Plasma protein regulation by thyroid hormone

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Abstract

Thyroid hormones (THs) regulate growth, development, differentiation and metabolic processes by interacting and activating thyroid hormone receptors (TRs). Although much progress has been made in our understanding of the transcriptional regulation of many TR target genes, little is known of the regulation of plasma protein gene expression by TRs. To investigate the role of TRs in plasma protein expression we used human hepatocellular carcinoma cell lines and carried out cDNA microarray analysis. Our results indicate that several plasma proteins including transferrin, prothrombin, angiotensinogen, haptoglobin, α-2-HS-glycoprotein α and β chain, complement, lipoproteins and fibrinogen are up-regulated by THs. Furthermore, clusterin, α-2-macroglobulin precursor, prothymosin α and α-fetoprotein were found to be down-regulated by THs.

Transferrin, an iron-binding protein expressed in all mammals, and mainly synthesized in the liver, was investigated further. Immunoblot and Northern blot analyses revealed that exposure of HepG2–TRα1 sub-lines and HepG2-Neo cells to tri-iodothyronine (T3) induced time- and dose-dependent increases in the abundance of transferrin mRNA and protein, with the extent of these effects correlating with the level of expression of TRα1. Nuclear run-on experiments indicate that this induction is functioning at the transcriptional level. Moreover, cyclohexamide treatment did not eliminate the induction of transferrin by TH. Thus, our results suggest that the induction of transferrin by TH is direct and may in fact be mediated by an as yet unidentified response element in the promoter region.


Introduction

Thyroid hormones (THs) regulate growth, development, differentiation and metabolic processes by interacting with TH receptors (TRs) that bind to specific DNA sequences in the regulatory regions of target genes (Cheng 2000). TRs are members of the steroid hormone and retinoic acid superfamily of ligand-dependent transcription factors. Two TR genes, TRα and TRβ, have been identified and located to human chromosomes 17 and 3, respectively (Lazar 1993). Each gene encodes at least two TR isoforms (TRα1 and 2, and TRβ1 and 2) that are generated as a result of alternative splicing and promoter choice (Lazar 1993, Wood et al. 1996).

Much progress has been made over the past few decades in our understanding of the transcriptional regulation of TR target genes but little is known about the role of TRs in plasma protein regulation (Lazar 1993, Cheng 2000, Zhang & Lazar 2000, Lin et al. 2001, 2002). Previously, we examined the expression of TRα1 and TRβ1 in nine hepatoma cell lines to investigate the role of TRs during carcinogenesis. We showed that endogenous TRβ1 is abundant in Mahlavu, SK-Hep-1 and HA22T cells, present in moderate amounts in J5, J7 and J3–28 cells, and expressed at low concentrations in HepG2, Hep3B and PLC/PRF/5 cells (Lin et al. 1994). We also demonstrated that the abundance of the Nm23-H1 protein in these cells is inversely correlated with that of TRβ1 (Lin et al. 1995).

A major site of plasma protein production is the hepatocyte, and various in vitro techniques have been used to modulate the production of these essential proteins. For example, Chang et al. (1983) reported the establishment of a new human hepatoma cell line, HA22T/VGH. This cell line had many of the properties of human hepatocellular carcinoma. Of particular interest, only five of the 15 plasma proteins investigated were detected in the medium of HA22T/VGH cells cultured for 10 days. However, when these cells and a clonal derivative, C5, were cultured in an aggregated form, all 15 plasma proteins were present in the culture medium.

HepG2, a well-differentiated hepatocellular carcinoma cell line, secretes all 15 plasma proteins. However, the relationship between TRα1 and plasma proteins expression remained undetermined due to the lack of cell lines
expressing TRα1 at a high level (Lin et al. 1996). Our data demonstrated (Lin et al. 1997) that the hepatoma cell line HepG2 stably expressed functional TRα1 and the presence of TR interacting cell-type specific factors. We are also investigating the regulation of TR target genes in the HepG2 stably expressed functional TRα1 in other studies. Thus, using HepG2 cell lines could serve as a model system to study the cell type-specific and TR isoform-specific regulation of the tri-iodothyronine (T3) target genes in liver. Therefore, in the present study we used isogenic cell lines derived from HepG2 that stably express high levels of wild-type TRα1 (HepG2-TRα1 cells).

Transferrin (Tf) is an essential mammalian plasma protein largely synthesized in the liver and then secreted into the bloodstream. Tf is a monomeric 80 kDa glycoprotein consisting of a polypeptide chain of 679 amino acids and two N-linked complex type glycan chains. The Tf molecule comprises two homologous domains, the N-terminal and C-terminal domains, each containing one iron-binding site (Kwok & Richardson 2002). The carbohydrate moiety is attached to the C-terminal domain. Together with ferritin, the iron-storage protein, and the Tf receptor, Tf is involved in maintaining iron homeostasis (Feelders et al. 1999). Tf circulates at ~2 to ~4 mg/ml in the blood, where its major function is to transfer iron between sites of absorption, storage and utilization throughout the organism. Iron is essential for cell proliferation, differentiation and metabolism (Feelders et al. 1999). Interestingly, Tf can also act as a growth factor for a variety of cells (Theisen et al. 1993).

The mechanisms of how TRα1 selectively maintain liver-specific gene transcription have not yet been elucidated. To investigate this phenomenon we utilized cDNA arrays to follow the modulation of plasma protein levels in isogenic hepatocellular carcinoma cell lines when treated with TH. Furthermore, the use of cells overexpressing TRα1 enabled us to investigate the effect of various receptor levels on the regulation of plasma protein levels. In particular, we were interested in elucidating how THs affected iron uptake. Therefore, we further studied the effect or regulation of Tf by THs. The other up-regulated plasma proteins are also under investigation in separate studies.

Materials and Methods

Cell culture

The human hepatoma cell line HepG2 was obtained from The American Type Culture Collection and was routinely grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. The TRα1 over-expressing cell line, HepG2-TRα1, has been described previously (Lin et al. 2000). In this study, three HepG2-TRα1-overexpressed clones (#1–3) were used. The serum was depleted of T3 (Td) as described previously (Lin et al. 1993). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

RNA preparation and labeling

Total RNA from HepG2-TRα1#1, #2 and #3 with or without T3 treatment was prepared using TRizol (Life Technologies, Rockville, MD, USA). To fluorescently label cDNA, 30 µg of total RNA from untreated cells and 50 µg of total RNA from treated cells were reverse-transcribed in the presence of Cy3-dUTP and Cy5-dUTP (Amersham Inc., Piscataway, NJ, USA) respectively. Labeled cDNAs were purified and re-suspended in hybridization buffer as described previously (Eisen & Brown 1999). The data were obtained from three independent experiments.

High-sensitivity cDNA arrays to investigate plasma protein gene expression

Pre-spotted cDNA microarrays, Human UniversoChip 8K cDNA arrays (Asia BioInnovations Corporation, Taipei, Taiwan), containing 7597 genes, were used to investigate the effect of T3 on the expression of plasma proteins.

Image and data analysis

Labeled cDNAs were hybridized to the arrays overnight at 70 °C. The arrays were washed as described previously (Eisen & Brown 1999). Hybridized slides were scanned using the GenePix 4000B scanner (Axon Instrument, Atlanta, CA, USA) and images were processed using the GenePix Pro 3·0 (Axon Instrument). Microarray data were analyzed using eGenomix V1·0 (Asia BioInnovations Corporation) and Excel (Microsoft, Seattle, WA, USA).

Immunoblot analysis

Cell lysates were fractionated by SDS–PAGE on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane (Amersham). The membrane was gently shaken for 2 h at room temperature in 5% (w/v) nonfat dried milk in Tris-Buffered saline (TBS), washed three times with TBS, and then incubated for 1 h with rabbit polyclonal antibodies to Tf (1:1000 dilution in TBS) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with mouse monoclonal antibody C4 to TRα1 (1:1000 dilution in TBS) (kindly provided by S.-Y. Cheng, NCI, Bethesda, MD, USA). After further washing, the membrane was incubated for 1 h with horseradish peroxidase conjugated to affinity-purified antibodies to either rabbit (1:2000 dilution in TBS) or mouse (1:2000 dilution in TBS) immunoglobulin (Santa Cruz Biotechnology). Immune complexes were then visualized by chemiluminescence with an ECL detection kit (Amersham).
The intensities of immunoreactive bands were quantitated by analysis with Image Gauge software (Fuji Film, Tokyo, Japan).

**Determination of the trans-activation activity of TRs**

T3-dependent trans-activation of TRs was assayed in the various HepG2 cell lines as described previously (Lin et al. 1996). Briefly, cells were transfected with a luciferase reporter plasmid (2 µg) containing Lys-TRE (a chicken lysozyme gene and consisting of two inverted repeats of the half-site binding motif separated by six nucleotides; thyroid hormone response element), along with a β-galactosidase plasmid (1 µg) to control for transfection efficiency. Transfected cells were subsequently incubated for 24 h in Td medium containing various concentrations of T3 (Sigma, St Louis, MO, USA), after which the activities of luciferase and β-galactosidase in cell lysates were measured (Flores-Morales et al. 2002). The activity of luciferase was normalized against β-galactosidase activity.

**Northern blot analysis**

Total RNA was extracted from cells with the use of TRizol reagent, and equal amounts of total RNA (20 µg) were analyzed on a 1.2% agarose–formaldehyde gel as described previously (Lin et al. 2002). This was then blotted onto a nitrocellulose membrane and subjected to Northern blot analysis as described previously (Lin et al. 2000). A full-length Tf cDNA fragment was amplified and labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham) by the polymerase chain reaction and used as a probe. The membrane was subsequently re-probed with a 32P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment to verify equal application of RNA to each lane. In some experiments cells were treated with T3 and subjected to Northern blot analysis. In other experiments cells were treated with T3, human β-actin, and linearized pGEM-T cDNA were vacuum-transferred onto nylon membranes using a slot blot apparatus (Amersham). The membranes were baked and pre-hybridized as described for Northern blots. The precipitated radiolabeled transcripts (~10^7 c.p.m.) were re-suspended in 2 ml of hybridization buffer containing 50% formamide, 5X SSC, 2.5X Denhardt’s solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS and 250 µg/ml salmon sperm DNA. Hybridization of radiolabeled transcripts to the nylon membranes was carried out at 42 °C for 72 h. The membranes were then washed with 1X SSC, 0.1% SDS for 1 h at 65 °C before autoradiography for 24 h at ~80 °C.

**Results**

**Expression and trans-activation activity of TRα1 in HepG2 cell lines**

To investigate the role of TRα1 in the regulation of plasma protein genes, we prepared isogenic lines from HepG2 cells that stably express wild-type TRα1 (HepG2-TRα1 clones #1, #2 and #3). As a control, HepG2 cells were transfected with the empty vector, yielding a cell line that expresses the Neo protein (HepG2-Neo cells). The expression of TRα1 in HepG2-Neo cells was not detectable in the immunoblot shown in Fig. 1A. However, endogenous levels were faintly observed after longer exposure times (data not shown). Prominent immunoreactive bands, corresponding to TRα1, were detected in HepG2-TRα1#1, #2 and #3 using the monoclonal antibody C4 (Fig. 1A). This antibody recognizes an epitope at the COOH terminus of TRα1 and TRβ1 (Bhat et al. 1995). Quantitation of the intensity of the immunoreactive bands revealed that the abundance of TRα1 protein in HepG2-wt and HepG2-TRα1 cells was 4–7-fold higher than that in HepG2-Neo cells.

A Lys-luciferase reporter construct, was used to compare the transcriptional activity of TRα1 in HepG2-TRα1#1, #2 and #3 to that of the HepG2-Neo cells (Fig. 1B). HepG2-TRα1#1 cells exhibited the highest trans-activity, which was increased in a T3-dependent manner. Clones #2 and #3 showed more moderate trans-activation of the reporter construct, while the control HepG2-Neo cells exhibited a low level of trans-activity. These results indicate that the level of TRα1 protein expression correlates well with its functional capacity to trans-activate the expression of downstream genes.

**Effects of T3 on the expression of plasma protein gene in HepG2-TRα1 cells**

HepG2-TRα1#1 over-expressing cells were chosen for cDNA microarray analysis of plasma protein gene...
expression. Time-course treatments with T₃ were analyzed at the 3, 12, 24 and 48 h stages and the results of the genes that displayed a greater than twofold modulation of expression are shown in Table 1. At the 48 h time point, the expression of 20 plasma protein genes was stimulated while five were repressed. Interestingly, four of these 25 genes are involved in blood coagulation while ten others function in inflammation. The expression of albumin and antitrypsin was not affected by T₃. Due to the medical significance of iron homeostasis and the lack of previous studies, the Tf gene was chosen for further analysis. Moreover, other up-regulated genes were also chosen for investigation in a separate study.

Effects of T₃ on the abundance of Tf mRNA and protein in HepG2 cell lines

The expression of the Tf protein (80 kDa) was compared between the various HepG2 cell lines after incubation in media containing various levels of T₃ across different time points (Fig. 2). Immunoblot analysis revealed that the exposure of the control HepG2-Neo cells to 100 nM T₃ resulted in a slight increase of Tf protein (see Fig. 2A and quantitated data in Fig. 2B). T₃ significantly increased the abundance of Tf in the HepG2-TRα1 stable cell lines. The amount of Tf increased approximately 1.5–2.5-fold after incubation of HepG2-TRα1#1, #2 and #3 cells with 10 nM T₃ for 24 h. A slightly greater but significant (2.1–2.7-fold) induction of Tf was observed when cells were incubated with 100 nM T₃ for 24 h. The T₃ induction increased further (∼3- to ∼5-fold) after 48 h incubation. These results indicate that the effect of T₃ in TRα1 over-expressing cells was time and dose dependent. Thus, the extent of induction for Tf protein expression by T₃ correlated with the level of expression of TRα1. Interestingly, the abundance of Tf protein did not significantly differ among the three TRα1-overexpressed cell lines in the absence of T₃ and may, in fact, be silenced (compare all lanes at 0 nM T₃ in Fig. 2A).

The effect of T₃ on the abundance of Tf mRNA was also examined by Northern blot analysis. A 2.3 kb Tf transcript was detected in all four cell lines examined (Fig. 3A). Exposure of HepG2-TRα1#1, #2 and #3 cells to 100 nM T₃ for 24 h resulted in a TRα1 dose-dependent and significant increase in the amount of Tf mRNA, with increases of 3.8–, 2.6– and 2.5-fold respectively. T₃ had little effect on the abundance of Tf mRNA in HepG2-Neo cells (Fig. 3A). Incubation of HepG2-TRα1 stably transfected cells with T₃ at 10 nM also increased the amount of Tf transcript levels similar to those when using 100 nM T₃. This indicates that Tf gene expression is very sensitive to the presence of small amounts of T₃ in the medium. In the absence of T₃ the abundance of Tf mRNA varied among the cell lines used in this study. Interestingly, it was higher in the Neo cells and this may be due to the silencing effect observed in the TR over-expressed cells (Figs 2 and 3). Thus, the effect of T₃ on the expression of Tf protein appears to be mediated, at least in part, at the mRNA level.

T₃ increases Tf mRNA by transcripitional stimulation

To further confirm the transcription level changes of Tf mRNA in response to T₃, we performed nuclear run-on
The two other cell lines highly expressing TR can specifically increase the number of transcripts encoding proteins involved in acute-phase response. Activation of TR, in the presence of T3, results in increased expression of Tf, a protein synthesis inhibitor, as demonstrated by Northern blots. The data clearly show that the increase in Tf mRNA transcription is due to T3-mediated induction of other messengers.

Table 1: T₃-regulated plasma protein genes in HepG2-TR cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession number</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orosomucoid 1</td>
<td>AA700876</td>
<td>0.96</td>
</tr>
<tr>
<td>Fibrinogen, A α polypeptide</td>
<td>AA011414</td>
<td>1.66</td>
</tr>
<tr>
<td>Coagulation factor II (thrombin)</td>
<td>H65052</td>
<td>2.03</td>
</tr>
<tr>
<td>Prothrombin precursor</td>
<td>T62131</td>
<td>1.50</td>
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<tr>
<td>Natural killer cells protein 4</td>
<td>AA458965</td>
<td>2.67</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>A985788</td>
<td>0.83</td>
</tr>
<tr>
<td>Complement component 1, s subcomponent</td>
<td>T62048</td>
<td>1.07</td>
</tr>
<tr>
<td>α 1-microglobulin/bikunin precursor</td>
<td>A1375135</td>
<td>1.02</td>
</tr>
<tr>
<td>Interleukin 12A (natural killer cell stimulatory factor 1)</td>
<td>A803360</td>
<td>1.69</td>
</tr>
<tr>
<td>β 2-microglobulin</td>
<td>A6670408</td>
<td>1.01</td>
</tr>
<tr>
<td>Serum amyloid A protein precursor</td>
<td>H25546</td>
<td>1.45</td>
</tr>
<tr>
<td>Coagulation factor X</td>
<td>N98524</td>
<td>0.80</td>
</tr>
<tr>
<td>α 2-HS-glycoprotein α and β chain</td>
<td>R92227</td>
<td>0.96</td>
</tr>
<tr>
<td>α 2-HS-glycoprotein</td>
<td>T59108</td>
<td>1.05</td>
</tr>
<tr>
<td>Complement component 4A</td>
<td>A664406</td>
<td>0.94</td>
</tr>
<tr>
<td>Apolipoprotein CI</td>
<td>AA87159</td>
<td>12.47</td>
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<tr>
<td>Apolipoprotein CII</td>
<td>NS3169</td>
<td>1.51</td>
</tr>
<tr>
<td>Apolipoprotein CIII</td>
<td>AA456474</td>
<td>1.82</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>A0359985</td>
<td>4.47</td>
</tr>
<tr>
<td>Transferrin</td>
<td>H69531</td>
<td>3.66</td>
</tr>
<tr>
<td>Clusterin</td>
<td>AA292226</td>
<td>0.74</td>
</tr>
<tr>
<td>α 2-macroglobulin precursor</td>
<td>A775447</td>
<td>0.82</td>
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<tr>
<td>α-fetoprotein</td>
<td>T59043</td>
<td>0.84</td>
</tr>
<tr>
<td>Human α 2-macroglobulin</td>
<td>H06571</td>
<td>0.43</td>
</tr>
<tr>
<td>Insulin-like growth factor-binding protein-2</td>
<td>H79047</td>
<td>0.61</td>
</tr>
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</table>

Genes with a greater than twofold increase or decrease are shown. The data represent the mean of three independent experiments. The cells were treated with or without 100 nM T₃ for the indicated time. Thereafter, RNA was isolated and cDNA microarray was performed as described in the Materials and Methods.

Experiments. In parallel with the results of Western and Northern blots, T₃ induced approximately a twofold increase in Tf mRNA transcription (Fig. 4). The expression levels of β-actin were used as an internal control, and pGEM-T vector as a negative control. The data clearly demonstrate that activation of TR, in the presence of T₃, can specifically increase the number of Tf transcripts engaged in active synthesis by polymerase II machinery, either through enhancement of transcript initiation or that of re-initiation.

Effects of T₃ and cyclohexamide on the abundance of Tf mRNA

In an effort to further delineate the regulatory action of T₃ on the expression of Tf, a protein synthesis inhibitor, cyclohexamide, was employed. Tf expression was assayed with and without cyclohexamide by Western blot analysis. The data show that blocking protein synthesis with the addition of cyclohexamide at various time points (6–24 h) did not ablate the T₃-transcriptional response of Tf (Fig. 5). This suggests that blocking protein synthesis does not completely inhibit the concomitant induction of Tf mRNA by T₃, indicating that the effect of T₃ on the regulation of Tf is direct, rather than via the T₃-mediated induction of other messengers.

Discussion

With the use of HepG2 cells, and the derived stable cell lines, HepG2-TRα1, we have shown that expression of many plasma proteins is modulated by T₃. The differential expression of TRα1 seen in these cell lines revealed that the positive regulation of several plasma proteins, including Tf, by T₃, depended on the level of TRα1 expression. It has been long recognized that liver is a target organ for T₃, and the derived TRα1#1 cells, which express TRα1 in the absence of cyclohexamide addition, did not greatly affect the transcriptional response of Tf to T₃. The two other cell lines highly expressing TRα1 (#2 and #3) showed similar results (Fig. 5). Blocking of protein synthesis with the addition of cyclohexamide at various time points (6–24 h) did not ablate the T₃-transcriptional response of Tf (Fig. 6). This suggests that blocking protein synthesis does not completely inhibit the concomitant induction of Tf mRNA by T₃. This indicates that the effect of T₃ on the regulation of Tf is direct, rather than via the T₃-mediated induction of other messengers.
Figure 2 Effect of T₃ on Tf protein expression in HepG2 cell lines. (A) HepG2-Neo and HepG2-TRα1 cells were incubated with Td medium in the absence or presence of 100 nM T₃ for 1 or 2 days, after which cell lysates (50 μg of protein) were subjected to immunoblot analysis with polyclonal antibodies to Tf. The position of the 80 kDa Tf protein is indicated. (B) The intensities of Tf bands on immunoblots shown in (A) were quantified, and the extent of T₃-induced activation of Tf expression was determined at each time point. Data are means ± S.E. of values from three independent experiments. Values are shown as fold of induction of Td control. Student’s t-test; *P<0.05, **P<0.01, T₃- vs Td-treated.
reported equal intensity of 47 kDa (TRα) and 55 kDa (TRβ1) protein bands from rat liver (Macchia et al. 1990).

Moreover, Flores-Morales et al. (2002) used THβ1-deficient mice and cDNA microarray to determine the target genes regulated by T3. They observed that T3 activated a large number of genes in the absence of TRβ1, suggesting the involvement of TRα1. They further suggested that TRα1 may, in TRβ1(−/−) mice, substitute for the absent receptor. All these studies indicate the important but neglected contribution of TRα1 in liver. Therefore, TRα1 must play some important roles not recognized previously.

As well as the role played by Tf in maintaining serum iron, the iron regulatory protein (IRP) also plays a major role in the regulation of iron homeostasis (Klausner et al. 1993); whereas the regulation of Tf by T3 was mediated, at least in part, at the transcriptional level, apparently as a result of a direct interaction of TRα1 with the promoter region of this gene. Thus our data indicate that, in the maintenance of iron homeostasis, both transcriptional and translational regulation of related genes may be equally important.
Several factors have been shown to control the expression of Tf protein. Adrian et al. (Adrian et al. 1986) reported that the 5’ region of the human Tf gene contains the heavy metal and glucocorticoid control elements. Their observation was based mainly on the 12 bp sequences in the Tf 5’ promoter region that were ~75% identical to the glucocorticoid receptor response element. In Sertoli cells (Huleihel et al. 2002), interleukin (IL)-1β and follicle-stimulating hormone (FSH) may induce Tf secretion, and thus may be involved in the regulation of spermatogenesis and spermiogenesis and in male fertility. In addition, interferon-γ induced a marked increase in Tf synthesis by macrophages, while IL-1, IL-6 and tumor necrosis factor-α produced a more modest increase (Barnum-Huckins & Adrian 2000).

Several groups have reported that T3 positively regulates serum ferritin in humans (Macaron & Macaron 1982, Van de Vyver et al. 1982, Takamatsu et al. 1985, Hashimoto & Matsubara 1989, Kubota et al. 1993, Barnum-Huckins & Adrian 2000). Elevated serum ferritin levels were observed in hyperthyroid individuals, and levels decreased significantly after antithyroid treatment which helped to normalize T3 levels (Macaron & Macaron 1982, Van de Vyver et al. 1982, Takamatsu et al. 1985, Kubota et al. 1993, Barnum-Huckins & Adrian 2000). Furthermore, administration of T3 to hypothyroid individuals produced a significant increase in the serum ferritin level (Takamatsu et al. 1985, Hashimoto & Matsubara 1989). Leedman et al. (1996) reported that T3 regulates the Iron response element (IRE) binding activity of the IRP. These links between T3 and the regulation of ferritin expression suggest that a positive correlation exists between the levels of thyroxine (T4)/T3 and ferritin in the serum. However, the molecular mechanisms involved in

![Figure 5](https://www.endocrinology.org/JOE_179_367-377.pdf)

Figure 5 Cyclohexamide (CHX) has a limited effect on the response of Tf to T3 activation. (A) HepG2-TRα1 cells were treated as described in Fig. 3 with or without 10 μg/ml cyclohexamide. After T3 activation for 24 h, total RNA was isolated and subjected (20 μg per lane) to Northern blot analysis. (B) The intensities of the Tf and GAPDH mRNA bands on blots were quantified, and the increase in abundance of Tf transcripts was determined at each time point. The data are displayed as a fold induction compared with those in the Td condition and the data are from three independent experiments. Student’s t-test; *P<0.05, **P<0.01, T3- vs Td-treated.
the hepatic regulation of ferritin expression by T₃ had not been determined.

We also studied the regulation of Tf protein expression by TRβ1–expressing HepG2 cells (data not shown). However, the extent of T₃-induced Tf expression mediated by TRβ1 was less than that induced by TRα1. The structures of TRα1 and TRβ1 differ predominantly in the A/B domain and this may be directly involved in the transcriptional regulation of Tf. Moreover, it has been shown that the transcriptional activity of TRs is modulated by a host of co-regulatory proteins (Lin et al. 1994, 1995, 1999, 2001, 2002). Chaudhary & Skinner (2001) reported that the optimal activation of the mouse Tf promoter by FSH requires the synergistic actions of the cAMP response element-binding (CREB) protein and basic helix–loop–helix (bHLH) factors. Furthermore, the co-integrator CBP/p300 appears to be involved in regulating FSH-mediated activation of the Tf promoter by linking bHLH and CREB activities.

Recently, cDNA microarray technology has become available (Feng et al. 2000, Flores-Morales et al. 2002) and we have utilized this powerful technique to study the effect of T₃ on the expression of hepatic plasma proteins. However, when the livers of T₃–treated mice were investigated previously by cDNA microarrays, plasma protein regulation was not discussed (Feng et al. 2000).

Figure 6  Time-dependent effect of cyclohexamide (CHX) on Tf mRNA expression. (A) HepG2-TRα1 cells were treated as described in Fig. 3 with or without 10 μg/ml cyclohexamide for the indicated time. After T₃ activation, total RNA was isolated and subjected (20 μg per lane) to Northern blot analysis. (B) The intensities of the Tf and GAPDH mRNA bands on blots were quantified, and the increase in abundance of Tf transcripts was determined at each time point. The data are displayed as a fold induction compared with those in Td media and the data are from three independent experiments. Student's t-test; *P<0.05, **P<0.01, T₃- vs Td-treated.
It is possible that this subset of genes was not represented on the gene–chip or the delayed time course of induction by T₃ was not investigated. It is also possible that endogenous TR expression in the liver is not high enough for an effect to be observed. Our data indicate that many plasma proteins including prothrombin, angiotensinogen, haptoglobin, complement, lipoproteins and fibrinogen are up-regulated by T₃ at least twofold in a hepatoma cell line that highly expresses TRα1. Thus, further investigation of the regulation of hepatic plasma proteins by T₃ is required to continue elucidating this important and until now relatively unappreciated mechanism.

Importantly, this study has begun to investigate the molecular effects of TH on the regulation of plasma proteins. Of particular medical importance are a few of the genes whose expression is modulated in our arrays. For example, human α₂-HS glycoprotein (AHSG) is involved in important functions such as inhibition of insulin receptor tyrosine kinase activity, inhibition of protease activities and regulation of calcium metabolism and osteogenesis. Mathews et al. (2002) reported that AHSG concentrations start to decrease within a few hours after the onset of acute myocardial infarction (AMI) and return to near normal concentrations during the recovery period (5–7 days after AMI). It is possible that TH may have a role in AMI by regulating the AHSG level.

The genes coding for apolipoprotein AI, CI, CII and CIII produce proteins involved in the transport of cholesterol and other lipids in the plasma and are expressed predominantly in liver and intestine (Rottman et al. 1991). Thus, TH plays an important role in the homeostasis of cholesterol in the vascular endothelium. Taylor et al. (1996) reported that functional positive and negative TH response elements coexist within the rat apolipoprotein AI promoter and both elements contribute to the control of apolipoprotein AI gene expression (Taylor et al. 1996). In our data it is indicated that TH also positively regulates apolipoprotein CI, CII and CIII. We also observed the above apolipoproteins regulated by T₃ (data not shown).

Acute phase amyloid A protein has been reported to be regulated by proinflammatory cytokines and steroid hormones in human aortic smooth muscle cells (Kumon et al. 2002). Lee et al. (2002) reported that haptoglobin gene (Hp) expression is up-regulated by all-trans-retinoic acid in the extrahepatic monocytic cell line, THP-1. Hp is also up-regulated by TH in HepG2 cells (Table 1). α(1)-Acid glycoprotein (osromucoid) is an acute phase protein expressed in most mammalian species and whose concentration raises two- to fivefold during an acute phase reaction (Hochepied et al. 2002). Our results indicate that TH induces several acute phase inflammatory proteins including orosomucoid, haptoglobin, amyloid A, complement component, AHSG, AI M and bikunin. Thus, TH may play an important role in the expression of several serum proteins during blood coagulation or the inflammatory response. Further study is required to dissect the regulatory mechanism by TH for these genes.

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