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Production of Human Recombinant Proapolipoprotein A-I in *Escherichia coli*: Purification and Biochemical Characterization

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ABSTRACT

A human liver cDNA library was used to isolate a clone coding for apolipoprotein A-I (Apo A-I). The clone carries the sequence for the prepeptide (18 amino acids), the propeptide (6 amino acids), and the mature protein (243 amino acids). A coding cassette for the proapo A-I molecule was reconstructed by fusing synthetic sequences, chosen to optimize expression and specifying the amino-terminal methionine and amino acids −6 to +14, to a large fragment of the cDNA coding for amino acids 15-243. The module was expressed in pOTS-Nco, an *Escherichia coli* expression vector carrying the regulatable λ P_L promoter, leading to the production of proapolipoprotein A-I at up to 10% of total soluble proteins. The recombinant polypeptide was purified and characterized in terms of apparent molecular mass, isoelectric point, and by both chemical and enzymatic peptide mapping. In addition, it was assayed *in vitro* for the stimulation of the enzyme lecithin: cholesterol acyltransferase. The data show for the first time that proapo A-I can be produced efficiently in *E. coli* as a stable and undegraded protein having physical and functional properties indistinguishable from those of the natural product.

INTRODUCTION

HUMAN APOLIPOPROTEIN A-I (apo A-I), the major protein constituent of high-density lipoproteins (HDL), is produced in liver and intestine as a precursor protein (preproapo A-I) which undergoes intracellular cleavage and is released into the plasma and lymph as a proprotein (Wu and Windmueller, 1979). Proapo A-I carries six additional amino acids (Arg-His-Phe-Trp-Gln-Gln) at the amino-terminal of the mature protein. It is cleaved in the vascular space by a specific protease to yield the mature molecule (Gordon *et al.*, 1983; Zannis *et al.*, 1983). Apo A-I is a single nonglycosylated polypeptide of known sequence, composed of 243 amino acid residues (Brewer *et al.*, 1978). In the presence of synthetic liposomes, it serves as cofactor for lecithin:cholesterol acyltransferase, which is responsible for the formation of most of the cholesteryl esters in plasma (Fielding *et al.*, 1972). Apo A-I and HDL may participate in "reverse cholesterol transport," from peripheral tissues to the liver for excretion from the body (Glomset, 1968).

Since accumulation of cholesterol in the arteries is the hallmark and presumably an important cause for atherosclerosis, stimulation of reverse cholesterol transport by supplementing apo A-I might delay the atherosclerotic process. Techniques to produce this protein in large quantities have been developed using blood plasma as starting material (Ross and Carson, 1985; Brewer *et al.*, 1986). Attempts to express mature apo A-I in recombinant bacteria proved difficult due to the instability of the protein (Lorenzetti *et al.*, 1986; Mallory *et al.*, 1987); moreover, even as a fusion protein, apo A-I was still very sensitive to

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degradation and could not be cleaved efficiently from the fusion (Lorenzetti *et al.*, 1986; Monaco *et al.*, 1987). To circumvent these problems, we expressed apo A-I as the proprotein. We show here that proapo A-I can be produced efficiently in *Escherichia coli* as a stable and undegraded molecule, having physical and functional properties identical to those of the natural product.

MATERIALS AND METHODS

Plasmids, strains, and media

Three plasmids, pBR322 (Bolivar *et al.*, 1977), pULB1221 (a multi-cloning site derivative of pULB1219, Cravador *et al.*, 1985), pOTS-Nco (Devare *et al.*, 1984), and three *E. coli* strains, MM294 (Lawn *et al.*, 1981), AR58, and AR120 (Mott *et al.*, 1985), were used. M33 minimal medium is described by Fujimoto *et al.* (1988).

RNA preparation; synthesis, cloning, screening, and sequencing of cDNA

Procedures to prepare and screen the human liver cDNA library were as described before (Jacobs *et al.*, 1985). Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystem Synthesizer, model 380A (Matteucci and Caruthers, 1981). DNA sequence analysis was performed by the methods of Maxam and Gilbert (1977) and Sanger *et al.* (1977).

Immunological detection of proapo A-I in bacterial extracts

Transformed bacterial strains were grown in 20 ml of rich (LB) or minimal medium supplemented with ampicillin (50 μg/ml) to an OD_{630} of 0.6. Induction of the λ P_L promoter was achieved either by shifting the temperature from 30°C to 42°C, or by adding nalidixic acid (60 μg/ml) to cells grown at 37°C (Mott *et al.*, 1985). Induction was performed for various periods of time as indicated in the figure legends.

One-milliliter aliquots of cultures were collected and centrifuged at 10,000 × *g* for 5 min. Pellets were resuspended in 50 μl of $NaDodSO_4$ sample buffer (50 m*M* Tris HCl pH 6.8 containing 2% $NaDodSO_4$, 6 *M* urea, 5% 2-mercaptoethanol, and 10% glycerol), boiled for 3 min at 100°C, and centrifuged for 10 min at 15,000 × *g*. Samples were then fractionated by electrophoresis on 12.5 or 7.5% $NaDodSO_4$-polyacrylamide gels as described by Laemmli (1970).

The proteins were blotted onto nitrocellulose sheets and tested for the presence of proapo A-I with antibodies raised against plasma apo A-I. The procedure follows Bollen *et al.* (1984), except that the second antibody, goat antimouse, was labeled with alkaline phosphatase instead of peroxidase, and that the chromogenic substrates were BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Boehringer) and NBT (4-nitrotetrazolium-chloride blue, Sigma), dissolved in 100 m*M* Tris-HCl pH 9.5, 100 m*M* NaCl, and 50 m*M* $MgCl_2$ at 66 and 33 μg/ml, respectively.

Purification of recombinant proapo A-I

Packed bacterial cells (usually 35 grams), derived from 15-liter fermentations in M33 medium, were resuspended in two volumes of ice-cold extraction buffer (20 m*M* Tris-HCl pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* PMSF, and 100 μg/ml merthiolate) and disrupted by a single passage through a French's pressure cell at 1,000 PSIG. The extract was centrifuged for 15 min at 4,000 × *g* to sediment membranes and aggregates then applied onto a Phenyl Sepharose column CL-4B (bed volume, 25 ml; sample, 100 ml). The column was then sequentially eluted with 250 ml of extraction buffer, 250 ml of the same buffer supplemented with 60% (vol/vol) propylene glycol, and 250 ml of the same buffer without NaCl and supplemented with 80% (vol/vol) propylene glycol. Fractions containing proapo A-I were pooled and then applied onto a PBE 74 chromatofocusing column (0.8 × 60 cm), which was eluted with two bed volumes of a buffer containing 25 m*M* imidazole-HCl pH 6.0 and 6 *M* urea, followed by 10 bed volumes of polybuffer 74 (dilution 1/10) pH 4.0, 6 *M* urea. The column was regenerated by washing with 50 m*M* Na-citrate pH 4.0, 1 *M* NaCl, and 6 *M* urea. Fractions were assayed for total protein content and apo A-I-related immunoreactivity, and analyzed by isoelectric focusing.

Chemical peptide mapping with BNPS-Skatol

Chemical cleavage with BNPS-Skatol was performed according to the procedure of Fontana (1972). Briefly, 5–10 μg of protein were dissolved in 100 μl of a solution of 0.15% (vol/vol) phenol in 50% (vol/vol) acetic acid. Then, 50 μl of a solution of 4.8 mg BNPS-Skatol per milliliter of glacial acetic acid were added, followed by an incubation of 72 hr at 25°C. Subsequently, 50 μl of 2-mercaptoethanol was added, followed by a second incubation of 5 h at 37°C. The samples were evaporated, redissolved in 100 μl of water, and extracted three times with 200 μl of ethyl acetate. The organic phases were discarded, whereas the aqueous phases were lyophilized and analyzed by acrylamide gel electrophoresis.

Stimulation of lecithin:cholesterol acyltransferase activity by recombinant proapo A-I

Human plasma apo A-I was purified according to Holmquist and Carlson (1977) and Holmquist and Brostrom (1979). Simple bilayer vesicles containing egg lecithin and 7(*n*)-^{3}H-labeled cholesterol (molar ratio 4:1) were prepared according to Batzri and Korn (1973). Highly

purified human plasma lecithin:cholesterol acyltransferase (35,000 units/mg) was prepared according to Holmquist (1987). Incubations and enzyme activity determinations were carried out in 50 m*M* phosphate buffer pH 7.4 as described by Piran and Morin (1979). Incubation mixtures (250 μl) contained per liter: 240 μmoles lecithin, 60 μmoles cholesterol, and 3 or 6 μmoles apolipoprotein.

Miscellaneous procedures

Protein concentrations were determined according to the procedure of Bradford (1976), using ovalbumin as a standard. Partial tryptic hydrolysis was performed according to the procedure of Amons *et al.* (1983). Human apo A-I-related immunoreactivity was quantified by radial immunodiffusion (Cheung and Albers, 1977), using commercial apo A-I (Sigma) as a standard. The relative immunoreactivity profile were determined by immunoturbidimetry (Shapiro *et al.*, 1980). Isoelectric focusing was carried out according to the procedure of Catsimpoolas (1968) in a Biorad rod electrophoresis apparatus, applying a self-generating gradient from pH 4 to pH 6. Linear and nonlinear regression analyses were performed with the StatView program (Brain Power Inc., USA).

RESULTS

Molecular cloning of Apo A-I cDNA and screening of the cDNA library

Total poly(A)$^+$RNA (25 μg) from human liver was transcribed into double-stranded cDNA (ds cDNA). Molecules resistant to S_1 nuclease were then fractionated on a 10–30% sucrose gradient to enrich for molecules larger than 0.8 kb. This ds cDNA fraction was inserted into the *Pst* I site of pBR322 by the dC:dG tailing procedure and used to transform *E. coli* MM294 cells. The library was screened by the colony hybridization method using a 22-base synthetic oligonucleotide probe derived from the sequence for apo A-I (Cheung and Chan, 1983) and coding for amino acids −22 to −15 of the signal peptide. Of the 30 clones hybridizing with the labeled probe, one, pNIV1602, was chosen for further analysis.

Characterization of the clone pNIV1602

Clone pNIV1602 carried an insert of 878 bp which could be isolated as a single fragment by digesting the plasmid with *Pst* I. The insert was mapped with various restriction enzymes and fully sequenced on each strand. The data (not shown) showed that pNIV1602 carries (i) a 19-bp 5′ noncoding sequence, (ii) a 75-bp sequence corresponding to the known signal and propeptide region of apo A-I, including the ATG initiation codon, (iii) the complete 729-bp sequence corresponding to the mature protein, followed by a TGA stop codon, and (iv) a 55-bp 3′ noncoding sequence that did not contain the poly(A) stretch. The sequence of the cDNA insert was identical to the sequence published by Cheung and Chan (1983), but differed from the sequence of Seilhamer *et al.* (1984) by a few bases and by the length of the 5′ and 3′ noncoding regions. The deduced amino acid sequence, however, was consistent with the published data (Cheung and Chan, 1983; Seilhamer *et al.*, 1984).

Construction of the recombinant plasmid pNIV1617

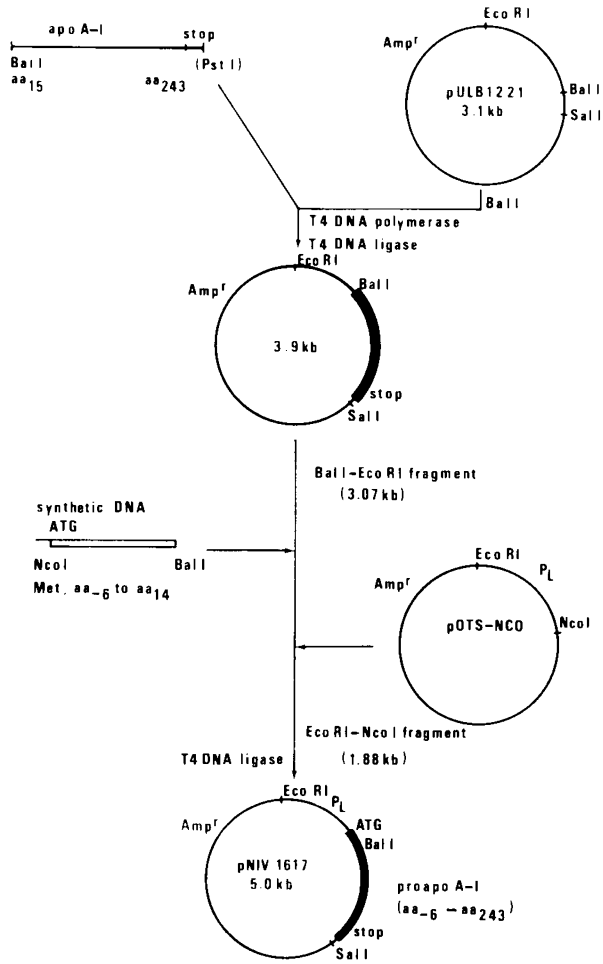
The construction proceeded in two steps. The first used the multi-cloning site plasmid pULB1221, linearized with *Bal* I, as an intermediate vehicle to subclone a blunt-ended 744-bp *Bal* I–*Pst* I DNA fragment derived from the clone pNIV1602 and coding for amino acids 15–243 of the apo A-I protein. The resulting plasmid was digested with *Eco* RI and *Bal* I and fused to (i) the 1,880-bp *Eco* RI–*Nco* I DNA fragment containing the regulatory regions derived from the expression vector pOTS-Nco (Devare *et al.*, 1984) and (ii) a 65/61-bp *Nco* I–*Bal* I synthetic DNA adaptor encoding the amino-terminal methionine and amino acids −6 to 14 of proapo A-I (Fig. 1). The resulting recombinant plasmid, pNIV1617, thus carried the strong regulatable λ P_L promoter upstream from the cassette coding for proapo A-I.

The synthetic adaptor was assembled from four oligonucleotides (Fig. 2) whose base sequence was designed by computer to minimize secondary structures at the 5′ end of the coding sequence (S.A.S.I.P. software package; Claverie, 1984). Triplets encoding amino acid residues −6, −1, 1, 3, 4, 5, 6, 7, 10, 11, and 14, therefore differed from the natural ones (Table 1), although the resulting amino acid sequence was preserved.

Plasmid pNIV1617 was characterized by restriction analysis, hybridization with labeled synthetic DNA fragments, and sequenced throughout the adaptor region. It was then introduced into *E. coli* AR58 (for thermal induction) and AR120 (for chemical induction).

Expression of proapo A-I

Cells carrying either the control plasmid, pOTS-Nco, or the pNIV1617 plasmid were grown at 30°C in minimal medium, for thermal induction, or at 37°C in rich medium, for chemical induction. Derepression of the λ P_L promoter was achieved either by shifting the temperature to 42°C or by adding nalidixic acid. Induction was performed for various periods of time as indicated in the legend to Fig. 3. Pelleted bacteria were lysed and the resulting extracts were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis, Western blotting, and immunodetection using antibodies raised against plasma apo A-I. Figure 3 shows that a single product, specifically recognized by anti-apo A-I antibodies, is expressed in the bacterial strains carrying plasmid pNIV1617, irrespective of the mode of induction.



The protein had a molecular mass of about 28,000 daltons which, within experimental limits, fitted the calculated molecular weight of proapo A-I.

Levels of proapo A-I produced in bacteria were estimated by single radial immunodiffusion. Under thermal induction conditions in M33 minimal medium, proapo A-I was expressed at about 10% of total soluble proteins versus 3.5% in LB medium. Chemical induction appeared to be less efficient, yielding expression levels on the order of 2% in LB medium and no expression in M33 medium.

Purification of recombinant proapo A-I

We used hydrophobic interaction chromatography to purify the recombinant proapo A-I. This approach allowed an excellent separation of recombinant molecules from other bacterial constituents (Fig. 4, peak III). Chromatofocusing on a PBE-74 column was used to purify the product further. As shown in Fig. 5, immunoreactive ma-

FIG. 1. Construction of plasmid pNIV1617. A 744-bp *Bal* I–*Pst* I fragment derived from clone pNIV1602, coding for amino acids 15–243 of apo A-I, was ligated into the unique *Bal* I site of pULB1221. From the resulting plasmid, the large *Eco* RI–*Bal* I fragment was fused to the 1,880-bp *Eco* RI–*Nco* I fragment derived from pOTS-Nco and to the synthetic adaptor *Nco* I–*Bal* I to regenerate a sequence coding for proapo A-I. Hybrid molecules were used to transform *E. coli* cells and transformants were selected for ampicillin resistance. The nucleotide sequence of the junctions between fragments and of the full synthetic fragment were determined.

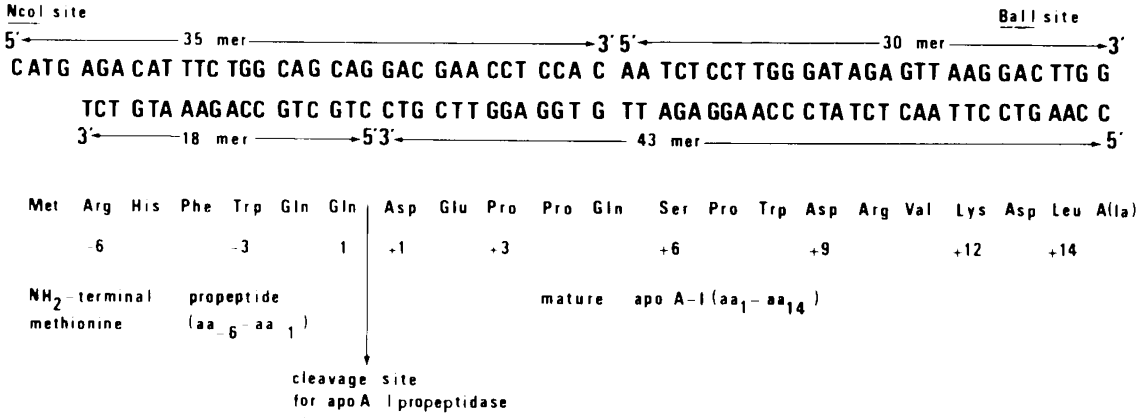


FIG. 2. Synthetic adaptor used to reconstruct the proapo A-I cDNA. Four oligomers were synthesized and assembled *in vitro* to generate a 65/61-bp fragment flanked by a *Nco* I and a *Bal* I sites on the 5′ and 3′ ends, respectively. The deduced amino acid sequence is indicated. The arrow shows the known cleavage site for apo A-I propeptidase (Gordon *et al.*, 1983; Zannis *et al.*, 1983).

TABLE 1. RELEVANT CHANGES IN CODONS USED TO RECONSTRUCT THE PROAPO A-I cDNA

Position in the protein	*Amino acid*	*Natural codon*	*Modified codon*
−6	Arg	CGG	AGA
−1	Gln	CAA	CAG
1	Asp	GAT	GAC
2	Glu	GAA	–
3 and 4	Pro	CCC	CCC and CCA
5	Gln	CAG	CAA
6	Ser	AGC	TCT
7	Pro	CCC	CCT
8	Trp	TGG	–
9	Asp	GAT	–
10	Arg	CGA	AGA
11	Val	GTG	GTT
12	Lys	AAG	–
13	Asp	GAC	–
14	Leu	CTG	TTG

Modifications in the codon usage were done in order to minimize secondary structures in the 5′ end of the proapo A-I cDNA. The software package S.A.S.I.P. developed by the Institut Pasteur (Paris) was used (Claverie, 1984).

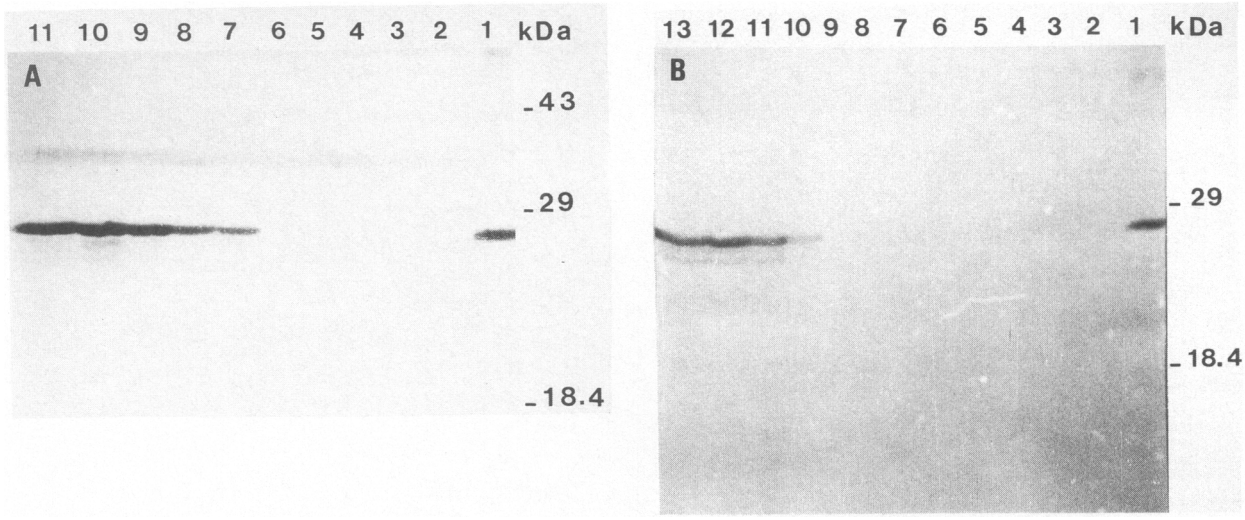


FIG. 3. Immunodetection of proapo A-I on Western blots. Protein extracts from induced and noninduced bacteria carrying plasmid pNIV1617 were prepared for electrophoresis on $NaDodSO_4$-polyacrylamide gels. Nitrocellulose blots were incubated with mouse anti apo A-I serum, followed by alkaline phosphatase-labeled goat anti-mouse and the chromogenic substrate. A. Time course of thermal induction of bacteria carrying pNIV1617 and grown in M33 minimal medium. Lane 1, Plasma derived apo A-I, 500 ng; lanes 2–6, samples taken at 1, 20, 40, 60, and 120 min in noninduction conditions (30°C); lanes 7–11, samples taken at 1, 20, 40, 60, and 120 min after induction (42°C). B. Time course of chemical induction (nalidixic acid) of bacteria carrying pNIV1617 and grown in rich LB medium. Lane 1, Plasma derived apo A-I, 500 ng; lanes 2–7, samples taken at 1, 30, 60, 120, 180, and 240 min in noninduction conditions (37°C); lanes 8–13, samples taken at 1, 30, 60, 120, 180, and 240 min after induction (37°C and nalidixic acid, 60 μg/ml).

terial (fractions 37–48) was resolved from contaminants and was identified by isoelectric focusing mainly as proapo A-I. Moreover, chromatofocusing resolved most apo A-I related isoforms.

Characterization of purified recombinant proapo A-I

Pure recombinant proapo A-I was analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis and isoelectric focusing. Recombinant proapo A-I behaved as a single polypeptide chain with an apparent molecular mass of (27.3 ± 1.1) kD. Mature apo A-I was slightly smaller, having an apparent molecular weight of (26.8 ± 1.1) kD. In isoelectric focusing (data not shown), the recombinant protein showed one major isoform and one minor isoform, having isoelectric points of 5.20 and 4.95, respectively. The relative migration distances of these isoforms coincided exactly with those of plasma proapo A-I and apo A-I, respectively. Partial chemical cleavage at tryptophan residues, was performed on recombinant proapo A-I (major isoform) and on plasma-derived apo A-I. Figure 6 shows the large peptides of expected molecular weight produced in both cases by cleavage at positions 8, 50, 72, and 108 in the proteins. In addition, digestions of recombinant proapo A-I and of plasma-derived apo A-I with trypsin in very mild conditions yielded similar patterns of fragments (data not shown). Thus, both types of experiments suggest that the recombinant molecule is structurally identical to its natural counterpart.

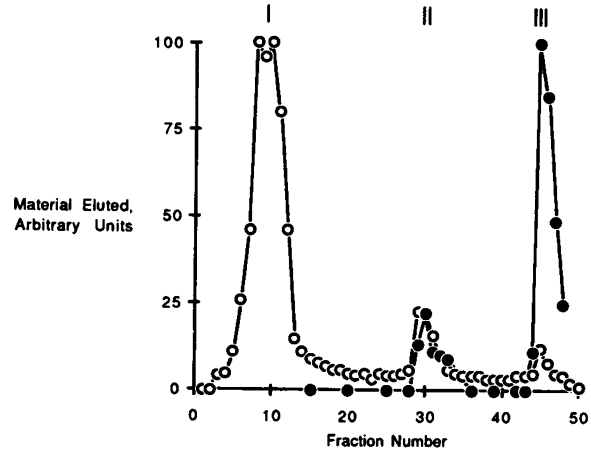


FIG. 4. Hydrophobic interaction chromatography. A total of 100 ml of cleared extract of bacterial cells derived from 15-liter fermentation in M33 medium was subjected to hydrophobic interaction chromatography as described in Materials and Methods. The eluate was assayed for total protein (○) and apo A-I-related immunoreactivity (●). Peak III contains the majority of the recombinant protein.

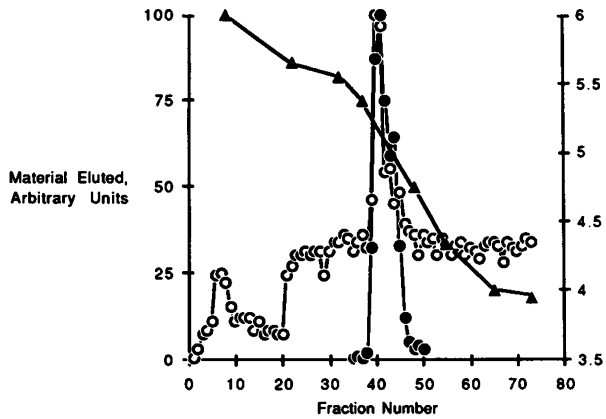


FIG. 5. Phenyl-sepharose chromatography. Peak III of the phenyl sepharose eluate was subjected to chromatofocusing as described in Materials and Methods. The eluate was assayed for total protein (○), apo A-I-related immunoreactivity (●), and pH (▲). Fractions 37–48 contain the purified recombinant product.

Functional analysis of recombinant proapo A-I

Recombinant proapo A-I (pI 5.20) and its minor isoform (pI 4.95) were both able to transform synthetic lecithin–cholsterol liposomes into substrates for the enzyme lecithin:cholesterol acyltransferase. As seen in Table 2, the activation capacity of recombinant apoproteins was similar to the one observed with authentic human plasma apo A-I.

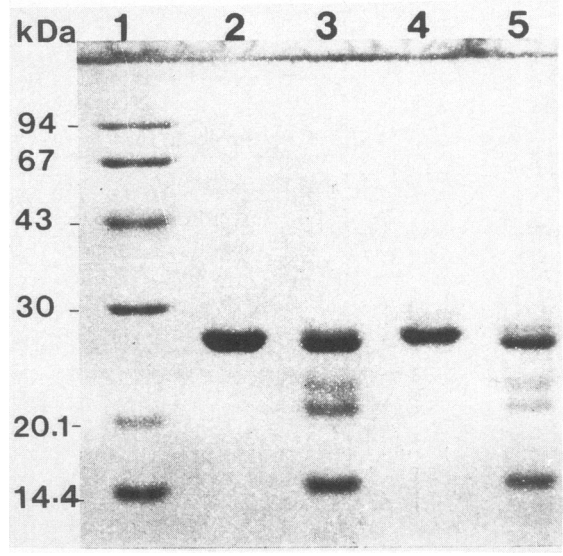


FIG. 6. Chemical and enzymatic characterization of proapo A-I. Purified recombinant proapo A-I and human plasma apo A-I were subjected to partial chemical peptide mapping as described in Materials and Methods. Lane 1, Molecular mass standards (Pharmacia, 14,400–94,000 daltons) indicated by arrows; lane 2, human plasma apo A-I, untreated; lane 3, human plasma apo A-I, cleaved; lane 4, recombinant proapo A-I, untreated; lane 5, recombinant proapo A-I, cleaved.

TABLE 2. STIMULATION OF LECITHIN:CHOLESTEROL ACYLTRANSFERASE ACTIVITY BY RECOMBINANT PROAPO A-I

Source	*LCAT (μl)*	*Apolipoprotein (μM)*	*LCAT activity (nmoles/hr × liters)*
Plasma apo A-I	25	0	2.5
	25	3	250
Recombinant proapo A-I	100	0	100
Major isoform (pI 5.20)	100	3	850
	100	6	1,600
Minor isoform (pI 4.95)	100	0	100
	100	3	700
	100	6	700

Synthetic liposomes (lecithin/cholesterol, 4:1) were incubated at 37°C for 2–4 hr, in a total volume of 250 μl, with LCAT (25 units/ml) and various amounts of apo A-I. The initial rate of cholesteryl ester formation (LCAT activity) was measured according to Piran and Morin (1979).

DISCUSSION

We have cloned a cDNA coding for human preproapo A-I; its deduced amino acid sequence fits the published data (Cheung and Chan, 1983; Seilhamer *et al.*, 1984) and is almost identical to that reported by Brewer *et al.* (1978). We constructed a module coding for the proapo A-I molecule and expressed it in *E. coli* under the control of the regulatable λ P_L promoter. Proapo A-I, produced at high level, was stable, largely undegraded, and indistinguishable from the natural product in terms of apparent molecular mass, isoelectric point, and partial peptide map. As expected, most of the material produced in *E. coli* was in the proprotein form, although the presence of an amino-terminal methionine cannot be excluded yet. The purified protein appeared to contain a minor isoform which coincided with mature apo A-I by isoelectric focusing. However, it is uncertain if this is due to deamidation or to conversion of the recombinant protein to mature apo A-I. The high level of expression observed for proapo A-I in bacteria contrasted with the relative failure (Lorenzetti *et al.*, 1986; our data, not shown) to achieve significant expression of a stable, undegraded mature apo A-I species in the same host. The dramatically increased expression associated with the presence of the propeptide sequence might be explained in several ways. Transcriptional or translational efficiencies might be increased due to the extra sequence. Alternatively, the proprotein species might fold more stably in bacteria than the mature protein and hence be more resistant to degradation. This last hypothesis seems more probable since pulse-chase experiments have shown that the half-life of mature apo A-I in bacteria is less than 10 min (Mallory *et al.*, 1987). Our data, in addition, offer direct evidence for the first time that recombinant proapo A-I is able to stimulate *in vitro* the enzymatic activity of lecithin:cholesterol acyltransferase, as efficiently as plasma-derived apo A-I. In this respect, proapo A-I might constitute an appropriate substitute to mature apo A-I for replacement therapy, provided that pharmacological studies in animal models confirm its functional efficiency.

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