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The Apolipoprotein(a) Promoter Contains a Retinoid Response Element

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Retinoids were previously shown to lower apolipoprotein(a) [apo(a)] mRNA levels, suggesting that the apo(a) promoter contains a retinoid response element (RRE). Scanning the apo(a) promoter for sequences related to the consensus RRE half-site (5′-PuG(G/T)TCA-3′) uncovered four sites that could potentially function as RREs at −2915, −1875, −1036, and −407. The activity of these sites was assessed by their ability to compete with a very strong consensus DR5 RRE for binding to retinoic acid receptor (RARα) and retinoid X receptor (RXRα) heterodimers using electrophoretic mobility-shift assays. Only the −1036 site (5′-TGACCTTGT-GATCC-′3) was an effective competitor of the DR5 RRE; therefore, it was designated as apo(a) RRE. Apo(a) RRE competed with DR5 RRE for RARα/RXRα binding with 1/10 the affinity of DR5 RRE, while a scrambled apo(a) RRE was inactive. These results suggested that apo(a) RRE is a potential candidate for mediating the effect retinoids have on apo(a) mRNA expression. © 1998 Academic Press

Key Words: **lipoprotein(a); retinoids; reinoid response element; RAR; RXR.**

Elevated plasma lipoprotein(a), [Lp(a)], is an independent risk factor for a variety of cardiovascular and cerebrovascular diseases (1). Lp(a) is produced extracellularly through the covalent association of two liver produced proteins- apolipoprotein (apo)B-100 and apo(a) (1). Since apoB-100 levels are usually in excess relative to apo(a), an effective way of lowering Lp(a) is through decreasing apo(a) production. Previously we have shown that retinoids can decrease apo(a) mRNA levels in primary hepatocyte cultures (2). This observation suggested that retinoid treatment may be a potential method for treating elevated plasma Lp(a).

To begin to address the mechanism by which retinoids attenuate apo(a) mRNA expression, the apo(a) promoter was scanned for the presence of retinoid response elements (RREs). Such elements have not been previously characterized in the apo(a) 5′-flanking region (3,4). Most RREs are composed of direct repeats (DR) of the consensus sequence, 5′-PuG(G/T)TCA-3′, with spacing between the DR ranging from 1 to 5 nucleotides (DR1 to DR5) (5). This analysis revealed the presence of four sites that could potentially function as RREs at: −2915; −1875; −1036 and; −407, relative to the major apo(a) transcription initiation site (3). The functionality of these sites was examined by their ability to compete with a strong consensus DR5 RRE (6) for binding to *in vitro* translated retinoic acid receptor (RARα) and retinoid X receptor (RXRα) heterodimers (7–9) in electrophoretic mobility shift assays. Only the apo(a) element at position −1036 (5′-TGACCTTGT-GATCC-′3) was an effective competitor of the DR5 RRE. This site was designated as apo(a) RRE.

METHODS

The oligonucleotide probes used in the electrophoretic mobility shift assays were as follows: DR5 RRE (DR5) (6), 5′-GGGGTACCTCAGGTCATCCTCAGGTCAGGTACCC-3′; DR5 mix, 5′-GGGGTACCGTACTGCTCTACGTCGGGTACCC-3′; apo(a) −2915, 5′-AAGAATGAACTTTATGAACAAA-3′; apo(a) −1875, 5′-AGTTGCAAGGTAAGAAAAGATCAAGAGAC-3′; apo(a) −1036, 5′-$(G)_5$TACCTCAAACTCCTGACCTTGTGATCCACCTGTCTTGGGTACC-3′; apo(a) −1036 mix, 5′-$(G)_5$TACCTCAAACCTCCGATGGTACATCGCTCTCGGTACC-3′ and; apo(a) −407, 5′-GCTCGTAAACTAAGACCTGAAAGGG-3′ Some oligonucleotides contained a KpnI site for cloning purposes (underscored above). In the above, negative numbers after "apo(a)" represent the location of the site relative to the major apo(a) transcriptional initiation site (3,4). The apo(a) promoter sequence beyond −1401 and up to −3,000 was obtained from Dr. Roberto Taramelli, Universita degli Studi di Catania, Catania , Italy. Complementary strands of the latter oligonucleotides were synthesized missing the last two 3′ C residues to allow for labeling by DNA

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Abbreviations used: RER, retinoid response element; apo(a) RER, apolipoprotein(a) retinoid response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; Pu, purine (A/G); Py, pyrimidine (T/C).

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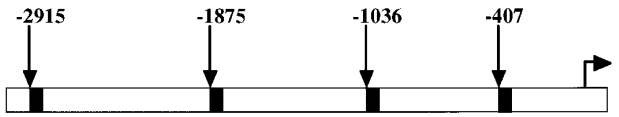
polymerase as detailed below. Full length zebrafish RARα and RXRα protein was prepared from their respective linearized plasmids (provided by Dr. M. Petkovich, Queen's University, Kingston, Ontario, Canada) using Promega's TNT Coupled Wheat Germ Extract system with the addition of [^{35}S]methionine. Protein concentration was quanititated by TCA precipitation. Radiolabeled probes were prepared by filling in the GG overhang at the 5′ ends of the double-strand annealed oligonucleotides using DNA polymerase Klenow fragment (Life Technologies) and [α-^{32}P]dCTP. Labeled double-stranded oligonucleotide probe (25 fmoles) was incubated with *in vitro* translated receptor proteins (100,000 cpm/protein) in 60 mM KCl, 20 mM HEPES, 3% Ficoll, 0.5 mM DTT, and 0.05 mg/ml dIdC in a 20 μl reaction volume at room temperature for 15 minutes. When used, cold competitor was added at 100 pmoles/sample or the concentration indicated in the figure. Aliquots of the reaction mixture (5 μl) were loaded onto 6% mini (Novex) or large 15×17 cm DNA Retardation gels (6% acrylamide, 3% glycerol, and 0.25× TBE) and electrophoresis carried out at 100 W for 45 minutes (minigels) or 2 hours (large gels). After electrophoresis, gels were dried down and exposed to either x-ray film (Amersham Hyperfilm MP) or a phosphoimager screen.

RESULTS

Our previous studies showing that retinoids decrease apo(a) mRNA expression (2) pointed to the presence of an uncharacterized retinoid response element (RRE) in the apo(a) promoter. Most RREs are composed of direct repeats (DR) of the consensus sequence *5′-PuG(G/T)TCA-3′* with spacing between the DR ranging from 1 to 5 nucleotides (DR1 to DR5) (5). In general, RARα/RXRα heterodimers bind RREs with much higher affinity than RARα or RXRα homodimers (10). This suggests that RARα/RXRα heterodimers are the biologically relevant transactivators of the retinoid response. To uncover potential RRE sites, the human apo(a) promoter (3,4) was scanned for the presence of the consensus RRE half-site described above. This analysis revealed the presence of four sites that could potentially function as REs (Fig. 1). These sites were all imperfect DR separated by 2 (DR2) to 4 (DR4) nucleotides. The four putative elements were analyzed for activity by competitive electrophoretic mobility shift assays. In these experiments, the apo(a) promoter elements were competed against a DR5 RRE (6) for binding to *in vitro* translated RARα and RXRα heterodimers. The DR5 element was chosen because it binds RARα/RXRα heterodimers with very high affinity (6), consequently, it can be used as a stringent test to screen the potential apo(a) RRE sites for binding to the retinoid receptors. As expected, RARα/RXRα bound strongly to the DR5 element (Fig. 2, lane 2). This tight binding was specific since it was almost completely competed with an excess of cold DR5, (Fig. 2, lane 3), but, not with a scrambled (mixed) DR5 sequence (Fig. 2, lane 4, *DR5M*). Competition experiments using the four putative apo(a) retinoid response elements (Fig. 2, lanes 5, 7, 8 and 9) demonstrated that only the element at position −1036 (Fig. 2, lane 5) competed with labeled DR5 for binding to RARα/RXRα. Furthermore, a scrambled (mixed) ver-



Concensus RRE:
Upper 5'-PuG(G/T)TCA-3'
Lower 5'-TGA(C/A)CPy-3'
apo(a) sites:
-2915 5'-TGAACT TTA TGAACA-3'
-1875 5'-C AAGGTA AGAA AAGATC-3'
-1036 5'-TGACCT TG TGATCC-3'
-407 5'-TAAACT AAGACCTGA-3'

FIG. 1. Potential retinoid response elements in the apo(a) promoter. The locations of four potential retinoid response elements in the apo(a) promoter are diagrammed relative to the major transcriptional start site (3). These sites were located by computer aided comparison of the apo(a) promoter and the consensus retinoid response element (RRE) half-site (11). Apo(a) promoter sequences beyond −1400 and up to −3,000 were obtained from Dr. Roberto Taramelli (see Methods). The upper- and lower-strand sequence of the consensus RRE half-site along with the upper-strand sequence of the potential apo(a) retinoid response elements [apo(a) RREs] are shown below the diagram. All four potential apo(a) RREs are located on the lower-strand relative to the consensus RRE element half-site upper-strand sequence. Direct repeats are underlined.

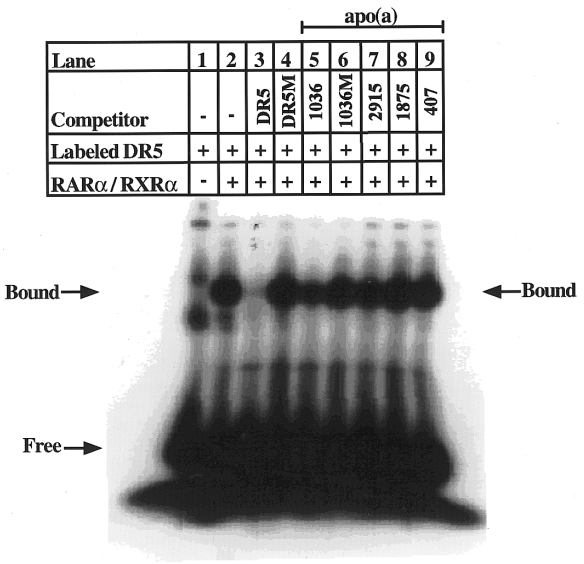


FIG. 2. Displacement of DR5 binding to RAR/RXR heterodimers by potential retinoid response elements from the apo(a) promoter displayed in *Fig. 1*. *In vitro* transcribed and translated zebrafish RARα and RXRα proteins (100,000 cpm/reaction) were incubated with radiolabeled consensus DR5 double-stranded oligonucleotide (25 fmoles) with or without cold competitor (100 pmoles) as detailed in Methods. Part of the reaction (15 μl) was resolved on Novex, 6%, DNA retardation gels. The gels were dried and exposed to Amersham Hyperfilm-MP. *DR5M* and *1036M* are mixed (scrambled) sequences of the RRE in DR5 and 1036, respectively.

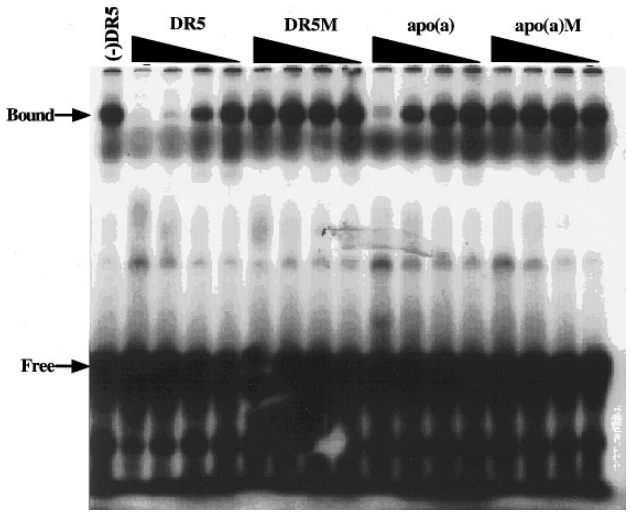


FIG. 3. Competitive binding of DR5 to RARα/RXRα heterodimers. For competition experiments, RARα and RXRα were incubated with radiolabeled DR5 (25 fmoles), and decreasing amounts of cold DR5, DR5M, apo(a) −1036 and apo(a) −1036M oligonucleotide. Cold oligonucleotide was present in the reaction mixture at 100, 10, 1, and 0.1 pmoles/reaction as represented by the decreasing wedge above the lanes. Reaction mixtures were resolved on 6% polyacrylamide DNA retardation gels (15 cm × 17 cm) containing glycerol and the bands visualized by autoradiography of the dried gels. *(−)DR5,* complete reaction mixture not containing cold DR5 or apo(a) −1036 (positive control).

sion of the apo(a) −1036 element did not compete with DR5 for binding to the receptors, (Fig. 2, lane 6, *1036M*). These results showed that of the four putative retinoid response elements in the apo(a) promoter, only the −1036 element was active at competing with DR5 for binding to RARα/RXRα, suggesting that it was responsible for the retinoid response of the apo(a) gene. This element is of the DR2 class (5) and will be referred to as the apo(a) retinoid response element (apo(a) RRE).

Relative to the consensus retinoid response element, apo(a) RRE is located on the lower strand of the apo(a) promoter and has the upper-strand sequence: 5′-TGACCTTGTGA*T*CC-′3. Apo(a) RRE differs from the consensus retinoid half-site lower-strand sequence by containing a T (italicized above) instead of C/A at the third position of the second half-site (Fig. 1).

The latter experiments determined that apo(a) RRE could compete with DR5 for binding to RARα/RXRα. To test the relative affinity of DR5 and apo(a) RRE for binding to RARα/RXRα, competition experiments were setup using labeled DR5 and increasing concentration of cold DR5 or apo(a) RRE. These results demonstrated strong binding of DR5 to RARα/RXRα, (Fig. 3, *(−)DR5*), that could be competed by cold DR5, in a dose dependent manner, (Fig 3, *DR5*), but, not by a scrambled (mixed) DR5, (Fig. 3, *DR5M*). Similarly, cold apo(a) RRE effectively competed with labeled DR5 in a concentration dependent manner, (Fig. 3, *apo(a)*), while a scrambled (mixed) apo(a) RRE was not active, (Fig. 3, *apo(a)M*). However, the intensity of the shifted band generated in these competition experiments showed that apo(a) RRE competed with DR5 for binding to RARα/RXRα at approximately 1/10 the affinity of DR5 itself. DR5. Indeed, quantitation of the latter bands indicated that DR5 and apo(a) RRE binding to RARα/RXRα was inhibited by their corresponding cold oligonucleotides with $IC_{50's}$ of approximately 50 nM and 500 nM, respectively. The weaker binding of apo(a) RRE to RARα/RXRα than DR5 may be due to the T-substitution in apo(a) RRE described above and shown in Fig 1. Additionally, the lower affinity of DR2s for RARα/RXRα than DR5s may also contribute to the difference in apo(a) RRE and DR5 binding to the receptors (6).

DISCUSSION

The ability of retinoids to lower Lp(a) levels by attenuating apo(a) mRNA steady-state levels (2) is consistent with the known transcriptional effects of retinoids (5, 11). Our finding of a retinoid response element at position −1036 in the human apo(a) promoter (apo(a) RRE), that specifically interacts with *in vitro* translated RARα/RXRα heterodimers, is further evidence that endogenous apo(a) gene expression is retinoid responsive.

There are several mechanisms that could explain the negative effect of retinoids on apo(a) mRNA expression. RARα/RXRα heterodimers could bind apo(a) RRE and directly attenuate transcription. Alternatively, the RARα/RXRα complex may not have a direct effect on inhibiting apo(a) gene transcription but instead could partially displace a strong, positive-acting, transcription factor from its *cis*-acting element within apo(a) RRE. It is also possible that RARα/RXRα could bind to a positive *trans*-acting factor and prevent its interaction with the apo(a) promoter. In addition, limited amounts of shared nuclear receptor coactivators could play a role in attenuating apo(a) mRNA levels (12–14).

In conclusion, the discovery of apo(a) RRE in the apo(a) promoter provides a starting point to further explore the mechanism by which retinoids act to lower apo(a) mRNA expression at the transcriptional level. Furthermore, apo(a) RRE may be used in a rational approach to develop potent retinoids capable of effectively lowering elevated plasma Lp(a) levels.

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