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Thyroid Hormone Receptor-Dependent Transcriptional Regulation of Fibrinogen and Coagulation Proteins

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Thyroid hormone (T_3) regulates growth, development, and differentiation. These activities are mediated by the nuclear thyroid hormone receptors (TRs), which belong to the steroid/TR superfamily of ligand-dependent transcription factors. The effect of T_3 treatment on target gene regulation was investigated in a TRα-overexpressing hepatoma cell line (HepG2-TRα), by performing cDNA microarrays. We demonstrate that 148 of the 7597 genes represented were up-regulated by T_3, including fibrinogen and several other components of the coagulation factor system. To confirm the microarray results, fibrinogen and a small number of the blood clotting components were further investigated using quantitative RT-PCR. The T_3-induction ratios observed with quantitative RT-PCR for factors such as thrombin (8-fold), coagulation factor X (4.9-fold), and hepatoglobin (30-fold) were similar to those observed by the cDNA microarray analysis. Further investigation, using HepG2-TRα (cell lines, revealed a 2- to 3-fold induction of fibrinogen transcription after 24 h of T_3 treatment. In addition, T_3 treatment increased the level of fibrinogen protein expression 2.5- to 6-fold at 48 h. The protein synthesis inhibitor, cycloheximide, did not inhibit the induction of fibrinogen by T_3, indicating that this regulation was direct. Furthermore, transcription run-on experiments indicate that the induction of fibrinogen by T_3 is regulated largely at the level of transcription. Similar observations were made on the regulation of fibrinogen by T_3 using rats that received surgical thyroidectomy (TX) as an *in vivo* model. These results suggest that T_3 plays an important role in the process of blood coagulation and inflammation and may contribute to the understanding of the association between thyroid diseases and the misregulation of the inflammatory and clotting profile evident in the circulatory system of these patients. (*Endocrinology* 145: 2804–2814, 2004)

THE THYROID HORMONE, T_3, is a potent mediator of many physiological processes, which include embryonic development, cellular differentiation, metabolism, and the regulation of cell proliferation (1–4). T_3 controls these processes in most, if not all, organs of the body. These activities are mediated by the nuclear thyroid hormone receptors (TRs), of which two principal types of TRs have been identified. These are referred to as TRα and TRβ, which are encoded on human chromosome 17 and 3, respectively (1, 4, 5). Both of these genes have alternative promoters allowing the generation of TRα1 and α2 as well as TRβ1 and β2 receptor isoforms (1–3, 5). TRs, as for many nuclear hormone receptors, are ligand-dependent transcription factors. These receptors are comprised of modular functional domains that mediate the binding of hormones (ligands), DNA binding, receptor homo- and heterodimerization, and interaction with other transcription factors and cofactors (1–5). It has been demonstrated that TRs regulate the transcription of target genes by binding to specific DNA elements in the promoter regions of these genes, referred to as thyroid hormone response elements (TREs). In the absence of T_3 ligand, TRs repress the expression of target genes, a phenomenon known as transcriptional silencing. This process is thought to be mediated by interaction via the ligand binding domain of the receptor with transcriptional corepressors, such as the silencing mediator of retinoic acid and TR (6). The binding of ligand is thought to induce dissociation of TRs from corepressors and to result in the recruitment of transcriptional coactivators such as steroid receptor coactivator and the subsequent activation of target gene expression (1).

Previously, we examined the expression and regulation of TR genes in nine human hepatoma cell lines (7). However, the mechanisms used by TRα1 to selectively maintain liver-specific gene transcription have yet to be elucidated. It is well established that the liver is a target organ for TRs and is also the primary site of synthesis of blood proteins involved in coagulation. In fact, Chamba *et al.* (8) reported on the high quantity of TRα1 and TRβ1 observed in normal human liver via Western blot analysis. Abundant levels of TRα1, TRα2, and TRβ1 proteins have also been demonstrated in human hepatocytes (8). HepG2 is a well-differentiated hepatocellular carcinoma cell-line, secreting all 15 plasma proteins and retaining many liver-specific functions. Thus, the HepG2 cell line is a suitable candidate for an *in vitro* model system to study the cell type-specific and TR isoform-specific regulation of T_3 target genes in the liver.

The cDNA microarray assay has proven to be a powerful tool for deciphering the mechanisms and studying the numerous facets of the cellular functions of TR in normal and aberrant situations. This technique, an excellent means for

Abbreviations: CHX, Cycloheximide; Ct, threshold cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Q-RT-PCR, quantitative RT-PCR; SD, Sprague Dawley; TBS, Tris-buffered saline; Td, depleted of T_3; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TX, thyroidectomy.

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identifying differentially expressed genes, was employed here to identify T_3 target genes in HepG2 cells overexpressing TRα1. In this study, we demonstrate the up-regulated expression of 148 genes by T_3 in a time course-dependent manner. Among these are genes involved in metabolism, detoxification, signal transduction, cell adhesion, and cell cycle. Surprisingly, a very high proportion of these genes are involved in the systemic/cellular inflammatory response, which is not traditionally associated with thyroid hormone function. Thus, we focused our study on this subset of genes, with particular attention being paid to fibrinogen.

Human fibrinogen is a circulating 340-kDa glycoprotein, primarily synthesized by hepatocytes. It is comprised of two symmetric half molecules, each consisting of one set of three different polypeptide chains termed Aα, Bβ, and γ. The molecule is highly heterogenous due to alternative splicing, extensive posttranslational modification, and proteolytic degradation. Fibrinogen is cleaved by thrombin to form fibrin, the most important component in the blood clotting reaction (9). The role of TR in the process of blood clotting is currently unknown.

This investigation examined the regulation of fibrinogen by T_3 in more detail. T_3 treatment increased the abundance of fibrinogen in HepG2 cell lines stably expressing TRα1, at both the RNA and protein level, compared with the control line. The use of a nuclear run on assay and cycloheximide (CHX), a protein synthesis inhibitor, established that the regulation of fibrinogen by T_3 occurred directly, without the requiring the synthesis of other proteins. Importantly, studies in TX rats revealed similar regulation of fibrinogen by T_3.

Materials and Methods

Cell culture

The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA)and was routinely grown in DMEM supplemented with 10% (vol/vol) fetal bovine serum. HepG2-TRα1 highly expresses TRα1 as previously described (10). The serum was depleted of T_3 (Td) as described (11). Cells were cultured at 37 C in a humidified atmosphere of 95% air-5% CO_2.

RNA preparation and labeling

Total RNA from HepG2-TRα1 no. 1, treated with or without T_3, was prepared using TRIzol (Life Technologies, Rockville, MD). For fluorescence labeling of cDNA, 30 μg total RNA from untreated cells and 50 μg total RNA from treated cells was reverse transcribed in the presence of Cy3-deoxyuridine 5-triphosphate and Cy5-deoxyuridine 5-triphosphate (Amersham Inc., Piscataway, NJ), respectively. Labeled cDNA was purified and resuspended in the hybridization buffer as described (12).

cDNA microarrays

Prespotted cDNA microarrays, Human UniversoChip 8K cDNA arrays (Asia BioInnovations Corporation, Taipei, Taiwan, Republic of China), containing 7597 genes, were used in all array experiments.

Image and data analysis

Labeled cDNA was hybridized to the arrays overnight at 70 C. The arrays were washed as previously described (12). Hybridized slides were scanned using the GenePix 4000B scanner (Axon Instrument, Union City, CA), and images were processed using the GenePix Pro 3.0 (Axon Instrument). Microarray data were analyzed using the eGenomix V1.0 (Asia BioInnovations Corporation) and EXCEL (Microsoft, Seattle, WA) software.

Immunoblot analysis

Cell lysates were fractionated by SDS-PAGE on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane (pH 7.9 membrane, Amersham). The membrane was blocked for 2 h at room temperature in 5% (wt/vol) nonfat dried milk in Tris-buffered saline (TBS). Next, the membrane was washed three times with TBS and then incubated for 1 h with rabbit polyclonal antibodies to fibrinogen (1:500 dilution in TBS) (DAKO, Copenhagen, Denmark) or with mouse monoclonal antibody C4 to TRα1 (1:1000 dilution in TBS) (kindly provided by S.-Y. Cheng, National Cancer Institute, Bethesda, MD). After further washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated, affinity-purified antibodies to either rabbit (1:1000 dilution in TBS) or mouse (1:1000 dilution in TBS) Ig (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were then visualized by chemiluminescence with an enhanced chemiluminescence detection kit (Amersham). The intensities of immunoreactive bands were quantitated by analysis with Image Gauge software (Fuji Film, Tokyo, Japan).

Northern blot analysis

Total RNA was extracted from cells with the use of TRIzol Reagent (Life Technologies), and equal amounts of total RNA (20 μg) were analyzed on a 1.2% agarose-formaldehyde gel as described (10, 13). The separated RNA molecules were then transferred to a nitrocellulose membrane and subjected to Northern blot analysis, as described (14), with a full-length fibrinogen cDNA fragment that was PCR-amplified and labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). The membrane was subsequently reprobed 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 CHX simultaneously for 12 or 24 h, followed by total RNA isolation and Northern analysis.

Nuclear run-on assay

To determine whether T_3 stimulation occurs at the transcriptional level, a nuclear run on assay was performed based on the method described previously (15). Subconfluent HepG2-TRα1 no. 1 cells were treated with or without 10 nM T_3 for 3 h. Cells were subsequently washed twice with ice-cold PBS, collected, and centrifuged at 500 $\times$ *g* for 5 min at 4 C. The pellet was gently resuspended 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 recentrifuged at 500 $\times$ *g*, and the resulting nuclear pellet was resuspended in 100 μl 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) was performed in a shaking water bath at 30 C for 30 min in a labeling buffer with 1 mM creatine kinase, 10 mM phosphocreakine, 1 mM CTP, ATP, GTP, and 100 μCi of [α-^{32}P] uridine 5′-triphosphate as described previously (16). Equal amounts (2 μg) of purified, denatured full-length fibrinogen, 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 prehybridized as described for Northern blots. The precipitated radiolabeled transcripts ($\sim$10^7 cpm) were resuspended in 2 ml hybridization buffer containing 50% formamide, 5$\times$ saline sodium citrate, 2.5$\times$ Denhardt's solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% sodium dodecyl sulfate, and 250 μg/ml salmon sperm DNA. Hybridization of radio-labeled transcripts to the nylon membranes was carried out at 42 C for 72 h. The membranes were then washed with 1$\times$saline sodium citrate, 0.1% sodium dodecyl sulfate for 1 h, at 65 C, before autoradiography for 24 h at $-$80 C.

Quantitative RT-PCR (Q-RT-PCR)

Total RNA was extracted from cells using TRIzol as described above. Subsequently, the first strand of cDNA was synthesized using the Superscript III kit for RT-PCR (Life Technologies). Briefly, total RNA was denatured at 65 C for 5 min in the presence of 0.5 μg oligo dT and 1 mM deoxynucleotide triphosphate. After chilling on ice for at least 1 min, reverse transcription was allowed to proceed at 25 C for 5 min in the presence of 1$\times$ first-strand buffer, 5 mM dithiothreitol, and 40 U ribo-

nuclease inhibitor. The reaction was then allowed to proceed at 50 C for another 60 min. The reaction was terminated by heat inactivation at 70 C for 10 min.

Real-time Q-RT-PCR was performed in a 25-μl reaction mixture containing 50 nM forward and reverse primers, 1× SYBR Green reaction mix (Applied Biosystems, Werrington, UK), and various amounts of template. The reaction was performed with preliminary denaturation for 10 min at 95 C to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 C for 15 sec, and annealing/extension at 60 C for 1 min. Fluorescence emitted by SYBR Green was detected on line by the ABI PRISM 7000 sequence detection system (Applied Biosystems). Studies have shown that initial copy number can be quantitated during real-time PCR analysis based on threshold cycle (Ct). The Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. Different amounts of template (16, 8, 4 ng) were used in the same reaction to ensure linear amplification. All PCR were done in duplicate on the same 96-well plate. For quantification of gene expression changes, theΔCt method was used to calculate relative-fold changes normalized against the ribosomal binding protein (RiboL35A) gene, as described in user bulletin number 2 (Applied Biosystems).

Animals

Male Sprague Dawley (SD) rats received TX at 6 wk of age, as described in previous reports (17–19). The rats were given 1% calcium lactate in their drinking water after surgery. Two weeks later, rats were injected with T_3 at 10 μg/100 g body weight, or with the control vehicle (2.5 mM NaOH in PBS), daily for an additional 2 wk. Rats were killed at the end of the experiment, and the serum was used for T_3 and TSH determination. The expression levels of several plasma proteins in the liver were analyzed by Q-RT-PCR or Western blot. All animal experimentation described in this study was conducted in accordance with the National Institutes of Health Guide and Chang-Gung Institutional Animal Care and Use Committee Guide for the Care and Use of Laboratory Animals.

Results

Microarray identified genes responsive to T_3 induction in HepG2 cells

To examine the consequences of TRα1 up-regulation on target gene expression, we established and used the HepG2-TRα1 cell line, which expresses high levels of TRα1 protein (10). Total RNA was isolated from HepG2-TRα1 and reverse transcribed into Cy5-labeled cDNA at several time points after T_3-induction (3, 12, 24, and 48 h). This cDNA was hybridized to the Cy3-labeled cDNA derived from the untreated HepG2-TRα1 cell line. For the microarray assays, Human Universo-Chip 8K cDNA arrays (Asia BioInnovations Corporation) containing 7597 cDNAs were used. In duplicate experiments, 2% (148 of 7597) of the genes represented were up-regulated by T_3.

Thyroid hormone is traditionally recognized as a hormone involved in metabolism-related events. Previous reports have identified a number of target genes that are regulated in the liver by T_3 (20–23). Although T_3 has been reported to increase the concentration of some plasma proteins, its function in the systemic/cellular inflammatory response is not well documented. Thus, it is surprising to find that inflammatory response-related genes constitute the largest subgroup in the T_3-activated hepatic gene cluster. These genes, which include fibrinogen, coagulation factor, heparin cofactor, haptoglobin, natural killer cells protein 4 precursor, CD40, and complement (Table 1), were selected for verification of the microarray analysis and further study.

Several coagulation- and inflammation-related proteins were used as verification of the microarray data

To confirm the microarray data, we chose to study several of the coagulation- and inflammation-related genes up-regulated by T_3. Real-time Q-RT-PCR confirmed the results from the cDNA microarray analysis (Table 1), with respect to the induction of coagulation-related proteins. This assay is a very sensitive tool in the analysis of gene expression profiling. Table 2 displays the induction of the selected proteins, in HepG2-TRα1 cells, when treated with T_3 for 12 and 24 h, compared with the control-lacking treatment. The Q-RT-PCR assay yielded very reproducible results in support of the microarray data. For example, haptoglobin was induced approximately 30-fold, whereas complement component 1, thrombin, and natural killer cells protein were induced 9.1-, 8.6-, and 8.3-fold, respectively. Table 2 shows that, in fact, T_3 represses plasminogen by 20%. In a similar manner, α-2-macroglobulin and α-fetoprotein were also down-regulated by T_3 (data not shown). These results indicate that T_3 may play an important role in the process of blood coagulation and/or in the inflammatory response. However, further study is required to elucidate the mechanisms by which T_3 regulates these genes.

Effects of T_3 on the abundance of fibrinogen protein and mRNA in HepG2 cells

Fibrinogen is comprised of two identical subunits, each of which is composed of three further subunits, referred to as Aα, Bβ, and γ. We investigated the expression of all three fibrinogen subunits (70, 56, and 48 kDa for Aα, Bβ, and γ chain, respectively) via Western and Northern blots (Figs. 1 and 2). The two HepG2 cell lines used in this study are referred to as HepG2-TRα1 no. 1 and no. 2, where the TRα1 protein was overexpressed approximately 10- and 3-fold, respectively, compared with the HepG2-Neo control cell line (Fig. 1A). Furthermore, the TRα1 expressed in both HepG2-TRα1 no. 1 and no. 2 cell lines is functional, as demonstrated in trans-activation assays using the TRα1-sensitive reporters Lap-TRE and Pal-TRE (data not shown).

T_3 treatment increased the abundance of fibrinogen in HepG2-TRα1 no. 1, and no. 2 cells, compared with the control, with fibrinogen Aα increased approximately 2.1- to 2.7-fold after incubation of HepG2-TRα1 no. 1 and no. 2 cells with 1 nM T_3 for 24 h. Incubation of these cells with 10 nM T_3 for 24 h led to a slightly greater (2.9- to 3.6-fold) induction of fibrinogen Aα. After 48 h incubation, the 10 nM T_3-induction was even higher (up to 6-fold). The application of 100 nM T_3 can induce a further 10–15% increase in the levels of fibrinogen expression (data not shown). A similar, but slightly less striking, induction was observed for fibrinogen Bβ and γ chain (Fig. 1, B–F). These results indicate that the effect of T_3 on fibrinogen expression was time- and dose-dependent, with the higher TRα1-expressing cell line, HepG2-TRα1 no. 1, consistently expressing higher levels of the fibrinogen Aα subunit. In contrast, the expression of fibrinogen protein in HepG2-Neo cells was largely unaffected by T_3 (data not shown). Thus, the extent of induction of fibrinogen protein by T_3 correlated with the level of expression of TRα1. The abundance of fibrinogen protein did

TABLE 1. T_3-up-regulated genes in TRα1-overexpressed cells

Gene name and molecular function	Clone identity		T_3 induction[a] (Hours after T_3 treatment)			
	Accession	gs Link	3 h	12 h	24 h	48 h
Inflammation						
S100 calcium-binding protein A8 (calgranulin A)	AA086471	Hs.100000	2.23	4.11	5.40	5.93
α-2-HS-glycoprotein	T59108	Hs.324746	1.42	3.58	6.05	6.95
α-2-HS-glycoprotein α and β chain	R92227	Hs.52256	1.31	3.74	5.45	7.59
CD40 receptor precursor	H98636	Hs.25648	0.93	2.31	4.34	5.42
Complement component 1, s subcomponent	T62048	Hs.169756	0.93	1.50	2.53	7.71
Complement component 4A	AA664406	Hs.170250	0.92	1.21	2.52	3.62
Deoxyribonuclease 1-like III protein (DNAS1L3)	T73558	Hs.88646	1.18	1.56	1.69	2.60
Herpesvirus entry mediator B (poliovirus receptor-related 2)	AA668508	Hs.183986	1.47	2.01	2.09	2.22
Human CX3C chemokine precursor	R66139	Hs.80420	1.28	2.32	2.39	2.98
Human renal cell carcinoma antigen RAGE-4	AI684696	Hs.55209	1.26	1.78	2.11	2.33
Human toll-like receptor 5 (TLR5)	N41021	Hs.114408	2.37	3.35	3.57	3.93
IL 12A	AI803560	Hs.673	1.22	2.23	2.59	2.79
Major histocompatibility complex, class I, C	AA464246	Hs.77961	1.30	1.47	2.39	5.33
Major histocompatibility complex, class I, F	AA988615	Hs.77961	1.08	1.46	1.84	3.13
Mannan-binding lectin serine protease 2	AA776328	Hs.119983	0.89	2.15	1.98	5.21
Natural killer cells protein 4 precursor	AA458965	Hs.943	2.19	7.06	10.56	11.53
Neutrolphil gelainase-associated lipocalin	AA401137	Hs.204238	0.65	0.83	2.62	3.55
NKG-2-D type integral membrane protein	AA397819	Hs.74085	0.94	2.02	1.15	2.17
Pentaxin-related gene, rapidly induced by IL-1 β	W48562	Hs.2050	1.05	1.44	2.62	2.53
Phospholipase A2 receptor 1, 180-kDa	AA086038	Hs.171945	1.22	1.48	2.11	2.74
Small inducible cytokine subfamily A (Cys-Cys), member 15	AI700237	Hs.272493	1.11	1.98	2.76	3.14
Tapasin (NGS-17)	T69304	Hs.179600	1.41	1.45	1.46	2.38
β-2-Microglobulin precursor	AA670408	Hs.75415	1.30	1.58	2.24	3.80
α-1-Microglobulin/bikunin precursor	AI375135	Hs.76177	1.42	2.63	4.90	5.57
Amphiphysin (128-kDa autoantigen)	H06541	Hs.173034	1.48	3.62	5.20	6.21
Coagulation factor X precursor	N98524	Hs.47913	1.06	1.56	3.02	2.59
Haptoglobin	AI985788	Hs.75990	1.02	0.84	4.03	15.65
Serum amyloid A protein precursor	H25546	Hs.181062	1.00	1.60	1.72	2.20
Plasma protein						
α-1-acid glycoprotein (Orosomucoid 1)	AA700876	Hs.572	1.40	3.50	6.01	7.98
Coagulation factor II (thrombin)	H65052	Hs.37926	1.37	3.70	4.47	5.66
Fibrinogen, A α polypeptide	AA011414	Hs.90765	1.11	7.39	10.73	20.12
Prothrombin precursor	T62131	Hs.76530	1.23	2.89	4.45	4.55

[a] Gene with a greater than 2-fold increase at 48-h induction. The expression ratios represent the mean of trplicate experiments.

TABLE 2. Expression profile of coagulation genes after T_3 treatment

Genes	Fold induction by T_3	
	12 h[a]	24 h[a]
Fibrinogen, A α polypeptide	6.17 ± 0.79	11.80 ± 0.76
Coagulation factor II (thrombin)	3.68 ± 0.19	8.57 ± 1.80
Coagulation factor X	2.10 ± 0.15	4.91 ± 1.63
Haptoglobin	1.21 ± 0.14	29.67 ± 6.33
Natural killer cells protein 4	3.87 ± 0.28	8.29 ± 1.77
CD40	1.91 ± 0.01	1.93 ± 0.02
Complement component 1, s subcomponent	2.34 ± 0.23	9.13 ± 0.28
Complement component 4A	1.28 ± 0.53	3.20 ± 0.14
Plasminogen	0.57 ± 0.00	0.20 ± 0.02

[a] The cells were treated with or without 10 nM T_3 for the indicated time. Thereafter, RNA was isolated, and Q-RT-PCR was performed as described in *Materials and Methods*.

The data represent the mean of three independent experiments.

not differ between the two HepG2-TRα1 cell lines in the absence of T_3 (Fig. 1, B and C).

The effect of T_3 on the abundance of fibrinogen mRNA was examined by Northern blot analysis. A 2.2-kb *fibrinogen Aα* transcript was detected in all cell lines examined (Fig. 2A). A dose-dependent increase in *fibrinogen* mRNA was observed when HepG2-TRα1 no. 1 and no. 2 cells were exposed to T_3 (10 nM) for 12 h. A 6.9- and 2.7-fold increase was identified in each cell line, respectively. RNA from the individual cell lines, incubated in Td medium and harvested immediately (0 h), was used as a control. Incubation of HepG2-TRα1 no. 1 and no. 2 cells with T_3 at 1 nM also increased the amount of *fibrinogen* transcript, comparable with using 10 nM T_3, indicating that *fibrinogen* gene expression is very sensitive to the presence of T_3 in the medium. A small amount of T_3 was enough to induce the expression of *fibrinogen* dramatically (Fig. 2A). Cells exposed to T_3 for 48 h demonstrated greater induction than those treated for 24 h (data not shown). The abundance of *fibrinogen* mRNA, in the absence of T_3, was very low in both cell lines (Fig. 2). Thus, at least part of the effect of T_3 on the expression of fibrinogen protein appears to be mediated at the mRNA level.

To analyze the time-dependent induction of fibrinogen, HepG2-TRα1 no. 1 cells were cultured with or without T_3 and investigated at earlier time points. To induce the maximal effect, 100 nM T_3 was used. As early as 3 h after T_3-treatment, *fibrinogen* mRNA increased about 1.5-fold. Thereafter, increases in *fibrinogen* mRNA expression of 2-, 3-, 6-, and 8-fold at 6, 12, 24, and 48 h, respectively, are demonstrated (Fig. 3). Thus, it appears that the induction of fibrinogen expression in these cells is not only sensitive, but responds quickly to treatment by T_3.

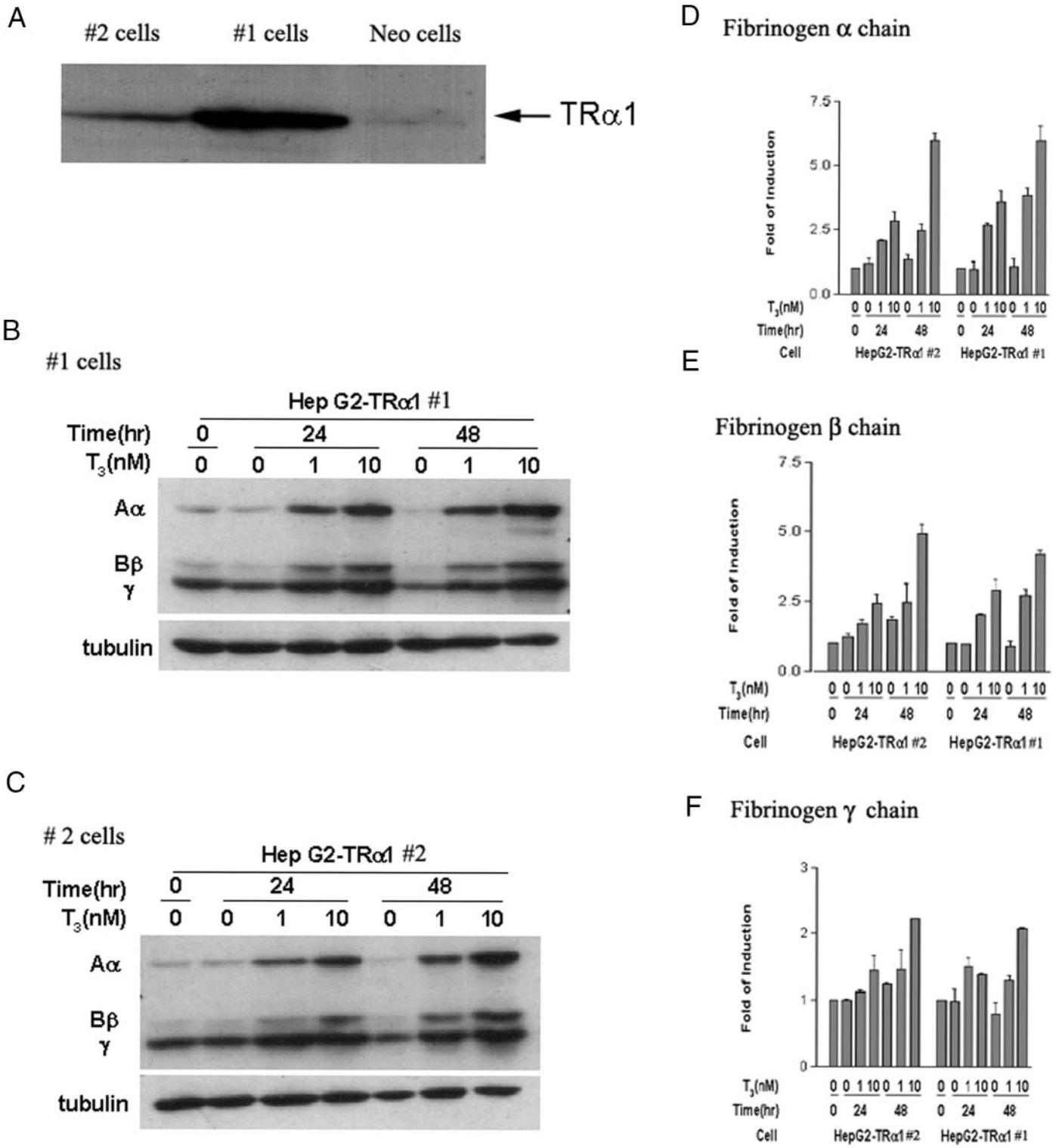

FIG. 1. Effect of T_3 on fibrinogen protein expression in HepG2 cell. A, TRα1 expression level in two HepG2 stable lines and HepG2-Neo cells. B and C, HepG2-TRα1 no. 1 or no. 2 cells were incubated with T_3-depleted medium in the absence or presence of 1–10 nM T_3 for 24 or 48 h, after which cell lysates (50 μg protein) were subjected to immunoblot analysis with polyclonal antibodies to fibrinogen (DAKO A0080). The positions of the 70-, 56-, and 48-kDa for Aα, Bβ, and γ fibrinogen subunits are indicated on the *left hand side of each blot*. D–F, The intensities of each fibrinogen subunit band were quantified, and the extent of T_3-induced activation was determined at each time point. Data are means ± SE of values from three independent experiments.

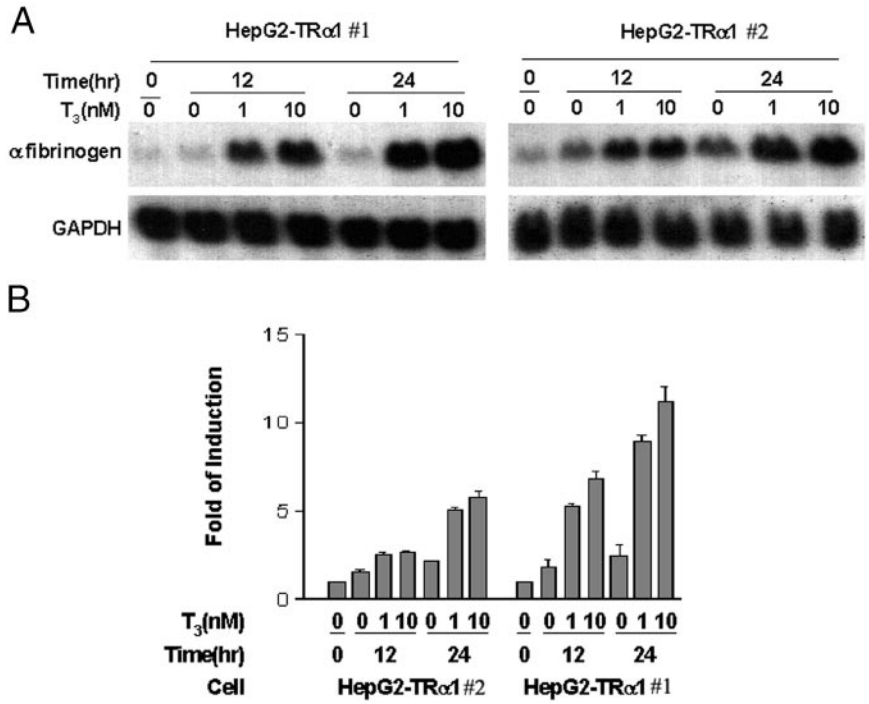

FIG. 2. Effect of T_3 on the abundance of *fibrinogen* mRNA in HepG2 cell lines. A, HepG2-TRα1 no. 1, and no. 2 cells were incubated for 12 or 24 h in the absence or presence of 1–10 nM T_3, after which total RNA was isolated and subjected (20 μg per lane) to Northern blot analysis with ^{32}P-labeled *fibrinogen* or GAPDH cDNA probes. The positions of the 2.2-kb *fibrinogen* and 1.0-kb GAPDH mRNAs are indicated. B, The intensities of the *fibrinogen* mRNA bands on blots similar to that shown in A were quantified, and the extent of the T_3-induced increase in the abundance of *fibrinogen* transcripts was determined at each point. Data are means ± SE of values from three independent experiments.

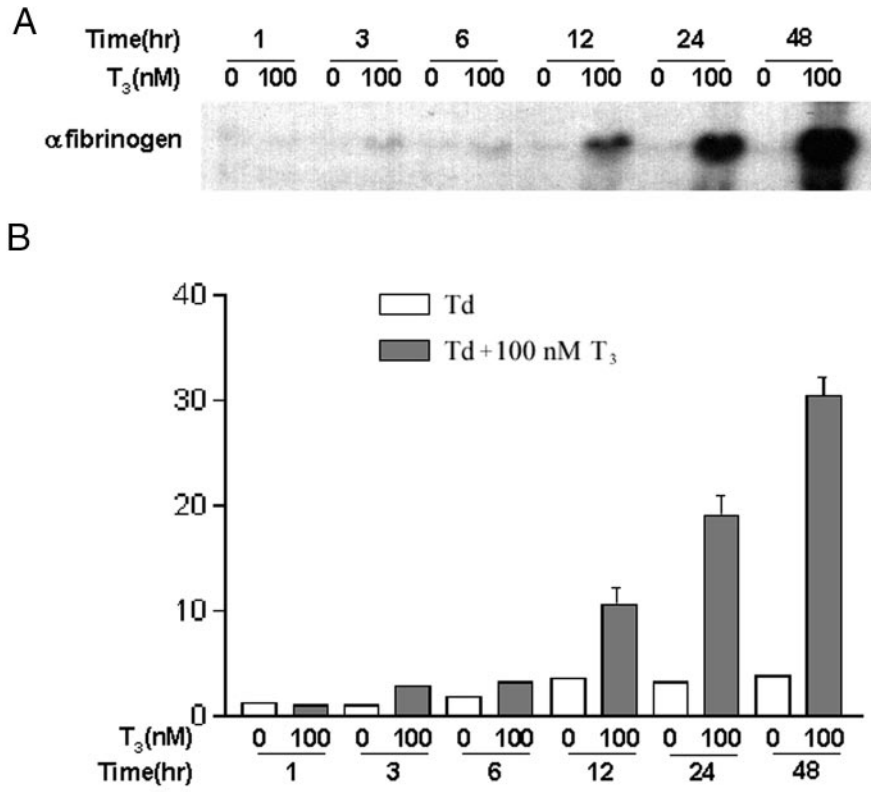

FIG. 3. Time-dependent induction of fibrinogen α chain by T_3. A, Expression of fibrinogen α chain in HepG2-TRα no. 1 was determined at 1, 3, 6, 12, 24, and 48 h in the absence (Td) or presence of 100 nM T_3 by Northern blot analysis. B, Quantitation of the result from A. Fibrinogen α chain was induced 2- to 3-fold by T_3 after 3 h treatment; and subsequently, the induction increased in a time-dependent manner.

T_3 increases fibrinogen mRNA levels by transcriptional stimulation

Most cellular effects of thyroid hormone are mediated via the augmentation of target gene transcription. Thus, we investigated the T_3-induced, steady-state level of *fibrinogen* mRNA via nuclear run-on assays of HepG2-TRα1 cells. The results are summarized in Fig. 4. In agreement with the results from Western and Northern blot analysis, the transcription of *fibrinogen* mRNA was increased by the addition of T_3. Specifically, a 2-fold induction of *fibrinogen* mRNA by T_3 was observed. β-Actin expression was used as an internal control, and pGEM-T vector was applied as a negative con-

trol. The data clearly demonstrated that activation of TR via the addition of T_3 can specifically increase the number of fibrinogen transcripts engaged in active synthesis by polII machinery, either through the enhancement of transcriptional initiation or reinitiation.

Effects of T_3 and CHX on the abundance of fibrinogen mRNA

In an effort to further elucidate the regulatory action of T_3 on the expression of *fibrinogen*, we investigated the effect of CHX, a protein synthesis inhibitor, on the induction of *fibrinogen* expression via T_3. Our results, over a 12- and 24-h period, demonstrate that the blocking of protein synthesis via CHX did not significantly affect the transcriptional response of *fibrinogen* to T_3 (Fig. 5). However, treatment of cells with 10 μg/ml CHX appears to decrease fibrinogen expression at 12 and 24 h. It may be due to a nonspecific cytopathic effect. The data suggest that activation of TRα1 by T_3 may regulate *fibrinogen* mRNA production directly, and the effect on transcription is not an indirect mechanism requiring the synthesis of other proteins to increase fibrinogen gene transcription. However, further study is required to elucidate the details involved in the direct regulation of *fibrinogen* expression by T_3.

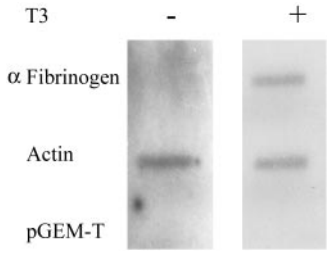

FIG. 4. Effect of T_3 on *fibrinogen* gene transcription rate, measured by nuclear run-on assay. After HepG2-TRα1 no. 1 cells were incubated with 10 nM T_3 for 3 h, total nascent RNA was labeled as described in *Materials and Methods* and probed against denaturized-linear plasmids containing the cDNAs indicated *at the left* of the figure. Actin and pGEM-T were used as internal controls.

T_3 induced fibrinogen and plasma protein expression in vivo

To determine the *in vivo* response of *fibrinogen* to T_3 treatment, two groups of male SD rats, at 6 wk of age, received surgical thyroidectomy (TX). One group (six individuals per group) of rats was injected with T_3 daily for 2 wk. The second set of rats was not treated with T_3 and formed the control group. The rats were killed at the end of the experiment, serum was collected for determination of T_3 and TSH levels, and livers were removed for Western blot analysis. T_3 levels in the serum from the control group after TX were about 0.4-fold in comparison to the group receiving T_3 (54 *vs.* 136 ng/dl). TSH levels of the TX control group were about 70-fold greater than those of the T_3-treated group (1.71 *vs.* 0.024 mIU/ml). The clotting time, from both the TX and TX+T_3 groups, was also measured using the activated partial thromboplastin time method, as described by Gottfried *et al.* (24). The activated partial thromboplastin time results were 34.3 and 16.7 sec in the TX and TX+T_3 groups, respectively. The prolonged time of coagulation observed in the TX group indicates a lower level of fibrinogen. Western blots also demonstrated that fibrinogen protein levels were augmented in the TX rats after addition of T_3, in comparison with the control group of rats. The rat Aα chain (~60 kDa) essentially

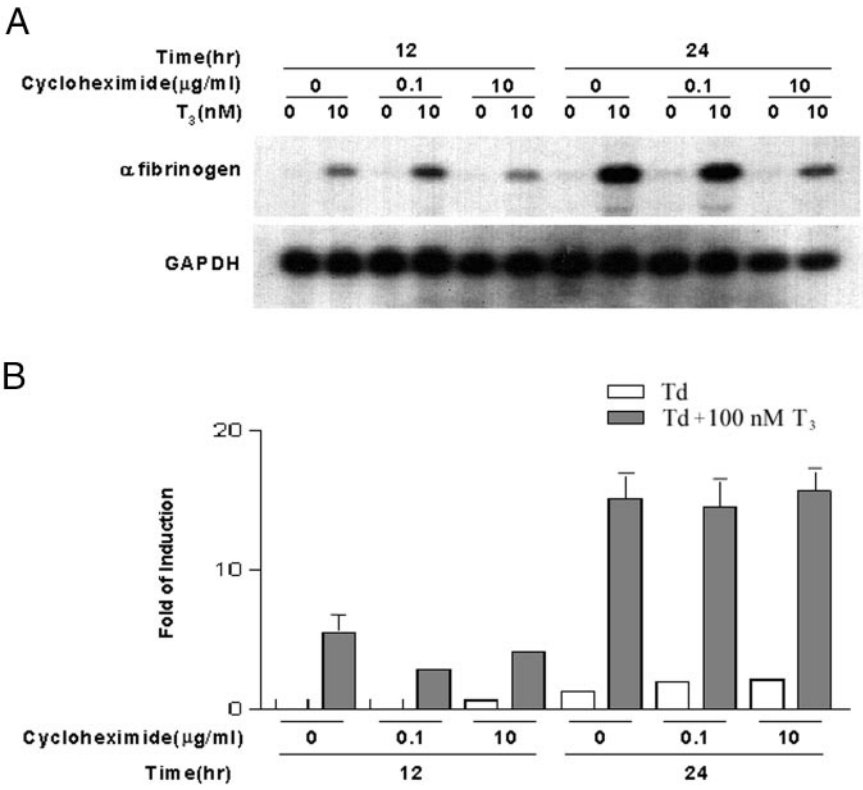

FIG. 5. CHX did not ablate the response of *fibrinogen* to T_3 activation. A, HepG2-TRα1 no. 1 cells were treated as described in Fig. 3 with or without 0.1 or 10 μg/ml CHX. After T_3 activation for various lengths of time, total RNA was isolated and subjected (20 μg per lane) to Northern blot analysis. B, The intensities of the *fibrinogen* and GAPDH mRNA bands were quantified, and the increase in abundance of *fibrinogen* transcripts was determined at each time point. These results are displayed as fold induction compared with those in control (Td) conditions. These results were derived from three independent experiments.

comigrates with the rat Bβ chain (Fig. 6A). On a reduced and denatured gel, rat fibrinogen has only two chains. The rat Bβ and γ chains comigrate with the human Bβ and γ chains in the SDS-PAGE (25–27). The level of fibrinogen proteins α, β level in the TX+T_3 group was about 3-fold higher than that in the control TX group (Fig. 6). These results further validate the *in vitro* cDNA microarray, Q-RT-PCR, and Northern and Western blot analyses that elucidate the regulation of fibrinogen expression at the mRNA and protein level by thyroid hormone. Moreover, the plasma proteins induced by T_3 in HepG2-TRα1 cells, as listed in Table 2, were also stimulated by T_3 in the livers of the TX rats (Table 3). This correlation further validates the use of the HepG2 cell line as a suitable model system in which to study the cell type-specific and TR isoform-specific regulation of the T_3 target genes in liver.

Discussion

To study the target genes regulated by T_3 in a TRα1-overexpressing hepatoma cell line, we performed c-DNA microarrays. Two percent of the genes represented on the array were up-regulated by T_3. Among the remaining T_3-induced genes are several components of coagulation- or inflammation-related factors, including fibrinogen, thrombin, coagulation factor X, tissue plasminogen activator, CD40, complement, and heparin cofactor II. In a previous investigation of livers taken from T_3-treated mice, plasma protein regulation genes were not identified using cDNA microarrays (28–31). It is possible that endogenous TR expression in the liver is too low for an effect to be observed in mice. On the other hand, TR isoform-specific regulation was observed in the knockout mouse model (32, 33). In addition, the T_3-regulated genes in liver, such as spot 14 (34) and malic enzyme (35), were not identified during our screen. Spot 14 was not detected in this investigation, because it was not represented on the gene-chip, and previous RT-PCR results (data not shown) indicate that malic enzyme is not expressed in the HepG2 cell line. Our data reveal that several plasma proteins, including prothrombin, angiotensinogen, haptoglobin, complement, lipoproteins, and fibrinogen, are up-regulated by T_3 at least 2-fold in a hepatoma cell line that highly expresses TRα1 as well as in the TX rats. Our array indicates that the other plasma proteins, such as plasminogen, α-2-macroglobulin, and α-fetoprotein, were down-regulated by T_3 (Table 2, and data not shown). Caturla *et al.* (36) reported that T_3 down-regulates the level of α-fetoprotein mRNA in the hepatoma cell line, HepG2. Their data suggest that T_3 controls α-fetoprotein gene expression, especially during the neonatal shut-off of the gene. Further investigation of the regulation of hepatic plasma proteins by T_3 is required to continue elucidating this important, but so far relatively unappreciated, mechanism.

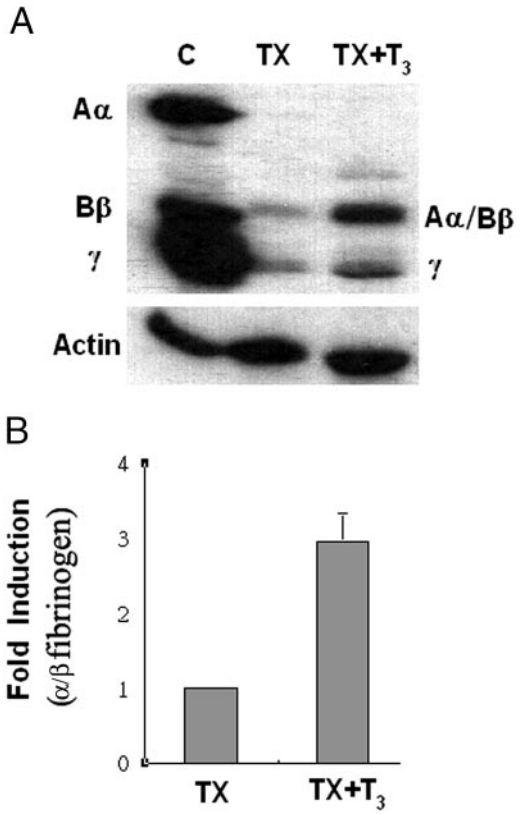

FIG. 6. Induction of fibrinogen expression by thyroid hormone in rat liver. A, Expression of fibrinogen in TX or TX+T_3 male SD rat liver was determined by Western blot analysis as described in *Materials and Methods*. Data are means ± SE of values from two independent experiments, three rats per group. C, Fibrinogen from human HepG2 no. 1 cells. The rat Aα chain (~60 kDa) essentially comigrates with the rat Bβ chain. B, The intensities of the Aα fibrinogen and actin bands on the blots were quantified. These results are displayed as fold induction compared with those in control (TX).

TABLE 3. Expression profile of coagulation genes in thyroidectomized rats

Genes	Fold induction by T_3
Fibrinogen, A α polypeptide	2.18 ± 0.62
Coagulation factor II (thrombin)	3.34 ± 0.06
Coagulation factor X	11.31 ± 0.81
Haptoglobin	1.74 ± 0.25
Natural killer cells protein 4	2.17 ± 0.88
CD40	2.11 ± 0.65
Complement component 1, s subcomponent	2.46 ± 0.32
Complement component 4A	1.64 ± 0.08
Plasminogen	0.66 ± 0.02

SD rats received surgical thyroidectomy. Two weeks after surgery, T_3 (10 μg/100 g body weight) was applied to the rats daily for additional 2 wk. RNA was isolated from liver, and Q-RT-PCR was performed as described in *Materials and Methods*.

To verify the results of the microarray experiments, nine genes (Table 2) were selected for Q-RT-PCR analysis at two time points (12 and 24 h). The increase in expression evident in the selected genes from the microarray was validated via Q-RT-PCR. Interestingly, all genes selected for further confirmation had no previously observed association with T_3 regulation. Although these genes were isolated from a human hepatoma cell line, they were up- or down-regulated in a similar manner in the TX rats. Several genes recently identified to be regulated by TRs, such as Na+/H+ exchanger (37), phosphoenolpyruvate carboxykinase (38), and apolipoprotein CI (39), were also observed in our array.

Fibrinogen is a circulating glycoprotein mainly secreted by hepatocytes. It is comprised of two symmetric half molecules, each containing three (Aα, Bβ, and γ) polypeptide chains. Interestingly, not all of the fibrinogen chains that are

synthesized are assembled into fibrinogen, and the remaining unassembled chains are not secreted. HepG2 cells contain surplus Aα and γ chains that accumulate as free γ chains and as an Aα-γ complex. The nonsecreted fibrinogen chains are degraded both by proteasomes and lysosomes. Therefore, the basal level of the three component chains of fibrinogen is not equal within the cell (40, 41). Using microarray assays, we demonstrate here the up-regulation of the Aα subunit in response to T_3 treatment. Temporal change in fibrinogen Aα with T_3 treatment was confirmed by Q-RT-PCR. However, the reason for the up-regulation of the Aα chain by T_3 to a greater extent than the γ chain is currently unknown. Furthermore, we investigated the modulation of expression of all three subunits of fibrinogen via Western blot analysis. The three related polypeptides were up-regulated by T_3 to a differing extent, with Aα being the strongest. Therefore, we concentrated our efforts on this subunit. Hertzberg *et al.* (42) have previously reported that addition of physiological concentrations (10 nM) of T_3 or T_4 to primary hepatocyte cultures produced 3-fold or greater increases in the rates of synthesis of fibrinogen. However, no detailed characterizations, such as Western or Northern blot analysis, were carried out. In addition, Miller *et al.* (43) used the GC cell line (a rat pituitary cell line expressing functional TRs) to investigate the transcriptional program underlying T_3-induced cell proliferation by cDNA microarrays. In this study, fibrinogen Aα expression was found to be up-regulated 4-fold by T_3. Alternatively, expression of other coagulation factors was not affected in this nonhepatocyte cell line. Niessen *et al.* (44) reported that thyroid hormone significantly increased the amounts of the coagulation proteins, factor II (1.28-fold), factor X (1.45-fold), and fibrinogen (2.17-fold). The plasma concentration ($P < 0.01$) of fibronectin, angiotensin-converting enzyme, and factor VIII-related antigen have been demonstrated to be significantly increased in hyperthyroid patients (45). A number of these factors were also observed to be similarly regulated in our experiments. The results published previously on fibrinogen are wholly consistent with ours, demonstrating that fibrinogen expression, particularly that of the Aα subunit, is increased with the addition of T_3 *in vitro* and *in vivo*.

The results we report here, particularly concerning the up-regulation of blood clotting factors with treatment of T_3, raise an interesting question. What are the potential physiological consequences, in response to increased T_3 (hyperthyroidism) or lowered T_3 (hypothyroidism) levels, on the coagulation of blood? Burggraaf *et al.* (46) have reported that excess T_3 was associated with elevated levels of plasma fibronectin and fibrinogen, whereas plasminogen was decreased. This finding is similar to our Q-RT-PCR results (Table 2). Moreover, the level of tissue plasminogen activator in hyperthyroidism was reduced, compared with control patients (47). It has also been reported that hyperthyroid patients may experience vascular endothelial dysfunction and decreased fibrinolytic activity in blood. This may explain the association between hyperthyroidism and thromboembolism (47). Marongiu *et al.* (48) reported that significantly increased plasma levels of fibrinogen and, in particular, Bβ 15–42, a specific product of fibrinogen metabolism induced by plasmin, were observed in hyperthyroid patients. The restoration of euthyroidism either by antithyroid drug treatment or by radioiodine caused a significant decrease of fibrinogen and B β specific product. Fibrinopeptide A and Bβ 15–42 are *in vivo* indicators of thrombin and plasmin activity. Furthermore, fibrinogen, fibrinopeptide A, and Bβ 15–42 were higher in patients with hyperthyroidism (Graves' disease) than in normal controls. After treatment, fibrinogen returned to normal levels (49). Moreover, an elevated plasma fibrinogen level has been identified as a risk factor for ischemic heart disease, because it indicates that the inflammatory profile has been altered (50). These data further indicate that modulation of T_3 levels (hyper- and hypothyroidism) are clinical conditions associated with an increased or decreased concentration of fibrinogen and a number of blood clotting factors.

The activation of the blood coagulation cascade usually induces other acute phase responses such as inflammation. We have shown here that T_3 also up-regulates several other inflammatory-related plasma proteins, such as haptoglobin, orosomucoid, and interleukin (Table 1). In addition, previous reports have found that plasma factor VIII levels are elevated in hyperthyroidism (51). Furthermore, significantly increased plasma concentrations ($P < 0.01$) of such proteins as fibronectin, angiotensin-converting enzyme, and factor VIII-related antigen were found in hyperthyroid patients (45). Interestingly, patients with moderate hypothyroidism, who were consistently shown to be at high risk for cardiovascular disease, have decreased fibrinolytic activity (52). In summary, patients with hyperthyroidism have the tendency to generate arterial thromboembolism (53). Alternatively, the blood of patients with hypothyroidism has been demonstrated to lack full coagulation ability. This suggests that modulation of the levels of T_3 is extremely important for the capability to control blood clot formation.

Apart from T_3, fibrinogen is also regulated by other factors. The nuclear receptor peroxisome proliferator-activated receptor α is involved in repression of the human fibrinogen-β gene (54). In addition, IL-6 stimulates expression of the human fibrinogen-β subunit in human primary hepatocytes and hepatoma HepG2 cells (54). Engström *et al.* (55) reported that hypercholesterolemia is associated with high plasma levels of five inflammation-sensitive plasma proteins (fibrinogen, α1-antitrypsin, haptoglobin, ceruloplasmin, and orosomucoid). These proteins seem to be involved in the cholesterol-related incidence of cardiovascular diseases. Moreover, insulin had an overall stimulating effect on the amounts of fibrinogen present in blood.

In summary, the use of DNA microarray technology allowed us to determine the downstream target genes of TRα1-dependent, T_3-regulated expression. The data presented here give greater insight into the action of TRα1 in hepatoma cell lines. Of greatest importance is the elucidation of the T_3 control of numerous coagulation and inflammation-related genes. Although these genes were isolated from a human tumor cell line, they were regulated similarly in rats. This may help to explain the association between thyroid diseases (hyper- and hypothyroidism) and the misregulation of the inflammatory and clotting profile. Further study is required to investigate the tumor-specific T_3 target genes.

Acknowledgments

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