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Isolation and characterization of cDNA clones for human apolipoprotein A-I

(cDNA library/oligonucleotide probe/M13 sequence analysis/lipoprotein metabolism)

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ABSTRACT **We have isolated cDNA clones encoding human apolipoprotein (apo) A-I. Twenty putative apo A-I cDNA clones were selected by screening 10,000 clones of an adult human liver cDNA library with an oligonucleotide probe. The probe was a mixture of synthetic 14-base-long DNA oligomers constructed to correspond to the codons for apo A-I amino acids 105–109. Four of these clones were examined further and showed 600- to 800-base-pair (bp) inserts. Preliminary restriction mapping and partial DNA sequence analysis indicated that the shorter inserts were a subset of the longer DNA inserts. DNA sequence analysis of the clone with an insert of ≈600 bp, designated pAI-113, revealed that it contained a DNA sequence corresponding to apo A-I amino acids 94–243. The DNA base sequence of this clone also contained a standard termination codon, polyadenylylation signal, and poly(A) tail. Partial DNA sequence of a second clone that contained an 800-bp insert, designated pAI-107, showed that it corresponded to apo A-I amino acids 18–243 and also included the 3′ untranslated region. Isolation of these cDNA clones will facilitate molecular analyses of apolipoproteins in normal and disease states.**

Apolipoprotein (apo) A-I is the major apoprotein of high density lipoproteins (HDL) and is a relatively abundant plasma protein with a concentration of 1.0–1.5 mg/ml (1–3). It is a single polypeptide chain composed of 243 amino acid residues of known primary amino acid sequence (4–6). apo A-I serves as a cofactor for the plasma enzyme lecithin–cholesterol acyltransferase, which is responsible for the formation of most cholesteryl esters in plasma (7–9). In tissue culture studies, apo A-I promotes cholesterol efflux from cells (10). In mammalian systems, apo A-I synthesis is thought to occur exclusively in liver and small intestine (11–15). Recent studies indicate that the primary translation product of rat apo A-I is 24 amino acids longer than its plasma counterpart (16). Similar observations also have been made for human apo A-I (17). In previous work, we have shown that apo A-I secreted by human intestine and liver consist of isoproteins 2 and 3 that are more basic than the major isoproteins 4 and 5 seen in plasma (18–20). Based on these observations, we have proposed that a conversion of the nascent apo A-I isoproteins 2(3) to 4(5) must occur in plasma.

A possible physiological significance of this post-translational apo A-I isoprotein 2(3) to 4(5) conversion has been suggested by our recent studies of patients with Tangier disease (21). Patients with this condition were shown to have very low plasma HDL and apo A-I levels despite normal synthesis and secretion of this apoprotein by the diseased intestine (21–23). Our studies have shown that the plasma form of Tangier disease apo A-I consists of ≈50% isoprotein 2 in contrast to normal plasma in which apo A-I isoprotein 2 is <1% of the total plasma apo A-I (21). Therefore, it appears that this disorder may involve a defect in a post-translational apo A-I isoprotein 2(3) to 4(5) conversion or a structural apo A-I mutation that either precludes normal apo A-I isoprotein conversion or results in a very unstable product.

In trying to better understand the processes that result in functional apo A-I, it not only will be important to study the co- and post-translational events described above but also to investigate the genomic organization and sequence of the apo A-I gene and its transcripts. To understand these processes and to explore the possibility of an apo A-I structural mutation in Tangier disease, it is necessary to isolate and study both normal and variant apo A-I genes. As a first step towards this goal, in this paper we report the isolation and characterization of bacterial clones containing portions of human apo A-I cDNA.

METHODS

Preparation of the Oligonucleotide Probe. The oligonucleotide mixture that was synthesized corresponds to the region of apo A-I containing amino acids 105–109 (Gln-Lys-Lys-Trp-Gln) (6). This region was selected because these amino acids are specified by relatively unambiguous codons. Fig. 1 shows the sequence of mRNA from 5′ to 3′ specifying apo A-I amino acids 105–109 as well as the cDNA sequence from 3′ to 5′. This cDNA sequence was synthesized by a solid-phase phosphate triester method by using the reaction conditions and procedures of Caruthers and colleagues (24, 25, 26). Briefly, a sample of 25 mg of functionalized silica gel, charged with 1.3 μmol of 5-*O*-dimethoxytritylthymidine attached by its 3′-OH group through ester linkage to the solid phase, was unblocked at the 5′-OH group with a Lewis acid (saturated $ZnBr_2$/aqueous CH_3NO_2). The sample was condensed with a mixture of 10 mg each of protected nucleoside phosphoramidites of cytosine and thymidine (5′-dimethoxytrityl-*N*,*N*,*N*,*N*-acrysoyldeoxycytidine-3′-(methoxy)-diethylaminophosphine and 5′-dimethoxytrityl-thymidine-3′-(methoxy)-diethylaminophosphine), which were activated with tetrazole. The nucleoside phosphoramidites used throughout this procedure were obtained from ChemGenes Corporation (Waltham, MA). Any unreacted 5′-OH of the silica gel-bound nucleoside was blocked subsequently by reaction with a large excess of a very reactive phosphite. The phosphites were next oxidized with iodine to phosphates as described (24). The cycle was repeated with the appropriate nucleoside phosphoramidite(s) until the last condensation was performed, subsequent to which the 5′-OH was not unblocked. At each point where an ambiguity in the DNA sequence existed, a mixture

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Abbreviations: apo, apolipoprotein(s); bp, base pair(s); HDL, high density lipoproteins.

Residue Number		105	106	107	108	109	
Amino Acid		Gln	Lys	Lys	Trp	Gln	
mRNA	5'	A AG	A AAG	A AAG	UGG	A CAG	3'
cDNA	3'	T TC	T TTC	T TTC	ACC	T GTC	5'

FIG. 1. apo A-I oligonucleotide probe. Sequences of mRNA and cDNA corresponding to apo A-I amino acid residues 105–109 are shown.

of derivatized nucleosides was employed as indicated in Fig. 1. By this procedure, the resultant probe was a mixture of 16 oligomers. All reactions were carried out in a filtration device fashioned from Teflon and stainless steel.

After the synthesis was completed, the methyl groups of the phosphodiesters were removed by treatment with thiophenol (24) and the ester bond joining the oligonucleotide to the support was cleaved by treatment with concentrated ammonia as were the base-protecting groups (24). The reaction products then were fractionated by preparative HPLC by using a Waters C-8 column. The sample was loaded in 0.1 M triethylammonium bicarbonate at pH 7.0 and eluted by a linear gradient up to 25% acetonitrile over 40 min. This procedure separates failure sequences from the desired trityl-oligomers, which emerge at the top of the gradient. Detritylation in 80% acetic acid for 20 min at room temperature then was followed by a second preparative HPLC under the same conditions. A dominant peak emerging approximately halfway through the gradient proved to be the desired tetradecamer mixture. This peak was subjected to polynucleotide kinase labeling at the 5′ terminus with [γ-^{32}P]ATP and run on a 20% polyacrylamide gel. The gel then was subjected to autoradiography, and over 95% of the oligomer ran as a 14-base long nucleotide. The overall yield from starting material was 9%.

Screening of the Adult Human Liver cDNA Library. The cDNA library used in these studies was generously provided by D. Woods, E. Prochownik, and A. Michelson of Children's Hospital Medical Center and has been described elsewhere (27). The clones were plated on 82-mm Petri dishes at a density of 1,000 bacterial colonies per dish. After growth and chloramphenicol amplification, the colonies were transferred to nitrocellulose filters and hybridized to the oligonucleotide probe that had been labeled at its 5′ terminus (28). The hybridization mixture contained 0.75 M NaCl, 0.15 M Tris·HCl (pH 8), 10 mM EDTA, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidone, 0.1% Ficoll, 0.1% sodium pyrophosphate, 0.1% $NaDodSO_4$, yeast tRNA (carrier) at 100 μg/ml, and 0.65 μg of 5′-labeled oligonucleotide probe with a specific activity of 5 $\times$ 10^8 cpm/μg. The filters were hybridized at 23°C for 16 hr. The filters then were washed in 0.9 M NaCl, 0.09 M sodium citrate, 0.05% sodium pyrophosphate, and 1% $NaDodSO_4$ sequentially at 23°C, 30°C, 40°C, and 50°C.

Plasmid DNA Preparations and Restriction Analysis. Bacterial clones were grown in Luria broth supplemented with tetracycline at 20 μg/ml. Plasmid DNA was isolated by using the alkaline lysis method (29) and was further purified on CsCl gradients. Restriction endonucleases were purchased from New England BioLabs and the conditions for enzymatic digestions were those recommended by the vendor.

DNA Sequence Analysis Methods. DNA sequence analysis was performed both by the chemical method of Maxam and Gilbert (30) and the enzymatic method described by Sanger (31). In the latter case, appropriate restriction fragments were excised from low-melting point agarose and were cloned either in M13mp8 or M13mp9 vectors (32). The recombinant single-stranded phage DNA was used as a template for the sequence analysis reactions, with either the M13 sequence analysis primer purchased from New England BioLabs or the mixture of 14-base-long oligonucleotides used for screening the library.

RESULTS

Identification of apo A-I cDNA Clones. Ten-thousand cDNA clones were transferred to nitrocellulose filters and were hybridized to the oligonucleotide probe as described. Initial washing of the filters at 23°C showed significant nonspecific hybridization, whereas washing at 30°C or 40°C substantially decreased this background, and positive clones could be identified. About 30 positive clones were observed after the 30°C wash, 20 of which maintained the hybridization signal after the 40°C wash. A 50°C wash totally abolished the signal from all clones. Four of the 20 clones selected after the 40°C wash were used for further analysis. The plasmid preparations from these four clones were subjected to digestion by a series of restriction enzymes. After *Pst* I digestion, all four plasmids were shown to have cDNA inserts of between 600 and 800 bp. Further digestion of

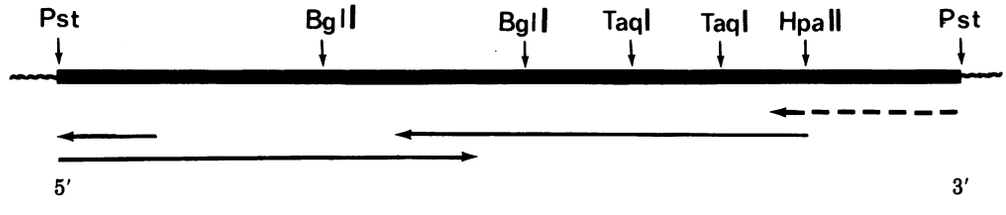

FIG. 2. Restriction sites of clone pAI-113 of apo A-I and strategy used for DNA sequence analysis. Only the positions of the relevant restriction sites are indicated. The horizontal arrows indicate the direction and extent of sequence determinations. Broken and intact line arrows represent sequences obtained by the Maxam and Gilbert (30) and Sanger (31) methods, respectively. The wavy line on either side of the clone represents the plasmid sequences. Calibration bar = 100 bp.

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AAG GCC AAG GTG CAG CCC TAC CTG GAC GAC TTC CAG AAG AAG TGG CAG GAG GAG ATG GAG
                          100                                     110
Lys-Ala-Lys-Val-Gln-Pro-Tyr-Leu-Asp-Asp-Phe-Gln-Lys-Lys-Trp-Gln-Glu-Glu-Met-Glu

CTC TAC CGC CAG AAG GTG GAG CCG CTG CGC GCA GAG CTC CAA GAG GGC GCG CGC CAG AAG
                          120                                     130
Leu-Tyr-Arg-Gln-Lys-Val-Glu-Pro-Leu-Arg-Ala-Glu-Leu-Gln-Glu-Gly-Ala-Arg-Gln-Lys

CTG CAC GAG CTG CAA GAG AAG CTG AGC CCA CTG GGC CAG GAG ATG CGC GAC CGC GCG CGC
                          140                                     150
Leu-His-Glu-Leu-Gln-Glu-Lys-Leu-Ser-Pro-Leu-Gly-Gln-Glu-Met-Arg-Asp-Arg-Ala-Arg

GCC CAT GTG GAC GCG CTG CGC ACG CAT CTG GCC CCC TAC AGC GAC GAG CTG CGC CAG CGC
                          160                                     170
Ala-His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala-Pro-Tyr-Ser-Asp-Glu-Leu-Arg-Gln-Arg

TTG GCC GCG CGC CTT GAG GCT CTC AAG GAG AAC GGC GGC GCC AGA CTG GCC GAG TAT CAC
                          180                                     190
Leu-Ala-Ala-Arg-Leu-Glu-Ala-Leu-Lys-Glu-Asn-Gly-Gly-Ala-Arg-Leu-Ala-Glu-Tyr-His

GCC AAG GCC ACC GAG CAT CTG AGC ACG CTC AGC GAG AAG GCC AAG CCC GCG CTC GAG GAC
                          200                                     210
Ala-Lys-Ala-Thr-Glu-His-Leu-Ser-Thr-Leu-Ser-Glu-Lys-Ala-Lys-Pro-Ala-Leu-Glu-Asp

CTC CGC CAA GGC CTG CTG CCC GTG CTG GAG AGC TTC AAG GTC AGC TTC CTG AGC GCT CTC
                          220                                     230
Leu-Arg-Gln-Gly-Leu-Leu-Pro-Val-Leu-Glu-Ser-Phe-Lys-Val-Ser-Phe-Leu-Ser-Ala-Leu

GAG GAG TAC ACT AAG AAG CTC AAC ACC CAG TGA GGA CGC CCG CCG CCG CCC CCC TTC CCG
                          240
Glu-Glu-Tyr-Thr-Lys-Lys-Leu-Asn-Thr-Gln stop

GTG CTC AGA ATA AAC GTT TCC AAA GTG GAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
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FIG. 3. DNA base sequence of clone pAI-113 of apo A-I.

these inserts with *Bgl* I revealed that each of these plasmid inserts contained two restriction sites for this enzyme. In each case, this resulted in DNA fragments of approximately 275 and 125 bp and a third fragment whose length varied from clone to clone between 200 and 400 bp.

DNA sequence analysis was undertaken to verify that these clones corresponded to apo A-I.

Sequence Analysis of Clones pAI-113 and pAI-107. Clone pAI-113 has been mapped with restriction enzymes (Fig. 2), and the complete sequence determination was shown to correspond to apo A-I amino acids 94–243 (Fig. 3). The sequence analysis also showed that clone pAI-113 contained the 3′ untranslated region as well as a portion of the poly(A) tail of the apo A-I mRNA. A second clone, pAI-107, which had a longer insert of ≈800 bp, was selected for partial DNA sequence analysis to confirm that this clone also corresponded to apo A-I. Sequence analysis of the region of DNA complementary to the 5′ end of the message revealed that this clone began with nucleotides corresponding to apo A-I amino acid 18. Sequence analysis of the other end of this clone showed that it terminated in the 3′ untranslated region as did the clone pAI-113 (data not shown).

DISCUSSION

In previous studies, we have shown that apo A-I is a relatively abundant secretory product of human fetal and adult liver in organ culture and of human hepatoma cells in tissue culture (18–20). In addition, we recently have shown that ≈1% of the protein that is synthesized in a cell-free translation system of human liver cytoplasmic poly(A)-containing RNA is apo A-I (17). This implies that apo A-I mRNA is abundant in a total liver mRNA preparation and therefore human liver cDNA libraries

should be enriched in clones that contain the apo A-I cDNA sequence. Based on the amino acid sequence for apo A-I (4–6), we designed and synthesized a mixture of oligonucleotides and used these as a probe to screen an adult human liver cDNA library in an attempt to isolate these apo A-I cDNA clones. Because the literature contains two reported sequences for apo A-I that differ in several respects (4–6), in the construction of the A-I probe, a region of the amino acid sequence was sought that was concordant between these two reported sequences. The region that spans apo A-I amino acids 105–109 was selected for the construction of the oligonucleotide probe.

It has been shown earlier that oligonucleotides 13–15 bases in length are sufficient for the detection of a unique gene in a genomic yeast DNA library (33). However, isolation of unique genes from mammalian DNA genomic libraries with such short DNA oligomers may be more difficult because of the greater complexity of the DNA in these libraries. On the other hand, screening of mammalian cDNA libraries constructed from a tissue that expresses the gene of interest circumvents the problem of the high DNA complexity present in genomic libraries. Therefore, we screened an adult human liver cDNA library with the oligonucleotide probe described above. To establish the hybridization condition for the screening, we used empirical formulas (34) to estimate that the temperature maximum of a perfectly matched 14-base-long DNA oligomer with a 50% content of G·C should be 51°C. This indicated that hybridization at 1 M salt at 31–36°C should satisfy the opposing requirements for sensitivity (low stringency) and specificity (high stringency). As predicted, screening of the cDNA bank with the mixture of labeled 14-base-long DNA oligomers at room temperature (low stringency) showed a high background of nonspecific hybridization. However, hybridization at 30–40°C (high stringency) increased the specificity without significantly affecting the sensitivity of the hybridization signal.

In this manner, 10,000 clones of the cDNA library were screened by using the probe under high-stringency hybridization conditions and 20 clones were identified as strongly hybridizing colonies. Further examination of 4 of these 20 positive clones showed that they contained *Pst* I inserts of between 600 and 800 bp in length. Restriction mapping analysis indicated that these inserts shared common restriction sites at similar positions. This implied that they may represent the same DNA sequence starting from the same 3′ end of the corresponding mRNA but terminating at different 5′ upstream positions. This is usually due to inefficient cDNA synthesis by reverse transcriptase during library construction.

To verify this point and to show that the DNA sequence contained in these clones was indeed the apo A-I cDNA sequence, the 600- and 800-bp inserts from the clones pAI-113 and pAI-107, respectively, were used for DNA sequence analysis. The DNA base sequence of clone pAI-113 coincided almost exactly with what one would predict from the sequence reported by Brewer *et al.* (6). The only discrepancy was at amino acid 147 where the DNA base sequence predicts a Glu rather than the Gln specified by Brewer *et al.* (6). Clones pAI-113 and pAI-107 both contain inserts that begin with the poly(A) region of the corresponding apo A-I mRNA and proceed through the untranslated region and the termination codon UGA and extend towards the 5′ end of the mRNA. The pAI-113 terminates one base before the triplet AAG which codes for the 94th amino acid of apo A-I. The pAI-107 terminates at the 18th amino acid of apo A-I. Examination of the other clones in search of a full-length cDNA transcript is necessary. One feature shared by most eukaryotic polyadenylylated mRNAs is the occurrence of the sequence A-A-U-A-A-A 12–22 nucleotides 5′ to the poly(A) tail (35). In the apo A-I mRNA, this signal hexanucleotide is located 35 bases after the last base of the termination codon and 14 bases before the polyadenylylation site.

apo A-I is an important structural constituent of HDL and the isolation of these clones should open several avenues of investigation of the structure and function of this locus in normal and disease states. An apo A-I cDNA clone can be used to probe genomic DNA and may allow the detection of restriction site polymorphisms in or closely linked to the apo A-I gene (36). Such polymorphism may have great clinical significance for the detection of abnormalities in lipoprotein metabolism caused by structural or regulatory apo A-I gene mutations (21, 37, 38). apo A-I polymorphic variants have been shown to exist at the level of the apo A-I protein (39, 40), and it may be possible to detect an even larger number of these variants at the DNA level. In addition, these cDNA probes should allow the isolation of the apo A-I gene from genomic libraries of normal people and patients with apo A-I defects in order to study the fine structure and inherited alterations of this gene. These probes will also make possible studies of the regulation of apo A-I gene expression at the transcriptional and post-translational level. Moreover, *in vitro* mutagenesis of the apo A-I gene and expression of those mutagenized genes in bacteria may provide a unique way to map the specific amino acids involved in lipid binding and in lecithin–cholesterol acyltransferase activation. Finally, production of native unmodified apo A-I from bacteria may be useful in understanding the nature of the post-translational modifications that this protein undergoes during its maturation and allow identification of factors involved in these modification processes.

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