type MCF-7 cellular protein profiles. The bands that displayed the greatest changes in intensity were not the cSHMT protein, which is a 55-kDa protein. Additionally, the changes were identical in all clonal cell lines that express a sense cSHMT construct or an HCF construct. The profile changes observed with increased cSHMT expression were not observed when the antisense cSHMT construct was expressed. This suggests that increases in cSHMT protein levels alter the expression of other genes in MCF-7 cells. Therefore, either increased cSHMT protein directly influences the expression of other genes, or cSHMT-induced perturbations in folate metabolism modulate the expression of other genes.

**DISCUSSION**

Our previous studies have demonstrated that chemical chelators inhibit cSHMT expression at the transcriptional level in a cell-specific manner (25). Our findings here, however, demonstrate that iron chelation by HCF induces cSHMT expression at the translational level. This latter alteration in cSHMT expression appears to be unrelated to the transcriptional effects induced by chemical iron chelators. This notion is supported by our studies demonstrating that chemical chelators act on cSHMT expression in a cell-specific fashion, whereas the effect of HCF on cSHMT expression may represent a more global mechanism. Therefore, chemical iron chelation and HCF-mediated iron chelation likely affect cSHMT expression by distinct mechanisms. Chemical iron chelators can sequester both the regulatory and functional iron pools, whereas HCF expression can influence only the regulatory iron pool. The HCF induction of cSHMT expression is likely due to changes in the iron regulatory pool, whereas chemical iron chelators may act by disrupting a cell-specific iron-binding protein that is important in cSHMT transcriptional regulation. We also conclude that HCF-induced increases in cSHMT expression do not occur by the same mechanisms that regulate ferritin and transferrin synthesis (i.e., mediation by IRPs) since cSHMT protein levels do not respond to elevations of iron in the culture medium. The data indicate that cSHMT expression responds only to decreases in the regulatory iron pool, and the effects of iron chelation on cSHMT expression are distinct depending on the primary catalyst for iron depletion.
TABLE II

**Effect of elevated cSHMT expression on de novo dTMP biosynthesis in human cultured cells**

Cells were plated to confluence and grown on medium lacking ribonucleosides, deoxyribonucleosides, thymidine, hypoxanthine, serine, and glycine. Experimental cells, but not control samples, were cultured with deoxouridine for 90 min. All cells were incubated for an additional 24 h with [3H]thymidine (1 mCi/ml). The incorporation of [3H]thymidine into DNA (cpm/µg) in cells incubated with and without dUrd was compared and taken as a ratio for each cell line to reflect each clone’s capacity to synthesize thymidylate de novo. These ratios were then compared with wild-type cell ratios. All values are normalized to wild-type cells that have a value of 1.0 relative to dUrd suppression. AS, antisense.

| Cell line Description | Relative dUrd suppression | p value<sup>a</sup> |
|-----------------------|---------------------------|--------------------|
| MCF-7                 | 1                         |                    |
| MCF-7-cSHMT2         | Increased expression of cSHMT cDNA | 1.7 | <0.001 |
| MCF-7-cSHMT21        | Increased expression of cSHMT cDNA | 2.0 | <0.001 |
| MCF-7-HCF1           | Increased expression of HCF cDNA | 1.5 | <0.005 |
| MCF-7-HCF2           | Increased expression of HCF cDNA | 1.7 | <0.001 |
| MCF-7-HCF5           | Increased expression of HCF cDNA | 2.0 | <0.001 |
| MCF-7-HCF6           | Increased expression of HCF cDNA | 2.1 | <0.001 |
| MCF-7-AS cSHMT11     | Expression of anti-cSHMT cDNA | 0.29 | <0.001 |
| SH-SYSY Control      | 1                         |                    |
| SH-SYSY-HCF1         | Increased expression of HCF cDNA | 2.2 | <0.001 |
| SH-SYSY-HCF2         | Increased expression of HCF cDNA | 2.0 | <0.001 |
| SH-SYSY-HCF3         | Increased expression of HCF cDNA | 1.4 | <0.002 |

<sup>a</sup> Student’s t test assuming unequal variances.

This study demonstrates a complex relationship between cellular iron status and folate metabolism and suggests that changes in cSHMT translation may be responsible for mediating the metabolic associations between iron and folate metabolism. The cSHMT enzyme catalyzes the interconversion of glycine and serine and is functionally poised to regulate the flux of one-carbon units to the different folate-dependent biosynthetic pathways in the cytoplasm by manipulating 5,10-methylene-THF pools. Our results indicate that increases in cSHMT activity cause changes in the flux of one-carbon units to the different folate-dependent biosynthetic pathways in MCF-7 cells. It is well established that impaired folate metabolism results in increased uracil content in DNA resulting from impaired thymidylate synthesis (43, 44). Increased DNA uracil content is associated with several cancers (45), and impaired de novo thymidylate biosynthesis has been implicated in the etiology of neural tube defects in Pax3 mice (46). Our studies indicate that the specific activity of cSHMT potentially influences the uracil content in DNA. In addition, increases in cSHMT-catalyzed serine synthesis may decrease 5-methylTHF availability and therefore impair homocysteine remethylation as demonstrated in cell culture systems (28). It is therefore possible that iron-mediated increases in cSHMT activity could be implicated in the development or prevention of certain clinical disorders by impairing homocysteine remethylation and enhancing dTMP synthesis.

Our data suggest that cSHMT is a key regulatory enzyme in folate metabolism. cSHMT displays robust changes in expression resulting from changes in iron status and metabolism (25) as well as from retinoic acid exposure (47). Therefore, cSHMT may be a homeostatic sensor that effects changes in folate metabolism when signaled by seemingly unrelated metabolic pathways. Additionally, these changes in cSHMT expression alter the expression of several proteins in MCF-7 cells, possibly due to changes in one-carbon metabolism, e.g., these genes may be methylation-sensitive. Alternatively, we recently demonstrated that cSHMT is an mRNA-binding protein (32). cSHMT may therefore influence alterations in protein expressions by directly modifying the translation of some mRNA species.

The results of this study provide evidence for a general mechanism whereby alterations in iron metabolism can affect changes in folate-mediated one-carbon metabolism. We propose that this mechanism is triggered by decreases in the regulatory non-ferritin bound iron pool. Several studies have demonstrated that HCF functions intracellularly to sequester iron into ferritin, whereas LCF is believed to function in the mineralization of iron at the core of ferritin. Increased expression of HCF in cell culture models lowers the free iron regulatory pool (19, 35). Physiologically, HCF (but not LCF) levels are increased in tissues >10-fold during pregnancy and cancer and as a result of ethanol exposure (48–50). These physiological states are also associated with altered folate metabolism (51–53); however, the specific mechanisms underlying these alterations have not been characterized. In addition, there are direct associations in the literature between iron deficiency and aberrations in folate metabolism. Here, we show that both human neuroblastoma and MCF-7 cells have increased cSHMT protein levels only when the HCF (but not LCF) protein is increased. In addition, we found that increased cSHMT modulates the flux of one-carbon units available for the folate-dependent biosynthetic reaction of thymidylate synthesis. The results of this study therefore reveal a physiological mechanism that may explain the parallels and associations between iron and folate metabolism.
