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The modulation of apolipoprotein E gene expression by 3,3′-5-triiodothyronine in $HepG_2$ cells occurs at transcriptional and post-transcriptional levels

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The regulation of the synthesis and secretion of apolipoprotein E (apoE) is incompletely understood. This study examines the mechanisms responsible for regulating apoE gene expression in $HepG_2$ cells by thyroid hormone (3,3′-5-triiodothyronine). The secretion rate of apoE was by thyroid hormone increased (1.5–1.8-fold) in pulse/chase experiments. Thyroid hormone doubled apoE mRNA concentration as determined by Northern-blot analysis. Inhibition of protein synthesis by cycloheximide increased the thyroid-hormone-induced stimulation of apoE mRNA. This suggests that the synthesis of new protein is not required for thyroid hormone to stimulate apoE mRNA. Actinomycin D was used to inhibit new transcription; there was a more rapid degradation of mature apoE mRNA in thyroid hormone-treated $HepG_2$ cells than in control cells, suggesting that thyroid hormone acts post-transcriptionally to regulate apoE gene expression. Cycloheximide blocked the action of thyroid hormone, suggesting that thyroid hormone regulates the turnover of apoE mRNA via the synthesis of *de novo* protein. Nuclear run-on transcription assays demonstrated that thyroid hormone stimulated apoE gene transcription threefold in 24 h. These findings indicate that the expression of the apoE gene is controlled at both transcriptional and post-transcriptional loci by the thyroid hormone.

Apolipoprotein E (apoE) is a major component of several classes of lipoproteins and plays an important role in cholesterol metabolism. Its principal function is the mediation of the cellular uptake of lipids by serving as a ligand for the apoB,E (low-density lipoprotein) receptor and for the remnant receptor (for review, see [1]). Although the liver appears to be the major site of apoE synthesis, a wide variety of peripheral tissues also produce this protein, in contrast to most apolipoproteins [2–5].

Several dietary and hormonal disturbances have been used to gain insight into the regulation of hepatic apolipoprotein synthesis, transport, lipid association and the secretion of lipoprotein particles. Hormonal stimuli affecting hepatic apolipoprotein synthesis by the rat liver *in vivo* include insulin [6], corticosteroid [7], sex steroids [8], and thyroid hormones [9, 10]. When injected into living rats, thyroid hormones induced an increase in hepatic apoAI mRNA [9, 11, 12] and, to a lesser extent, in apoAIV mRNA [9, 11]. The change in the transcription rates of apoAI and apoAIV paralleled those of their mRNA concentrations [11]. However, Strobl et al. found that the major effect of thyroid hormones on apoAI gene expression was post-transcriptional, leading to increased stability of nuclear apoAI mRNA [13]. The synthesis of apoB48 by hyperthyroid rat liver was also greater than in hypothyroid rat liver, at the expense of apoB100 due to an enhanced post-transcriptional introduction of a stop codon into apoB mRNA [14].

The expression of the apoE gene in the liver is known to be increased *in vivo* by fasting [15], a sucrose diet [16], injection of glucagon or cAMP [17], but is inhibited by a fish oil diet [18]. Hyperthyroid rats have much lower plasma apoE levels than hypothyroid or euthyroid rats [12, 19], and this is paralleled by a lower [^{3}H]leucine incorporation into liver apoE [9]. However, Wilcox and Heimberg [10] found that the apoE production by perfused liver from hyperthyroid rats was not depressed. There appears to be little or no change in the hepatic apoE mRNA concentration following exposure to thyroid hormone [12, 13, 19]. The relationship between apoE mRNA synthesis and its abundance in hyperthyroidism is therefore probably complex, and the interplay of stimulatory and inhibitory factors may depend on the dose of thyroid hormone and the duration of hyperthyroidism.

However, it is not clear whether the alterations in apolipoprotein metabolism that occur in thyroid disorders are due to a direct effect of thyroid hormone itself or to secondary changes (i.e. changes in the circulating levels of other factors or metabolic fuel). We therefore investigated the direct effect of thyroid hormone on the expression of the apoE gene in cultured human hepatoma cell line, $HepG_2$. This cell line retains the capacity to synthesise and secrete many liver-specific proteins, including apolipoproteins [20–23]. $HepG_2$ cells have been used to investigate apoE secretion under various metabolic conditions [24–26]. Although $HepG_2$ cells bear fewer thyroid hormone receptors than do normal liver

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Abbreviations. apoE, apolipoprotein E; DMEM, Dulbecco's modified Eagle's medium; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

cells, these receptors have identical thyroid hormone binding activity [27]. These cells have been successfully used to study the effect of thyroid hormones on thyroxin-binding protein and α_1-glycoprotein synthesis [28] and on apoB gene expression [29].

The present study investigates the modulation of apoE gene expression in $HepG_2$ cells by thyroid hormone. Thyroid hormone had a specific effect on the expression of the apoE gene, and appeared to act occurring at both transcriptional and post-transcriptional levels.

MATERIALS AND METHODS

Materials

Chemicals of the highest purity were obtained from Merck, Serva Fine Biochemicals, Sigma Chemical Co, Pharmacia Fine Chemicals Inc. and Boehringer Mannheim. Dulbecco's modified essential medium (DMEM), antibiotics, fetal calf serum, methionine-free Eagle's minimum essential medium and Dulbecco's phosphate-buffered saline were obtained from Gibco BRL (European division). Thyroid hormone (3,3',5-triiodothyronine), dexamethasone, actinomycin D and cycloheximide, free-fatty-acid bovine serum albumin and RNase-free DNase I were obtained from Boehringer. L-[^{35}S]Methionine (1000 Ci/mol), [*methyl*-^{14}C]methylated proteins, [α-^{32}P]dCTP, [γ-^{32}P]ATP, Amplify, Nylon Hybond N+, filters Hyper film-MP, Royal X-Omat films and multi-primer kits were purchased from Amersham International. Acrylamide, *N,N'*-methylene bisacrylamide (electrophoresis grade) and protein markers were obtained from BioRad. Protein-A−Sepharose CL4B and Protein-G−Sepharose 4 Fast Flow for immmunoprecipitation were purchased from Pharmacia. Anti-(human apoE) antiserum was purchased from Immuno-France. Anti-(human α_1-glycoprotein) antiserum was purchased from Behring Diagnostic (France).

Cell culture

The human hepatocarcinoma cell line $HepG_2$ [30] was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (penicillin, streptomycin). When the cells were approximately confluent, the DMEM/fetal bovine serum was removed and the cells were carefully washed with NaCl/P_i, pH 7.5 to remove residual fetal bovine serum proteins, hormones and lipoproteins. The $HepG_2$ cells were then grown for 24 h in serum-free medium supplemented with 2% (mass/vol.) albumin and thyroid hormone was then added with dexamethasone (1 μM). Many studies, particularly those related to lipoprotein synthesis, have used albumin-containing media [31, 32]. The cells appeared to survive for at least 96 h under these conditions and cell growth appeared to be minimal [23].

For mRNA half-life studies, $HepG_2$ cells were incubated for 24 h in the presence or in the absence of thyroid hormone, at which time actinomycin D (5 μg/ml) was added. Total RNA was then recovered at various times from 0−8 h after the addition of actinomycin D. $HepG_2$ cells used to study the effect of cycloheximide were cultured in the serum-free medium. Cells were then incubated for 24 h in the presence of 0.5 μM thyroid hormone and 35 μM cycloheximide alone or in combination and harvested. Cycloheximide was used at a concentration 35 μM which inhibited [^{35}S]methionine incorporation into trichloroacetic-acid-precipitable proteins by >35% compared with control cells (data not shown).

Pulse/chase studies and immunoprecipitation

$HepG_2$ were cultured in DMEM with 2% (mass/vol.) bovine serum albumin for 24 h. The cells were then incubated in DMEM plus albumin in the presence or in the absence of thyroid hormone for 24 h. To study the synthesis of α_1-glycoprotein, the cells were washed three times with 2 ml methionine-free minimum essential medium and incubated for 3 h with the same medium containing [^{35}S]methionine (70−140 μCi/dish). In pulse/chase experiments, cells were preincubated for 2 h in methionine-free medium containing [^{35}S]methionine (400 μCi/dish). The medium was then removed, and the labeled cells were washed three times with methionine-free medium. The medium was replaced with non-radioactive medium (DMEM containing 0.5 mM methionine) and apoE secretion was chased for up to 2 h. At the end of the incubation, media and cells were collected and treated as previously described [17]. Antiproteases [phenylmethylsulfonyl fluoride ($PhMeSO_2F$), aprotinin, leupeptin and pepstatin] were added to cell lysates. Immunoprecipitations were carried out by the method of Andus et al. [33]; 5 μl anti-(human apoE) antiserum and 2 μl anti-(human α_1-glycoprotein) antiserum were used for 1 ml medium or 0.8−1 ml lysed cells.

After solubilization, immunoprecipitated proteins were submitted to electrophoresis. SDS/PAGE was performed under reducing conditions [34] using 12% slab gels for α_1-glycoprotein and 10% gels for apoE (Protean II slab cell). [*methyl*-^{14}C]Methylated proteins were run as molecular mass markers. The gels were soaked in sulfosalicylic acid/trichloroacetic acid/acetic acid/methanol/glycerol (90/15/125/250/2; mass/mass/vol./vol./vol.) for 30 min and then in amplifying fluorographic reagent (Amplifly) for 30 min. The gels were dried and autoradiographed with Hyperfilms-MP in a Kodak cassette with intensifying screens at −80°C for one or several days. Autoradiograms were scanned using an LKB 2202 Ultroscan laser densitometer connected to a 2220 recording integrator. Protein was assayed according to Lowry et al. [35].

Northern blot analysis

Total RNA was isolated from $HepG_2$ cells using guanidium isothiocyanate [36]. The total RNA content was measured by spectrophotometry, and its integrity was assessed by agarose gel electrophoresis; 10 μg total RNA/lane were separated on 1% agarose/2.2 M formaldehyde gels and transferred onto nylon filters [18]. The filters were hybridized at 42°C with appropriate cDNA probes for apoE [37] labeled with [α-^{32}P]dCTP (resulting specific activity around 10^8−10^9 cpm/μg cDNA) by the multi-primer technique. The apoE cDNA used included the apoE polymorphic sites and specified cysteine (112), arginine (145) and arginine (158) in these positions. Thus, the apoE corresponding to this cDNA represented phenotype E3 [37]. The hybridization solution contained 50% formamide, 0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, 5× Denhardt's solution, 0.1% SDS and 100 μg/ml denaturated herring sperm DNA. The membranes were washed twice for 15 min each in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at room temperature, and for 1 h at 60−65°C in 0.3 M NaCl, 0.03 M sodium citrate and autoradiographed. The blots were rehybridized to a 28S rRNA probe

labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP [38]. The relative abundance of the mRNA was calculated by quantitative scanning using an LKB laser densitometer.

Nuclei isolation and run-on assays

Nuclei were prepared from $HepG_2$ cells by lysis in an isotonic solution containing 0.1% Triton X-100 with 12 strokes in a Dounce homogenizer fitted according to Marzluff et al. [39] The nuclei were then sedimented through a sucrose gradient, suspended in storage buffer (40% glycerol, 50 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, and 0.1 mM EDTA).

Nuclear run-on experiments were performed essentially as described by Antras et al. [40]. Nuclei (2×10^7) were incubated in 200 µl reaction mixture containing 20% glycerol, 100 mM Tris/HCl pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 1.2 mM dithiothreitol, (DDT), 0.1 mM $PhMeSO_2F$, 0.35 M $(NH_4)_2SO_4$, 4 mM $MnCl_2$, 10 mM creatine phosphate, 10 µg/ml creatine phosphokinase, 500 unit/ml RNasin, 1 mg/ml heparin sulfate, 1 mM each ATP, GTP and CTP, and 250 µCi [α-^{32}P]UTP (400 Ci/mmol) at 30°C for 30 min. The mixture was then treated with 2 units RNase-free DNase I for 30 min at 37°C, followed by 30 min at 37°C with 1% SDS and 100 µg/ml proteinase K. Nascent RNAs were extracted in 3 vol. 8 M guanidine/HCl pH 5.0 containing 20 mM sodium acetate, 1 mM dithiothreitol, 0.5% (mass/vol.) lauryl sarcosine. Yeast tRNA (50 µg) was added to each sample and precipitated by adding 0.6 vol. ethanol for 12 h at −20°C. After centrifugation at $12000\times g$ at −10°C for 30 min, the pellet was dissolved in 0.5 ml 7 M guanidine/HCl pH 7.0, containing 20 mM sodium acetate, 20 mM EDTA, 1 mM dithiothreitol and 0.5% lauryl sarcosine; 25 µl 2 M sodium acetate pH 4.5 was added to each tube, followed by 0.6 vol. ethanol, and transcripts were allowed to precipitate for 2 h at −20°C. This last step was repeated once.

Equal amounts of labeled RNA species (70×10^6 cpm) were then hybridized for 3 days at 42°C with 10 µg various linearized plasmids (pUC 19, β-actin, and apoE) immobilized on nitrocellulose (Schleicher & Schüll BA 85); 10 µg plasmid was found to represent a sufficient excess of DNA when tested with 5−20 µg DNA. The filters were washed twice with 0.3 NaCl, 20 mM H_2NaPO_4 and 2 mM EDTA at 37°C for 15 min and incubated with 5 µg/ml RNase A in the same solution for 30 min at 37°C and finally autoradiographed at −70°C for five days.

RESULTS

Effect of thyroid hormone on α_1-glycoprotein secretion in $HepG_2$ cells

Kobayaski et al. [28] showed that incubating $HepG_2$ cells with thyroid hormone for 24 h or 48 h induced the synthesis and secretion of α_1-glycoprotein without affecting albumin secretion. We checked that our culture conditions, which were designed to obtain the best lipoprotein secretion [23, 31, 32], did not alter the response to thyroid hormone by first investigating the effect of thyroid hormone on α_1-glycoprotein and albumin synthesis and secretion.

$HepG_2$ cells incubated for 24 h with 0.5 µM thyroid hormone were pulse-labeled for 3 h with [^{35}S]methionine and cell lysates. The α_1-glycoprotein in the culture medium was immunoprecipitated with anti-(human α_1-glycoprotein) antiserum. Two forms of α_1-glycoprotein were identified after electrophoresis on 12% SDS/PAGE (Fig. 1). The apparent molecular mass of the form found in the medium was higher than that of the intracellular form, according to Andus et al. [33]. The cellular form was never detected in the $HepG_2$ medium. The two molecular entities differ in their carbohydrate moieties: the intracellular form (39 kDa) contains a high proportion of mannose, the medium form (47−51 kDa) contains complex oligosaccharides [33]. Quantification of the autoradiograms by scanning densitometry indicated that [^{35}S]methionine incorporation into α_1-glycoprotein was 2.2-fold higher in thyroid-hormone-treated $HepG_2$ cells (4.6 ± 0.4 arbitrary units) than in control cells (2.2 ± 0.4 arbitrary units). The albumin secretion, assessed by the immuno-dot technique with anti-(human albumin) antiserum, did not change (not shown).

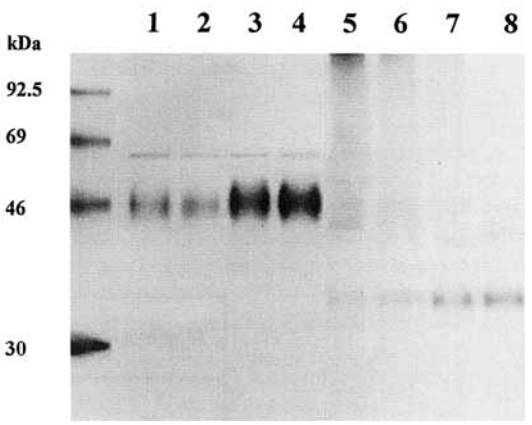
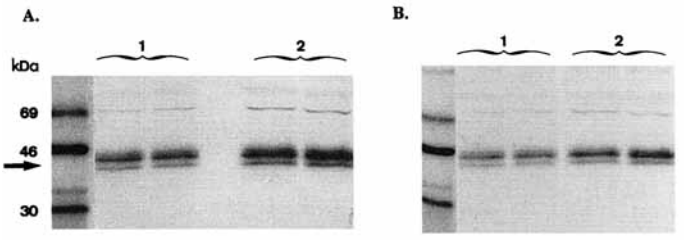


Fig. 1. Incorporation of [^{35}S]methionine into cellular and extracellular immunoprecipitated α_1-glycoprotein. $HepG_2$ cells were cultured in serum-free DMEM for 24 h in the presence or in the absence of 0.5 µM thyroid hormone. They were then incubated with [^{35}S]methionine (70−140 µCi/dish) for 3 h. Cell extracts and culture medium were analyzed for newly synthesized α_1-glycoprotein by immunoprecipitation with an anti-(human α_1-glycoprotein) antiserum and separated on a 12% SDS/PAGE. Three independent experiments were performed in triplicate. The autoradiogram shows immunoprecipitates from the same amount of trichloroacetic-acid-precipitable ^{35}S from extracts of control and thyroid-hormone-treated cells. Lanes 1 and 2, media from control cells; lanes 3 and 4, media from thyroid-hormone-treated cells; lanes 5 and 6, control cell extracts; lanes 7 and 8, thyroid-hormone-treated cell extracts. The mobilities of molecular mass standards are indicated on the left of the autoradiogram.

These results agreed with those described by Kobayashi et al. [28] and indicated that our culture conditions were suitable for studying the effect of thyroid hormone on $HepG_2$ cells.

Changes in apoE mRNA and protein synthesis

The effect of thyroid hormone on apoE gene expression, was assessed in $HepG_2$ cells grown for 24 h in serum-free medium supplemented with 2% albumin. Thyroid hormone was then added and the cells were incubated for a further 24 h. Fig. 2 (A, B) shows bands with the appropriate mobilities of apoE isoforms in the medium, in agreement with Fazio et al. [26]. More [^{35}S]methionine was incorporated into immunoprecipitated apoE from the medium of cells cultured with thyroid hormone in pulse/chase experiments than into control cell apoE. Quantification by laser scanning densitometry indicated that the rate of apoE secretion was signifi-



C.

Chase period (min)	CONTROL	T_3	Elevation of apo E secreted in %
60	3.5 ± 0.25	5.2 ± 0.7	48 %
120	2.6 ± 0.1	4.6 ± 0.2	75 %

Fig. 2. Pulse/chase analysis of secreted apoE. $HepG_2$ cells were incubated in the presence or in the absence of 0.5 μM thyroid hormone for 24 h and were pulsed for 2 h with [^{35}S]methionine (400 μCi/dish). The cells were then washed and chased with 0.5 mM unlabeled methionine for 60 and 120 min. The labeled media were collected at the end of each chase period. Medium apoE was immunoprecipitated with a specific anti-(human apoE) antiserum and analysed by 10% SDS/PAGE. The autoradiogram of the electrophoresis gels for a representative experiment is shown in the upper panel. (A) Chase period, 60 min, (B) chase period, 120 min; lanes 1, control cells; lanes 2, thyroid-hormone-treated cells. Equal volumes of media (containing the same amount of trichloroacetic-acid-precipitable ^{35}S) were electrophoresed. The mobilities of molecular mass standards are indicated on the left of the autoradiograms. The arrows indicates the apoE secreted forms. The intensities of apoE bands were analysed by densitometry. (C) Values shown in the table are for the amount of apoE (in arbitrary units, means ± SD) in control and thyroid-hormone-treated (T) cells. Three independent experiments were performed in triplicate.

cantly ($P < 0.05$) stimulated by thyroid hormone. ApoE recovery was 1.5-fold above control level at the end of a 1-h chase and 1.8-fold higher at the end of a 2-h chase (Fig. 2C).

The mechanisms by which thyroid hormone stimulates apoE production were investigated by measuring apoE mRNA in control and thyroid-hormone-treated $HepG_2$ cells. A representative Northern blot analysis of RNA isolated from thyroid-hormone-treated and control cells using an apoE cDNA probe is shown in Fig. 3. $HepG_2$ cells were incubated in the presence of two concentrations of thyroid hormone, 0.5 μM and 1 μM, for 24 h. Correction of the apoE signal (obtained by scanning densitometry) for the 28S rRNA signal showed that apoE mRNA levels were increased 2.4-fold after incubation with 0.5 μM thyroid hormone and 2.2-fold after incubation with 1 μM thyroid hormone. Longer incubation (48 h) indicated that the apoE mRNA concentration was doubled with 0.5 μM and increased 1.8-fold with 1 μM thyroid hormone (not shown).

Effect of inhibiting protein synthesis on apoE mRNA levels

We determined whether new protein synthesis was required to stimulate apoE mRNA induction by thyroid hormone, by exposing control and thyroid-hormone-treated $HepG_2$ cells to cycloheximide (Fig. 4). Northern blot analysis of cellular RNA indicated that cycloheximide did not prevent induction of apoE mRNA. Incubating $HepG_2$ cells with cycloheximide alone approximately doubled apoE mRNA, while the β-actin mRNA content remained constant (Fig. 4). This stimulatory effect of cycloheximide itself has been described for the low-density lipoprotein receptor mRNA in human fibroblasts [41]. The effect of the two effectors (cycloheximide and thyroid hormone) was found to be additive, giving a 3.4-fold increase in apoE mRNA. These findings suggest that thyroid hormone does not require additional protein synthesis to stimulate an increase in apoE mRNA concentration.

Effect of thyroid hormone on mRNA half-life

The increase in the steady-state mRNA concentration may be due to increases in apoE gene transcription or enhanced mRNA stability. We therefore determined whether the increase in apoE mRNA after thyroid hormone treatment was due to enhanced formation or diminished degradation of mRNA. Actinomycin D, which blocks transcription of RNA gives an accurate measurement of mRNA half-life over a short period [42]. We measured successive levels of apoE mRNA after blocking RNA synthesis with actinomycin D (25 μg/ml) in control and thyroid-hormone-stimulated cells. ApoE mRNA was estimated by Northern blot analysis after 0−8 h of actinomycin D treatment. Results obtained from one of three such experiments are displayed in Fig. 5. There was a gradual decrease in apoE mRNA, as assessed by Northern hybridization with the human apoE cDNA probe. The mRNA half-lives ($t_{1/2}$) were calculated, assuming first-order kinetics. The concentration of apoE mRNA declined with a half-time of 1 h in the thyroid-hormone-induced culture ($r = 0.881$), as compared with 7 h in the control cells ($r = 0.934$; Fig. 5A).

A control gene encoding the structural protein β-actin was used to check the decay patterns of the steady-state β-actin in thyroid-hormone-treated and control $HepG_2$ cells (Fig. 5D). Thyroid hormone did not alter the half-life of β-actin mRNA. The effect of thyroid hormone was therefore

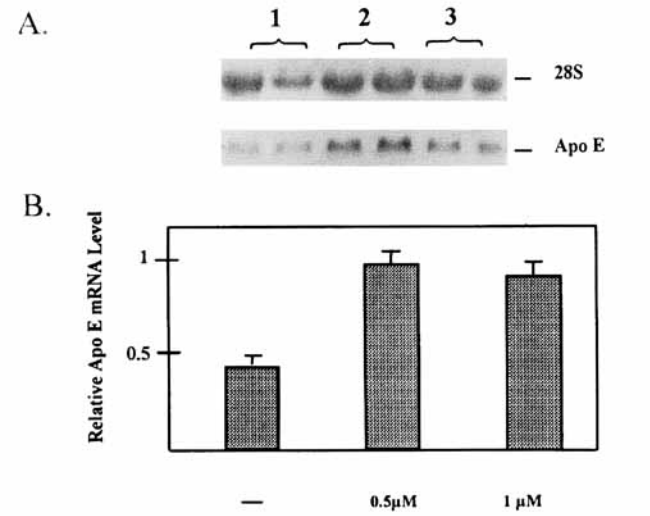


Fig. 3. Dose-dependent effects of thyroid hormone on apoE mRNA in $HepG_2$ cells. (A) 10 μg of total cellular RNA isolated from control cells (lane 1) and cells treated with 0.5 μM (lane 2) and 1 μM (lane 3) thyroid hormone were analyzed in duplicate by Northern blotting as described in Materials and Methods. Membranes were hybridized with a human cDNA probe for apoE and the 28S rRNA probe. The intensities of the bands corresponding to apoE and the internal control, 28S, was measured by scanning densitometry of the autoradiograms. (B) Quantification of the apoE transcripts. The relative amounts of the apoE transcripts in A were quantified by calculating the ratio of the apoE-band integrated densities to those of 28S rRNA. The values are means ± SD for three separate experiments performed in duplicate. Concentration of thyroid hormone is shown at the bottom.

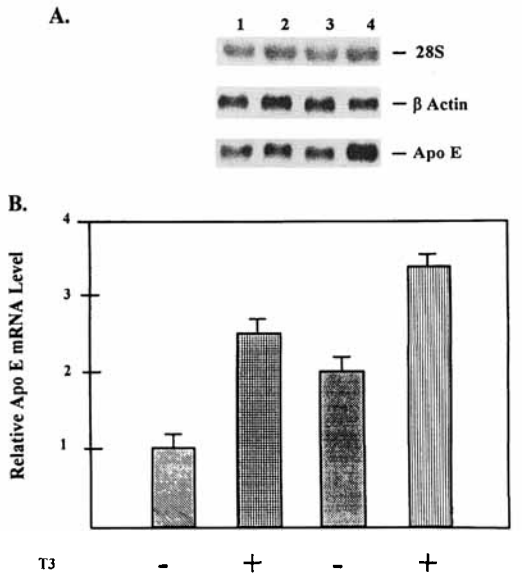


Fig. 4. Effect of cycloheximide on the induction of apoE mRNA by thyroid hormone. $HepG_2$ cells were cultured in serum-free DMEM for 24 h; 0.5 μM thyroid hormone and 35 μM cycloheximide were then added to the medium and the cells were incubated for a further 24 h. Total cellular RNA was then isolated from control cells, thyroid-hormone-treated cells, cycloheximide-treated cells and cycloheximide + thyroid-hormone-treated cells. (A) RNA samples (10 μg) were then analyzed by Northern blot as indicated in Fig. 3. The intensity of the bands corresponding to apoE and the internal controls, 28S and β-actin, were measured by scanning densitometry. Lane 1, control cells; lane 2, thyroid-hormone-treated cells (T3); lane 3, cycloheximide-treated cells (CHX); lane 4, cycloheximide + thyroid-hormone-treated cells. (B) The results are expressed as amount of apoE mRNA after correcting for the 28S signal, relative to control cells arbitrarily designated as 1. Results are the means ± SD of three independent experiments performed in triplicate.

specific to apoE mRNA and the induction of apoE mRNA by thyroid hormone in $HepG_2$ cells cannot be ascribed to a thyroid-hormone-induced stabilization of apoE mRNA.

We then tested the effects of cycloheximide on the response of apoE mRNA to thyroid hormone to determine whether the decrease of apoE mRNA half-life by thyroid hormone required the *de novo* synthesis of other gene products (Fig. 5B). The addition of cycloheximide to thyroid-hormone-treated cells brought the half-life of apoE mRNA back to 7 h. In contrast, cycloheximide alone had no effect on the half-life of apoE mRNA (Fig. 5C). Similar results were obtained with 25 μg/ml of the specific transcription inhibitor 5,6-dichloro-1-β-d-ribofuranosyl benzimidazole, which inhibits the elongation step (results not shown).

Effect of thyroid hormone on relative transcription rates of apoE gene

The doubling in apoE mRNA level and the marked decrease in apoE mRNA half-life induced by thyroid hormone in $HepG_2$ cells suggest that the hormone has an effect on apoE gene transcription. Nuclear run-on assays were performed using isolated nuclei to determine whether the changes observed were due to changes in the rate of transcription. $HepG_2$ nuclei were isolated from control cells and from cells treated with thyroid hormone for 6 h, 12 h and 24 h. Initiated transcripts were elongated *in vitro* using [α-^{32}P]UTP, as described in Materials and Methods. Radiolabeled nuclear RNA was hybridized to pUC19 (as negative control), apoE cDNA and β-actin cDNA immobilized on nitrocellulose membranes (Fig. 6A).

Quantification of the autoradiogram data by scanning densitometry showed that the ratio apoE/β-actin was increased 1.5-fold at 6 h, 2-fold at 12 h, and 3-fold at 24 h in thyroid-hormone-treated cells (Fig. 6B), indicating that thyroid hormone stimulated apoE gene transcription.

DISCUSSION

Many studies, principally performed in the rat, have reported that changes in thyroid hormone status have profound effects on plasma lipid transport and apolipoprotein metabolism. The patterns of the changes in chronic and acute hyperthyroidism are different and the results are conflicting. The results of our studies on the human cell line $HepG_2$ indicate that the abundance of apoE mRNA is doubled in response to thyroid hormone (Fig. 3). There is a parallel increase in the secretion of apoE by 48% and 75%, (Fig. 2). The discrepancy between *in vivo* experiments and the present results could be explained by a higher catabolism of plasma apoE in hyperthyroid rats than in normal rats, as it has been demonstrated that thyroid hormone increased the synthesis and secretion of hepatic triacylglycerol lipase in $HepG_2$ cells [43] which is involved in the clearance of chylomicron remnants and intermediate-density lipoprotein by the liver [44]. Thyroid hormone also increases the apoE-mediated uptake of remnants by stimulating expression of low-density-lipoprotein receptor [19].

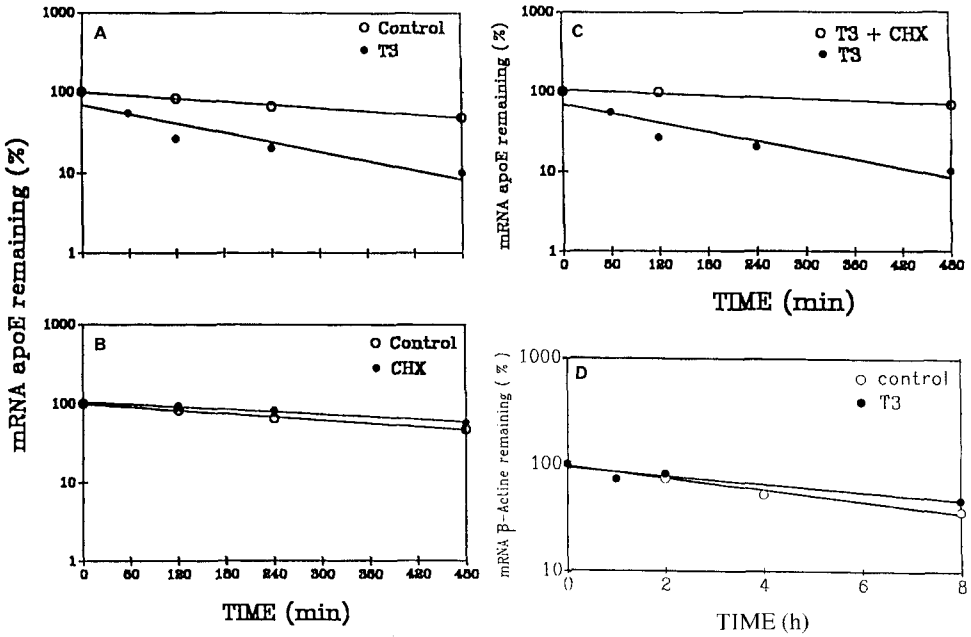


Fig. 5. Decay of apoE mRNA in control and thyroid-hormone-treated $HepG_2$ cells. In this representative experiment, cells were cultured in serum-free DMEM for 24 h; thyroid hormone (T3, 0.5 μM) and cycloheximide (CHX, 35 μM) were then added to the medium and the cells were incubated for a further 24 h. Actinomycin D (5 μg/ml) was added to either control cells or treated cells and total cellular RNA was isolated at the time indicated. RNA samples were analyzed by Northern blotting and quantified as indicated in Fig. 3. Data are expressed as percentages of remaining apoE mRNA relative to the initial values before actinomycin D treatment (time zero is 100%) and are plotted logarithmically against time after obtaining the results of densitometric analysis. The first-order decay rate constants were used to calculate the half-life values. (A, B, C) ApoE mRNA decays; (D) β-actin mRNA decay.

A recent report described the effect of thyroid hormone on apoE gene expression in $HepG_2$ cells [45]. The authors suggested that apoE gene expression does not appear to be significantly influenced by thyroid hormone. However, the culture conditions were not clearly described and it is possible that differences in cell culture conditions and/or the concentrations of thyroid hormone (50 nM) could explain the discrepancy between the results reported above and our own.

Modulation of mRNA stability is a potential way of controlling mRNA levels. It has been demonstrated, that the phorbol ester 12-tetradecanoyl-phorbol 13-acetate stabilizes apoE mRNA during the differentiation of THP_1 cells into macrophages [46]. We evaluated the potential post-transcriptional loci for controlling apoE mRNA by measuring the half-life of apoE mRNA. The specific transcription inhibitor, actinomycin D, was added to the medium of control and thyroid-hormone-treated $HepG_2$ cells and apoE mRNA was measured. Mature apoE mRNA was degraded more rapidly in thyroid-hormone-treated cells than in control cells (Fig. 5). The half-life of apoE mRNA in control $HepG_2$ was about 7 h and about 1 h in thyroid-hormone-treated cells. Similar destabilizing effect of thyroid hormone has been reported for transcript containing the growth hormone gene in transient transfection experiments in a gonadochorionic cell line [47] but, in this case, the slight reduction in transcript half-life (−28%) was less than that of the messenger SV40-3′-UTR/FR (−60%) used as a control, indicating a non-specific effect. In contrast, our experiments, indicated that the thyroid hormone effect was specific, as the half-life of β-actin mRNA was unchanged (Fig. 5).

Cycloheximide alone increases the apoE mRNA content of control cells but does not appear to increase message stability (Figs 4 and 5B). This suggests that the biosynthesis of factor inhibiting apoE gene expression has been blocked.

Protein synthesis is reported to be involved in the thyroid-hormone-induced increases in the hepatic mRNA of spot 14 [48], hydroxymethylglutaryl-CoA reductase [49] and fatty acid synthase [50]; cycloheximide does not block the thyroid hormone effect. Our results indicate that thyroid hormone can increase apoE gene expression without ongoing protein synthesis (Fig. 4). An identical phenomenon has been described for the action of thyroid hormone on the malic enzyme mRNA in fetal chicken hepatocytes in primary culture [50].

Cycloheximide does not affect apoE mRNA stability directly (by inhibiting the translation of apoE mRNA, or the stability of polyribosome formed from apoE mRNA) but by inhibiting the translation of some protein involved in its degradation. Cycloheximide which, alone, does not affect apoE mRNA half-life, blocked instability of apoE mRNA induced by thyroid hormone. This indicates that the destabilizing effect of thyroid hormone does not result from a direct effect of thyroid-hormone-receptor/mRNA interaction, but needs ongoing synthesis of another protein factor. The specificity of such a factor which stimulates apoE mRNA degradation would presumably depend on the presence of a specific RNA sequence which allows interaction between mRNA and the thyroid-hormone-induced protein.

Our experiments demonstrate that thyroid hormone induces an increase in apoE mRNA concentration (Figs 3 and 4) and a decrease in its half-life (Fig. 5). These results suggest that, in addition to its indirect effect on apoE mRNA stability, thyroid hormone stimulates the expression of apoE gene at the transcriptional level. We confirmed this in run-

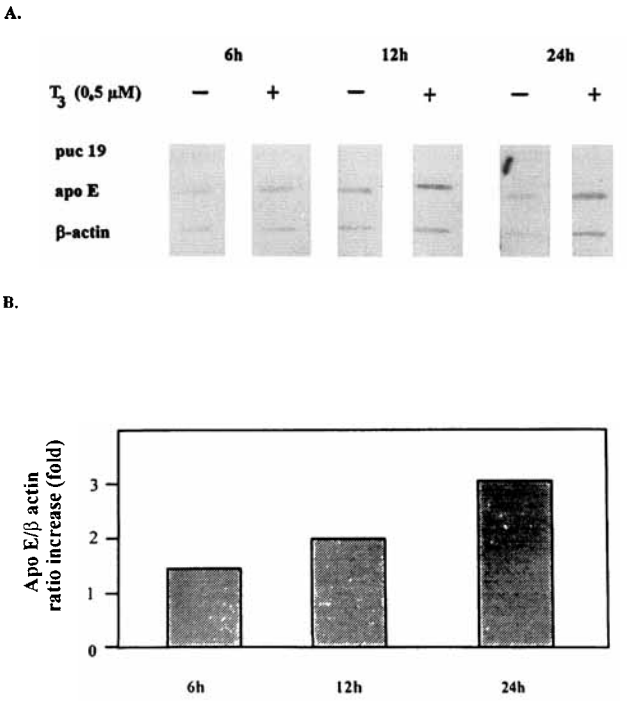


Fig. 6. Nuclear run-on analysis of apoE gene transcription in $HepG_2$ cells after thyroid hormone treatment. (A) Representative nuclear run-on assay. Nuclei were isolated from $HepG_2$ cells and incubated in the presence or absence of 0.5 μM thyroid hormone (T_3) for the indicated times. Nuclear RNA was labeled with [^{32}P]UTP and transcripts were hybridized to denatured apoE, β-actin or vector (pUC19) cDNA slot-blotted to filters as described in Materials and Methods. The autoradiograms were exposed for 5 days. (B) Densitometric analysis of changes in apoE transcription rate. Signal intensities were quantified by densitometry of the autoradiograms. The apoE/β-actin ratio increased from cells exposed to 0.5 μM thyroid hormone at each time over apoE/β-actin ratio from control cells. Nuclear run-on analysis were performed in two or three separate experiments for each time point.

on experiments on isolated nuclei from $HepG_2$ cells. ApoE gene transcription was stimulated about threefold by thyroid hormone alone. Thus the apoE gene is transcriptionally regulated by thyroid hormone (Fig. 6). The disparity between the strong destabilizing effect of thyroid hormone on apoE mRNA and the apoE gene transcription rate could be explained by the fact that run-on analysis does not take into account the efficiency of processing and export of mRNA precursors into the cytoplasm. Therefore, the increase of the transcriptional rate of apoE by thyroid hormone could result from the rapid turnover of apoE mRNA. Regulation of apoE mRNA transcription by its turnover rate is also suggested by experiments performed on livers from a mutant strain of rats with induced analbuminemia [51]. These experiments demonstrated that specific apoE transcription was higher in the analbuminic rat than in the control animals in which the pattern of apoE mRNA concentration was opposite. The effect of thyroid hormone on the apoE transcription rate does not seem to include the proximal (−200/+1) promoter, since transient transfection experiments showed no stimulatory effect after a 12-h or 24-h incubation in the presence of thyroid hormone (data not shown).

We believe that this is the first time that thyroid hormone has been shown to exert an important regulatory effect on apoE gene expression in human cell lines. This effect is both transcriptional and post-transcriptional and the mechanisms by which thyroid hormone regulates these steps appear to differ. The stimulation of transcription by thyroid hormone is independent of on-going protein synthesis, but additional protein synthesis is required for the instability of apoE mRNA induced by thyroid hormone. There are A+U-rich sequences in the 3′ untranslated region of many unstable mRNA such as c-fos or c-myc [52]. Serum and other mitogens can stimulate the degradation of c-fos mRNA in quiescent fibroblasts [53], and this effect is blocked by protein synthesis inhibitors [54]. However, apoE mRNA does not contain A+U-rich sequences, which implies the existence of other structural elements capable of specifying the rapid turnover of apoE mRNA. Further studies are clearly needed to elucidate the molecular details of the mechanism by which thyroid hormone alters apoE gene expression post-transcriptionally.

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