Regulation of fibronectin by thyroid hormone receptors

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Abstract

Thyroid hormones regulate growth, development, differentiation, and metabolic processes by interacting with and activating thyroid hormone receptors and associated pathways. We investigated the triiodothyronine (T₃) modulation of gene expression, in human hepatocellular carcinoma cell lines, via a PCR-based cDNA subtraction method. Here we present further data on one of the T₃-upregulated genes, fibronectin (FN). We demonstrate that the induction of FN protein expression by T₃ in TRα₁ and TRβ₁ over-expressing cells was time and dose-dependent at the mRNA and protein levels. Blockade of protein synthesis by cycloheximide almost completely inhibited the concomitant induction of FN mRNA by T₃, indicating that T₃ indirectly regulates FN. Furthermore, nuclear-run on and FN promoter assay clearly demonstrated that the presence of T₃ can specifically increase the number of FN transcriptional initiations. In addition, we further confirmed that the up-regulation of FN by T₃ was mediated, at least in part, by transforming growth factor-β (TGF-β), because the induction of FN was blocked in a dose-dependent manner by the addition of TGF-β neutralizing antibody. In an effort to elucidate the signaling pathways involved in the activation of FN by T₃, we demonstrated the involvement of the mitogen activated protein kinase/c-Jun N-terminal kinase/p38 MAPK (MAPK/JNK/p38) pathway. Although T₃ induces the expression of TGF-β, neither wild-type nor dominant-negative Smad3 or Smad4 over-expression affected the activation of FN by T₃. Thus, we demonstrate that T₃ regulates FN gene expression indirectly at the transcriptional level, with the participation of the MAPK/JNK/p38 pathway and the TGF-β signaling pathway but independent of Smad3/4.

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Introduction

The subtractive cDNA hybridization method has been used to elucidate the regulation of molecular processes during hormone treatment, cell differentiation, embryonic development, drug application and malignant transformation. This technique is a powerful approach for the identification, cloning and detailed study of relevant subsets of differentially expressed genes of interest. Recently, an improvement to this technique has been used to selectively amplify differentially expressed target cDNA fragments and simultaneously suppress the amplification of non-target DNA (Kuang et al. 1998). This new procedure overcomes previous difficulties associated with differences in mRNA abundance by incorporating a hybridization step to normalize the transcripts. We utilized the PCR-based cDNA subtraction method to examine the triiodothyronine (T₃) regulation of liver protein levels. Among these are genes including fibronectin (FN), fibrinogen, elongation factor, heat shock protein, protein disulfide isomerase, glucocorticoid receptor AF-1 specific elongation factor, and ribosomal protein L37 involved in metabolism or signal transduction. Several groups (Shirakami et al. 1986, Watzke et al. 1987) reported that thyroid hormones (THs) positively regulate plasma levels of FN by unknown mechanisms. In addition, Baumgartner-Parzer et al. (1997) also showed that hyperthyroidism is associated with elevated plasma levels of FN. Thus, we focused our study on the
mechanism of how FN was regulated by THs. This initial investigation, in hepatocellular carcinoma cell lines, demonstrated that FN was up-regulated by T₃.

THs regulate growth, development, differentiation, and metabolic processes by interacting with TH receptors (TRs) that, in turn, 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 mapped to human chromosomes 17 and 3 respectively (Lazar et al. 1994). Each gene encodes at least two TR isoforms (TRα1, TRα2, and TRβ1, TRβ2) that are generated as a result of alternate splicing and/or promoter choice (Wood et al. 1996). The mechanisms involved in the maintenance of liver-specific gene transcription by TRα1 and TRβ1 have not yet been fully elucidated. HepG2, a well-differentiated hepatocellular carcinoma cell-line, has been well-characterized and has been reported to secrete all 15 known liver-specific plasma proteins. Thus, we used HepG2 to derive isogenic cell lines stably expressing high levels of wild-type TRα1 and β1 (HepG2-TRα1 and -TRβ1 cells) (Lin et al. 2000). These lines have proved to be a useful tool when investigating the target genes of THs.

FN mediates a wide variety of key interactions between cells and the extra-cellular matrix (ECM) and plays a significant role in cell adhesion, migration, growth, differentiation and the maintenance of normal cell morphology (Hynes 1985a,b, Pankov & Yamada 2002). FN is an important glycoprotein that usually occurs as a dimer composed of two, nearly identical, ~250 kDa subunits covalently linked near their C termini by a pair of disulfide bonds (Pankov & Yamada 2002). Although FN exists as a single gene, alternative splicing of a single pre-mRNA can generate as many as 20 variants in the human (Islami et al. 2001, Srebrow et al. 2002). These alternative splice products may contain at least three regions (ED-A, ED-B, and IIICS) of the primary transcript (Borsi et al. 1990). Interestingly, it has been reported that transforming growth factor-β (TGF-β) preferentially increases the accumulation of the FN isoforms containing the ED-A sequence in cultured normal human fibroblasts (Balza et al. 1988). FN isoforms can be classified as either soluble plasma FN or as the less-soluble cellular FN (Sekiguchi et al. 1986).

Plasma FN is synthesized predominantly in the liver by hepatocytes and displays a relatively simple splicing pattern. On the other hand, cellular FN consists of a much larger and more heterogeneous group of FN isoforms, resulting from cell-type and species-specific splicing patterns.

The control of FN expression by T₃ and the specific isoforms of TR has not been investigated in a cellular context. We report here that T₃ up-regulates FN expression at the transcriptional and translational level in HepG2-TRα1 and -TRβ1 cells. Furthermore, the effect of T₃ on the level of FN expression requires the de novo synthesis of cellular proteins. In addition, we found that the up-regulation of FN by T₃ is mediated, at least in part, by the TGF-β and/or MAP kinase (MAPK) signaling pathways. The MAP family is also the TGF-β downstream signaling mediator (Hocevar et al. 1999). Finally, we demonstrate that T₃ does not utilize the TGF-β signaling components, Smad3/4 in the control of FN expression.

Materials and methods

Cell culture

The human hepatoma cell line, HepG2, was obtained from the American Type Culture Collection (Manassas, VA, USA) and was routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. TRα1 and β1 over-expressing cell lines have been described previously (Lin et al. 2000). In this study, two HepG2-TRα1- (#1 and #2) and one TRβ1-over-expressing clones were used. The serum was depleted of T₃ (Td) as described (Samuels et al. 1979). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Subtractive hybridization

Total RNA from HepG2-TRα1#1, with or without T₃ treatment, was prepared using TRIzol (Life Technologies, Rockville, MD, USA). The PCR-based cDNA subtraction method was carried out according to the manufacturer’s protocol (Clontech, Palo Alto, CA, USA). The subtracted cDNAs were ligated into the pGEM-T vector. Subsequently, the clones were screened for differentially expressed genes using the PCR-Select Differential Screening kit (Clontech).
**Immunoblot analysis**

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA). 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 FN (1:1000 dilution in TBS) (Transduction, Lexington, KY, USA), or with mouse monoclonal antibody C4 to TRα1 (1:1000 dilution in TBS) (kindly provided by S-Y Cheng, NCI, NIH, Bethesda, MD, USA) (Bhat et al. 1995). After further washing, the membrane was incubated for 1 h with horseradish peroxidase conjugated to affinity-purified antibodies to either rabbit (1:1000 dilution in TBS) or mouse (1:1000 dilution in TBS) immunoglobulin (Santa Cruz Biotechnology). Detergent-solubilized cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane and probed with antibodies to FN (1:1000 dilution in TBS) (Transduction, Lexington, KY, USA), or with mouse monoclonal antibody C4 to TRα1 (1:1000 dilution in TBS) (kindly provided by S-Y Cheng, NCI, NIH, Bethesda, MD, USA) (Bhat et al. 1995). After further washing, the membrane was incubated for 1 h with horseradish peroxidase conjugated to affinity-purified antibodies to either rabbit (1:1000 dilution in TBS) or mouse (1:1000 dilution in TBS) immunoglobulin (Santa Cruz Biotechnology). Immune complexes were then visualized by chemiluminescence with an ECL detection kit (Amersham). The intensities of the 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-activity of TRs was assayed in the various HepG2 cell lines as described previously (Kyriakis & Avruch 1996). Briefly, cells were transfected with a luciferase reporter plasmid (2 µg) containing the Lys-TRE or FN 508 promoter (2 µg, a gift from Kinichiro Oda, Science University of Tokyo, Noda, Japan) (Suzuki et al. 1998), along with a β-galactosidase plasmid (1 µg) to control for transfection efficiency or TR expression vector. 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. Equal amounts of total RNA (20 µg) were analyzed on a 1.2% agarose-formaldehyde gel as described (Lin et al. 2002). This was then blotted onto a nitrocellulose membrane and subjected to Northern blot analysis as described (Lin et al. 2000). A full-length FN cDNA fragment was amplified, labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham) by the PCR 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 10 µg/ml cycloheximide (Sigma) simultaneously for 12 or 24 h, followed by total RNA isolation and Northern analysis.

**Nuclear run-on assay**

Sub-confluent HepG2-TRα1#1 cells were treated with or without 100 nM T3 for 3 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 resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40, and allowed to swell and lyse on ice for 10 min. The lysate was recentrifuged at 500 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 was performed 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 [α-32P]UTP as described previously (Liao et al. 1995). The reaction was incubated in a shaking water bath at 30 °C for 30 min. Equal amounts (2 µg) of purified, denatured full-length FN, 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 radio-labeled transcripts (~107 cpm) were resuspended in 2 ml hybridization buffer containing 50% formamide, 5 × SSC, 2.5 × Denhardt’s solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 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 × SSC and 0.1% SDS for
Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

To determine the expression of TGF-β, Q-RT-PCR was carried out. 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). 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), and various amounts of template. Fluorescence emitted by SYBR Green was detected on line by the ABI PRISM 7000 sequence detection system (Applied Biosystems). All PCRs were carried out in duplicate on the same 96-well plate. For quantification of gene expression changes, the ΔCt 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). The Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background.

TGF-β protein assay

To determine the effect of T₃ on the expression of TGF-β protein, TGF-β1 Emax ImmunoAssay System (Promega Corporation, Madison, WI, USA) was used. Briefly, HepG2-TRα1#1 cells were treated with or without 10 to 100 nM T₃ for various times. The supernatant was collected for TGF-β determination according to the manufacturer’s instructions.

Determination of the effect of exogenous Smad on FN expression

To determine the exogenous effect of Smad on the expression of FN, we transfected HepG2-TRα1#1 cells (1~2×10⁵ per 60-mm dish) with wild-type or dominant-negative Smad plasmid using Lipofectamine (Gibco BRL). Twenty-four hours after transfection, the cells were lysed and Western blot analysis performed. Smad plasmids were a gift from Dr P T Dijke (Ludwig Institute, Uppsala, Sweden).

Results

Expression and trans-activation activity of TRα1 and TRβ1 in HepG2 cell lines

We used a PCR-based cDNA subtraction method to identify the induction of genes by T₃ in HepG2 cells. FN was selected because the control mechanism of its expression by T₃ and TR has not been studied. To further investigate the molecular regulation by T₃ of the FN gene, we used isogenic HepG2 cell lines that stably express wild-type TRα1 (HepG2-TRα1 clones #1, #2) and TRβ1 (HepG2-TRβ1). As a control, HepG2 cells were transfected with the empty vector, yielding a cell line that expresses the Neo protein (HepG2-Neo cells). Prominent immunoreactive bands, corresponding to TRα1 or TRβ1 were detected in HepG2-TRα1#1, #2, and HepG2-TRβ1 using the monoclonal antibody C4 (Fig. 1). This antibody recognizes an epitope at the COOH-terminus of TRα1 and TRβ1 (Bhat et al. 1995). Quantitation of the immunoreactive bands revealed that the abundance of TRα1 protein in HepG2-TRα1 cells was four- to sevenfold that in HepG2-Neo cells. Endogenous levels of TR in HepG2-Neo cells were only faintly observed after longer exposure times.

A Lys-luciferase reporter construct (containing the thyroid hormone response element (TRE) of the chicken lysozyme gene, a 6 nt inverted repeat), was used to compare the transcriptional activity of TRα1 in HepG2-TRα1#1 and #2 and of TRβ1 in HepG2-TRβ1 with that of the HepG2-Neo cells (Fig. 1B). HepG2-TRα1#1 and HepG2-TRβ1 cells exhibited higher trans-activity (five- to sevenfold), which increased in a T₃-dependent manner. TRα1#2 displayed moderate trans-activation of the reporter construct (three-to fourfold), while the control HepG2-Neo cells exhibited a low level of trans-activity (Fig. 1B). These results indicate that the isogenic cell lines over-express TRα1 or TRβ1 and the level of expression correlates well with their functional capacity to trans-activate the expression of downstream genes.

Effects of T₃ on the abundance of FN protein and mRNA in HepG2-TRα1 and TRβ1 cell lines

Next, we were interested in the effect of the TRs on the level of FN protein expression when the HepG2 isogenic cell lines were incubated in media containing various levels of T₃ across different time...
points (Fig. 2). T3 significantly increased the abundance of FN in the HepG2-TRα1#1, #2 and TRβ1 stable cell lines as compared with the HepG2-Neo control cell line. FN levels increased approximately 1.5- to 2.5-fold after incubation of HepG2-TRα1#1, #2, and TRβ1 cells with 10 nM T3 for 24 h. In addition, 100 nM T3 for 24 h gave a slightly greater and more significant induction (2.2- to 2.8-fold) of FN protein. Moreover, after 48 h incubation in 100 nM T3, FN activation was further elevated (≈2.5- to 4.2-fold). These results indicate that the effect of T3 on the level of FN in TRα1 and β1 over-expressing cells was time- and dose-dependent. In addition, immunoblot analysis revealed that exposure of control HepG2-Neo cells, expressing endogenous levels of TR proteins, to 100 nM T3, resulted in little effect on the expression of FN protein (Fig. 2). Thus, the extent of FN protein induction by 100 nM T3 correlated with the level of TR protein expression.

Northern blot analysis was utilized to examine the response of FN mRNA expression to the exogenous addition of T3. In all three cell lines investigated, a 9 kb FN transcript was detected (Fig. 3A). Exposure to 100 nM T3 for 24 h resulted in a significant induction in the expression of FN mRNA in HepG2-TRα1#1, #2 and TRβ1 cells, with increases of 2.8-, 2.6- and 2.5-fold respectively. This suggests that the augmented expression of FN mRNA in response to T3 corresponds to the amount of TR in the individual line. The highest levels of T3 (100 nM) used in this experiment did not significantly increase the level of FN expression above that already displayed in the 10 nM T3 experiment (Fig. 3B). These results indicate that FN gene expression may be very sensitive to the presence of small amounts of T3 in the medium. Applying 10 nM T3 for 24 h or 48 h, to the HepG2-TRα1 cell line, increased FN levels 1.5- and 2.0-fold respectively. However, when the cells were exposed to 100 nM T3 for 24 h or 48 h FN mRNA expression increased 2.5- and 3.5-fold respectively. This indicates that the action of T3 in TRα1 is different from that in TRβ1. FN mRNA levels were less sensitive to the presence of T3 in the TRβ1 cell line compared with the TRα1 and TRβ1 stable cell lines. Furthermore, T3 had little effect on the abundance of FN mRNA in HepG2-Neo cells (Fig. 3). Thus, the effect of T3 on the expression of FN protein appears to be mediated, at least in part, at the mRNA level.

Effects of T3 and cycloheximide (CHX) on the abundance of FN mRNA

In an effort to further delineate the regulatory action of T3 on the expression of FN mRNA, a protein synthesis inhibitor, CHX, was employed. Induction of FN mRNA expression by T3, in the presence or absence of CHX, was observed for
both time periods in HepG2-TRα1#1 cells. The transcriptional response of FN mRNA to T₃ over 12- and 24-h periods was greatly reduced in the presence of CHX (Fig. 4). Similar results were observed in the other two TR over-expressing cell lines (data not shown). These data indicate that blocking protein synthesis almost completely inhibits the induction of FN transcription by T₃. It follows from this result that de novo protein synthesis may be required for this activation to occur.

**T₃ induces the expression of FN at the transcriptional level**

To further confirm that regulation of FN expression by T₃ occurred at the transcriptional level we performed nuclear run-on experiments. T₃ induced an approximate 2.5-fold increase in FN mRNA transcription (Fig. 5), as observed in the Western and Northern blots. 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 T₃ can specifically increase the number of FN transcripts in HepG2-TRα1#1 cells.

To further support this conclusion, we carried out reporter assays. We used the FN 5’-flanking region encompassing nucleotides −508/+18 (Suzuki et al. 1998) and then placed it upstream of the luciferase reporter gene in pGL2. Using this reporter, we determined the effect of trans-activation of FN by T₃. As shown in Fig. 6, at
100 nM T₃, FN promoter had the highest activity (approximately 2.2-fold activation). Therefore, the FN promoter was sensitive to T₃ treatment.

**Effects of T₃, TGF-β and MAP kinase on the abundance of FN protein**

In an effort to elucidate the signaling pathways involved in the activation of FN by T₃, we demonstrated the involvement of the TGF-β and/or MAPK/c-Jun N-terminal kinase/p38 MAP kinase (MAPK/JNK/p38) pathway. Our data indicate that treatment of cells with T₃ for various times induces TGF-β expression from 1.7- to 3.3-fold at the mRNA level (Table 1). Similarly, treatment of cells with 10 to 100 nM T₃ for various times induces TGF-β expression from 1.5- to 3.8-fold at the protein level (Table 2). This is consistent with the report by Miller et al. (2001). We further confirmed that the induction of FN by T₃ was mediated by TGF-β (Fig. 7). Figure 7 indicates that the induction of FN by T₃ was blocked in a dose-dependent manner by the addition of TGF-β neutralizing antibody but not by the control antibody. Furthermore, it has been demonstrated that TGF-β signaling is mediated by members of the MAPK family in the induction of FN expression (Hocevar et al. 1999). To determine if the role of T₃ or recombinant TGF-β on the induction of FN in HepG2-TRα1#1 cell lines is
mediated by MAPKs, we investigated the effect of several MAPK inhibitors. PD98059 is a selective inhibitor of MAPK kinase (MEK) 1/2 that blocks extracellular signal-regulated kinase (ERK) activation, whereas SB203580 inhibits both JNK and p38 MAPK, and SB202190 is a specific inhibitor of p38 MAPK. These inhibitors were applied individually to HepG2-TRα1#1 cells 3 h before the addition of 100 nM T3. After incubation for an additional 24 h, the induction of FN protein in the absence of inhibitors was ~threefold, as seen previously (Fig. 2). FN was also stimulated ~threefold by TGF-β. However, the activation of FN by T3 or TGF-β was completely abolished after the addition of 15 µM SB203580 or SB202190 (Fig. 8). In contrast, this inhibition was not observed with the application of the inhibitor PD98059 (10 µM). These data indicate that activation of FN by T3 or TGF-β is mediated, at least in part, by the JNK or p38, but not the MEK/ERK pathway.

**Stimulation of FN by T3 is not mediated by the Smad 3/4-dependent pathway**

The MAPK family has been implicated in the events downstream of TGF-β signaling (Marais & Marshall 1996). TGF-β signaling is mediated by the phosphorylation and activation of either Smad2 or Smad3, which allows the formation of a heterodimeric complex with Smad4. This complex subsequently translocates into the nucleus, where it acts on the promoters of target genes (Itoh et al. 2000). Therefore, we investigated the possible role of Smad3/4 in the JNK/p38-mediated activation of FN protein by T3. Both wild-type and dominant-negative forms of Smad3 and Smad4 were...
transiently over-expressed in HepG2-TRα1#1 cells to observe their effect on T₃-mediated FN activation. Interestingly, neither the over-expression of wild-type nor dominant-negative Smad3 or Smad4 affected the activation of FN by T₃ (Fig. 9). This indicates that these components of the TGF-β signaling pathway are not involved in the T₃-mediated transcriptional activation of FN.

Discussion

We reported here the isolation of T₃-responsive genes using a PCR-based cDNA subtraction method in HepG2 cell lines over-expressing TRα1 protein. One gene (FN, the extra-cellular matrix component) that was significantly induced by T₃ was targeted because several groups (Shirakami et al. 1986, Watzke et al. 1987, Baumgartner-Parzer et al. 1997) have reported a significant positive correlation between plasma concentrations of FN and total THs. However, no detail of the regulation mechanism has been reported. HepG2, a well-differentiated hepatocellular carcinoma cell-line, secretes all 15 plasma proteins (Chang et al. 1983) and preserves many liver-specific functions. Thus, the HepG2 cell line serves as a suitable model system to study the cell type-specific and TR isoform-specific regulation of T₃-target genes in the liver (Lin et al. 1997). The liver is the major target organ of THs (Cheng 2000) and is the main site of FN secretion (Pankov & Yamada 2002). However, the mechanisms of how TRs selectively maintain liver-specific gene transcription have not yet been elucidated. Furthermore, the use of cells over-expressing TRs enabled us to investigate the regulation of plasma protein levels in response to variations in the levels and isoforms of the TR receptors. Therefore, in this study we investigated the molecular regulation of FN by T₃ in isogenic HepG2 cell lines.
Although this study investigated the induction of FN by T₃ in human hepatoma cell lines, similar results have also been observed in animal and primary cell culture models. Murata et al. (1990) showed that FN mRNA was decreased by half in thyroidectomized rats, while administration of physiological doses of thyroxine or T₃ for 5–6 days restored FN mRNA to control levels. Moreover, administration of pharmacological doses of thyroid hormones induced a further increase in the abundance of FN mRNA. Subsequently, Lee et al. (1992) demonstrated that TH promotes FN gene expression in primary cultured rat hepatocytes, yet inhibits FN synthesis in cultured human skin fibroblasts. Furthermore, in hepatocytes, these authors demonstrated that TH directly enhanced FN gene expression without requiring de novo protein synthesis. In contrast, we demonstrate the need for de novo protein synthesis for the induction of FN by T₃ in HepG2 cell lines. The discrepancy between these results may be due, in part, to the previous authors using dot blot hybridization analysis with monoclonal antibodies to FN. Tubulin was used as an internal control. (B) The intensities of bands in (A) were quantified and the extent of T₃-induced activation of FN expression was determined in each lane. Data are means ± S.E of values from three independent experiments. Values are shown as fold induction of the T₃-control. *P < 0.05, **P < 0.01, T₃-compared with TGF-β Ab-treated (Student's t-test).
further as the previous authors did not present their dot blot data, although it would be very difficult to determine the effect of CHX on FN expression as their data indicate that T3 did not induce FN mRNA expression (see Fig. 4 of their paper). Our data clearly show that FN mRNA induction by T3 is directed, de novo protein synthesis required. Additionally, studies in humans and animal models further support our findings. Watzke et al. (1987) studied the plasma concentration of FN in 13 untreated thyroid carcinoma patients after total thyroidectomy. FN levels were found to be significantly decreased compared with those of healthy volunteers. Following oral administration of L-thyroxine for 6 weeks, the same patients showed significantly increased levels of FN compared with untreated patients. In support of this data, fibronectin expression in hypothyroid animals is reduced to about 40% in the midbrain compared with untreated animals (Calloni et al. 2001). Furthermore, in vivo animal studies demonstrated that THs promote the expression of the FN gene in the rat liver (Lee et al. 1992).

In this report we examined the potential role played by TGF-β in the enhanced expression of FN by T3. This pathway was investigated, as there is evidence of a close relationship between T3 and TGF-β. A previous report (Miller et al. 2001) and ours have demonstrated that treatment with T3 for 12 h or more induces TGF-β expression. Furthermore, an increased TGF-β plasma concentration was reported to be associated with high plasma T3 levels in elderly patients with non-thyroidal illnesses (Corica et al. 1998). In addition, TGF-β signaling...
has been reported to be mediated by members of the MAPK family (Hocevar et al. 1999). The MAPK family incorporates the ERK pathway (Marais & Marshall 1996) and two stress activated pathways, the JNK and p38 pathways (Kyriakis & Avruch 1996, Woodgett et al. 1996). Consistent with our results, the JNK pathway is stimulated rapidly by TGF-β in a human fibrosarcoma cell line, and this activity is essential for TGF-β-mediated FN induction (Hocevar et al. 1999). Similar to our results, these authors demonstrated that Smad4 is not required for TGF-β-mediated FN induction. Interestingly, there are several activator protein 1 (AP1) binding sites in the FN promoter region, which are the target of downstream signaling following TGF-β activation (Hocevar et al. 1999). We speculate that T3 induces TGF-β and the JNK/p38 pathway to phosphorylate AP1, allowing it to bind to the API sites in the FN promoter and thus activate FN expression.

Several other factors have been demonstrated to affect the expression of FN. Recombinant human interleukin-1 administered to rats was shown to increase plasma FN levels, concomitant with increased FN in the liver (Hagiwara et al. 1990). In addition, human cytomegalovirus (HCMV)-infected fibroblasts exhibited a progressive loss of cellular FN (Pande et al. 1990). These authors demonstrated that this decrease occurred at the transcriptional level and suggested that HCMV-encoded and/or -induced factors may induce these alterations. Vitamin A-deficient rats had increased levels of FN in their serum and it was demonstrated that regulation by vitamin A is at the level of transcription (Kim & Wolf 1987). Interestingly, all-trans retinoic acid (RA) induces FN expression, up to 130%, at the transcriptional level in bovine lens epithelial cells (Shanker & Sawhney 1996). Insulin-like growth factor-I (IGF-I) and insulin induce the level of FN in the culture media of smooth muscle cells (SMC) whereas only IGF-I upregulates FN in cell lysates from glomerular mesangial cells (Tamaroglio & Lo 1994). The liver regeneration process after surgery has been demonstrated to increase FN mRNA threefold (Caputi et al. 1995). Ehretsmann et al. (1995) reported that treatment of the human fibrosarcoma cell line HT-1080 with dexamethasone (DEX) results in the induction of FN protein and mRNA synthesis. These authors, through the use of nuclear run-on experiments, demonstrated that the DEX-dependent induction of FN occurs primarily at the post-transcriptional level. Vollmer et al. reported (1995) that FN is an estrogen-repressed protein in rat endometrial adenocarcinoma cells. This research indicates that different steroid hormones can affect the production of FN in the liver in different ways.

Our results have demonstrated that T3 plays an important role in the expression of FN protein at the transcriptional and post-transcriptional level. The induction of FN by T3 was demonstrated to be indirect as there was an absolute requirement for de novo protein synthesis. We further elucidated that FN activation by T3 is mediated, at least in part, by the TGF-β, JNK/p38 pathway. In addition, we established that the TGF-β pathway, in particular Smad3 and Smad4, was not implicated in FN induction by T3. Further study is required to dissect the regulatory cascade induced by the T3 activation of fibronectin expression.
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