Intracellular levels of the light (L) and heavy (H) ferritin subunits are regulated by iron at the level of message translation via a modulated interaction between the iron regulatory proteins (IRP1 and IRP2) and a 5'-untranslated region. Iron-responsive element (IRE). Here we show that iron and interleukin-1β (IL-1β) act synergistically to increase H- and L-ferritin expression in hepatoma cells. A GC-rich cis-element, the acute box (AB), located downstream of the IRE in the H-ferritin mRNA 5'-untranslated region, conferred a substantial increase in basal and IL-1β-stimulated translation over a similar time course to the induction of endogenous ferritin. A scrambled version of the AB was unresponsive to IL-1. Targeted mutation of the AB altered translation; reverse orientation and a deletion of the AB abolished the wild-type stem-loop structure and abrogated translational enhancement, whereas a conservative structural mutant had little effect. Labeled AB transcripts formed specific complexes with hepatoma cell extracts that contained the poly(C)-binding proteins, iso-αCP1 and -αCP2, which have well defined roles as translation regulators. Iron influx increased the association of αCP1 with ferritin mRNA and decreased the αCP2-ferritin mRNA interaction, whereas IL-1β reduced the association of αCP1 and αCP2 with H-ferritin mRNA. In summary, the H-ferritin mRNA AB is a key cis-acting translation enhancer that augments H-subunit expression in Hep3B and HepG2 hepatoma cells, in concert with the IRE. The regulated association of H-ferritin mRNA with the poly(C)-binding proteins suggests a novel role for these proteins in ferritin translation and iron homeostasis in human liver.

The mechanisms governing the regulation of ferritin mRNA translation are complex, but their elucidation is critical to understanding iron homeostasis. Iron and oxidative stress are known to modulate the first stage of translation of ferritin mRNAs when the 43 S ribosome subunit attaches to the 5' cap-specific M7GpppN in the 5'-UTR of L- and H-ferritin mRNAs (1–4). The iron regulatory proteins (IRP1 and IRP2, iso-IRPs) play a central role in regulating ferritin mRNA translation. During conditions of intracellular iron chelation with desferrioxamine (DesF) and oxidative stress, the IRPs bind with higher affinity to the conserved iron-response element (IRE) RNA stem loop 40 nucleotides (nt) downstream of the 5' cap sites of the L- and H-ferritin mRNAs (1). This translational repression event prevents attachment of the small ribosome subunit to the 5' cap sites of the L- and H-ferritin mRNAs and inhibits ferritin translation (2). In contrast, after iron influx the iso-IRPs are released from the 5' cap IREs, and ferritin translation is no longer inhibited, increasing the cellular iron storage capacity (2, 3). The IRP2 knock-out mouse, which is characterized by unregulated ferritin mRNA translation and ferritin accumulation in neurons and gut epithelial cells in a gene-dose manner, validated these observations in vivo (4). Recently zinc and cadmium were reported to interfere with the RNA binding activity of IRP-1, extending the spectrum of IRP binding modulators to these two metal elements (5).

Thyroid hormone (T3), which displaces iso-IRPs from the iso-IREs in iron-loaded cells, increases ferritin translation (6). Other factors also regulate ferritin expression via altered IRP-IRE interactions. These include phospholipids, endothelial growth factor and thyrotropin-releasing hormones, each of which modulates the phosphorylation status of the iso-IRPs (7). The thyrotropin-releasing hormone/endothelial growth factor–induced changes in ferritin subunit synthesis were IRP-dependent in one pituitary cell line and IRP-independent in another, suggesting that other sequences within L- and H-ferritin...
mRNAs contribute to translation regulation (2). IRP-independent ferritin subunit synthesis is also induced in human epidermal A431 cells when they are infected with *Neisseria meningitidis* (8). These data indicate that mechanisms other than the IRP-IRE interaction can modulate L- and H-ferritin mRNA translation.

Interleukin-1 (IL-1) appears to control the rate of L- and H-ferritin subunit synthesis at the second stage of 43 S ribosome translation scanning, immediately upstream from the start codon before the complete 80 S ribosome translates the open reading frame into protein (9–11). We have shown previously that IL-1β stimulates the rate of L- and H-ferritin subunit translation in both hepatomas (11). In particular, IL-1β induced both L- and H-ferritin mRNAs and activated their recruitment to the polyribosome from stored ribonucleoproteins (11). A distinct 63-nt G+C-rich RNA sequence 105 nt downstream from the H-ferritin mRNA IRE was found to confer 2–3-fold IL-1β-dependent enhancement of the translation of hybrid H-ferritin-chloramphenicol acetyltransferase (CAT) reporter mRNAs in human HepG2 hepatoma cells (13). This IL-1β-dependent translation enhancement element encodes a core 25-nt consensus motif (CGCGCGCAACGCACGCGCGCCGG, the acute box (AB)), homologous to sequences encoded in the 5′-UTRs of several hepatic acute phase reactant mRNAs, including α-1 acid glycoprotein (AGP), α-1 antitrypsin (α1AT), and haptoglobin (14, 15). In both hepatitis A and B, a homologous L-ferritin AB also conferred translation enhancement to reporter mRNA (16). A third transcript containing the AB is the Alzheimer amyloid precursor protein (APP), which confers IL-1β-induced regulation of APP translation (9). Based on the fact that repression of upstream IRE-dependent translation by DesF was dominant over the IL-1β-induced stimulation of ferritin translation (9), we suggested previously that the AB operated to enhance the 60 S ribosome joining step of ribosome translational scanning of H-ferritin mRNA by the 43 S ribosome according to the Kozak model (10) rather than providing an internal ribosome entry site (IRES).

Current models for ferritin translation only incorporate iron-dependent IRP-1 and IRP-2 binding to the 5′-UTR of H-ferritin mRNA for modulating the interaction with the incoming ribosome and translation initiation factors (12). There are currently no reports identifying other RNA-binding proteins that may selectively interact with the H-ferritin mRNA 5′-UTR via the AB to regulate basal ferritin translation. The poly(C)-binding proteins (PCBPs) are structurally diverse family, including heterogeneous nuclear ribonucleoprotein K and αCP1–4 (17). They bind mRNA sequences that contain either a single C run (heterogeneous nuclear ribonucleoprotein K) or stretch of Cs (αCPs) via their K-homology (KH) domains (18). The PCBPs have been implicated in regulation of mRNA stability, translation silencing (mainly through interactions with 3′-UTR sequences), and enhancement. The PCBPs family members bind to each other and to other mRNA binding proteins, including AUF-1 (AU-rich element RNA-binding protein) and PABP, which are important in modulating decay of globin mRNAs (19). αCP2 regulates translation of poliovirus mRNA via a specific IRES (20, 21) and 15 lipoxigenase mRNA translation via a C-rich element in the 3′-UTR (17, 22). Pertinent to this report, human HepG2 and Hep3B hepatoma cells are reported to contain αCP1 and αCP2 that bind 3′-UTR elements of erythropoietin and tyrosine hydroxylase mRNAs (23).

To determine the structural and functional characteristics of the AB in the regulation of ferritin translation, we tested the effects of mutations of the AB element on IL-1- and iron-dependent translation in two different human liver cell lines. We found that the predicted shape and sequence of the AB element, independent of the IRE, was critical for maintaining enhanced base-line translation in addition to an IL-1β-induced increase in translation. In a time course the AB responded to IL-1β signals at the same time that endogenous ferritin was induced. Transfections using an H-ferritin promoter and 5′-UTR sequences together with either the wild-type AB or an equivalent length scrambled AB demonstrated that the AB was a novel basal and IL-1-dependent translational enhancer. Furthermore, in RNA gel-shift (RNA electrophoretic mobility shift assay (REMSA)) and UV cross-linking experiments we demonstrated that the H-ferritin mRNA AB cis-element interacts specifically with recombinant αCP1 and that both αCP1 and αCP2 associate with H-ferritin mRNA in vivo. These data provide evidence that the AB is an important contributor to H-ferritin expression in human liver cells, responsible for enhancing both basal and IL-1-mediated translation and also identified the PCBPs (αCP1 and αCP2) as novel H-ferritin mRNA-binding proteins that may act in a coordinate manner with the IRPs to control the overall rate of ferritin translation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human hepatoma HepG2 (ATCC HB-8065) and Hep3B (ATCC HB-8064) cells were grown in Dulbecco’s minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine, essential amino acids, penicillin, and streptomycin (Invitrogen) (7).

**Metabolic Labeling and Immunoprecipitation**—Cells were incubated in Dulbecco’s minimal essential medium with IL-1β, IL-1α, IL-6 (0.5 ng/ml, Invitrogen), iron-transferin (Fe₃₆₀₀₀TD) (10 μM), or desferrioxamine mesylate (50 μM DesFe) for different time periods. Cells were washed in methionine-deficient media (RPMI 1640, Invitrogen) and then grown in the presence of 25–100 μCi/ml [³⁵S]methionine for 30 min and subsequently washed twice in phosphate-buffered saline at 4 °C. Equal numbers of cells (10⁶ cells) were lysed in 1 ml of lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.01% Nonidet P-40, 0.1 mM Na orthovanadate, 2 mM phenylmethylsulfonyl fluoride). Total protein synthesis was measured by the amount of isobutyl[³⁵S]methionine incorporated into trichloroacetic acid-precipitable material. 20 μg of human ferritin or α1AT-IgG was added to each sample for immunoprecipitation assay as described previously (9). After extensive washing, immunoprecipitated H- and L-ferritin and α1AT (21, 18, and 55 kDa, respectively) were separated by denaturing PAGE (7.5% polyacrylamide, 15% acrylamide, 0.1% SDS, 0.1 M sodium phosphate, pH 7.2, gels or on 15% Laemmli-SDS gels) and visualized by autoradiography with Amplify-fluorographic enhancer (Amersham Biosciences) on Kodak XAR-5 film. densitometric scanning of the autoradiographs was performed with a Bio-Rad model 620 video densitometer.

**Plasmid Constructs for Transient Transfections**—pUC-HFHER (a gift from Dr. J. Drysdale, Tufts University School of Medicine, Boston, MA) containing a 454-bp SstI fragment from the H-ferritin gene cloned into PUC12 (9, 13) comprises 162 nt of H-ferritin gene sequence upstream of the PUC12 (9, 13) cap site and 292 bp of the first exon containing 5′ leader sequences. All subsequent constructs were derived from H-ferritin sequences encoded by pUC-HFHER, and their identity was confirmed by double-stranded DNA sequencing to preclude the presence of artificial AUG sites upstream of the CAT initiation codon. HIRECAT contains 302 nt. A SstI-StyI fragment from pUC-HFHER ligated into the unique SstI and HincII regulatory domains. For transient transfections, pUC-HFHER was cotransfected with plasmid DNA and hiroferritin constructs. To prepare pSV2CAT-derived constructs, pSV2(Ac)CAT and pSV2(Bt)CAT were prepared by digesting the 63 bp of StyI-DdeI fragment from the 5′-UTR of the H-ferritin mRNA (previously 5′ end filled by Klenow polymerase) (18) into a unique StuI site of pSV2CAT, residing 42 nt downstream of the SV40 early T-antigen.
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**Fig. 1.** Plasmid constructs generated for transfection reporter gene studies. Panel A, H-ferritin mRNA 5′-UTR nucleotide sequence (GenBank accession number HUMFHC122) demonstrating IRE and AB stem loops, Sty1 and Ddel restriction sites, and CAC deletion (Δ3) mutant and C-A point (Mt) mutant. Panel B, pSV2CAT constructs (Ac, wild-type (wt) sense orientation of Sty1/Ddel fragment (63 nt); rei, wild-type antisense of Sty1/Ddel fragment (63 nt); Mc, 175C→A point mutant of Sty1/Ddel fragment (63 nt); Δ3, 175C/AC deletion mutant of Sty1/Ddel fragment (60 nt) where transcription control was via the SV-40 promoter (SV-40 pr). Panel C, HIRECAT comprises the Sst1/Sty1 H-ferritin gene sequence, containing the H-ferritin promoter (H-ferr pr) for transcription control and the IRE. Panel D, 5′-UTRCAT comprises the Sst1/Ddel H-ferritin gene sequence, containing the H-ferritin promoter and the AB regions.

**Transfected Transfections—**HepG2 and Hep3B hepatoma cells (10⁷ cells) were transiently transfected with 10 µg of each H-ferritin 5′-UTR-derived construct and 5 µg of pSVLlac as a control using either calcium phosphate-precipitated DNA (13, 20) or by Lipofectamine (Invitrogen) according to manufacturer’s recommendations (9). After transfection the cells were passaged to equal density into 10-cm² flasks before exposure of the cells to iron (2.5 mM Fe₂Tf) and/or cytokines (IL-1β, IL-6, 1 ng/ml), RNA extracted using the AGP 5′-UTR cRNA probes (negative controls). A 63-nt H-ferritin AB deletion cRNA of the H-ferritin AB (Mc). Labeled cRNA transcripts were produced as previously described (25) with [α-32P]UTP (Amersham Biosciences). AGP 5′-UTR oligo probes were employed as templates to transcribe AGP 5′-UTR cRNA probes (negative controls). A 63-nt H-ferritin AB cRNA (A146-A200; Fig. IA) identical to the H-ferritin AB of pSV2/Ac/CAT was transcribed for UVXL (25). Labeled cRNA probes had a specific activity of ~5 x 10⁶ cpm/µg of RNA. Ferritin IRE transcripts were prepared as described (23).

**RNA 2 Structure Predictions—**Structural plots were determined by mfold3.1 (www.bioinfo.rpi.edu/applications/mfold/old/rna/form.1.cgi).

**RNase T1 Characterization—**10⁴ cpm (±10 µg) of 32P-labeled H-ferritin AB riboprobe (33 nt) was digested with RNase T1 for 30 min at 37 °C over a concentration range sufficient to completely digest heat-denatured cRNA (not shown). Reactions were terminated in sample buffer (95% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol), and RNA fragments were resolved by denaturing PAGE (20%) acrylamide:bisacrylamide, 7 µ urea gels) before autoradiography.

**Cytosplasmic Protein Extracts—**Cytosplasmic protein extracts of HepG2 cell cultures were prepared as described (7). Briefly, cells were scraped from culture dishes in chilled phosphate-buffered saline, centrifuged at 450 x g for 4 min at 4 °C, washed again in phosphate-buffered saline, and then incubated for 20 min in cold cytoplasmic extraction buffer (10 mM HEPES, 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol) containing freshly added protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 2 µg/ml aprotinin). Lysates were cleared by centrifugation at 4 °C for 10 min at 12,100 x g, and the supernatant was snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentra-
Iron Is Required for IL-1β Induction of L- and H-ferritin Subunit Synthesis—To investigate the relative impact of IL-1β and iron (Fe₄Tf) on the synthesis of ferritin subunits in vitro, we incubated HepG2 cells with each ligand over a time course of 2–6 h. Synthesis of L- and H-ferritin subunits was increased by 4-fold by IL-1β relative to untreated counterparts at 6 h (Fig. 2,A and D). A combination of Fe₄Tf and IL-1β increased ferritin synthesis to ~20-fold above control (Fig. 2, A and D), suggesting a synergistic effect of the two ligands when combined. The synthesis of α₁AT, another liver protein remained unchanged (see Fig. 2B and the line in Fig. 2D). The unchanged α₁AT levels are depicted by the line in panel D relative to the modulated levels of ferritin synthesis measured by densitometry. Intracelluar iron chelation with desferrioxamine mesylate (DesF) inhibited ferritin subunit synthesis even when IL-1β was present (Fig. 2A, lane 5) without affecting α₁AT synthesis.
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Ferritin synthesis is induced by IL-1. These data are consistent with other reports that demonstrate iron uptake as measured by 59Fe-transferrin uptake (data not shown). IL-1β (0.5 ng/ml) or Fe2Tf (10 μM) for 24 h. Error bars, S.D.; n = 3. Panel C, AB-HIRECAT with a 63-bp wild-type AB DNA cassette ligated into the 5′-UTR PstI site of HIRECAT. Scr-HIRECAT with a 63-bp Scr acute box cassette ligated into the 5′-UTR PstI site of HIRECAT. Panel D, CAT reporter expression (enzyme-linked immunosorbent assay) in AB-HIRECAT (black bars) relative to Scr-HIRECAT (gray bars) in HepG2 transfectants incubated for 16 h with either no ligand (i), IL-1α (ii), IL-1β (iii), IL-6 (iv), Fe-Tf (v), or DesF (vi). Error bars, S.E.; n = 6. Panel E, (ii) IL-1β 3′ end of the entire sequence. Panel F, time course of CAT reporter expression (enzyme-linked immunosorbent assay) in 5′-UTR CAT transfectants stimulated with and without IL-1α or IL-1β (0.5 ng/ml) for 2, 6, and 24 h. Panel B, the relative effect of IL-1β and iron (Fe2Tf) on CAT gene expression in HIRECAT and 5′-UTR CAT transfectants with and without IL-1β (0.5 ng/ml) or Fe2Tf (10 μM) for 24 h. Error bars, S.D.; n = 3. Panel C, AB-HIRECAT with a 63-bp wild-type AB DNA cassette ligated into the 5′-UTR PstI site of HIRECAT. Scr-HIRECAT with a 63-bp Scr acute box cassette ligated into the 5′-UTR PstI site of HIRECAT. Panel D, CAT reporter expression (enzyme-linked immunosorbent assay) in AB-HIRECAT (black bars) relative to Scr-HIRECAT (gray bars) in HepG2 transfectants incubated for 16 h with either no ligand (i), IL-1α (ii), IL-1β (iii), IL-6 (iv), Fe-Tf (v), or DesF (vi). Error bars, S.E.; n = 6. Panel E, 2′ structure prediction plot of AB-HIRECAT where b is the 5′ end of the AB insert, and e is the 3′ end of the AB insert; *, 5′ end of the entire sequence. Panel F, 2′ structure prediction plot of Scr-HIRECAT where b is the 5′ end of the Scr insert, and e is the 3′ end of the Scr insert; *, 5′ end of the entire sequence. CAP, chloramphenicol.

Contribution of the AB Element to Ferritin Translation in HepG2 Cells Stimulated with Iron and IL-1β: the AB Confers Enhanced Base-line Translational Enhancement—To determine the relative contribution of the AB versus the IRE to the ferritin translational enhancement described above, we generated constructs of the 5′-UTR of the H-ferritin gene (HIRECAT and 5′-UTRCAT) for transfections. The HIRECAT construct harbors a CAT reporter gene driven by the natural H-ferritin promoter and encodes 144 nt of the H-ferritin 5′-UTR, containing an IRE stem-loop 30 nt from the 5′ cap site but lacking the IL-1β-responsive AB sequence (Fig. 1C). The 5′-UTR-CAT transfectants encode the full-length H-ferritin mRNA 5′-UTR driven by the natural H-ferritin gene promoter and contain both the IRE stem loop and the AB domain (Fig. 1I).

When HepG2 cells were transfected with 5′-UTRCAT and treated with either IL-1α or IL-1β, we found that there was no increase in CAT translation at 2 h (Fig. 3A). However, at 6–24 h both basal and IL-1-stimulated CAT translation increased severalfold (~4-fold for IL-1α and ~3-fold IL-1β) (Fig. 3A). The data in these experiments closely paralleled the time course we had observed for IL-1β induction of endogenous H-ferritin expression in the same cells (Fig. 2E).

Iron induced an 8-fold increase in CAT gene expression in HIRECAT transfectants (Fig. 3B), but IL-1β had no effect. These data confirmed that a functional IRE was not sufficient
to confer IL-1β-dependent translation to a CAT reporter mRNA. As before, IL-1β conferred a 2.6-fold increase in CAT gene expression in the 5'-UTRCAT transfectants (Fig. 3B). Interestingly, the fold induction of iron-dependent translation was lower for 5'-UTRCAT construct (3-fold) than for HIRECAT (8-fold) when compared with untreated control. This maybe
because the base-line CAT expression was higher for the 5'-UTR CAT transfectants relative to the HIRECAT transfectants (−2−2.5-fold, n = 3), such that the absolute change induced by iron was substantially smaller. The expected greater maximal induction of 5'-UTR CAT expression under conditions of iron influx was not observed as seen with endogenous ferritin levels (Fig. 2), suggesting that other regions of the ferritin mRNA maybe involved in translational regulation (e.g. 3'-UTR) (26, 27).

A Scr 63-nucleotide spacer was generated (Fig. 3C) to directly investigate if the presence of the wild-type AB was required to confer basal and IL-1-dependent translational enhancement by H-ferritin 5'-UTR sequences. In transfection studies using HepG2 cells, we consistently observed that chimeric H-ferritin-CAT mRNA derived from AB-HIRECAT transfectants was translated 3-fold more efficiently than H-ferritin-CAT mRNA derived from Scr-HIRECAT transfectants (Fig. 3D). In AB-HIRECAT transfectants, IL-1α, IL-1β, and iron induced CAT reporter translation (2-fold relative to untreated (n = 6)), whereas only iron remained active in Scr-HIRECAT counterparts. We concluded that the presence of the wild-type AB upstream of the CAT start codon increased reporter translation, whereas an equivalently located scrambled insert was unable to confer either increased base-line or IL-1-dependent translation relative to HIRECAT. These data indicated that the AB was active as a sequence and/or structure-specific translation enhancer element (compare Fig. 3, E and F).

The H-ferritin AB Domain Is an Autonomous Base-line Translation Enhancer That Also Mediates IL-1β-induced Translation—The HIRECAT and 5'-UTR CAT based transfections described above provided the first evidence that the AB element in the H-ferritin mRNA conferred increased base-line translation in addition to being an IL-1β-responsive translational enhancer. To further characterize the H-ferritin AB-mediated translation regulation, we generated a series of constructs for comparative analysis. The wild-type Ac transcript (pSV2(Ac)CAT) harbors a chimeric 137-nucleotide 5'-UTR consisting of viral sequences with the AB inserted in the sense orientation upstream of the CAT reporter start codon, whereas rev (pSV2(rev)CAT) was used for an antisense comparison. These constructs encode the same length 5'-UTR (137 nt) and transcribe a hybrid CAT gene under the translational control of sense (Ac) and antisense (rev) acute box sequences (63 nt). In HepG2 cells, the Ac construct induced a 2.2-fold increase in basal CAT expression compared with the parental vector, pSV2CAT (Fig. 4A, n = 9). IL-1β induced a reproducible increase in CAT expression by 75% in cells transfected with the wild-type Ac construct (Fig. 4A, n = 6). No significant change in CAT expression was observed with the IL-1β-treated antisense (rev) transfectants or the control transfectants (pSV2CAT) (Fig. 4A). Of particular interest, the antisense construct also decreased basal expression relative to control (Fig. 4A) despite the fact that the predicted 2 structure (28) for the rev stem-loops is similar (with a $\Delta G = \pm 40$ kcal/mol). RNase protection assay confirmed no change in CAT mRNA levels with IL-1β treatment, indicating that the effect observed was at the level of translation (Fig. 4B). Taken together these data indicate that the H-ferritin mRNA AB is a significant translational enhancer that not only confers IL-1β-dependent CAT expression but also acts as a powerful base-line translational enhancer.

Originally the pSV2(rev)CAT construct was employed to exclude the possibility that the AB was a transcriptional enhancer. Gillies et al. (29) have shown that transcriptional enhancer elements operate in an orientation-independent fashion. Our data consistently demonstrate that the AB element is active only when inserted in front of a heterologous start codon in the sense orientation (as in the pSV2(Ac)CAT construct), a finding consistent with the presence of novel translational enhancer activity.

To further investigate the function of the AB on translational efficiency, HepG2 cells were transfected with either pSV2CAT or pSV2(Ac)CAT, and the CAT activity was standardized to CAT mRNA (as measured by slot-blotted CAT mRNA levels) (16). In these experiments, the H-ferritin AB increased CAT expression by 2.8-fold, as shown by the change in the slope of kinetic induction for pSV2CAT relative to pSV2(Ac)CAT gene expression (Fig. 4C). In other experiments we determined the maximal induction of translation mediated by the AB from the reaction slopes of graphs of CAT expression from cells transfected with either the Ac or rev or parental vector. We found that pSV2(Ac)CAT lysates accumulated 3.5-fold more CAT than pSV2(rev)CAT and that pSV2(Ac)CAT lysates accumulated CAT 2.2-fold faster than the parental pSV2CAT lysates (data not shown).
Translation Enhancement by the H-ferritin mRNA AB in a Second Hepatoma Cell Line (Hep3B Cells)—To investigate the cell line specificity of the translation enhancement of the H-ferritin AB, Hep3B hepatoma cells were transfected with pSV2(Ac)CAT and pSV2CAT. Similar to HepG2 cells, the AB increased CAT expression by 3-fold (corrected against CAT mRNA levels using slot-blot analysis as above) (Fig. 5A).

A marked dose- and time-dependent induction of CAT expression was evident when cells transfected with pSV2(Ac)CAT were treated with IL-1β (~4-fold increase in response, Fig. 5, B and C). In contrast, no significant change in CAT activity was observed with the pSV2CAT transfectants (Fig. 5, B and C). These data confirm that the effects of the AB in these two human liver cell lines are very similar, although there is a
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FIG. 6. Wild-type configuration of the H-ferritin mRNA AB enhancer is required to maintain both IL-1β-dependent- and base-line translation enhancement. Panel A, HepG2 cells were transfected with 10 μg of either pSV2/AcCAT (wild-type, Ac), pSV2/Δ3CAT (Δ3), pSV2/McCAT (Mc), or pSV2CAT (Vec) and treated with and without IL-1α, IL-1β, or IL-6 at 1 ng/ml. The CAT activity was determined as above and expressed as CAT activity per mg of CAT mRNA. Error bars, S.D.; n = 4. Panel B, HepG2 cells were transfected with 10 μg of either pSV2/AcCAT (Ac), pSV2/revCAT (rev), or pSV2/Δ3/CAT (Δ3) and treated with and without either IL-1β or IL-6 (1 ng/ml). The CAT activity was assessed by the percent [14C]CAP (chloramphenicol) acetylated using TLC (13). n = 4. Panel C, HepG2 cells were transfected with 10 μg of Ac, Δ3, Mc, rev, or Vec together with pRSVLuc (5 μg) (as control), and CAT was activity determined over a 100-min time course as above. CAT activity ([3H]-labeled chloramphenicol) was standardized against luciferase activity. Panel D, predicted 2° structure of the H-ferritin AB stem-loop (33 nt) (28). Panel E, RNase T1 digestion of H-ferritin AB RNA (33 nt). RNA fragments of undigested- and RNase T1-digested [32P]-labeled H-ferritin AB cRNAs were resolved by denaturing PAGE (20% sequencing, 7 M urea gels). Lane 1, size markers; lane 2, undigested cRNA; lane 3, 1 unit (U) RNase T1; lane 4, 0.5 units of RNase T1; lane 5, 0.1 units of RNase T1; lane 6, size markers, where the 20-mer is present at twice the concentration. n = 3. A and B are molecular weight markers, constituting a DNA ladder.

consistently larger effect on translation in the Hep3B cells. The greater induction by IL-1β of pSV2/Ac/CAT in Hep3B cells may reflect the fact that base-line translation is lower in Hep3B cells compared with HepG2 cells, and greater induction of mRNA translation in Hep3B cells is required to achieve the same final endpoint ferritin levels as in HepG2 cells.

To verify the importance of the IL-1β pathway in mediating the AB-mediated translation enhancement, an IL-1β-neutralizing Ab (Invitrogen) was added. The typical IL-1β-induced increase in CAT expression (~2.5–4-fold) was markedly reduced in the presence of IL-1β-inactivating Ab (Fig. 5D). Inclusion of normal rabbit serum in all samples had no influence in the pattern of induction (data not shown). These data are consistent with a role for IL-1β receptor signaling in mediating translation of H-ferritin mRNA in Hep3B cells via the AB element.

Effect of Mutations and Deletions within the H-ferritin mRNA AB Sequence on Translation—To investigate the effects of mutations within the AB region on translational efficiency, we generated two key mutations of the wild-type H-ferritin sequence: (i) pSV2/Δ3CAT (Δ3) and (ii) pSV2/McCAT (Mc) (Fig. 1, A–B). The Δ3 vector encodes a CAC deletion (Δ173–175) from the loop region of the AB stem-loop and is predicted to substantially alter folding of the AB domain (Supplemental Fig. 1C). The Mc construct contains a C177 to A175 mutation in the loop that maintains correct folding of the AB (Supplemental Fig. 1B). In transfected HepG2 cells, base-line CAT activity of Δ3 was reduced by 6–7-fold relative to Ac and reduced 2-fold relative to parenteral vector (Fig. 6A). IL-1α and IL-1β increased CAT gene expression in the Mc transfectants (Fig. 6A), similar to that observed in Ac transfectants. We also investigated the effects of IL-6 on CAT activity. Multiple experiments showed that IL-6 caused a modest reduction (10–20%) in Ac-CAT and Mc-CAT translation in pSV2/Ac/CAT and pSV2/Mc/CAT transfections (see Fig. 6A, in which n = 8, and Fig. 6B, where n = 5).

Base-line CAT expression was consistently 2-fold higher in Mc transfectants than in Δ3 transfectants and ~30% above that of the parental vector (Vec). These data illustrated the ligand specificity of IL-1α/β versus IL-6 and the importance of maintenance of the AB stem-loop structure and sequence for preservation of the translational enhancement. Interestingly, the AB antisense construct pSV2/rev/CAT and Δ3 each abrogated base-line and IL-1β/IL-6-treated CAT expression similarly (Fig. 6B).

We next examined all of the constructs together in a time course experiment from representative lysates (n = 4) taken from the complete set of HepG2 transfectants. The Ac and Mc constructs generated 3–4-fold more CAT gene expression than pSV2CAT (Fig. 6C). Both Δ3 and rev transfectants exhibited 3–4-fold less CAT activity after normalization than pSV2CAT alone (Fig. 6C). The rank order for the available AB elements as translational regulators of downstream CAT reporter expression was Ac = Mc > Vec > Δ3 = rev. Taken together, these data illustrate the potency of the H-ferritin AB element as a
The H-ferritin mRNA AB domain is bound by proteins from HepG2 cells. Panel A, REMSA shows that the AB (33 nt) forms RNA-protein complexes selectively with extracts of HepG2 cells; lanes 2–9 include varying amounts of unlabeled (cold) competitor RNA, where the numbers represent molar excess of cold probe (10–200-fold). Arrows denote two specific H-ferritin RNA-protein complexes (RPC). H-AB, H-ferritin AB; L-AB, L-ferritin AB; Δ3, Δ3 mutant H-ferritin AB; Mc, Mc H-ferritin AB. Panel B, UVXL with HepG2 cell extract (10 µg) incubated with 32P-labeled H-ferritin AB cRNA riboprobe (63 nt) with or without excess unlabeled (cold) H-ferritin AB cRNA (lane 2), poly(A) (lane 3), or poly(C) (lane 4). M denotes 13C M, markers. Panel C, ImageQuant analysis of the intensity of the 43-, 48-, and 68-kDa complexes after competition with excess cold competitor RNA in panel B, with the data graphed as the % relative intensity to the most intense complex within a specific M group. Error bars represent S.D. Panel D, effect of IL-1β on binding to the AB element in UVXL assay. The bottom panel shows a Western blot of the membrane using actin Ab to control for variation in loading. Panel E, IP-RT-PCR assay demonstrates that αCP1 and αCP2 associate with H-ferritin mRNA in vivo. RT-PCR was performed with H-ferritin-specific primers on RNA isolated from beads (lanes 6–11) and from supernatant cell extracts (lanes 1–5). Controls included beads alone (lane 6), a RT reaction (lane 12), IP-RT-PCR using αCP1 and αCP2 Abs with total (protein free) HepG2 RNA (lane 15), and RT-PCR with HepG2 RNA (lane 17). M, molecular markers. Panel F, Western blot showing the effect of IL-1β and Fe2Tf on intracellular ferritin levels in HepG2 hepatoma cells treated with either IL-1β (lane 1), Fe2Tf + IL-1β (lane 2), Fe2Tf (lane 4), or control (lane 3; control). The numbers denote the M, of L- and H-ferritin subunits.

The H-ferritin mRNA AB is a Target for Proteins from HepG2 Cells, Including the Poly(C)-binding Proteins 1 and 2 (αCP1 and αCP2)—Analysis of the AB and surrounding sequences permitted folding of the H-ferritin AB element into a stable stem-loop, with the loop comprising the sequence CCACCC, where the terminal CG nucleotides are base-paired (Supplemental Fig. 1A). A single-stranded 5-nt poly(C) sequence (C144-C148) was identified upstream of the stem-loop but within the AB region. A CCCCUCUC motif (C190-C197) was also identified 2 nt downstream of the stem loop (Fig. 1A). Another sequence with a poly(C) consensus element, C102-C107 (Fig. 1A), resides outside the AB region but within the H-ferritin mRNA 5′-UTR. The presence of these poly(C) stretches raise the possibility that the PCBPs may bind the AB and the H-ferritin 5′-UTR and function to regulate translational efficiency, similar to that observed for 15 lipoxygenase (22) and poliovirus RNA (20) (for review, see Ref. 17).

Given these transfection data, we further examined the predicted 2° structure of the wild-type H-ferritin AB region (Fig. 6D). To obtain direct physical data concerning folding of the H-ferritin AB mRNA, we labeled the 33-nt H-ferritin AB cRNA for use in RNase T1 assays. RNase T1 cuts RNA specifically at the 3′ end of guanosine (G) residues and adjacent nucleotides in single-stranded RNA, GpN, and we predicted that it could potentially cleave at eight GpN sites in the probe. Interestingly, however, RNase T1 digestion demonstrated a single major product at Mw ~ 32 nt (Fig. 6E, n = 3), indicating that the 33-nt H-ferritin AB was highly resistant to RNase T1 degradation. This suggests that the H-ferritin cRNA probe folded into a higher order structure that is cleaved only once at the GC bond at the 5′ end to generate the 32-nt cRNA. After thermal denaturation at 95 °C for 10 min, the AB RNA structure was completely digested (data not shown).
detectable complexes with the probe (Fig. 7A, lane 5), suggesting that both H- and L-ferritin ABs have a similar affinity for HepG2 cell RNA-binding proteins. A 200-fold excess of unlabelled homologous cRNA of the acute phase reactant, AGP, did not compete for formation of RPC1 and -2 (Fig. 7A, lane 6), emphasizing the specificity of the interaction. Of interest, a 200-fold excess unlabelled Δ3 and Mc cRNAs (Fig. 7A, lanes 7 and 8, respectively) did not compete as efficiently as unlabelled H- or L-ferritin AB cRNAs, consistent with a sequence and structure-specific requirement for the trans-acting factors. It is noteworthy that cold Mc RNA competed more efficiently than cold Δ3 mRNA, consistent with the cis-regulatory data, demonstrating a sequence and structural requirement for optimal function of the AB as a translational enhancer (Fig. 6). An increase in complex formation was observed in the presence of a 200-fold excess of an unlabeled IRE cRNA (Fig. 7A, lane 9), suggesting that either there are important protein-protein interactions between iso-IRPs and proteins forming RPC1 and -2, or the IRPs compete with other RNA-binding proteins to bind to the AB.

UVXL-Western assay with a HepG2 cell cytoplasmic extract and the 32P-labeled H-ferritin AB riboprobe (63 nt) demonstrated several RPCs of M, 43–68 (Fig. 7B, lane 1). These complexes were effectively competed by excess unlabeled AB cRNA (Fig. 7B, lane 2) and C but not by excess poly(A) (Fig. 7B, lane 3 and C). However, excess poly(C) significantly reduced formation of the 68-, 48-, and 43-kDa complexes (Fig. 7B, lane 4 and C). Varying concentrations of unlabeled tRNA (0–1 μg) had no effect on the RNA-protein complex (data not shown). The relative intensities of each of the complexes determined by ImageQuant analysis and plotted (Fig. 7C) highlights that the 43-kDa complex was reduced the most by competition with poly(C) RNA, consistent with the known M, of aCP1/aCP2 at ~43 kDa (17). Probing the same membrane with aCP1 and aCP2 Abs confirmed that both proteins were present in the extracts and that excess competitor tRNA did not effect their concentrations in samples (Supplemental Fig. 2A). Taken together, these data raised the possibility that the AB could be a target for binding by aCP1 and/or aCP2.

We next examined the effects of IL-1β on RPC formation from HepG2 cells treated over a 16-h time course using UVXL-Western assays. IL-1β treatment decreased binding of the RPCs (43–48 kDa) over the 16-h period (by ~40% as determined by PhosphorImager-ImageQuant analysis), whereas actin levels remained constant (Fig. 7D). In addition, IL-1β had little or no effect on the binding interaction between IRE and IRP in hepatoma cells (data not shown). To determine whether treatment with IL-1β altered total cellular levels of aCP1 and aCP2 protein, we examined lysates from the cells from the 16-h IL-1β time course. We consistently observed that IL-1β did not change the intracellular levels of aCP1 and aCP2 in HepG2 cells (Supplemental Fig. 2B). These data indicated that IL-1β signals modulated binding to the AB region rather than altering absolute levels of aCP1 and aCP2.

To investigate the association of aCP1 and aCP2 with H-ferritin mRNA in vivo and to investigate the effects of iron on this interaction, we utilized an IP-RT-PCR assay in HepG2 cells. H-ferritin mRNA could be detected in all HepG2 lysate supernatants (Fig. 7E, lanes 1–5), producing an amplicon of 290 bp, the size of the positive H-ferritin control (Fig. 7E, lane 17). aCP2 co-immunoprecipitated with H-ferritin mRNA in lysates from untreated HepG2 cells (Fig. 7E, lane 8), whereas association of H-ferritin mRNA with aCP1 was not detected (Fig. 7E, lane 7). However, aCP1 preferentially associated with H-ferritin mRNA in lysates from HepG2 cells treated with 10 μM iron (Fig. 7E, lane 9). Residual aCP2 binding remained under these conditions (Fig. 7E, lane 10). In the presence of IL-1β neither aCP1 nor aCP2 co-immunoprecipitated with H-ferritin mRNA (Fig. 7E, lanes 11–12) even in the presence of iron (Fig. 7E, lanes 13–14). This is consistent with the data in Fig. 7D in which IL-1β reduced complexes at 43–48 kDa, which are predicted to contain one or more of the PCBP5s. Western blotting confirmed that the HepG2 cells exhibited a consistent physiologic response to ligand; that is, an increase in both H- and L-ferritin protein levels in response to iron and IL-1β, with an augmented response when added together (Fig. 7F). Taken together these data suggest that aCP1 and aCP2 both associate with H-ferritin mRNA in vivo and that the interaction is regulated by the prevailing intracellular iron concentration.

Given the IP-RT-PCR data demonstrating in vivo association of the aCPs with H-ferritin mRNA, we next sought to examine the binding between recombinant aCP1 and ferritin mRNA. In REMSA assays recombinant aCP1 bound avidly to the AB probe (Fig. 8A), whereas no significant binding was observed with high concentrations of GST protein alone. In UVXL assays, recombinant aCP1 binding to the H-ferritin AB probe was effectively competed with excess unlabeled H-ferritin AB and poly(C) mRNAs but not with poly(A) mRNA (Fig. 8B). Excess tRNA (1 μg) did not compete with the aCP1-ferritin mRNA interaction (data not shown). An aCP1 Western blot confirmed that these changes were unrelated to alteration in the input concentration of aCP1 (Fig. 8B, right-hand panel, aCP1 at 43
These data suggest a specific direct interaction of αCP1 with H-ferritin AB mRNA and support our sequence homology observations and IP-RT-PCR results.

**Discussion**

These studies have defined the role of the H-ferritin AB cis-element within the 5′-UTR as a significant translational regulatory element and identified αCP1 and αCP2 as novel trans-acting factors that associate with the H-ferritin mRNA in vivo. The data indicate that the H-ferritin mRNA AB is a substantial base-line translation enhancer element of H-ferritin mRNA in addition to being IL-1α and IL-1β responsive in two separate hepatoma cell lines, HepG2 and Hep3B. Before this work, the only RNA-protein interaction controlling ferritin synthesis that had been well characterized was the regulated binding between the IRE RNA stem-loop and the iso-IRPs.

The functional RNA stem-loops predicted for H-ferritin mRNA 5′-UTR (Fig. 6) and is predicted not to alter the H-ferritin AB mRNA stem-loop structure in H-ferritin mRNA additional to the better characterized IRE.

The antibody-mediated down-regulation of the H-ferritin AB cis-element within the 5′-UTR as a significant translational regulatory element and identified αCP1 and αCP2 as novel trans-acting factors that associate with the H-ferritin mRNA in vivo. The data indicate that the H-ferritin mRNA AB is a substantial base-line translation enhancer element of H-ferritin mRNA in addition to being IL-1α and IL-1β responsive in two separate hepatoma cell lines, HepG2 and Hep3B. Before this work, the only RNA-protein interaction controlling ferritin synthesis that had been well characterized was the regulated binding between the IRE RNA stem-loop and the iso-IRPs.

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configuration. The Mc mutant enhanced base-line reporter gene regulation and IL-1β-induced translation in hepatoma Hep3B cells, although this effect was less than in HepG2 cells (Figs. 5 and 6). It will be of interest to determine whether natural mutations exist in the human ferritin AB domain and if so whether they are associated with a clinical phenotype reflective of aberrant ferritin homeostasis.

The PCBPs aCP1 and aCP2 are members of the heterogeneous nuclear ribonucleoprotein K-homology domain family of RNA-binding proteins (17) and regulate the expression of a variety of transcripts, including α-globin, tyrosine hydroxylase, and erythropoietin (23, 41–45) as well as regulating translation of 15-lipoxygenase (22) and human Papillomavirus (17, 20). We have recently identified in vivo association of aCP1 with several transcripts including the human androgen receptor (18), p21WAF1 (46), and renin (47). In the latter, aCP1 plays an important role in regulating the stability of renin mRNA. Identification of this diverse set of key mRNA targets for the CPs has focused attention on determining their functional role in human pathology.

Our in vivo association of aCP1 and aCP2 with H-ferritin mRNA from HepG2 cells suggests for the first time that these proteins bind to one or more of the motifs distributed throughout the H-ferritin mRNA (Fig. 1A) and that they may play an important role(s) in the regulation of ferritin gene expression. In particular, putative PCBP binding motifs in the AB region include a 5-base poly(C) stretch from +144 to +148 nt, a CCCTCTCC motif at +190 to +197 nt from the H-ferritin mRNA 5′ cap site, and a further stretch from +102 to +107 nt. Our REMSA and UVXL studies confirmed that aCP1 binds the AB domain mRNA, consistent with this region being an important target within the 5′-UTR. Most interestingly, our data indicate that association of PCBP with H-ferritin mRNA is an iron- and IL-1β-dependent phenomenon. Thus, regulation of PCBP-ferritin mRNA interactions by intracellular iron and oxidative stress may be critical determinants of the overall ferritin subunit translational rate and protein synthesis.

Our data are consistent with a model whereby the AB is a docking site for 60 S ribosome subunit entry (48). Interestingly, DesF causes translational repression, which exerts a dominant effect over the IL-1β induction of ferritin subunit synthesis (13) (Fig. 2). This is because iron chelation by DesF suppresses ferritin translation at the first stage by inducing a high affinity interaction between IRP1/IRP2 and the 5′ cap-specific IREs, which prevents IL-1β from exerting its effects. This suggests that IL-1β-induced ferritin translation is dependent upon the pre-formation of a scanning complex, which is a requirement for establishing ferritin synthesis during 60 S ribosome entry 5′ of the H-ferritin AUG codon (13).

According to the scanning model of translation the position of RNA stem-loops within the 5′-UTR is a critical determinant for translational repression of mRNAs (10, 34, 30). Goossen and Hentze (49) provided firm experimental support for a model whereby the AB is a “functional domain” (49). Ostareck et al. (22) showed that aCP1 is involved in inhibiting 60 S ribosome joining when binding to the 15-lipoxygenase 3′-UTR (22). Reduced binding of aCP2 to the AB in the H-ferritin mRNA may likewise promote 60 S ribosome subunit joining and enhanced translation, although the mechanisms by which the PCBP are involved in ferritin translation will require further study.

Binding by the IRPs may prevent the AB from forming an IRES and could explain its function in the context of ribosome scoring (50–53). Consistent with this model, aCP2 is known to promote translation of poliovirus mRNAs after infection of HeLa cells by selectively interacting with domain IV of an IRES in the 5′-UTR of the virus RNA (20). However, many endogenous eukaryotic mRNAs, including the vascular endothelial growth factor, are translated from IRESs downstream from the 5′ cap sites of their mRNAs (5′-cap-independent translation) (54–56). These data emphasize the need to evaluate the functional role of aCP1 and aCP2 in H-ferritin translation mediated via the AB domain.

Maintenance of efficient ferritin translation by the cytokine-responsive AB domain is consistent with the fact that ferritin provides cytoprotection to liver cells through stress-responsive pathways mediated both via cytokines (16) and accelerated by iron (heme)-catalyzed oxidative stress (57). Ferritin is at high abundance in the brain to protect neuronal cells from metal catalyzed oxidative damage during inflammation (9, 58, 59). Ferritin is also present in amyloid plaques of AD, suggesting a neuroprotective role in the final stages of neuronal death during this disease process (9, 58). Translational activation through the ferritin AB domain also provides a mechanism for iron sequestration from the bloodstream into liver and spleen as occurs in rat models in the anemia of chronic disease and inflammation (16, 60). Taken together, these data support a model for the maintenance of high basal ferritin levels by dual control of ferritin mRNA translation in the presence of intracellular iron through the upstream IRE and the downstream AB domain. Certainly regulation of ferritin mRNA by 5′-UTR-specific AB domains remains key to our understanding of the regulation of ferritin expression during inflammation, where it plays a role as the universal iron storage protein that protects cells from metal-catalyzed oxidative stress (16, 57, 61). The studies described here provide novel insight into the function of the AB and lay the foundation for future work that will investigate the precise functional role of the AB. It will be of interest to determine whether the AB works in a cooperative manner with the IRE and to delineate how the PCBP integrate with the IRPs to coordinate regulation of iron homeostasis in cells under a variety of clinical states.

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