

The present invention relates to new recombinant DNA sequences comprising a sequence coding for human proapolipoprotein A-I, to cloning and expression vectors containing these DNA sequences, to host cells transformed with these expression vectors, as well as to production processes for human proapolipoprotein A-I using new recombinant DNA sequences, vectors and transformants.

Human apolipoprotein A-I (apo A-I) is the main protein constituent of high density lipoproteins (HDL) and of lymph chylomicrons. The liver and the small intestine are primary centres for the synthesis of apo A-I. Apo A-I is synthesized in these organs in the form of a protein precursor (preproapo A-I). Cotranslational cleavage of the prepeptide takes place within the cell and the proapo A-I is secreted in the plasma and lymph.

Proapo A-I contains six additional amino acids (Arg-His-Phe-Trp-Gln-Gln) which are attached to the terminal amino group of the apo A-I. On reaching the vascular region, proapo A-I is cleaved in vivo by a specific proteolytic enzyme (apo A-I propeptidase) to give mature apo A-I.

Mature apo A-I is a single non glycosylated polypeptide of known sequence, composed of 243 amino acids (H.B.BREWER et al., Biochem.Biophys.Res.Commun.80, (1978), 623-630); it serves as a cofactor for the plasma enzyme of lecithin: cholesterol acyltransferase, which is responsible for the formation of most cholesterol esters in the plasma. Defects in apolipoprotein structures or biosynthesis may result in disorders of the plasma lipid transport system and in the development of coronary artery disease. It has been shown that low levels of apo A-I and of HDL in plasma constitute a high risk factor in heart attacks (myocardial infarction) and in other atherosclerotic vascular diseases. Mutations in the gene coding for apo A-I in particular, have been associated with reduced HDL levels as well as premature coronary artery diseases.

Apo A-I and HDL are the major plasma constituents that participate in the transfer of cholesterol from peripheral

tissues (arteries) to the liver (also known as reverse cholesterol transport) to be excreted from the system. Since accumulation of cholesterol in the arteries is the characteristic feature and most important mechanism in atherosclerosis, stimulation of reverse cholesterol transport by means of apo A-I may retard and reverse the atherosclerotic process and hence reduce the incidence of heart attacks.

Maturation of proapo A-I into apo A-I can occur quantitatively outside the cell with a residence time in the blood of less than 12 hours. Since proapo A-I is the major, if not the only, precursor of mature apo A-I, it could be used in replacement therapy whenever HDL levels decrease, for example in hereditary or acquired deficiencies. Because of the therapeutic utility of apo A-I in general, researchers have been looking for techniques which would enable apo A-I to be produced in large quantities. Conventionally, such techniques comprise purification of apo A-I from blood plasma.

Several publications (P.CHEUNG and L.CHAN, *Nucleic Acids Res.* 11, (1983), 3703-3715; J.J.SEILHAMER et al., *DNA*, 3, (1984), 309-317 and Japanese Patent Application N° 96998/86) have shown that the complementary DNA coding for preproapo A-I can be obtained by well-known genetic engineering techniques.

R.LORENZETTI et al., (*FEBS Lett.* 194, (1986), 343-346) have shown that apo A-I can be expressed in E.Coli as an uncleavable protein fused to beta-galactosidase. However, attempts to express mature apo A-I in the form of an unfused protein have remained unsuccessful.

In the above-mentioned Japanese Patent Application 96998/86, the expression is described of a protein similar to human apolipoprotein A-I in E.Coli, which has been transformed with a pHAIE-I plasmid containing the structural apo A-I gene under the control of the tac promoter. However, the structural gene is incomplete, and consists of the codons for amino acids +4 to +243, preceded by an ATG translation initiation codon. As a result, the expression product obtained contains N-terminal methionine (corresponding to the ATG

codon) which might cause secondary effects when used in therapy.

J.B. MALLORY et.al. (J.Biol.Chem. 262, (1987), 4241-4247; PCT International Patent Applications WO 86/04920 and WO 87/02062) describe the expression of human apolipoprotein A-I in Chinese hamster ovary cells (animal cell culture). The construction used contains the complete human preproapolipoprotein A-I gene. The Chinese hamster ovary cells appear to transform the proapo form into the mature apo form, since only 5 to 10% of the secreted apo A-I protein is proapo A-I, the remainder being the mature apo A-I protein. The productivity of the system is relatively low, since 0.5×10^6 cells secrete only 25-30 $\mu\text{g/ml}$ of apo A-I in 24 hours. In PCT International Patent Application WO 87/02062, some examples relate to expression of human apolipoprotein A-I in other hosts, such as E. coli (bacterial host) and S. cerevisiae (yeast). However, none of the constructions used contains the genetic information for the proapo form, and the expressed protein is not human proapolipoprotein A-I.

The present invention concerns the means and methods of preparing human proapolipoprotein A-I by means of recombinant DNA technology; that is to say, mature proapo A-I protein which is susceptible to cleavage by the specific proteolytic apo A-I propeptidase enzyme. The term "mature", as used herein, is understood to mean not only proapo A-I as such, but also the corresponding protein, of which the first amino acid is methionine the presence of which is due to the ATG translation initiation codon in the expression convector construction.

The present invention relates to the construction of cloning and expression vectors containing a DNA sequence encoding human proapo A-I, so as to enable the amplification, and hence expression, of the mature human proapo A-I protein as well as met-proapo A-I, its fused conjugates or its signal N-terminal conjugates. Similarly, the present invention concerns viable cell cultures or micro-organisms genetically altered by virtue of their harbouring such vectors, and

capable of producing human proapo A-I polypeptide. In addition, the present invention provides human proapo A-I in a physical state distinct from that which is found in, or isolated from, a natural source or environment; by virtue of its method of preparation, the human proapo A-I is essentially free from usual endogenous proteins and other native materials or substances.

The present invention concerns the production of human proapo A-I by means of the recombinant DNA technique in all its aspects and should not be interpreted as being limited to specific details described herein and encompassed within the scope of the invention.

One fundamental aspect of the present invention concerns the production of a protein which consists of or contains mature human proapo A-I and which, on account of this, can be converted, in vitro and in vivo, into mature human apo A-I by means of the proteolytic action of apo A-I propeptidase. The human proapo A-I product may be used as such for therapeutic purposes; met-proapo A-I may also be used if the presence of the methionyl radical is pharmaceutically acceptable, because this product may be converted naturally and efficiently in the blood stream by naturally occurring propeptidase to provide authentic mature human apo A-I. If desired, human proapo A-I may be produced in selected micro-organisms or cell cultures which are capable of processing N-terminal methionine, and which, consequently, are capable of producing human proapo A-I in a form which does not contain N-terminal methionine. Alternatively, whether or not it contains an N-terminal methionyl radical, human proapo A-I may be cleaved in vitro to obtain mature human apo A-I, which can be used for therapeutic applications. The same applies to fusion conjugates and conjugates including the N-terminal signal of proapo A-I; cleavage produces authentic human apo A-I lacking the N-terminal methionine.

A second important aspect of the present invention lies in the use of a modified sequence encoding at least part of the human proapo A-I molecule, this modified coding sequence

improving the translational efficiency by virtue of the reduction or even prevention of hairpin structure formation. It is possible that R. LORENZETTI et.al. (FEBS Lett. 194, (1986), 343-346) were not able to detect expression of an unfused mature apo A-I protein because their DNA constructions were susceptible to forming hairpin structures to an appreciable extent, leading to very inefficient expression. Surprisingly, it has been found that efficient expression can be ensured by appropriate modification of a few codons in the coding sequence for amino acids - 6 to +14 of human proapo A-I. The words "appropriate modification" as used herein imply that some of the natural codons are replaced by other codons which, according to the genetic code, represent the same amino acids, and further imply that all the modifications taken together lead to a reduction in, or even prevention of, hairpin structure formation. It is important to note that the modifications to the DNA sequence have no effect on the nature of the propeptidase cleavage site, since the amino acid sequence recognized by the propeptidase is preserved in the construction.

On the protein level, the present invention relates to human proapo A-I as a product of a genetically modified cell culture or micro-organism. This human proapo A-I may be in the form of mature proapo A-I, in the form of mature proapo A-I preceded by a methionine residue, in the form of a fused protein such as for example beta galactosidase-proapo A-I protein and in the form of the preproapo A-I compound. The product obtained is essentially free of usual endogenous proteins and other native materials or substances common to the natural source (blood plasma) of the protein concerned. The cell culture or microorganism used in the production is not limited to specific cell lines and organisms: both prokaryotic and eukaryotic cells can be utilized, including animal and human cell lines. For illustrative purposes only, we give herein as an example, expression in E. coli bacterium (as representative of prokaryotic cells) and in the yeast

Saccharomyces cerevisiae as well as in insect cells infected by baculovirus (as representative of eukaryotic cells).

On the protein level, the present invention also relates to human apo A-I obtained by treatment of the human proapo A-I prepared as above, with apo A-I propeptidase. This human apo A-I will be identical to the authentic form of human apo A-I and will be lacking in any N-terminal methionine residue.

On the application level, the present invention relates to pharmaceutical compositions containing the above human proapo A-I and/or apo A-I, and containing in addition, at least one pharmaceutically acceptable carrier, solvent, diluent or excipient, suitably selected in accordance with the route of administration and the normal requirements for the formulation of pharmaceutical compositions.

On the DNA level, the present invention relates to a recombinant DNA sequence comprising a sequence which codes for human proapo A-I, in which part of the natural coding sequence has been replaced by a DNA fragment coding for the same amino acids but consisting of a different nucleotide sequence, so as to reduce or prevent formation of hairpin structures. In a specific preferred embodiment, the natural sequence coding for the amino acids -6 to +14 of proapo A-I has been replaced by the sequence (only one strand shown):

5' -ATGAGACATTTCTGGCAGCAGGACGAACCTCCACAATCTCCTTGGGATAGAGTTAAGG
ACTTG-3' the said sequence comprising an additional ATG translation initiation codon and modified codons for the amino acid residues -6, -1, +1, +3, +4, +5, +6, +7 +10, +11 and +14.

On the DNA level, the present invention also relates to replicable cloning vectors and expression vectors comprising the aforesaid recombinant DNA sequence which codes for human proapo A-I. The present invention more especially relates to the recombinant plasmids pULB9291, pULB9292, pULB9296, pULB9299, pNIVI612 and pNIV1613, the construction of which is described hereinafter. The first mentioned plasmids pULB9291 and pULB9292 contain the modified proapo A-I structural gene preceded by an ATG codon and under the control of the phage lambda P_L promoter. These plasmids are suitable expression

vectors for E. coli and direct the synthesis of human proapo A-I, which can be cleaved by the propeptidase to generate authentic mature human apo A-I. When the plasmid pULB9296 is introduced into E. coli, it directs the expression of a fused protein containing beta-galactosidase and human proapo A-I under control of the lac promoter region of E. coli. Since the fused product still contains the propeptidase cleavage sequence, it can be cleaved to yield authentic mature human apo A-I.

The plasmid pULB9299 is an expression vector which is suitable for yeast species and contains ARG3 promoter and translation terminator regions to ensure efficient expression in yeast. Once again, the human proapo A-I obtained may be split up by propeptidase to provide authentic mature human apo A-I.

The pNIV1612 plasmid contains the proapo A-I structural gene fused to the DNA sequence of the E. Coli OmpA protein signal peptide and under the control of the lpp (lipoprotein) and of the lac promoter-operator. In the construction, the sequence coding for the signal peptide of ompA precedes the proapo A-I sequence without the ATG translation initiation codon. The plasmid is an appropriate secretion vector for E. coli and directs the synthesis of human proapo A-I which can be secreted into the periplasm without N-terminal methionine. The pNIV1613 plasmid is a transfer vector for introducing the human proapo A-I cDNA sequence into baculovirus. It comprises the polyhedrin promoter of baculovirus and the structural gene of proapo A-I, including the ATG translation initiation codon. In conjunction with the wild strain of baculovirus (Autographa Californica nuclear polyhedrosis virus, AcNPV), the pNIV1613 plasmid directs the synthesis of proapo A-I in insect cells, and can be found free of methionine following post-translational modifications in insect cells.

Finally, the present invention also relates to cell cultures and micro-organisms which have been transformed by a cloning vector or a translation vector as described above, and

in particular, to transformed cell cultures and transformed micro-organisms which are capable of producing human proapo A-I . The expression "transformed", as used herein, has the broad meaning of "genetically modified" and is certainly not limited to the narrow concept of "bacterial transformation". Depending on the nature of the vector employed and the host selected, any compatible genetic engineering technique for obtaining the desired genetic modification can be used, including transfection, transduction etc.

Cell cultures and micro-organisms prepared according to the invention may be used for industrial scale production of human proapolipoprotein A-I by fermentation.

The invention described herein has been carried out using, among others, the MM 294 strain of micro-organism E. coli K12 (endoA thi⁻, hsdR, supE); this strain has been deposited with the American Type Culture Collection (ATCC n° 33625) without restriction as regards access. However, various other microbial strains may be used, including known strains of E. coli such as E. coli B, E. coli x 1776 (ATCC n° 31537, deposited on 3rd July 1979, without restriction), E. coli AR 58, N99 derivative (ATCC n°33956) with cIII⁻, ci 857, bio⁻ and delta H functions devoid of lambda, sold by PL-PHARMACIA (J.E.MOTT et al., Proc.Natl.Acad.Sci,USA, 82, (1985), 88-92; G.DEVARE et al., Cell, 36, (1984), 43-49), E. coli JM101 (ATCC n° 33876) (J.MESSING et al., Nucleic Acids Res. 9, (1981), 309-321), or E. coli JA 221 (lpp⁻, hdsM⁺, trpE5, leuB6, lacY, recAI/F', lacI^q, lac⁺, pro⁺) (J. GHAYEB et al., EMBO J. 3, (1984), 2437-2442), or other microbial strains many of which have been deposited and are accessible at recognized micro-organism depository institutions, such as the American Type Culture Collection (ATCC) -- cf the ATCC catalogue listing. These other microorganisms include, for example, Bacilli such as Bacillus subtilis and other Enterobacteriaceae among which may be mentioned as examples Salmonella Typhimurium and Serratia marcesans, utilizing plasmids that can replicate and express heterologous gene sequences.

Expression plasmids for use with bacteria, e.g. E. coli, are usually obtained from plasmid pBR322 used as a vector (deposited in the ATCC under accession number 37017) and appropriately inserting the heterologous gene sequence together with translational start and stop signals in the correct reading frame with a functional promoter, taking advantage of natural or synthetically created restriction sites. The vector will carry one or more phenotypic selection characteristic genes and an origin of replication to ensure amplification within the host. Once again, the heterologous insert can be aligned so as to be expressed at the same time as a fused presequence, derived for example from the lac system genes.

Baculovirus expression vectors, for example pACRP6 and pAcYM1 (Y.MATSUURA et al., J.Gen.Virol.68, (1987), 1233-1250) and wild type baculovirus (Autographa californica nuclear polyhedrosis virus, AcNPV) are now widely used. They are described in detail in the literature and can be obtained mainly from the Texas Agricultural Experiment Station.

According to the present invention, various insect cells may also be used as host for compatible expression vectors, such as for example Spodoptera frugiperda cells Sf9 (M.D. SUMMER and G.E. SMITH. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas University, College Station, (1987); ATCC CRL 1711).

According to the present invention, various yeast strains may also be used, hosting compatible expression vectors, such as the plasmid YRp7 (D.T. STINCHCOMB et al., Nature, 282, (1979), 39-43) which is capable of selection and replication in both E. coli and yeast, particularly Saccharomyces cerevisiae.

Yeast strains which may be used are strain RH218 (G.MIOZZARI et al., J.Bacteriol.134, (1978), 48-59) deposited with the American Type Culture Collection without restriction (ATCC No. 44076), the 10S44c strain (T.CABEZON et al., Proc.Natl.Acad.Sci.USA, 81, (1984), 6594-6598) which has the leu2-3, leu2-112, pep 4-3 genotype (M.HOYLAERTS et al., FEBS

Lett.204, (1986), 83-87) and strain Ic 1697d (argJ, leu2-1) an arginine bradotroph (ATCC No.20631).

To express a heterologous gene such as the cDNA for human proapo A-1 in yeast, it is necessary to construct a plasmid vector containing four components.

The first component is the fragment which allows for transformation of both E. coli and yeast and which must consequently contain a selectable gene from each organism. This can be the gene responsible for ampicillin resistance derived from E. coli (cf. "AmpA") and the gene leu2 from yeast. This component also requires an origin of replication from each organism to be maintained as a plasmid DNA in both organisms. This can be the E. coli origin from pBR322 and the ars1 origin from chromosome III of yeast or the origin of replication from 2 μ circle DNA.

The second component of the plasmid is a sequence situated at 5' from a highly expressed yeast gene in order to promote transcription of a structural gene placed downstream. The sequence situated at 5' can be that from the yeast TDH3 or ARG3 genes. The fragment is constructed in such a way as to remove the TDH3 or ARG3 structural sequences, which are replaced with a sequence containing alternative restriction sites, such as NcoI or BamHI restriction sites, for the convenient attachment of this sequence situated at 5' in the structural gene.

The third component of the system is a structural gene constructed in such a way that it contains both an ATG translational start and translational stop signal.

The fourth component is a yeast DNA sequence containing the yeast gene sequence situated at 3', which contains the appropriate signals for transcription termination and polyadenylation. For example, plasmids directing the production of human proapo A-I in yeast can be constructed by inserting gene fragments for the human proapo A-I polypeptide into the BamHI site of expression plasmid pRIT 10774 as described in European Patent Application No.151102.

Additional characteristics of the invention will furthermore become apparent in the course of the following description of preferred constructions for vectors containing a recombinant DNA sequence according to the invention and of the conditions in which these vectors can be used. Reference will be made on this occasion to diagrams in which :

- Figures 1A and B show the nucleotide and amino acid sequence of human preproapo A-I. The nucleotide sequence of the human preproapo A-I messenger RNA was determined from DNA sequence analysis of the cDNA clone pULB1609. Predicted amino acids in the signal peptide, propeptide and mature apo A-I polypeptide are shown and are numbered from the first amino acid residue of the apo A-I protein. The region corresponding to the synthetic DNA probe used to isolate the clone is underlined. Single-letter abbreviations used for designating amino acids are as follows :
A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenyl-alanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophane; and Y, tyrosine.
- Figure 2 represents the synthetic scheme of an oligonucleotic adapter for the construction of a DNA fragment coding for the 6 amino acids of the propeptide and for the first 14 amino acids of mature apo A-I polypeptide, together with the initiation ATG codon. The arrows indicate the oligonucleotides used to synthesize the 65/61 base pair (bp) NcoI-BalI fragment.
- Figure 3 shows the construction of plasmid pULB9291 which carries the P_L lambda regulatory regions and the proapo A-I nucleotide sequence.
- Figure 4 shows the construction of plasmid pULB9296 which carries the E. coli lac promoter and a beta-galactosidase sequence fused with that of proapo A-I.

- Figure 5 shows the construction of plasmid pULB9299 which carries the yeast ARG3 regulatory regions and the proapo A-I nucleotide sequence.
- Figures 6A, B and C show the construction of plasmid pNIV1612 which carries the lac and the lpp regulatory regions, the sequence coding for the ompA signal peptide and the proapo A-I nucleotide sequence.
- Figure 7 shows the construction of plasmid pNIV1613 which carries the polyhedrin gene regulatory region and the proapo A-I nucleotide sequence.

Detailed description of the invention.

RNA preparation : Human liver total RNA was prepared by the guanidinium chloride method (R.A. COX, Methods Enzymol.XII, part B, (1968), 120-129). This total RNA preparation was then passed onto an oligo(dT)-cellulose column to obtain the total polyA⁺RNAs (T.MANIATIS et al., in Molecular Cloning (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982). 200 µg of polyA⁺RNAs were obtained from 10 g of human liver.

Synthesis in vitro of the complementary DNA (cDNA).

The reverse transcription reactions, starting from 0.1 to 5 µg of total polyA⁺RNAs, were primed with oligo (dT)₁₂₋₁₈ (approximately 1 µg; source: BOEHRINGER). The single-stranded cDNA was then transformed into the double stranded molecule (cDNAds) with the same reverse transcriptase enzyme. cDNAds preparations, usually 1 µg, were treated with S1 nuclease to create blunt ended extremities. The procedures are well-known in the art and described in detail by T. MANIATIS et al., loc.cit. cDNAds was then oligo(dC)-tailed by the technique described by L.VILLA-KOMAROFF et al., (Proc.Natl.Acad.Sci.USA, 75, (1978)), 3727-3731). Usually, 100 ng of cDNAds are treated with the enzyme terminal deoxynucleotidyl transferase. In general, 15-base-long extensions are added to the 3' ends of the cDNAds molecules.

Cloning of the tailed cDNAs into the plasmid vector pBR322.

pBR322 plasmid DNA was linearized by the enzyme PstI and oligo(dG)-tailed as described by R.M. LAWN et al. (Nucleic Acids Res. 9, (1981), 6103-6114). Oligo(dC)-tailed cDNAs were mixed with oligo(dG)-tailed pBR322 DNA in equimolar proportions. Usually, 50 μ g of the mixture were hybridized to recircularize the plasmid. Conditions are well-known in the art and are described in detail by R.M. LAWN et al., loc.cit. The hybridized mixture was then used to transform competent E. coli cells, strain MM 294, for example according to the method described by R.M. LAWN et al., loc.cit. Several hundred transformants were obtained by selecting for growth on tetracycline medium, the resistance to this antibiotic having been conferred by the pBR322 plasmid. Transformants were also assessed for sensitivity to ampicillin. Those which are sensitive contain a chimeric plasmid since insertion of foreign DNA into the vector inactivates the ampicillin gene.

Screening of the cDNA library.

E. coli transformants were screened with 32 P 5' end labelled synthetic oligonucleotides corresponding to a fragment of the apo A-I gene. The nucleotide sequence of human apo A-I is known (see P CHEUNG and L. CHAN, loc.cit and J.J. SEILHAMER et al., loc.cit); it is therefore practical to synthesize chemically by the method of N.D. SINHA et al., (Nucleic Acids Res. 12, (1984), 4539-4557), an oligonucleotide probe having a length of 22 bases corresponding to the 5' end of the gene. The selected sequence is as follows : 5'-GCTGCGGTGCTGACCTTGGCCG-3'. Before being used in hybridization experiments, the synthetic oligonucleotide was phosphorylated at its 5' end by means of T4 polynucleotide kinase (P-L Biochemicals) and [γ - 32 P] ATP before being used for hybridization experiments. Conditions for labelling and for hybridization are well-known in the art and are described in detail by T. MANIATIS et al., loc.cit. and by A. BOLLEN et al., (DNA, 2, (1983), 255-264).

Construction of the expression plasmids.

Methods for DNA preparation and DNA fragment isolation, as well as the conditions for restriction enzyme analysis and for ligation of fragments are well known in the art and are described in detail by R.M.LAWN et al., loc. cit. and by T.CABEZON et al., loc. cit. and are applicable herein.

Synthesis of fragments connecting the promoter of expression vectors to the 5' end of the gene are described below.

Synthesis of the NcoI-BalI fragment

Figure 2 shows in detail the principles which are the bases for the design of the 35-mer, 30-mer, 18-mer and 43-mer oligonucleotides used to synthesize the 65/61 bp NcoI-BalI fragment. The 30-mer and 18-mer synthetic fragments were phosphorylated at their 5' ends using T4 polynucleotide kinase (P-L Biochemicals). 1 μ g of each oligonucleotide, including the non-phosphorylated 35-mer and 43-mer, were hybridized for 3 minutes at 95°C in 300 mM sodium acetate (pH 7.0) and then allowed to cool slowly to 4°C. The hybridization mixture was used as such in the cloning procedure for the construction of the expression plasmids.

DNA sequencing.

DNA sequence analysis was carried out according to the method described by A.M. MAXAM and W. GILBERT (Proc.Natl.Acad.Sci.USA, 74, (1977), 560-564) and by F.SANGER et al., (Proc.Natl.Acad.Sci.USA, 74, (1977), 5463-5467).

Protein analysis

Cell pellets having an optical density of 1 unit at 630 nm (1 OD₆₃₀) were obtained at various stages during the fermentation of strains carrying the human proapo A-I expression plasmids, pULB9291, pULB9292, pULB9296 and pULB9299 (transformed into E. coli strain AR58 and JM101 and into yeast strain 10S44c respectively). Each sample was resuspended in a pH 6.8, 50 mM Tris-HCl buffer, containing 2%

sodium dodecyl sulphate (SDS), 6 M urea, 10% glycerol and 5% 2-mercaptoethanol, and was boiled for 3 minutes. Samples were subjected to electrophoresis in polyacrylamide gels (U.K. LAEMMLI, (Nature, 227, (1970), 680-685). Total proteins were visualized by Coomassie Blue staining and synthesized human proapo A-I was identified by immunological recognition following electrophoretic transfer (see A. BOLLEN et al., loc.cit.).

Construction of recombinant vectors for the expression of human proapo A-I in bacteria, yeast and baculovirus infected insect cells.

1. Initial cDNA clone for human preproapo A-I: pULB1609 (Figure 1)

Several hundred transformants, derived from the cloning of cDNAs corresponding to human liver polyA⁺ RNA, into the pstI site of pBR322, were screened by means of the 22-mer apo A-I synthetic probe described above. One of the clones gave an intense hybridization signal during the experiment. This clone, designated pULB1609, was isolated and the DNA insert present in the recombinant plasmid was characterized by DNA sequence analysis. Its length corresponds to 878 base pairs (bp); it encodes the complete preproapo A-I polypeptide. As shown in Figure 1, the cloned cDNA fragment carries 5' and 3' non-coding regions (19 bp and 55 bp respectively), the 54 bp sequence coding for the precursor peptide (aa-24 to aa-7), the 18 bp sequence coding for the propeptide (aa-6 to aa-1) and the 732 bp sequence coding for mature apo A-I (aa1 to aa243) and including the translational stop codon. The protein sequence, deduced from the DNA sequence, corresponds perfectly well with the amino acid sequence found for the preproapo A-I from the protein and from independently isolated cDNA clones (W.C. BARKER et al., Comp.Biochem.Physiol. 57B, (1977), 309-315; Japanese Patent Application No.96998/86; P.CHEUNG and L.CHAN, Loc.cit. and J.J. SEILHAMER et.al., loc.cit.).

2. Construction of a bacterial expression vector containing the human proapo A-I cDNA sequence: pULB9291 (Figures 2 and 3).

pULB9291, a plasmid producing human proapo A-I, was constructed by placing a segment derived from clone pULB1609 behind the regulatable P_L lambda promoter (Fig.3). The construction of this expression plasmid required the synthesis of DNA fragments comprising an NcoI restriction site, an ATG translation initiation codon and the nucleotide sequence encoding the amino acids at the amino end of the human proapo A-I structural gene, up to the first unique restriction site, BalI (Figure 2).

This adapter was synthesized by chemical procedures (N.D.SINHA et al., loc.cit.). Four synthetic oligonucleotides were prepared and after having been hybridized they coded for methionine corresponding to the ATG translation initiation codon, for the 6 amino acids

corresponding to the propeptide and for the first 14 amino acids of mature human apo A-I (Figure 2). The synthetic adapter was designed to minimize the formation of secondary structures at the 5' end of the gene. In order to achieve this, the codons selected to encode amino acid residues -6, -1, 1, 3, 4, 5, 6, 7, 10, 11 and 14 did not correspond to the natural codons observed in the cDNA of the pULB1609 clone.

The synthetic adapter described above was used to join a 744 bp DNA fragment, derived from pULB1609 to the P_L lambda promoter in the expression plasmid pULB1221.

The construction of expression vector pULB1221 is described in European Patent Application 186,643. It comprises three major steps starting from plasmid pCQV2. Plasmid pCQV2 has been described by C.QUEEN in J.Mol.Appl.Genet.2, (1983), 1-10 and is freely available.

The choice of this particular vector is not compulsory; any other vector having a promoter and an

appropriate NcoI site downstream to it can be used. Approximately 0.1 μ g of synthetic fragments, such as described above, were ligated by means of T4 DNA ligase to approximately 1 μ g of the 744 bp BalI-PstI fragment derived from pULB1609 and approximately 1 μ g of the vector pULB1221 cut with NcoI and BalI.

Prior to the ligation, the 744 bp BalI-PstI fragment was treated with T4 DNA polymerase so as to convert the protruding 3' ends into blunt ends. The procedure is well-known in the art and is described in detail by T. MANIATIS et al., loc. cit.

After having been amplified in competent cells of the AR58 strain of E. coli, reconstructed plasmids were characterized by restriction site analysis and DNA sequence of the synthetic fragments and of the junction sites. A recombinant plasmid, pULB9291, satisfied all the criteria since it had the fragments in the correct orientation and order; it was used for expression studies.

3. Construction of a bacterial expression vector containing the fused sequences corresponding to beta-galactosidase and human proapo A-I : pULB9296. (figure 4)

In this construction, the DNA sequence coding for human proapo A-I was fused in the correct reading frame, downstream from the DNA sequence of beta-galactosidase. The beta-galactosidase gene is present on a freely available E. coli expression plasmid, pUR288 (U. RUTHER and B.MULLER-HILL, EMBO J.2, (1983), 1791-1794) which carries an efficiently inducible lac promoter and appropriate restriction sites into the beta-galactosidase sequence. The appropriate recombinant plasmid was constructed as shown in Figure 4. First of all, pUR288 plasmid DNA was successively cut with BamHI, treated with T4 DNA polymerase and cut once again with SalI. Secondly, an 805 bp DNA fragment was isolated from pULB9291 by successively digesting with KpnI, treating with T4 DNA

polymerase and finally digesting with SalI. The two fragments were ligated together, in molar proportions, with T4 DNA ligase and the resulting plasmid was used to transform the competent cells of the E. coli JM101 strain, a strain which is widely and freely available (ATTC 33876). Transformants were checked by restriction analysis for the correct orientation of the human proapo A-I sequence with respect to the beta-galactosidase gene and for the presence of a reconstituted BamHI site at the junction of the two sequences. This indicated that the human proapo A-I sequence was indeed fused downstream from the beta-galactosidase DNA sequence and in the correct reading frame. One of the transformants, containing the plasmid pULB9296, satisfied all the criteria and was used for expression experiments.

4. Construction of a yeast expression plasmid carrying the human proapo A-I cDNA sequence : pULB9299 (Figure 5).

In this construction, the cDNA sequence coding for human proapo A-I was cloned between the promoter and terminal signals carried by a yeast expression plasmid. The yeast expression vector pRIT 10774 was selected for this experiment. The construction of expression plasmid pRIT 10774 is described in European Patent Application 151,102. It was constructed starting, on the one hand from plasmid pRIT 10749, which has been deposited under the terms of the Budapest Treaty in the ATCC under accession number 39133 and, on the other hand, from E.coli-S.cerevisiae shuttle vector YEp13, described by J.R.BROACH et al., in Gene, 8, 1979, 121-133 and which is available from the ATCC under accession number 37115. Vector pRIT 10774 can replicate in both E. coli and yeast and carries the promoter and transcription terminator of ornithine carbamoyl transferase (ARG3) separated by a unique BamHI restriction site convenient for the insertion of a foreign DNA having its own ATG translation initiation codon. In addition, the vector carries yeast

2 μ sequences, metabolic markers for selection in yeast and the AmpA selection marker for shuttling in E. coli. It is not the only vector which can be used for the expression of human proapo A-I in yeasts : any other yeast vector carrying regulatory signals can be used and will yield qualitatively similar results. The construction illustrated in fig.5 proceeded as follows. pRIT 10774 plasmid DNA was linearized by means of the enzyme BamHI and treated with T4 DNA polymerase.

On the other hand, an 810 bP DNA fragment was derived from pULB9291 by digestion with the enzymes Asp718 and SalI, followed by treatment with T4 DNA polymerase. This fragment encodes human proapo A-I and includes the ATG translation initiation codon. The two fragments, obtained as described above, were ligated together in equimolar proportions with T4 DNA ligase and the mixture was used to transform E. coli MM294 competent cells. Transformants were checked by restriction analysis to verify the correct orientation of the proapo A-I DNA sequence with respect to the ARG3 promoter sequence. One of the transformants contained a recombinant plasmid, pULB9299, which satisfied this condition. The plasmid pULB9299 was amplified in E. coli and used to transform spheroplasts of the yeast Saccharomyces cerevisiae strain 10S44c (pep4-3, leu2-3, leu2-112); (T. CABEZON et al., loc.cit.,).

The use of strain Ic 1697d (ATCC No.20631) would lead to qualitatively similar results. Yeasts transformants were then assayed for expression of human proapo A-I.

5. Construction of a bacterial secretion vector containing the human proapo A-I cDNA sequence: pNIV1612 (Figures 6A, B and C).

In this construction, the DNA sequence coding for human proapo A-I was fused, in the correct reading frame,

downstream from the DNA sequence of the E. coli OmpA protein signal peptide. The secretion vector pIN-III-ompA-2 (J. GHAYEB et al., EMBO J.3, (1984), 2437-2442) was selected for this test. This vector and its host strain E. coli JA221 are available on request from the Department of Biochemistry of the State University of New York at Stony Brook.

The secretion vector contains a powerful lpp (lipoprotein) promoter, a lac promoter-operator fragment, the lac I sequence of the lac repressor and appropriate restriction sites situated immediately after the sequence coding for the ompA gene signal peptide. The appropriate recombinant plasmid was constructed as shown in figures 6A, B and C. First of all, the secretion vector pIN-III-ompA-2 was linearized with EcoRI and treated with T4 DNA polymerase. Secondly, an 805bp DNA fragment was removed from pULB9291 by successively digesting with the restriction enzymes KpnI and SalI, followed by treatment with T4 DNA polymerase. This fragment encodes human proapo A-I and contains the ATG translation initiation codon. The two fragments obtained, as described above, were ligated together, in equimolar proportions by means of T4 DNA ligase, and the mixture was used to transform E. coli JA221 strain competent cells grown on M9 medium (J.H.MILLER, in "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1972, p.431.) containing 20 mg/l of tryptophane, 20 mg/l of leucine, 2 g/l of lactose and 50 mg/l of ampicillin. Transformants were selected for their ampicillin resistance and screened with an 18-mer synthetic oligonucleotide (the same as was used in the synthesis of the adapter as shown in figure 2).

The selected transformants were checked by restriction analysis to verify the correct orientation of the proapo A-I DNA sequence with respect to the signal peptide sequence carried by the secretion vector (reconstituted EcoRI site at the junction of the two

sequences). One of the transformants carried a recombinant plasmid which satisfied this condition. The extra sequence derived from the construction with the adapter, underlined in the following nucleotide sequence,

5' GTA GCG GAG GCC GCT GAA TTC ATG AGA CAT TTC TGG 3'
Val Ala Gln Ala Ala Glu Phe Met Arg His Phe Trp

aa₆

was removed by directed mutagenesis. For this purpose, the following 24-mer oligonucleotide sequence

signal peptide --- > < --- proapo A-I
5' GTA GCG GAG GCC AGA CAT TTC TGG 3'
Val Ala Gln Ala Arg His Phe Trp

lacking the 12 extra bases, was synthesized and used to remove the 12-base extra sequence derived from the adapter.

The Amersham system was used for the mutagenesis. This system is based on the method of F.ECKSTEIN et al. (Nucleic Acids Res. 13, (1985), 8765-8785). The method gives a high yield of plaques and the highest available efficiency : up to 95%.

First of all, the region of the DNA to be mutagenized was cloned in an M13 vector. For this purpose, the XbaI-BalI DNA fragment of the recombinant plasmid pIN-III-ompA-2 carrying the proapo A-I gene was inserted in the M13mp19 vector cut by XbaI and HindII. Vector M13mp19 is commercially available; it can be obtained from Amersham (Buckinghamshire, Great Britain). The mutagenic 24-mer oligonucleotide was then hybridized with the single-strand template and extended by the Klenow fragment of DNA polymerase in the presence of T4 DNA ligase, to produce a mutant heteroduplex.

Selective removal of the non-mutant strand was made possible by the incorporation of a thionucleotide into the mutant strand during the in vitro synthesis, and

cleavage of the non-phosphorothionated strand by NCiI. Such cleavages present sites for exonuclease III, which digests the non mutant strand. The mutant strand was then used as a template for reconstructing the double-stranded circular molecule, thus creating a homoduplex mutant molecule. The mutagenesis was verified by sequencing the junction between the signal peptide and the beginning of the proapo A-I gene. The recombinant plasmid, pNIV1612, was reconstructed by ligating the XbaI-HindIII fragment of the pIN-III-ompA-2 vector with the XbaI-BalI fragment from which the 12 extra bases had been deleted and with the BalI-HindIII fragment of the proapo A-I gene.

The three fragments were ligated with T4 DNA ligase and the mixture was used to transform E. coli JA221 competent cells, as described above. Transformants were selected for their ampicillin resistance and screened with the above-mentioned 18-mer oligonucleotide. One of the transformants carried a recombinant plasmid pNIV1612. In the final construction, the sequence coding for the ompA signal peptide preceded the complete proapo A-I sequence without the ATG codon (figures 6A, B and C). E. coli transformants were then assayed for the expression of human proapo A-I.

6. Construction of a transfer vector for the introduction of human proapo A-I cDNA sequence into baculovirus: pNIV1613. (figure 7).

The pNIV1613 plasmid, carrying the human proapo A-I sequence was constructed by placing a segment derived from the clone pULB9291 downstream from the polyhedrin gene promoter of the baculovirus (Figure 7). The baculovirus transfer vector pAcRP6 (Y. MATSUURA et al., J.Gen. Virol., 67, (1986), 1515-1529) was used in these experiments; it can be obtained from the Department of Microbiology and Immunology of the University of Ottawa. The plasmid carries the polyhedrin gene promoter up to

nucleotide -7 in the 5' leader sequence; it lacks the polyhedrin ATG codon and the first 170 nucleotides of the polyhedrin coding sequence. An appropriate BamHI site is located downstream from nucleotide -7. The construction depicted in Figure 7 proceeded as follows. pACRP6 plasmid DNA was linearized with BamHI. On the other hand an 810 bp DNA fragment was removed from pULB9291 by digesting with the restriction enzymes Asp718 and SalI. This fragment encodes human proapo A-I and contains the ATG translation initiation codon. The two fragments, in equimolar proportions, were treated together with T4 DNA polymerase, ligated with T4 DNA ligase and used to transform E. coli AR58 strain competent cells. Transformants were selected for their ampicillin resistance, screened by means of a ^{32}P -labelled synthetic 35-mer oligonucleotide (see Figure 2) corresponding to part of the proapo A-I DNA sequence, and checked by restriction analysis to verify the correct orientation of the proapo A-I DNA sequence with respect to the polyhedrin gene promoter. One of the transformants carried a recombinant plasmid, pNIV1613, which satisfied this condition; it was used for expression experiments.

7. Production of human proapo A-I by transformed micro-organisms.

20 ml cultures of strain AR58 or JM101 of E. coli transformed with pULB9291 and pULB9296 respectively, were grown in rich medium supplemented by 50 $\mu\text{g/ml}$ ampicillin (LB broth, see T. MANIATIS et al., loc.cit. for experimental details) until the optical density reached 0.6 at 630 nm. In the case of pULB9291, induction of the P_L lambda promoter was achieved by changing the initial growth conditions of the culture from 30°C to 42°C, so as to inactivate the repressor of the P_L lambda promoter (M. ROSENBERG et al., Methods Enzymol. 101, (1983), 123-138). Induction was carried out for 20 minutes.

In the case of pULB9296, induction of the lac promoter was achieved by adding to the culture, incubated at 37°C, the chemical inducer IPTG (isopropyl-beta-D-thiogalactoside) until a final concentration of 1 mM was reached (L.LORENZETTI et al., loc.cit.) Induction was carried out for 60 minutes.

On the other hand, 20 ml cultures of yeast cells 10S44c, transformed with pULB9299, were grown at 30°C in yeast nitrogen base medium (Difco) supplemented with glucose (1%) until the optical density reached 0.3 at 630nm. No inducer was necessary since the expression is constitutive in this particular case.

One ml samples of the above cultures were taken and centrifuged at 15,000 g for 5 minutes. The pellets obtained were lysed in boiling sodium dodecyl sulphate (SDS) as follows. The pellets were resuspended in 50 μ l of SDS sample buffer (50 mM of Tris-HCl, pH 6.8, 2% SDS, 6M urea, 5% 2-mercaptoethanol and 10% glycerol) and boiled for 3 minutes at 100°C. Extracts were then centrifuged at 15,000 g for 10 minutes. The samples were then ready for electrophoretic analysis on 15% or 7.5% polyacrylamide gels, in the presence of SDS, in denaturing conditions (U.K. LAEMMLI, loc.cit.).

After electrophoresis, the polyacrylamide gels were washed briefly with 40 ml of distilled water and with 40 ml of 50 mM sodium phosphate buffer at pH 7.5. The proteins were then transferred electrically from the gels onto nitrocellulose sheets for 2 hours at 60 V and 1.5 A in the same phosphate buffer (T.CABEZON et al., loc cit.) The nitro cellulose sheets were saturated with albumin (1%) in 50 mM sodium phosphate buffer at pH 7.5, then incubated at ambient temperature over night in the presence of rabbit anti-human apo A-I serum at a dilution of 1/500 (and of mouse anti-beta-galactosidase monoclonal antibodies in the case of pULB9296) in the same buffer without albumin.

The sheets were washed five times with 40 ml of the same phosphate buffer and were then incubated in 40 ml of phosphate buffer containing 10 μ g/ml of goat anti-rabbit (or anti-mouse) serum and labelled with peroxylase. After 4 hours of incubation at ambient temperature, the sheets were washed again five times with 40 ml phosphate buffer and finally revealed by adding 50 ml of a chromogenic substrate solution (10 mg diaminobenzidine, 100 μ l of 10% urea peroxide and 100 mM Tris-HCl at pH 7.6).

In the case of pULB9291 and pULB9299, a single product was found reacting with the anti-human apo A-I antibodies. It had a molecular weight consistent with that of standard natural apo A-I. In the case of pULB9296, a fused polypeptide reacting with anti-human apo A-I serum and with anti-beta-galactosidase serum was found at the expected size for the sum of the beta-galactosidase and proapo A-I polypeptides (144kDa). These sizes were previously determined from a calibration curve based on the migration of molecular weight standards placed on the same gels as the cell extracts.

In another experiment, 20 ml cultures of E. coli strain JA221, transformed with pNIV1612 were grown in rich medium supplemented with 50 μ g/ml of ampicillin (LB broth, see T.MANIATIS et al., loc.cit.,) until the optical density reached 0.6 at 630 nm. Induction of the lac promoter was performed by adding to the culture, incubated at 37°C, the chemical inducer IPTG (isopropyl-beta-D-thiogalactoside) to a final concentration of 2mM. Induction was performed for 60 minutes. One ml samples of the cultures were taken and centrifuged at 15,000 g for five minutes. The pellets obtained were submitted to a mild osmotic shock in order to release the periplasmic fraction (D.KOSHLAND and D.BOTSTEIN, Cell, 20, (1980), 749-760). The released fraction was resuspended in the above-mentioned SDS sample buffer, but without urea, boiled, centrifuged and subjected to electrophoresis on

12.5% polyacrylamide gel in the presence of SDS, followed by immunodetection after electrophoretic transfer. A fraction of the synthesized proapo A-I was found in the cell and not in the periplasm. This was due either to the fact that some proapo A-I was not secreted or that the efficiency of the osmotic shock was not optimal. The main fraction of proapo A-I was found to be released into the medium after the osmotic shock, indicating that the protein was indeed secreted by the cells.

8. Production of human proapo A-I by baculovirus infected insect cells.

The recombinant plasmid pNIV1613 has been used in conjunction with the wild strain baculovirus to coinfect Spodoptera frugiperda cells in culture. Screening of recombinant viruses, lacking polyhedrin, gave recombinant plaques. The recombinant virus, purified from a plaque, was used to infect insect cells. The procedure is well-known in the art and is described in detail by M.D. SUMMERS and G.E. SMITH in "A manual of methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas University, College Station, (1987)". The recombinant virus was assayed immunologically for the production of proapo A-I, following electrophoretic transfer and by electrophoresis on a 12.5 % polyacrylamide gel in the presence of SDS, after lysis of the cells with the RIPA buffer (0.05 M Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl, 1 % Triton 100, 0.1 % SDS, 0.1 % deoxycholate and 1 mM of phenylmethylsulphonyl fluoride (PMSF)) and treatment with boiling sodium dodecyl sulphate. A single product reacting with the anti-human apo A-I antibodies was found. It had a molecular weight consistent with that of standard natural apo A-I and the expressed protein represented the major constituent of the total protein content. The concentration of proapo A-I per litre of culture, measured by single radial immunodiffusion

(G. MANCINI et al., Immunochem.2, (1965), 235-254) was estimated as being approximately 100 mg.

9. Cytoplasmic production of human proapo A-I in *E.coli*.
Use of a defined minimal medium.

The plasmid pULB9292 was used for the cytoplasmic production of human proapo A-I in *E. coli* in a minimal medium. pULB9292 was constructed by exchanging the fragment EcoRI-NcoI of the plasmid pULB9291 coding for the P_L lambda promoter, with same fragment EcoRI-NcoI of the plasmid pOTS. (M.ROSENBERG et al., Methods Enzymol.101, (1983),123-138). The fragment EcoRI-NcoI of the pOTS vector (G.DEVARE et al., Cell,36,(1984)43-49) also contains the efficient regulatable P_L promoter of phage lambda. 20 ml cultures of *E. coli* strain AR58 transformed with pULB9292 were grown in a defined minimal medium. The composition of the minimal medium was (per litre): 3g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 3 g KH_2PO_4 ; 0.5 g NaCl; 1 g NH_4Cl ; 1.37mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 29.5 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 236 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 10 g glucose; 1 mg vitamin B1; 50 mg ampicillin; LB broth 1/20 (v/v). Cells were grown in this minimal medium until the optical density reached 0.5 at 630 nm. Induction of the P_L lambda promoter was achieved by changing the initial growth conditions of the culture from 30°C to 42°C in order to inactivate the repressor of the P_L lambda promoter (M.ROSENBERG et al., loc.cit.). Induction was performed for 60 minutes. One ml samples of the cultures were taken and passed through the French's press or were centrifuged at 15,000 g for 5 minutes. The total cell extract obtained or the pellet were treated with boiling SDS as described in paragraph 7. After electrophoresis and electrophoretic transfer, a single product was found which reacted with the anti-human apo A-I antibodies. It had a molecular weight consistent with that of standard natural apo A-I. The amount of expressed proapolipoprotein A-I in the defined

minimal medium represented 13.5% of the total proteins content, i.e. an estimated concentration of proapo A-I of about approximately 270 mg per litre of culture.

10. Isolation, purification and characterization of human proapo A-I produced by transformed micro-organisms.

10.1 Isolation and purification.

Crude extracts of the recombinant proapo A-I were centrifuged at 4,000 g for 15 minutes and the pellet was discarded. The supernatant was centrifuged at 100,000 g for two hours. The resulting pellet was resuspended in a minimal volume of a buffer (TEN100) consisting of 20 mM of Tris-HCl, pH 7.5; 1mM of EDTA, 100 mM of NaCl; 1.75 µg/ml of PMSF and 100 µg/ml of sodium merthiolate (sodium salt of ethyl-mercuri-thiosalicylic acid) and the volumes of the suspension and of the supernatant were adjusted separately to the original extract volume by means of the same buffer. Protein was then precipitated from the two suspensions using increasing concentrations of isopropyl alcohol. The precipitated protein fraction from each suspension which contained the major part of the immunoreactivity corresponding to human apo A-I, was determined by radial immunodiffusion (G. MANCINI et al., loc. cit.), using a commercial apo A-I standard. Each fraction thus obtained was further purified by chromatography on a Sephacryl S200 column, using the same buffer as eluent. 0.9 ml fractions were collected and the total quantity of protein having an immunoreactivity corresponding to that of apo A-I was determined in each fraction by radial immunodiffusion. The molecular weight of the eluted protein in the fractions was determined by calibrating the column with standards having known molecular weights such as aldolase, bovine serum, ovalbumin, albumin, chymotrypsinogen and cytochrome

C, under identical conditions. The purity of the proapo A-I per mg of total protein in the principal fractions containing recombinant proapo A-I, expressed in mg of protein having the same immunoreactivity as a commercial apo A-I standard, was estimated as being 95 %.

10.2. Characterization.

10.2.1. Physical properties of recombinant proapo A-I.

When the recombinant proapo A-I, purified and isolated from the supernatant and from the pellet from centrifuging at 100,000 g, is subjected to isoelectric focusing according to the process of N. CATSIMPOOLAS (Anal. Biochem. 26, (1969), 54-62) and an autogenerated gradient of pH 4 to pH 6 is applied, a single band is obtained having an estimated isoelectric point of 4.95. Human plasma apo A-I appears to be slightly more acidic and has an estimated isoelectric point of 4.75. This difference of 0.2 pH units between the values of isoelectric points of recombinant proapo A-I and plasma apo A-I is in good agreement with the known difference between the values of isoelectric points for plasma apo A-I and plasma proapo A-I, which has been reported as being equal to 0.17 (G.L. MILLS et al., Lab.Tech.BiochemMol.Biol.14, (1984), 244-245).

As regards molecular weight, both the pellet and supernatant recombinant proapo A-I consist of a single polypeptide chain having identical molecular weights equal to 29.9 ± 1.4 kDa. Human plasma apo A-I

appears to be slightly smaller, having a molecular weight equal to 29.3 ± 1.3 kDa.

10.2.2. Peptide mapping of recombinant proapo A-I with BNPS-skatol.

Chemical cleavage was carried out by means of 3-bromo-3-methyl-2-[(2-nitrophenyl)thio] -3H-indole (BNPS-skatol) according to the process by A. FONTANA (Methods Enzymol. 25, (1972), 419-423).

5 to 10 μ g of purified protein preparations were dissolved in 100 μ l of a 0.15 % (v/v) solution of phenol in 50 % (v/v) aqueous acetic acid solution. 50 μ l of a solution of 4.8 mg of BNPS-skatol per ml of glacial acetic acid were then added, followed by incubation at 25°C for 72 hours. 50 μ l of 2-mercaptoethanol were then added, followed by incubation a second time at 37°C for 5 hours. The samples were evaporated, redissolved in 100 μ l of water and extracted 3 times with 200 μ l of ethyl acetate. The organic phases were discarded and the aqueous phases were lyophilized and analysed by electrophoresis on SDS-polyacrylamide gel.

In the case of chemical cleavage by BNPS-skatol, the number and size of the fragments derived from apo A-I can be more or less predicted since, under the experimental conditions used, the BNPS-skatol selectively cuts after the tryptophane residues. Assuming an efficiency of 100 % at each cleavage site, the largest fragment which may be expected is a C-terminal fragment of 15.4kDa. The molecular weights of the remaining

fragments are between 0.5 and 5.3 kDa and are consequently too small to be detected.

In the case of incomplete cleavage, the 15.4 kDa fragment will be "elongated" in the direction of the N-terminus, yielding fragments of 20.7 kDa, 23.1 kDa and 27.6 kDa respectively. These predictions are very successfully achieved for human plasma apo A-I as well as for the various purified preparations of recombinant proapo A-I.



CLAIMS for the contracting States : AT, BE, CH, DE, FR, GB,
GR, IT, LI, LU, NL, SE.

1. Recombinant DNA sequence coding for human proapolipoprotein A-I and capable of reducing or preventing the formation of hairpin structures, characterized in that it comprises (a) a synthetic DNA fragment having the sequence (only one strand shown):
5'-ATGAGACATTTCTGGCAGCAGGACGAACCTCCACAATCTCCTTGGGATAGAG
TTAAGGACTTG-3', coding for the amino acids -6 to +14 of human proapolipoprotein A-I and comprising an added ATG translation initiation codon and modified codons for the amino acid residues -6, -1, +1, +3, +4, +5, +6, +7, +10, +11 and +14 and (b) downstream from the synthetic DNA fragment, the natural DNA sequence coding for the amino-acids +15 to +243 of human proapolipoprotein A-I.
2. Replicable cloning vector, characterised in that it contains the recombinant DNA sequence according to Claim 1.
3. Expression vector, characterized in that it contains the recombinant DNA sequence according to Claim 1 operably linked to a regulatory DNA sequence which regulates the expression of the said recombinant DNA sequence.
4. Expression vector according to Claim 3, characterized in that the regulatory DNA sequence comprises the phage lambda P_L promoter region.
5. Expression vector according to Claim 3, characterized in that the recombinant DNA sequence is fused downstream from the beta-galactosidase DNA sequence and in that the regulatory DNA sequence comprises the E. coli lac promoter region.



6. Expression vector according to Claim 3, characterized in that the regulatory DNA sequence comprises the yeast ARG3 promoter and transcription terminator regions.
7. Expression vector according to Claim 3, characterized in that the recombinant DNA sequence lacking the ATG codon is fused downstream from the DNA sequence of the E. coli Ompa protein signal peptide, and in that the regulatory DNA sequence comprises the lpp promoter, the lac promoter-operator and the lac I sequence of the lac repressor.
8. Expression vector according to Claim 3, characterized in that the regulatory DNA sequence comprises the baculovirus polyhedrin gene promoter region.
9. Cell culture or micro-organism transformed with an expression vector according to any one of Claims 3 to 8.
10. Process for producing human proapolipoprotein A-I characterized in that a transformed cell or microorganism according to Claim 9 is cultured under appropriate conditions, and in that the human proapolipoprotein A-I thus produced is recovered.



CLAIMS for Contracting States : ES

1. Process for the preparation of a recombinant DNA sequence coding for human proapolipoprotein A-I and capable of reducing or preventing the formation of hairpin structures, characterized in that the natural DNA sequence coding for amino acids +15 to +243 of human proapolipoprotein A-I is ligated downstream from a synthetic DNA fragment having the sequence (only one strand shown) :
5'-ATGAGACATTTCTGGCAGCAGGACGAACCTCCACAATCTCCTTGGGATAGAGTTAAGGACTTG-3' coding for the amino acids -6 to +14 of human proapolipoprotein A-I and comprising an additional ATG translation initiation codon and modified codons for the amino acid residues -6, -1, +1, +3, +4, +5, +6, +7, +10, +11 and +14.
2. Process for the preparation of a replicable cloning vector, characterized in that the recombinant DNA sequence prepared according to the process in Claim 1 is introduced into an appropriate vector.
3. Process for the preparation of an expression vector, characterized in that the recombinant DNA sequence prepared according to the process of Claim 1 is operably ligated to a regulatory DNA sequence which regulates the expression of the said recombinant DNA sequence.
4. Process according to Claim 3, characterized in that the regulatory DNA sequence comprises the phage lambda P_L promoter region.
5. Process according to Claim 3, characterized in that the recombinant DNA sequence is fused downstream from the beta-galactosidase DNA sequence and in that the regulatory DNA sequence comprises the E. coli lac promoter region.



6. Process according to Claim 3, characterized in that the regulatory DNA sequence comprises the yeast ARG3 promoter and transcription terminator regions.
7. Process according to Claim 3, characterized in that the recombinant DNA sequence lacking the ATG codon is fused downstream from the DNA sequence of the E. coli Ompa protein signal peptide and in that the regulatory DNA sequence comprises the lpp promoter, the lac promoter-operator and the lac I sequence of the lac repressor.
8. Process according to Claim 3, characterized in that the regulatory DNA sequence comprises the baculovirus polyhedrin gene promoter region.
9. Preparation process for a cell or micro-organism, characterised in that a cell or micro-organism is transformed with an expression vector prepared according to a process such as defined in any one of Claims 3 to 8.
10. A cell culture or micro-organism transformed with an expression vector prepared according to a process such as defined in any one of Claims 3 to 8.
11. Process for the production of human proapolipoprotein A-I, characterized in that a transformed cell or micro-organism according to Claim 10, is cultured under suitable cultivating conditions and in that the human proapolipoprotein A-I thus produced is recovered.