Interaction of oestrogen and peroxisome proliferator-activated receptors with apolipoprotein(a) gene enhancers

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A high plasma concentration of lipoprotein(a) [Lp(a)] confers an increased risk for the development of coronary heart disease. Hormones, such as oestrogen, are some of the few compounds known to reduce plasma Lp(a) levels. A putative enhancer region, located at the DHII DNase I hypersensitive site approx. 28 kb upstream of the apolipoprotein(a) [*apo(a)*] gene, contains a number of sequences similar to the binding half-sites for nuclear hormone receptors, such as the oestrogen receptor and the peroxisome proliferator-activated receptor (PPAR). The 180 bp core DHII enhancer increased the activity of the *apo(a)* promoter by over 7-fold in reporter-gene assays in HepG2 cells *in vitro*. Almost 60% of this increase was lost in the presence of co-transfected oestrogen receptor and oestrogen. In contrast, co-transfection with PPAR α increased the effect of the DHII enhancer on *apo(a)* transcriptional activity by approx. 70% and

could overcome the inhibitory effect of the oestrogen receptor on *apo(a)* transcription. Gel mobility-shift assays showed that oestrogen receptor protein bound to one half of a sequence corresponding to a predicted oestrogen receptor response element. PPAR α also bound to this site and competed with oestrogen receptors for binding. In addition, PPAR α bound to a separate site that comprised part of a direct repeat of nuclear hormone receptor half-sites. The results suggest that nuclear hormones affect plasma Lp(a) concentrations by binding to the sequences within the DHII enhancer, thereby altering the amount by which the enhancer increases the transcription of the *apo(a)* gene.

Key words: androgen, gene transcription, lipoprotein(a).

INTRODUCTION

Lipoprotein(a) [Lp(a)] is a plasma lipoprotein that consists of a particle similar to low-density lipoprotein covalently attached to a unique protein known as apolipoprotein(a) [*apo(a)*] [1]. There is a large variation in the plasma Lp(a) concentration between individuals, almost all of which is determined by inherited sequences at or close to the *apo(a)* gene locus [2–4]. *Apo(a)* bears a strong structural resemblance to plasminogen [5]. Indeed, the *apo(a)* gene is adjacent to the plasminogen gene on chromosome 6 [6] and probably arose from it by gene duplication. The *apo(a)* gene exhibits considerable size polymorphism related to the presence of different numbers of a tandemly repeated sequence similar to that of the fourth of the Kringle structures found in plasminogen [5,7]. There is a general inverse relationship between the number of these Kringle 4 repeats and plasma Lp(a) levels [8], resulting from the less efficient processing of the larger *apo(a)* isoforms and the consequently reduced availability of *apo(a)* for incorporation into Lp(a) [9,10]. However, these differences cannot account for all of the variation in Lp(a) concentrations, since the same sized *apo(a)* isoforms can be associated with widely different levels of *apo(a)* in plasma [11].

It is now recognized that Lp(a) is a significant independent risk factor for the development of atherosclerosis, probably through the ability of *apo(a)* to compete with plasminogen for binding to fibrin. Many studies have shown an association of high Lp(a) levels with arterial disease and stroke [12]. Furthermore, transgenic mice expressing the human *apo(a)* gene develop arterial lesions when fed a high-fat diet [13] and Lp(a) has been detected in human fatty streaks [14]. Thus discovering mechanisms that could reduce Lp(a) concentrations in plasma could be of considerable clinical benefit. Most lipid-lowering drugs and therapies

have little effect on Lp(a) concentrations. However, some steroid hormones are known to reduce Lp(a) levels [15]. For instance, Lp(a) concentrations are reduced by as much as 50% in postmenopausal women on hormone-replacement therapy [16–18], and are increased in orchidectomized men [19].

In both monkeys [20] and man [21] the abundance of *apo(a)* mRNA transcripts of the same size can vary greatly between individuals. Also, the reduction in plasma *apo(a)* concentration produced by administration of pharmacological doses of oestrogen and testosterone to transgenic mice containing the *apo(a)* genomic region is accompanied by a similar reduction in the hepatic *apo(a)* mRNA content [22]. These results suggest that the effect of steroid hormones on Lp(a) levels could be mediated by changes in the rate of transcription of the *apo(a)* gene. The immediate 5'-flanking region of the *apo(a)* gene has been cloned, but shows only weak promoter activity [23,24]. Natural sequence changes in the region are not associated with any large differences in plasma Lp(a) concentration and its activity is not influenced by steroids [25]. However, there are two upstream regions between the *apo(a)* and plasminogen genes that enhance *apo(a)* promoter activity in reporter-gene constructs *in vitro* [26]. These correspond to two DNase I hypersensitive sites, DHII and DHIII [27]. The DHII enhancer contains two sites that bind the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) [26]. A separate study has located the chromosomal region responsible for the oestrogen effect in transgenic mice to a sequence close to one of the PPAR sites in the DHII enhancer, though binding of the oestrogen receptor to this sequence could not be demonstrated [28]. PPAR binds as a heterodimer with retinoid X receptor (RXR) to direct repeats (DRs) of the nuclear hormone receptor half-sites separated by one nucleotide (DR1 sites). The oestrogen receptor, on the other hand, usually binds as a homodimer to

Abbreviations used: *apo(a)*, apolipoprotein(a); DR, direct repeat; ERE, oestrogen receptor response element; ETYA, eicoso-5,8,11,14-tetraenoic acid; IR, inverted repeat; Lp(a), lipoprotein(a); PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor.

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inverted repeats (IRs) of the half-sites separated by three nucleotides (IR3 sites). However, examples from other genes suggest that it is possible that hormone receptors can influence transcriptional activity through an interaction between a hormone receptor half-site and certain neighbouring sites, such as those for Sp1 [29–31].

In this study we have examined the ability of PPAR α and oestrogen receptors to influence the enhancer activity of the DHII region in reporter-gene constructs. The possibility of competition between the two receptors was investigated and the sequence responsible for the binding of the oestrogen receptor has been identified.

EXPERIMENTAL

Materials

Plasmid pSG5mPPAR α , containing the full-length cDNA of the mouse PPAR α gene, was provided by Dr J. Tugwood (AstraZeneca, Macclesfield, Cheshire, U.K.). The plasmid containing the full-length human RXR α cDNA, pSG5hRXR α , was provided by Dr A. Dejean (Institut Pasteur, Paris, France). The wild-type oestrogen receptor was expressed from the HEGO plasmid, which was obtained from Dr P. Chambon (Faculte de Medecine, Strasbourg, France). The expression vector containing the human androgen receptor DNA, pCMVhAR, was a gift from Dr E. Wilson (University of North Carolina, Chapel Hill, NC, U.S.A.). Reporter-gene constructs containing the core enhancer regions cloned upstream of the basic (–98 to +130) apo(a) promoter driving the luciferase gene were prepared as described previously [26]. All antisera used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). ETYA (eicosa-5,8,11,14-tetraynoic acid), 9-*cis* retinoic acid, oestrogen (17 β -oestradiol) and androgen (4-androsten-17 β -ol-3-one) were from Sigma-Aldrich (Poole, Dorset, U.K.).

Transient transfections of HepG2 cells and the measurement of reporter gene activity

Human hepatoma HepG2 cells, at a density of 0.5×10^6 cells/24 mm well, were transfected using the Lipofectin reagent (Life Technologies, Paisley, U.K.) essentially as described previously [26]. Up to 8 μ g of nuclear hormone receptor expression vectors were added to each well. Within each experiment, the total amount of DNA added was kept constant by the addition of pSG5 vector (Stratagene Europe, Amsterdam, The Netherlands). Transfection efficiency was controlled by the co-transfection of 0.5 μ g of CMV- β -galactosidase vector (where CMV is cytomegalovirus; BD Clontech UK, Basingstoke, Hants, U.K.). The cells were incubated for 24 h in medium containing charcoal-treated serum and 100 nM nuclear hormones as indicated. ETYA (20 μ M) and 9-*cis* retinoic acid (100 nM) were always added to cells transfected with PPAR α and RXR α . Cell lysates were prepared and the corrected reporter-gene activity measured as described in [26].

Electrophoretic mobility-shift assays

Double-stranded oligonucleotide probes, with 4 bp overhangs, were synthesized with the sequences shown in Figures 4 and 5 (see below). The sequence used for an authentic peroxisome proliferator response element (PPRE) was 5'-CATGCGATATCAT-GACCTTTGTCTAGGCCTCTAG-3', and for the oestrogen receptor response element (ERE) was 5'-TAATTCAGGTCAC-TGTGACCTGATCT-3'. The probes were labelled by fill-in with Klenow enzyme incorporating the appropriate [α - 32 P]dNTPs.

Figure 1 Effect of oestrogen receptors on enhancer activity

HepG2 cells were transfected with reporter-gene constructs containing the core DHII or DHIII enhancer regions upstream of the apo(a) promoter driving the luciferase gene. Where indicated (+) the cells were co-transfected with an oestrogen receptor expression plasmid (ER) and incubated with 100 nM oestrogen (E) in the medium. Results are shown as means $\pm$ S.E.M. of four independent experiments. *, Statistically significant ($P < 0.05$, Students *t* test) effect of ER + E on the DHII construct.

Electrophoretic mobility-shift reactions were performed as adapted [26] from Kleiwer et al. [32], using 400 ng of poly(dI-dC) per reaction throughout. Oestrogen receptor, PPAR α and RXR α proteins were synthesized *in vitro* using the TNT T7-coupled transcription/translation kit (Promega, Madison, WI, U.S.A.). Supershift reactions were performed using 200 ng of specific antiserum per reaction.

RESULTS

Reporter-gene assays

Hepatoma HepG2 cells were transfected with reporter-gene constructs containing the apo(a) promoter driving the luciferase gene. Placing the DHII enhancer upstream of the promoter increased expressed luciferase activity over 7-fold (Figure 1). Co-transfection with an expression vector for the oestrogen receptor, activated by incubation with oestrogen in the medium, decreased this activity by over 60%. Oestrogen and its receptor had no effect on the activity of the promoter alone, nor on the activity of a construct containing the core region of the other enhancer (DHIII) found upstream of the apo(a) gene (Figure 1).

The effect of increasing amounts of expression vectors for various nuclear hormone receptors on the activity of the DHII reporter-gene construct is shown in Figure 2(A). Co-expression of the oestrogen receptor produced a concentration-dependent inhibition of enhancer activity. The maximum effect was achieved with 2 μ g/ml expression vector DNA. The androgen receptor also reduced activity but required a higher concentration to achieve the same effect. In contrast, an expression vector for PPAR α produced a concentration-dependent increase in DHII enhancer activity, with a maximum increase of about 70%, again achieved with 2 μ g/ml vector DNA (Figure 2A). No such increase was observed using a vector containing the reversed PPAR α sequence.

An effect of PPAR α on the DHII-enhancer reporter construct was observed by co-transfecting with a PPAR α expression vector alone (Figure 3). This confirms our previous observation that HepG2 cells contain significant quantities of endogenous RXR, the heterodimeric partner required for PPAR α binding to DNA,

Figure 2 Concentration-dependence of the effects of hormone receptors on DHII enhancer activity

HepG2 cells were transfected with a reporter-gene construct containing the core DHII enhancer region upstream of the apo(a) promoter driving the luciferase gene. (A) Cells were co-transfected with the indicated concentrations of expression vectors for PPARα (●), PPARα reversed (○), the androgen receptor (Δ) or the oestrogen receptor (■). Values are the averages of results from two independent experiments, which did not differ by more than 15%, and are expressed as a percentage of the activity obtained with the reporter construct alone. (B) Cells were co-transfected with 1 μg/ml of the oestrogen receptor vector together with the indicated concentrations of PPARα vector (●), or 1 μg/ml of PPARα vector with the indicated concentrations of oestrogen receptor vector (■). Values are means ± S.E.M. of results from three separate experiments, expressed as a percentage of the activity with the constant vector alone.

Figure 3 Nuclear hormone receptors and DHII enhancer activity

HepG2 cells were transfected with a reporter-gene construct containing the core DHII enhancer region upstream of the apo(a) promoter driving the luciferase gene. They were co-transfected with expression vectors for PPARα, RXRα or the oestrogen receptor (ER) and incubated with 100 nM oestrogen (E) as indicated. Results are shown as means ± S.E.M. from 10 independent experiments. *, Statistically different ($P < 0.01$, Student's *t* test) from none. †, Statistically different ($P < 0.01$, Student's *t* test) from ER + E.

but little PPARα [33]. Nevertheless, co-transfection with a vector containing RXRα produced an additional response, suggesting that endogenous RXR was limiting. The addition of oestrogen to the medium had no effect on the reporter activity of the DHII construct, confirming that HepG2 cells do not express oestrogen receptors. Activity was inhibited merely by co-expression of the oestrogen receptor, indicating the presence of some endogenous activators, and was further reduced by oestrogen in the medium (Figure 3).

The inhibition produced by the oestrogen receptor was completely overcome by co-transfection with PPARα plus RXRα (Figure 3), and promoter activity reached levels similar to those with PPARα plus RXRα alone. This suggested that there could be competition between the receptors. Experiments in which increasing amounts of one vector were co-transfected in the presence of a sub-optimal concentration of the other (Figure 2B) were inconclusive in this respect. At all concentrations the inhibition produced by the oestrogen receptor was lower than in the absence of PPARα plus RXRα, but the data were not consistent enough to establish any difference in apparent affinity. Similarly the effect of PPARα plus RXRα was greater in the presence than in the absence of the oestrogen receptor, but there was no obvious difference in the concentration dependency.

Gel mobility-shift assays

DNase I footprinting had revealed that the 180 bp DHII enhancer contained two protein-binding regions [26]. One, called footprint I, was located between -76 bp and -112 bp from the 3' end of the enhancer and contained a possible DR1 binding site (Figure 4). It bound several HepG2 nuclear proteins and gave a weak supershifted band if incubated with a general anti-PPAR antibody. However, in the current studies we were unable to detect any retarded bands corresponding to bound PPARα when a labelled probe with the footprint I sequence was incubated with a mixture of *in vitro*-synthesized PPARα and RXRα. The only visible radioactive complexes were produced by the reticulocyte lysate alone (Figure 4A, lane 1). There was no supershifted band with an antibody specific for PPARα (Figure 4A, lane 3). When incubated with a known PPRE the same lysate containing PPARα/RXRα gave a specific band which was supershifted with the anti-PPARα antibody (Figure 4B). Similarly there was no evidence for the binding of the oestrogen receptor to footprint I (Figure 4A, lanes 4–6), despite the expected binding to a known ERE (Figure 4B, lanes 4–6).

The second footprint, footprint II, was located between -137 bp and -178 bp from the 3' end of the enhancer and contains four possible nuclear receptor binding half-sites (Figure 5). Incubation of *in vitro*-synthesized PPARα/RXRα with a labelled probe encompassing the region gave a strong retarded band (Figure 5A, lane 2) that was not present with reticulocyte lysate alone (Figure 5A, lane 1). Surprisingly there was no visible supershifted band with the anti-PPARα antibody (Figure 5A, lane 3). Nevertheless an excess of unlabelled footprint II probe competed with the labelled probe (Figure 5A, lane 5), as did the authentic PPRE (Figure 5A, lane 6), confirming specific PPARα binding to the region. Two of the potential half-sites form a DR1 site. Destroying this site in the unlabelled probe by mutation (mA) prevented it from competing with the labelled footprint II probe (Figure 5A, lane 7), indicating that the site was required for maximal PPARα binding. Mutating one of the other sites (mB) did not prevent competition (Figure 5A, lane 8), showing that this site was not necessary for PPARα binding. Mutating the fourth site (mE), reduced but did not eliminate the amount of radioactive probe in the complex (Figure 5A, lane 11), suggesting that this site was also involved in PPARα binding. Clearly an interaction between site A and site E is not essential for binding since mutation of either alone reduced competition. The observation that the amount of radioactive probe in the complex was apparently reduced by mutating site E (Figure 5A, lane 11) or sites A and B (Figure 5A, lane 9) compared with that in the adjacent lane in which both A and E were mutated (Figure 5A, lane 10) is consistent with independent binding of PPARα/RXRα to the two sites.

Figure 4 Gel mobility-shift assays with probe I, PPRE and ERE

In vitro synthesized PPAR α (P), RXR α (R) and oestrogen receptor (ER), or lysate incubated without vector (—) were incubated with end-labelled probe I, corresponding to footprint I in the DHII enhancer (A), or labelled authentic PPRE or ERE (B). An antibody to PPAR α (α P) or the oestrogen receptor (α E) was included where indicated. Shifted (a, c) and supershifted (b, d) complexes are arrowed. The sequence of probe I is shown above, numbered from the 3' end of the enhancer, with the positions of the possible nuclear hormone receptor half-sites shown by arrows.

In vitro-synthesized oestrogen receptor gave only a weak retarded band when incubated with the labelled footprint II probe (Figure 5B, lanes 1 and 5). However, inclusion of an anti-oestrogen receptor antibody produced a dense supershifted band that was not present if a non-immune serum was employed (Figure 5B, lane 3). Thus competition studies were performed using supershifted complexes. Incubation with an excess of unlabelled footprint II probe or an authentic ERE essentially eliminated the radioactive probe from the complexes (Figure 5B, lanes 8 and 9), confirming specific binding of the oestrogen receptor. Unlabelled footprint I probe did not displace the labelled footprint II probe (Figure 5B, lane 7), supporting the earlier direct observation of a lack of binding of the oestrogen receptor to the footprint I region. Mutating site A in the unlabelled probe prevented the displacement of the labelled probe from the complexes (Figure 5B, lane 11). Mutating sites B or E did not prevent competition (Figure 5B, lanes 12 and 13). These results, together with those in which the mutations were combined (Figure 5B, lanes 14–17), were entirely consistent with the oestrogen receptor binding to site A alone, with no contribution from other sequences in the region.

To confirm direct competition between PPAR α and oestrogen receptors for binding to footprint II, studies were performed in which the amount of each protein in the incubation mixture was varied. First the labelled footprint II probe was incubated with a fixed amount of PPAR α /RXR α , together with increasing concentrations of the oestrogen receptor. Complexes containing oestrogen receptors were detected using the anti-oestrogen

receptor antibody. The addition of oestrogen receptor produced a concentration-dependent increase in the labelled supershifted complexes containing the oestrogen receptor and a corresponding decrease in the labelled complexes containing PPAR α /RXR α (Figure 6, lanes 1–4). Conversely, there was a concentration-dependent displacement of oestrogen receptors from the complexes by PPAR α /RXR α (Figure 6, lanes 5–9).

DISCUSSION

Previous studies have identified a number of elements that are involved in the regulation of *apo(a)* gene transcription, including the promoter region immediately upstream of the gene and two putative enhancers in the intergenic region between the *apo(a)* and plasminogen genes. It is known that some steroid hormones can reduce plasma Lp(a) levels *in vivo* [15,18,34], and the oestrogen effect has been localized to the DHII enhancer region about 28 kb upstream of the *apo(a)* gene [28]. This corresponds to a DNase I-hypersensitive site that is presumed to be a region of open chromatin sensitive to DNase I digestion and favourable for the binding of transcription factors. The full enhancer is orientation-sensitive, being active in the direction found in the native DNA [26]. This may be relevant, since the plasminogen gene, which is closer and transcribed in the opposite direction to the *apo(a)* gene, is not sensitive to oestrogen. This suggests that there could be some insulating elements between the DHII enhancer and the plasminogen gene and that the DHII enhancer may represent the last element capable of regulating the *apo(a)*

Figure 5 Gel mobility-shift assays of footprint II in the core region of the DHII enhancer

Probe II was synthesized as a synthetic double-stranded oligonucleotide spanning footprint II (-137 to -178) of the DHII enhancer. The positions of the possible nuclear hormone receptor half-sites are shown by the arrows. Probes containing mutations that destroyed binding to these sites (mA, mE, mB) were synthesized with the sequences shown in lower case below. *In vitro*-synthesized PPARα (P), RXRα (R) and oestrogen receptor (ER), or lysate incubated without vector (-) were incubated with end-labelled probe II, together with an antibody to PPARα (αP) or the oestrogen receptor (αE) where indicated. Unlabelled competitor oligonucleotides were included at a 100-fold molar excess as shown. I and II refer to unlabelled probes I and II respectively and PPRE and ERE to unlabelled oligonucleotides containing authentic PPARα or oestrogen receptor-binding sites. mA, mE and mB are unlabelled probe II with the mutations at positions A, E and B, with double and triple mutants as shown. Shifted complexes are denoted by a and supershifted complexes by b with arrows beside the gels.

gene [28]. The 186 bp core of the DHII enhancer is remarkably well conserved. No base changes were observed on sequencing the region from over 20 subjects (L. H. Puckey and B. L. Knight, unpublished work). Since the subjects were chosen to cover the whole range of Lp(a) concentrations, from 0 to over 300 mg/dl, the region is clearly not involved in determining the common genetic differences in Lp(a) concentration between individuals. However, the conservation could indicate that it has an important modulating function by binding factors that mediate the effect of drugs and hormones. In support of this, we have shown here that activated oestrogen and androgen receptors inhibit DHII enhancer activity in reporter-gene assays *in vitro*.

The DHII enhancer was shown previously to bind PPAR, though the isoform involved was not determined [26]. There are three currently known PPAR isoforms, all of which are thought to be important regulators of lipid metabolism [35]. Each is activated by specific ligands that switch the structure of the PPAR from an inactive or repressive form to an active form capable of binding co-activators. The fibrate group of lipid-lowering drugs are ligands for PPARα. A number of studies have investigated the effects of fibrates on plasma Lp(a) levels, but the results have been inconsistent, ranging from a decrease [36] to an increase [37] or no effect at all [36]. Our experiments reported here show that co-expression of a vector for PPARα produced a moderate increase in DHII enhancer activity of reporter-gene constructs in HepG2 cells *in vitro*. This is consistent with the general transcriptional activating properties of the receptor.

Activation by PPARα in HepG2 cells was concentration-dependent and its maximal effect required co-transfection with RXR and stimulation with ligand. Most striking was the observation that co-transfection with vectors for PPARα and RXRα overcame the inhibition produced by oestrogen receptors, suggesting that they competed for binding to DNA or co-factors.

Footprint analysis of the 186 bp core DHII enhancer showed two protein-binding areas, known as footprint I and footprint II, each of which contained two sequences that were essential for maximum enhancer activity [26]. PPAR binding to both footprints was demonstrated in gel-shift assays using HepG2 cell nuclear extracts with a supershift produced by a non-specific anti-PPAR antibody [26]. In the current studies we have concentrated on the effects of PPARα and have examined the binding of *in vitro*-synthesized PPARα rather than that of a whole nuclear extract. We could detect no binding of PPARα to an oligonucleotide probe containing the footprint I sequence, either directly or after addition of a specific antibody for PPARα. This suggests that the binding observed previously was not that of PPARα but one of the other isoforms, probably that of PPARδ, since liver contains little PPARγ [38].

In contrast, footprint II clearly bound *in vitro*-synthesized PPARα in gel-shift assays. Although the shifted band could not be supershifted with the anti-PPARα antibody, binding was confirmed by competition with an excess of unlabelled authentic PPRE. The sites of PPARα binding were determined by

Figure 6 Competition between PPAR α and the oestrogen receptor for binding to probe II

Lanes 1–4, labelled probe II was incubated with a fixed amount of *in vitro*-synthesized PPAR α plus RXR α (P/R; 2 μ l) and increasing amounts of *in vitro*-synthesized oestrogen receptor (ER; 1, 2 and 4 μ l). Lanes 5–9, labelled probe II was incubated with a fixed amount of *in vitro*-synthesized oestrogen receptor (2 μ l) and increasing amounts of *in vitro*-synthesized PPAR α (1, 2 and 4 μ l) plus RXR (2, 4 and 6 μ l). Complexes containing the oestrogen receptor were identified as a supershifted band (arrow) by incubation with an antibody against the oestrogen receptor (α E).

mutating possible sequences in unlabelled probe II and examining the effect on the shifted band. Mutating one site, site B, in the unlabelled probe did not prevent it from competing with the labelled native probe for binding to PPAR α , indicating that site B was not involved in PPAR α binding. Mutating site A or site E, on the other hand, prevented the unlabelled probe from completely displacing the labelled unmutated probe from PPAR α , though in each case the intensity of the shifted band was reduced, suggesting that some competition had occurred. The simplest explanation of these results is that PPAR α bound independently to sites A and E. Site A is a DR1 sequence, which would be predicted to bind PPAR α . Site E is part of a possible IR6 sequence that was proposed by Boffelli et al. [28] to be responsible for the oestrogen effect. It encompasses a possible nuclear hormone half-site, but there is no obvious DR1 sequence in the region between sites A and B.

As with PPAR α , there was no evidence for binding of oestrogen receptors to footprint I. However, there was strong evidence for binding to footprint II. The presence of *in vitro*-synthesized oestrogen receptor alone did not produce a pronounced band of shifted labelled probe II, but the further addition of an anti-oestrogen-receptor antibody gave an intense supershifted band that was not present with non-immune serum. Again, binding

could be confirmed by competition with unlabelled authentic ERE. The increase in intensity of the shifted band in the presence of the non-immune serum suggests that the usual conditions were not optimal for binding the oestrogen receptor to this region, which could explain the inability of Boffelli et al. [28] to demonstrate binding in their experiments. Mutation analysis was clearer than that for PPAR α . Unlabelled probe containing mutations in sites B or E almost completely displaced labelled unmutated probe from the complexes, whereas probe with a mutation at site A did not compete. Thus the oestrogen receptor bound to site A but not to sites B or E. This was unexpected since sites A and E form a possible IR sequence that might be expected to bind oestrogen receptors. The mutations in site A would have destroyed a possible IR sequence that could be the site of oestrogen receptor binding.

Studies showing the mutual displacement of oestrogen receptors and PPAR α /RXR from their complexes with probe II confirmed the conclusion that they bind to the same site. Overall, the results suggest that footprint II contains two independent sites for the binding of PPAR α /RXR α , one of which can also bind oestrogen receptors. This would predict that PPAR α could overcome the effect of the oestrogen receptor, but that oestrogen receptors would not completely abolish the effects of PPAR α , which is entirely consistent with the competition experiments performed with the reporter-gene constructs. We have no information on the binding site for the androgen receptor. It is possible that it binds to the same site as the oestrogen receptor, but the consensus half-site (AGAACA) is sufficiently different from that for the oestrogen receptor or PPAR α (AGGTCA) [39] to suggest that this may not be so. There are no matching sequences within the two active footprints.

In tissues such as the liver, which contain appreciable amounts of PPAR α [38], the oestrogen receptor could compete with PPAR α for binding to footprint II. However, it is unlikely that the oestrogen receptor acts merely by counteracting the positive transcriptional activity of PPAR α . HepG2 cells contain little PPAR α [33], yet oestrogen receptors produced a marked inhibition of enhancer activity, indicating that it has a direct repressive effect on transcription. From one extreme to the other, the interaction of oestrogen receptors and PPAR α can alter DHII enhancer activity over 5-fold. Thus the region has the potential for significant modulation of *apo(a)* gene transcription and could be an important site for pharmacological intervention.

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