A proximal tissue-specific module and a distal negative regulatory module control apolipoprotein(a) gene transcription

Sarita NEGI, Saurabh K. SINGH[1], Nirupma PATI[2], Vikas HANDA[3], Ruchi CHAUHAN and Uttam PATI[4]

Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

The *apo(a)* [apolipoprotein(a)] gene is responsible for variations in plasma lipoprotein(a), high levels of which are a risk factor for atherosclerosis and myocardial infarction. The *apo(a)* promoter stimulates the expression of reporter genes in HepG2 cells, but not in HeLa cells. In the present study, we demonstrate that the 1.4 kb *apo(a)* promoter comprises two composite regulatory regions: a distal negative regulatory module (positions − 1432 to − 716) and a proximal tissue-specific module (− 716 to − 616). The distal negative regulatory module contains two strong negative regulatory regions [polymorphic PNR (pentanucleotide repeat region) and NREβ (negative regulatory element β)], which sandwich the postive regulatory region PREβ (positive regulatory element β). The PNR was shown to bind to transcription factors in a tissue-specific manner, whereas the ubiquitous transcription factors hepatocyte nuclear factor 3α and GATA binding protein 4 bound to NREβ to repress gene transcription. The proximal tissue-specific module contains two regulatory elements: an activating region (PREα) that activates transcription in HepG2 cells, and NREα, which is responsible for repressing the *apo(a)* gene in HeLa cells. NREα binds to a HeLa-specific repressor. These multiple regulatory elements might work co-operatively to finely regulate *apo(a)* gene expression. Although the tissue-specific module is required for *apo(a)* gene activation and repression in a tissue-specific manner, the combinatorial interplay of the distal and proximal regulators might define the complex pathway(s) of *apo(a)* gene regulation.

Key words: apolipoprotein(a), GATA binding protein 4 (GATA4), hepatocyte nuclear factor (HNF3), negative regulatory module, tissue-specific regulatory element, transcriptional regulation.

INTRODUCTION

Apo(a) [apolipoprotein(a)] is a large, highly polymorphic glycoprotein which is synthesized predominantly by the liver and forms a unique protein constituent of the atherogenic lipoprotein particle Lp(a) [lipoprotein(a)] [1,2]. Elevated plasma Lp(a) levels have been shown to represent a significant independent risk factor for premature atherosclerosis, myocardial infarction and cerebrovascular stroke [3,4]. Lp(a) concentrations are predominantly genetically determined, and more than 90 % of this variation is determined by the *apo(a)* genetic locus [5,6].

Functional analysis of the chimpanzee and human *apo(a)* promoter sequences has led to the identification of sequence variations in the 1.4 kb 5′-flanking region that are responsible for elevated transcriptional activity in the chimpanzee [7]. The immediate 1.4 kb 5′-flanking region of the *apo(a)* gene contains sufficient promoter/enhancer elements to drive transcription of the luciferase gene in transfected HepG2 cells [8]. The minimal *apo(a)* promoter region (− 98 to + 130) has been mapped by deletion analysis, and basal transcription of the *apo(a)* gene was shown to be dependent on the binding of the liver-enriched transcription factor HNF1α (hepatocyte nuclear factor 1α) [9]. Retinoids decrease the level of apo(a) mRNA in primary hepatocytes, and a retinoid response element at position − 1177 was identified as a potential candidate for mediating this effect of retinoids [10]. Aspirin reduces apo(a) production and suppresses apo(a) mRNA expression in human hepatocytes, and the promoter region from − 30 to + 138 was observed to be critical for the effect of aspirin [11]. A 64 bp AT-rich region upstream of the *apo(a)* gene (− 703 to − 640) has been shown by us to bind to multiple liver-specific factors and to activate *apo(a)* gene transcription, and we predicted that the upstream region of the *apo(a)* promoter might contain a strong negative regulatory region [12]. These studies suggest that the 1.4 kb 5′-flanking region of the *apo(a)* promoter might contain the requisite regulatory elements that determine the tissue-specific expression of the *apo(a)* gene.

The *apo(a)* promoter stimulates the expression of reporter genes in HepG2 cells, but not in HeLa cells [8,12]. It is important to identify regulatory modules, and the regulators that control these modules, for expression of the *apo(a)* gene in a tissue-specific manner. In the present paper, we have identified two composite regulatory regions in the *apo(a)* promoter: a distal negative regulatory module (positions − 1432 to − 716) and a proximal tissue-specific module (− 716 to − 616). The distal negative regulatory module comprises two negative regulatory regions [PNR (pentanucleotide repeat) and NREβ (negative regulatory element β)], and a positive regulatory region, PREβ (positive regulatory element β). PNR was shown to bind to factors in a tissue-specific manner, whereas HNF3α and GATA4 (GATA binding protein 4) bind to NREβ to repress gene transcription. The proximal tissue-specific module contains two regulatory elements: an activating region (PREα) that activates transcription in HepG2 cells [12],

Abbreviations used: AP1, activator protein 1; apo(a), apolipoprotein(a); EMSA, electrophoretic mobility-shift assay; GATA4, GATA binding protein 4; HNF, hepatocyte nuclear factor; Lp(a), lipoprotein(a); NRE, negative regulatory element; PNR, pentanucleotide repeat; PNR-8 (etc.), PNR containing eight repeats of the pentanucleotide sequence TTTTA (etc.); PRE, positive regulatory element; RBP, retinol-binding protein; SV40, simian virus 40.

[1] Present address: International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India.

[2] Present address: Institute of Biochemistry (FB 08), Justus Liebig University, Giessen, Germany.

[3] Present address: National Institute of Immunology, New Delhi 110067, India.

[4] To whom correspondence should be addressed (e-mail uttam@mail.jnu.ac.in).

and a negative regulatory region (NREα) that represses gene transcription specifically in HeLa cells. These multiple regulatory elements might work co-operatively to closely regulate *apo(a)* gene expression. Although the tissue-specific module is required for *apo(a)* gene activation and repression in a tissue-specific manner, the combinatorial interplay of the distal and proximal regulators might define the complex pathways(s) of *apo(a)* gene regulation.

MATERIALS AND METHODS

Plasmid construction

All cloning was carried out using the pGL2-Promoter Vector (Promega Corp.), which contains the SV40 (simian virus 40) promoter inserted upstream of the luciferase gene. Insertion of the test fragment 5′ to the SV40 promoter affects the transcription level of the luciferase gene. The 3′ deletion constructs were generated by PCR using a common sense primer (− 1432; 5′-*ATTGGTACCGCTA*GCGGAAAGATTGATACTATG CTT) and various antisense primers (− 716, 5′-*ATAGATC*AGGGGGTGGTGGCTC; − 893, 5′-*ATAGATCT*GCTGCGGCACTAGAATTGC; − 993, 5′-*ATAGATCT*AGGAAAGAAAAAAAAATA; − 1193, 5′-*ATAGATCT*CAGGCCAATGTGGTGAA; − 1348, 5′-CTTCAACCGGGGTGAGAGTCTC). The PCR products were subcloned into the *Kpn*I/*Bgl*II site of the pGL2-Promoter. PNR (− 1432 to − 1348) was PCR amplified and subcloned at the *Sma*I site of pGL2pr. PREβ (− 1311 to − 1236) was amplified using the primer pair 5′-TCC*CCCGGG*CTCCTGGGTTCAAGT (sense) and 5′-CGG*GGTACC*AATTAGCCAGGCGTG (antisense) and subcloned at the *Sma*I/*Kpn*I site of pGL2pr. NREβ (− 995 to − 893) was amplified using primers (5′-AT*GGTACC*TGAGACATTCTTGCTCTG (sense) and 5′-*ATAGATCT*GCTGAGGCACTAGAAT (antisense) and was subcloned at the *Kpn*I/*Bgl*II site of pGL2pr. NREα (− 643 to − 616) was prepared by annealing the primers 5′-TTTTGAAGATAATAAAAGATTTTCACTT and 5′-AAGTGAAAATCTTTTATTATCTTCAAAA, and was subcloned at the *Sma*I site of pGL2pr.

Cell culture, transient transfections, and luciferase assay

Human hepatoma carcinoma HepG2 and human epithelial carcinoma HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10 % (v/v) fetal bovine serum at 37 °C in a 5 % CO_2 atmosphere. The cells were transfected using lipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer's directions in duplicate or triplicate in 60 mm dishes with 1 μg of reporter plasmid. pRSVβ-GAL was also transfected as a standard. In co-transfection experiments, 1–2 μg of pCMV-HNF3α, pCMV-GATA4 or pCMV-HNF3β was transfected along with the reporter construct. After 3 h of transfection, fresh medium was added. The cells were harvested after 30 h by washing twice with ice-cold PBS and scraping the cells into 200 μl of lysis buffer. The lysates were assayed immediately or frozen at − 70 °C. Luciferase activity was determined using 20 μl of the lysate and 100 μl of Luciferin solution (substrate). The luminescence was measured immediately on a luminometer (Turner Design). The β-galactosidase activity of the extracts was determined as described previously [12]. Luciferase activities were corrected for differences in transfection efficiency between different dishes by dividing luciferase activity by β-galactosidase activity.

Nuclear extract preparation

Nuclear extracts were prepared from HepG2 and HeLa cells as described by Dignam [13]. The cells (1.5×10^7) were harvested and centrifuged at 250 ***g*** for 10 min. The pellet was washed with PBS and again centrifuged at 250 ***g*** for 10 min. The resulting pellet was resuspended in 5 vol. of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF). It was then incubated on ice for 10 min and centrifuged at 250 ***g*** for 10 min. The pellet was again resuspended in 3 vol. of buffer A, Nonidet P-40 was added to a final concentration of 0.05 % and the pellet resuspension was homogenized with 20 strokes of a tight-fitting Dounce homogenizer to release the nuclei. The suspension was then centrifuged at 250 ***g*** for 10 min to pellet the nuclei. The pellet was resuspended in 1 ml of buffer C [5 mM Hepes, pH 7.9, 26 % (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF]. NaCl was added to a final concentration of 300 mM. After mixing well, the suspension was incubated on ice for 30 min, then centrifuged at 24 000 ***g*** for 20 min. All centrifugation steps were carried out at 4 °C. Aliquots of the supernatant were stored at − 70 °C. The protein concentration of the nuclear extract preparation was estimated using a Bradford protein detection kit (Bio-Rad).

DNase I footprinting analysis

For footprinting analysis, end-labelled probes were generated by PCR amplification. The reaction was performed with 0–100 μg of nuclear extract and 2 μg of salmon sperm DNA in 1 × binding buffer [10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.05 mM dithiothreitol, 50 mM NaCl, 4 % (v/v) glycerol and 1–2 μg of non-specific DNA] and incubated for 5 min on ice. The ^{32}P-labelled probe (3×10^4 c.p.m.) was then added to the nuclear extract in a total volume of 75 μl and incubated on ice for 30 min. After completion of the incubation, 75 μl of Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$ and 10 mM $MgCl_2$) was added and the mixture was incubated at room temperature for 1 min. RQ1 DNase I (0.04 unit) was then added and the sample was incubated at room temperature for 1 min. Digestion was terminated by adding 135 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1 % SDS and 100 μg/ml yeast tRNA) at 37 °C. Following phenol/chloroform extraction and ethanol precipitation, the digested DNA was analysed on a urea/6 % (w/v)-polyacrylamide gel. The gel was then dried and exposed to autoradiography.

EMSA (electrophoretic mobility-shift assay) and antibody supershift assay

For gel retardation analysis, oligonucleotides were labelled with [γ-^{32}P]ATP by polynucleotide kinase, and probes were prepared by either PCR amplification or annealing oligonucleotides. Binding reactions were prepared by adding 5–10 μg of nuclear extracts to 20 000–50 000 c.p.m. of probe in 1 × binding buffer and incubation for 30 min on ice. Reaction mixtures were then loaded on a 4 % (w/v) native polyacrylamide gel and run at 100 V for 2 h. Gels were dried and autoradiographed. For competition assays, a 100-fold molar excess of unlabelled probe was added to the reaction. PNR-8 (i.e. PNR containing eight repeats of the pentanucleotide sequence TTTTA) was prepared by utilizing genomic DNA from an individual homozygous for PNR-8 as a template for PCR, and PNR-5 was prepared by annealing primers containing five repeats of the pentanucleotide sequence [$(TTTTA)_5$]. Mutant PREβ was prepared by annealing the primers 5′-TCCAGCC*GAAG*TAGTAGCT*TCCT*TTACAGG and 5′-CCTGTAA*AGGA*AGCTACTACTA*CTTC*GGCTGGA. Mutant NREα was prepared by annealing the primers 5′-TTTTGAAGATAAT*GTTG*GATT TTCACTT and 5′-AAGTGAAAATC*CAAC*ATTATCTTCAAAA.

For antibody supershift assays, 1 μg of antibody was added to the reaction mixture 1 h prior to the addition of probe. Antibodies

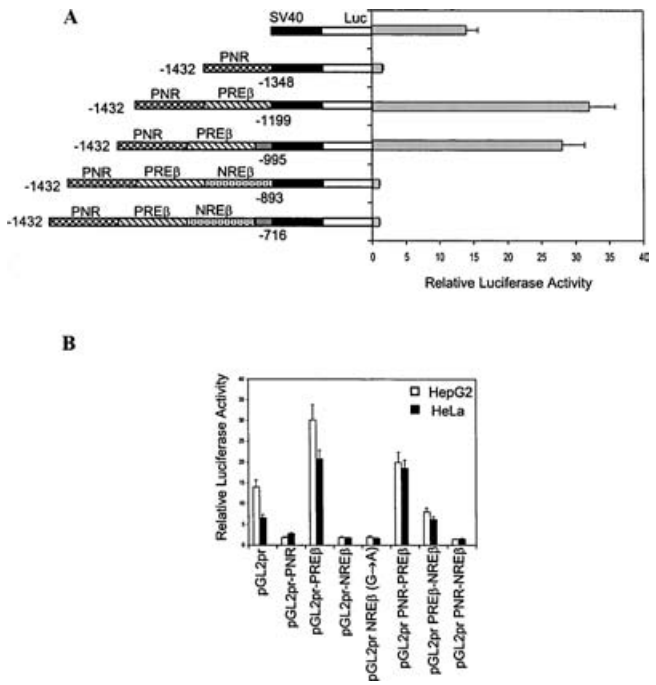

Figure 1 Identification of multiple regulatory elements in the distal region of the 1.4 kb *apo(a)* promoter

(**A**) Deletion analysis of the negative regulatory module (positions −1432 to −716) was performed. A series of 3′ deletion constructs was made in the pGL2-Promoter vector, all containing an identical *apo(a)* promoter 5′ end (−1432), but deleted to a variable extent at the 3′ end (−716, −893, −993, −1199 and −1348). A 1 μg portion of plasmid DNA was transiently transfected into HepG2 cells along with the reference DNA, and a luciferase reporter gene assay was performed as described in the Materials and methods section. The negative regulatory module contains three regulatory elements, PNR, NREβ and PREβ. (**B**) Comparison of the transcriptional strength of PNR, PREβ and NREβ. A 1 μg portion of reporter gene construct was transiently transfected into HepG2 and HeLa cells. PNR and NREβ repressed luciferase activity by 9-fold, and PREβ stimulated luciferase activity by 2.5-fold, in HepG2 cells. In HeLa cells, PNR could repress transcription by only 2-fold, whereas PREβ and NREβ showed similar activity as was observed in HepG2 cells. pGL2pr-(PNR–PREβ) stimulated luciferase activity by 2.2-fold in HepG2 and HeLa cells, whereas pGL2pr-(PREβ–NREβ) repressed the luciferase activity by only 2-fold in both cell types. pGL2pr-(PNR–NREβ) reduced luciferase activity by 10-fold in both cell types. This indicates that PNR is a tissue-specific negative regulatory region, and that NREβ is a strong negative regulatory region and could completely neutralize activation by PREβ.

against GATA4 and AP1 (activator protein 1) were from Santa Cruz Biotechnology, and antibodies against HNF3α and HNF3β were gifts from Dr R. H. Costa (University of Chicago, Chicago, IL, U.S.A.).

RESULTS

A strong negative regulatory module (−1432 to −716) is located in the distal *apo(a)* promoter

In a previous study, we suggested that a negative regulatory module might be located at positions −1432 to −706 of the *apo(a)* promoter [12]. To verify the role of this region in transcriptional regulation, a fragment (−1432 to −716) was subcloned into the pGL2-Promoter vector (containing a SV40 promoter and a luciferase reporter gene), which was transiently transfected into HepG2 cells followed by a reporter gene assay. The negative regulatory module repressed luciferase activity by 14-fold in HepG2 cells compared with the control vector (pGL2-Promoter) (Figure 1A).

To localize the regulatory elements that are present within the negative regulatory module (−1432 to −716), a series of 3′ deletion constructs was made in the pGL2-Promoter vector (see the Materials and methods section). These constructs were transiently transfected into HepG2 cells and the luciferase assay was performed. The relative luciferase activities of the constructs revealed that multiple *cis*-acting DNA sequence elements might be present within the negative regulatory module (Figure 1A).

The construct −1432 to −1348, spanning a PNR region, exhibited 10-fold less activity compared with that observed with the control vector (pGL2-Promoter), suggesting that the PNR element might be a negative regulatory region. The construct −1432 to −1199 showed a 30-fold enhancement of luciferase activity, indicating that a positive regulatory region might be located in the region −1348 to −1199. The construct −1432 to −995 exhibited slightly reduced luciferase activity, whereas the construct −1432 to −893 repressed luciferase activity by 28-fold. These results suggested that a strong negative regulatory region is located in the region −995 to −893. Overall, these observations suggested that three DNA regulatory elements are present within the negative regulatory module, contributing to transcriptional regulation of the *apo(a)* gene: PNR (−1432 to −1348), a negative regulatory region; PREβ (−1348 to −1199), a positive regulatory region; and NREβ, (−995 to −893), a negative regulatory region. The combined repressive activity of PNR and NREβ was stronger than the positive effect of PREβ.

In order to determine the strength of these regulatory elements, PNR, NREβ and PREβ were subcloned both independently and in different combinations into the pGL2-Promoter vector and transiently transfected into HepG2 and HeLa cells (Figure 1B). PNR and NREβ both repressed luciferase activity by approx. 9-fold, and PREβ stimulated luciferase activity by 2.5-fold, in HepG2 cells. However, in HeLa cells, PNR caused only 2-fold repression, whereas PREβ and NREβ showed equivalent activities. It is suggested that PNR is a tissue-specific NRE, whereas PREβ and NREβ are non-tissue-specific positive and negative regulatory regions respectively.

Transient transfection assays with different fusion products of these regulatory elements (PREβ–NREβ, PNR–NREβ, and PNR–PREβ) were also conducted. The construct PREβ–NREβ repressed transcription by 2-fold in both HepG2 and HeLa cells, indicating that NREβ is a strong negative regulatory region and could completely mask activation by PREβ. PNR–PREβ resulted in 2.2-fold transcriptional activation in HepG2 and HeLa cells. This indicated that PNR is a weak negative regulatory element in comparison with NREβ and has a minor effect on activation by PREβ. The PNR–NREβ fusion product resulted in equivalent repression in both HepG2 and HeLa cells.

PNR shows differential repression and binding to transcription factors in HepG2 and HeLa cells

The repression of transcription by the PNR element was observed to be 9-fold in HepG2 cells but only 2-fold in HeLa cells, suggesting that it might function in a tissue-specific manner. The PNR element is highly polymorphic, and contains 8–11 repeats of a pentanucleotide sequence, $(TTTTA)_n$ [14]. The repeat number has an inverse relationship with the Lp(a) level. In order to identify the negative regulators that might bind to the allelic PNR element, we performed EMSA and DNase I footprinting assays. A 85 bp fragment (−1432 to −1348) containing the PNR-10 sequence was 5′-^{32}P-labelled and was incubated with an increasing amount of HepG2 nuclear extract in the presence of poly(dI · dC). The DNA–protein complex was digested with DNase I and the digested fragments were subjected to denaturing PAGE followed

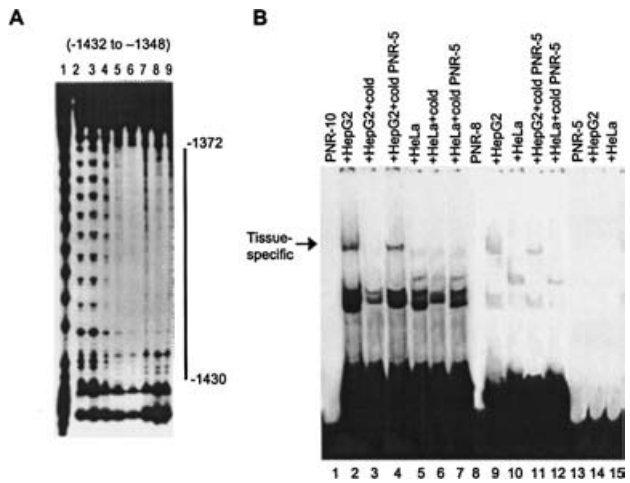

Figure 2 The PNR element shows differential binding to HepG2 and HeLa nuclear extracts

(**A**) DNase I footprinting analysis of the PNR (−1432 to −1348) with 10, 30, 40, 60, 75 and 100 μg of HepG2 nuclear extract (lanes 4, 5, 6, 7, 8 and 9 respectively); lane 1 shows the A + G sequencing reaction; lanes 2 and 3 represent the control without nuclear extract. A large footprint is present, extending from positions −1430 to −1372. (**B**) Gel shift assay of the PNR-10 element. Differential binding to HepG2 and HeLa nuclear extracts was observed (lanes 2 and 5 respectively). Binding was completely abolished on addition of a 100-fold excess of unlabelled ('cold') PNR-10 DNA (lanes 3 and 6). PNR-8 showed faint binding (lanes 9 and 10), whereas PNR-5 did not bind at all (lanes 14 and 15) in either HepG2 or HeLa cells. PNR-5 failed to compete with the factors that were bound to PNR-10 or PNR-5 (lanes 4, 7, 11 and 12). Lanes 1, 8 and 13 represent the control lanes for PNR-10, PNR-8 and PNR-5.

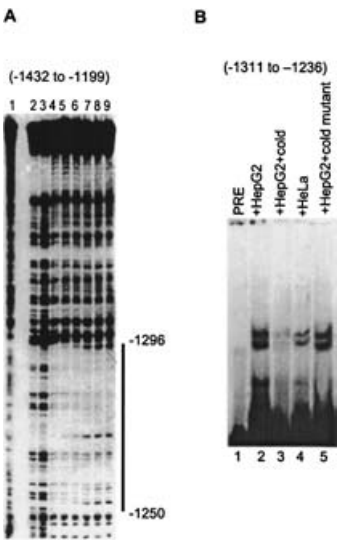

Figure 3 PREβ is a non-tissue-specific regulatory element

(**A**) DNase I footprinting of region −1432 to −1199 with 10, 20, 40 and 50 μg of HepG2 nuclear extract (lanes 4, 5, 6 and 7 respectively); lane 1 represents an A + G sequencing reaction; lanes 2 and 3 represent the control without nuclear extract. A broad footprint (−1296 to −1250) was observed. (**B**) Gel shift assay of PREβ (−1311 to −1236) with HepG2 and HeLa nuclear extracts (lanes 2 and 4 respectively). Two retarded bands were observed with both HepG2 and HeLa nuclear extracts. The binding was specific, as it was competed with a 100-fold excess of unlabelled ('cold') PREβ (lane 3), but not by a 100-fold excess of mutant unlabelled probe (lane 5); lane 1 is a control.

by exposure to X-ray film. A single footprint was observed between positions −1430 and −1372, covering the entire ten repeats (Figure 2A). As the PNR-10 sequence was completely protected from DNase I, we then analysed the EMSA pattern of PNR-10 by utilizing both HepG2 and HeLa nuclear extracts. In EMSA with a HepG2 nuclear extract, three retarded bands were observed, whereas only two retarded bands were observed with a HeLa nuclear extract (Figure 2B). Thus HepG2 cells might contain an additional tissue-specific factor that binds to the PNR element.

To check the specificity of the retarded bands, a competition assay was also performed. The intensity of the retarded bands was reduced by the addition of a 100-fold excess of unlabelled probe. We utilized two mutants, PNR-8 and PNR-5, for the binding experiment. PNR-8 showed faint binding, whereas PNR-5 did not bind to the factors at all in either HepG2 or HeLa cells. PNR-5 was chosen as a competitor that failed to compete with transcription factors bound to PNR-10.

PREβ does not show differential binding to regulators in HepG2 and HeLa cells

PREβ activated transcription from a heterologous promoter in a non-tissue-specific manner. To identify the activators that might bind to PREβ, DNase I footprinting and gel shift assays were performed. An end-labelled 234 bp fragment (−1432 to −1199) was incubated with an increasing amount of HepG2 nuclear extract and treated with DNase I. The digested DNA–protein complex was run on denaturing PAGE. We observed a 47 bp region (−1296 to −1250) that was protected by the HepG2 nuclear extract, suggesting this region to be the site for binding of the activators (Figure 3A). To confirm that the footprint region was actually involved in the binding of proteins, two end-labelled primers flanking the protected region were utilized to PCR-amplify the PREβ (−1311 to −1236) fragment for gel retardation analysis. In EMSA, PREβ was shown to form two DNA–protein complexes of identical mobility with both HepG2 and HeLa nuclear extracts (Figure 3B). These results demonstrated that PREβ is a non-tissue-specific PRE.

To confirm the specificity of the retarded bands, a competition assay was performed. Binding was reduced on adding a 100-fold excess of the unlabelled probe. A mutant PREβ probe was generated in which the putative interleukin-6 response element [8] and an adjacent palindromic sequence were modified. No difference in binding was observed on adding the unlabelled mutant probe in the gel shift reaction. This suggested that the region −1283 to −1268 plays an important role in DNA–protein interactions.

NREβ binds to HNF3α and GATA4

NREβ repressed transcription in both HepG2 and HeLa cells. In order to identify the regulators that bind to NREβ, DNase I footprinting and gel shift analysis were performed. DNase I footprinting of a 5′ labelled 349 bp fragment (−1140 to −792) resulted in a broad footprint covering a 96 bp region (−995 to −900), suggesting that multiple factors bind to this region (Figure 4A).

NREβ displays 83 % identity with the consensus-binding motif for GATA at positions −973 to −978 (in an antisense direction) [15] and 90 % identity with that for HNF3α at positions −949 to −939 [16] (Figure 5A). NREβ contains a *Bst*NI restriction enzyme site that results in cleavage into two fragments of 26 bp (−995 to −970) and 77 bp (−969 to −893). The 26 bp region contained the binding site for GATA and the 77 bp region contained the binding site for HNF3α. To observe if these 26 bp and 77 bp fragments of NREβ could bind to transcription factors

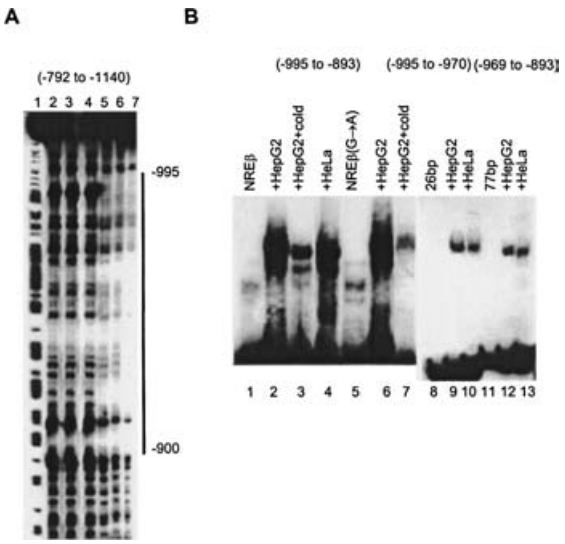

Figure 4 NREβ binds to two factors in HepG2 and HeLa cells

(**A**) DNase I footprinting of region − 792 to − 1140 with 10, 20 or 40 μg of a HepG2 nuclear extract showed a footprint from − 995 to − 900 (lanes 5, 6 and 7 respectively); lanes 2–4 represent control DNA without nuclear extract, and lane 1 represents an A + G reaction. (**B**) Gel shift assay of NREβ (− 995 to − 893) with HepG2 and HeLa nuclear extracts (lanes 2 and 4 respectively) showed two retarded bands, which were competed completely with a 100-fold excess of unlabelled ('cold') NREβ (lanes 3 and 5). NREβ(G → A) also showed similar binding to HepG2 cells (lane 6) and was competed with a 100-fold excess of unlabelled NREβ(G → A) (lane 7). Gel shifts of *Bst*NI fragments of NREβ were also performed: a 26 bp fragment (− 995 to − 970) showed one retarded band with the HepG2 and one retarded band with the HeLa nuclear extract (lanes 9 and 10), and a 77 bp fragment (− 969 to − 893) showed a single retarded band with both HepG2 and HeLa nuclear extracts (lanes 12 and 13).

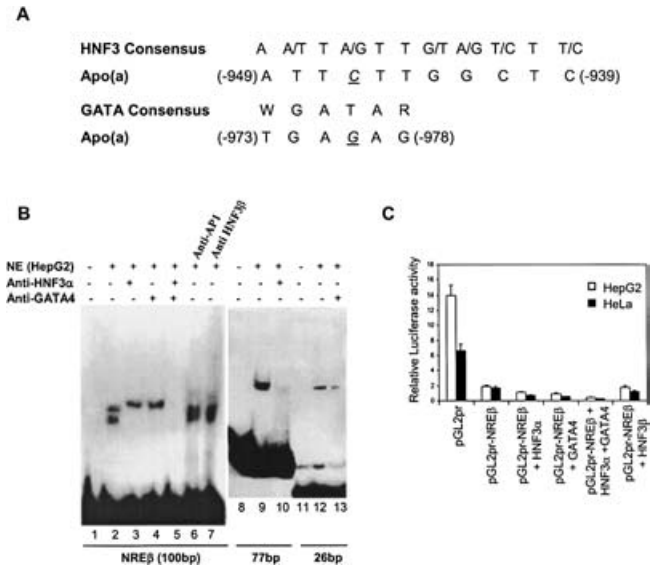

Figure 5 NREβ binds to HNF3α and GATA4 and represses transcription

(**A**) Comparison between the binding sites for HNF3 and GATA in the *apo(a)* promoter and the consensus sequence. Letters in italics correspond to nucleotides differing from the consensus. (**B**) Supershift assay for NREβ with antibodies against HNF3α and GATA4. Binding was enhanced on adding antibodies against HNF3α or GATA4 (lanes 3 and 4); lane 1 is a control; lane 2 shows a binding reaction of NREβ with a HepG2 nuclear extract. The retarded bands were completely eliminated on adding both antibodies (lane 5), whereas binding was not altered on addition of anti-AP1 or anti-HNF3β antibodies (lanes 6 and 7). Anti-HNF3α antibody eliminated the retarded band obtained with the 77 bp fragment (lane 10), and anti-GATA4 antibody eliminated the retarded band obtained with the 26 bp fragment (lane 13). (**C**) Co-transfections were performed in HepG2 and HeLa cells. Cells were transiently transfected with 1 μg of pGL2pr-NREβ with or without 1 μg of HNF3α or GATA4 expression vector. Both HNF3α and GATA4 repressed the luciferase activity induced by NREβ, by 12- and 15-fold respectively. pGL2pr-NREβ was also co-transfected with HNF3α and GATA4 simultaneously, resulting in 50-fold reduced luciferase activity, indicating a co-operative interaction. Co-tranfection was also performed with HNF3β expression vector, which had no effect on luciferase activity.

present in HepG2 and HeLa cells, gel shift assays were performed. Gel shift analysis showed two closely migrating retarded bands for NREβ and a single band each for the 26 and 77 bp fragments (Figure 4B). We concluded that two *trans*-factors of approximately the same molecular mass bind to NREβ. The putative transcription factors HNF3α (49 kDa) and GATA4 (48 kDa) that were presumed to recognize NREβ are of approximately the same molecular mass. NREβ and both of its fragments showed the same binding pattern with a HeLa nuclear extract (Figure 4B).

To confirm that HNF3α and GATA4 can bind to NREβ, supershift assays were performed. The intensity of a retarded band observed with NREβ was enhanced if antibodies against HNF3α or GATA4 were added to the binding reactions. Addition of both antibodies resulted in complete elimination of the retarded complexes. The addition of irrelevant anti-AP1 or anti-HNF3β antibodies to the binding reaction had no effect on the binding pattern. Further, supershift assays were also carried out with the 26 bp and 77 bp fragments. Anti-GATA4 antibodies decreased the intensity of the DNA–protein complex obtained with the 26 bp fragment, whereas anti-HNF3α antibodies decreased the intensity of the retarded complex observed with the 77 bp fragment (Figure 5B). Since the binding sites for the two factors are close together, there might be steric hindrance of the binding of one factor by the other. The anti-HNF3α antibody bound to HNF3α and inhibited its binding to its consensus sequence, and hence removed the steric hindrance caused by the binding of HNF3α. Thus GATA4 could bind to its consensus sequence with higher affinity, giving a band of enhanced intensity. Similarly, quenching of GATA4 by its antibody increased the binding of HNF3α to its consensus sequence. Moreover, anti-HNF3α and anti-GATA4 antibodies eliminated the retarded bands observed with the 77 bp and 26 bp fragments respectively, confirming that HNF3α and GATA4 are the factors that bind specifically to their cognate sequences in NREβ.

The 5′-flanking region of the *apo(a)* gene shows a G → A polymorphism at position − 914 [8]. This does not affect the HNF3α and GATA4 binding sequences. An oligonucleotide with an A at position − 914 was synthesized to generate a mutant NREβ(G → A) probe, which was utilized in gel shift analysis. It showed a similar binding pattern as was obtained with NREβ (Figure 4B). The mutant NREβ(G → A) was subcloned into pGL2-Promoter and transiently transfected into HepG2 and HeLa cells. It also did not show any differential repression. We concluded that the G → A polymorphism at position − 914 does not affect the binding of transcription factors or the repressive activity of NREβ (Figure 1B).

HNF3α and GATA4 repress gene transcription in a non-tissue-specific manner

Since the supershift assay revealed that GATA4 and HNF3α could bind to their cognate binding sites in NREβ, we tested their effects on transcription in both HepG2 and HeLa cells. An NREβ–luciferase reporter construct was co-transfected along with cDNA for HNF3α or GATA4 into both HepG2 and HeLa cells. HNF3α and GATA4 both had a strong inhibitory effect on transcription (Figure 5C). NREβ repressed transcription by 9-fold, and overexpression of HNF3α and GATA4 further repressed luciferase

activity by 12- and 15-fold respectively. The co-transfection of HNF3α and GATA4 together in the presence of NREβ repressed luciferase activity by 50-fold. A co-transfection experiment was conducted with a control plasmid containing HNF3β cDNA and pGL2pr-NREβ. Unlike the transcriptional repression that was, independently, observed with HNF3α and GATA4, HNF3β failed to induce any transcriptional repression, thus confirming the specificity of transcriptional repression by HNF3α. These results suggested that HNF3α and GATA4 negatively regulate transcription in a co-operative manner (Figure 5C).

The proximal tissue-specific module contains a 28 bp HeLa-specific repressor element

We have previously identified an AT-rich HepG2-specific activating region (PREα; -716 to -640) in *apo(a)* that was shown to bind to multiple HepG2-specific factors, but failed to bind to any HeLa-specific factors [12]. However, an extended region (-716 to -616) was shown in a gel shift assay to bind to a single factor in HeLa cells (Figure 6B). As PREα did not bind to any transcription factor in HeLa cells, it was inferred that the new region (NREα; -640 to -616) might bind to a HeLa-specific factor. The expression of many tissue-specific genes is negatively regulated in non-permissive cells by the presence of a repressor molecule [17,18]. We inferred that NREα might contain the regulatory element that is active in HeLa cells only.

To identify the role of this element in transcriptional regulation, oligonucleotides were synthesized with the sense and antisense sequences to generate a 28 bp region (-643 to -616). This fragment was subcloned into the pGL2-Promoter vector and utilized for transient transfection. NREα repressed luciferase activity by 4-fold in HeLa cells, whereas there was no repression in HepG2 cells (Figure 6C), thus suggesting that NREα (-643 to -616) is a HeLa-specific NRE. DNA elements with a perfect or imperfect dyad symmetry sequence play an important role in transcriptional regulation, as they are known to bind to transcription factors [19,20]. NREα also contains an imperfect dyad symmetry of 22 bp (-640 to -619; TGAAGATAATAAAAGATTTTCA) that could potentially recognize the single HeLa-specific transcriptional repressor.

The HeLa-specific NRE was examined further for its ability to interact with nuclear proteins from expressing and non-expressing cells. NREα showed a single retarded band with a HeLa nuclear extract and no retarded band with a HepG2 nuclear extract. The retarded band observed with HeLa cells corresponded to the HeLa-specific retarded band observed with the -716 to -616 fragment (Figure 6B). The complex observed was specific, as it was completely eliminated by the addition of a 100-fold excess of the unlabelled probe. Further, a mutant probe was generated by synthesizing an oligonucleotide with a GTTG sequence at positions -627 to -630 that might hinder the formation of a cruciform structure. The complex was unaffected by addition of an excess of the unlabelled mutant probe, suggesting that this region plays a crucial role in DNA–protein interactions. It was inferred that NREα binds to a HeLa-specific repressor molecule that might be involved in tissue-specific repression of the *apo(a)* gene in HeLa cells.

To locate the sequences involved in binding, DNase I footprinting of a 219 bp fragment (-716 to -498) was performed with a HeLa nuclear extract, and a 41 bp footprint spanning the region -656 to -616 was observed (Figure 6A). The footprint also exhibited protein-induced DNase I hypersensitivity sites, indicating close proximity of the binding factor and the *cis*-element. The protected region was found to be extended further on the distal side when compared with the 28 bp region involved

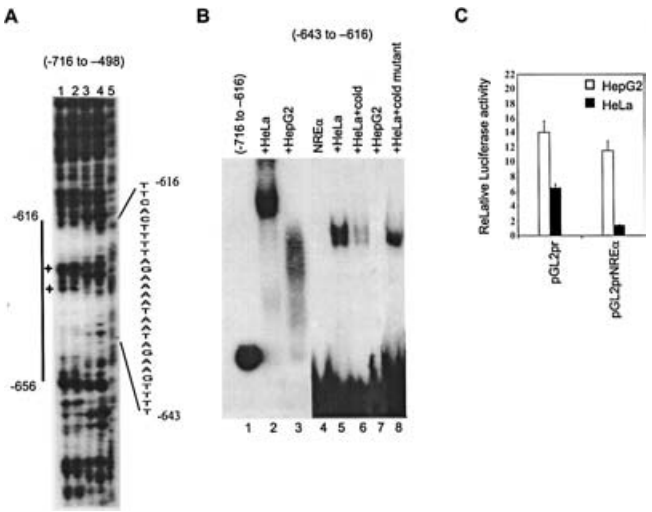

Figure 6 NREα is a HeLa-specific negative regulatory region

(**A**) DNase I footprinting of region -716 to -498 with 40, 30, 20 or 10 μg of HeLa nuclear extract (lanes 1–4 respectively) revealed a footprint between positions -616 and -656 with two hypersensitive sites; lane 5 represents the A + G reaction. The sequence represents the palindrome present within the footprint region. (**B**) Gel shift assay of the tissue-specific activating region (-716 to -616) showed multiple bands with a HepG2 nuclear extract and a distinct band with a HeLa nuclear extract (lanes 2 and 3 respectively); gel shift of NREα (-643 to -616) showed single band with the HeLa nuclear extract and no retarded band with the HepG2 nuclear extract (lanes 5 and 7 respectively); lanes 1 and 4 represent control probes. The complex is specific, as it was competed out by a 100-fold excess of unlabelled ('cold') probe (lane 6), but not with a mutant probe (lane 8). (**C**) Relative luciferase activity of NREα in HeLa and HepG2 cells. NREα repressed luciferase activity by 6-fold in HeLa cells, but had no effect in HepG2 cells, suggesting that it is a HeLa-specific repressor.

in binding in EMSAs. This may be explained by DNase I binding with the minor groove of DNA in which the DNA binding surface covers approx. 10 bp, resulting in an extended footprint. Several *cis*-elements have been reported to possess palindromic sequences and have been found to extrude into cruciform structures, which provide an alternative site for the binding of *trans*-acting factors. The formation of such a structure could also explain the extended footprint.

DISCUSSION

Apo(a) is synthesized in the liver, and its level in plasma varies to a large extent in the human population. The 1.4 kb *apo(a)* promoter shows tissue-specific basal activity, and a few studies have shown a clear relationship between 5′ alleles and plasma Lp(a) levels [21]. Therefore the factors responsible for the determination of tissue specificity might be located in this region. The precise control of tissue-specific genes is achieved by co-operative and concerted interactions of various transcription factors with their cognate binding sites, with each other and with the transcription initiation complex. Various regulatory modules, each of which contains multiple regulatory elements, interact with each other to provide the combinatorial regulation of gene transcription [22]. Multiple positive and negative regulatory regions that interact with liver-specific and ubiquitous transcription factors have been observed in various liver-specific genes [23,24].

Our analyses of the 1.4 kb *apo(a)* promoter demonstrate that the hepatic expression of apo(a) is governed by a complex interplay of tissue-specific and non-tissue-specific multiple positive and negative regulatory regions, and by co-operative interactions

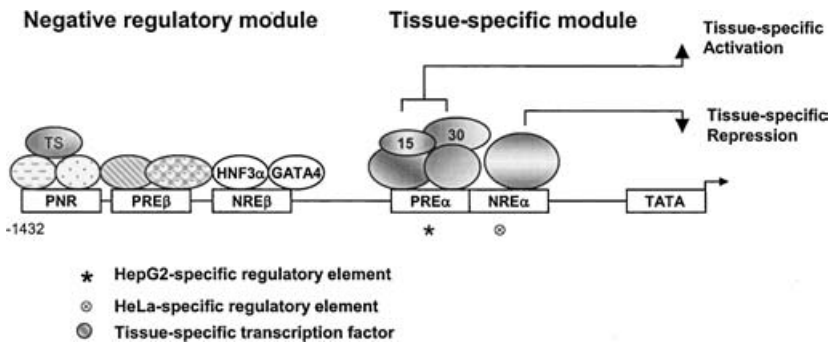

Figure 7 Multiple regulators that bind to the distal negative regulatory module and the proximal tissue-specific module in the *apo(a)* promoter

The 1.4 kb 5′-flanking region of the human *apo(a)* promoter can be divided into two regulatory modules. (i) A tissue-specific regulatory module that comprises PREα (HepG2-specific activating element) and NREα (HeLa-specific negative regulatory region). This module might be involved in determining the expression of *apo(a)* in expressing or non-expressing cells. (ii) A negative regulatory module, composed of PNR (weak negative regulatory region), PREβ (a strong negative regulatory region) and NREβ (a strong negative regulatory region), which binds to HNF3α and GATA4. The two negative elements (PNR and NREβ) flank PREβ and neutralize its influence, giving an overall negative effect.

among the factors binding to these regions. We have identified two distinct composite regulatory modules in the *apo(a)* promoter, a distal negative regulatory module (positions -1432 to -716) and a proximal tissue-specific regulatory module (-716 to -616), that contribute to transcriptional activity. The negative module is a composite element and is composed of two strong negative regulatory regions, PNR and NREβ, and a positive regulatory region, PREβ. The two negative elements flank PREβ and neutralize its positive effect, resulting in an overall negative effect. Two non-tissue-specific negative regulatory regions have been found to be essential for the tissue-specific expression of *ADH1C* (the gene encoding alcohol dehydrogenase1C) [25]. Furthermore, the androgen receptor gene promoter contains a composite negative regulatory region, and the repressor function involves a co-ordinate interaction between NFI (nuclear factor I) and at least two other nuclear factors [26]. The mouse albumin gene contains an enhancer element that is a composite of at least three functional regions: a negative region that suppresses an otherwise positive region, a liver-specific enhancer element and a third region that by itself is inactive but, in conjunction with the remaining elements, overrides the effect of the negative region [27].

The PNR region has been shown to be polymorphic, and its repeat number has an inverse relationship with the plasma Lp(a) level, i.e. individuals with higher Lp(a) levels have a lower PNR number, and vice versa [14]. Further, PNR is a strong negative regulatory region, and it showed differential factor binding patterns in HepG2 and HeLa cells. Regulatory elements that interact with liver-specific and ubiquitous transcription factors have been observed in various liver-specific genes [23,24].

The second non-tissue-specific negative regulatory region, NREβ, binds to HNF3α and GATA4 and represses *apo(a)* gene transcription in a ubiquitous manner. A co-transfection experiment utilizing both HNF3α and GATA4 resulted in further repression of transcription in both HeLa and HepG2 cells. A co-operative model of early hepatic gene activation had been proposed, in which GATA4 has been shown to co-operate with an immediately adjacent HNF3β element to induce liver-specific albumin expression [28] and contribute to the liver-enriched expression of the homeobox gene *Hex* [29]. These transcription factors participate in the co-ordinate expression of several liver-specific genes [30,31]. HNF3 plays a dual role in glucagon gene transcription, by direct activation and by impairing Pax6-mediated transactivation of the glucagon promoter [32]. The mouse α-fetoprotein promoter is repressed in HepG2 hepatoma cells by HNF3 [(FOXA (forkhead box A1)] through an indirect mechanism [33]. GATA4 is expressed ubiquitously and acts both as an activator [34,35] and as a repressor of the *ABCG5* (ATP-binding cassette G5) promoter [36]. A G$\rightarrow$A polymorphism was observed at position -914 of NREβ. We have now shown that both NREβ and its G$\rightarrow$A allele bind to HNF3α and GATA4, and repressed gene transcription from a SV40 promoter in both HeLa and HepG2 cells, thus supporting an earlier observation that *apo(a)* gene transcription is dependent on nucleotide polymorphism in its 5′-region [21].

The multiple factors that bind to these elements might interact with one another in modulating complex *apo(a)* gene expression, although, using time-dependent super-shift analysis, we failed to demonstrate that these regulators interact with each other via protein–protein interactions. A possible protein–protein interaction pattern of the type 'repressor–activator–repressor contact' in the PNR (-1432 to -1348), PREβ (-1311 to -1236) and NREβ (-995 to -893) regions might dictate the overall outcome of *apo(a)* gene expression in the liver. The repression of transcription by the -1432 to -716 fragment could then be explained by the fact that two negative regulatory regions (PNR and NREβ) might neutralize the effect of the 'sandwiched PREβ'. Multiple NREs have been identified in the promoters of the human collagen [37], interleukin-4 [38] and β-globin [39] genes that repress transcription. The expression of *ALDH3* (the aldehyde dehydrogenase 3 gene) is controlled by multiple regulatory processes. For both inducible and constitutive *ALDH3* expression, two distal negative regulatory regions interact with a proximal activating regulatory element [40].

The tissue-specific module of the *apo(a)* gene promoter (-616 to -703) contains a HepG2-specific activating element (PREα) that we described previously [12]. It is interesting that we have now identified a HeLa-specific repressing element (NREα) adjacent to the HepG2-specific element. NREα binds to a HeLa-specific repressor and was able to repress transcription in HeLa but not in HepG2 cells. This factor might be responsible for the tissue-specific repression of the *apo(a)* gene in HeLa cells. There might be more than one mechanism whereby the expression of certain genes is restricted to one cell type. This could be best explained by postulating the existence of repressors or tissue-specific activators. These mechanisms are obviously not mutually exclusive. Tissue-specific expression of a large number of genes is mediated via tissue-specific positive *trans*-acting factors [41]. Further, transcriptional repressors are found in the cells where

the regulated gene is not expressed [17,18]. The repression of myelin proteolipid protein gene expression is mediated through the combinatorial action of both general and cell type-specific negative regulatory regions in non-expressing cells [42]. The simultaneous involvement of a repressor and an activator in transcriptional regulation, as well as tissue-specificity, has been reported for the RBP (retinol-binding protein) gene. RBP is expressed in human hepatoma but not in HeLa cells. It has a non-tissue-specific enhancer and a tissue-specific repressor (not present or non-functional in hepatoma cell lines) in the 5′ flanking region of promoter [43]. The tissue-specific region of the alcohol dehydrogenase gene activates transcription in hepatoma cells, but represses transcription in HeLa cells [25].

In conclusion, the human *apo(a)* promoter can be divided into two modules, a tissue-specific module and a negative regulatory module (Figure 7). The proximal tissue-specific module (positions −716 to −616), containing a HepG2-specific activating region and a HeLa-specific negative regulatory region, might determine the expression of the *apo(a)* gene in hepatic or non-hepatic cells. The distal negative module (positions −1432 to −716) comprises multiple regulatory elements. Interactions between the multiple regulators could be of a complex nature. The hepatic expression of the human multidrug resistance protein 2 gene is also regulated by a putative silencer element and a liver-specific PRE [44]. The smooth muscle cell-specific expression of this gene is controlled by complex combinatorial interactions between multiple general and tissue-specific proteins [45]. Further, the tissue-specificity of L-pyruvate kinase transgenes results from the combinatorial effects of proximal promoter and distal activator regions [46]. Also, *in vivo* analysis of two striated muscle actin promoters revealed combinations of multiple regulatory modules required for skeletal and cardiac muscle-specific gene expression [47]. We suggest that the unique expression programme of the *apo(a)* gene might be imparted by the combinatorial action of the two modules, and probably other regulatory elements as well, and is fine-tuned by co-operative interactions between liver-specific and ubiquitous transcriptional factors binding to these regulatory elements. Work is in progress to characterize the regulators that bind to these elements.

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