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Dual tissue-specific expression of apo-AII is directed by an upstream enhancer

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ABSTRACT

Apolipoprotein-AII (apo-AII) is one of a family of evolutionarily related proteins which play a crucial role in lipid transport and metabolism. The serum levels of human apo-AII have been shown to be inversely correlated to the incidence of coronary heart disease and its expression to be limited to the liver and intestine. Here we demonstrate that this dual tissue-specificity involves DNA sequences located in a 259 bp region centred 782 bp upstream from the transcription initiation site. These sequences function in an orientation-independent manner and are absolutely required for transcription from the apo-AII promoter. The regulatory region contains sequences which are homologous to the apo-AI, β-globin and immunoglobulin gene promoters and to the immunoglobulin heavy-chain enhancer.

INTRODUCTION

There is a growing body of evidence that the tissue specific and developmental regulation of individual genes is controlled by unique combinations of specific and non-specific cis and trans-acting elements. While relatively little is known about these latter factors, DNA sequences associated with a number of genes have been identified which are responsible for the control of spatial and temporal transcription. Such elements have been found within the promoter region [1-2], within exons [3], up to 6 Kb upstream of the transcription start site [4] and both 5' and 3' of the translation initiation site [5]. In addition, it has been shown that their action can be either orientation independent [6-8] or orientation dependent [9]. However, to date, the majority of these cis-acting elements have been identified in higher eukaryotic genes whose expression is limited mainly to a single tissue-type. The apolipoprotein gene family, on the other hand, is transcribed in a range of tissue types [10-14] and so provides an opportunity to study how restricted but multiple tissue specific expression is controlled.

Knowledge of these regulatory mechanisms is of particular interest given the central role played by the apolipoproteins in lipid transport and metabolism and the intimate association of related defects with the pathogenesis of cardiovascular disease. While apolipoprotein deficiencies have been identified as the cause of a

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limited number of lipid disorders, it is the combination of several, individually innocuous, genetic defects which probably is the main influence on the etiology of atherosclerosis. The control of apo-AI and apo-AII gene expression is of particular interest since these proteins represent the main structural components of high density lipoprotein (HDL) particles whose plasma concentrations appear to be inversely correlated to the incidence of coronary disease [15-21]. In this regard, we have previously identified a restriction fragment length polymorphism (RFLP) that links an apparently normal allele of the apo-AI/apo-CIII gene complex with a predisposition to hyperlipidemia and cardiovascular disease [22-23]. An RFLP linked to the apo-AII gene has also been recently described [24]. This variant associates with apo-AII that, although normal, is present at high circulating levels in the plasma [25], thus indicating a possible linkage to a regulatory mutant. Detailed knowledge of the elements involved in the regulation of apolipoprotein gene expression are therefore important both in molecular biological and clinical terms.

The human apo-AII gene is transcribed exclusively in the adult liver and intestine [26-30] and the resulting transcripts appear to be identical [31]. Our recent demonstration that in 6 to 12 week embryos apo-AII is the only apolipoprotein not expressed in the gut [14], indicates the expression of this gene is also subject to developmental regulation. A series of experiments was therefore designed to identify those cis-acting elements of the human apo-AII gene that may be required for this developmental and tissue-specific expression. Apo-AII gene sequences were linked to the 3' terminal exon of the human α1-globin gene and the resulting hybrid constructs introduced into hepatic (Hep-G2 and Hep-3B), intestinal (CaCo-2) and non apo-AII producing (HeLa, DS-1 and EJ138) tissue culture cell lines. These transient expression experiments defined an "enhancer-like" element upstream of the apo-AII gene that directs dual tissue-specific expression.

MATERIALS AND METHODS

General Procedures

Purification of DNA, ligation reactions, restriction enzyme digestion, Bal31 digestion, gel electrophoresis, transfer of RNA to nitrocellulose, nick translation and filter hybridization were performed according to established procedures as described by Maniatis et al. [32].

Construction of Plasmids

All plasmids were constructed and isolated by standard recombinant DNA tecniques [32] using DNA fragments of the human apo-AII gene present in the clone pAIITSλ4.0 [33].

pαAII/5'1171. An EcoR1/HincII fragment covering the apo-AII gene from position

-1171 to +1168 was isolated from plasmid pAIITSX4.0 [33] and ligated into pSVα1Fd [34] between the StuI and BstEII sites. The correct integration of the insert was tested by a series of analytical restriction enzyme digestions.

pαAII/5'1171 and 912. A HindIII/HincII fragment, covering the apo-AII gene from position -912 and +1168 was isolated from plasmid pAIITSX4.0 [33] and ligated between the StuI and BstEII sites of pSVα1Fd [34]. The correct integration of the insert was tested by a series of analytical restriction enzyme digestions.

pαAII/5'675, 210 and 117. pAIITSX4.0 was digested with EcoR1 followed by a time course of Bal31 nuclease digestion. The DNA was then digested with HincII and inserted between the StuI and BstEII sites of pSVα1Fd [34]. Those recombinants containing apo-AII sequences upstream of the HincII site were identified by colony hybridization. The orientation of the inserts and the length of apo-AII 5' flanking sequence each recombinant contained was determined by sequencing the plasmid DNA [35,36]. Three recombinants were finally isolated containing 675, 210 and 117 bp of apo-AII 5' flanking sequence.

pαAII/5'210/5'857-912, 791-912, 383-912(R), 653-793(R). The sequences present between 857 and 912 bp upstream of the apo-AII transcription start site are contained in a RsaI/HindIII restriction fragment of pAIITSX4.0 (Fig.3); those between 791 and 912 in a HinfI/HindIII fragment; those between 383 and 912 in a BglII/HindIII fragment and those between 653 and 793 in a HinfI/AluI fragment. These fragments were introduced into the unique EcoR1 site of pαAII/5'210 in the correct orientation relative to the apo-AII sequence already present within this plasmid. The resulting recombinants were respectively, pαAII/5'210/5'857-912, 791-912, 383-912 and 653-793. In addition, the HindIII/BglII and HinfI/AluI fragments were also introduced in the reverse orientation to generate pαAII/5'210/5'383-912R and 653-793R, respectively. The correct integration of the inserts was checked by a series of analytical restriction enzyme digestions.

pαAII/5'210/SV40. A 100 bp StuI/EcoR1 fragment isolated from pSVα1Fd containing the SV40 enhancer and origin (Fig.1) was inserted into the unique EcoR1 site of pαAII/5'210. The orientation of the fragment was not determined.

Cell Growth

Hep-G2, Hep-3B [37-40], DS-1 (Goss S.J., Sir William Dunn School of Pathology, Oxford) and EJ138 [41,42] cells were grown at 37°C under 5% CO_2 in MEM medium supplemented with 10% FCS and 2 mM glutamine. DMEM medium supplemented with 2 mM glutamine and 10% or 20% FCS was used to grow HeLa [43] or CaCo-2 [44-47] cells respectively. In continuous culture, cells were harvested by trypsinization, seeded at a 1 : 5 dilution and fed with fresh medium every day. All cell lines were

grown in 80 cm^2 tissue culture flasks (Nunc) and 150 x 15 mm tissue culture dishes (Lux).

DNA Transfection

Hep-G2, Hep-3B, HeLa, CaCo-2, DS-1 and EJ138 cells were transfected with plasmid DNA by the calcium phosphate co-precipitation method [48] with the following modifications: 3 hrs before transfection the cells were fed with fresh medium, 100 μg of each plasmid was used to transfect two 150 x 15 mm dishes of subconfluent cells. Transfection was for 4 hrs after which the medium was replaced.

Preparation of RNA

Total cellular RNA was prepared by lysis of cells in 5 M guanadinium isothiocyanate, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.1 M β-mercaptoethanol [49]. RNA was pelleted through a cushion of 5.7 M CsCl, 0.1 M EDTA in a Beckman SW50 rotor at 28,000 rpm and 20°C for 24 hrs [50,51]. RNA pellets were resuspended in sterile distilled water containing 10 mM ribonucleoside-vanadyl complex (Biolabs), ethanol precipitated and stored as aqueous solutions at -20°C.

RESULTS

Determination of the approximate 5' border of an element involved in the tissue-specific expression of the apo-AII gene

The majority of the cis-acting regulatory elements which have been implicated in tissue-specific expression are located either within the transcribed portion of the gene they control or in its 5' flanking sequence. Therefore, as the starting point of our search for equivalent control regions associated with apo-AII gene expression, we used a 2339 bp restriction fragment which stretched from 1171 bp upstream of the apo-AII CAP site [31] to within 163 bp of the polyadenylation point.

The construction of the plasmids used to determine the approximate 5' boundary of sequences involved in the direction of the tissue-specific expression of the apo-AII gene is described in Figure 1. Each construct represents a hybrid gene containing almost the entire transcribed region of the apo-AII gene, lacking only the last 163 bp of the 3' terminal exon (AII E4) containing the polyadenylation signal. This signal is replaced with its equivalent from the human α1-globin gene by the fusion of the remaining 5' portion of AII E4 to 218 bp representing the 3' portion of the 3' terminal exon of α1-globin (α1 E3). Therefore, the resulting 3' terminal exon (AII E4/α1 E3) of the hybrid gene only contains sequences that had themselves originally been part of such exons. Consequently the possibility of the mRNA transcribed from the hybrid gene being unstable due to the presence of incompatible and inappropriate exon sequences is minimized.

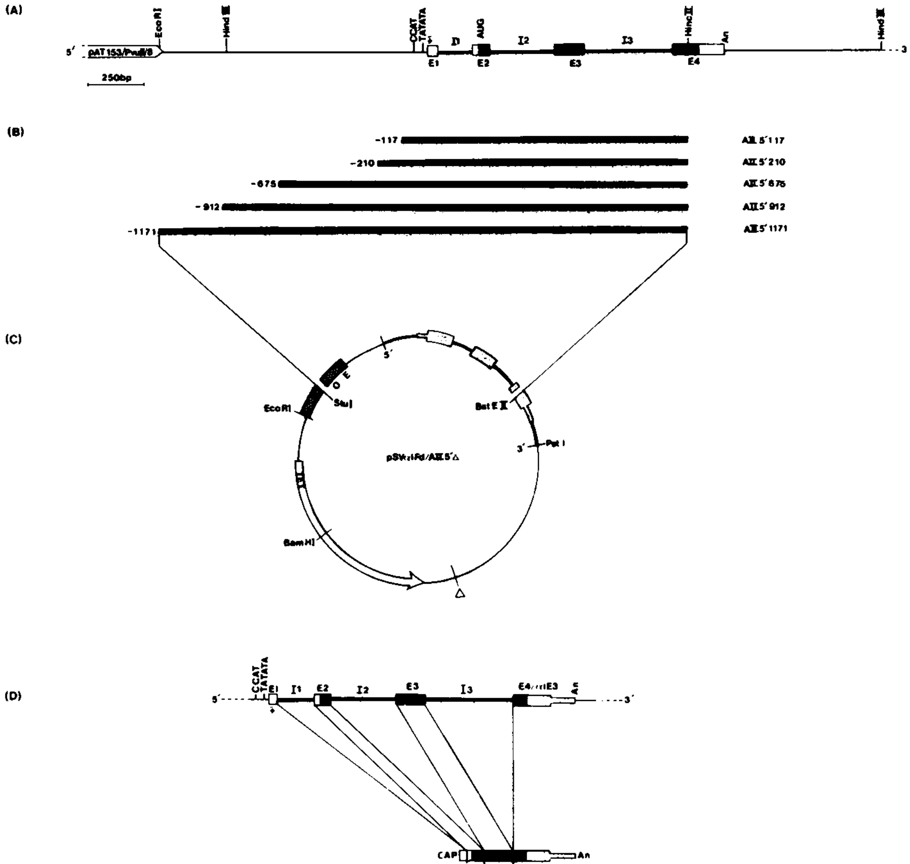
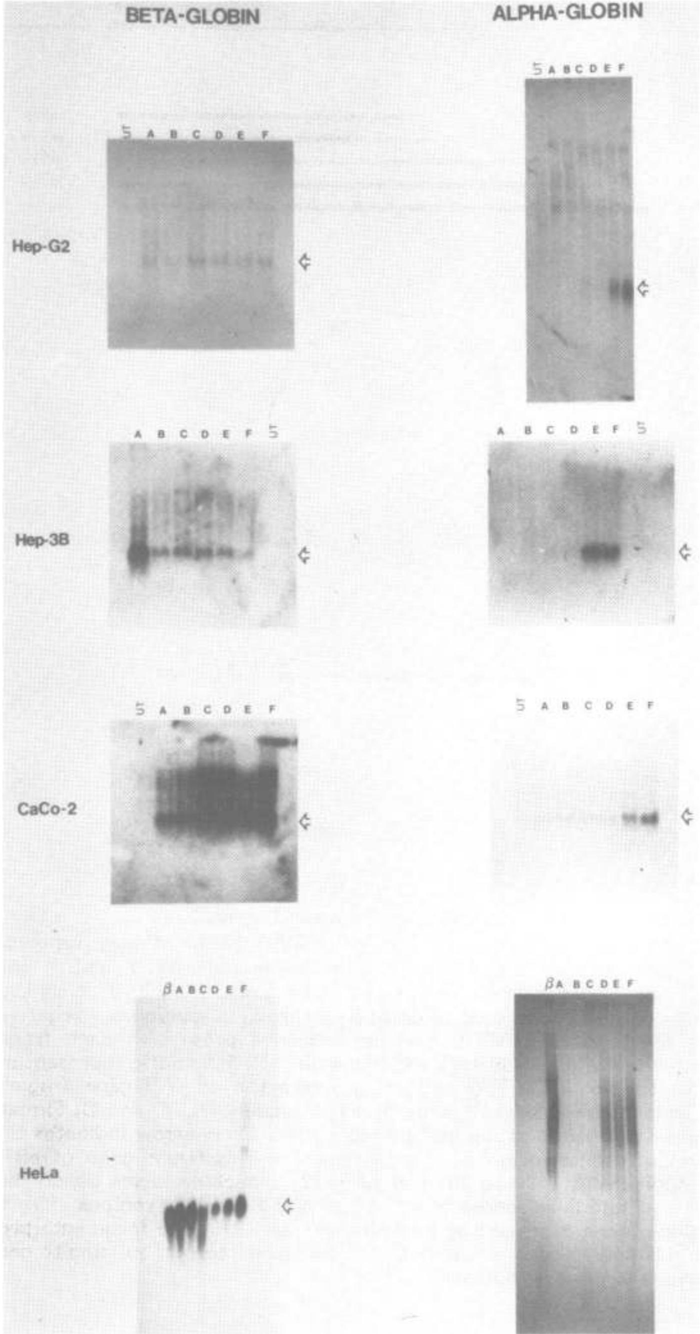


Figure 1. Construction of 5' deletions of the apo-AII gene.
(A) Restriction map of the subclone pAIITSX4.0 [33]. Boxes represent exons, connecting filled bars denote introns. Open boxes indicate 5' and 3' untranslated sequences, filled boxes coding sequences, thin lines 5' and 3' flanking regions. (B) Fragments of pAIITSX4.0 used to generate expression subclones. Negative numbers indicate the length of apo-AII 5' flanking sequence present in each fragment (see Materials & Methods for details of construction). (C) Schematic representation of the expression vector pSVα1Fd [34] indicating where the apo-AII gene fragments were inserted. Cross hatched boxes represent SV40 sequences. E and O, Simian virus 40 (SV40) enhancer and origin of replication sequences. Open arrow indicates the position and direction of transcription of the tetracycline resistance gene of pBR322. , deletion of nucleotides 1426 to 2490 of pBR322. Speckled boxes denote human α1-globin exons and bold lines represent introns, 5' and 3' flanking regions. (D) The hybrid apo-II/α1-globin gene produced by inserting the apo-AII gene fragments presented in (B) into the α1-globin vector presented in (C). The pattern of splicing to generate the mature hybrid transcript is indicated.


BETA-GLOBIN
ALPHA-GLOBIN
Hep-G2
Hep-3B
CaCo-2
HeLa
5 A B C D E F
A B C D E F 5
βA B C D E F

A series of hybrid gene constructs was generated, differing only in the length of apo-AII gene 5' flanking sequence they contained. Five recombinant plasmids were made: pαAII/5'1171, 912, 675, 210 and 117 containing respectively 1171, 912, 675, 210 and 117 bp of apo-AII gene sequence upstream of the transcription initiation site. The SV40 origin and enhancer sequences present in the parent plasmid vector pSVα1Fd (pSVOα_1p3d in Mellon et al [34]) were eliminated during the production of these constructs (see Fig.1) with the result that the only eukaryotic promoter elements they contained were those of the human apo-AII gene. This series of plasmids was transfected into logarithmically growing cultures of HeLa, Hep-3B, Hep-G2 and CaCo-2 cells. As a negative control the religated vector plasmid containing no apo-AII sequences, but otherwise identical to the deletion series, was also transfected into these cells. All the recombinants were co-transfected with an equal quantity of a plasmid (pHβ5'SVBglII) containing a functional copy of the human β-globin gene, the transcription of which served as a measure of transfection efficiency. This plasmid contains the SV40 origin of replication and also expresses SV40 large T-antigen and so has the necessary machinery to replicate. The α1-globin containing plasmids do not contain the SV40 origin of replication and therefore are not influenced by the presence of large T-antigen. pHβ5'SVBglII is the human equivalent of the rabbit globin construct pβ5'SVBglII described by Grosveld et al [52].

Total RNA was extracted 48 hrs after transfection and analysed by Northern-blotting (see Fig.2) and quantitative densitometry. This indicated that pαAII/5'1171 and 912, which contain respectively 1171 and 912 bp of apo-AII gene 5'-flanking DNA, are the only plasmids from which significant amounts of α1-globin sequences are transcribed. The size of these α1-globin containing transcripts is consistent with their expression being under the control of the normal apo-AII promoter and their pattern of

Figure 2. Northern blots of total RNA extracted from cells transfected with apo-AII 5' deletion/α1-globin gene constructs.
Autoradiograms of duplicate Northern blot filters hybridized with β and α1-globin specific probes. Hep-G2, Hep-3B, CaCo-2 and HeLa cells were transfected with the parent vector, pSVα1Fd lacking the StuI/BstEII fragment (A); pαAII/5'117 (B); pαAII/5'210 (C); pαAII/5'675 (D); pαAII/5'912 (E) and pαAII/5'1171 (F). The same cells were also cotransfected with pHβ5'SVBglII. Total RNA was extracted from the cells 48 hrs later and 35 µg analysed on each of two Northern blots following electrophoresis through formaldehyde containing 1.2% agarose gels. RNA extracted from cells which had either not been transfected (UT) or transfected only with pHβ5'SVBglII (β) was similarly analysed. The RNA on one of the duplicate blots was hybridized to an α1-globin specific probe while the other was hybridized to a probe specific for β-globin. The α1-globin probe was a nick-translated BstEII/PstI fragment isolated from pSVα1Fd (Fig.1). The β-globin probe was a nick-translated AccI/EcoR1 fragment isolated from pHβ5'SVBglII (see [94]). The position of the mature apo-AII/αI-globin transcript is arrowed. Autoradiography was performed at -70°C using an intensifying screen and preflashed Kodak X-O-mat S film. Exposures were on average 5 days.

Figure 3. Construction of 3' deletions of the apo-AII gene.
(A) Restriction map of the subclone pAIITSX4.0 (see Fig.1 for conventions used). (B) Fragments of pAIITSX4.0 used to generate expression subclones. The bars on the right represent the apo-AII gene fragment used to produce the parent plasmid vector, pαAII/5'210 (Fig.1). The bars on the left represent the additional apo-AII gene fragments introduced into pαAII/5'210 in order to try and rescue its transcriptional activity. These fragments were all introduced in their correct orientation relative to the apo-AII sequences already present. However the HindIII/BglII fragment was also introduced in the reverse orientation, as indicated by the arrowhead. Negative numbers indicate the region of apo-AII 5' flanking sequence present in each fragment as measured from the transcription initiation site [31]. (C) Schematic restriction map of the expression vector pSα1Fd [34] indicating where the apo-AII gene fragments were inserted (see Fig.1 for conventions used).

processing being as predicted (Fig.1D). pαAII/5'675, 210 and 117, which contain 675, 210 and 117 bp of 5' flanking DNA respectively, do not produce detectable quantities of transcripts containing α1-globin. The production of α1-globin sequences by pαAII/5'1171 and 912 is specific for the apo-AII producing cell lines Hep-3B, Hep-G2 and CaCo-2. This suggests that the 5' boundary of sequences required for the tissue-specific expression of apo-AII in both liver and intestine lies between 912 and 675 bp upstream of the transcriptional start site of the gene.

It is difficult to rigorously exclude differential mRNA stability as contributing to these results. However this cannot account for the loss of activity following deletion of 5' flanking sequences since identical transcripts are involved. Therefore, it seems likely that these results reflect different rates of transcription initiation.

Determination of the approximate 3' border of sequences involved in the tissue-specific expression of the apo-AII gene

The construction of the plasmids employed in the identification of the approximate 3' boundary of the sequences identified above which are involved in the tissue-specific expression of apo-AII is described in Figure 3.

The series of constructs generated were based on pαAII/5'210 which had been previously shown to be non-functional (see Fig.2). Increasing amounts of additional apo-AII 5' flanking sequences to those already present were introduced into this plasmid in an attempt to rescue its tissue-specific transcriptional activity. The 5' boundary of the sequences required for such transcription had been shown to lie between 912 and 675 bp upstream of the transcription initiation site. Therefore the 5' end of all the additional apo-AII 5' flanking sequences introduced into pαAII/5'210 was marked by the HindIII site 912 bp 5' of the CAP site. Their 3' ends were defined by the RsaI, HinfI and BglII cleavage sites 55, 121 and 529 bp downstream respectively of this HindIII site. Each of these three fragments was introduced in its correct orientation upstream of the apo-AII 5' flanking sequences already present in pαAII/5'210 (see Fig.3). Thus the HindIII/RsaI, HindIII/HinfI and HindIII/BglII fragments gave rise respectively to pαAII/5'210/5'857-912, 791-912 and 383-912. The HindIII/BglII fragment was also inserted in the reverse orientation relative to its normal association with the first 210 bp of apo-AII 5' flanking sequence (pαAII/5'210/5'383-912R).

These plasmids were transfected into HeLa, Hep-3B, Hep-G2 and CaCo-2 cells as described previously. The parent plasmid, pαAII/5'210, was transfected separately into each cell line as a negative control and all the constructs were co-transfected with pHβ5'SVBglII in order to control for transfection efficiency. Total RNA was extracted 48 hrs after transfection and analysed by Northern blotting (see Fig.4) and quantitative densitometry. These results demonstrate that pαAII/5'210/5'383-912 and 383-912R are the only plasmids from which α1-globin sequences are efficiently transcribed. However pαAII/5'210/5'791-912 appears to retain some transcriptional activity although this is only a fifth of that of pαAII/5'210/5'383-912 and 383-912R. pαAII/5'210/5'857-912, on the other hand, fails to produce a detectable level of transcripts containing α1-globin. The transcriptional activity of αAII/5'210/5'383-912 is the same as that of 383-912R and both are specific for the apo-AII producing cell lines Hep-3B, Hep-G2 and CaCo-2. pαAII/5'210/5'383-912 was subsequently also transfected into the embryonic fibroblast line DS-1 and the bladder carcinoma line EJ138. These two human cell lines do not produce apo-AII and, like HeLa cells, failed to express the hybrid gene which was introduced into them (data not shown).

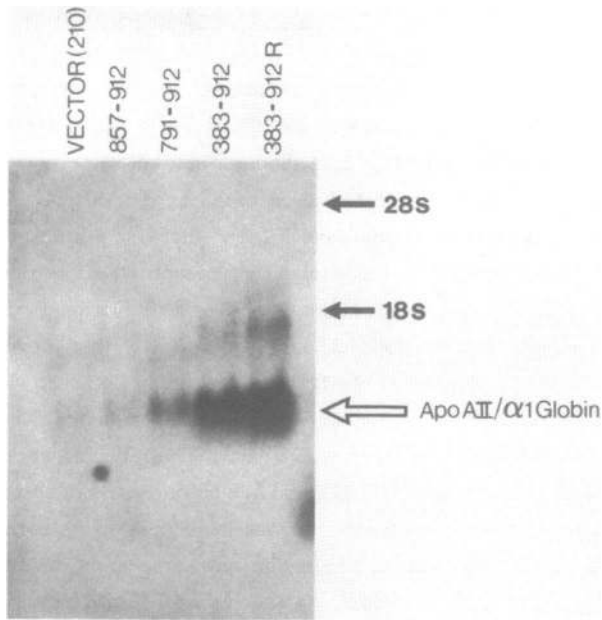


Figure 4. Northern blot of total RNA extracted from Hep-G2 cells transfected with apo-AII 3' deletion/ α1-globin constructs.
Autoradiogram of a Northern blot filter hybridized with an α1-globin probe. Hep-G2 cells were transfected with the parent plasmid vector, pαAII/5'210 (210); pαAII/5'210/5'857-912 (857-912); pαAII/5'210/5'791-912 (791-912); pαAII/5'210/5'383-912 (383-912) and pαAII/5'210/5'383-912R (383-912R). The same cells were also co-transfected with pHβ5'SVBglII. Total RNA was extracted from these cells and analysed by Northern blotting as described previously (Fig.2). Only the duplicate filter hybridized with the α1-globin probe is presented. The positions of the 28S and 18S ribosomal RNA species are indicated. These RNAs are 6333 and 2366 nucleotides long respectively [95]. A similar pattern of hybridization was obtained when RNA extracted from transfected Hep-3B and CaCo-2 cells was analysed. However, RNA extracted from transfected HeLa cells gave no hybridization signal as did that extracted from EJ138 and DS-1 cells transfected with pαAII/5'210/5'383-912 (see Fig.2 for experimental details and conventions used).

The results presented in Figures 2 and 4, when combined, implicate the 116 bp of 5' flanking sequence lying between 675 and 791 bp upstream of the transcription initiation site in the direction of the tissue-specific expression of the apo-AII gene (see Fig.5). In addition, the 121 bp immediately 5' of this region directs tissue-specific transcription at approximately 20% of the maximum observed level (Fig.4). It is possible therefore that the upstream cis-acting regulatory sequences involved in the specificity of apo-AII gene expression span both of these regions. The activity of these sequences, like that of viral and cellular enhancers, appears to be orientation

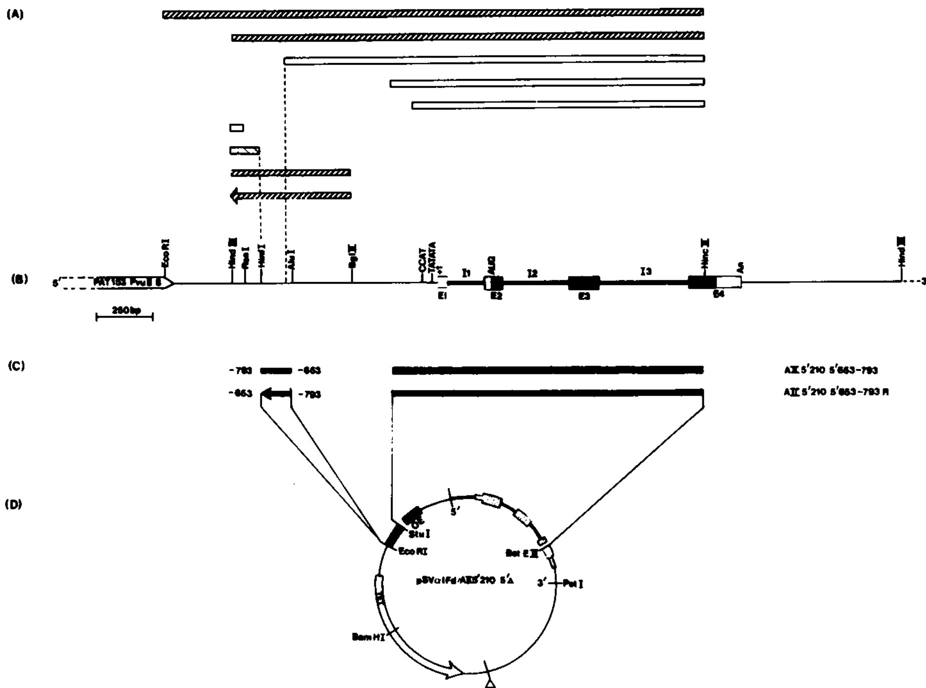


Figure 5. Construction of apo-AII gene deletions for fine mapping of the enhancer region.
(A) Summary of the results produced by the first two series of apo-AII gene deletion experiments. Tightly stripped bars indicate regions of the apo-AII gene that can, either in isolation or in combination with the apo-AII sequences present in pαAII/5'210, cause the tissue-specific expression of the apo-AII/α1-globin hybrid. The broadly stripped bar indicates a region of the gene which can direct transcription at a level a fifth of that directed by the tightly stripped regions. Open bars denote areas of the gene which do not direct a detectable level of transcription. The comparison of these results implicates the area indicated by the vertical dotted lines in the tissue-specific expression of the apo-AII gene. (B) Restriction map of pAIITSλ4.0 (see Fig.1 for conventions used). (C) Fragments of pAIITSλ4.0 used to generate expression subclones (see Fig.3 for conventions used). (D) Schematic restriction map of the vector pSVα1Fd [34] indicating where the apo-AII gene fragments were inserted (see Fig.1 for conventions used).

independent [53-59]. Thus the cell-specific transcription of apo-II in both liver and intestine appears to be controlled, at least in part, by one or more "enhancer-like" elements.

Evidence that the enhancer sequences involved in the tissue-specific expression of the apo-AII gene lie between 653 and 912 bp upstream of the transcription initiation site

Since the importance of the 116 nucleotides located between 675 and 791 bp upstream of the apo-AII transcription initiation site had so far only been implied, this

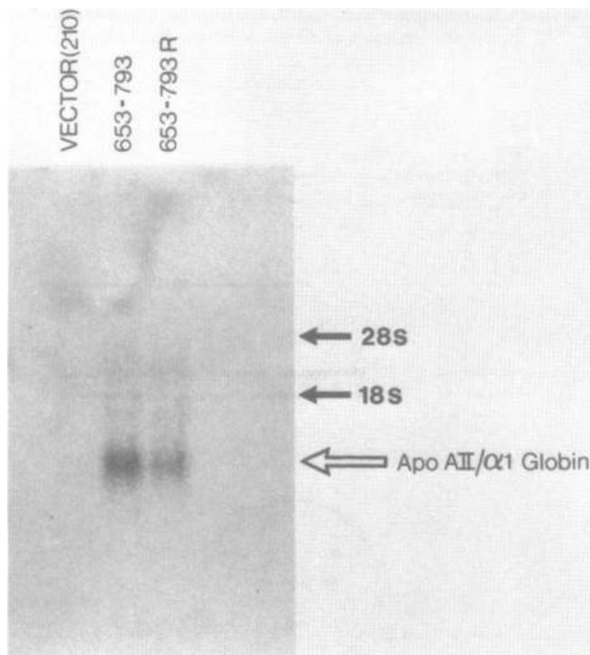


Figure 6. Northern blot of total RNA extracted from Hep-3B cells transfected with recombinants designed to finely map the enhancer region of the apo-AII gene. Autoradiogram of a Northern blot filter hybridized with an α1-globin specific probe. Hep-3B cells were transfected with the parent plasmid vector, pαAII/5'210 (210); pαAII/5'210/5'653-793 (653-793) and pαAII/5'210/5'653-793R (653-793R). The same cells were also cotransfected with pHβ5'SVBglII. Total RNA was extracted and analysed as described in Figure 2. Only the duplicate filter hybridized with the α1-globin probe is presented. A similar pattern of hybridization was obtained when RNA extracted from transfected Hep-G2 and CaCo-2 cells was analysed. However, RNA extracted from transfected HeLa cells gave no hybridization signal (see Fig.2 for experimental details and conventions used).

region was used in isolation to try and rescue the tissue specific transcriptional activity of pαAII/5'210. This sequence is contained within a 140 bp HinfI/AluI fragment which was introduced into pαAII/5'210 exactly as the fragments described previously (see Fig.5). Two constructs were generated, pαAII/5'210/5'653-793 contained the HinfI/AluI restriction fragment in its correct orientation relative to the 5' flanking sequences already present in the parent plasmid vector, while in pαAII/5'210/5'653-793R it was inserted in the reverse orientation. These two plasmids were transfected separately into HeLa, Hep-3B, Hep-G2 and CaCo-2 cells as before. The parent plasmid, pαAII/5'210, was again included as a negative control and all three constructs were co-transfected with pHβ5'SVBglII.

Transcripts were analysed by Northern blotting (Fig.6) and quantitative densitometry. This indicated that both orientations of the HinfI/AluI fragment could rescue, to the same extent, the tissue-specific expression of pαAII/5'210. However although the HinfI/AluI fragment retains the enhancer properties of the HindIII/BglII fragment, it, like the HindIII/HinfI fragment immediately upstream (Fig.4), confers only a fifth of the transcriptional activity directed by the larger fragment (Fig.4 cf Fig.6).

The pattern of expression of all the deletion constructs analysed appears identical in all three apo-AII producing cell lines. Consequently in both liver and intestine the same DNA sequence seems to be important in causing the expression of the apo-AII gene. The relatively low enhancer activity of the HinfI/AluI fragment indicates that it does not contain all this regulatory DNA sequence. The remainder may reside immediately upstream in the HindIII/HinfI fragment (Fig.3) as these sequences also appear to confer a low level of tissue-specific expression on the apo-AII gene (Fig.4). Therefore this regulatory sequence probably lies in the 259 bp between the HindIII and AluI restriction recognition sites, 912 and 653 bp upstream, respectively of the apo-AII transcription initiation point (Figs. 5 and 8).

Evidence that the apo-AII enhancer confers cell-specific expression on the apo-AII gene

The sequences between 912 and 653 bp upstream of the transcription initiation site have been shown to have enhancer properties and to be involved in the tissue-specific expression of apo-AII. However this has always been in conjunction with the apo-AII sequences present in pαAII/5'210. Therefore it could be possible that the apo-AII "enhancer", like those linked to the immunoglobulin and insulin genes [1-2] is associated with elements in the promoter region which also exhibit a pronounced host cell preference. Therefore, in order to determine whether pαAII/5'210 contained sequences that could independently direct tissue specific expression, its transcriptional activity was rescued not by the apo-AII "enhancer", but by the non-specific SV40 enhancer. This and other viral enhancers have been shown to be incapable of overriding the specificity of expression associated with a number of cellular genes [1-2].

A StuI/EcoR1 fragment isolated from pSVα1Fd (Fig.1) containing the SV40 enhancer and origin was cloned into pαAII/5'210 in exactly the same position upstream of the apoAII promoter as was the apo-AII "enhancer-like" element (Figs.3 and 5). The resulting construct, pαAII/5'210/SV40, was then transfected into HeLa and Hep-3B cells. The parent plasmid, pαAII/5'210, was introduced separately as a negative control and both constructs were cotransfected with pHβ5'SVBglII to check for variations in transfection efficiency. Total RNA was extracted and analysed by

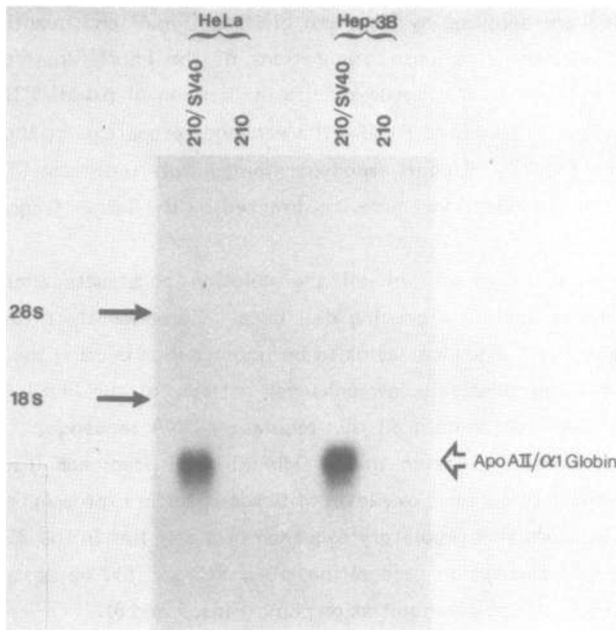


Figure 7. Northern blot of total RNA extracted from HeLa and Hep-3B cells transfected with pαAII/5'210/SV40
Autoradiogram of a Northern blot filter hybridized with an α1-globin specific probe. HeLa and Hep-3B cells were transfected with the parent plasmid, pαAII/5'210 or pαAII/5'210/SV40 (see text and Materials & Methods for details of construction). The cells were also cotransfected with pHβ5'SVBglII. Total RNA was extracted, Northern blotted and analysed by hybridization to β-globin (data not shown) or α1-globin specific probes (see Fig.2 for experimental details and conventions used).

Northern blotting (Fig.7) and quantitative densitometry. This indicated that the SV40 enhancer causes that part of the apo-AII gene present in pαAII/5'210 to be expressed both in HeLa and in Hep-3B cells. Therefore, while the apo-AII enhancer rescues expression in a tissue specific manner, the SV40 enhancer rescues expression non-specifically. Thus the apo-AII gene, stretching from 210 bp upstream to 1168 bp downstream of the transcription start site, appears to contain no elements that can independently direct tissue specific expression. Rather, it is the apo-AII enhancer centred at 782 bp upstream that confers such specificity, either independently or in conjunction with other apo-AII sequences. In addition, the presence of apo-AII/α1-globin hybrid transcripts in HeLa cells discounts the possibility that mRNA instability accounted for the results previously obtained with these cells (Fig.2).

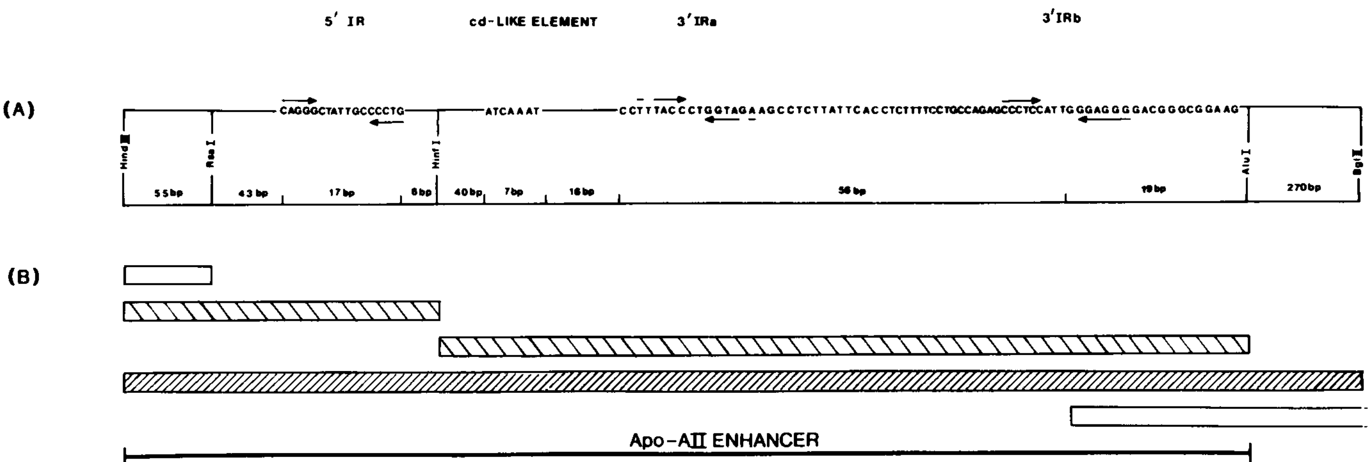


Figure 8. Structure of the apo-AII enhancer
(A) Schematic representation of the organization of the apo-AII enhancer region indicating the position of potentially important sequences. The 3' BglII site is located 383 bp upstream of the apo-AII transcription initiation site [31]. Inverted repeats are marked by converging pairs of arrows. (B) Summary of the results which indicate this region of the apo-AII gene is involved in tissue-specific expression (see Fig.5 for conventions used).

DISCUSSION

The introduction of a series of deletion and substitution mutants of the apo-AII gene into human cells in culture has allowed the identification of a cellular "enhancer-like" element upstream of the transcription initiation site. The "enhancer" appears to be absolutely required for tissue specific transcription from the apo-AII promoter, directing expression both in liver and intestine derived cells but not in bladder, fibroblast or cervical epithelial cell lines. Two other examples of dual tissue-specific expression have been similarly studied, one in Drosophila [60-61] and one in man [8]. These reports demonstrated that while the expression of the human antithrombin III gene in both liver and kidney cells is directed by a single enhancer region of about 800 bp, different cis-acting regulatory regions appear to direct transcription of the Drosophila yolk protein I gene in fat body and ovarian follicle tissue.

In the case of some immunoglobulin genes [2,62-65] and the rat insulin gene [1], it has been demonstrated that promoter sequences display cell-type specificity independently of their associated enhancers. The human apo-AII promoter region, like those of the chicken lysozyme [4] and α-crystallin [66] genes, does not exhibit this type of specificity in the cells used in these transient expression studies. Rather, the replacement of the apo-AII enhancer with that of SV40 leads to comparable expression from the apo-AII promoter both in apo-AII producing Hep-3B and non-producing HeLa cells.

The human apo-AII "enhancer-like" element contains none of the consensus sequence motifs which have been previously identified in cellular enhancers [67-70] However, the minimal apo-AII enhancer fragment (-653 to -912) identified in this study does contain the sequence 5'-ATCAAAT-3' (Fig.8). This is very similar to the highly conserved octanucleotide 5'-ATGCAAAT-3' ("cd-element") found upstream of the immunoglobulin heavy chain promoter and, in the reverse orientation, within the heavy chain enhancer and upstream of the kappa-light chain promoter [71]. In all three locations this sequence has been implicated in tissue specific expression [2,62-65,72] and appears to bind both lymphoid specific and non-tissue-specific protein factors [68,70,73-75]. One nucleotide downstream of the "cd-like element", the enhancer region of apo-AII contains the sequence 5'-ACCAGGTGCC-3'. This is homologous to the consensus sequence, 5'-GCCAGGTGGC-3', of a group of related motifs which also appear to be involved in the activity of the immunoglobulin enhancers [67,68,70,76].

The other obvious structural feature of the apo-AII enhancer sequence is the presence of three pairs of inverted repeats, one at its 5' end and two at its 3' end (Fig.8). Between the 3' IRs, the sequence 5'-TTCACCTCTTTTCC-3' contains homologies both with a viral enhancer consensus sequence [77] and with a region ("E1")

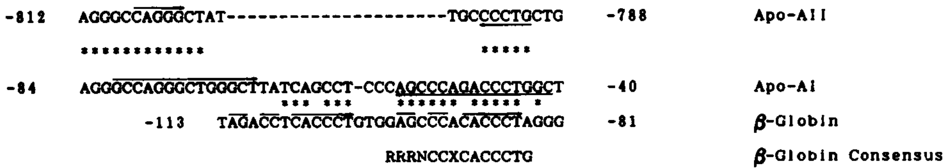


Figure 9. Gene sequences homologous to the apo-AII enhancer.
Sequence comparison of the apo-AII enhancer 5' inverted repeat (5'IR) and homologous sequences close to the CAP site of the human β-globin and apo-AII genes. Repeats are marked by arrows and sequence identity is indicated by asterisks. R = purine; X = A, T or C; N = any nucleotide. The β-globin consensus sequence is as described by Dierks et al [96].

implicated in the tissue-specific activity of the insulin gene enhancer [78]. The 5' inverted repeat, 812 bp upstream of the CAP site, shares significant homology to repeat regions of both the human apo-AI and the human β-globin genes which are only 41 bp and 85 bp 5' of the transcription initiation site respectively (Fig.9). Mutations within this region of the β-globin gene have been linked with β-thalassemia [79,80] while a naturally occurring mutation within the homologous region of the human apo-AI gene has been implicated in hyperlipidemia [81]. The element within the apo-AI gene promoter represents one of a number of apolipoprotein gene sequences which share homologies with the apo-AII enhancer [Sharpe et al., in preparation]. This is not surprising since the apo-AII gene belongs to a well characterized gene family that is thought to have arisen from a common ancestor [33,82-84] and whose expression might therefore conceivably involve common control mechanisms. Indeed when potential regulatory sequences of the apo-AII gene were compared to those of the other apolipoprotein genes [33,85], a region of common homology was found. In the apo-AII gene this lies between 469 bp and 495 bp upstream of the transcription initiation site. Consequently, in terms of the apo-AII gene deletions employed in this study, it was both absent from actively transcribed constructs and present in others which were non-functional. Therefore if this sequence is involved in some common aspect of the regulation of apolipoprotein gene expression, it appears either unable to act in isolation and/or is relevant to a process other than basic transcriptional competence. Further downstream from the region of common homology, 355 and 304 bp 5' of the CAP site of the gene, there are potential viral enhancer core sequences, 5'-CTTTCCA-3' [86]. These are unlikely to have any functional significance since they were absent from all the constructs in which the tissue-specific expression of the apo-AII gene was rescued.

While the functional importance of the various sequences within the apo-AII

enhancer which we have discussed is unclear, it is interesting to note that when this region is divided into two parts, one containing the 5' pair of inverted repeats and the other the "cd-like element" together with the two 3' pairs of inverted repeats, each retains only approximately 20% of the activity of the intact enhancer. Therefore it seems likely that this element has at minimum a bipartite structure, the components of which cooperate to give the apo-AII enhancer its full activity. The region of common homology, 495 bp upstream of the CAP site, may be involved in this synergistic process.

The regulatory element identified in this study is clearly vital to the dual tissue-specific transcription of the human apo-AII gene. Mutations within it will therefore represent prime candidates for genetic lesions contributing to abnormal apo-II levels. These levels are of critical importance given that they appear to have a strong negative correlation with the prevalence of myocardial infarction [87] and peripheral vascular disease [88].

A number of apolipoprotein gene RFLPs have been found associated with abnormalities within the lipid transport system [22,23,89-92]. Scott et al [25] have identified such a polymorphism of the apo-AII gene that is linked both to high plasma apo-AII levels and altered HDL composition. Moreover, in inbred strains of mice, a locus tightly linked to the apo-AII gene has been shown to confer resistance to diet induced hyperlipidemia and atherosclerosis [93]. In all these studies the proteins involved appear normal, indicating the causative lesions may lie in regulatory regions such as the apo-AII enhancer.

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