Regulation of ATP-Binding Cassette Transporter A1 Transcription by Thyroid Hormone Receptor†

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ABSTRACT: Transcriptional regulation of the ATP-binding cassette transporter (ABCA1) gene is complex. It involves multiple transcription start sites and the binding of several different transcription factors to the ABCA1 promoter region. Cholesterol- and oxysterol-mediated up-regulation of ABCA1 transcription includes the binding of the liver X receptor and retinoid X receptor (LXR/RXR) heterodimer to the DR-4 element of the ABCA1 promoter. In this study we show that another nuclear hormone receptor, thyroid hormone receptor (TR), can suppress ABCA1 transcription. Electrophoretic mobility shift assays using both purified proteins and isolated nuclear extracts from primary human fibroblasts and 293T cells demonstrate that the TR/RXR heterodimer is able to bind to the DR-4 element of the ABCA1 promoter. This binding is also demonstrated in vivo by chromatin immunoprecipitation studies. Luciferase assays from 293T cells transfected with TRβ or LXRα expression plasmids show that TR, together with its ligand T3, suppresses ABCA1 transcriptional activity, even in the presence of LXR-activating oxysterols. Finally, competition between TR/RXR and LXR/RXR heterodimers to suppress or activate ABCA1 transcription is shown to be dynamic and dependent on the amount of nuclear receptor present in the cells. These data identify a novel regulatory mechanism for ABCA1 and suggest new strategies to modify its expression.

Liver X receptor (LXR)[1] is a member of a nuclear receptor protein family regulating several genes involved in lipid and carbohydrate metabolism, inflammation, and other complex metabolic systems (*1*, *2*). LXR exists in two isoforms, α and β. It functions mainly as a heterodimer with the retinoid X receptor (RXR), which binds to a DR-4 element (direct repeat of hexameric AGGTCA or its derivative, with a 4-base spacing) (*3*, *4*). LXR may up- or down-regulate the transcription rates of individual target genes (*1*, *2*). Among those strongly up-regulated is the ATP-binding cassette transporter A1 (ABCA1) gene, whose product is a major determinant of high-density lipoprotein (HDL) concentration in plasma (*5*−*8*). Transcription of ABCA1 is also stimulated by oxysterols and retinoic acid, the ligands for LXR and RXR, respectively (*9*−*11*).

Thyroid hormone receptor (TR), a second nuclear receptor protein, also exists in multiple isoforms (α-1, α-2, β-1, β-2, β-3) (*12*, *13*). TRα plays a key role in postnatal development and cardiac metabolism. Among other actions, TRβ regulates multiple steps in hepatic metabolism as well as thyroid hormone levels (*13*). In addition to binding a promoter DR-4 element as a heterodimer with RXR, TR may also bind as a monomer or TR/TR homodimer (*14*, *15*). TR suppresses transcription from >80% of mouse liver genes responsive to thyroid hormone (*16*).

The regulation of ABCA1 expression is determined by multiple mechanisms. Effects at the level of protein and mRNA stability have been described (*17*−*21*). Selection between multiple transcriptional start sites by which DR-4 elements are spliced in to or out of the promoter is dynamically regulated (*22*−*24*). Though ABCA1, like other genes regulated by LXR, seems likely to respond competitively to the presence of other RXR heterodimers, this has not been previously studied, despite very wide interest in the expression of this clinically significant gene (*5*, *6*, *25*). In the present study, the ability of TR and LXR to compete for binding at the DR-4 site of ABCA1 is described.

EXPERIMENTAL PROCEDURES

Recombinant Proteins, Antibodies, and Promoter Constructs. Recombinant human RXRβ was obtained from Biomol. Recombinant chicken TRα was from Santa Cruz Biotechnology. Antibodies against nuclear receptor proteins (sc-553x against RXRα, sc-772x against TRα/β, and sc1201x against LXRα/β) were from Santa Cruz, and polyclonal anti-FLAG antibody was from Sigma. A human ABCA1 promoter insert (bases −844 to +188, numbering based on ref *26*) was prepared by PCR using primers with a *Hin*dIII site

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[1] Abbreviations: ABCA1, ATP-binding cassette transporter A1; EMSA, electrophoretic mobility shift assay; HDL, high-density lipoprotein; LXR, liver X receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; 22(*R*)-OH,22(*R*)-hydroxycholesterol.

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in the 5′-overhang and cloned into pGL3B-Basic vector (Promega) expressing the firefly luciferase gene. Site-directed mutagenesis of the ABCA1 DR-4 site (AGGTTActatAGGTCA) was carried out using the QuikChange mutagenesis kit (Stratagene) with the mutated oligo (5′-cgcgAGGTTActatCTGCAGaagcctgtgctctc-3′) (*10*). A promoter construct containing two consensus DR-4 elements was a gift from Dr. Paul Webb (University of California, San Francisco).

Cell Culture, Transfections, and Luciferase Assays. Primary human skin fibroblasts and transformed human embryonal kidney (293T) cells were cultured in DMEM medium containing 10% fetal calf serum and gentamycin (100 μg/mL). Transient transfections in 293T cells were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For the luciferase assays, 293T cells (1.5×10^5 cells/well in 24-well plates) were seeded each day prior to transfection. Serum-free medium supplemented with 2 mg/mL of T3-free bovine serum albumin (Sigma) was used for transfection. In some experiments, 10 μM 22(*R*)-hydroxycholesterol (Sigma) or 200 nM triiodothyronine (T3, Sigma) was included. The cells were transfected with 0.5 μg of expression plasmid for human TRβ or mouse LXRα (generous gifts from Dr. Keith Yamamoto, University of California, San Francisco, and Dr. Ronald Evans, Salk Institute, San Diego, respectively), 0.5 μg of firefly luciferase plasmid (pGL3-basic, Promega) under the control of experimental promoter sequence, and 0.1 μg of internal control plasmid pRL-TK (Promega). This plasmid expresses renilla luciferase under the control of thymidine kinase promoter. The cells were harvested 24 h following transfection, and expression of luciferases was assayed using a dual luciferase assay system (Promega). To make a stable LXRα overexpressing cell line, we cloned the FLAG-tagged LXRα into the pcDNA3 expression vector (Invitrogen), which contains the neomycin resistance gene for selection. Since the 293T cells already contained a neomycin resistance gene, we were unable to use this cell line. For this reason a stable cell line was generated from human embryonal kidney 293 cells by selecting the transfected cells with 1 mg/mL neomycin.

RT-PCR. RNA from 293T cells was isolated using RNeasy kit (Qiagen) and reverse transcribed using random hexamers with SuperScript II enzyme (Invitrogen) according to the manufacturer's instructions. PCR from 40 ng of transcribed original RNA was performed with Taq polymerase (Qiagen) using SYBR Green as fluophor in the Mx3000P real-time PCR system (Stratagene). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a control. PCR conditions were 95 °C for 2 min, and then 40 cycles of 95 °C for 15 s to 60 °C for 1 min. Melting curve analysis and separation of PCR products on agarose gel were used to validate the method. The PCR primers used were ABCA1 forward (5′-cgaagccacaaaaacatt-3′), ABCA1 reverse (5′-cctcataccagttgagagacttgat-3′), GAPDH forward (5′-cgagatccctccaaaatcaa-3′), and GAPDH reverse (5′-catgcgtccttccacgataccaa-3′).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from skin fibroblasts or 293T cells as previously described (*27*). The oligonucleotides containing the consensus DR-4 sequence (5′-agcttcAGGTCAcaggAGGTCAgagag-3′, Santa Cruz), wild-type ABCA1 DR-4 oligo (5′-gggAGGTTActatAGGTCActcga-3′), or mutated ABCA1 DR-4 oligo (5′-gggTAGTTActatACATCActcga-3′) were annealed to their reverse counterparts and labeled with ^{32}P. Assays (in 20 μL total volume) were carried out in 20 mM Hepes pH 7.9, 20 mM KCl, 1 mM spermidine, 0.1% NP-40, 3 μM dithiothreitol, and 10% glycerol, together with 1 μg of poly(dI-dC), 2×10^5 dpm-labeled probe, and nuclear extract or recombinant nuclear protein as described for individual experiments. After 30 min at room temperature, fractionation was carried out by electrophoresis on 6% nondenaturing polyacrylamide gels. In some experiments, excess cold oligonucleotide was added to the reaction mixture for 10 min prior to the addition of labeled probe. In other experiments, antibody protein (4 μg) was first added, and the reaction mixture was incubated overnight at 4 °C prior to the addition of labeled probe.

Chromatin Immunoprecipitation Assays. Precipitation was carried out as described by Boyd and Farnham (*28*) with slight modifications. 293T cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cells from ten 6-cm dishes transfected with TRβ or an equal number of cells stably expressing FLAG-LXRα were suspended in buffer containing 3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-HCl pH 7.4, and 0.1% NP-40, Dounce homogenized, and centrifuged at 3000*g* for 5 min at 4 °C. The resulting pellet was resuspended in PBS containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS and sonicated to yield chromatin fragments with an average length of 500 bp. After preclearing with blocked Pansorbin staphylococcus cells (CalBiochem), the cell extract was incubated overnight with 5 μg of antibody or nonimmune rabbit IgG. After coprecipitation of nuclear protein and its associated DNA, the Pansorbin cells were washed twice for 10 min at room temperature with 50 mM Tris-HCl pH 8.0, 2 mM EDTA, and 0.2% w/v sarkosyl, and four times with 100 mM Tris-HCl pH 9.0, 500 mM LiCl, 1% NP-40, and 1% deoxycholic acid. Cross-linked material was eluted with 1% SDS in 0.1 M $NaHCO_3$. Cross-linking was reversed in 0.3 M NaCl for 4 h at 67 °C. After ethanol precipitation, proteins were digested with proteinase K and DNA precipitated after phenol−chloroform extraction. The DNA was used as a template for PCR performed with rTth polymerase (Applied Biosystems). ABCA1 oligonucleotides (5′-cccaactccctagatgtgtc-3′ forward, 5′-ccactcactctcgctcgca-3′ reverse) spanning the promoter DR-4 element were used in the PCR.

RESULTS

Immunodepletion of Fibroblast Extracts. The possible association of different orphan receptor proteins with the DR-4 sequence of ABCA1 was first determined by immunodepletion, using nuclear extract from unstimulated fibroblasts. After preincubation with individual nuclear protein antibodies or nonspecific IgG, the reaction mixture was incubated with ^{32}P-labeled oligonucleotide and fractionated by EMSA on nondenaturing polyacrylamide gels. Following autoradiography, the signal from different incubations was quantitated by computerized densitometry. A significant depletion of label was obtained with anti-RXR and anti-TR antibodies, and a smaller effect was obtained with anti-LXR antibody (Figure 1). While abundant RXR protein was detected in fibroblast nuclear extract, TR and LXR levels were barely detectable. For this reason, subsequent experiments comparing TR and LXR competitive binding were

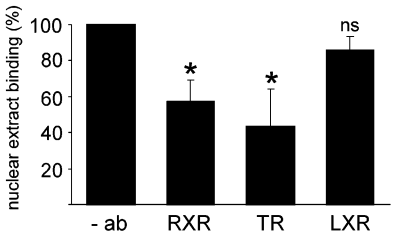

FIGURE 1: Depletion of nuclear extract binding to ABCA1 oligonucleotide with different antibodies. Nuclear extracts from noninduced human primary skin fibroblasts were isolated. EMSA was carried out as described under Experimental Procedures. The band intensity of the formed heterodimer was quantitated in the absence of antibody (−ab) or upon incubation with antibodies against RXRα, TRα/*β*, or LXRα/*β*. Results are shown relative to control and are from three independent experiments performed in triplicate. ∗, $p < 0.005$ by Student's *t*-test compared to control; ns, not significant.

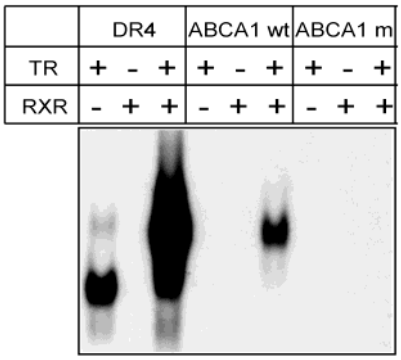

FIGURE 2: Binding of purified TRα and RXR*β* to ABCA1 promoter. A radioactively labeled consensus DR4 element (DR4) or the same element from ABCA1 gene (ABCA1 wt) was incubated with purified nuclear receptors either alone or in combination. The reactants were separated on a nondenaturing polyacrylamide gel, and the dried gel was exposed to X-ray film. A mutated ABCA1 DR4 element (ABCA1 m) was used to control the specificity of binding.

carried out either using recombinant nuclear proteins or 293T cells overexpressing TR or LXR.

EMSA with Purified Proteins. To obtain further information on the type of complexes formed between RXR and TR with the ABCA1 DR-4 element, recombinant RXR*β* and TRα proteins were incubated alone or in combination with DNA containing a consensus DR-4 sequence, or with wild-type or mutant ABCA1 promoter DR-4 element. When both TR and RXR were present, an intense band was seen with the consensus and wild-type oligonucleotides (Figure 2). Its migration rate was identical in both cases. This band was absent when mutant DNA was used. When only RXR was present, no binding to DNA was detected. This indicates that a RXR/RXR homodimer was not formed. Finally, the consensus DR-4 oligonucleotide, but not the wild-type or mutant ABCA1 sequences, formed products when only TR was present (Figure 2). On the basis of published results (*29*), the majority of these bands represent the TR monomer, and the weaker band represents the TR/TR homodimer. These data indicate that the heterodimer TR/RXR binds strongly and specifically to ABCA1 promoter. Differences in reactivity between the wild-type ABCA1 and consensus sequences probably reflect differences between DR-4 elements or the flanking sequences of these oligonucleotides.

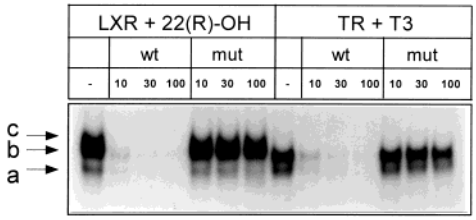

FIGURE 3: EMSA with nuclear extracts from transfected 293T cells. Nuclear extract from cells overexpressing LXRα with 22(*R*)-OH or TR*β* with T3 was prepared, and extracts were incubated with labeled oligonucleotide containing wild-type ABCA1 DR4 element. This binding was competed with increasing molar amounts (10-, 30- or 100-fold excess) of either cold wild-type (wt) or mutated (mut) oligo.

EMSA with Extracts of Overexpressing Cells. Nuclear extracts from either TR- or LXR-overexpressing 293T cells were incubated with labeled oligonucleotide whose sequence included the DR-4 element of ABCA1. In the case of LXR, three bands (a, b, and c) were observed (Figure 3, Figure 4A lower panel). These bands were diminished in the presence of unlabeled wild-type, but not mutant, oligonucleotide. In the presence of extract from TR-overexpressing cells, only bands a and b were present.

To further identify products containing TR, simultaneous experiments were carried out in which nuclear extracts from control or from LXR- or TR-overexpressing cells were preincubated with antibodies to TR or RXR, or with nonspecific IgG, prior to incubation with the labeled wild-type ABCA1 oligonucleotide. The presence of RXR antibody generated a supershifted band (f) in each case (Figure 4A). TR antibody generated an additional shifted band (d) in TR-overexpressing cells.

The absence of band c from control cell extract suggests that in the control 293T cells, most of the complex formed (bands a and b) between nucleoproteins and wild-type ABCA1 DR-4 oligonucleotide does not contain LXR. When the intensities of bands a and b were scanned, there was a 25% ± 3% ($n = 14$, $p < 0.001$) decrease in the intensity of band a in LXR-overexpressing cells. This may indicate that band a represents a heterodimer other than LXR/RXR and this complex is partially in competition with the newly formed LXR/RXR complex (band c) in LXR-overexpressing cells. The appearance of band f in the presence of anti-RXR antibody indicates that this complex contains RXR. Anti-TR antibody generated a similar shifted band, as did anti-RXR antibody in the TR-overexpressing cells. Two additional bands, band d present in TR-overexpressing cells in the presence of anti-TR and band e in RXR-overexpressing cells in the presence of anti-RXR, remain unidentified.

To further define the nature of complexes a and b in Figures 3 and 4A, we incubated either control or LXR-overexpressing nuclear extracts with purified TRα. Addition of increasing amounts of TRα led to the formation of a complex migrating with the same mobility as that of purified TR/RXR (band g in Figure 4B). Put together, these results from EMSA experiments indicate the following: (i) LXR/RXR complex (band c) is absent from basal cells and is detected in LXR-overexpressing cells. (ii) In TR-overexpressing cells, a portion of nuclear complexes a and/or b contains TR since they can be supershifted with anti-TR antibody (band f). However, the TR/RXR complex (band g)

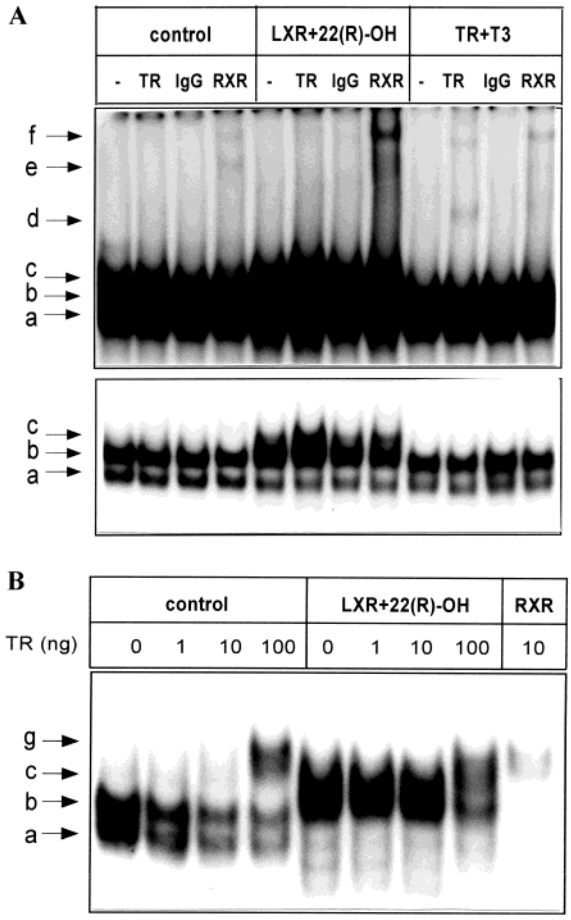

FIGURE 4: (A) EMSA with specific antibodies. Nuclear extracts from nontransfected cells (control) or cells overexpressing TRβ or LXRα were preincubated overnight with antibodies against nuclear receptors and then with the labeled ABCA1 DR4 oligonucleotide. Nonimmune rabbit IgG (IgG) was used as a negative control. Short exposure of the lower part of the gel (containing bands a, b, and c) allowed bands a, b and c to be distinguished. Shifted complexes (bands d, e, and f) detected on longer exposure are indicated with arrows. (B) Nuclear extracts from nontransfected cells (control) or cells overexpressing LXRα were preincubated with increasing amounts of purified TRα. Purified TRα and RXRβ were used on the right-most lane to compare the mobilities of the nuclear complexes to that of TR/RXR.

has a slower migration than either complex a or b. (iii) The complexes a and b (not representing TR/RXR or LXR/RXR) still remain to be identified.

Chromatin Immunoprecipitation Assays. To study the in vivo binding of nuclear complexes to the ABCA1 DR-4 element, we performed chromatin immunoprecipitation (ChIP) studies from control and TR- and LXR-overexpressing cells. Since available anti-LXR antibodies were ineffective to precipitate the cross-linked DNA−protein complexes (data not shown), anti-FLAG antibody was used to immunoprecipitate FLAG-tagged LXR from overexpressing cells. In TR-overexpressing cells, the binding of the TR/RXR complex to the DR-4 element in ABCA1 promoter was shown (Figure 5). In LXR-overexpressing cells, this element was occupied by the LXR/RXR complex. In control cells, only the binding of RXR to the DR-4 element was observed.

Taken together, the results from both EMSA and ChIP assays demonstrate that both TR/RXR and LXR/RXR complexes can bind the ABCA1 DR-4 element. The inability to detect band c (corresponding to LXR/RXR) in EMSA

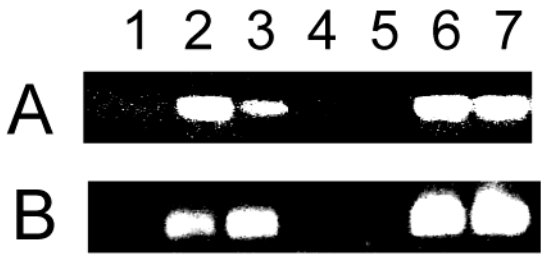

FIGURE 5: Chromatin immunoprecipitation (ChIP) assays. The cross-linked TRβ or LXRα overexpressing cells were subjected to immunoprecipitation with antibodies against TR, RXR, or FLAG, or with nonimmune rabbit IgG, as described under Experimental Procedures. The co-immunoprecipitated DNA was subjected to PCR using oligonucleotides spanning the DR4 element of ABCA1 promoter. Panel A shows TRβ-overexpressing cells, and panel B shows LXRα-overexpressing cells. Lane 1, negative PCR control using no sample in the ChIP assay; lane 2, RXR antibody; lane 3, TR antibody (panel A) or FLAG antibody (panel B); lane 4, rabbit IgG; lane 5, no antibody; lane 6, put on sample (1:100 dilution of the sonicated genomic DNA used for the immunoprecipitation); lane 7, positive PCR control with 100 ng of isolated genomic DNA.

from control cells indicates that this was not a major complex in nonstimulated cells. Hu and co-workers have reported the presence of the LXR/RXR complex in uninduced 293T cells using ChIP assay (*30*). A possible explanation for the differences observed is the antibodies used. Neither the results of Hu et al. nor our own results allow determination of the proportion of the DR-4 elements occupied by the LXR/RXR or TR/RXR heterodimer. It is also possible that some promoter sites are unoccupied or possibly occupied by other RXR heterodimers. While further research is needed to resolve this issue, the data shown here suggest the TR/RXR heterodimer to be present in the nuclei of nonstimulated cells.

Effects of LXR and TR Expression on Transcription Rates. The ability of ABCA1 wild-type promoter, the same sequence with mutations in the DR-4 element, or a promoter construct with two consecutive consensus DR-4 motifs to activate gene expression was studied by means of luciferase assays. As expected (*9*, *10*, *31*), LXRα overexpression markedly increased ABCA1 promoter activity over that in control cells, and this activity was further increased in the presence of oxysterol (Figure 6A). A major finding in the present studies was the 3-fold reduction in luciferase activity seen with the wild-type ABCA1 promoter sequence in the presence of TR and T3. This indicates that TR is an effective suppressor of LXR-stimulated transcription activity, even when oxysterol is present. Another unexpected finding was that in the presence of the consensus DR-4 element, and in contrast to the effect seen with wild-type ABCA1, TR + T3 stimulated transcription. This suggests that while TR binds to both DR-4 sequences, its kinetic effect, positive or negative, is mediated by the fine structure of the site. Finally, replacement of the ABCA1 wild-type promoter with the mutant DNA fragment almost completely abolished all of the effects of both TR and LXR in these studies, indicating that the observed effects are mediated directly by the DR-4 site. The repression of oxysterol-induced ABCA1 promoter activity by TR and T3 was also detected in ABCA1 mRNA levels (Figure 6B). The magnitudes of oxysterol induction in control cells, LXR overexpression, and TR + T3-mediated repression were very similar to those observed in luciferase assays.

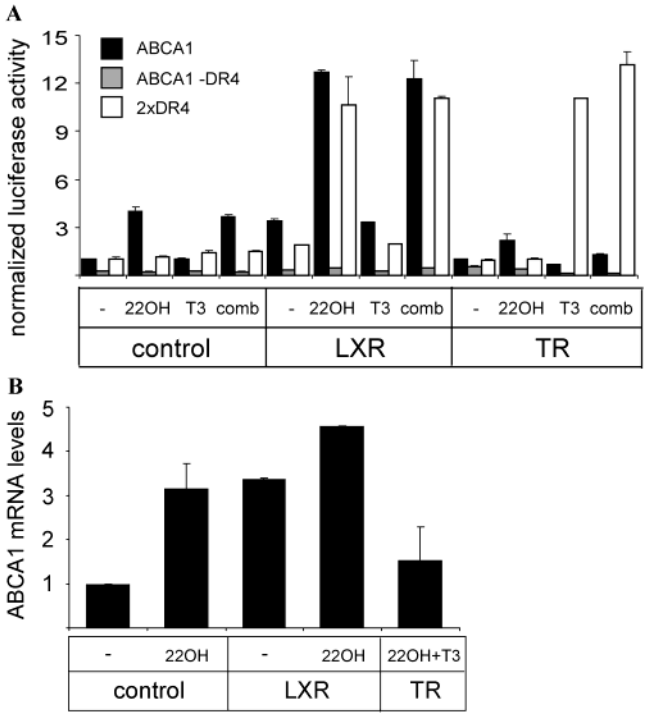

FIGURE 6: (A) Luciferase activity of ABCA1 promoter. 293T cells were transfected with firefly luciferase cDNA preceded by either wild-type ABCA1 promoter (black bars), ABCA1 promoter containing a mutated DR4 element (gray bars), or a promoter having two consensus DR4 elements (white bars). The cells were cotransfected with a plasmid expressing LXRα (LXR) or TRβ (TR) and incubated with LXR ligand 22(*R*)-OH (22OH), TR ligand T3 (T3), or their combination (comb.). After 24 h the cells were harvested and the luciferase activities measured. The results from a typical experiment performed in duplicate are shown and are represented relative to the wild-type ABCA1 promoter activity in nontransfected (control) cells, which is given the value of 1. (B) ABCA1 mRNA levels. 293T cells were transfected and treated as described above. RNA was isolated 24 h post-transfection and used in RT-PCR assays to determine the ABCA1 mRNA levels. The average results of two experiments performed in duplicate are shown and are represented relative to the ABCA1 mRNA levels in nontransfected (control) cells.

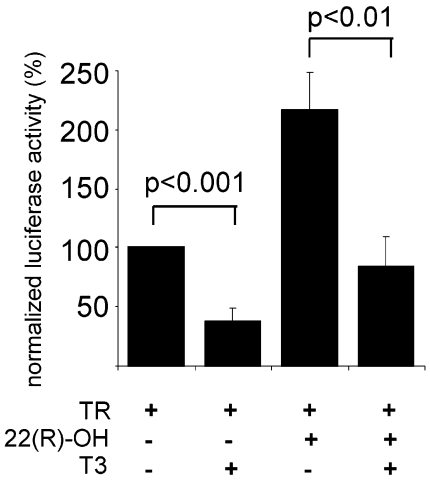

FIGURE 7: Inhibition of ABCA1 promoter activity by TR overexpression. 293T cells were transfected with wild-type ABCA1 promoter construct together with 500 ng of TRβ and incubated with either T3 or 22(*R*)-OH alone or in combination. After 24 h the luciferase activities were measured. The results are from four independent experiments performed in duplicate and are shown relative to ABCA1 promoter activity in TR-overexpressing cells in the absence of ligands.

The suppression of ABCA1 promoter activity by TR overexpression in the presence of T3 was 61 ± 12% in the presence of oxysterol and 62 ± 11% in the absence of oxysterol ($n = 4$). In TR-overexpressing cells, oxysterol up-regulated ABCA1 promoter activity only 2.2 ± 0.3-fold, while in control cells the corresponding stimulus was 3.8 ± 0.2-fold (Figure 7). These data provide a further illustration of the dynamic relationship between TR and LXR in 293T cells.

Competition between TR and LXR for the Regulation of ABCA1. To further quantify the efficiency of TR as an inhibitor of LXR-mediated transcription, 293T cells were transfected with increasing levels of TR expression plasmid in the presence of oxysterol to up-regulate LXR-dependent transcription activity (Figure 8A). Increased expression of TR in the presence of T3 led to a progressive decrease in the ABCA1 promoter activity. Comparable results were obtained when these experiments were performed in the absence of the LXR-activating oxysterol ligand (data not shown). To study the effects of LXR, TR was expressed at a level (100 ng of plasmid DNA) sufficient to reduce endogenous promoter activity by 50%. Increasing amounts of LXR expression plasmid were transfected into these cells. As more LXR cDNA was added, increasing activation of ABCA1 transcription was found (Figure 8B). At the same time, the ability of TRβ to suppress luciferase activity was diminished. These dose-dependent effects of TR and LXR were most likely the direct effect of increasing the amount of nuclear receptors in the cells, since increasing the amount of transfected plasmid led to an increased amount of expressed protein (Figure 8C, left panel). Since the levels of nuclear receptors were undetectable from nontransfected cell lysates prepared from a single 24-well plate, the presence of endogenous receptors was demonstrated from isolated nuclear extracts (Figure 8C, right panel). The right panel demonstrates also that overexpression of TR or LXR in the cells does not change the endogenous expression level of the other receptor. Taken together, the data in the luciferase studies confirm that ABCA1 promoter activity in intact cells represents a balance between the activating effect of LXR and the suppressive action of TR.

DISCUSSION

Regulation of the expression of ABC transporter proteins is recognized to be exceptionally complex. It includes effects at the transcriptional, translational, and protein levels (*9*, *17*, *18*, *20*, *21*, *32*). Among transcriptional effects, we and others demonstrated the presence of multiple transcriptional start sites (*22*−*24*). These allow the splicing of response elements (including DR-4) in to or out of the promoter sequence. The stimulatory effects of oxysterols and retinoic acid mediated via LXR on this regulatory element have been well and extensively defined (*9*, *10*, *33*). These seem to be particularly important in hepatocytes and in the lipid-filled macrophages of atherosclerotic lesions, where cellular oxysterol levels are substantial (*34*, *35*). An additional level of regulation involves the ability of different nuclear receptor proteins to heterodimerize with RXR and bind the same response element (*36*, *37*). When this manuscript was under revision, Costet

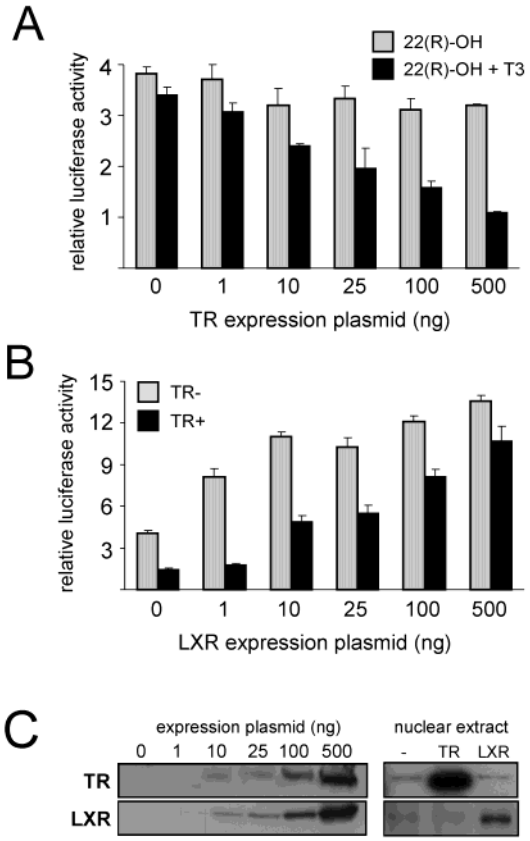

FIGURE 8: Effect of increasing TR and LXR overexpression on the ABCA1 promoter activity. (A) Dose-dependent inhibition of ABCA1 promoter activity by TR. 293T cells were transfected with increasing amounts (0−500 ng) of TRβ expression plasmid. The cells were incubated for 24 h with LXR ligand 22(*R*)-OH in the absence (gray bars) or in the presence (black bars) of TR ligand T3. The luciferase activity compared to basal cells (no overexpression, no ligands; given the value of 1) is shown from a representative experiment performed in duplicate. (B) A dose-dependent activation of ABCA1 promoter activity by LXR. 293T cells were transfected with increasing amounts (0−500 ng) of LXRα expression plasmid either in control medium (gray bars) or cotransfected with 100 ng of TRβ plasmid in T3-containing medium (black bars). All the cells were incubated for 24 h with 22(*R*)-OH. The luciferase activity compared to basal cells (no transfection, no ligands; given the value of 1) is shown from a representative experiment performed in duplicate. (C) Levels of nuclear receptors from transfected cells. 293T cells were transfected with increasing amounts of expression plasmids for TR or LXR, and the expressed proteins were detected from cell lysates using Western blotting with specific antibodies (left panel). Levels of nuclear receptor proteins were demonstrated from nuclear extracts of nontransfected (−) or TR- or LXR-transfected cells (right panel).

et al. identified a novel interaction of retinoic acid receptor RAR/RXR heterodimer on the ABCA1 DR-4 element (*38*). This further demonstrates that several nuclear receptor complexes can compete for the binding in the same element.

LXR and TR affect the HDL levels in animals and humans. Compared to wild-type mice, in LXRα, LXRβ, or double knock-out mice the HDL levels were reduced (*39*, *40*). Administration of T3 reduced the HDL levels in wild-type mice, whereas T3 deprivation increased these levels (*41*). These changes were abrogated in TRβ-deficient mice, indicating the importance of this nuclear receptor for the HDL levels in mice. Even though the changes in HDL levels in humans are not as pronounced, overt hypothyroidism leads to unchanged or increased HDL levels, whereas human subjects with hyperthyroidism have either unchanged or decreased HDL levels (*42*). These changes have been attributed to the effect of thyroid hormone on the cholesteryl ester transfer protein and hepatic lipase activities, as well as effects on apolipoprotein A−I expression (*41*, *42*). The data presented in this paper adds ABCA1, the main regulator of HDL biogenesis, to the list of genes regulated by thyroid hormone.

Whether the TR/RXR or LXR/RXR heterodimers bind to a given DR4 element in a physiological setting is determined by at least three factors: the relative abundance of each nuclear receptor protein, the affinity of each for its response element, and the availability of ligand (oxysterol or T3). Both the LXRβ and TRβ isoforms are ubiquitously expressed, whereas the α isoforms of both nuclear receptors have a more restricted expression pattern (*3*, *12*, *43*−*45*). Thus, in most tissues/cell types there is always at least one isoform of both TR and LXR present to heterodimerize with a more abundant RXR. Both cell types used in this study, human primary skin fibroblasts and 293T cells, have been previously used to study the effects of TR and LXR (*24*, *46*−*48*). Further, the transfection efficiency of 293T cells is high, making it a feasible system to manipulate the expression levels. In nonstimulated 293T cells, the levels of both TR and LXR were very low compared to RXR (data not shown). By transfecting an increasing amount of either TR or LXR expression plasmid into these cells, we were able to demonstrate a dose-dependent response in ABCA1 promoter activity (Figure 8). Furthermore, an increased binding of LXR/RXR heterodimer to the DR-4 element in LXR-overexpressing cells was observed (Figures 3 and 4).

Comparison between the ABCA1 wild-type and the DR-4 consensus sequences in this study indicates that differences either within the element itself or within the 5′- and 3′-flanking sequences of the DR-4 element must play a considerable role in both the direction and the magnitude of the response. TR activated transcription from a consensus DR-4 sequence but inhibited transcription from that of ABCA1 (Figure 6A). The LXR ligand did not activate the consensus DR-4 element in the absence of LXR overexpression, in sharp contrast to the ABCA1 promoter sequence. Further, the binding of the TR/RXR complex was stronger to the consensus DR-4 element than to the ABCA1 promoter element (Figure 2). Finally, in contrast to the ABCA1 DR-4 element, the consensus element also exhibited significant TR homodimer and TR/TR heterodimer binding.

In these studies, the ligands of each receptor protein (oxysterol in the case of LXR, T3 in the case of TR) intensified the effects on ABCA1 promoter activity but did not change its direction. That is, oxysterol increased activation by LXR, and T3 intensified inhibition by TR (Figures 6 and 8). In addition, significant crosstalk was seen between these pathways, since T3 significantly inhibited the ability of oxysterol to activate LXR, presumably by enhancing the recruitment of TR to the DR-4 site. Such effects are noteworthy not only because they illustrate the likely complexity of regulation of ABCA1 in vivo, but also because they suggest novel interventions. For example, T3 analogues might regulate ABCA1 but not SREBP-1c transcription, even though both genes utilize LXR and a DR-4 element as major constituents (*49*, *50*). In summary, competition between LXR and TR within the ABCA1 promoter describes a novel

regulatory mechanism and could offer new directions for therapeutic intervention of a key gene of human lipid metabolism.

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