DNA binding and mutagenesis in vitro established that the −67/−55 region of the apoA-II (apolipoprotein A-II) promoter contains a thyroid HRE (hormone response element), which strongly binds RXRα (retinoid X receptor α)/T3Rβ (thyroid receptor β) heterodimers and weakly T3Rβ homodimers, but does not bind other homo- or heterodimers of RXRα or orphan nuclear receptors. Transactivation was abolished by point mutations in the thyroid HRE. In co-transfection experiments of HEK-293 cells, the −911/+29 human apoA-II promoter was transactivated strongly by RXRα/T3Rβ heterodimers and in the presence of RA (9-cis retinoic acid) or T3 (tri-iodothyronine). Homopolymeric promoters containing either three copies of the −73/−40 (element AIIAB) or four copies of the −738/−712 (element AIJ) apoA-II promoter could be transactivated by RXRα/T3Rβ heterodimers in COS-7 cells only in the presence of T3 or RA respectively. RXRα/T3Rβ heterodimers and USF2a (upstream stimulatory factor 2a) synergistically transactivated the −911/+29 apoA-II promoter in the presence of T3; USF2a also enhanced the activity of a GAL4–T3Rβ fusion protein in the presence of T3 and suppressed the activity of a GAL4–RXRα fusion protein in the presence of RA. These findings suggest a functional specificity of the two HREs of the apoA-II promoter for retinoids and thyroids, which is modulated by synergistic or antagonistic interactions between RXRα/T3Rβ heterodimers and the ubiquitous transcription factor USF2a.

Key words: apolipoprotein, atherosclerosis, gene regulation, hormone nuclear receptor, thyroid hormone-responsive element, transcriptional synergism.

INTRODUCTION

Human apoA-II (apolipoprotein A-II) is a major protein component of HDL (high-density lipoprotein) [1,2]. The prevailing view has been that apoA-II may alter the composition and functions of HDL and in some instances contribute to atherogenesis. Earlier genetic studies in mice and humans suggested that apoA-II may be a risk factor for atherosclerosis [3–5]. However, subsequent studies did not substantiate this point [6–8]. On the other hand, overexpression of human apoA-II in transgenic mice induced low plasma HDL and post-prandial hypertriglyceridaemia [9].

ApoA-II synthesis is restricted to the liver and, to a much lesser extent, to the intestine [10]. The −903 to −33 apoA-II promoter region contains a set of eight proximal and middle, and six distal regulatory elements [11–15]. The distal −911/−614 regulatory region (elements AIJ–AIIN) acts as a strong hepatic transcriptional enhancer of the proximal apoA-II promoter in vitro and in vivo [12]. Several factors contribute to the apoA-II gene transcription, and it appears that there is synergism between factors that are bound to the proximal promoter and the apoA-II enhancer in the transactivation of the apoA-II promoter/enhancer cluster [16].

The heat-stable transcription factor USF (upstream stimulatory factor) binds to three sites on the regulatory elements AIAB, AIH and AIIL [17,18]. All three E-boxes present in the proximal and distal USF-binding sites were necessary for transactivation by USF2a [18]. The cholesterol-regulated transcription factors SREBP-1 (sterol-regulated-element-binding protein-1) and SREBP-2 also bind to five and four sites respectively and transactivate the human apoA-II promoter in vitro [19,20]. The SREBP-binding motif in element AIAB (−73/−40) overlaps with the binding site of USF [20].

ApoA-II gene transcription is also regulated by orphan and ligand-dependent nuclear receptors that bind to the element AIJ of the apoA-II enhancer [21,22]. This element contains an imperfect direct repeat AGGGTA(A)AGGTGG between nt −721/−733 on the non-coding strand with DR-1 (direct repeat with 1 nucleotide spacing) and binds orphan receptors HNF-4 (hepatic nuclear factor-4), ARP-1 (apoA-I regulatory protein 1), EAR-2 (vErb-A related protein) and EAR-3 and ligand-dependent

Abbreviations used: apoA-II, apolipoprotein A-II; ARP-1, apoA-I regulatory protein 1; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; DR-1, direct repeat with 1 nucleotide spacing; EAR, vErb-A related protein; EMSA, electrophoretic mobility-shift assay; HDL, high-density lipoprotein; HEK-293 cells, human embryonic kidney 293 cells; HNF-4, hepatocyte nuclear factor-4; HRE, hormone response element; RA, 9-cis retinoic acid; RXRα, retinoid X receptor α; SREBP, sterol-regulated-element-binding protein; T3, tri-iodothyronine; T3Rβ, thyroid receptor β; Tk, thymidine kinase minimal promoter; USF, upstream stimulatory factor; WT, wild-type.

1 These authors have contributed equally to this work.
2 To whom correspondence should be addressed (e-mail kardasis@imbb.forth.gr).
nuclear receptors [21–23]. In a previous study, we have shown that the distal element AIJ, as well as the proximal element AIIB, bind heterodimers of RXRα and T3Rβ (thyroid receptor β) [23].

In the present study, we present evidence that the proximal apoA-II regulatory region AIIB contains functional thyroid HRE (hormone response element) which strongly binds RXRα/ 

\[ T_3R\beta \]

heterodimers and mediates transactivation of the apoA-II promoter in response to T3 (tri-iodothyronine). By utilizing homopolymeric promoters in co-transfection experiments, we established a functional specificity of the two HREs of the apoA-II promoter (elements AIJ and AIIB) for retinoids and thyroids respectively. Furthermore, the specificity of these ligands in the induction of the apoA-II promoter is modulated by positive and negative interactions of USF2a with nuclear receptors T3Rβ and RXRα respectively.

**MATERIALS AND METHODS**

Reagents were purchased from the following sources: polyclonal anti-RXRα and anti-T3Rβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and anti-mouse horseradish peroxidase-conjugated secondary antibody was from Chemicon International (Temecula, CA, U.S.A.). Restriction enzymes and modifying enzymes (T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I and calf intestinal alkaline phosphatase) were purchased from Minotech (Crete, Greece) and New England Biolabs. Polycl(dI/dC), acetyl-CoA, dNTPs, [\( ^{32}P\)]dCTP and [\( ^{14}C\)]chloramphenicol were purchased from Minotech (Crete, Greece). All reagents for cell culture [DMEM (Dulbecco’s modified Eagle’s medium), foetal calf serum, L-glutamine and penicillin–streptomycin] were purchased from Invitrogen/Life Technologies (Carlsbad, CA, U.S.A.). o-Nitrophenyl-galactopyranoside was purchased from Sigma–Aldrich. All other chemicals were obtained from commercial sources at the purest grade available.

**Plasmid construction**

Plasmids (\(-911/ +29\)) A-II-CAT (where CAT stands for chloramphenicol acetyltransferase), (\(-911/ +29\)) AI A-II-CAT that contains a deletion of regulatory element AIJ, (\(-911/ +29\)) AIIAB A-II-CAT that contains a deletion of regulatory element AIIB, (\(-911/ +29\)) BM1 A-II-CAT that contains nucleotide substitutions in element AIIB, A-II(J)tk-CAT (where tk stands for thymidine kinase minimal promoter) that contains the tk under control of four copies of the apoA-II regulatory element AIJ and (\(-80/ +29\)) A-II-CAT have been described previously [16,18,22]. Plasmid A-II (AB)tk-CAT that contains the tk under control of three copies of the apoA-II regulatory element AIIB was constructed by inserting a synthetic double-stranded oligonucleotide containing the \(-73 \rightarrow -40\) regulatory region of human apoA-II at the XbaI site of the vector pBlCAT2. The sequence of the sense strand of the oligonucleotides used for cloning is 5’-CTGACCATGTCATCAGGAGGGTGGTAT-3’. The number and orientation of the inserted oligonucleotides were verified by sequencing. The expression vectors pMTC-HNF-4, pMTC-ARF-1, pMTC-RXRα, pMTC-T3Rα and pMTC-T3Rβ have been described previously [21,24]. The expression vector for human USF2a was described previously [18]. Plasmids GAL4(DBD)-RXRα (D/E) and GAL4(DBD)-T3Rβ (D/E) expressing hybrid proteins consisting of the DBD (DNA-binding domain) of the yeast transactivator GAL4 and domains D (hinge) and E (ligand-binding domain) of human RXRα and chicken T3Rβ respectively were gifts from Dr H. Gronemeyer (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France).

**Cell cultures, transient transfections and CAT assays**

Human hepatoma HepG2 cells, monkey kidney COS-7 cells and HEK-293 (human embryonic kidney 293) epithelial cells were cultured in DMEM, supplemented with 10% (v/v) foetal bovine serum, L-glutamine and penicillin–streptomycin at 37 °C, in a 5% CO₂ atmosphere. For experiments involving treatment of the cell culture with T3 or RA (9-cis retinoic acid), cells were grown in DMEM containing 5% of charcoal-stripped serum. Transient transfections were performed using the Ca3(PO4)2 co-precipitation method [25]. Forty hours after transfection, cells were harvested and lysed in 1× lysis buffer included in the luciferase assay kit, followed by a freeze–thaw cycle and centrifugation at 9500 g for 5 min at room temperature (25 °C). CAT and β-galactosidase assays were performed as described previously [26,27]. Luciferase assays were performed using the luciferase assay kit from Promega, following the manufacturer’s instructions.

**Expression of proteins in COS-7 cells**

The day before transfection, COS-7 cells were seeded at a density of 5×10⁵ cells/100 mm dish. The next day, COS-7 cells were transfected with 17 µg each of the expression vectors for the hormone nuclear receptors HNF-4, ARF-1, RXRα, RARα and T3Rβ or left untransfected. Forty hours after transfection, cells were harvested and lysed in 1× lysis buffer included in the luciferase assay kit, followed by a freeze–thaw cycle and centrifugation at 9500 g for 5 min at room temperature (25 °C). Cell debris was removed by centrifugation at 4 °C for 5 min in a microfuge and the supernatant was aliquoted and stored at −70 °C.

**Gel electrophoretic mobility-shift assay (EMSA)**

Rat liver and HepG2 nuclear extracts were generated as described previously [28]. Gel EMSAs were performed as described previously [28]. Sense and anti-sense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labelled with Klenow fragment of DNA polymerase I and [\( ^{32}P\)]dCTP. The sequence of the sense oligonucleotides, used as probes, is as follows:

A-II (\(-73/-40\)) WT (wild-type): 5’-CTGATCCCATAGTCGTCACCTGACAGGGAGGGTGGTAT-3’;

A-II (\(-73/-40\)) BM1: 5’-CTGATCCCATAGTCAGGGAGGGTGGTAT-3’;

The underlined sequence in oligonucleotide BM1 indicates a mutation. For supershift assays, 1 µl of polyclonal anti-RXRα or anti-T3Rβ antibody was added to the reaction mixture before the addition of the probe and was left on ice for 10 min.

**RESULTS AND DISCUSSION**

The regulatory element AIIB \((-73/-40\)) of the human apoA-II promoter binds RXRα/T3Rβ heterodimers strongly and specifically and T3Rβ homodimers weakly.

The proximal region of the human apoA-II promoter between nt \(-73 \rightarrow -40\) contains overlapping binding sites for the transcription factors USF and SREBP [18,19] (Figure 1A). This region
contains an imperfect DR-1 HRE between nt −67 and −55 having the sequence 5'-AGTCCTGTCACCT-3'. To characterize this DR-1 HRE further in terms of nuclear receptor binding, DNA-binding experiments were performed using the −73/−40 region of the apoA-II promoter as probe and extracts from COS-7 cells expressing the human HNF-4, ARP-1, RXRα, RXRα/RARα, RXRα/TβR and TβR nuclear receptors. This analysis showed that element −73/−40 (previously designated AIIAB) binds RXRα/TβR heterodimers strongly and TβR homodimers weakly, but does not bind any of the orphan (HNF-4 and ARP-1) or the other ligand-dependent (RXRα/RARα) nuclear receptors tested (Figure 1B). Importantly, a five-nucleotide substitution in the middle of this DR-1 HRE (mutation BM1; Figure 1A) diminished greatly the binding of the RXRα/TβR heterodimers or TβR homodimers to the mutated −73/−40 probe (Figure 1B). Immunoblotting analysis showed that all receptors utilized in the gel EMSAs of Figure 1(A) were expressed at comparable levels in COS-7 cells (results not shown).

Binding of nuclear receptors to the AIIAB (−73/−40) DNA probe was also investigated by DNA EMSA using nuclear extracts from rat liver or HepG2 cells as well as competition and supershift assays. As shown in Figure 1(C), incubation of the −73/−40 A-II probe with rat liver nuclear extracts resulted in the formation of two DNA–protein complexes (lane 1). One of them had the same electrophoretic mobility as RXRα/TβR heterodimers expressed in COS-7 cells (cf. lanes 1 and 3). The mobility of this complex was supershifted by antibodies against RXRα (lane 4), TβR (lane 5) or both (lanes 2 and 6), thus confirming the presence of RXRα and TβR in this complex. A similar pattern of DNA-protein complexes was observed using nuclear extracts from HepG2 cells and the −73/−40 apoA-II probe (Figure 1D, lane 1). The formation of both complexes was competed by the WT −73/−40 apoA-II probe (Figure 1D, lane 2). These results indicate that the −73/−40 apoA-II promoter region contains an HRE that binds RXRα/TβR heterodimers present in nuclear extracts from liver cells.

**Transactivation of the −911/+29 apoA-II promoter by RXRα/TβR heterodimers in the presence of T3 and RA**

To investigate the role of this proximal HRE in apoA-II promoter regulation by retinoids and thyroids, transient transfections were performed on HEK-293 cells. The activity of the −911/+29 human apoA-II promoter/enhancer is low in these cells owing
to the absence of liver-specific factors and thus induction by retinoids and thyroids can be easily scored. As shown in Figure 2(B), co-transfection of HEK-293 cells with a reporter plasmid containing the −911/+29 apoA-II promoter, fused with the CAT gene along with expression vectors for human RXRα and T3Rβ, resulted in strong transactivation of this promoter in the presence of the ligands RA and T3 (33- and 54-fold respectively). Importantly, this ligand-dependent transactivation of the apoA-II promoter by RXRα/T3Rβ heterodimers was totally abolished by the BM1 mutation that disrupts the proximal HRE.

In previous studies, we had established that the distal element AIIJ of the human apoA-II promoter is recognized by the orphan nuclear receptors HNF-4, ARP-1, EAR-2 and EAR-3 [21]. HNF-4 transactivated, whereas ARP-1, EAR-2 and EAR-3 repressed apoA-II promoter activity in HepG2 cells [21]. The same element (AIIJ) is also bound strongly by RXRα/RARα heterodimers and weakly by RXRα homodimers as well as by heterodimers of ultraspireacle, which is a Drosophila analogue of RXRα, with T3Rβ [22,23]. Combination of RXRα/RARα heterodimers or RXRα homodimers in the presence of RA or by agonists of RXRα, but not by agonists of RARα increased apoA-II promoter activity as well as the steady-state apoA-II mRNA levels and apoA-II secretion in HepG2, COS and SL2 cells [22]. In agreement with our previous findings, deletion of element AIIJ of the apoA-II enhancer (−911/+29 A-II CAT), decreased significantly (by 80%) the response to RA, whereas the response to T3 remained high (36-fold) (Figure 2B).

Our findings strongly suggested that in addition to the distal HRE which mediates apoA-II gene regulation by retinoids, the apoA-II promoter contains a proximal HRE that mediates apoA-II promoter induction specifically in response to thyroid hormone.

**Specificity of the proximal −67−55 and the distal −721−733 HREs of the apoA-II promoter for thyroids and retinoids respectively**

To investigate further the specificity of the two HREs present on the apoA-II promoter (elements AIIAB and AIIJ) for retinoids and thyroids, transient transfections were performed in COS-7 cells using reporter constructs containing multiple copies of each HRE linked to a heterologous promoter. As shown in Figure 3, a reporter construct containing three tandemly spaced apoA-II AB regulatory elements linked to the tk (Figure 3A) was transactivated strongly by RXRα/T3Rβ heterodimers in the presence of T3 (16-fold). In contrast, a minimal transactivation of this artificial promoter was observed by RXRα/T3Rβ heterodimers in the presence of RA or by T3Rβ homodimers in the presence of T3 (2.2- and 1.8-fold respectively). These results confirmed that the proximal AIIAB-regulatory element contains a functional thyroid HRE.

![Figure 3 Transactivation of the All(AB)3tk in COS-7 cells by RXRα/T3Rβ heterodimers in the presence of T3](image-url)
In gel EMSAs, the apoA-II element AIIJ bound RXRα/T, Rβ heterodimers in COS-7 cells, but did not bind RXRα or T, Rβ homodimers in accordance with previous analyses using heterodimers of T, Rβ with the *Drosophila* RXRα homologue ultraspiracle [23]. Furthermore, the addition of the ligands (RA or T3) did not change the affinity or the specificity of the nuclear receptors for the AIIJ element. As shown in Figure 4(B), RXRα/ T, Rβ heterodimers transactivated the AII(J)tk strongly in the presence of RA but not in the presence of T3. Similar observations were made in HEK-293 cells (results not shown).

The findings of Figures 3 and 4 indicate that the two HREs present on the human apoA-II promoter, i.e. the proximal element AIAB and the distal element AIIJ, both bind RXRα/T, Rβ heterodimers with high affinity and specificity and respond selectively to thyroids and retinoids respectively.

**Ligand-dependent synergistic transactivation of the apoA-II promoter by RXRα/T, Rβ heterodimers and the ubiquitous transcription factor USF2a**

The regulatory element AIAB (−73/−40) is a composite element that contains overlapping binding sites for RXRα/T, Rβ heterodimers (in the present study) SREBP [19] and USF [18]. Transcription factor USF appears to be the predominant bHLH/ZIP factor in liver nuclear extracts [29] and has three isoforms designated USF1, USF2a and USF2b that are encoded by two different genes, with apparent molecular masses of 43, 44 and 38 kDa respectively [30–32]. Although ubiquitously expressed, USF has been involved in the tissue-specific expression of many genes [32,33]. The USF binding site in element AIAB (GTCACTTG) contains the CANNTG consensus E-box motif, which is recognized by basic helix–loop–helix transcription factors. USF2a binds to two additional elements of the apo-A-II enhancer (elements AIIK and AIIIL) [18]. Binding of USF to the element AIIK was found to be enhanced by the simultaneous binding of the orphan nuclear receptor HNF-4 to the adjacent element AIIJ [18]. Co-operative binding of USF and HNF-4 was shown to result in the synergistic transactivation of the apoA-II promoter, possibly owing to physical and functional interactions between these two transcription factors [18].

In agreement with previous findings, USF2a strongly transactivated the −911/+29 apo-A-II promoter in HEK-293 cells (Figure 5), in COS-7 cells or in HepG2 cells (results not shown). Interestingly, co-expression of USF2a and RXRα/T, Rβ heterodimers in HEK-293 cells resulted in a strong (39-fold), T3-dependent, synergistic transactivation of the apoA-II promoter. In the absence of USF2α, transactivation of the apoA-II promoter by RXRα/T, Rβ heterodimers in this experiment was 9-fold in the presence of T3, whereas transactivation by USF2a alone was 8-fold. This finding suggested functional interactions between RXRα/T, Rβ heterodimers and USF2a, which modulate the response of the apoA-II promoter to thyroids. Deletion of regulatory element AIAB (−911/+29 ΔAB AII-CAT) abolished the transactivation of the apoA-II promoter by RXRα/ T, Rβ heterodimers or USF2a as well as the synergism between these transcription factors. In contrast, deletion of element AIIJ of the apoA-II enhancer had no effect on the synergistic transactivation of the apoA-II promoter by RXRα/T, Rβ heterodimers and USF2a.

The results presented in Figure 5 imply that functional interactions between USF2a and RXRα/T, Rβ heterodimers bound to overlapping sites on regulatory element AIAB may modulate apoA-II gene transcriptional induction in response to thyroid hormone.

---

**Figure 4** Transactivation of the AII(J)tk in COS-7 cells by RXRα/T, Rβ heterodimers in the presence of RA

(A) Gel EMSAs using the −73/−40 apoA-II probe (element AIJ) and extracts of COS-7 cells transfected with RXRα and T3Rβ in the absence or in the presence of the corresponding ligands RA and T3 are shown. An arrow shows the positions of the band shifted by RXRα/T, Rβ heterodimers. (B) COS-7 cells were transiently co-transfected with 3 μg of the AII(J)tk CAT plasmid along with 2 μg of GMV pGAl plasmid and 0.5 μg of the indicated pMT2 plasmids expressing human RXRα and human T3Rβ or the expression vector pMT2 alone in the presence or absence of RA and T3. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means ± S.E.M. from at least two independent transfections performed in duplicate are presented in the form of bar graphs. Fold transactivations achieved by nuclear receptors in the absence or in the presence of their ligands are shown in parentheses on top of each bar. (C) Schematic representation of the AII(J)tk CAT construct used in the transactivation experiments of (B).

A similar analysis was performed in COS-7 cells using an artificial promoter containing four copies of the apoA-II-regulatory element J linked to the tk [AII(J)4tk-CAT] (Figure 4C [23]).
GAL4-based transactivation assays established ligand-dependent positive and negative interactions of USF2a with T3Rβ and RXRα respectively

To investigate further potential functional and physical interactions between USF2a and nuclear receptors RXRα and T3Rβ, a GAL4-based transactivation system was employed. For this purpose, we utilized hybrid proteins consisting of the DBD of the yeast transactivator GAL4 fused with the hinge and the ligand-binding domains (domains D and E respectively) of RXRα and T3Rβ (Figure 6A). In transient transfections of HEK-293 cells, GAL4(DBD) RXRα(D/E) and GAL4(DBD) T3Rβ(D/E) hybrid proteins transactivated efficiently a homopolymeric promoter consisting of five tandem GAL4-binding elements placed in front of the minimal E1B promoter (pG5B-Luc) in a ligand-dependent manner (Figure 6B). Co-expression of GAL4(DBD) RXRα(D/E) and USF2a in HEK-293 cells caused a marked inhibition of the RA-responsive AII(J)4tk-CAT homopolymeric promoter or the AII(J)4tk-CAT promoter co-operatively with RXRα/T3Rβ heterodimers in response to RA. In contrast, USF2a transactivated the −80/+29 apoA-II promoter co-operatively with RXRα/T3Rβ only in the presence of T3 (Figure 8).

In summary, the findings of Figures 5–8 suggest that functional interactions between USF2a and the nuclear receptors RXRα and T3Rβ may modulate the response of these receptors to their ligands RA and T3 in a negative or positive manner respectively. A putative model of regulation of the apoA-II gene by nuclear receptors and USF2a is shown schematically in Figure 9.

The molecular mechanism by which USF2a interferes with the transactivation function of RXRα/T3Rβ heterodimers in the presence of their ligands (RA and T3) is not yet known. Crystalllographic studies of nuclear receptors in the absence or in the presence of their ligands have established that binding of the ligand to a hydrophobic cleft in the ligand-binding domain of the nuclear receptor causes a conformational change in this domain that results in the replacement of co-repressors bound to the activation domain of RXRα/T3Rβ heterodimers in the activated state. On the other hand, it is possible that interaction of RXRα with USF2a could result in the stabilization of the ligand-binding domain of RXRα in the repressed state.

Is direct binding of USF2a to the apoA-II promoter essential for its regulatory effect on apoA-II promoter induction by thyroids or its repression by retinoids? The apoA-II promoter contains three regulatory sites present on elements AB, K and L, which were shown previously to bind efficiently USF2a [18]. The experiment of Figure 5 showed that USF2a transactivated the −911/+29 apoA-II promoter that contains all three sites strongly (8-fold) in the absence of co-expressed RXRα/T3Rβ heterodimers. Deletion of the proximal element AB totally abolished the transactivation of the −911/+29 apoA-II promoter by USF2a, whereas an apoA-II promoter containing only the element AIIAB...
Specificity of apoA-II HREs for retinoids and thyroid.

Figure 6 Positive and negative interactions of USF2a with T3Rβ and RXRα respectively in GAL4 transactivation assays

(A) Schematic representation of the expression vectors and the reporter used in the transactivation experiment of (B) are shown. In (B) HEK-293 cells transiently co-transfected with 3 µg of the pG5B-Luc reporter are shown along with 2 µg of CMV pGal plasmid and 0.5 µg of GAL4(DBD) RXRα(D/E), GAL4(DBD) T3Rβ(D/E) and USF2a in the presence or absence of T3 and/or RA as indicated at the bottom of the graph. Forty hours after transfection, cells were harvested and the relative luciferase activity was determined in the cell lysate. Means ± S.E.M. from at least two independent transfections performed in duplicate are presented in the form of a bar graph. Fold transactivation is shown in parentheses on top of each bar.

was transactivated by USF2a only by 4-fold. These findings suggest that binding of USF2a to multiple sites on the apoA-II promoter, particularly to element AB, is essential, and that optimal transactivation of the apoA-II promoter by this factor is probably achieved by co-operative interactions between USF2a molecules bound to proximal and distal sites. On the other hand, the GAL4-based transactivation experiments of Figure 6 as well as the experiment involving the artificial AI(J)4tk-CAT reporter that lacks USF2a-binding sites (Figure 7) favour a complementary function of USF2a, i.e. its ability to modulate positively or negatively the activity of the ligand-bound hormone nuclear receptors RXRα and T3Rβ in a DNA-binding-independent fashion. This co-activator–co-repressor function of USF2a seems to be determined by the identity of the HRE as well as by the ligand. Based on our findings, we could hypothesize that USF2a may regulate the retinoid or thyroid inducibility of other genes that contain HREs but no USF2a binding sites on their promoters, but this hypothesis needs to be investigated further experimentally.

Figure 7 Inhibition of RA signalling by USF2a

HEK-293 cells were transiently co-transfected with 3 µg of the AI(J)4tk-CAT plasmid along with 2 µg of CMV pGal plasmid and 0.5 µg of vectors expressing RXRα, T3Rβ and USF2a in the presence or absence of RA and T3. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means ± S.E.M. from at least two independent transfections performed in duplicate are presented in the form of a bar graph. Fold transactivation is shown in parentheses on top of each bar.

Figure 8 Activation of T3 signalling by USF2a

HepG2 cells were transiently co-transfected with 3 µg of (−80/ +29) All-CAT plasmid along with 2 µg of CMV pGal plasmid and 0.5 µg of vectors expressing RXRα, T3Rβ and USF2a in the presence or absence of T3. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means ± S.E.M. from at least two independent transfections performed in duplicate are presented in the form of a bar graph. The fold transactivation is shown in parentheses on top of each bar.
uninduced and induced states of transcriptional activity of RXRα or a co-repressor, which can play important roles during the repression of USF2α in the cells could quench out a co-activator function positively or negatively. For instance, overexpression of USF2α is that USF2α could interact physically and α independent mode of regulation of RXRα transcriptional activity by USF2α is that USF2α could interact physically and functionally with nuclear receptor co-activators and modulate their function positively or negatively. For instance, overexpression of USF2α in the cells could quench out a co-activator or a co-repressor, which can play important roles during the uninduced and induced states of transcriptional activity of RXRα/T,Rβ heterodimers. In fact, it has been shown previously that USF2α interacts functionally with the p300 co-activator to regulate the expression of the Fl,F2 ATP synthase α-subunit [35]. More recently, it was shown that USF2α interacts physically and functionally with the peroxisome-proliferator-activated receptor γ-co-activator 1 to inhibit the transcription of carnitine palmitoyltransferase-I [36]. Again, such a role for USF2α could have a major impact in the regulation of gene expression by steroid/thyroid hormones.

Functional interactions between USF and ligand-dependent nuclear receptors have been reported to regulate the response of cathespins D to oestrogens, a process that may be relevant to breast cancer [37]. Cathespin D is a ubiquitous lysosomal protease, which is closely associated with a poor clinical outcome for patients with breast cancer [37]. The cathespin D promoter contains binding site for USF-1 and USF-2. Point mutations within the USF-binding site in the cathespin D promoter abolished USF binding and reduced the levels of both basal transcription and oestrogen-stimulated transcription of the cathespin D gene in MCF-7 cells [37].

Potential role of thyroids in triacylglycerol metabolism

ApoA-II is an important protein component of HDL. Genetic studies in mice and humans have pointed out that apoA-II may be a risk factor for atherosclerosis [3–4]. ApoA-II overexpression in the liver may alter the structure and functions of HDL and may alter the risk for coronary artery disease through a mechanism that is not fully understood at the present time. In a previous study, it was shown that apoA-II gene transcription in rat liver was increased transiently following a single saturating dose of T₃ [38]. It has also been shown previously that administration of T₃ to hyperthyroid animals increased hepatic triacylglycerol secretion [39,40]. This observation may be relevant to recent findings showing that overexpression of human apoA-II in transgenic mice decreased low plasma HDL and caused post-prandial hypertriglyceridaemia [8] owing to incorporation of apoA-II in very-low-density lipoprotein and inhibition of lipoprotein lipase. These observations indicate that alterations in apoA-II gene expression by thyroids may be a contributing factor in hypertriglyceridaemia observed in diseases such as Type II diabetes.

This work was funded by a grant from the Greek Ministry of Research and Technology. D.K. and V.Z. were supported by Institute of Molecular Biology and Biotechnology of Crete internal funds, and G.K. was partially supported by a graduate fellowship in the context of the Inter-Departmental Graduate Program in Molecular Biology and Biomedicine of the Departments of Medicine and Biology of the University of Crete. V.Z. was also supported by an NIH grant (HL33952). We also thank Dr H. Gronemeyer for reagents and P. Papakosta for excellent technical assistance.

REFERENCES

Specificity of apoA-II HREs for retinoids and thyroids


23 Hatzivassiliou, E., Cardot, P., Zannis, V. I. and Mitsialis, S. A. (1997) Ultraspiracle, a Drosophila retinoic X receptor α homologue, can mobilize the human thyroid hormone receptor to transactivate a human promoter. Biochemistry 36, 9221–9231


