Thyroid Hormone Modulates the Interaction between Iron Regulatory Proteins and the Ferritin mRNA Iron-responsive Element*

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The cytoplasmic iron regulatory protein (IRP) modulates iron homeostasis by binding to iron-responsive elements (IREs) in the transferrin receptor and ferritin mRNAs to coordinate regulation of transferrin receptor mRNA stability and ferritin mRNA translational efficiency, respectively. These studies demonstrate that thyroid hormone (T3) can modulate the binding activity of the IRP to an IRE in vitro and in vivo. T3 augmented an iron-induced reduction in IRP binding activity to a ferritin IRE in RNA electrophoretic mobility shift assays using cytoplasmic extracts from human liver hepatoma (HepG2) cells. Hepatic IRP binding to the ferritin IRE also diminished after in vivo administration of T3 with iron to rats. In transient transfection studies using HepG2 cells and a human ferritin IRE-chloramphenicol acetyltransferase (H-IRE-CAT) construct, T3 augmented an iron-induced increase in CAT activity by ∼45% RNase protection analysis showed that this increase in CAT activity was not due to a change in the steady state level of CAT mRNA. Nuclear T3 receptors may be necessary for this T3-induced response, because the effect could not be reproduced by the addition of T3 directly to cytoplasmic extracts and was absent in CV-1 cells which lack T3 receptors. We conclude that T3 can functionally regulate the IRE binding activity of the IRP. These observations provide evidence of a novel mechanism for T3 to up-regulate hepatic ferritin expression, which may in part contribute to the elevated serum ferritin levels seen in hyperthyroidism.

The iron regulatory protein (IRP,† previously known as the iron-responsive element-binding protein, IRE-BP; and iron-responsive factor, IRF) is a trans-acting RNA-binding protein which binds with high affinity to conserved stem-loop structures, iron-responsive elements (IREs), present in the ferritin, transferrin receptor (TfR), and erythroid 5-aminolevulinate synthase mRNAs (1–3). The IRP serves a central role in the regulation of iron (Fe) homeostasis (1). In the absence of iron, the IRP binds to the IRE in the 5′-untranslated region (5′-UTR) of ferritin and erythroid 5-aminolevulinate synthase mRNAs and represses translation (4–6). Binding of the IRP to IREs in the 3′-untranslated region (3′-UTR) of TfR mRNA stabilizes the mRNA and prevents its degradation (7–9). In iron-replete states, the reverse holds, which results in increased ferritin translation and decreased TfR mRNA stability. This reciprocal regulation is achieved at the post-translational level and is independent of new protein synthesis (10).

Two IRPs have been defined in various human and rat tissues (3, 11, 12). The most widely expressed and abundant IRP in human tissues is IRP1 (1, 3). A second human IRP (IRP2) has been described recently. IRP2 is 57% identical with IRP1 at the amino acid level and 2–10 times less abundant than IRP1 in most tissues, except in the brain (3). In contrast to IRP1, cellular concentrations of IRP2 are inversely regulated by iron levels due to iron-dependent regulation of the half-life of IRP2 protein (3). The relative contribution of each of these species to iron homeostasis remains to be elucidated. The two rat IRPs have been designated BP1 and BP2 (12) (also known as IRF and IRFb, respectively) (11) and may represent rodent counterparts for IRP1 and IRP2. Significant functional differences exist between BP1 and BP2. In particular, BP2 does not have functional aconitase activity and, in contrast to BP1, levels of BP2 protein are regulated by iron (12). In addition, IRFb (and presumably BP2) is expressed most abundantly in intestine, brain, and kidney (11).

Thyroid hormone (T3) plays a central role in differentiation, development, and maintenance of body homeostasis (13). The actions of T3, like the steroid hormones, are mediated through intracellular T3 receptor proteins (TRs) (14, 15) which act predominantly to modulate transcription by binding to specific T3-responsive elements in target genes (16). Recent studies have demonstrated, however, that T3 also has important effects at the post-transcriptional level to regulate the expression of several genes, including the β-subunit of thyrotropin (TSHβ) (17), the thyrotropin releasing hormone receptor (18), and the retinoid-X receptor (19). To date, there is little understanding of the molecular mechanisms underlying these T3-induced changes in mRNA stability.

Several groups have documented an association between T3 levels and ferritin expression. In earlier reports, hypothyroidism produced by thyroidectomy was associated with increased rat hepatic ferritin content, which was found to be due to post-transcriptional changes in the ferritin synthetic rate (20, 21). More recently, however, and in contrast, hyperthyroid rats with elevated T3 and T4 levels were found to have an increased
liver ferritin protein synthesis rate (38% above control) (22). Part of this increase may be due to elevated IRE-mediated ferritin translation, although T₃ has also been shown to increase the transcription rate of H-ferritin mRNA in rat C6 glioma cells (23), raising the possibility of a transcriptional effect in the liver. Of particular interest, are reports from several groups in which T₃ was shown to positively regulate serum ferritin measurements in humans (24–28), similar to the changes reported in the rat (22). Elevated serum ferritin levels were observed in hyperthyroid individuals, and levels decreased significantly after antithyroid treatment with normalization of T₃ levels (24–26, 28). Furthermore, administration of T₃ to hypothyroid individuals produced a significant increase in the serum ferritin level (26, 27), although the cause of the T₃-induced increase in the serum ferritin level in humans is unknown, increased synthesis of ferritin in the liver may well be an important contributor. These links between T₃ and the regulation of ferritin expression suggest that a positive correlation exists between the levels of T₃/T₄ and ferritin in the serum. However, the molecular mechanisms involved in the hepatic regulation of ferritin expression by T₃ remain to be determined.

We reasoned that a component of the effect of T₃ on ferritin expression in the liver was due to T₃-induced modulation of IRP binding to the ferritin IRE. Therefore, we used 1) the RNA electrophoretic mobility shift assay (REMSA) and 2) transient transfection assays to investigate the regulation of IRE-dependent gene expression by T₃, in vivo and in vitro. Our results demonstrate that T₃ can functionally regulate the binding activity of the human and rat IRP to a ferritin IRE. These data provide evidence for a role of T₃ in the post-transcriptional regulation of iron-responsive genes and new insights into the action of T₃ in the modulation of iron homeostasis. Furthermore, these data may, in part, explain the positive association between serum levels of T₃ and ferritin.

**EXPERIMENTAL PROCEDURES**

Preparation of Tissue and Cell Extracts—Frozen Sprague-Dawley rat liver was homogenized in 5 volumes of ice-cold 10 mM HEPES (pH 7.5), 40 mM KC1, 3 mM MgCl₂, 1 mM dithiothreitol, 0.32 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 2 µg/ml aprotonin (Buffer A) with a Teflon homogenizer. After removal of nuclei by low speed centrifugation, the supernatant was centrifuged at 100,000 × g for 1 h, and the supernatant was frozen rapidly in dry ice and stored at −70°C. Some animals received a single intraperitoneal injection of either T₃ (20 µg/100 g body weight), ferric ammonium citrate (FAC) (2.3 mg/100 g body weight), or a combination of both, 8 h prior to preparing the extract. Cultured cells (HepG2, human liver carcinoma; CV-1, monkey kidney) were grown in Dulbecco's modified Eagle's medium and 10% fetal bovine serum supplemented with penicillin and streptomycin at 37°C in 5% CO₂. Forty eight h before harvesting, the cells were washed with phosphate-buffered saline, and incubated with T₃-deficient hypothyroid medium. In the 16–24 h prior to harvesting, cells were grown for various times in the absence or presence of combinations of FAC (110 µM), desferrioxamine (DF) (100 µg/ml), Desferal, Ciba Geigy), and T₃ (ether 1 or 100 µM, Sigma). Cells were harvested by lysis in the same buffer as above plus 5% glycerol and 0.2% Nonidet P-40, but without the sucrose. After removal of the nuclei, the cells were centrifuged at 100,000 × g, and the supernatant was stored in aliquots at −70°C. T₃ was removed from fetal bovine serum for use in hypothyroid medium as described (29). Protein concentrations were determined in duplicate by the Bradford method (Pierce).

Preparation of RNA Transcripts—Plasmid pGem-IRE was a 118-base sense transcript prepared with SP6 RNA polymerase from a Smal-digested rat l-subunit ferritin pseudogene (pG6L-66). It contains the first 65 bases of the 5'-UTR (large hatching), including a conserved IRE stem-loop (IRE), 33 bases of the 5'-flanking sequence (small hatching), and 20 bases from pGEM2. pGEM-vec is pGEM-IRE linearized with Smal but transcribed with T7 polymerase to produce a 45-base transcript containing vector sequence only. The H-ferritin genomic clone, pUC-HFER, contains a 458-base SstI fragment from the H-ferritin gene cloned into pUC12. The insert comprises 164 bases of the H-ferritin gene sequence upstream of the 5' cap site and 294 bases of the first exon including the 5'-UTR. The 302-base SstI-Syl fragment from pUC-HFER was ligated into the unique SstI and HindIII sites in the polyclinker of pUC12CAT to produce H-IRE-CAT. pUC12CAT contains the CAT gene inserted into the HindIII site of pUC12. H-IRE-CAT has the H-ferritin promoter and 5'-UTR in front of the CAT open reading frame, and the transcript has a correctly positioned IRE. 182 bases of the H-ferritin promoter is present in H-IRE-CAT. 142 bases of H-ferritin mRNA as part of a hybrid H-ferritin/CAT transcript.

**Fig. 1. Plasmids for generation of RNA probes and transfection.** pGEM-IRE is a 118-base sense transcript prepared with SP6 polymerase from a Smal-digested rat l-subunit ferritin pseudogene (pG6L-66). It contains the first 65 bases of the 5'-UTR (large hatching), including a conserved IRE stem-loop (IRE), 33 bases of the 5'-flanking sequence (small hatching), and 20 bases from pGEM2. pGEM-vec is pGEM-IRE linearized with Smal but transcribed with T7 polymerase to produce a 45-base transcript containing vector sequence only. The H-ferritin genomic clone, pUC-HFER, contains a 458-base SstI fragment from the H-ferritin gene cloned into pUC12. The insert comprises 164 bases of the H-ferritin gene sequence upstream of the 5' cap site and 294 bases of the first exon including the 5'-UTR. The 302-base SstI-Syl fragment from pUC-HFER was ligated into the unique SstI and HindIII sites in the polyclinker of pUC12CAT to produce H-IRE-CAT. pUC12CAT contains the CAT gene inserted into the HindIII site of pUC12. H-IRE-CAT has the H-ferritin promoter and 5'-UTR in front of the CAT open reading frame, and the transcript has a correctly positioned IRE. 182 bases of the H-ferritin promoter is present in H-IRE-CAT, and it transcribes the first 142 bases of H-ferritin mRNA as part of a hybrid H-ferritin/CAT transcript. The identity of all constructs was confirmed by double-stranded DNA sequencing to preclude the presence of artificial AUG sites upstream of the CAT initiation codon.

Preparation of RNA Transcripts—Linearized templates (Smal-digested) were used with either T7 or SP6 RNA polymerase (Promega) in transcription reactions containing [α-32P]UTP (800 Ci/mmol; DuPont NEN), as described (30, 33), to produce transcripts with a specific activity of approximately 2 × 10⁶ cpm/µg of RNA. Full-length transcripts were isolated on 5% urea/acylamide gels, eluted for 3 h at 25°C in 0.5 mM ammonium acetate, 1 mM EDTA, and ethanol-precipitated to recover the RNA as described (30, 33). Unlabeled RNA transcripts were synthesized as above but with 2.5 µM UTP, purified on agarose gels, and quantitated by spectrophotometry. RNA Electrophoretic Mobility Shift Assay (REMSA)—Binding reactions were performed as described (30, 33) with 5–10 µg of cytoplasmic extract protein and 10¹⁰–¹⁰¹⁵ cpm of RNA (~0.5 ng) in Buffer A in a total volume of 15–25 µl. Following incubation at 25°C for 30 min, 1 unit of RNase T1 (Boehringer Mannheim) was added for 10 min, followed by the addition of heparin (final concentration, 5 mg/ml) (Sigma) for an additional 10 min. Samples were subjected to electrophoresis on 4% native polyacrylamide gels (acrylamide/methylene bisacrylamide ratio, 70:1), which were dried and analyzed by autoradiography at −70°C. In some assays, extracts were preincubated for 10 min at 25°C with nonspecific and specific competitor sense unlabelled RNA (100-fold excess) before addition of the radiolabeled RNA. A Betagen 603 Blot Analyzer (Betagen Corp., Waltham, MA) was used to quantitate the radioactivity present in each lane. UV Cross-linking of RNA-Protein Complexes—RNA-protein binding
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RESULTS

IRP-IRE Complex Is Species-specific In T₃-responsive Tissues—To determine if there were qualitative differences between the IRP-IRE RNA-protein complex (RPC) in the T₃-responsive tissue and cells selected for use in this study, cytoplasmic extracts from rat liver and human hepatoma (HepG2) cells were used in REMSA with labeled pgem-IRE. Fig. 2A shows that the nature of the IRP-IRE complex is species-specific. In HepG2 cells, a single complex was seen (IRP1, lane 1). A second human IRP has recently been identified, IRP2 (3), although it co-migrates with IRP1 in HepG2 cells. With the exception of the brain, there is –2–10 times more IRP1 than IRP2 in human tissues (3). In rat liver (lanes 3–6), two characteristic RPCs, denoted BP1 and BP2 (12, 30), were identified, both of which represent specific bona fide IRP-IRE complexes. Accumulated evidence from several reports suggests that BP1 and IRP1 are species homologues, and that BP2 and IRP2 are likely rat and human counterparts, respectively (3, 11, 12).

We then confirmed that the cytoplasmic regulation of IRP binding to the ferritin IRE was preserved in these cells. The IRP-IRE binding activity of the IRP is typically increased in vitro by reducing agents, such as 2-mercaptoethanol (2-ME) (37). As predicted, 2-ME (2%) increased the binding of the IRP to IRE RNA with both HepG2 and rat liver cytoplasmic extracts (Fig. 2A, lanes 1 and 2). The binding reactions were incubated for 30 min at room temperature prior to sequential addition of RNase T1 and heparin, as described under “Experimental Procedures.” The binding mixtures were analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel (REMSA). In lanes 2 and 4, 2-ME (2%) was added to the reaction mixture at the beginning of the incubation. A 100-fold excess of specific (unlabeled pgem-IRE, lane 5) or nonspecific (pgem-vec, lane 6) competitor RNA was incubated with the extract for 10 min at 22°C prior to addition of labeled probe. B, UV cross-linking analysis of rat liver IRE-IRP complexes. Arrows at BP1 and BP2 denote rat RPCs containing IRPs and IRE (11, 12). Following incubation with RNase T1 and heparin, 32P-labeled RPCs were UV-cross-linked as described under “Experimental Procedures.” The complexes were treated with RNase A at 37°C for 15 min. After addition of SDS-sample buffer and boiling, the cross-linked products were separated by 7% SDS-PAGE and analyzed by autoradiography. RNase A, incubation with RNase A after UV-cross-linking; the positions of the molecular weight markers are indicated.

Fig. 2. The IRP-IRE complex is species- and tissue-specific. A, REMSA was performed using 32P-labeled pgem-IRE and 5 μg of different cytoplasmic extracts: HepG2 cells (lanes 1 and 2) and rat liver (lanes 3–6). The binding reactions were incubated for 30 min at room temperature prior to sequential addition of RNase T1 and heparin, as described under “Experimental Procedures.” The binding mixtures were analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel (REMSA). In lanes 2 and 4, 2-ME (2%) was added to the reaction mixture at the beginning of the incubation. A 100-fold excess of specific (unlabeled pgem-IRE, lane 5) or nonspecific (pgem-vec, lane 6) competitor RNA was incubated with the extract for 10 min at 22°C prior to addition of labeled probe. B, UV cross-linking analysis of rat liver IRE-IRP complexes. Arrows at BP1 and BP2 denote rat RPCs containing IRPs and IRE (11, 12). Following incubation with RNase T1 and heparin, 32P-labeled RPCs were UV-cross-linked as described under “Experimental Procedures.” The complexes were treated with RNase A at 37°C for 15 min. After addition of SDS-sample buffer and boiling, the cross-linked products were separated by 7% SDS-PAGE and analyzed by autoradiography. RNase A, incubation with RNase A after UV-cross-linking; the positions of the molecular weight markers are indicated.

IRE binding activity of the IRP is typically increased in vitro by reducing agents, such as 2-mercaptoethanol (2-ME) (37). As predicted, 2-ME (2%) increased the binding of the IRP to IRE RNA with both HepG2 and rat liver cytoplasmic extracts (Fig. 2A, lanes 1 and 2). Interestingly, the BP2 RPC in rat liver was consistently abolished in the presence of 2-ME (Fig. 2A, lane 4), and the intensity of the BP1 RPC was increased. A 100-fold excess of an unlabeled specific competitor RNA (pgem-IRE) competed efficiently for binding to the IRP and abolished BP1 and BP2 RPC formation (lane 5). However, addition of excess...
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**Fig. 3.** T₃ modifies IRP binding to a ferritin IRE in iron-loaded rat liver cells in vivo. REMSA (4% PAGE) was performed using 5 µg of rat liver cell cytoplasmic extract and ³²P-labeled pgem-IRE. Male Sprague-Dawley rats (n = 14) were given a single intraperitoneal injection with either vehicle alone (lane 1, n = 3), T₃ (20 µg/100 g body weight, lane 2, n = 3), FAC (2.3 mg/100 g body weight, lane 3, n = 4), or both (lane 4, n = 4). The rats were killed 8 h later, and liver cytoplasmic extracts were prepared and used in REMSA with labeled pgem-IRE as described in Fig. 2. Each lane, with the exception of lane 5, represents an analysis of liver extract from a different rat. A portion of the reaction mixture from lane 4 was incubated separately with 2-ME (2%) prior to PAGE analysis (lane 5). Arrows at BP1 and BP2 denote rat liver cell RPCs containing IRPs and IRE (compare with Fig. 2).

unlabeled nonspecific competitor RNA, either growth hormone (data not shown) or pgem-vec (lane 6), did not affect either RPC. The two bands at the bottom of each lane are nonspecific.

To confirm the size of the two rat RPCs, BP1 and BP2, a portion of the reaction mixture from Fig. 2A, lane 3, was analyzed after UV cross-linking and digestion with RNase A (100 µg/ml) for 30 min at 37 °C. Two RPCs were identified which migrated at the positions predicted for the BP1 and BP2 proteins (~90–95 and ~105 kDa; respectively; Fig. 2B) (11, 12). We concluded that these cells contained IRPs, BP1 and BP2, which were of the predicted size and which displayed appropriate regulation with reducing agents.

**T₃ Modifies IRP-IRE Complex in Rat Liver**—To address our hypothesis, we first investigated whether T₃ affected the IRE-IRP complex in rat liver in vivo by REMSA using cytoplasmic extracts from Sprague-Dawley rats injected intraperitoneally with either vehicle alone (n = 3), T₃ (n = 3), ferric ammonium citrate (FAC) (n = 4), or a combination of both (n = 4), together with labeled pgem-IRE. Administration of T₃ alone had no significant effect on the BP1 and BP2 RPCs (compare lane 1 and 2). Animals injected with FAC alone showed a decrease in the BP1 complex (~30%) and complete loss of the BP2 complex (Fig. 3, compare lane 3 and 1). When FAC and T₃ were injected together, BP1 expression decreased further, and BP2 expression returned (lane 4). There was a reduction in total binding activity when T₃ was combined with FAC (compare lane 3 and 4, ~30%, Betagen scan). Although the RPCs are generally more intense in Fig. 3 than in Fig. 2A due to longer autoradiography exposure time, the relative ratios of BP1 to BP2 are similar throughout. These T₃-induced changes in IRE binding activity of BP1 and BP2 appear to be a post-translational event, because in the presence of FAC and T₃, maximal IRE binding activity was recovered in vitro by treatment with 2-ME (2%) (lane 5). These findings, which are representative of 3 different REMSA experiments, provided evidence that T₃ could regulate the binding of the IRP to the ferritin IRE and thus modulate hepatic ferritin expression.

**T₃ Modifies IRE Binding Activity of IRP in Iron-replete HepG2 Cells**—The data from the in vivo studies suggested that T₃ was able to modify, either directly or indirectly, the binding activity of the IRP to a ferritin IRE. Furthermore, the in vivo results supported the accumulated data for humans where elevated T₃ levels are associated with increased serum ferritin levels (24–28). We then examined whether T₃ had an effect on IRP binding activity in vitro using human HepG2 cells. This hepatoma cell line has been used extensively to characterize IRP-IRE interactions, and HepG2 cells contain well-characterized thyroid hormone receptors (TRs) (38). HepG2 cells were plated into 6-cm dishes, cultured in hypothyroid medium (T₃-deficient) (29) for 36 h, and thereafter in the presence or absence of combinations of FAC, desferrioxamine (Df), and T₃, for various times, prior to harvesting. Cytoplasmic extracts from the cells were mixed with labeled pgem-IRE and analyzed by REMSA. Fig. 4 is a representative REMSA (n = 4) showing that the IRE-IRP complex was diminished to ~85% of control (Betagen quantitation) after incubation with 110 µM FAC for 16 h (lane 2). Although co-culture with 100 nM T₃ for 16 h diminished the complex further to 75% (lane 3), the most significant changes were seen with shorter incubations. When 100 nM T₃ was added to the medium for 4 h, the intensity of the complex was reduced to approximately 60% of control (lane 4), while incubation for only 2 h reduced it to approximately 50% of control (lane 5). This effect was only evident when the HepG2 cells were iron-replete and was similar to that observed in T₃-treated rat liver extracts described above.

Similar experiments were conducted using HepG2 cells after the addition of Df (100 mg/ml) in the presence and absence of T₃. However, we were unable to detect any effect of T₃ on the IRE-IRP complex in iron-depleted cells using REMSA (data not shown).

**T₃ Augments Iron-induced CAT Gene Expression in HepG2 Cells**—H-IRE-CAT Transfectants—These in vivo and in vitro REMSA results showed that T₃ was able to reduce IRP binding to a ferritin mRNA IRE. To investigate whether this T₃-induced effect on IRP binding activity observed in REMSA studies was associated with functional changes in IRE-dependent gene expression, we utilized a transient transfection assay in HepG2 cells with a plasmid containing a human H-ferritin IRE inserted upstream of a CAT reporter gene driven by the H-ferritin promoter (H-IRE-CAT). Previous studies with this and similar constructs containing ferritin IREs have demonstrated that changes in CAT activity represent changes in translational efficiency of ferritin mRNA and not changes in the rate of transcription (32, 39). Furthermore, no typical T₃-response elements are present within the sequence of the H-IRE-CAT plasmid.

HepG2 cells, cultured in hypothyroid medium, were transiently transfected with H-IRE-CAT and then incubated in the presence or absence of various combinations of FAC, T₃, and Df.
After 24 h, the cells were harvested and the level of [3H]lactochyloraphenol, a measure of the CAT activity, was measured. The results shown in Fig. 5 are from a representative experiment, although similar results have been observed in all other HepG2 experiments (n = 3) and in H-IRE-CAT transfectants of rat GH3 pituitary cells.2 In the absence of iron loading, no increase in CAT activity was seen after the addition of 100 nM T3 alone (lane 2). As expected, incubation with 110 μM FAC alone increased CAT activity (lane 3). However, co-culture of 100 nM T3 with iron further increased the CAT activity (lane 4) approximately 35–45%. Even co-culture of 1 nM T3 with iron increased CAT activity (lane 5), consistent with a dose-response relationship. In contrast, incubation of the cells with Df (100 mg/ml) reduced the CAT activity by ~50% from basal (lane 6), reflecting high affinity binding of the IRP to the IRE and reduced translational efficiency. Addition of 100 nM T3 did not further decrease the CAT activity significantly (lane 7). The 3-fold overall difference in translational efficiency of H-IRE-CAT after stimulation with FAC and Df is consistent with similar findings in other recent reports (5, 6, 11).

To ensure that the increased CAT activity induced by T3 was not a consequence of increased transcription of H-IRE-CAT, CAT mRNA was quantitated by slot blotting and RNase protection analysis. CAT mRNA levels were measured in RNA extracted from the same H-IRE-CAT HepG2 transfectants by slot blotting. The slot blot was performed in duplicate for two separate loadings of 4 and 20 μg of RNA. Compared to control (hypothyroid medium alone), no significant increase in CAT mRNA was detected after the addition of T3 to the cultured cells (Fig. 6A). The radioactivity in each of the bands was quantitated, and no increase in counts was detectable in the T3-treated samples (data not shown). These results are consistent with the data in

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2 P. Leedman, unpublished observation.
bated various concentrations of $T_3$ ($10^{-5}$ to $10^{-9} \text{ M}$) with HepG2 cell cytoplasmic extracts containing labeled pgem-IRE probe, at the commencement of the reaction. As can be seen in Fig. 8, addition of $T_3$ directly to the cytoplasmic reaction mixture (lanes 2–4), did not modify IRP binding activity. These data support our results in CV-1 cells and are consistent with the notion that in liver cells $T_3$ acts via nuclear TRs to facilitate displacement of the IRP from the ferritin IRE.

**DISCUSSION**

The molecular mechanisms governing the regulation of ferritin gene expression by iron are based on regulated changes in the IRE binding activity of the IRPs. Here we provide the first evidence that $T_3$, a hormone critical for maintaining body homeostasis, can modulate the binding activity of the IRP to a ferritin IRE both in vitro and in vivo. Our REMSA results indicate that $T_3$ acts post-translationally to augment the iron-induced displacement of the rat and human IRP from an IRE present in the 5′-UTR of ferritin mRNA. Furthermore, this $T_3$-induced effect was associated with a similar sized functional increase in IRE-dependent gene expression (~40–50%), as demonstrated in transfection studies using a human ferritin IRE-CAT construct in human hepatoma cells. Our results suggest that $T_3$ can, possibly in a TR-dependent manner, functionally regulate the IRE binding activity of the IRP.

Our data provide further evidence that the binding activity of the IRP can be modulated by agents other than iron and the redox state. Recent data indicate a direct association with the nitric oxidin/nitric oxide synthase pathway, in which increases in NO activates IRP binding to IREs in ferritin and TFR mRNAs (39). The reactive oxygen intermediate hydrogen peroxide ($H_2O_2$) has recently been shown to increase binding of the IRP, resulting in reduced ferritin synthesis and increased transferrin receptor expression (41). In contrast to activation of IRP by iron depletion which is okadaic-acid-insensitive, induction of IRP by $H_2O_2$ is okadaic-sensitive suggesting the involvement of stress-induced kinase/phosphatase pathways (41). Changes in the phosphorylation status of the IRP mediated by protein kinase C may provide another level of regulation (42).

$T_3$, however, is the first endocrine hormone that has been shown to modulate the IRP-IRE interaction and ferritin translation, and our studies suggest that nuclear TRs are possibly involved, as evidenced by the absence of effect in CV-1 cells which lack endogenous nuclear TRs. Moreover, $T_3$ was unable, even at high concentrations ($10^{-5} \text{ M}$), to reproduce the effect when added directly to the cytoplasmic extracts. This IRE-dependent action of $T_3$ on ferritin translation differs from other agents, such as interleukin 1, which modify ferritin translation through IRE-independent mechanisms (43). It is not known which TR isoform(s) (40) is involved in mediating this $T_3$-induced effect, and this question requires further investigation.

A significant body of evidence exists showing a positive correlation between the serum levels of $T_3/T_4$ and ferritin (24–28) in individuals with thyroid abnormalities. All of these studies documented elevated serum ferritin levels in patients with hyperthyroidism which normalized when the $T_3/T_4$ levels returned to normal. Interestingly, the positive relationship between serum ferritin and $T_3/T_4$ levels holds in hypothyroidism as well. A similar positive relationship between serum ferritin and $T_3/T_4$ levels has been observed in rats rendered hypo- and hyperthyroid (22). Furthermore, the hepatic ferritin synthesis rate increased significantly in hyperthyroid rats (22), consistent with the increased serum ferritin levels. These observations are consistent with our own data. To date, there are no human data to suggest that serum ferritin levels rise in hypothyroidism. Thus, differences exist between the recent data and the results presented herein compared to the earlier reports documenting increased rat hepatic ferritin synthesis in hypothyroidism (20, 21). The reasons for this are unclear, but may relate, in part, to the nature of thyroid dysfunction utilized in each study (e.g. thyroidectomy versus $T_3$ supplementation).

Interestingly, however, the earlier work did document that the changes induced by $T_3$ were at the post-transcriptional level which would be consistent with our results (21). The IRP is one of two trans-acting RNA-binding proteins whose binding activity is modified by $T_3$. The other is an 80–85-kDa pituitary protein that recognizes a specific region within the 3′-UTR of TSHβ mRNA, an anterior pituitary hormone (33). Remarkably, the RNA binding site for this $T_3$-regulated pituitary trans-acting factor within TSHβ 3′-UTR mRNA contains features of a ferritin IRE. The TSHβ 3′-UTR sequence similarity with a ferritin IRE extends over 12 nucleotides (9 of 12 nucleotides, which includes the loop and a portion of the IRE stem, Fig. 9). To investigate whether this sequence could compete with the IRE for IRP binding, REMSA studies were performed. These showed that excess unlabeled TSHβ 3′-UTR mRNA could compete efficiently with labeled pgem-IRE for IRP binding in pituitary cells (33). Further, pgem-IRE competed efficiently with TSHβ 3′-UTR mRNA for binding of this $T_3$-regulated trans-acting factor (33). In summary, TSHβ mRNA has an IRE-like element which can interact with pituitary factors, including the IRP, in a $T_3$-dependent manner. The cellular consequences of interactions between the IRP with other mRNAs sharing sequence similarity with the IRE is under further investigation.

Our results are consistent with a model of iron homeostasis in which IRE-dependent ferritin gene expression is positively...
Fig. 9. Nucleotide sequence alignment of the rat ferritin IRE with rat TSHβ 3′-UTR region. The stem-loop structure depicts the rat ferritin IRE contained within the pgm-IRE plasmid (30). The six-membered loop contains five nucleotides that are almost invariant (boxed nucleotides) (44). The 12-nucleotide region of sequence similarity comprises the sequence between the two lines. Nucleotide differences between the IRE and the consensus region sequence are indicated with arrows.

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regulated by T₃. T₃ alone did not alter expression at the transcriptional or post-transcriptional level. However, in iron-replete cells, there was a significant T₃ effect to up-regulate ferritin gene expression, through modulation of the IRP binding activity and enhanced IRE-dependent translation. These results provide further insight into a rapidly emerging model for the regulation of iron homeostasis, by providing evidence that T₃ acts post-translationally to augment displacement of the IRP from a ferritin IRE. Other endocrine hormones may also modify IRP binding activity and have profound metabolic effects (e.g. retinoic acid, glucocorticoids etc.). Further experiments are in progress to investigate this possibility. Given the important role that both T₃ and iron play in the maintenance of body homeostasis, further elucidation of the control mechanisms governing the interactions between T₃ and IRE-dependent gene expression will be an important goal of future studies.

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