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-TR α 1 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 TR α 1. 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.

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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, TR α and TR β , have been identified and located to human chromosomes 17 and 3, respectively (Lazar 1993). Each gene encodes at least two TR isoforms (TR α 1 and 2, and TR β 1 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 TR α 1 and TR β 1 in nine hepatoma cell lines to investigate the role of TRs during carcinogenesis. We showed that endogenous TR β 1 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 TR β 1 (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 TR α 1 and plasma proteins expression remained undetermined due to the lack of cell lines

expressing TR α 1 at a high level (Lin *et al.* 1996). Our data demonstrated (Lin *et al.* 1997) that the hepatoma cell line HepG2 stably expressed functional TR α 1 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 TR β 1 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 TR α 1 (HepG2-TR α 1 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 TR α 1 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 TR α 1 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 TR α 1 over-expressing cell line, HepG2-TR α 1, has been described previously (Lin *et al.* 2000). In this study, three HepG2-TR α 1-overexpressed clones (#1–3) were used. The serum was depleted of T₃ (Td) as described previously

(Samuels *et al.* 1979). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

RNA preparation and labeling

Total RNA from HepG2-TR α 1#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 TR α 1 (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

T_3 -dependent trans-activity 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 T_3 (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 - 32 P]dCTP (3000 Ci/mmol; Amersham) by the polymerase chain reaction and used as a probe. The membrane was subsequently re-probed with a 32 P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment to verify equal application of RNA to each lane. In some experiments cells were treated with T_3 and 10 µg/ml cyclohexamide (Sigma) simultaneously for 0.5 or 12 h, followed by total RNA isolation and Northern analysis.

Nuclear run-on assay

Sub-confluent HepG2-TR α 1#1 cells were treated with or without 100 nM T_3 for 24 h. Cells were subsequently washed twice with ice-cold PBS, collected, and centrifuged at 500 *g* for 5 min at 4 °C. The pellet was gently re-suspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40, allowed to swell and lyse on ice for 10 min. The lysate was re-centrifuged at 500 *g*, and the resulting nuclear pellet was re-suspended in 100 µl of labeling buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 140 mM KCl, 14 mM β -mercaptoethanol, 1 mM MnCl₂ and 20% glycerol. *In vitro* transcription using the nuclear pellet (100 µl) in labeling buffer with 1 mM creatine kinase, 10 mM phosphocreatine, 1 mM CTP, ATP, GTP,

and 100 µCi of [α - 32 P]UTP as described previously (Liao *et al.* 1995) was performed in a shaking water bath at 30 °C for 30 min. Equal amounts (2 µg) of purified, denatured full-length Tf, 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 (~10⁷ 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 TR α 1 in HepG2 cell lines

To investigate the role of TR α 1 in the regulation of plasma protein genes, we prepared isogenic lines from HepG2 cells that stably express wild-type TR α 1 (HepG2-TR α 1 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 TR α 1 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 TR α 1, were detected in HepG2-TR α 1#1, #2 and #3 using the monoclonal antibody C4 (Fig. 1A). This antibody recognizes an epitope at the COOH terminus of TR α 1 and TR β 1 (Bhat *et al.* 1995). Quantitation of the intensity of the immunoreactive bands revealed that the abundance of TR α 1 protein in HepG2-wt and HepG2-TR α 1 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 TR α 1 in HepG2-TR α 1#1, #2 and #3 to that of the HepG2-Neo cells (Fig. 1B). HepG2-TR α 1#1 cells exhibited the highest trans-activity, which was increased in a T_3 -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 TR α 1 protein expression correlates well with its functional capacity to trans-activate the expression of downstream genes.

Effects of T_3 on the expression of plasma protein gene in HepG2-TR α 1 cells

HepG2-TR α 1#1 over-expressing cells were chosen for cDNA microarray analysis of plasma protein gene

Figure 1 Expression and trans-activity of TRα1 in HepG2 cell lines. (A) Immunoblot analysis of the expression of TRα1 in transfected HepG2 cells. Lysates (100 µg of protein) of HepG2-TRα1#1, #2 and #3, and HepG2-Neo cells were subjected to immunoblot analysis with monoclonal antibody C4 (Bhat *et al.* 1995) as described in Materials and Methods. The position of the 47 kDa TRα1 protein is indicated. Tubulin was used as an internal control. (B) T_3 -dependent trans-activity of TRs in the various HepG2 cell lines. Cells were transfected with a luciferase reporter plasmid containing the Lys-TRE, as well as with a β -galactosidase plasmid to control for transfection efficiency. They were subsequently incubated for 24 h in Td medium containing the indicated concentrations of T_3 , after which the activities of luciferase and β -galactosidase in cell lysates were measured. The activity of luciferase was normalized on the basis of the activity of β -galactosidase. Data are means $\pm$ S.E. of four independent experiments, each performed in duplicate, and are expressed in light units. The ordinate indicates fold activation and non-induced = 1.

expression. Time-course treatments with T_3 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 T_3 . 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 T_3 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 T_3 across different time points (Fig. 2). Immunoblot analysis revealed that the exposure of the control HepG2-Neo cells to 100 nM T_3 resulted in a slight increase of *Tf* protein (see Fig. 2A and quantitated data in Fig. 2B). T_3 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 T_3 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 T_3 for 24 h. The T_3 induction increased further ($\sim$ 3- to $\sim$ 5-fold) after 48 h incubation. These results indicate that the effect of T_3 in TRα1 over-expressing cells was time and dose dependent. Thus, the extent of induction for *Tf* protein expression by T_3 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 T_3 and may, in fact, be silenced (compare all lanes at 0 nM T_3 in Fig. 2A).

The effect of T_3 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 T_3 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. T_3 had little effect on the abundance of *Tf* mRNA in HepG2-Neo cells (Fig. 3A). Incubation of HepG2-TRα1 stably transfected cells with T_3 at 10 nM also increased the amount of *Tf* transcript levels similar to those when using 100 nM T_3 . This indicates that *Tf* gene expression is very sensitive to the presence of small amounts of T_3 in the medium. In the absence of T_3 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 T_3 on the expression of *Tf* protein appears to be mediated, at least in part, at the mRNA level.

*T_3 increases *Tf* mRNA by transcriptional stimulation*

To further confirm the transcription level changes of *Tf* mRNA in response to T_3 , we performed nuclear run-on

Table 1 T_3 -regulated plasma protein genes in HepG2-TR cells

	Molecular function	Accession number	Time			
			3 h	12 h	24 h	48 h
Genes						
Orosomucoid 1	Acute-phase protein/cardiovascular function	AA700876	0.96	2.40	3.87	28.97
Fibrinogen, A α polypeptide	Coagulation	AA011414	1.66	8.07	8.84	18.20
Coagulation factor II (thrombin)	Coagulation	H65052	2.03	3.94	6.54	12.80
Prothrombin precursor	Coagulation	T62131	1.50	3.47	5.14	11.15
Natural killer cells protein 4	Inflammation	AA458965	2.67	4.73	10.50	8.71
Haptoglobin	Inflammation	AI985788	0.83	0.77	4.02	8.31
Complement component 1, s subcomponent	Inflammation	T62048	1.07	2.21	3.87	7.60
α 1-microglobulin/bikunin precursor	Inflammation	AI375135	1.02	1.70	3.69	7.59
Interleukin 12A (natural killer cell stimulatory factor 1)	Inflammation	AI803560	1.69	3.05	4.20	6.63
β 2-microglobulin	Inflammation	AA670408	1.01	1.11	1.53	6.59
Serum amyloid A protein precursor	Inflammation	H25546	1.45	2.35	1.67	6.23
Coagulation factor X	Coagulation	N98524	0.80	1.47	2.94	5.91
α 2-HS-glycoprotein α and β chain	Inflammation/immune response	R92227	0.96	2.86	4.08	5.31
α 2-HS-glycoprotein	Inflammation/immune response	T59108	1.05	2.49	4.09	4.51
Complement component 4A	Inflammation/immune response	AA664406	0.94	1.53	3.34	4.35
Apolipoprotein CI	Metabolism/lipid	AA873159	12.47	26.79	26.14	4.06
Apolipoprotein CIII	Metabolism/lipid	N53169	1.51	3.53	4.12	3.54
Apolipoprotein CII	Metabolism/lipid	AA456474	1.82	3.19	4.94	2.80
Angiotensinogen	Blood pressure regulation	AI359985	4.47	4.67	7.94	2.11
Transferrin	Transporter/binding protein/iron	H69531	3.66	5.21	6.82	2.07
Clusterin	Transporter	AA292226	0.74	0.53	0.40	0.22
α 2-macroglobulin precursor	Inflammation/tissue remodeling	AA775447	0.82	0.55	0.38	0.18
α -fetoprotein	Cell matrix protein	T59043	0.84	0.52	0.28	0.15
Human α 2-macroglobulin	Inflammation/tissue remodeling	H06571	0.43	0.36	0.29	0.14
Insulin-like growth factor-binding protein-2	Binding protein	H79047	0.61	0.34	0.12	0.05

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 T_3 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, T_3 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 T_3 , 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 T_3 and cyclohexamide on the abundance of *Tf* mRNA*

In an effort to further delineate the regulatory action of T_3 on the expression of *Tf*, a protein synthesis inhibitor, cyclohexamide, was employed. *Tf* expression was induced via T_3 , in the presence or absence of cyclohexamide simultaneously for 24 h in HepG2-TR α 1#1 cells. Blocking of protein induction, via cyclohexamide addition, did not greatly affect the transcriptional response of *Tf* to T_3 . The two other cell lines highly expressing TR α 1 (#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 T_3 -transcriptional response of *Tf* (Fig. 6). This suggests that blocking protein synthesis does not completely inhibit the concomitant induction of *Tf* mRNA by T_3 . This indicates that the effect of T_3 on the regulation of *Tf* is direct, rather than via the T_3 -mediated induction of other messengers.

Discussion

With the use of HepG2 cells, and the derived stable cell lines, HepG2-TR α 1, we have shown that expression of many plasma proteins is modulated by T_3 . The differential expression of TR α 1 seen in these cell lines revealed that the positive regulation of several plasma proteins, including *Tf*, by T_3 depended on the level of TR α 1 expression. It has been long recognized that liver is a target organ for TRs. In fact, Chamba *et al.* (1996) reported that the abundance of TR α 1 and TR β 1 in normal human liver is 0.8 vs 1.08 absorbance units by Western blot analysis. Their results revealed abundant TR α 1, TR α 2 and TR β 1 protein in human hepatocyte. Macchia *et al.* (1990) also

Figure 2 Effect of T_3 on Tf protein expression in HepG2 cell lines. (A) HepG2-Neo and HepG2-TR α 1 cells were incubated with Td medium in the absence or presence of 100 nM T_3 for 1 or 2 days, after which cell lysates (50 μ g 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 T_3 -induced activation of Tf expression was determined at each time point. Data are means $\pm$ 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, T_3 - vs Td-treated.

Figure 3 Effect of T_3 on the abundance of *Tf* mRNA in HepG2 cell lines. (A) HepG2-TR α 1 or HepG2-Neo cells were incubated for 24 h in the absence or presence of T_3 , after which total RNA was isolated and subjected (20 μ g per lane) to Northern blot analysis with 32 P-labeled *Tf* or GAPDH cDNA probes. The positions of the 2.3 kb *Tf* and 1.0 kb GAPDH mRNAs are indicated. (B) The intensities of the *Tf* mRNA bands shown in (A) were quantified, and the extent of the T_3 -induced increase in the abundance of *Tf* transcripts was determined at each point. Data are means $\pm$ S.E. of values from three independent experiments. Student's *t*-test; ** $P < 0.01$, T_3 - vs T_d -treated.

Figure 4 Effect of T_3 on *Tf* gene transcription rate, measured in nuclear run-on assay from HepG2-TR α 1 cells. Nuclei were isolated after cells were treated with or without 100 nM T_3 for 24 h. *In vitro* transcription using the nuclear pellet labeling with [α - 32 P]UTP. Total labeled nascent RNA was labeled as described in Materials and Methods and probed against denatured linear plasmids containing the cDNAs indicated at the left of the Figure. Actin and pGEM-T were used as internal controls.

reported equal intensity of 47 kDa (TR α 1) and 55 kDa (TR β 1) protein bands from rat liver (Macchia *et al.* 1990). Moreover, Flores-Morales *et al.* (2002) used TH β 1-deficient mice and cDNA microarray to determine the target genes regulated by T_3 . They observed that T_3 activated a large number of genes in the absence of TR β 1, suggesting the involvement of TR α 1. They further suggested that TR α 1 may, in TR β 1(-/-) mice, substitute for the absent receptor. All these studies indicate the important but neglected contribution of TR α 1 in liver. Therefore, TR α 1 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 T_3 was mediated, at least in part, at the transcriptional level, apparently as a result of a direct interaction of TR α 1 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.

Figure 5 Cyclohexamide (CHX) has a limited effect on the response of *Tf* to T_3 activation. (A) HepG2-TR α 1 cells were treated as described in Fig. 3 with or without 10 μ g/ml cyclohexamide. After T_3 activation for 24 h, total RNA was isolated and subjected (20 μ g 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, T_3 - vs Td-treated.

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)-1 α , β 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 T_3 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 T_3 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 T_3 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 T_3 regulates the Iron response element (IRE) binding activity of the IRP. These links between T_3 and the regulation of ferritin expression suggest that a positive correlation exists between the levels of thyroxine (T_4)/ T_3 and ferritin in the serum. However, the molecular mechanisms involved in

Figure 6 Time-dependent effect of cyclohexamide (CHX) on *Tf* mRNA expression. (A) HepG2-TR α 1 cells were treated as described in Fig. 3 with or without 10 μ g/ml cyclohexamide for the indicated time. After T_3 activation, total RNA was isolated and subjected (20 μ g 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; * $P < 0.05$, ** $P < 0.01$, T_3 - vs Td-treated.

the hepatic regulation of ferritin expression by T_3 had not been determined.

We also studied the regulation of *Tf* protein expression by TR β 1-expressing HepG2 cells (data not shown). However, the extent of T_3 -induced *Tf* expression mediated by TR β 1 was less than that induced by TR α 1. The structures of TR α 1 and TR β 1 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 T_3 on the expression of hepatic plasma proteins. However, when the livers of T_3 -treated mice were investigated previously by cDNA microarrays, plasma protein regulation was not discussed (Feng *et al.* 2000,

Weitzel *et al.* 2001, Flores-Morales *et al.* 2002, Sadow *et al.* 2003). It is possible that this subset of genes was not represented on the gene-chip or the delayed time course of induction by T_3 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 T_3 at least twofold in a hepatoma cell line that highly expresses TR α 1. Thus, further investigation of the regulation of hepatic plasma proteins by T_3 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 α 2-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 T_3 (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 (orosomucoid) is an acute phase protein expressed in most mammalian species and whose concentration raises two- to fivefold during an acute phase reaction (Hochebied *et al.* 2002). Our results indicate that TH induces several acute phase inflammatory proteins including orosomucoid, haptoglobin, amyloid A, complement component, AHSG, A1 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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