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The isolation of cDNA clones for human apolipoprotein E and the detection of apoE RNA in hepatic and extra-hepatic tissues

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We have isolated cDNA clones coding for apolipoprotein E (apoE) from a cDNA library prepared from adult human liver mRNA. Mixtures of 128 different oligonucleotides, 17 residues long were synthesised to be complementary to regions of the mRNA corresponding to amino acids 1-6 and 151-156. Five independent apoE clones were selected by direct screening of 5000 recombinants with the two oligonucleotide mixtures. Two overlapping clones contain the 3'-untranslated sequence, the entire coding sequence and an additional 30 bases 5' to the amino terminus of the mature protein. The DNA sequence has been determined spanning the known sites of amino acid substitutions which account for the observed protein polymorphism of apoE. Using the clones as probes in Northern blot analysis of total human liver and kidney RNAs and leucocyte poly(A)$^+$ RNA we have detected a single species of mRNA in liver and kidney of 1.2 kb and two larger species in leucocyte RNA. The level of expression of the mRNA in kidney is ~10% of that in liver while the level of apoE RNA sequences in the leucocytes is <1% of that in the liver.

Key words: apolipoprotein E/expression/sequence

Introduction

Apolipoprotein E (apoE) is a protein of 299 amino acids important in the lipid transport system of man (Rall *et al.*, 1982a). It is found in very low density lipoprotein, chylomicrons, intermediate density lipoprotein, remnant particles and some sub-classes of high density lipoprotein (reviewed in Brown *et al.*, 1982). ApoE mediates the recognition of some of the lipid particles by its interaction with a liver lipoprotein receptor distinct from the low density lipoprotein (LDL) receptor (Mahley *et al.*, 1981).

There is extensive apoE polymorphism detectable on isoelectric focussing (IEF), caused by a combination of post-translational modification and amino acid substitution (Utermann *et al.*, 1977; Zannis and Breslow, 1981; Breslow *et al.*, 1982b; Rall *et al.*, 1982b). Four alleles have been described. E3 is the most common and is related to the others as follows: E4 has arginine instead of cysteine at position 112, E2 has cysteine instead of arginine at position 158 and E2* has cysteine instead of arginine at position 145. Some of these variants affect the interaction of lipoproteins with the 'apoE receptor' (Schneider *et al.*, 1981; Rall *et al.*, 1982b). These may contribute to the delayed clearance of lipoprotein, the development of hyperlipidaemia, and premature vascular disease. For instance, homozygosity for E2 is a predisposing factor to the development of type III hyperlipidaemia (Utermann *et al.*, 1977; Zannis and Breslow, 1981; Breslow *et al.*, 1982a), and homozygosity for E4 may possibly predispose to the development of type V hyperlipidaemia (Ghiselli *et al.*, 1982). It is likely that further variants exist, some of which may not be detectable on IEF due to neutral amino acid substitutions. Such variants may be associated with hyperlipidaemia or premature vascular disease. We have isolated cDNA clones coding for apoE for use as potential polymorphic DNA markers for the apoE locus, and as an aid to the direct sequence analysis of variable sites in the apoE of hyperlipidaemic individuals.

There have been several recent reports that monocyte-derived macrophages (Basu *et al.*, 1981, 1982a), adrenal and kidney (Blue *et al.*, 1983) synthesise apoE. Avian kidney has been reported to synthesise other apoproteins as well as apoE (Blue *et al.*, 1982), but human kidney does not synthesise apoA1 or apoB (Blue *et al.*, 1983), and baboon kidney contains no RNA sequences homologous to apoA1 cDNA (Cheung and Chan, 1983). These data have given rise to the hypothesis of 'reverse cholesterol transport', in which it is postulated that peripheral tissues synthesise apoproteins which participate in the formation of high density lipoproteins (HDL) allowing transport of cholesterol from these tissues to the liver (Basu *et al.*, 1982b). We have analysed the expression of apoE in extra-hepatic tissues to study this hypothesis.

Results

When this work was started, only the N-terminal amino acid sequence and the sequence of two internal peptides of apoE were known (Weisgraber *et al.*, 1981). From the known sequences, two regions were selected for synthesis of the corresponding nucleotide sequence. Seventeen-nucleotide long DNA sequences (17-mer) complementary to the mRNA coding for amino acids 1-6 (probe E1), and 151-156 (probe E2), were synthesised. In any position where ambiguity existed, all possible nucleotide sequences were synthesised. One hundred and twenty eight different sequences were thus synthesised for each 17-mer, to ensure that the correct sequence would be present (Figure 1).

Initially, 5 x 1000 recombinants were screened directly with a mixture of both probes (E1 and E2). Figure 2 shows an autoradiograph of a filter containing 1000 recombinants hybridised to this mixture with several positives. A total of 25 recombinants was analysed further by growing in ordered array, which were screened with the two probes separately. Of these, five recombinants positive with the central probe (E2) were picked, of which one, designated A1, was also positive with the amino-terminal probe E1. These recombinants were colony purified and plasmid DNA prepared. No false positives were observed.

The insert sizes of the two largest clones are 780 bp (A1)

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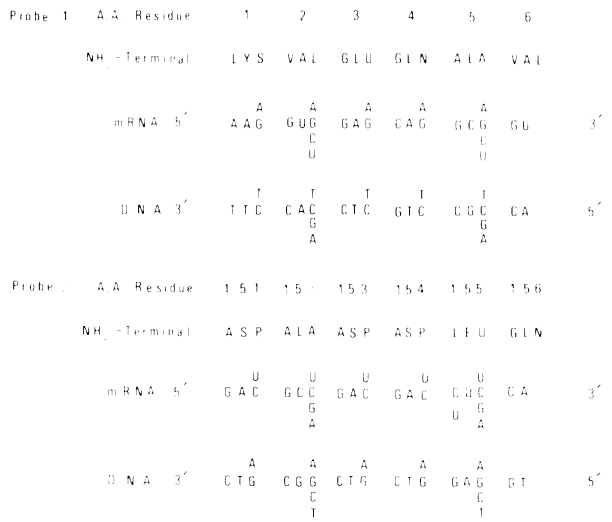

Fig. 1. Oligonucleotide design. The oligonucleotides were designed to be complementary to the mRNA coding for amino acids 1–6 (E1) and 151–156 (E2).

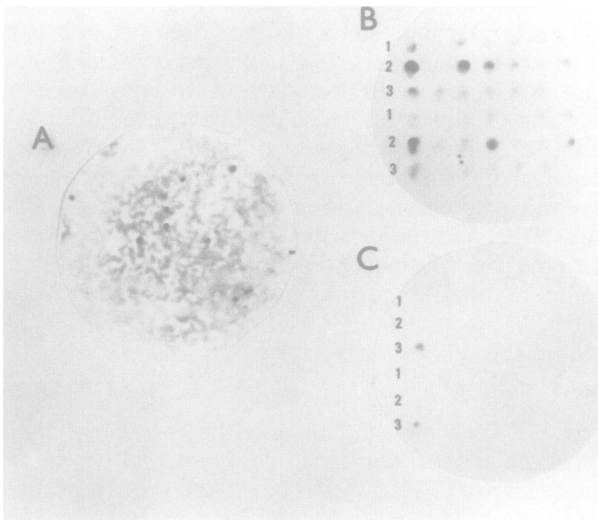

Fig. 2. Library screening. **(a)** Primary screening of 1000 colonies. **(b)** Secondary screening in ordered array with E2 after colony purification. **(c)** A duplicate of **(b)** screened with E1.

and 930 bp (B4). Preliminary restriction mapping of the clones indicated a considerable overlap and suggested the alignment shown in Figure 3. To confirm the identity of the clones the sequencing strategy outlined in Figure 3 was employed. Sequence information was usually derived from both strands and often from both clones by a combination of chemical cleavage and dideoxy chain terminator methods. The sequence of the cDNA clones from amino acids 45 to 221 is presented in Figure 4.

This region contains the sites of arginine and cysteine interchange which account for the known protein polymorphism. Within this region the sequences of A1 and B4 are identical, and both code for the apoE allele E3. The DNA sequence around the positions of known arginine and cysteine interchange is shown in Table I. In each case, a single base interchange of C and T could account for the amino acid substitution.

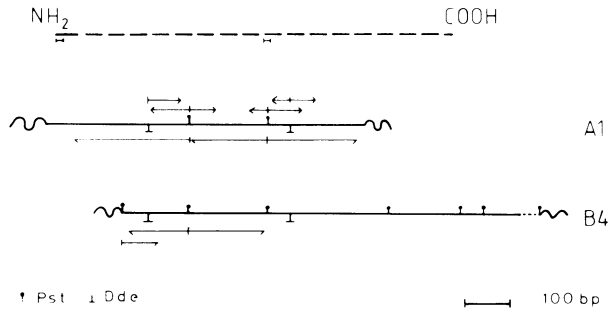

Fig. 3. Restriction enzyme map of the clones A1 and B4 showing the sequencing strategy employed. → represents Maxam and Gilbert and ⇀ M13 dideoxy sequencing, --- shows the poly(A) tail and ∿ plasmid sequence.

To investigate the expression of the *apoE* gene in different tissues total RNA or poly(A)$^+$ RNA was prepared from liver, kidney and leucocytes. Northern blots of adult human liver and kidney total RNA give a reproducible single band at 1.2 kb (Figure 5). Densitometric scanning of the autoradiographs indicates that the apoE mRNA sequences in kidney are present at a level ~10% of that in liver. However, in leucocyte poly(A)$^+$ RNA, two hybridising bands larger than the liver mRNA are detected, of 1.4 and 2.7 kb. The levels of apoE RNA sequences in leucocytes cannot be calculated accurately, but we estimate that it is ~1% or less of that in the liver.

Discussion

Over the last few years, many genes have been identified and isolated, using oligonucleotides whose sequence has been predicted from the amino acid sequence of a protein. A common way of using these oligonucleotides is as a primer for cDNA synthesis by reverse transcriptase. This cDNA can then be cloned directly or used as a probe (Agarwal *et al.*, 1981; Shoulders and Baralle, 1982). Conditions have recently been described where oligonucleotides can be used directly as probes for gene sequences in a cDNA library (Wallace *et al.*, 1981; Suggs *et al.*, 1981). Mixtures of up to 64 different oligomers have been successfully used (Woods *et al.*, 1982). We show here that mixtures of up to 128 different oligonucleotides can be used to detect specific sequences. The use of two oligonucleotides constructed to hybridise to different regions of the same cDNA clone is also helpful for aligning and orientating the cDNA clones. The use of an amino-terminal probe in addition to the central probe was responsible for the successful isolation of clones coding for the major portion of the mRNA.

The clones identified by the probes are 780 bp and 930 bp long and together span the 3′-untranslated and coding regions to just beyond the amino terminus of the mature protein. The partial sequence of the clones so far determined (Figure 4) confirms their identity as apoE cDNA by comparison with the published amino acid sequence (Rall *et al.*, 1982a).

It would be extremely useful to be able to distinguish the alleles of apoE at the DNA level. Examination of the DNA sequence around the known polymorphic sites in the apoE protein (Table I) suggests a *Hha*I restriction enzyme cleavage site in the gene for E4 at amino acid 112 which should be ab-

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45
    gln val gln glu glu leu leu ser ser gln val thr gln glu
    CAG GTG CAG GAG GAG CTC CTC AGC TCC CAG GTC ACC CAG GAA

60
leu arg'ala leu met asp glu thr met lys glu leu lys ala tyr
CTG AGG GCG CTG ATG GAC GAG ACC ATG AAG GAG TTG AAG GCC TAC

75
lys ser glu leu glu glu gln leu thr pro val ala glu glu thr
AAA TCG GAA CTG GAG GAA CAA CTG ACC CCG GTG GCG GAG GAG ACG

90
arg'ala arg'leu ser lys glu leu gln ala ala gln ala arg'leu
CGG GCA CGG CTG TCC AAG GAG CTG CAG GCG GCG CAG GCC CGG CTG

105

gly ala asp met glu asp val cys gly arg*leu val gln tyr arg*
GGC GCG GAC ATG GAG GAC GTG TGC GGC CGC CTG GTG CAG TAC CGC

120

gly glu val gln ala met leu gly gln ser thr glu glu leu arg'
GGC GAG GTG CAG GCC ATG CTC GGC CAG AGC ACC GAG GAG CTG CGG

135

val arg*leu ala ser his leu arg*lys leu arg lys arg'leu leu
GTG CGC CTC GCC TCC CAC CTG CGC AAG CTG CGT AAG CGG CTC CTC

150

arg*asp ala asp asp leu gln lys arg leu ala val tyr gln ala
CGC GAT GCC GAT GAC CTG CAG AAG CGC CTG GCA GTG TAC CAG GCC

165

gly ala arg*glu gly ala glu arg*gly leu ser ala ile arg*glu
GGG GCC CGC GAG GGC GCC GAG CGC GGC CTC AGC GCC ATC CGC GAG

180

arg*leu gly pro leu val glu gln gly arg*val arg'ala ala thr
CGC CTG GGG CCC CTG GTG GAA CAG GGC CGC GTG CGG GCC GCC ACT

195

val gly ser leu ala gly gln pro leu gln glu arg'ala gln ala
GTG GGG TCC CTG GCC GGC CAG CCG CTA CAG GAG CGG GCC CAG GCC

210                                          221

trp gly glu arg'leu arg*ala arg'met glu glu met
TGG GGC GAG CGG CTG CGC GCG CGG ATG GAG GAG ATG
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Fig. 4. The DNA sequence of clones A1 and B4 in the region of overlap. Known sites of amino acid substitution are shown as __, arginine codons which could become cysteine codons by a single base change are shown as *, and those which cannot are shown as '.

sent from the gene for E3. Similarly there should be a *Hha*I site in the gene for E3 at amino acid 158 which would be absent from E2. This raises the possibility of a direct DNA-based diagnosis of the important apoE alleles analogous to the *Dde*I or *Mst*II site affected by the mutation responsible for sickle cell anaemia (Chang and Kan, 1982). However neither *Hha*I nor its isoschitzomer *Cfo*I cuts methylated CpG sites and when used with human leucocyte DNA only a small fraction of DNA is digested. We believe that it is this which has made it impossible for us to achieve a reproducible signal in Southern blotting experiments.

All the variants of apoE so far reported have been due to cysteine and arginine interchanges. It is interesting that of the 23 arginine codons present in the cDNA sequence shown in Figure 4, 13 could be changed to cysteine by the alteration of the first base from C to T. Some of these sites are within the region believed to interact with the 'apoE receptor' (Rall *et al.*, 1982b) and could therefore be of functional significance.

The sequence of an apoE cDNA clone isolated from the same cDNA library has been published recently (Breslow *et*

Table I. The sequence of the known sites of amino acid substitutions in apoE

	DNA sequence of the polymorphic sites in apoE						
	110						115
				Cys			
E3	GAG	GAC	GTG	TGC	GGC	CGC	CTG
E4			G	CGC			
				Arg			
				145			
				Arg			
E3	CGC	AAG	CTG	CGT	AAG	CGG	CTC
E2*				T			
				Cys			
	155						
				Arg			
E3	CTG	CAG	AAG	CGC	CTG	GCA	GTG
E2				T			
				Cys			

*Hha*I restriction enzyme sites are underlined.

al., 1982b). This clone also coded for the apoE allele E3 but comparison of the published sequence with that in Figure 4 shows a difference of ~5%. These differences are usually in the third position of a codon and none of them results in an amino acid alteration. We have recently become aware of the fact that these authors have re-sequenced their clone and the revised sequence eliminates these differences (J.Breslow, personal communication).

Several determinations have shown that the length of the apoE mRNA is ~1.2 kb: the length of cDNA represented in our clones A1 and B4 is 1.04 kb, this leaves a maximum of 160 bp for the signal sequence 5′-untranslated region and the poly(A) tail. The usual length of poly(A) tail is 50–100 bp from which we infer that ~60–110 bp of 5′-untranslated and signal sequence are missing from our clones.

The Northern blot shown in Figure 5 using liver and kidney RNAs demonstrates that kidney RNA contains a highly homologous sequence of the same size as the mRNA in liver. This adds further weight to the reports that tissues other than liver and intestine synthesise apoE. The level of expression in kidney is ~10% of that in liver. In the absence of reliable estimates of apoE mRNA levels in liver it is not possible to estimate the mRNA level in kidney by this analysis although other methods should allow this to be done. It will also be possible to sequence the kidney RNA by primer extension to see if it is identical to the sequence expressed in liver. It is of considerable interest that leucocyte RNA contains homologous sequences larger than those seen in liver or kidney. The high stringency washing conditions employed make it unlikely that the sequences detected in leucocytes are the product of a different gene. Furthermore, Southern blots of restriction enzyme digests of genomic DNA only detect a single copy gene. The immunological data of Basu *et al.* (1981, 1982a) suggest that leucocytes do not synthesise apoE until the monocytes have been loaded with cholesterol; the possibility that unstimulated monocytes contain precursors of apoE mRNA is currently under investigation.

Materials and methods

Screening of the adult human liver cDNA library

Two mixtures of 17-nucleotide long oligonucleotides (Figure 1) were synthesised using a solid-phase phosphotriester method (Markham *et al.*, 1980;

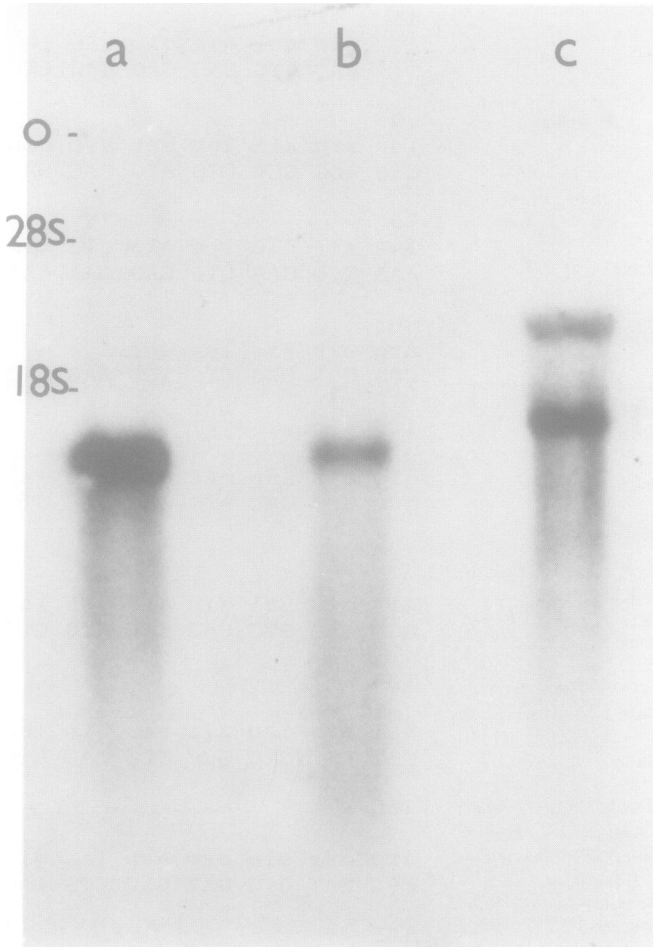

Fig. 5. Northern blot of human liver and kidney RNAs. 10 μg total liver RNA **(a)**, and 25 μg total kidney RNA **(b)** and **(c)** 20 μg of leucocyte poly(A)$^+$ RNA were loaded. Densitometric scanning of the autoradiograph showed peaks and the ratio between the area under the kidney peak to that under the liver peak was 0.36. It follows that the level found in kidney is 0.36/2.5 x 100 = 14%.

Gait *et al.*, 1980; Edge *et al.*, 1981).

The cDNA library was constructed by G-C tailing into the *Pst*I site of pKT218 (Talmadge *et al.*, 1980). The library was plated at a density of 1000 colonies per 82 mm nitrocellulose disk (Schleicher and Schuell), and was screened with each mixture of oligonucleotides separately and together, labelled to a specific activity of 10^7 c.p.m./μg with [γ-^{32}P]ATP >5000 Ci/mmol (Amersham) using T4 polynucleotide kinase (PL Biochemicals) (Woods *et al.*, 1982).

Analysis of DNA

Plasmid DNA was isolated from bacteria by the alkaline lysis method (Ish-Horowicz and Burke, 1981) digested with restriction enzymes as suggested by the manufacturer (Bethesda Research Laboratories) and analysed on agarose or acrylamide gels as appropriate. Restriction fragments were 5′ end-labelled with T4 polynucleotide kinase, using the forward reaction with [γ-^{32}P]ATP (Amersham), or 3′ end-labelled with cordycepin 3000 Ci/mmol (Amersham) using terminal transferase (PL). Appropriate DNA fragments were isolated and sequenced using the Maxam-Gilbert technique (Maxam and Gilbert, 1980). Some restriction fragments of the inserts were subcloned into M13 phage strains mp8 and mp9 (Messing and Vieira, 1982), and the recombinant single-strand phage were sequenced using the M13 universal primer (New England Biolabs) (Sanger *et al.*, 1977). Sequencing through G-C tails was accomplished by the use of a higher concentration of DNA polymerase I Klenow fragment (Boehringer), 5 units per four reactions instead of the usual 0.8 units.

RNA isolation and Northern blots

Fresh human adult liver or kidney was obtained from a cadaver donor, cut into 1 cm cubes, frozen in liquid nitrogen and stored at −70°C. Frozen tissue

was homogenised in 8 M urea, 4 M LiCl at room temperature and RNA prepared (Auffray and Rougeon, 1980). Leucocytes were prepared from 100 ml of whole blood by centrifugation through a Lymphoprep (Nyegaard) step gradient (Boyum, 1968). Usually 2 x 10^8 cells were obtained, 30% of which were monocytes the remainder being lymphocytes; these were washed in 0.9% saline, the cell pellet collected and resuspended in 4 M guanidine thiocyanate and RNA prepared (Chirgwin *et al.*, 1979). The RNA was enriched for poly(A)$^+$ RNA by one passage over an oligo(dT)-cellulose column (Aviv and Leder, 1972).

The yield of poly(A)$^+$-enriched RNA was 5% of the starting material. The RNA was denatured in 50% formamide and 7% formaldehyde and was fractionated by electrophoresis on a 1% agarose gel in 7% formaldehyde and transferred to a nitrocellulose filter (Thomas, 1980). The filter was hybridised with 5 x 10^7 c.p.m./μg of the apoE clone B4, 'nick-translated' with [α-^{32}P]-dCTP 400 Ci/mmol using a kit from Amersham. Hybridisation was performed in 3 x SSC, 0.1% SDS, 1 x Denhardt's solution and 50 μg/ml of both poly(A) and herring sperm DNA at 65°C for 24 h. Post-hybridisation washes were performed at 0.1 x SSC at 65°C. Size markers were provided by ethidium bromide staining of the gel, allowing visualisation of 28S, 23S, 18S and 16S rRNAs. Densitometric scanning of autoradiographs was performed with a Helena Quick Scan R and D.

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