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Retinoids Increase Human Apolipoprotein A-II Expression through Activation of the Retinoid X Receptor but Not the Retinoic Acid Receptor

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Considering the link between plasma high-density lipoprotein (HDL) cholesterol levels and a protective effect against coronary artery disease as well as the suggested beneficial effects of retinoids on the production of the major HDL apolipoprotein (apo), apo A-I, the goal of this study was to analyze the influence of retinoids on the expression of apo A-II, the other major HDL protein. Retinoic acid (RA) derivatives have a direct effect on hepatic apo A-II production, since all-*trans* (at) RA induces apo A-II mRNA levels and apo A-II secretion in primary cultures of human hepatocytes. In the HepG2 human hepatoblastoma cell line, both at-RA and 9-*cis* RA as well as the retinoid X receptor (RXR)-specific agonist LGD 1069, but not the RA receptor (RAR) agonist ethyl-*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]-benzoic acid (TTNPB), induce apo A-II mRNA levels. Transient-transfection experiments with a reporter construct driven by the human apo A-II gene promoter indicated that 9-*cis* RA and at-RA, as well as the RXR agonists LGD 1069 and LG 100268, induced apo A-II gene expression at the transcriptional level. Only minimal effects of the RAR agonist TTNPB were observed on the apo A-II promoter reporter construct. Unilateral deletions and site-directed mutagenesis identified the J site of the apo A-II promoter mediating the responsiveness to RA. This element contains two imperfect half-sites spaced by 1 oligonucleotide. Cotransfection assays in combination with the use of RXR or RAR agonists showed that RXR but not RAR transactivates the apo A-II promoter through this element. By contrast, RAR inhibits the inductive effects of RXR on the apo A-II J site in a dose-dependent fashion. Gel retardation assays demonstrated that RXR homodimers bind, although with a lower affinity than RAR-RXR heterodimers, to the AII-RXR response element. In conclusion, retinoids induce hepatic apo A-II production at the transcriptional level via the interaction of RXR with an element in the J site containing two imperfect half-sites spaced by 1 oligonucleotide, thereby demonstrating an important role of RXR in controlling human lipoprotein metabolism. Since the J site also confers responsiveness of the apo A-II gene to fibrates and fatty acids via the activation of peroxisome proliferator-activated receptor–RXR heterodimers, this site can be considered a plurimetabolic response element.

apo A-I and apo A-II are the major protein components of HDL, the levels of which in plasma are inversely correlated with the incidence of coronary artery disease (16). apo A-II exists in plasma as a homodimer linked by an intradisulfide bond at residue 6 (7). The apo A-II protein sequence (7), cDNA sequence (29, 35, 60, 66), and genomic structure (30, 36, 62, 66) have been determined, and the human gene has been localized on chromosome 1 (28, 37, 45, 52). In humans and other mammals, such as rats and mice, apo A-II is produced mainly by the liver and to a lesser extent by the intestine (23, 57). The transcription of apo A-II requires the action of a set of transcription factors binding to regulatory elements in the promoter (sites A to N) (8, 10, 38, 43, 61, 63, 68) as well as in the first intron of its gene (6). The association, reported in certain mouse strains, between elevated apo A-II levels and increased HDL size and HDL cholesterol levels suggests that the plasma concentration of apo A-II might affect HDL structure and function (13, 51). apo A-II could furthermore compete for the binding of HDL to the putative HDL receptor (15). Recent in vivo studies demonstrate that the overexpression of mouse apo A-II in transgenic mice enhances the development of atherosclerotic lesions (20, 69), whereas overexpression of human apo A-II in human apo A-I transgenic mice leads to a reduced level of protection against atherosclerosis (59). This in vivo effect could be linked with the in vitro observations of a decreased capacity of apo A-II to stimulate cholesterol efflux (2) and lecithin-cholesterol acyltransferase activity (67) and suggests that apo A-II might reduce the effects of HDL protective against atherosclerosis. This hypothesis is further supported by the differential effects on the development of atherosclerosis of the two main lipoprotein (Lp) particles composing HDL, LpA-I, containing only apo A-I, and LpA-I:A-II, containing both apo A-I and A-II. Numerous data suggest that LpA-I plays the crucial role in the protection against atherosclerosis, whereas LpA-I:A-II is less efficient (55).

The expression of the major HDL apos, apo A-I and A-II, is subject to regulation by hypolipidemic drugs, hormones, and nutritional agents in vivo (reviewed by Fielding and Fielding [16]). Several of these agents are ligands for and/or modulators of the activity of transcription factors belonging to the nuclear hormone receptor superfamily, which have been shown to con-

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trol the expression of the apo A-I and apo A-II genes (reviewed in references 25 and 72). The nutritionally derived vitamin A derivatives are a good example of compounds acting via receptors of the nuclear hormone receptor family (reviewed in references 14, 19, 41, 65, and 73 and references therein). Three RARs, termed RARα, -β, and -γ, and three RXRs, termed RXRα, -β, and -γ, are classically thought to transduce the effects of RA on gene expression. RARs are activated by both isomers of RA, at- and 9c-RA, whereas only 9c-RA binds and activates RXRs. RARs bind preferentially as heterodimers with RXR to DR-5 or DR-2 elements, whereas RXR homodimers bind to DR-1 elements. Recently an alternative RA-responsive pathway involving orphan receptors, such as LXR (71) and NGFI-B–Nur77 or the closely related receptor NURR1 (17, 53), has been described. Through heterodimerization with RXR, these orphan receptors are capable of mediating RXR-dependent, 9c-RA-dependent activation of transcription.

Whereas previous studies mainly addressed the regulation of apo A-I gene expression by retinoids, relatively little is known about their effects on apo A-II gene expression (3, 24, 56, 70, 74). Given the important role of apo A-II in HDL physiology, we initiated more detailed studies, first, to investigate the effects of retinoids on human apo A-II gene expression and, second, to elucidate the molecular mechanisms underlying the regulation of the apo A-II gene by retinoids. In this report, we demonstrate that retinoids increase hepatic production of apo A-II in humans. Furthermore, we show that this effect is due to the induction of apo A-II gene expression at the transcriptional level in the hepatocyte. Finally, we show that this effect of retinoids is mediated through binding of the nuclear hormone receptor RXR to an RXR-RE, localized in the J site of the 5′ upstream regulatory sequence of the apo A-II gene.

MATERIALS AND METHODS

Abbreviations. apo, apolipoprotein; RA, retinoic acid; at-RA, all-*trans* RA; 9c-RA, 9-*cis* RA; RAR, RA receptor; RXR, retinoid X receptor; mRXR, mouse RXR; RXR-RE, RXR response element; LXR, liver X receptor; NGFI-B, nerve growth factor IB; TTNPB, ethyl-*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl-1-propenyl]-benzoic acid; TK, thymidine kinase; PPAR, peroxisome proliferator-activated receptor; CAT, chloramphenicol acetyltransferase; DR-1, DR-2, and DR-5, direct repeats spaced by 1, 2, and 5 nucleotides respectively; HDL, high-density lipoprotein(s); DMSO, dimethyl sulfoxide; SD, standard deviation(s); wt, wild type; ARP-1, apolipoprotein regulatory protein 1.

Materials. at-RA was purchased from Sigma, St. Louis, Mo. 9c-RA, TTNPB (47), LGD 1069, and LG 100268 (4, 5) were kind gifts from M. Boehm of Ligand Pharmaceuticals.

Cloning and construction of recombinant plasmids. A 3-kb *Hin*dIII fragment containing the apo A-II gene promoter was cloned from a human placenta genomic DNA library in Charon 4A (a kind gift from D. Stehelin). A fragment containing the sequence from base −911 to +160 of the apo A-II promoter was excised by *Bgl*I digestion, blunted, *Hin*dIII digested, and subsequently cloned into the *Hin*dIII and blunted *Xba*I sites of pBLCAT5 (44). This construct was designated −911/+160AII-CAT. The construction of the various 3′-distal enhancer deletion vectors as well as the site-directed mutagenesis of the J site RXR-RE was described previously (68).

The J_{wt} and J_{mt} oligonucleotides (wt and mutated, respectively) containing bases −737 to −715 of the apo A-II gene promoter were cloned into the *Bam*HI-*Bgl*II sites of pIC20H (49), digested with *Hin*dIII and subcloned upstream of the TK promoter in pBLCAT4 (44) to generate J_{wt1}-TK-CAT, J_{wt3}-TK-CAT (containing one and three copies of the J_{wt} site, respectively), and J_{mt1}-TK-CAT (containing one copy of the J_{mt}-site).

A CMV-β-Gal vector (46) was used as an internal control for transfection efficiency in mammalian cells (46). The expression vectors pSG5mRXRα and pSGmRARα, used in transfections, have been described by Leid et al. and were a kind gift of P. Chambon (42). The vectors used for insect cell transfection, pPADH (a kind gift of A. Courey) (11) and pPADH-hRXRα (31), have been described previously.

Cell culture, measurements of apo A-II and apo E protein, and RNA analysis. The human hepatoma cell line, HepG2, and HeLa cells were obtained from ECACC (Porton Down, Salisbury, United Kingdom). Cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium, supplemented with 10% fetal calf serum, at 37°C in a humidified atmosphere of 5% CO_2–95% air. The medium was changed every other day. Human liver specimens were collected for transplantation at the Moscow Medical Center from physically healthy multiorgan donors who died after severe traumatic brain injuries. Permission to use the remaining, untransplanted parts of the donor livers for scientific research was obtained from the Ministry of Health of the Russian Federation. Hepatocytes were obtained by a two-step collagenase perfusion as previously described (39). Cells were resuspended in minimal essential medium with Earle's salts (Gibco/BRL, Paisley, United Kingdom) supplemented with 10% FCS–2 mM L-glutamine–gentamicin (50 mg/ml), seeded at a density of 1.5×10^5 cells per cm^2 in 60-mm-diameter plastic culture dishes coated with 20 mg of rat tail collagen type I (Sigma), and incubated in a humified atmosphere of 5% CO_2–95% air at 37°C. Medium was renewed after a 4-h adhesion period. After 20 h the medium was discarded and RA (in DMSO, final concentration 0.5% [vol/vol]) was added at the indicated concentrations in serum-free medium. *Drosophila* Schneider (SL2) cells were grown at 25°C in Shields and Sang M3 insect medium supplemented with 10% charcoal-treated fetal calf serum in the presence of penicillin (100 U/ml) and streptomycin (100 mg/ml).

Human apo A-II and apo E concentrations in culture medium of primary human hepatocytes was measured as previously described (68). RNA extraction, Northern blot (RNA) hybridizations, and measurements of apo A-II mRNA were performed as described previously (68). The cDNA clone 34B6 (50) coding for the human acidic ribosomal phosphoprotein PO (34) was used as a control.

Transient transfections and expression assays. Transfections were performed at 50 to 60% confluency by the calcium phosphate coprecipitation procedure with a mixture of plasmids which contained, in addition to the CAT reporter plasmids (1 to 5 μg per 60-mm-diameter culture dish) and expression vector(s) (200 ng), 0.5 μg of cytomegalovirus-driven β-galactosidase expression vector as a control for transfection efficiency. All samples were complemented, with pSG5 plasmid, to an equal total amount of plasmid DNA. After 4 h, cells were washed with phosphate-buffered saline and incubated for another 16 h with the various retinoids or vehicle (DMSO) in fresh medium containing 5% calf serum delipoproteinized by ultracentrifugation in KBr (1.21 g/ml) and subsequently treated with AG-1-X8 resin (Bio-Rad) plus activated charcoal. For transfections of the *Drosophila* Schneider (SL2) cells, approximately 2.5×10^6 cells were transfected in a 30-mm-diameter plate by the calcium phosphate method as described previously (31), with the luciferase reporter plasmid, the expression vectors, and an ADH-β-Gal internal control vector. The total amount of DNA in different transfection mixtures was equalized by the addition of pPADH plasmid. After transfection, the cells were lysed by four freeze-thaw cycles in 100 μl of 0.25 M Tris-HCl (pH 8.0)–1 mM dithiothreitol. Luciferase, CAT, and β-galactosidase assays were performed on the extracts as described previously (58). All transfection experiments were performed at least three times.

Electrophoretic mobility shift assays. For RXR-binding studies, a synthetic double-stranded oligonucleotide spanning nucleotides −737 to −715 of the human apo A-II gene 5′ upstream regulatory sequence was used (5′-gatCCT TCAACCTTTACCCTGGTAGA-3′) (AII-J_{wt}) as well as an oligonucleotide containing two point mutations into the potential AII-RXR-RE (5′-gatCCT TCAAGCTTTACTCTGGTAGA-3′) (AII-J_{mt}). pSG5mRARα, pSG5hRARβ, pSG5mRXRγ, and pSG5mRXRα (42) proteins were synthesized in vitro with the rabbit reticulocyte lysate system (Promega). The molecular weight and quality of the in vitro-translated proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. RAR and/or RXR (2-μl volume) were preincubated in a total volume of 20 μl for 15 min on ice with 2.5 μg of poly(dI-dC) and 1 μg of herring sperm DNA in TM buffer (10 mM Tris-HCl [pH 7.9], 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40, and 1 mM dithiothreitol). For competition experiments, increasing amounts of cold oligonucleotide AII-J_{wt} or AII-J_{mt} (from 50- to 400-fold molar excess) were included just before adding T4-polynucleotide kinase end-labelled oligonucleotide. After 15 min of incubation at room temperature, DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25× TBE buffer at 4°C (18).

Statistical analysis. Data are expressed as means ± SD unless stated otherwise. Student's *t* test, analysis of variance, and the Mann-Whitney U test were used to analyze statistical significance.

RESULTS

Retinoids increase apo A-II mRNA and protein secretion in primary human hepatocytes. The regulation of apo A-II expression by retinoids was studied in primary cultures of human hepatocytes. The addition of at-RA (10 μM) for 24 h to the culture medium had a pronounced effect on apo A-II mRNA levels (Fig. 1A). No change in the expression of the control 34B6 mRNA levels was observed under these conditions (Fig. 1A). The induction of apo A-II mRNA levels was accompanied by a significant increase in apo A-II secretion in the culture medium (Fig. 1B). In contrast, apo E secretion in the culture medium remained constant under these conditions (Fig. 1B).

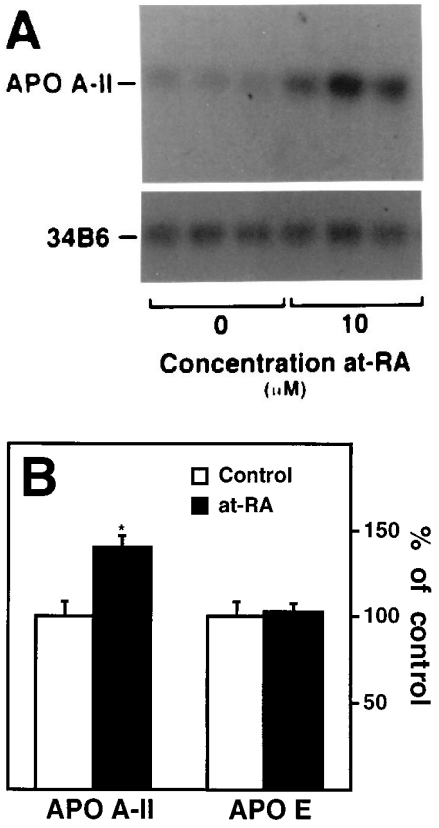

FIG. 1. RA increases apo A-II mRNA levels (A) and protein secretion (B) in primary cultures of adult human hepatocytes. (A) Human hepatocytes were isolated and treated for 24 h with 10 μM at-RA. Total RNA (5 μg) was subjected to electrophoresis, transferred to a nylon membrane, and hybridized to apo A-II (top panel) or 34B6 (bottom panel) cDNA as described in Materials and Methods. (B) Human hepatocytes were treated for 24 h with at-RA or solvent (Control [DMSO]). apo A-II and apo E protein concentrations were measured in medium as described in Materials and Methods. Values are expressed relative to controls and represent the mean ± SD of three points. A value statistically significantly different from the control value ($P < 0.05$ by the Mann-Whitney U test) is indicated by an asterisk.

Next, in order to determine which RAR mediates the effects of RA on apo A-II expression, apo A-II mRNA expression was analyzed in the human hepatoblastoma cell line HepG2 after treatment with various retinoids (Fig. 2). The addition of both 9c-RA and the RXR-specific agonist LGD 1069 induced apo A-II mRNA levels in a dose-dependent fashion. In contrast, the RAR-specific agonist TTNPB had no effects on apo A-II mRNA levels at concentrations up to 10^{-5} M, whereas the panagonist at-RA induced apo A-II mRNA levels slightly at the highest dose tested only.

The increase in apo A-II gene expression after retinoid treatment is due to an increase in apo A-II gene transcription. To study whether retinoids induce apo A-II gene expression at the transcriptional level, the human apo A-II promoter was cloned in front of the CAT reporter gene to generate the −911/+160AII-CAT reporter construct. This construction was transfected in the human hepatoblastoma cell line HepG2, and the cells were treated with different retinoids. apo A-II promoter-driven CAT activity increased by ~50% after incubation with either at-RA, 9c-RA, or the RXR-specific compounds LGD 1069 and LG 100268, whereas no effect was observed after addition of the RAR-specific compound TTNPB. The RSV promoter-driven CAT activity remained unchanged under these conditions (DMSO, 100% ± 9%; at-RA, 92% ± 11%; 9c-RA, 102% ± 7%; TTNPB, 92% ± 11%; LGD 1069, 107% ± 6%; and LG 100268, 92% ± 10%). These results clearly indicate that the increase in apo A-II production in human liver after retinoid treatment is rather specific for compounds which activate RXR and occurs, at least partially, at the transcriptional level.

In order to explore the relative contributions of the RARs and RXRs in the induction of the apo A-II promoter by at-RA and 9c-RA, mRXRα, mRARα, or both mRXRα and mRARα were cotransfected with the apo A-II promoter vector and cells were subsequently treated with RA derivatives or vehicle (Fig. 3). Compared with basal unstimulated conditions (relative CAT activity, 100% ± 11%), cotransfection of mRARα had little effect on apo A-II promoter-driven CAT activity (98% ± 13%). Furthermore, no induction of CAT activity was observed when the apo A-II promoter was cotransfected with mRARα in the presence of the various activators. In contrast, both mRXRα (228% ± 25%) as well as mRXRα and mRARα cotransfection (180% ± 12%) induced apo A-II promoter activity substantially, which could be further enhanced by at-RA, 9c-RA, LGD 1069, and LG 100268 but not by TTNPB. Together with the RNA data (Fig. 2), these experiments indicate that the effects of RA on apo A-II gene expression in HepG2 cells are mediated mainly by RXR.

Delineation of an RXR-RE in the regulatory sequences of the apo A-II gene. Next, studies were performed to delineate the *cis*-acting regulatory sequences within the 5′URS of the apo A-II gene, implicated in the induction of apo A-II gene transcription by RA. Since apo A-II promoter activity is largely determined by elements in the distal enhancer (elements I to N) (63), 3′ site-by-site recurrent deletions of the distal enhancer region were cloned in front of the basal apo A-II promoter and the effects of at-RA or 9c-RA in the presence or absence of mRXRα were analyzed after transfection in HepG2 cells (Fig. 4). Both at-RA and 9c-RA treatment induced the CAT activity of the construct containing the entire regulatory region (nucleotide −911 to +160) as well as that of constructs containing sites N to I and N to J. However, upon deletion of site J (plasmids containing only sites N to K and N to L) the induction by RA was completely abolished. Similarly, cotrans-

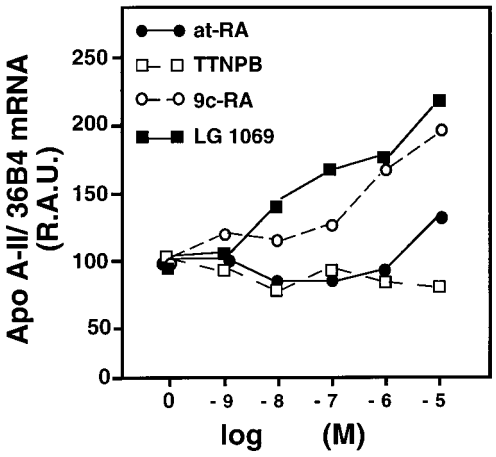

FIG. 2. Effects of RA derivatives on apo A-II mRNA levels in the HepG2 hepatoblastoma cell line. HepG2 human hepatoblastoma cells were treated for 24 h with the indicated concentrations of at-RA, 9c-RA, LGD 1069, and TTNPB. RNA was prepared, and 10 μg of total RNA was dot blotted on a nylon membrane, hybridized to apo A-II and 34B6 cDNAs, and quantified as described in Materials and Methods. R.A.U., relative absorbance units.

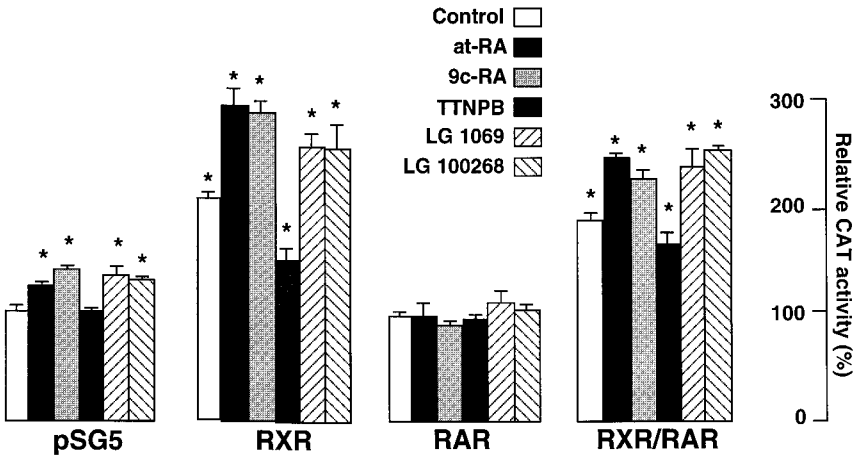

FIG. 3. RXR induces apo A-II gene expression at the transcriptional level via the apo A-II promoter. HepG2 cells were transiently transfected with the −911/+160AII-CAT plasmid in the presence of cotransfected pSG5 control vector, mRXRα, mRARα, and mRXRα and mRARα together (in equimolar ratio) and treated for 16 h with 1 μM at-RA, 9c-RA, LGD 1069, LG 100268, TTNPB, or vehicle (DMSO). CAT activity was measured and expressed (mean ± SD) as described in Materials and Methods. Values statistically significantly different from control values ($P < 0.05$ by analysis of variance) are each indicated by an asterisk.

fection of the mRXRα expression vector only activated the constructs containing the J site, which are capable of responding to RA (Fig. 4). The addition of at-RA to cells cotransfected with mRXRα and the various apo A-II promoter constructs did result in a further stimulation relative to that with mRXRα alone only on constructs containing the J site (Fig. 3 and 4). These data suggest the presence of a potential retinoid RE in the J site, a regulatory element located between −734 and −716 bp from the transcription start site of the human apo A-II gene, which has previously been shown to bind liver nuclear proteins (9, 38, 68).

Interestingly, the J site contains two imperfect copies of a motif related to the consensus steroid hormone receptor binding half-site TGACCT arranged as DR-1. To investigate whether this DR-1 located in the J site represents the functional RE mediating the observed effects of RXR and retinoids on apo A-II gene transcription, the DR-1 site was mutated (Fig. 5A) and its activity was compared with the activity of the

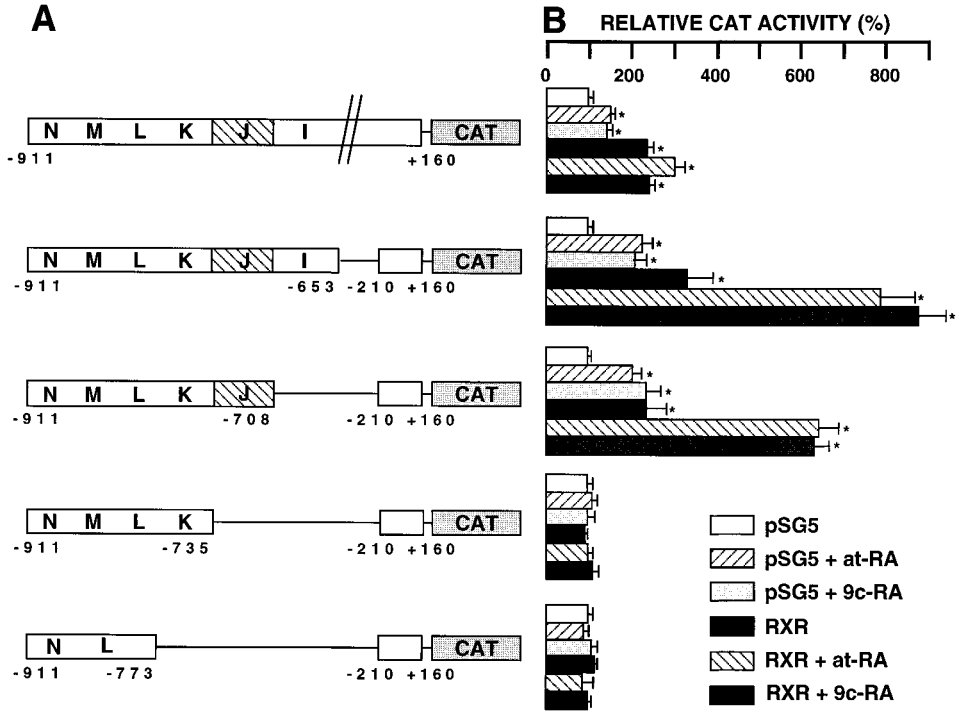

FIG. 4. The induction of apo A-II gene transcription by at-RA, 9c-RA, or RXR is mediated by the apo A-II promoter J site in the apo A-II promoter. HepG2 cells were transfected with different apo A-II gene promoter deletion constructs as schematically depicted (A), in the presence of cotransfected mRXRα or pSG5 vector plasmid. Cells were treated with at-RA or 9c-RA (1 μM) or vehicle (DMSO) and CAT activity was measured and expressed (mean ± SD) (B) as described in Materials and Methods. Values statistically significantly different from those for vehicle-treated, pSG5-cotransfected apo A-II promoter constructs are each indicated by an asterisk.

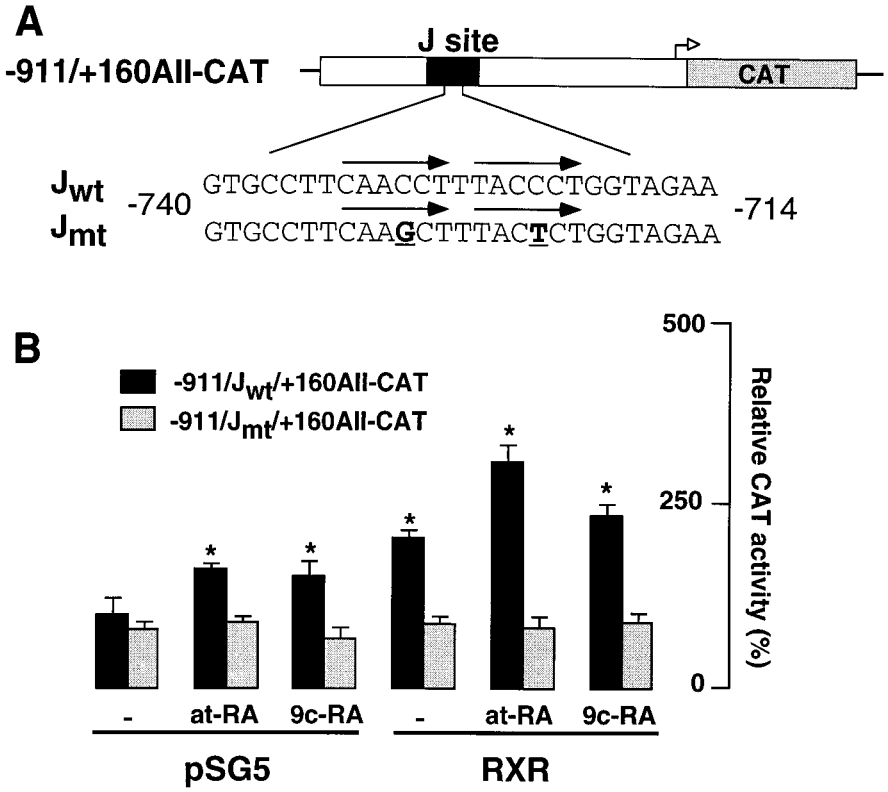

FIG. 5. The J site in the apo A-II promoter contains a functional RXR-RE: effects of point mutation in the J site. (A) Schematic representation of the −911/+160AII-CAT construct. The wt and the mutated apo A-II J sites (J_{wt} and J_{mt}, respectively) are indicated. (B) Effects of at-RA, 9c-RA, and mRXRα on the expression of apo A-II promoter containing a wt (−911/J_{wt}/+160AII-CAT) or mutant (−911/J_{mt}/+160AII-CAT) AII-RXR-RE. HepG2 cells were transfected with the indicated reporter plasmids in the presence of cotransfected mRXRα or pSG5 vector plasmids. Cells were treated with either RA derivative (1 μM) or vehicle (DMSO) and CAT activity was measured and expressed as described in Materials and Methods. Values statistically significantly different from control values (with pSG5) are each indicated by an asterisk.

wt construct (−911/+160AII-CAT) transfected in HepG2 cells (Fig. 5B). Mutation of the J site DR-1 sequence resulted not only in a loss of inducibility of apo A-II promoter regulation by RXR and the two RA derivatives (Fig. 5B) but also in a decrease in baseline level CAT activity. This decrease in basal CAT activity suggests that the site not only mediates the transcriptional response to retinoids but also is a strong site driving the basal expression of the apo A-II promoter.

RAR inhibits transactivation through the J site, which functions as a strict RXR-RE. To prove that the J site could function as an RXR-RE in front of a heterologous promoter, the J_{wt} site was cloned as a mono- and trimer in front of the heterologous TK promoter to generate the constructs J_{wt1}-TK-CAT and J_{wt3}-TK-CAT, respectively (Fig. 6A). Upon cotransfection of the J_{wt1}-TK-CAT construct with mRXRα into HepG2 cells, it was evident that the J site could transmit retinoid activation to this heterologous promoter after cotransfection of mRXRα but not mRARα (Fig. 6B). The TK-CAT vector (data not shown) or the J_{mt1}-TK-CAT was not activated by mRXRα in the presence of RA derivatives (Fig. 6B).

In a further experiment, HeLa cells were transfected with the J_{wt3}-TK-CAT construct in the presence of mRXRα and/or mRARα and the influence of at-RA, 9c-RA, and RAR- or RXR-selective agonists was analyzed (Fig. 7). Similar to the case with the J_{wt1}-TK-CAT construct, the addition of the different agonists by themselves did not activate J_{wt3}-TK-CAT expression to a large extent in HeLa cells. Upon cotransfection of mRXRα, treatment with at-RA, 9c-RA, and RXR agonists LGD 1069 and LG 100268 resulted in a substantial increase in CAT activity (10.5-, 8.2-, 7.8-, and 6.8-fold, respectively) (Fig. 7). RXR in the presence of the RAR agonist TTNPB only marginally induced CAT activity (2.1-fold; no statistical significance). Treatment with at- and 9c-RA or the RXR and RAR agonists in the presence of cotransfected mRARα did not activate J_{wt3}-TK-CAT expression in HeLa cells (Fig. 7). Cotransfection of mRXRα with an equimolar ratio of RAR did not result in any additional effect relative to cotransfection of mRXRα alone. Taken together, these data strongly argue that the J site of the apo A-II gene is a bona fide RXR-RE (but not an RAR-RE) which mediates the induction of apo A-II gene transcription through RXR activation.

In order to explore the potential inhibitory effects of RAR on the transcriptional activation of the J site by RXR in more detail, increasing amounts of RAR were cotransfected with a constant amount of RXRα expression and J_{wt3}-TK-CAT reporter vector in the presence of 9c-RA. Under these conditions RXR-stimulated CAT expression was significantly inhibited in a dose-dependent manner by cotransfection of mRARα (Fig. 8). This effect already became evident when the ratio of cotransfected RAR exceeded that of RXR by threefold. In contrast, cotransfection of RXR with increasing amounts of mPPARα stimulated the expression of the J_{wt3}-TK-CAT construct (data not shown). Furthermore, when increasing amounts of RAR were cotransfected with a constant amount of RXRα in the presence of an RAR-RE-containing DR-5′–

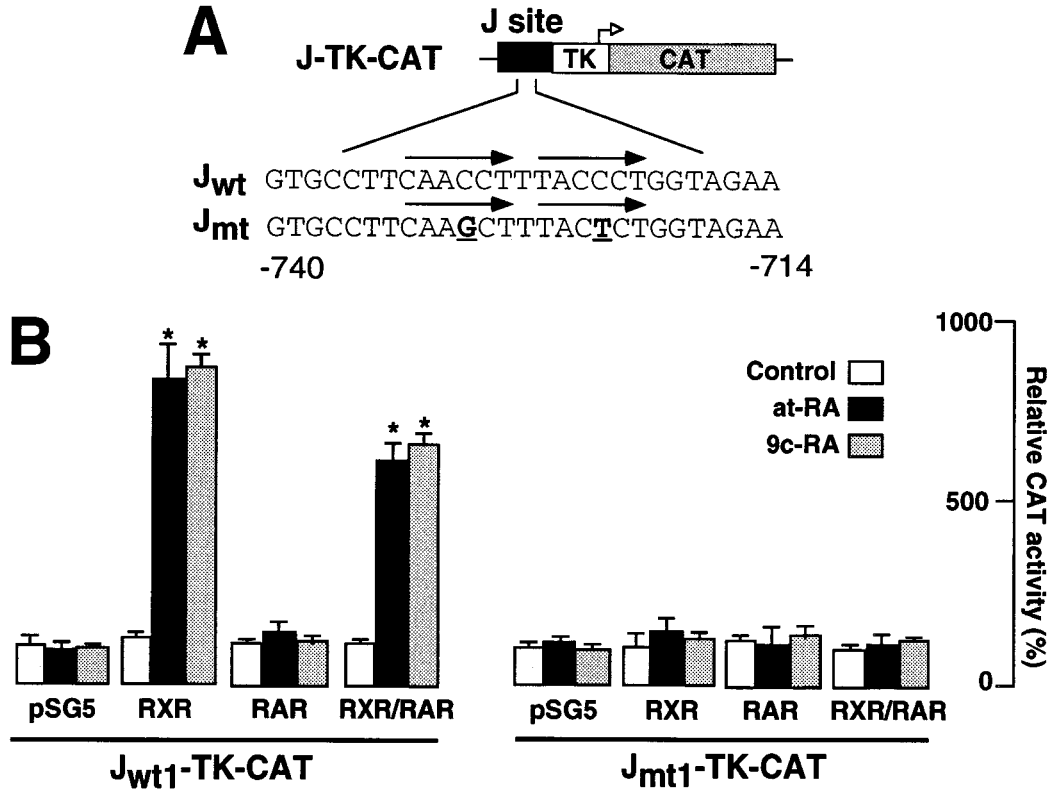

FIG. 6. The J site in the apo A-II promoter contains a functional RXR-RE: effects of the J site on a heterologous promoter. (A) Schematic representation of the J-TK-CAT construct. The wt as well as the mutated apo A-II J sites (J_{wt} and J_{mt}, respectively) are indicated. (B) RXR, but not RAR, in the presence of at- or 9c-RA activates the wt (J_{wt1}) but not the mutant (J_{mt1}) J site when cloned upstream of the heterologous TK promoter. HepG2 cells were cotransfected in the presence mRXRα, mRARα, mRARα and mRXRα together, or pSG5 vector plasmids. Cells were treated with at-RA, 9c-RA (1 μM), or vehicle and CAT activity was measured and expressed as described in Materials and Methods. Values statistically significantly different from control values (pSG5-cotransfected constructs) are each indicated by an asterisk.

TK-CAT reporter vector, CAT expression was stimulated by RAR cotransfection (Fig. 8).

To demonstrate that this site can function as an RXR-RE irrespective of the presence of other nuclear receptors, we cotransfected the J_{wt3}-TK-CAT vector together with RXR in cells known to contain low levels of nuclear receptors, such as COS cells and *Drosophila* SL2 cells. In both cell types RXR by itself slightly induced CAT activity. The addition of at-RA (10 μM) to the culture medium of COS or SL2 cells resulted in a strong activation of CAT activity, suggesting that RXR could activate the J site independently of the presence of other nuclear receptors (Fig. 9).

RAR-RXR heterodimers bind with a relative affinity higher than that of RXR homodimers to the AII-RXR-RE in the J site. Next, it was investigated whether RXR could bind to the AII-J_{wt} site (or AII-RXR-RE) by electrophoretic mobility shift assays. The incubation of a double-stranded oligonucleotide corresponding to the AII-J_{wt} and spanning sequences from −737 to −715 relative to the transcription initiation site of the apo A-II gene with in vitro-produced mRXRα resulted in the formation of a barely visible, retarded complex (Fig. 10). Similar weak binding was observed when mRXRα was replaced with mRXRγ (data not shown). This demonstrates that the AII-J_{wt} site is capable of binding different RXR homodimers, although with a low affinity. Binding of human RARβ-mRXRα (Fig. 10) or mRARα-mRXRα (data not shown) heterodimers to the A-II-J_{wt} site was much stronger, despite the inability of RAR-RXR heterodimers to activate through the apo A-II-J_{wt} site. On a labelled double-stranded oligonucleotide containing the mutated AII-J_{mt} site (AII-RXR-RE_{mt}) no binding of either mRXRα or -β homodimers or human RARβ/mRXRα or mRARα-mRXRα heterodimers was observed, thereby confirming and extending the results of our transfection experiments (data not shown).

DISCUSSION

In view of the evidence linking elevated levels of HDL cholesterol and a protective effect against the development of coronary artery disease as well as the suggestion that retinoids might induce the expression of apo A-I, a factor protective against coronary artery disease, one of the goals of our research was to determine whether retinoids regulate the expression of apo A-II, another major HDL protein which has been suggested to antagonize the protective action of apo A-I. Treatment of primary human hepatocytes with at-RA resulted in a significant increase in apo A-II gene expression and corresponding protein production. Similarly, in HepG2 cells the addition of at-RA, 9c-RA, or LGD 1069 but not TTNPB induced apo A-II mRNA levels. To understand the molecular mechanisms underlying this regulation, transient-transfection experiments were performed and showed that RA derivatives activate the apo A-II gene promoter. By unilateral 3′ deletion and site-directed mutagenesis experiments, we localized the RA-responsive region in the J site (63) of the apo A-II distal enhancer region.

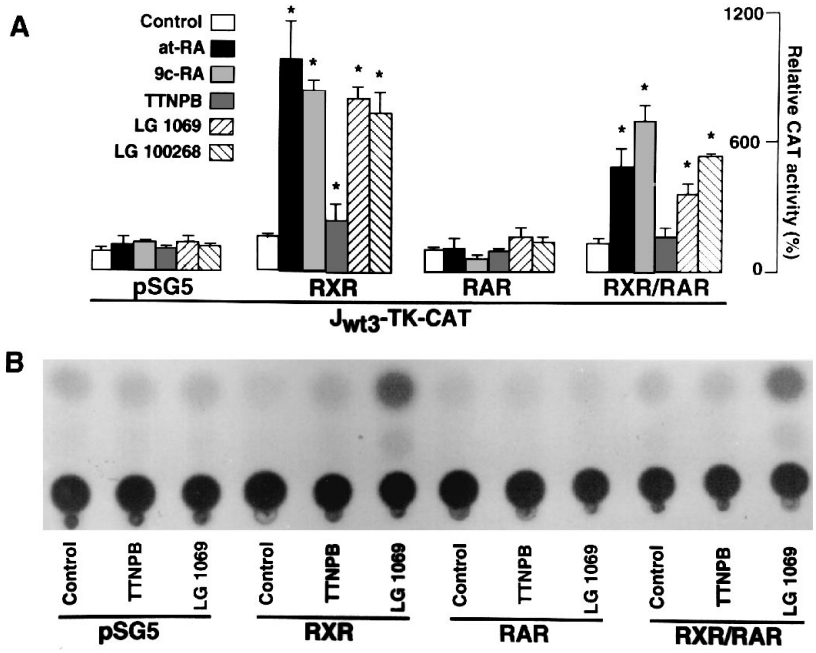

FIG. 7. RXR but not RAR can activate a triple repeat of the apo A-II J site in front of a heterologous promoter. (A) HeLa cells were cotransfected with the J_{wt3}-TK-CAT plasmid in the presence of mRXRα, mRARα, mRXRα and mRARα together, or pSG5 vector plasmids. Cells were treated with at-RA, 9c-RA, LGD 1069, LG 100268, or TTNPB (each at 1 μM) or with vehicle (DMSO) and CAT activity was measured and expressed as described in Materials and Methods. Values statistically significantly different from control values (with pSG5) are each indicated by an asterisk. (B) CAT assay demonstrating the effects of cotransfection of mRXRα, mRARα, mRXRα and mRARα together, or pSG5 vector plasmids on the expression of J_{wt3}-TK-CAT treated with LGD 1069 (1 μM), TTNPB (1 μM), or vehicle (DMSO) in HeLa cells.

The effects of retinoids on gene transcription are generally mediated by transcription factors belonging to the nuclear hormone receptor gene superfamily, which interact with specific REs in the regulatory regions of genes. These regulatory elements are classically composed of derivatives of a canonical hexameric DNA recognition motif (PuGGTCA) arranged either as a DR or as a palindromic sequence or inverted repeat. Retinoid signalling may occur through three distinct pathways: via formation of either RXR-RAR heterodimers, RXR-RXR homodimers, or heterodimers of orphan receptors, such as LXR or NGFI-B with RXR. The RXR-RAR heterodimer mediates the effects of at-RA and 9c-RA on gene expression through a functional but asymmetrical interaction with DR-5 or DR-2. In this complex, RXR occupies the 5′ half-site and RAR occupies the 3′ half-site of the DR-5 (or DR-2) RE (32, 54). In this conformation, RXR is unable to bind its ligand and can be considered a silent nonactivating partner (32). In the second pathway, constituted of LXR (or RLD-1)-RXR or NGFI-B (or Nur77)-RXR heterodimers, RXR becomes an active ligand-binding heterodimeric partner (in contrast to its role as a silent partner in the RXR-RAR heterodimers), mediating the response of target genes to 9c-RA (1, 17, 53, 71). Finally, RXR can also mediate 9c-RA action by binding through a DR-1 element under the form of an RXR homodimer (48, 56).

The apo A-II J site contains two imperfect copies of a motif related to the consensus nuclear receptor half-site TGACCT DR-1 and binds RXR homodimers in electrophoretic mobility shift assays. Accordingly, by transient-transfection studies and by the use of RXR- and RAR-specific compounds, it was shown that only RXR and not RAR is capable of mediating the RA-dependent transcriptional activation of the apo A-II promoter. The existence of a distinct RXR signalling pathway mediated by RXR homodimers through specific RXR-REs (i.e., DR-1-type elements) was demonstrated in the rat (48) and mouse cellular retinol-binding protein type II gene (48) and the human apo A-I gene (56), indicating that RXR, but not RAR induces activity via these putative RXR-REs. Using RAR- and RXR-specific ligands, Davis et al. confirmed the existence of an RXR-specific signalling pathway under more physiological conditions in pituitary cells (12). Furthermore,

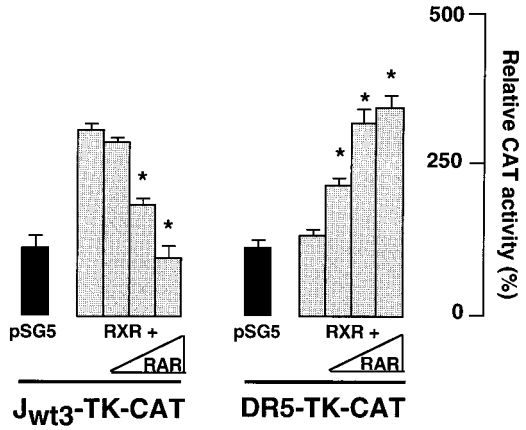

FIG. 8. Increasing amounts of RAR inhibit the transcriptional activation of the J site by RXR. HepG2 cells were cotransfected with the J_{wt3}-TK-CAT plasmid in the presence of mRXRα (200 ng) and increasing amounts of mRARα (gray bars) (0, 200, 600, and 1,200 ng). Cotransfection of pSG5 vector plasmid served to determine the basal level of expression of the J_{wt3}-TK-CAT plasmid, which was arbitrarily set at 100 (black bar). All cells were treated with 9c-RA (1 μM) and CAT activity was measured and expressed as described in Materials and Methods. Values statistically significantly different from control values (with RXRα only) are each indicated by an asterisk.

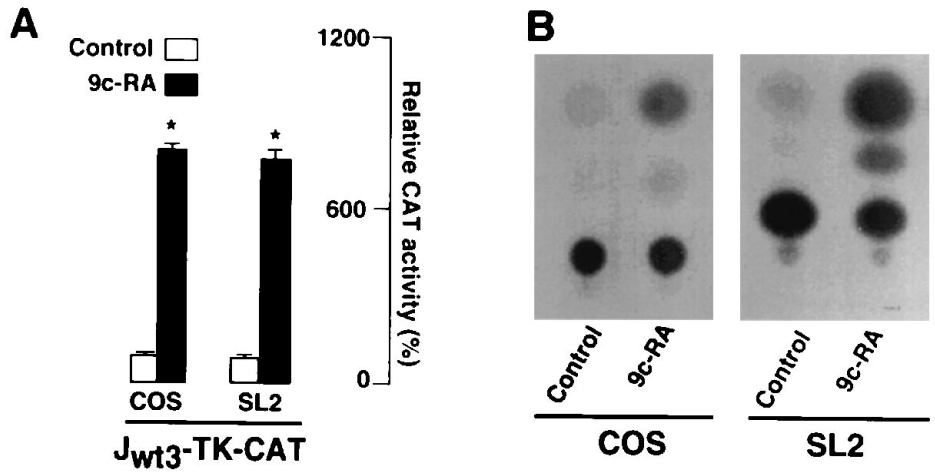

FIG. 9. Induction of transcriptional activation by RXR through the J site is independent of the presence of other nuclear hormone receptors. (A) COS cells or *Drosophila* SL2 cells were cotransfected with the J_{wt}-TK-CAT plasmid and the appropriate mRXRα expression vector. Cells were treated with 9c-RA (10 μM) or vehicle (DMSO) and CAT activity was measured and expressed as described in Materials and Methods. Values statistically significantly different from values for untreated cells are each indicated by an asterisk. (B) CAT assay demonstrating the effects of cotransfection of mRXRα and treatment with 9c-RA (10 μM) or vehicle (DMSO) in COS or SL2 cells.

RXR homodimers are capable of activating REs composed of a DR-1 in *Saccharomyces cerevisiae* (21) and in *Drosophila* SL2 cells (the present investigation), which both lack endogeneous RAR. Our mRNA (Fig. 2) and transfection data (Fig. 3 to 9) provide further support for the existence of an RXR-specific signalling pathway which can control the expression of the human apo A-II gene in liver.

When a multimerized apo A-II J site was used to drive a heterologous TK promoter-driven reporter vector, RAR overexpression inhibited transcription. Therefore, although RXR-RAR heterodimers are capable of binding to the apo A-II J site, they are incapable of activating transcription through this site. This substantiates the previous observation that, in sharp contrast to their activating effects on a DR-5, RXR-RAR heterodimers are incapable of activating transcription through a DR-1 site and in fact might even act as potent repressors of ligand-mediated transcription activation by RXR homodimers on this site (26, 32, 40, 48). The conversion of RAR from an activator to an inhibitor of RA-dependent transcription has been shown to be due to its polarized binding to the upstream half-site of the DR-1 element (32). This renders the heterodimeric complex incapable of responding to either at-RA or 9c-RA and furthermore blocks the binding of 9c-RA to RXR (32). Recently, this allosteric regulation has been shown to involve the nuclear receptor corepressor, which is only released from RAR-RXR heterodimers when bound on a DR-5 but not on a DR-1 RE (33). Regulation of gene expression by RXR in response to RA must therefore occur independently of RAR and should require a high ratio of RXR to RAR, taking the inhibitory effects of this last receptor into account. In view of our RNA data, which show distinctive effects on apo A-II expression of RXR and RAR agonists, and under the assumption that the J site represents the only RA-RE in the apo A-II promoter, the results of this study suggest that RXR-mediated RA signalling occurs in vivo. This suggests hence that in human hepatocytes sufficiently high concentrations of RXR might be present, allowing them to drive the formation of RXR homodimers in favor of RAR-RXR heterodimers. It can furthermore not be excluded at this stage that RA treatment alters the relative levels of RXR and RAR proteins in the hepatocyte, favoring RXR-mediated signalling. Alternatively, it is possible

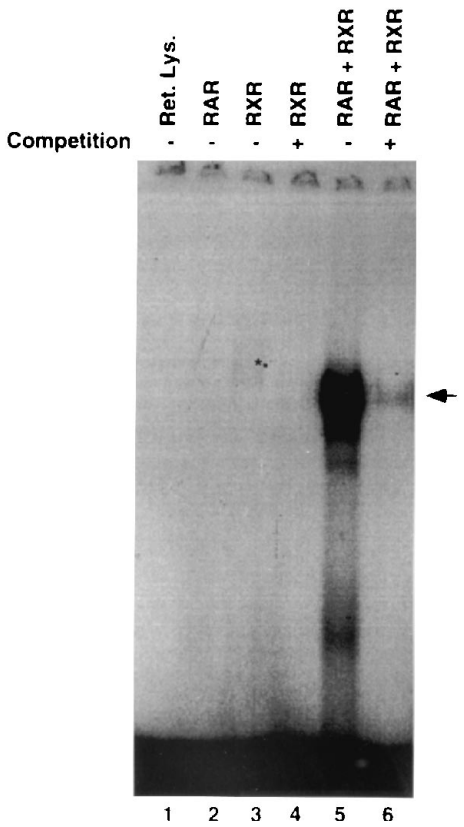

FIG. 10. RXR homodimers and RAR-RXR heterodimers bind to the AII-J_{wt} site. Gel retardation assays were performed on end-labelled AII-J_{wt} site oligonucleotide in the presence of in vitro-transcribed and -translated human RARβ (RAR), mRXRα (RXR), or unprogrammed reticulocyte lysate (Ret. Lys.) in the presence (+) or absence (−) of 100-fold molar excess of unlabelled wt AII-J_{wt} (competition) oligonucleotide as described in Materials and Methods. The complex formed by RXR homodimers is indicated by the asterisk, whereas the RXR-RAR heterodimer is indicated by the arrow.

that the apo A-II promoter contains additional cryptic RAREs which could be stimulated by one of the RA signalling pathways (RXR-RXR, RXR-RAR, LXR-RXR, or NGFI-B-RXR).

It is interesting to note that both the RXR agonists LGD 1069 and LG 100268 and 9c-RA activate transcription through the DR-1 of the J site. In addition, at-RA, an RAR-specific activator, is also capable of activating apo A-II expression albeit to a lesser extent. This phenomenon, which has been observed in three different cell lines, is most likely due to isomerization of at-RA to other metabolites such as 9c-RA (22), although alternative molecular explanations cannot be ruled out. The apo A-II promoter is furthermore also transcriptionally activated by RXR in the absence of RA or other exogenous stimuli. Such a ligand-independent activation has been observed with other nuclear hormone receptors and could be due to (i) the inherent activity of the transcriptional activating functions of RXR (64), (ii) the presence of low concentrations of a natural ligand(s), constitutively activating RXR in the cells, or (iii) the fact that RXR forms functionally active heterodimeric complexes with other receptors such as, for example, PPAR (27) or LXR (71). Unlike RAR, these other receptors would not inhibit activation of RXR by RA metabolites. However, our transfection experiments with *Drosophila* SL2 cells showed that RXR and RA alone can induce transcription from the A-II J site. These data hence demonstrate that RXR can function independently of interacting receptors such as RAR or PPAR, which are not present in *Drosophila* cells under basal conditions.

In addition to RXR homodimers, which mediate the RA response of the apo A-II gene, other nuclear receptors, some of which (such as EAR-2, EAR-3, ARP-1, and PPAR) RXR can heterodimerize with, have been shown to interact with the apo A-II J site (9, 38, 68). Hepatocyte nuclear factor 4 and PPAR induce, whereas EAR-2, EAR-3, and ARP-1 reduce, apo A-II gene transcription upon binding to this element (38, 68). Therefore, the J site of the apo A-II promoter might function as a composite hormonal and nutritional RE which can be activated by multiple (interacting) signalling pathways. In fact, it is tempting to speculate that several nutritional pathways converge at this site. Changes in nutritional intake of vitamin A derivatives affect apo A-II gene expression via activation of RXR. Furthermore, the induction of apo A-II promoter activity by dietary fatty acid derivatives and the fibrate class of hypolipidemic drugs is also mediated by this site, via activation of the nuclear receptor PPAR (68). The convergence of two important metabolic pathways on the J site hence warrants a more detailed investigation to study the combined effects of various nutritional compounds on apo A-II expression. Such studies are currently under way in our laboratory.

From these data as well as from previous studies from our laboratory (3) and other laboratories (24, 56, 70, 74), it becomes increasingly evident that retinoids have important effects on lipid transport and lipoprotein physiology. Despite the fact that the molecular mechanisms underlying the regulation of gene expression by retinoids have been characterized in great detail, very few in vivo and/or clinical studies have addressed the eventual regulatory role of retinoids on lipid metabolism. Considering HDL metabolism, Karathanasis and coworkers demonstrated that RXRα binds to the A site in the apo A-I promoter and activates transcription in response to RA (56, 70). In vitro studies with the HepG2 cell lines (3) as well as with rat (3) or cynomolgus monkey (24) primary hepatocytes confirmed that apo A-I gene expression is enhanced by the addition of RA to the cell culture medium. However, in vivo data from studies with rats seem to suggest an inhibitory effect of retinoids on apo A-I gene expression (3, 74). From the data in the current study, it is evident that retinoids not only control the expression of the apo A-I gene but also affect the transcription of apo A-II, the other major protein component of HDL, via the interaction of RXR through the J site of the apo A-II gene. Further studies are, however, necessary to evaluate whether this regulation observed in cultured cells bears any significance for the clinical situation.

In conclusion, the results from these studies show that apo A-II production increases in the liver as a result of an RXR-mediated induction of apo A-II gene transcription. The regulation of the apo A-II gene by retinoids is another indication that these compounds have important effects on lipid metabolism.

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