Functional specificity of two hormone response elements present on the human apoA-II promoter that bind retinoid X receptor α /thyroid receptor β heterodimers for retinoids and thyroids: synergistic interactions between thyroid receptor β and upstream stimulatory factor 2a

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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 RXR α (retinoid X receptor α)/T₃R β (thyroid receptor β) heterodimers and weakly T₃R β homodimers, but does not bind other homo- or heterodimers of RXR α or orphan nuclear receptors. Transactivation was abolished by point mutations in the thyroid HRE. In co-transfection experiments of HEK-293 (human embryonic kidney 293) cells, the –911/+29 human apoA-II promoter was transactivated strongly by RXR α /T₃R β heterodimers 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 AIIJ) apoA-II promoter could be transactivated by RXR α /T₃R β heterodimers in COS-7 cells only

in the presence of T₃ or RA respectively. RXR α /T₃R β 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–T₃R β fusion protein in the presence of T₃ and suppressed the activity of a GAL4–RXR α 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 RXR α /T₃R β 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 AIIJ–AIIIN) 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 AIIAB, AIIK and AIIJ [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 AIIAB (–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 AIIJ of the apoA-II enhancer [21,22]. This element contains an imperfect direct repeat AGGGTA(A)AGGTTG between nt –721/–733 on the non-coding strand with DR-1 (direct repeat with 1 nucleotide spacing) and binds orphan receptors HNF-4 (hepatocyte 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; RXR α , retinoid X receptor α ; SREBP, sterol-regulated-element-binding protein; T₃, tri-iodothyronine; T₃R β , thyroid receptor β ; tk, thymidine kinase minimal promoter; USF, upstream stimulatory factor; WT, wild-type.

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nuclear receptors [21–23]. In a previous study, we have shown that the distal element AIII, as well as the proximal element AIIAB, bind heterodimers of the *Drosophila* homologue of RXR α (retinoid X receptor α) ultraspiracle with T₃R β (thyroid receptor β) [23].

In the present study, we present evidence that the proximal apoA-II regulatory region AIIAB contains a functional thyroid HRE (hormone response element) which strongly binds RXR α /T₃R β 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 AIII and AIIAB) 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 T₃R β and RXR α respectively.

MATERIALS AND METHODS

Reagents were purchased from the following sources: polyclonal anti-RXR α and anti-T₃R β 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 Minotect (Crete, Greece) and New England Biolabs. Poly(dI/dC), acetyl-CoA, dNTPs, [α -³²P]dCTP and [¹⁴C]chloramphenicol were purchased from Amersham Biosciences (Uppsala, Sweden). All reagents for cell culture [DMEM (Dulbecco's modified Eagle's medium), foetal bovine serum, trypsin-EDTA, PBS, 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) Δ J A-II-CAT that contains a deletion of regulatory element AIII, (–911/+29) Δ AB A-II-CAT that contains a deletion of regulatory element AIIAB, (–911/+29) BM1 A-II-CAT that contains nucleotide substitutions in element AIIAB, A-II(J)₄tk-CAT (where tk stands for thymidine kinase minimal promoter) that contains the tk under the control of four copies of the apoA-II regulatory element AIII and (–80/+29) A-II-CAT have been described previously [16,18,22]. Plasmid A-II (AB)₃tk-CAT that contains the tk under the control of three copies of the apoA-II regulatory element AIIAB was constructed by inserting a synthetic double-stranded oligonucleotide containing the –73 to –40 regulatory region of human apoA-II at the *Xba*I site of the vector pBLCAT2. The sequence of the sense strand of the oligonucleotides used for cloning is 5'-CTAGAGATATCCATAGTCCTGTACCTGACAGGGGTGGGTAT-3'. The number and orientation of the inserted oligonucleotides were verified by sequencing. The expression vectors pMT2-HNF-4, pMT2-ARP-1, pMT2-RXR α , pMT2-RAR α and pMT2-T₃R β have been described previously [21,24]. The expression vector for human USF2a was described previously [18]. Plasmids GAL4(DBD)-RXR α (D/E) and GAL4(DBD)-T₃R β (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 RXR α and chicken T₃R β 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 μ g each of the expression vectors for the hormone nuclear receptors HNF-4, ARP-1, RXR α , RAR α and T₃R β or left untransfected. Forty hours after transfection, cells were collected in 40 mM Tris/HCl (pH 7.4)/1 mM EDTA (pH 8)/0.15 M NaCl and pelleted by low-speed centrifugation. Cells were resuspended in 400 μ l of whole-cell extract lysis buffer [20 mM Tris/HCl, pH 7.4/0.4 M KCl/2 mM DTT/10 % (v/v) glycerol and protease inhibitors] and were broken by three freeze-thaw cycles. 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 [α -³²P]dCTP. The sequence of the sense oligonucleotides, used as probes, is as follows:

A-II (–73/–40) WT (wild-type): 5'-CTGAATCCATAGTC-CTGTACCTGACAGGGGGTGGGTAT-3';

A-II (–73/–40) BM1: 5'-CTAGATCCATAGTCAGAGTACCTGACAGGGGGTGGGTAT-3'.

The underlined sequence in oligonucleotide BM1 indicates a mutation. For supershift assays, 1 μ l of polyclonal anti-RXR α or anti-T₃R β 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 AIIAB (–73/–40) of the human apoA-II promoter binds RXR α /T₃R β heterodimers strongly and specifically and T₃R β homodimers weakly

The proximal region of the human apoA-II promoter between nt –73 and –40 contains overlapping binding sites for the transcription factors USF and SREBP [18,19] (Figure 1A). This region

Figure 1 Binding of hormone nuclear receptors to the regulatory element AIIAB of the human apoA-II promoter

(A) Schematic representation of the $-911/-33$ apoA-II promoter region and the sequence of the WT and mutated $-73/-40$ region (element AIIAB). The nucleotide substitutions were introduced in residues $-63/-59$ of element AIIAB and the mutation was designated BM1. Binding of transcription factors SREBP and USF1/USF2 to the apoA-II promoter is shown by brackets. Arrows show the position of the HRE within the $-73/-40$ region. (B) DNA-binding gel EMSAs using WT and mutated oligonucleotides AIIAB ($-73/-40$) as probes and extracts of COS-7 cells expressing HNF-4, ARP-1, RXR α , RXR α /RAR α , T $_3$ R β and RXR α /T $_3$ R β . A double-stranded oligonucleotide corresponding to the WT and mutated apoA-II region was labelled at both ends with the Klenow fragment of the DNA polymerase I and [α - 32 P]dCTP and incubated with $1 \mu\text{l}$ of COS-7 extracts expressing nuclear receptors as indicated at the top of the Figure. Protein-DNA complexes were analysed by native polyacrylamide gels and detected by autoradiography. Note strong binding of RXR α /T $_3$ R β heterodimers, weak binding of T $_3$ R β and no binding of RXR α , RXR α /RAR α and orphan nuclear receptors HNF-4 and ARP-1. Also note that binding of RXR α /T $_3$ R β was greatly diminished by mutations within the HRE of element AIIAB. (C, D) DNA-binding gel EMSAs, competition and supershift assays using the WT oligonucleotide $-73/-40$ as probe. (C) Gel EMSAs and supershift assays using the $-73/-40$ apoA-II probe, rat liver nuclear extracts (RLNE) or extracts of COS-7 cells transfected with RXR α and T $_3$ R β and anti-RXR α or anti-T $_3$ R β antibodies. Arrows show the position of the band shifted by RXR α /T $_3$ R β heterodimers and supershifted by the anti-RXR α and anti-T $_3$ R β antibodies. (D) Gel EMSAs using nuclear extracts obtained from HepG2 cells and the WT oligonucleotide $-73/-40$ as probe. Competition was performed using 100-fold molar excess of the unlabelled WT $-73/-40$ apoA-II-oligonucleotide.

contains an imperfect DR-1 HRE between nt -67 and -55 having the sequence 5'-AGTCCTGTACCT-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, RXR α , RXR α /RAR α , RXR α /T $_3$ R β and T $_3$ R β nuclear receptors. This analysis showed that element $-73/-40$ (previously designated AIIAB) binds RXR α /T $_3$ R β heterodimers strongly and T $_3$ R β homodimers weakly, but does not bind any of the orphan (HNF-4 and ARP-1) or the other ligand-dependent (RXR α /RAR α) 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 RXR α /T $_3$ R β heterodimers or T $_3$ R β 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 RXR α /T $_3$ R β heterodimers expressed in COS-7 cells (cf. lanes 1 and 3). The mobility of this complex was supershifted by antibodies against RXR α (lane 4), T $_3$ R β (lane 5) or both (lanes 2 and 6), thus confirming the presence of RXR α and T $_3$ R β 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 (lane 2). These results indicate that the $-73/-40$ apoA-II promoter region contains an HRE that binds RXR α /T $_3$ R β heterodimers present in nuclear extracts from liver cells.

Transactivation of the $-911/+29$ apoA-II promoter by RXR α /T $_3$ R β heterodimers in the presence of T $_3$ 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

Figure 2 Transactivation of the WT and mutated $-911/+29$ apoA-II promoter in HEK-293 cells by RXR α /T $_3$ R β heterodimers in the presence of T $_3$ and RA

(A) ApoA-II promoter CAT constructs used in the transfection experiments shown in (B). In the $(-911/+29)$ AII(Δ J) CAT construct, the element AIIJ was deleted. In the $(-911/+29)$ BM1 AII CAT construct, the element AIIAB ($-73/-40$) was mutated as shown in Figure 1(A). (B) HEK-293 cells were transiently co-transfected with 3 μ g of the $-911/+29$ apoA-II CAT plasmids along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of the indicated pMT2-RXR α , and pMT2-T $_3$ R β vectors or the expression vector pMT2 alone in the presence or absence of T $_3$ and/or RA as indicated. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means $\pm$ 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.

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 RXR α and T $_3$ R β , resulted in strong transactivation of this promoter in the presence of the ligands RA and T $_3$ (33- and 54-fold respectively). Importantly, this ligand-dependent transactivation of the apoA-II promoter by RXR α /T $_3$ R β 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 RXR α /RAR α heterodimers and weakly by RXR α homodimers as well as by heterodimers of ultraspiracle, which is a *Drosophila* analogue of RXR α , with T $_3$ R β [22,23]. Combination of RXR α /RAR α heterodimers or RXR α homodimers in the presence of RA or by agonists of RXR α , but not by agonists of RAR α increased apoA-II promoter activity as

Figure 3 Transactivation of the AII(AB)₃tk in COS-7 cells by RXR α /T $_3$ R β heterodimers in the presence of T $_3$

(A) Schematic representation of the AII(AB)₃tk CAT construct used in the transactivation experiments of (B). (B) COS-7 cells were transiently co-transfected with 3 μ g of the AII(AB)₃tk CAT plasmid along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of the indicated pMT2 plasmids expressing human RXR α and human T $_3$ R β or the expression vector pMT2 alone in the presence or absence of RA and T $_3$. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means $\pm$ S.E.M. from at least two independent transfections performed in duplicate are presented in the form of a bar graph. Fold transactivations achieved by the nuclear receptors in the absence or in the presence of their ligands are shown in parentheses on top of each bar.

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 ($-911/+29$ Δ J A-II CAT), decreased significantly (by 80%) the response to RA, whereas the response to T $_3$ 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 RXR α /T $_3$ R β heterodimers in the presence of T $_3$ (16-fold). In contrast, a minimal transactivation of this artificial promoter was observed by RXR α /T $_3$ R β heterodimers in the presence of RA or by T $_3$ R β homodimers in the presence of T $_3$ (2.2- and 1.8-fold respectively). These results confirmed that the proximal AIIAB-regulatory element contains a functional thyroid HRE.

Figure 4 Transactivation of the AII(J)₄tk in COS-7 cells by RXRα/T₃Rβ heterodimers in the presence of RA

(A) Gel EMSAs using the -738/-712 apoA-II probe (element AIIJ) 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/T₃Rβ heterodimers. (B) COS-7 cells were transiently co-transfected with 3 μg of the AII(J)₄tk CAT plasmid along with 2 μg of CMV βGal 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 the 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)₄tk 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]).

In gel EMSAs, the apoA-II element AIIJ bound RXRα/T₃Rβ heterodimers expressed in COS-7 cells, but did not bind RXRα or T₃Rβ homodimers in accordance with previous analyses using heterodimers of T₃Rβ 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/T₃Rβ heterodimers transactivated the AII(J)₄tk 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 AIIAB and the distal element AIIJ, both bind RXRα/T₃Rβ 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/T₃Rβ heterodimers and the ubiquitous transcription factor USF2a

The regulatory element AIIAB (-73/-40) is a composite element that contains overlapping binding sites for RXRα/T₃Rβ 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 AIIAB (GTCACCTG) 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 apoA-II enhancer (elements AIIK and AIIJ) [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 apoA-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/T₃Rβ heterodimers in HEK-293 cells resulted in a strong (39-fold), T₃-dependent, synergistic transactivation of the apoA-II promoter. In the absence of USF2a, transactivation of the apoA-II promoter by RXRα/T₃Rβ 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/T₃Rβ heterodimers and USF2a, which modulate the response of the apoA-II promoter to thyroids. Deletion of regulatory element AIIAB (-911/+29 ΔAB AII-CAT) abolished the transactivation of the apoA-II promoter by RXRα/T₃Rβ 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/T₃Rβ heterodimers and USF2a.

The results presented in Figure 5 imply that functional interactions between USF2a and RXRα/T₃Rβ heterodimers bound to overlapping sites on regulatory element AIIAB may modulate apoA-II gene transcriptional induction in response to thyroid hormone.

Figure 5 Ligand-dependent synergistic transactivation of the apoA-II promoter by RXR α /T $_3$ R β heterodimers and the ubiquitous transcription factor USF2

HEK-293 cells were transiently co-transfected with 3 μ g of the (-911/+29) A-II CAT, (-911/+29) Δ AB A-II CAT or (-911/+29) Δ J A-II CAT plasmids along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of the indicated expression vectors for RXR α , T $_3$ R β and USF2a in the presence and absence of T $_3$ and/or RA as indicated. Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means $\pm$ 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.

GAL4-based transactivation assays established ligand-dependent positive and negative interactions of USF2a with T $_3$ R β and RXR α respectively

To investigate further potential functional and physical interactions between USF2a and nuclear receptors RXR α and T $_3$ R β , 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 RXR α and T $_3$ R β (Figure 6A). In transient transfections of HEK-293 cells, GAL4(DBD) RXR α (D/E) and GAL4(DBD) T $_3$ R β (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) RXR α (D/E) and USF2a in HEK-293 cells caused a marked inhibition of the RA-dependent transactivation of the pG5B reporter. In contrast, co-expression of GAL4(DBD) T $_3$ R β (D/E) and USF2a caused a strong synergistic transactivation of the pG5B reporter in a T $_3$ -dependent manner.

To investigate further the negative and positive crosstalk between USF2a and the RA or T $_3$ -signalling pathways, transient transfections were performed in HEK-293 and HepG2 cells using the RA responsive AII(J) $_4$ -CAT homopolymeric promoter or the -80/+29 apoA-II promoter containing a single copy of element AIIAB. As shown in Figure 7, USF2a totally abolished the transactivation of AII(J) $_4$ homopolymeric promoter by RXR α /T $_3$ R β heterodimers in response to RA. In contrast, USF2a transactivated the -80/+29 apoA-II promoter co-operatively with RXR α /T $_3$ R β only in the presence of T $_3$ (Figure 8).

In summary, the findings of Figures 5–8 suggest that functional interactions between USF2a and the nuclear receptors RXR α and T $_3$ R β may modulate the response of these receptors to their ligands RA and T $_3$ 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 RXR α /T $_3$ R β heterodimers in the presence of their ligands (RA and T $_3$) is not yet known. Crystallographic 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 function 2 of this receptor by co-activators [34]. We could speculate that the interaction of T $_3$ R β with USF2a may result in the stabilization of the ligand-binding domain of T $_3$ R β in the activated state. On the other hand, it is possible that interaction of RXR α with USF2a could result in the stabilization of the ligand-binding domain of RXR α 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 RXR α /T $_3$ R β 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

Figure 6 Positive and negative interactions of USF2a with $T_3R\beta$ and $RXR\alpha$ 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 μ g of the pG5B-Luc reporter are shown along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of GAL4(DBD) $RXR\alpha$ (D/E), GAL4(DBD) $T_3R\beta$ (D/E) and USF2a in the presence or absence of T_3 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 $\pm$ 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 AII(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 $RXR\alpha$ and $T_3R\beta$ 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 μ g of the AII(J)₄tk CAT plasmid along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of vectors expressing $RXR\alpha$, $T_3R\beta$ and USF2a in the presence or absence of RA and T_3 . Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means $\pm$ 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 T_3 signalling by USF2a

HepG2 cells were transiently co-transfected with 3 μ g of (-80/+29) AII-CAT plasmid along with 2 μ g of CMV β Gal plasmid and 0.5 μ g of vectors expressing $RXR\alpha$, $T_3R\beta$ and USF2a in the presence or absence of T_3 . Forty hours after transfection, cells were harvested and the CAT activity was determined in the cell lysate. Means $\pm$ 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.

Figure 9 Putative model of apoA-II gene regulation by RXRα/T₃Rβ in response to retinoids and thyroids and the potential role of USF2a as a modulator of these processes

White boxes show regulatory elements AIII and AIIAB of the apoA-II promoter/enhancer. RXRα/T₃Rβ heterodimers are represented by light grey ovals. USF2a is represented by dark grey ovals. Double arrows show interactions between transcription factors. +, Positive interaction, which leads to transcriptional activation; −, negative interaction, which leads to transcriptional repression. Single arrows of various widths indicate the magnitude of the transcriptional response.

An alternative hypothesis that could explain the DNA-binding-independent mode of regulation of RXRα/T₃Rβ transcriptional activity by USF2a is that USF2a could interact physically and functionally with nuclear receptor co-activators and modulate their function positively or negatively. For instance, overexpression of USF2a 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/T₃Rβ heterodimers. In fact, it has been shown previously that USF2a interacts functionally with the p300 co-activator to regulate the expression of the F₁F₀ ATP synthase α-subunit [35]. More recently, it was shown that USF2a interacts physically and functionally with the peroxisome-proliferator-activated receptor γ-co-activator 1 to inhibit the transcription of carnitine palmitoyl-transferase-1β [36]. Again, such a role for USF2a 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 cathepsin D to oestrogens, a process that may be relevant to breast cancer [37]. Cathepsin D is a ubiquitous lysosomal protease, which is closely associated with a poor clinical outcome for patients with breast cancer [37]. The −124/−104 region of the cathepsin D promoter contains binding sites for USF-1 and USF-2. Point mutations within the USF-binding site in the cathepsin D promoter abolished USF binding and reduced the levels of both basal transcription and oestrogen-stimulated transcription of the cathepsin 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.

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