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Human apolipoproteins AI, AII, CII and CIII. cDNA sequences and mRNA abundance

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ABSTRACT

The structure and function of the genes encoding the polypeptide components of plasma lipoproteins are of interest because of the central role they play in the regulation of lipid metabolism. We have now completed our previous studies on the human apoAI gene and furthermore isolated and sequenced cDNA clones for apoAII, CII and CIII. The nucleotide sequences show the signal peptides of apoAII, CII and CIII to be 18, 22 and 20 amino acids in length, respectively, and in addition that prepro apoAII bears a classical propeptide structure of 5 amino acids. The amino acid homology detected between apoCII and pro-apoAI is discussed, as is the gene arrangement of the 5' non-coding region of apoAI mRNA. The relative liver mRNA levels of the 4 apolipoproteins analysed in this study have been estimated and compared with their corresponding plasma products. The data reported here provide an essential basis for further studies of structural and functional alleles of apo AI, AII, CII and CIII genes.

INTRODUCTION

Lipoproteins form part of a complex system ensuring that tissue requirements for lipids (primarily triglycerides and cholesteryl esters) are met, whilst maintaining their plasma levels within acceptable limits. The lipoproteins consist of a hydrophobic core (cholesteryl ester and triglycerides) surrounded by a surface coat containing phospholipids, unesterified cholesterol and proteins called apolipoproteins. The latter are responsible for solubilizing the lipids and, in addition, some may interact with many of the enzymes involved in lipid metabolism and with specific cellular receptors. Defects in one or more of the many components involved in lipid transport and metabolism can lead to the formation of high plasma lipid levels as shown by a wide variety of genetic diseases (1).

Hyperlipoproteinemia is a common metabolic disorder, occurring in at least 10% of the UK population (2), and has been strongly implicated as an important factor in the incidence and severity of cardiovascular disease (3,4). In rare cases it is known to be caused by a structural gene muta-

tion [e.g. apoCII and LDL receptor (5,6)]. In the majority of cases, however, classical biochemical techniques have failed to reveal the molecular defect underlying the hyperlipidemia.

An alternative approach to understanding the molecular basis of these disorders is through a detailed analysis of the genetic organisation of the affected individuals by means of recombinant DNA techniques. Preliminary studies (7,8) have already shown that variations of genotype in the population associated with the apoAI gene, are correlated with the occurrence of some of these disorders. Further progress will only be possible after we have a complete knowledge of some of the structure of the genes involved. In these studies, we have therefore precisely defined the gene arrangement of the 5' non-coding region of apoAI mRNA. In addition, we have expanded our capability of detecting variations in other regions of the genome by isolating cDNA clones for apoAII, CII and CIII. Concomitantly, it has been possible to search for similarities in these proteins, e.g. signal and propeptide sequences, which may have functional significance. This was of particular interest, as previous studies (9,10) revealed that both rat and human apoAI have a most unusual propeptide structure, the function of which is unknown.

The influence of environmental factors (i.e. diet, cigarette smoking) on the rate of synthesis of human apolipoprotein mRNAs has not been closely studied. The availability of apolipoprotein cDNA clones should eventually make it possible to correlate the response, at the mRNA level, of individuals with different combinations of apolipoprotein alleles, to different environmental conditions. As a first step towards this goal, we have examined the relative levels of apoAI, AII, CII and CIII transcripts in a human liver sample.

MATERIALS AND METHODS

(a) Construction of the cDNA libraries

Aliquots of two amplified libraries were kindly supplied by Drs. A.K. and D.R. Bentley (11). The two libraries differed in that, whereas library 1 was prepared using double-stranded cDNA that was restricted with HaeIII, library 2 was prepared with full length double-stranded cDNA that had been treated with nuclease S1 and then repaired by "filling in" with the Klenow fragment of DNA polymerase I (12).

(b) Oligonucleotide synthesis

The initial isolation of apoAII, CII and CIII cDNA clones involved the use

TABLE 1

Synthetic oligonucleotide probes used to screen recombinant clones. Y indicates pyrimidine, R indicates purine. A unique oligonucleotide was prepared for apoCII (see Figure 5) according to the data of Mykelobost et al (14), while a mixture of 8 and 16 were synthesised for apoAII and CIII, respectively.

Protein	ApoAII	ApoCIII
Amino acid sequence	13 17 Q Y F Q T	63 67 E F W D L
mRNA	5' CARTAYTTYCARAC 3'	5' GARUUYUGGGAYYU 3'
Oligonucleotide	5' CARTAYTTYCARAC 3'	3' CTYAARACCCTRRA 5'

of specific oligonucleotides. The choice of oligonucleotide used in each case involved converting the known amino acid sequence, by using the genetic code, into its corresponding nucleotide sequence and then identifying a stretch between 14 and 17 nucleotides in length that contained few sequence ambiguities (see Table 1).

Each of the oligonucleotides was synthesised, simultaneously as a mixture, using the solid-phase phosphotriester method developed by Gait and colleagues (12,13). The radioactive oligonucleotide probes were prepared by the phosphorylation of 5' ends with [γ -^{32}P] ATP (7,000 Ci/mmol) using T4 polynucleotide kinase (Boehringer). The degree of labelling was maximized by performing the reaction with twice the molar concentration of the label to the oligonucleotide.

(c) Screening of the cDNA libraries

Colonies were transferred to sterilized Whatman 541 paper circles, amplified with chloramphenicol and prepared for hybridization according to Gergen et al (15). Prehybridization, hybridization and washing conditions for the screening of cDNA libraries with the oligonucleotide probes were essentially as described (16,17). Thus, the hybridization with the apoAII and CII probes proceeded at 32°C and 47°C, respectively, in the presence of approximately 10^5cpm of 5' end-labelled oligonucleotide per ml of the hybridizing solution.

For the screening of libraries with probes prepared from subclones pPA 1.8 (9), M13 AII and M13 CIII (see below), the plasmid DNA bound paper circles were prehybridized, hybridized and washed as described (12).

(d) Subclone construction

M13AI, pPAI 1.8 and pRHAI 5.7 are either cDNA or genomic subclones containing apoAI sequences that have been previously described (10,12). The methods used to construct and isolate the other subclones are as follows: (i) M13S AI - double-stranded M13AI DNA was restricted with BamHI and EcoR1 and ligated to BamHI/EcoR1 restricted and phosphatased M13 Mp701 vector (18). As the cloning sites of the two vectors used in this procedure are arranged in opposite orientations, the desired recombinant would contain a "turned around" (i.e. complementary) insert. The structure of the resulting recombinant was confirmed by dideoxy sequencing (19) using the universal primer (20), as was that of M13S 5', M13 AII and M13 CIII. (ii) M13S 5' - approximately 8μg of pPA 1.8 was restricted with HindIII, radioactively labelled (20), re-restricted with HinfI and then subjected to polyacrylamide gel electrophoresis. The desired product was eluted from the gel, purified, re-restricted with AluI and cloned into the SmaI site of M13mp9 vector (21). (iii) M13 AII and pS AII - this involved isolating an EcoR1/HaeIII fragment from pAII (see Results) by the procedure described by Girwitz et al (22) and then re-restricting it with AluI, before cloning into either SmaI-restricted and phosphatased M13mp9 vector or PvuII restricted and phosphatased pAT153/PvuII/8 vector (23). (iv) M13 CIII (a gift from M H Murphy) - the primary source of DNA for this subclone was obtained by isolating a 1.5 Kb PvuII fragment (see Results) from pRHAI 5.7 as described (22). This DNA fragment was then sonicated (24) and size fractionated on a 1% agarose gel. All products >400 bp were eluted (22), purified by phenol/chloroform extraction and finally concentrated by ethanol precipitation. The 5' overhanging ends of the purified DNA fragments were repaired by "filling in" with the Klenow fragment of DNA polymerase I prior to ligation into the SmaI restricted and phosphatased site of M13mp8 (21).

(e) Sequencing of cDNA clones

The fragments were obtained either by 3' end labelling whole plasmid digests and fractionating the fragments in polyacrylamide gels or by first purifying selected large molecular weight fragments by agarose gel fractionation and electroelution (22). The sequence was determined by the chemical degradation procedure (25).

(f) RNA blotting

Transfer of RNA from electrophoretic gels containing formaldehyde (26) to Gene Screen membranes and subsequent hybridization were carried out under conditions specified by the manufacturer (New England Nuclear, Boston, USA). The M13S AI and M13S 5' probes were prepared as previously described (12).

(g) Mapping of pRHA1 5.7

pRHAI 5.7 (10) was restricted with PvuII, fractionated by size on an 0.8% agarose gel and then prepared for hybridization with the CIII oligonucleotide mixture (see Table 1) as described (27). The hybridization proceeded at room temperature for 18 hrs with 8 x 10^6 cpm of the labelled oligonucleotide probe in a volume of 15 mls. Following hybridization, the gel was washed at room temperature for 1 hr.

RESULTS

The 5' non-coding sequence of apoAI mRNA

To define precisely the gene organization of the 5' non coding region of apoAI mRNA, cDNA library 2 was screened for full length apoAI cDNA clones. The probe used to screen this library was a Sau96I fragment, isolated from pPAI 1.8 (10), which contains the nucleotides between 694-805 of the apoAI gene (see Fig. 2). DNA was prepared from two of the apoAI cDNA clones, pAIA and pAIB, detected with this probe. The nucleotide sequence of the 5' termini of both cDNA inserts is shown in Figure 1. When compared with the gene sequence presented in Figure 2, it became apparent that:

i) the nucleotide sequence of the 5' non-coding region of apoAI mRNA is
5' GACTGCGAGAAGGAGGTCCCCCACGGCCCTTCAGG 3'

ii) in the gene this non-coding region is interrupted by an intron of 197 bp, as previously proposed (10).

The terminal 5' nucleotide of both cDNA inserts is located at position 470 in the gene sequence presented in Figure 2. However, as we cannot neglect the possibility that a few 5' nucleotides are lost in the cloning procedure, it is not possible to conclude that this is the true 5' terminus. At best, we can only infer by comparison with other structural genes (28) that the 5' terminus of apoAI liver mRNA is encoded by a nucleotide between positions 467-470, that is 30-33 bp downstream of the 3' adenosine residue of the "TATA box" (29).

However, Karathanasis et al (32) have recently isolated a cDNA clone, pAI-121, that contains, in addition to the nucleotides coding for the 5'

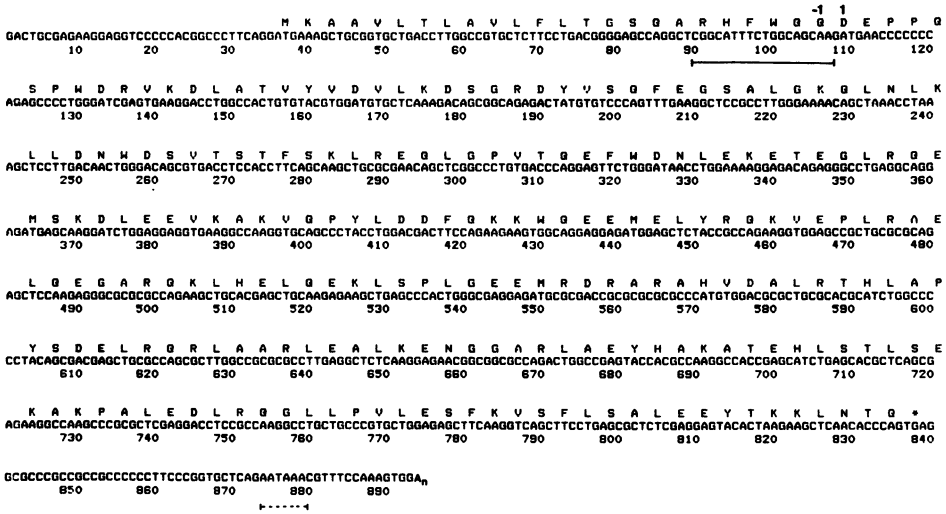

Figure 1: Complete nucleotide sequence of human apoAI cDNA

The sequence is shown in the mRNA sense and therefore reads in the 5' to 3' direction. The inferred amino acid sequence is represented by the nomenclature of Dayhoff (30). The dotted lines indicate the "AATAAA" sequence (31) and the A_n at position indicates the start of the poly(A) tail. The solid line preceding the start of the mature protein, position 1, indicates the propeptide sequence.

non-coding region of apoAI liver mRNA, sequences corresponding to nucleotides 409-471 of the apoAI gene. Thus, this clone contains the only identified "TATA box"-like structure of the apoAI gene and predicts that it encodes a 5' non-coding region of 96 nucleotides in length. This conflicts with our previous oligonucleotide primer extension studies, which showed that this region was a maximum of 70 nucleotides long (10,12). In order to resolve this apparent conflict, we examined whether any sequence between 335-456 of the apoAI gene forms part of apoAI liver mRNA. Total liver RNA blottings were carried out using the following probes:

i) subclone M13S 5', which contains an AluI fragment spanning nucleotides 335-456 of the apoAI gene

ii) M13S AI, which contains sequences coding for amino acids 66-81 of mature apoAI protein.

Hybridization to apoAI mRNA was detected only with M13S AI (see Figure 3). This demonstrates that the apoAI sequences present in M13S 5' do not form part of the major species of apoAI mRNA found in adult liver.

Figure 2: The nucleotide sequence of the 5' end of the apoA1 gene

The dotted lines at position 436-443 indicate the only "TATA" box-like sequence (29) found in the 5' flanking region of the structural gene. The arrow at position 470 indicates the putative capping site of apoAI liver mRNA; see text for further details. This sequence differs at positions 309, 321, 392, 400, 560, 611, 616 and 650 from that recently published (32). We are confident of our sequence and therefore further studies will need to be undertaken to discern whether these are genuine polymorphisms. The smaller dotted line spanning positions 470-484 and 682-701 indicate the nucleotides encoding for the 5' non-coding region of apoAI liver mRNA. The solid line between positions 943-960 indicate the propeptide sequence. The arrows after positions 484 and 744 indicate the exon/intron splice junctions; the arrows before positions 682 and 932 denote the intron/exon splice junctions. It should be noted that the glycine (G) residue at position 931 is coded by the triplet 5' GGG 3'.

Human apoAII

A human apoAII cDNA clone pAII, was isolated, by screening cDNA library 1 with an apoAII specific oligonucleotide mixture (see Table 1). This clone carried a 342 bp insert that contained sequences corresponding to nucleotides 1 to 197 shown in Figure 4. In addition, pAII contained nucleotides that did not correspond to apoAII sequences. From sequence analysis, it was clear that this was due to the ligation of three HaeIII cDNA fragments in pAT153/PvuII/8. pAII was used to construct a subclone, M13 AII, that contained only sequences corresponding to nucleotides 78-190 shown in Figure 4. This subclone was then used to isolate further apoAII cDNA clones from library 2, two of which were subsequently sequenced (Figure 4).

The nucleotide sequence of apoAII confirms, with only one exception, the amino acid sequence of the mature protein reported by Brewer et al

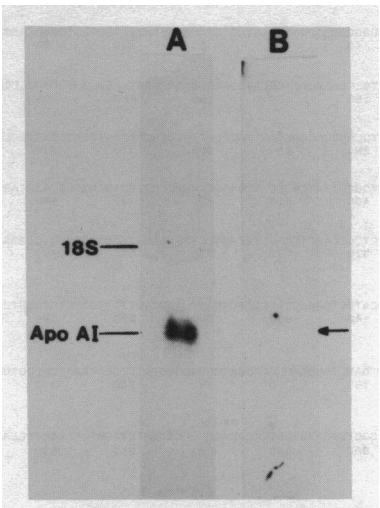

Figure 3: Autoradiograph of 2 Northern blots of total human liver RNA

A, was hybridized with subclone M13S AI which contains 47 nucleotides coding for amino acids 66-81 of mature apoAI; B, was hybridized with subclone M13S 5' which contains nucleotides 335-456 of the apoAI gene. The arrow indicates the location of apoAI mRNA. The specific activities of the probes were approximately equal. The filters were washed at low stringency (final wash 2 x SSC).

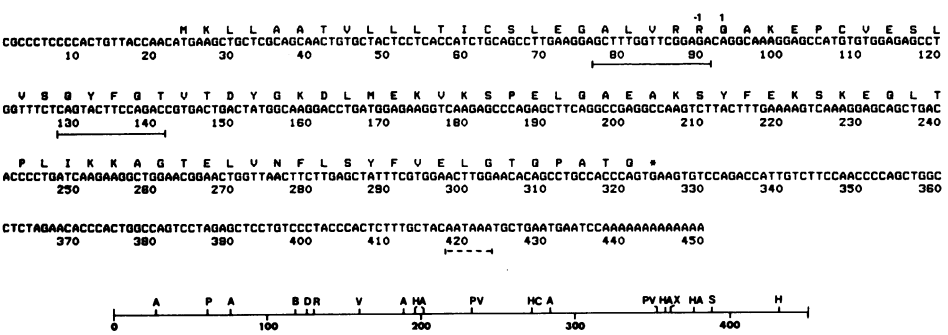

Figure 4: Nucleotide sequence and restriction enzyme map of human apoAII cDNA

See Figure 1 for details of the conventions used to present the features of the nucleotide sequence. The solid line spanning positions 128-141 indicate the nucleotides corresponding to the oligonucleotide probe. The lower section of the Figure shows a physical map of restriction endonuclease cleavage sites present in the cDNA insert where A = AluI, P = PstI, B = BstnI, D = DdeI, R = RsaI, V = AvaII, HA = HaeIII, Pv = PvuII, HC = HindII, X = XbaI, S = SacI and H = HinfI.

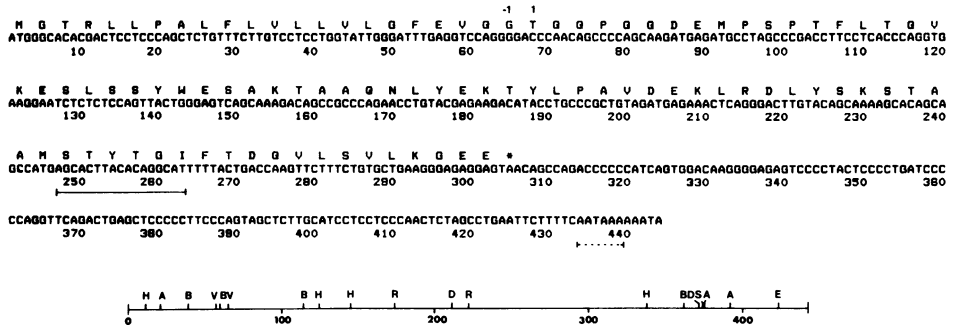

Figure 5: Nucleotide sequence and restriction enzyme map of human apoCII cDNA

See Figure 1 and 4 for details of the conventions used to present both the features of the nucleotide sequence and the restriction enzyme map of apoCII cDNA. Note that E = EcoR1.

(33). This difference occurs at amino acid 35; whereas direct amino acid sequencing apparently shows it to be a glutamine residue, the DNA sequence from two independently isolated clones both predict it to be glutamic acid.

The cDNA sequence predicts that human apoAII is initially synthesized with a 23 amino acid NH_2 terminal extension. Comparison of this sequence with the structure of other prepeptides (34) and prepropeptides (35) suggests that the first 18 amino acid residues of this NH_2 terminal could be defined as a prepeptide and the following five as a pro-segment.

Recently, two partial amino acid sequences of the prepropeptide structure of apoAII have been published (36,37). There are however differences in the sequence of the propeptide. The amino acid sequence predicted from the cDNA sequence shown in Figure 4 is in complete agreement with that reported by Gordon et al (37).

Human apoCII

17 putative human apoCII cDNA clones were isolated by screening cDNA library 2 with an apoCII specific oligonucleotide (see Table 1). Two of these, pCIIA and pCIIB, were characterized and used to determine the nucleotide sequence of apoCII cDNA shown in Figure 5.

Three amino acid sequences are published for apoCII (38,39,40). There are considerable differences between them. The amino acid sequence predicted from the apoCII cDNA sequence is in complete agreement with that reported by Hospattankar et al (40). Clearly, further studies will need to

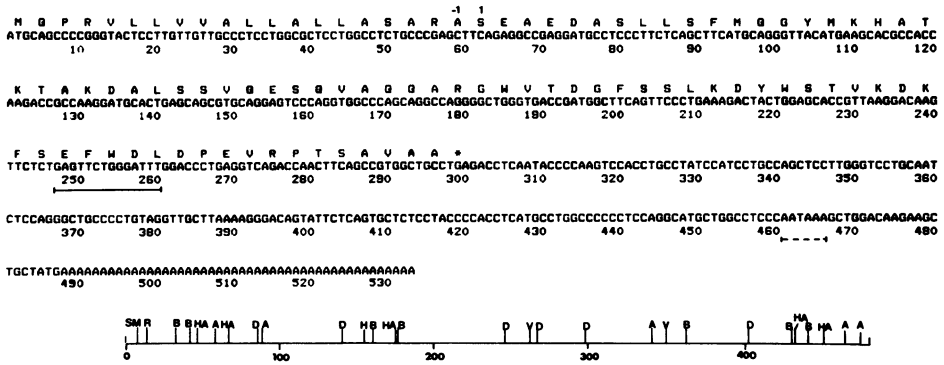

Figure 6: Nucleotide sequence and restriction enzyme map of human apoCIII cDNA

See Figure 1 and 4 for details of the conventions used to present both the features of the nucleotide sequence and the restriction enzyme map of apo-CIII cDNA. Note SM = SmaI.

be undertaken to determine whether the differences reported by Jackson et al (38) and Bradley et al (39) are genuine polymorphisms.

Interestingly, the sequence of amino acids 5-12 inclusive, of mature apoCII bears a remarkable resemblance to amino acids -2 to 6, inclusive, of pro-apoAI (see Table 3 and Discussion).

Human apoCIII

We used the previously described subclone, pRHAI 5.7, which carries part of the human apoAI and CIII gene (10,41), to produce a probe, M13 CIII, to screen cDNA library 2 for apoCIII cDNA clones. pRHAI 5.7 was restricted with PvuII, size fractionated on an agarose gel and then hybridized to a specific CIII oligonucleotide mixture (see Table 1). A PvuII fragment of 1.5 Kb was identified and used as the primary source of DNA to produce subclone M13 CIII.

Five apoCIII cDNA clones were isolated from cDNA library 2. The size of the inserts varied from 190-550 nucleotides in length. Clones pCIIIA and pCIIIB containing inserts of 520 and 550 bp, respectively, were sequenced.

The nucleotide sequence of apoCIII is shown in Figure 6. It predicts that the amino acid sequence of apoCIII is different at four positions from that previously reported (42). These differences are clustered between amino acids 31-39. Protein sequencing apparently shows the sequence in this region to be QSQQVAAQQ, while the cDNA sequence predicts it to be QESQVAQQA.

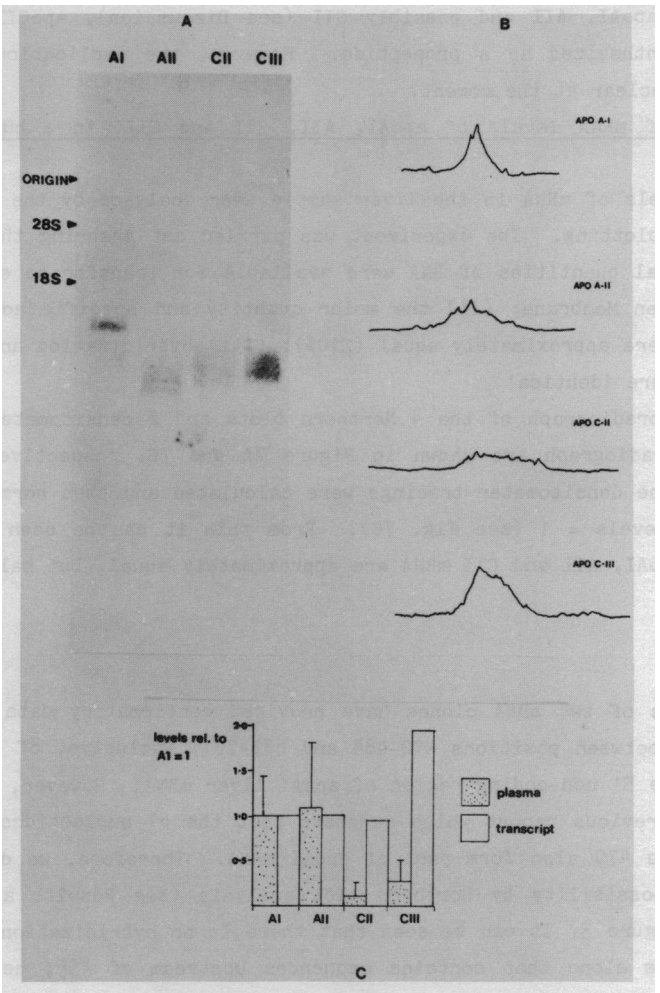

Figure 7: Comparison of apoAI, AII, CII and CIII mRNA levels in a human liver sample

A) Autoradiograph of 4 Northern blots of total human liver RNA (20µg) hybridized with different apolipoprotein cDNA probes. The apoAI, AII, CII and CIII probes were 3' end labelled anti-sense fragments prepared from equal quantities of pAIA, pS AII, pCIIA and pCIIIA plasmid DNA, respectively. The fragments ranged from 113-240 bp in length and incorporated very similar (±10%), as assayed by a number of Cherenkov counts per minute, quantities of radioactivity.

B) Densitometer tracings of the autoradiograph shown in A of the Figure.

C) Comparison of plasma levels of apolipoproteins with the corresponding mRNA abundance in liver. Both protein and mRNA levels are calculated relative to apoAI, i.e. apoAI = 1. The transcript levels are derived from A and B, while the protein levels, expressed in molar terms (i.e. AII calculated from the monomer) are derived from reference 1.

Unlike apoAI, AII and possibly CII (see Discussion), apoCIII is not initially synthesized as a propeptide. However, the implication of this finding is unclear at the moment.

Comparison of mRNA levels of apoAI, AII, CII and CIII in a human liver sample

The levels of mRNA in the liver sample were analysed by the technique of Northern blotting. The experiment was carried out ensuring the following: (i) equal quantities of RNA were available for transfer to each strip of Gene Screen Membrane; (ii) the molar quantity and specific activity of each probe were approximately equal (±10%); (iii) hybridization and washing conditions were identical.

The autoradiograph of the 4 Northern blots and a densitometer tracing of the autoradiograph are shown in Figure 7A and 7B, respectively. The area under the densitometer tracings were calculated and then normalized to ApoAI mRNA levels = 1 (see Fig. 7C). From this it can be seen that the levels of apoAI, AII and CII mRNA are approximately equal, but half that of apoCIII.

DISCUSSION

Analysis of two cDNA clones have provided confirmatory data that the nucleotides between positions 470-484 and 682-701, inclusive, of the apoAI gene form the 5' non-coding region of apoAI liver mRNA. However, this contradicts a previous report which suggests that the 61 nucleotides upstream of nucleotide 470 also form part of apoAI mRNA. Therefore, we decided to check this possibility by Northern blot analysis (see Results and Figure 3). From Figure 3, it can be seen that there is no hybridization to apoAI mRNA with the clone that contains sequences upstream of 456; hence, this region does not form part of the major species of apoAI mRNA found in adult liver. Whether this is true for other tissues, such as fetal liver or intestine, still needs to be investigated. Thus, in the absence of any direct RNA hybridization data from Karathanasis et al., we can only speculate about the origin of the cloned insert of pAI-121. Perhaps, it is a low abundant RNA polymerase II transcript that originates from the initiation of a weak upstream promoter (43), or, alternatively, part of a RNA polymerase III transcript (44).

We have detected no significant homology between the amino acid sequences in any of the apolipoprotein signal peptide analysed in this paper (see Table 2). This is in contrast to previous studies (9,10) which

TABLE 2

Comparison of the amino acid sequences of rat and human apolipoprotein pre-peptide sequences. X denotes an unknown residue.

Rat apoAI (10)	MKAAVLAVALVFLTGCQA
Rat apoAIV (45)	MFLKAVVLXVALVAXXXXQA
Human apoAIV (46)	MXLXAVVLXLALVAVALXXA
Human apoAI	MKAAVLTLAVLFLTGSQA
Human apoAII	MKLLAATVLLLTICSLEG
Human apoCII	MGTRLLPALFLVLLVLGFEVQG
Human apoCIII	MQPRVLLVVALLALLASARA

observed strong homology in this region between human apoAI with both rat apoAI and IV. Similarly, we do not observe in either apoAII, CII or CIII such a marked degree of preference in the codon usage for the various amino acids as was seen with apoAI (10).

There is a remarkable resemblance between the NH_2 terminus of apoCII and those amino acids of apoAI believed to be involved in the unique proteolytic processing of pro-apoAI to mature apoAI (9,10). This similarity is highly suggestive and it could be inferred that amino acids 1-6 of apoCII may constitute a propeptide structure that undergoes similar processing to apoAI. However, the substrate specificity of the enzyme believed to be involved in this processing is unknown and it is quite possible that the cleavage may require more than the primary structure homology. Furthermore, it is known that apoCII found in fasting plasma includes amino acids 1 to 6.

TABLE 3

Comparison of the amino acid sequences following the signal peptide of human and rat apolipoproteins. The human sequences were predicted from the nucleotide sequences of the respective cDNAs, while the rat sequence was previously determined by peptide sequencing of the *in vitro* product (9). X denotes an unknown residue, while * indicates the amino acid reported to be at the NH_2 terminus of the predominant form of the protein found in normal plasma.

	SIGNAL ←														
Rat apoAI	G	C	Q	A	X	E	F	X	Q	Q	D*	E	P	Q	S	Q
Human apoAI	G	S	Q	A	R	H	F	W	Q	Q	D*	E	P	P	Q	S
Human apoCII	E	V	Q	G	T*	Q	Q	P	Q	Q	D	E	M	P	S	P
Human apoAII	S	L	E	G	A	L	V	R	R		Q*	A	K	E	P	C
Human apoCIII	S	A	R	A							S*	E	A	E	D	A

We have presented data on the levels of 4 apolipoprotein mRNAs in a liver sample (see Fig.7). The data suggest that the relative contribution of liver biosynthesis to plasma levels of apoCII and CIII could be greater than it is for apoAI and AII, and that the difference in estimated plasma half life of each of these apolipoproteins [i.e. 3.7±0.35, 4.4±0.42, 1.25, 1.25 days for apoAI, AII, CII and CIII (47,48)] may be reflected in the ratio between transcript and plasma concentration. For example, from Figure 7C it is clear that the relative levels of apoCII mRNA are half that of apoCIII which correlates with their relative protein levels in plasma. Nevertheless, we cannot generalize from the results derived from just one liver sample taken at one time point, or neglect the possibility that what we are really seeing are differences in mRNA stability. For a full interpretation of our results, it is necessary to undertake similar studies in many samples from both liver and intestine obtained under more controlled conditions. On these we could fully investigate the effect of environmental conditions, e.g. fat and carbohydrate feeding, at the RNA level, in both normal and hyperlipidaemic patients. The study presented in this paper shows that such an approach in the future is clearly feasible.

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