PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 1/21, 15/70, 15/72, C07K 1/22, 14/775, A61K 38/17

A1

(11) International Publication Number: **WO 95/25786**

(43) International Publication Date: 28 September 1995 (28.09.95)

(21) International Application Number: PCT/US95/03826

(22) International Filing Date: 21 March 1995 (21.03.95)

(30) Priority Data:
215,749 21 March 1994 (21.03.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 215,749 (CIP)
Filed on 21 March 1994 (21.03.94)

(71) Applicant *(for all designated States except US)*: AMERICAN CYANAMID COMPANY [US/US]; One Cyanamid Plaza, Wayne, NJ 07470 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MALIK, Sohail [PK/US]; 3B Sixth Street, Englewood Cliffs, NJ 07632 (US). MAHLBERG, Florence [FR/FR]; 11, rue de Moulineaux, F-92150 Suresnes (FR). KARATHANASIS, Sotirios [GR/US]; 567 Route 9W, Grandview, NY 10960 (US).

(74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, US, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: PRODUCTION OF HUMAN APOLIPOPROTEIN A1 IN A BACTERIAL EXPRESSION SYSTEM

(57) Abstract

Biologically active recombinant apolipoprotein A1 (apoA1) is produced in a T7 RNA polymerase/promoter bacterial expression system that optimizes yield and facilitates subsequent purification of the protein. ApoA1 cDNA is cloned into an *Escherichia coli* plasmid containing a bacteriophage T7 RNA polymerase promoter; the recombinant plasmid is introduced into an *E. coli* strain containing inducible bacteriophage T7 RNA polymerase; T7 RNA polymerase is induced in the strain; and the resulting expresses apoA1 is isolated from the culture. The apoA1 protein may be a fusion protein that includes an additional sequence that facilitates purification. Recombinant apoA1 is useful in the treatment and in the prevention of various circulatory related diseases.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

PRODUCTION OF HUMAN APOLIPOPROTEIN A1 IN A BACTERIAL EXPRESSION SYSTEM

This is a continuation-in-part of U.S. Serial No. 08/215,749, filed March 21, 1994, now pending.

Field of the Invention

This invention relates to the production of pure, stable, mature and biologically active human apolipoprotein A1 (apoA1) in high yield and large quantities using a bacterial expression system. ApoA1 plays a role in various circulatory processes, including the stimulation of reverse cholesterol transport and the prevention of various conditions involving circulatory disturbances, including atherosclerosis, restenosis, and septic shock.

Background of the Invention

Epidemiological and genetic studies have implicated decreased plasma levels of high density lipoproteins (HDL) in the pathogenesis of atherosclerosis, while increased HDL levels have been correlated with protection against coronary heart disease and have been associated with longevity (Karathanasis, S., *Monogr.Hum.Gen.* **14**:140, 1992). HDL has been demonstrated to stimulate cholesterol efflux from cells, and it has been hypothesized that HDL acts as a carrier for reverse cholesterol transport, i.e., the delivery of cholesterol from the peripheral tissues to the liver for disposal (Karathanasis, *supra*). This hypothetical mechanism is consistent with the inverse correlation observed between the size of total body

cholesterol pools and plasma HDL level and between HDL levels and coronary heart disease. HDL may alternatively be a marker of a certain pattern of lipid transport and metabolism that protects against the development of atherosclerosis. Thus, HDL levels can be used to assess the risk that an individual will develop atherosclerosis.

Apolipoprotein Al (apoA1) is the major protein constituent of HDL. It is a relatively abundant plasma protein (~1 to 1.5 mg/ml) (see Karathanasis, et al., *Proc. Natl. Acad. Sci. USA*, 80:6147-6151, 1983; Cheung, P., and Chan, L., *Nucl. Acids Res.*, 11:3703-3715, 1983, and the references cited therein). ApoA1 has been shown to be necessary for the extraction of cholesterol from ascite cell membranes, and the protein is a potent activator/cofactor of lecithin:cholesterol acyltransferase, a plasma enzyme that catalyzes the conversion of cholesterol and phosphatidylcholine to cholesterol esters and lysophosphatidylcholine (*Cheung*, supra).

ApoA1 is synthesized predominantly in the liver and the small intestine in mammals, but has been found in a variety of other tissues in the rooster (*Cheung*, supra). The cDNA for human hepatic apoA1 has been cloned by different laboratories (Karathanasis, et al., *supra*; Cheung and Chan, *supra*). The predicted primary translation product of apoA1 mRNA is a preproprotein that undergoes intra- and extracellular proteolytic processing to produce the major apoA1 isoprotein form observed in plasma. Deficiencies in apoA1 are associated with abnormalities in lipoprotein metabolism that result in low plasma HDL levels and may contribute to the development of premature atherosclerosis. Indeed, genetic deficiencies in apoA1 and HDL are associated with intracellular cholesterol accumulation and premature atherosclerosis.

Furthermore, apoA1 may be be useful in the treatment or the prevention of other disease processes such as, for example, to inhibit progression of aortic atherosclerosis in rabbits (Akira et al., American Heart Assoc. abstracts, 1992, page 76); to protect erythrocytes against the generation of procoagulant activity (Epand et al., Abstract # 493, American Society for Biochemistry and Molecular Biology, 1994); and to reverse the inhibitory effect of oxidized LDL on endothelium-dependent arterial relaxation (Matsuda et al., *Circ.Res.* 72:1103, 1993). See also, Tytler et al., American Heart Assoc. abstracts, 1992, page 76 and Burkey et al., American Heart Assoc. abstracts, 1992, page 38.

In addition to playing some role in cholesterol transport and atherosclerosis, apoA1 appears to play a role in restenosis and septic shock. Restenosis is the narrowing or stricture of blood vessels, typically after angioplasty or surgical correction of the condition. Septic shock, associated with both gram-negative and gram-positive infections, is characterized by inadequate vasomotor tone resulting in profound hemodynamic disturbances involving a marked decrease in peripheral vascular resistance. Recently, purified apoA1 has been shown to prevent endotoxin-induced monokine release by human low and high-density lipoproteins (Flegel, W.A., et al., *Infection and Immunity*, 61:5140-5146, 1993). Furthermore, transgenic rats expressing high levels of apoA1 exhibited a decrease in aortic smooth muscle cell proliferation following balloon angioplasty (Burkey et al., *Circul.* 86:1876, 1992).

It is believed that, because of their involvement in cholesterol metabolism, apoA1 and HDL are important in protection against coronary artery disease. However, it has not been possible to obtain sufficiently large quantities of biologically active apoA1, particularly for assessing the mode of action of apoA1 including its role in lipid metabolism, and for the elucidation of its crystal structure. Isolation of apoA1 from plasma is arduous and risky. The protein has been expressed in Chinese hamster ovary cells, but only relatively low levels of 30% of secreted protein have been achieved (Mallory, J.B., et al., *J. Biol. Chem.*, 262:4241-4247, 1987). Previous attempts to express the apoA1 sequence in *E. coli* were unsuccessful due to the instability of the recombinant protein product. Attempts to express the protein as a β-galactosidase fusion protein also produced an unstable product (*ibid*).

Expression of other proteins in *E. coli* has resulted in variable yields. Citovsky, et al., reported a yield of only 100 to 200 pg *Agrobacterium* VirE2 protein/10^9 cells (Citovsky, V., et al., *Proc.Nat.Acad.Sci.USA* 86:1193-1197, 1989). Breyer, et al. achieved an level of expression of the $ß_2$ adrenergic receptor of only 0.01% of the total protein (Breyer, R.M., et al., *EMBO J.* 9:2679-2684, 1990). In a preparation of recombinant cytochrome C reductase, Campbell obtained 0.172 mg/ml protein in the crude extract (Campbell, W.H., *Plant Physiol.* 99:693-699, 1992)

Thus, there is a need in the art for methods and compositions that provide large quantities of pure, stable, and mature apoA1 at a reasonable cost.

Brief Description of the Drawing

Figure 1 is a graphic illustration of the concentration of recombinant apoA1 in plasma samples from mice administered recombinant apoA1 intraperitoneally (x), subcutaneously (circles), or intravenously (triangles).

Summary of the Invention

A method is provided for producing biologically active apolipoprotein A1 (apoA1) in a bacterial expression system at high levels.

The method comprises:

(a) subcloning apoA1 cDNA into a recombinant *Escherichia coli* plasmid to obtain a recombinant apoA1 fusion protein-encoding plasmid, wherein the cDNA is operatively linked to a bacteriophage T7 RNA polymerase promoter and is operatively linked to a DNA sequence encoding an additional amino acid sequence that facilitates purification of proteins;

(b) transferring the recombinant apoA1 fusion protein-encoding plasmid to *E. coli* cells of a strain containing inducible bacteriophage T7 RNA polymerase, to yield transformed *E. coli* cells;

(c) inducing the T7 RNA polymerase and growing the transformed cells in a culture for a sufficient time and under appropriate conditions to produce a biologically active recombinant apoA1 fusion protein;

(d) harvesting and lysing the cells to produce a soluble fraction containing the recombinant apoA1 fusion protein; and

(e) isolating recombinant apoA1 fusion protein from the soluble fraction.

In a preferred embodiment, the additional sequence comprises a cluster of positively charged amino acid residues, i.e., arginine, histidine, lysine and mixtures thereof. Most preferably, the added sequence comprises histidine residues, and particularly 6 to 10 histidine residues. The use of this sequence allows one-step purification using a metal affinity

or chelating column such as a nickel (II)-nitrilotriacetic acid agarose or a metal chelating Sepharose® column charged with nickel (II). Following purification, the additional sequence may be proteolytically removed from the recombinant fusion protein. The final product may also be extracted with organic solvents and subjected to an additional chromatographic step to reduce the amount of any bound lipid.

In another aspect, the present invention provides methods for preventing or treating atherosclerosis and cardiovascular restenosis in animals, preferably mammals and most preferably humans. The subject is administered a treatment effective amount of biologically active recombinant apoA1 protein that has been prepared according to the present invention. The treatment effective amount can be provided over a sustained period of time to yield at least a 10% increase in the total plasma level of apoA1 in the subject. In the case of restenosis, the treatment effective amount of recombinant apoA1 is administered before, during, after, or any combination thereof, surgical intervention procedures such as, for example, angioplasty.

Detailed Description of the Invention

The present invention encompasses methods and compositions that allow the production of human apolipoprotein Al (apoA1), which also includes the apoA1 fusion proteins described herein, in high yields in *Escherichia coli* using a T7 polymerase-promoter expression system. An apoA1 gene is introduced into a plasmid vector, the vector is introduced into living *E. coli* cells, and large amounts of apoA1 are produced and isolated from the culture.

In accordance with the invention, a gene for apoA1 is cloned into a vector so that it is operatively linked to a promoter recognized by bacteriophage T7 RNA polymerase. Enhanced expression of the apoA1 gene is achieved by introducing the plasmid into an *E. coli* strain that contains one or more copies of the gene encoding T7 RNA polymerase, using a modification of the method described by Studier, F.W., and Moffatt, B.A., *J. Mol. Biol.* **189**:113-130, 1986. The DNA encoding the viral polymerase either permanently resides within the *E. coli* or is introduced into cells transiently by infection with a specialized phage.

DNA encoding apoA1 for introduction into the bacterial vector may be directly

isolated from mammalian tissue using procedures well known in the art such as, for example, mechanical shearing, enzymatic shearing, or phenol extraction, followed by gel electrophoresis. Alternatively, the DNA may comprise recombinant cDNA prepared from cloned genomic libraries. (See, for example, Karathanasis et al., *Proc. Natl. Acad. Sci. USA*, 80:6147-6151, 1983; Cheung et al., cited above). Any clone or subclone containing the complete sequence of mature apoA1, including a polymerase chain reaction (PCR) product synthesized from a clone, may be introduced into an *E. coli* vector for the purpose of bacterial expression of biologically active apoA1.

A "vector" is any self-replicating DNA molecule that can transfer a DNA segment between host cells and includes plasmid cloning vectors. Any vector containing a bacteriophage T7 RNA polymerase promoter and adjacent cloning sites may be employed such as, for example, pT or pET plasmid cloning vectors described by Ausubel, F.M., et al., eds., *Short Protocols in Molecular Biology*, 2nd ed., John Wiley & Sons, New York, 1992, Figure 16.2.1, page 16-7; plasmids derived by inserting bacteriophage T7 DNA or T7 DNA plus *lac, trp,* or *tac* DNA, or other DNA used as a marker or to promote transcription, into restriction sites such as *Bam*HI on *E. coli* plasmids, including, but not limited to, pBR322 as described by Studier and Moffatt, cited above; or vectors commercially available from Novagen (Madison, WI 53711). pET vectors such as pET3, pET5, pET9, pET11, pET12, pET14, pET15, pET16, pET17, and pET19 to pET25 plasmids are preferred, especially the pET-11 series (pETlla-d), pET-15b, pET16b, pET-19b, the pET21 series, pET22b(+), the pET24 series, and pET25b(+). The pET11 series is particularly preferred, including, but not limited to, plasmid pET11d.

Preferably, apoA1 is expressed as a fusion protein containing additional amino acid sequences that facilitate purification of the polypeptide, particularly by cation or anion exchange, affinity, or immunoaffinity chromatography. This is achieved by cloning apoA1-encoding DNA so that DNA sequences encoding the desired additional amino acids are fused in frame to the 5' or 3' terminus of the apoA1 protein coding sequence. Examples of such additional sequences include without limitation clusters of charged amino acids from 3 to 15 residues in length; sequences derived from glutathione-S-transferase that bind glutathione; maltose binding protein; and protein A. Preferably, the additional sequence comprises a

cluster of positively charged amino acids i.e., arginine, histidine, lysine and mixtures thereof, which facilitates rapid and inexpensive purification by, for example, metal chelation chromatography; most preferably, the sequence comprises 6 to 10 histidine residues. The vectors may be designed so that the additional sequences may optionally be proteolytically removed after purification.

The expression vector described above contains the gene for apoA1 and is introduced into *E. coli* cells of a strain containing an inducible copy of the T7 RNA polymerase gene, such as a strain having a chromosomal copy of the enzyme under *lac* control. Transcription of the gene is induced, for example, by the addition of a chemical to, or physical manipulation of, the bacterial media. Typical hosts are lysogens of bacteriophage λ*DE3*. These strains contain the polymerase gene under the control of an inducible *lacUV5* promoter. In this system, the addition of isopropylthiogalactose (IPTG) to the culture induces the polymerase, which in turn transcribes the target DNA in the plasmid. A number of *E. coli* strains are commercially available for this purpose, including lysogenic BL21(DE3) and HMS174(DE3) strains. BL21(DE3) strains are particularly preferred when pET plasmids are used. Special mention is made of BL21(DE3)pLysS.

After induction of T7 RNA polymerase, the *E. coli* strain is grown for a sufficient time and under conditions suitable to produce mg/liter amounts of apoA1 in the culture. The time sufficient for apoA1 expression may be, for example, from about 2 to about 5 hours; suitable conditions include the optical density at which induction is initiated, which may range, for example, from about 0.3 to about 1.0 in small-scale production and from about 10 to about 25 in large-scale fermentor production, and aeration, which is achieved by agitating the cultures at speeds ranging, for example, from about 100 to about 350 rpm, and preferably from about 200 to about 300 rpm, using conventional microbiological shakers.

Any type of solidified or liquid medium that will support growth and reproduction of *E. coli* is useful in cultures for practicing the method of the invention, but liquid medium is preferred to facilitate isolation of expressed proteins produced therein. Numerous suitable bacterial media are known to those skilled in the art. Typical media include both minimal and rich media. Minimal media include mixtures of magnesium sulfate

and a carbon source, typically sugar or glycerol, with M9 medium containing disodium phosphate, monobasic potassium phosphate, ammonium chloride, sodium chloride and, optionally, calcium chloride, M63 medium containing ammonium sulfate, monobasic potassium phosphate, and ferrous sulfate; or a medium containing ammonium sulfate, monobasic potassium phosphate, dipotassium phosphate, and sodium citrate. Thiamine, Casamino acids (Difco, Detroit, MI 48232-7038), L amino acids, and antibiotics may be added, if required. Rich media include H medium containing tryptone and sodium chloride; L-broth containing tryptone and sodium chloride; LB medium containing tryptone, yeast extract, sodium chloride, and sodium hydroxide, NZC broth containing NZ amine A (Hunko Sheffield), sodium chloride, magnesium chloride and Casamino acids (Difco); Superbroth containing tryptone, yeast extract, sodium chloride, and sodium hydroxide; tryptone broth containing tryptone and sodium chloride; TY medium containing tryptone, yeast extract, and sodium chloride; and TYGPN medium containing tryptone, yeast extract, glycerol disodium phosphate and potassium nitrate.

After induction of T7 RNA polymerase and further growth of the cultures, *E. coli* cells are typically harvested by centrifugation, lysed, and separated into soluble and particulate fractions. Suitable lysis methods include without limitation sonication, osmotic shock, enzymatic digestion, mechanical fragmentation, and combinations thereof. Recovery of the soluble fraction may be achieved by, for example, centrifugation or by decantation of the aqueous layer from the cell debris. The recombinant apoA1 is isolated from the soluble fraction.

Purification of apoA1 may be achieved using any method known in the art. Such methods include without limitation as dialysis, gel filtration, ion exchange chromatography, affinity chromatography, electrophoresis, or combinations thereof. When additional sequences are fused to the expressed apoA1 sequence, purification from the soluble fraction may be achieved in a single step using, for example, metal chelation chromatography, affinity chromatography, immunoaffinity chromatography, and anion- and cation-exchange chromatography. It will be understood that the purification method used will depend upon the additional sequence that is added. For example, when 6-10 histidine residues are fused to the apoA1 sequence, metal chelation chromatography, and preferably

Ni(II)nitrilotriacetic acid agarose chromatography, may be used to purify the recombinant apoA1. Alternatively, when the added fused sequence comprises glutathione-S-transferase, affinity chromatography on a glutathione resin may be used to purify the recombinant apoA1 fusion protein.

Following purification of the recombinant apoA1 as described above, the additional sequence may be proteolytically removed from the polypeptide. In general, proteases useful in removing additional sequences include without limitation thrombin and factor X. When the additional sequences comprise aminoterminal histidine residues, removal may be achieved by incubation of the purified product with thrombin (Sigma Chemical Co., St. Louis, MO). Typically, 1 mg of purified recombinant apoA1 is incubated with 200 units of thrombin in cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% (v/v) β-mercaptoethanol). ApoA1 from which the additional sequence has been cleaved is then further purified using a second round of chromatography, such as, for example, metal chelation chromatography.

Further purification may also be performed, with or without proteolytic removal of additional sequences, by extracting with organic solvents to remove bound lipid partially or totally, including lipopolysaccharide. Suitable solvents include without limitation ethanol, chloroform, methanol, and mixtures of any of the foregoing. Extraction can be performed directly on lyophilized material or on apoA1 that has been precipitated at 4°C in 90% ethanol at pH 6. This purification step may be optionally combined with chromatography on a MonoQ column using the FPLC system (Pharmacia).

The methods of the present invention provide extraordinarily high yields of stable, bioactive recombinant apoA1. T7 RNA polymerase synthesizes RNA at a rate several times that of *E. coli* RNA polymerase and terminates transcription less frequently. T7 RNA polymerase is also highly selective for transcription initiation using its own promoter sequences and does not initiate transcription from *E. coli* DNA sequences. Furthermore, T7 RNA polymerase is resistant to many antibiotics, such as, for example, rifampicin, that inhibit *E. coli* RNA polymerase.

According to the present invention, apoA1 accumulates to greater than 25%, and preferably about 30% to about 40%, of the total bacterial protein in the culture. Yields

of at least 1 mg apoA1 per liter of culture, preferably about 2 to 3 mg apoA1 per liter, and most preferably greater than about 5 mg apoA1 per liter and about 5 to about 8 mg protein per liter of culture can be achieved. In comparison to expression of other proteins using similar T7 RNA polymerase/promoter systems, these yields are high in both small-scale benchtop cultures and in large, commercial fermentors. Economical production of apoA1 may thus be achieved on a large scale.

The methods and compositions of the present invention can be used for the treatment of cardiovascular conditions in animals, and preferably in mammals, such as, for example, humans. For example, to prevent or treat atherosclerosis, and particularly aortic atherosclerosis, recombinant apoA1 can be administered in an amount effective to increase the total level of plasma apoA1 in the subject, i.e., the sum of endogenous and recombinant apoA1, by at least about 10%, and most preferably, by at least about 20%, over a sustained period of time such as, for example, from about one month or to less than about ten years or more. Dosing may be at any suitable interval ranging from multi dosing daily to daily, weekly, monthly, or semi-annual dosing. This amount will depend upon the age, weight, sex, sensitivity, medical history, and the like of the individual. This amount can be determined experimentally by methods well known in the art, such as, for example, by establishing a matrix of dosages and frequencies and assigning a group of experimental subjects to each point in the matrix.

ApoA1 can be administered by any route, such as, for example, subcutaneous, intramuscular, intravenous, enteral, by-inhalation, transmuscosal, and the like. The recombinant apoA1 can be formulated in any suitable solution or dosage form, such as, for example, sterile saline or sterile phosphate-buffered saline. Typical formulations include from 0.1 to 50 mg apoA1. Excipients, preservatives, and other additives known in the art may also be included in the formulations. Other cardiovascular drugs can be co-administered with apoA1. Vehicles and devices for controlled-release of apoA1 may also be used, including subdermal pellets, pumps, and the like. Oral formulations that protect the protein as it traverses the gastrointestinal tract may also be prepared.

The present invention also encompasses methods for the prevention of cardiovascular restenosis, which often follows surgical intervention such as, for example,

angioplasty. A restenosis prevention effective amount of recombinant apoA1 may be administered to an animal, preferably a mammal, and most preferably a human, in conjunction with the surgical procedure, i.e., before, during, after, or any combination thereof, in an amount effective to prevent restenosis. Typically, dosing will be accomplished in a single unit, but multiple dosing as described above may be necessary. This amount will depend upon the age, weight, sex, sensitivity, medical history, and the like of the individual. This amount can be determined experimentally by methods well known in the art, such as, for example, by establishing a matrix of dosages and frequencies and assigning a group of experimental subjects to each point in the matrix.

The invention also encompasses the use of recombinant apoA1 in the prevention and the treatment of septic shock. An anti-septic shock effective amount of recombinant apoA1 may be administered to a subject at risk of developing septic shock, or to a subject exhibiting symptoms of septic shock. This amount will depend upon the age, weight, sex, sensitivity, medical history, and the like of the individual. This amount can be determined experimentally by methods well known in the art, such as, for example, by establishing a matrix of dosages and frequencies and assigning a group of experimental subjects to each point in the matrix.

The recombinant apoA1 of the present invention may also be used in the protection of erythrocytes against the generation of procoagulant activity and in the treatment or prevention of thrombosis by administration of anti-procoagulant or anti-thrombosis effective amounts which can be determined and dosed as described above.

The recombinant apoA1 of the present invention can also be used to reverse the inhibitory effect of oxidized LDL on endothelium-dependent arterial relaxation by administration of reversal effective amounts which can be determined and dosed as described above.

Description of the Preferred Embodiments

The following examples illustrate the invention without limitation:

Example 1: Cloning of ApoA1 cDNA

ApoA1 cDNA was subcloned in a T7 expression vector.

A segment of apoA1 CDNA encoding mature apoA1 was subcloned into pETlld-6His (Hoffman, A., and Roeder, *R.G., Nucl. Acids Res.* 19:6337-6338, 1991. In this vector, proteins are expressed under T7 control as fusion products comprising an additional sequence of six histidine residues at the amino terminus. The histidine residues facilitate subsequent purification by adsorption of the fusion protein to nickel chelating resins. Briefly, tags containing 6 histidines are inserted between the NcoI and BamHI sites of the bacterial expression vector pET11d obtained from Novagen (Madison, WI).

To introduce apoA1 cDNA into this vector, a polymerase chain reaction (PCR) product encompassing the mature segment of apoA1 was synthesized using the following synthetic primers:

5'-AGCCGAGGAT CCTCACTGGG TGTTGAGCTT-3' SEQ ID NO 1

5'-CTTTCGCATA TGGATGAACC CCCCCAGAGC-3' SEQ ID NO 2

These primers introduce *Bam*Hl and *Nde*l restriction sites at the 3' and 5' ends, respectively, of the PCR product.

The template for the PCR amplification was pAl-101 (Karathanasis, et al., *supra*), a plasmid vector bearing an apoA1 cDNA which includes the entire coding sequence for mature apoA1 (Cheung and Chan, *supra*). This plasmid was isolated by screening an adult human liver cDNA library cloned into bacteriophage λ and transformed into *E. coli* strain LE392. Large scale growth of clones was carried out in 250 ml a broth containing 1% NZ-amine (an enzymatic digest of casein)/O.5% yeast extract/0.01% casamino acid/10 mM $MgSO_4$. Recombinant phage was recovered and precipitated with polyethylene glycol and was purified on CsCl step gradients. Mapping using conventional digestion with restriction enzymes followed by electrophoresis showed that the insert of clone pAI-101 contains the entire untranslated region and part of the poly(A) tail of apo A-I mRNA.

Conditions for the PCR were those recommended by the manufacturer, Perkin Elmer, (Norwalk, CT). The following amplification steps were repeated 30 times: 94°C, 1 minute; 55°C, 2 minutes; 72°C, 2 minutes. The PCR product was verified by its size (ca. 950 base pairs) as determined by electrophoresis in 1% agarose gel and by the presence of predicted *Sac*I and *Stu*I restriction sites.

The *Nde*I- and *Bam*HI-cleaved PCR product was ligated into PETlld-6His vector restricted at the same sites. The resulting recombinant plasmid, designated pET-His.A1, was transformed into transformation-competent *E. coli* DH5a (GIBCO-BRL, Grand Island, NY), a strain that yields antibiotic-resistant transformants at high efficiency when exposed to plasmids carrying antibiotic resistant genes. The resulting recombinant clones were screened for the presence of the apoA1-specific PCR product by colony hybridization employing ^{32}P-end-labelled primer as a probe. Isolated plasmid DNA from positive clones was analyzed by restriction digestion with *Nde*I and *Bam*HI, and the reading frame was verified by direct DNA sequencing. A plasmid including the 950-base pair PCR product was selected for transformation of BL21(DE3)pLysS *E. coli* (Novagen, Madison, WI) for final expression of recombinant apoA1. The *E. coli* strain containing the plasmid was deposited in the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Drive, Rockville, MD 20852, USA, and bears accession number A.T.C.C. 69581.

Example 2: Expression of Recombinant ApoA1

E. coli strain BL21(DE3)pLysS was transformed with pET-6His.A1, which had been prepared according to the method of Example 1 above, using standard transformation procedures. The transformed cells were grown in LB media containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 ml 1N NaOH per liter. When the culture reached an optical density (O.D.) at 595 nm of 0.3, the cells were induced with 2 mM isopropylthiogalactose (IPTG) for 3 hours. Total cell lysates were obtained by resuspending cells in 100 μl Laemmli sample buffer (1% sodium dodecyl sulfate, SDS), sonicating for 30 seconds at a sonicator setting of 6, and boiling for 10 minutes, after which they were analyzed by SDS-PAGE. Induced cell extracts were found to contain recombinant apoA1 protein at levels

corresponding to 30% to 40% of the total bacterial protein. The identity of recombinant apoA1 in extracts was established by the appearance of a band with the expected molecular weight of about 28 kilodaltons only upon IPTG induction; furthermore, this band reacted specifically with anti-apoA1 antibodies (Sigma, St. Louis, MO) upon Western blotting.

To purify recombinant apoA1, a one-liter culture of BL21(DE3)pLysS *E. coli* harboring pET6His.A1 was grown to an O.D. of 0.3 at 37°C and then was induced with 2 mM IPTG for 3 hours at 30°C. After harvesting by centrifugation, the cells were lysed by suspension at 4°C in 20 mM Tris-HCl, pH 7.9 buffer containing glycerol, 0.2 mM EDTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 M KCl, and pepstatin and antipain each at 20 pg/ml. The suspension was made 0.1% with respect to Nonidet P-40, held on ice for 10 minutes, and sonicated briefly.

Total cell extracts were separated into soluble and membrane fractions by centrifugation, and the fractions were analyzed by SDS-PAGE. This analysis revealed that about 40% of the total apoA1 was recovered in the soluble fraction. Accordingly, apoA1 was purified from this fraction by one-step affinity chromatography on a Ni^{2+}-NTA agarose column (Qiagen, Studio City, CA). The protein was adsorbed batchwise onto about 2 ml of Ni^{2+}-NTA agarose by incubation for 1 hour at 4°C. After extensive washing with 20 mM imidazole in lysis buffer (25 mM Tris, pH 8.0 and 0.5 M NaCl), ApoA1 was eluted with 100 mM imidazole in lysis buffer. A near homogeneous preparation of recombinant apoA1 was obtained, with a total yield from the soluble fraction corresponding to 8 mg of pure protein per liter of bacterial culture.

Example 3: Purification of Large Quantities of ApoA1

E. coli BL21(DE3)pLysS cells harboring pET6His.A1 were grown in 10-liter fermentors at 30°C with agitation at 300 rpm in modified M9 media containing Na_2HPO_4, KH_2PO_4, NH_4Cl, NaCl and $CaCl_2$ supplemented with casaminoacids, glucose and ampicillin (0.1 mg/ml).

2mM IPTG was added to induce the cells when the optical density of the culture reached 20, and the culture was harvested after attaining an optical density of 35.

ApoA1 was then purified by chromatography of bacterial extracts on metal-chelating Sepharose columns (Pharmacia LKB, Piscataway, NJ) charged with Ni^{2+}. Frozen cells that had been harvested from 9 liters of culture were homogenized in 1 liter of lysis buffer. The extract was centrifuged to remove the cell debris, and the resulting supernatant (1.5 L) was adsorbed overnight batchwise onto 400 ml of the nickel-charged resin. The resin was extensively washed by batchwise incubation with the lysis buffer, followed by 5 mM imidazole in the same buffer. The resin was then packed into a column and was washed with several column volumes of 25 mM imidazole in lysis buffer. The bound protein was slowly eluted with 0.1 M EDTA in lysis buffer. The yield was about 1.5 g of highly purified apoA1 at a concentration of about 5 mg/ml.

Example 4: Comparison of Yields

The experiments described below were performed to compare the protein yields of apoA1 obtained using the T7 RNA polymerase/promoter system described in Examples 2 and 3 above with the yields obtained for different proteins using similar expression systems.

Transcription factor TFIIB, a factor required by eukaryotic RNA polymerase II for accurate transcription initiation, was expressed in the bacterial system by introducing an *Nde*I restriction site into plasmid pB3 containing TFIIB, subcloning the mutagenized cDNA into 6His-pET11d to obtain PIIB6His, and transforming the recombinant plasmid into *E. coli* BL21(DE3)pLysS as described by Malik, et al., *Proc. Natl. Acad. Sci.* **88**:9553-9557 (1991). Yields of about 100 μg/liter culture were obtained using the bacterial expression system, representing a significant improvement over isolation from eukaryotic cells, but much less than the milligram/liter quantities of apoA1 obtained in the practice of the present invention.

Thus, the yields of apoA1 obtained using the methods and compositions of present invention were highly increased over the yields observed with other recombinant proteins previously expressed. *See*, for example, Citovsky, V., et al., *Proc. Nat. Acad. Sci.* **86**:1193-1197, 1989; Breyer, R.M. et al., *EMBO J.* **9**:2679-2689, 1990; and Campbell, W.H., *Plant Physiol.* **99**:693-699, 1992, discussed above.

Example 5: Clinical Delivery of ApoA1

Recombinant apoA1 was prepared and was purified according to the method of Example 3, with two additional purification steps. First, the material eluted from the Ni^{+2}-charged Sepharose column was extracted with ethanol. The ethanol-extracted material was then subjected to chromatography on a MonoQ column in 6M urea, using the FPLC system (Pharmacia).

The purified protein was formulated as a 20 mg/ml solution in sterile water. A single injection of 100 μl, corresponding to 2 mg apoA1, was administered to individual mice intraperitoneally, subcutaneously, and intravenously. Plasma samples were obtained from the mice over a 25-hour period following administration. The concentration of recombinant human apoA1 in the plasma samples was determined by a turbidometric assay, using a commercially available kit (Cat.# 356-A, Sigma Chemical Co., St. Louis, MO). This immunoassay discriminates between endogenous murine apoA1 and exogenously administered recombinant human apoA1.

Results of this experiment are illustrated in Figure 1. Intraperitoneal or intravenous administration resulted in an initial high level of plasma apoA1, which declined with at least biphasic kinetics. Subcutaneous administration, by contrast, resulted in a lower but more uniform plasma level of apoA1.

Example 6: Comparison of Natural and Recombinant ApoA1

Recombinant apoA1 (containing 6 aminoterminal histidine residues) was prepared and purified according to the methods of Examples 3 and 5. 5 mg of this material and 5 mg of apoA1 purified from human plasma (prepared as described in Brewer et al., *Meth.Enzymol.* **128**:223, 1986) were radiolabelled with ^{125}I in the presence of 6M urea, using the McFarland procedure. Following iodination, the unbound ^{125}I and the free urea were removed by dialysis against a physiological buffer.

The ^{125}I-labelled apoA1 preparations derived from human plasma and recombinant *E. coli* were individually reconstituted into HDL particles by overnight incubation with reconstituted HDL (R-HDL) to form ^{125}I-R-HDL. The ^{125}I-labelled R-HDL particles, containing either plasma- derived or recombinant apoA1, were individually

administered to mice by intravenous injection. Plasma samples were obtained at regular intervals, and ^{125}I-R-HDL was measured.

Clearance curves were plotted, allowing the derivation of kinetic constants. For the intervals after 120 minutes, clearance was modelled as a single pool, using the SAAM2 Simulation, Analysis and Modelling Package (University of Washington). The results are shown in Table 1 below.

TABLE 1

Kinetic Constants

Source of apoA1	k(0.1)	$t_{½}$
	Pools/hr	hrs/pool
Plasma	0.14	7.3±0.4
Recombinant	0.14	7.1±0.4

Recombinant apoA1 and apoA1 purified from human plasma were comparable with respect to the calculated kinetic parameters.

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

WE CLAIM:

1. A method for the preparation of biologically active apolipoprotein A1 (apoA1), said method comprising:

(a) subcloning apoA1 cDNA into a recombinant *Escherichia coli* plasmid to obtain a recombinant apoA1 fusion protein-encoding plasmid, wherein said cDNA is operatively linked to a bacteriophage T7 RNA polymerase promoter and is operatively linked to a DNA sequence encoding an additional amino acid sequence that facilitates purification of proteins;

(b) transferring said recombinant apoA1 fusion protein-encoding plasmid to *E. coli* cells of a strain containing inducible bacteriophage T7 RNA polymerase, to yield transformed *E. coli* cells;

(c) inducing said T7 RNA polymerase and growing said transformed cells in a culture for a sufficient time and under appropriate conditions to produce a biologically active recombinant apoA1 fusion protein;

(d) harvesting and lysing said cells to produce a soluble fraction containing said recombinant apoA1 fusion protein; and

(e) isolating said recombinant apoA1 fusion protein from said soluble fraction.

2. A method as defined in claim 1, further comprising (f) proteolytically removing at least a portion of said additional amino acid sequence from said recombinant apoA1 fusion protein.

3. A method as defined in claim 1, wherein step (f) comprises removing all of said additional amino acid sequence to yield recombinant apoA1 protein.

4. A method as defined in claim 1, wherein said isolated recombinant apoA1 fusion protein is bound to lipid, and said method further comprises (f) extracting said recombinant apoA1 fusion protein to decrease the amount of said bound lipid.

5. A method as defined in claim 3, wherein said isolated apoA1 protein is bound to lipid, and said method further comprises (g) extracting said apoA1 protein to decrease the amount of said bound lipid.

6. A method as defined in claim 1, wherein said *E. coli* plasmid comprises a pT or pET plasmid.

7. A method as defined in claim 4, wherein said pET plasmid is selected from the group consisting of pET3, pET5, pET9, pET11, pET12, pET14, pET15, pET16, pET17, pET19, pET20, pET21, pET22, pET23, pET24, pET25, and derivatives of any of the foregoing.

8. A method as defined in claim 6, wherein said pET plasmid comprises pET11d-6His.

9. A method as defined in claim 1, wherein said *E. coli* strain is selected from the group consisting of BL21(DE3), HMS174(DE3), and derivatives of any of the foregoing.

10. A method as defined in claim 9, wherein said strain is BL21(DE)pLysS.

11. A method as defined in claim 1, wherein step (c) produces at least about 2 milligrams of said recombinant apoA1 fusion protein per liter of culture.

12. A method as defined in claim 11, wherein step (c) produces from about 2 to about 3 milligrams of said recombinant apoA1 fusion protein per liter of culture.

13. A method as defined in claim 11, wherein step (c) produces at least about 5 milligrams of said recombinant apoA1 fusion protein per liter of culture.

14. A method as defined in claim 13, wherein step (c) produces from about 5 to about 8 milligrams of said recombinant apoA1 fusion protein per liter of culture.

15. A method as defined in claim 1, wherein step (c) includes adding isopropylthiogalactose to said culture.

16. A method as defined in claim 1, wherein said amino acid sequence that facilitates purification comprises a cluster of positively charged amino acid residues.

17. A method as defined in claim 16, wherein said cluster comprises from 6 to 10 histidine residues.

18. A method as defined in claim 1, wherein said isolating comprises single-step chromatography.

19. A method as defined in claim 18, wherein said single-step chromatography comprises Ni(II) affinity chromatography.

20. A method as defined in claim 19 wherein said chromatography comprises Ni(II)-nitrilotriacetic acid agarose chromatography.

21. A method for the preparation of biologically active apoA1, said method comprising:

(a) subcloning apoA1 cDNA into a recombinant *Eschericia coli* plasmid to obtain an recombinant apoA1-encoding plasmid, wherein said cDNA is operatively linked to a bacteriophage T7 RNA polymerase promoter and is operatively linked to a DNA sequence encoding 6 histidine residues fused in frame to the 5' terminus of the apoA1 protein-coding sequence;

(b) transferring said recombinant apoA1 fusion protein-encoding plasmid to *E. coli* cells of strain BL21(DE)pLysS containing an inducible bacteriophage T7 RNA polymerase, to yield transformed *E. coli* cells;

(c) inducing said T7 RNA polymerase and growing said transformed cells in a culture for a sufficient time and under appropriate conditions to produce a biologically active recombinant apoA1 fusion protein;

(d) harvesting and lysing said cells to obtain a soluble fraction containing recombinant apoA1 fusion protein;

(e) isolating said recombinant apoA1 fusion protein from said soluble fraction using Ni(II)-nitrilotriacetic acid agarose chromatography; and

(f) extracting said isolated recombinant apoA1 fusion protein with ethanol.

22. A method as defined in claim 21, further comprising (g) proteolytically removing said histidine residues to produce apoA1.

23. A method for preventing or treating atherosclerosis in an animal in need of such treatment, said method comprising administering to said animal a treatment amount of a recombinant apoA1 protein prepared by a method as defined in claim 1 effective to increase the total plasma level of apoA1 protein in said animal by at least about 10%.

24. A method as defined in claim 23, wherein said administering is performed over a time interval from about one month to about ten years.

25. A method for preventing cardiovascular restenosis in an animal in need of such treatment, said method comprising administering to said animal a restenosis prevention effective amount of a recombinant apoA1 prepared by a method as defined in claim 1.

26. A method as defined in claim 25, wherein said administering is performed before, during, after, or any combination thereof, said animal has undergone surgical angioplasty.

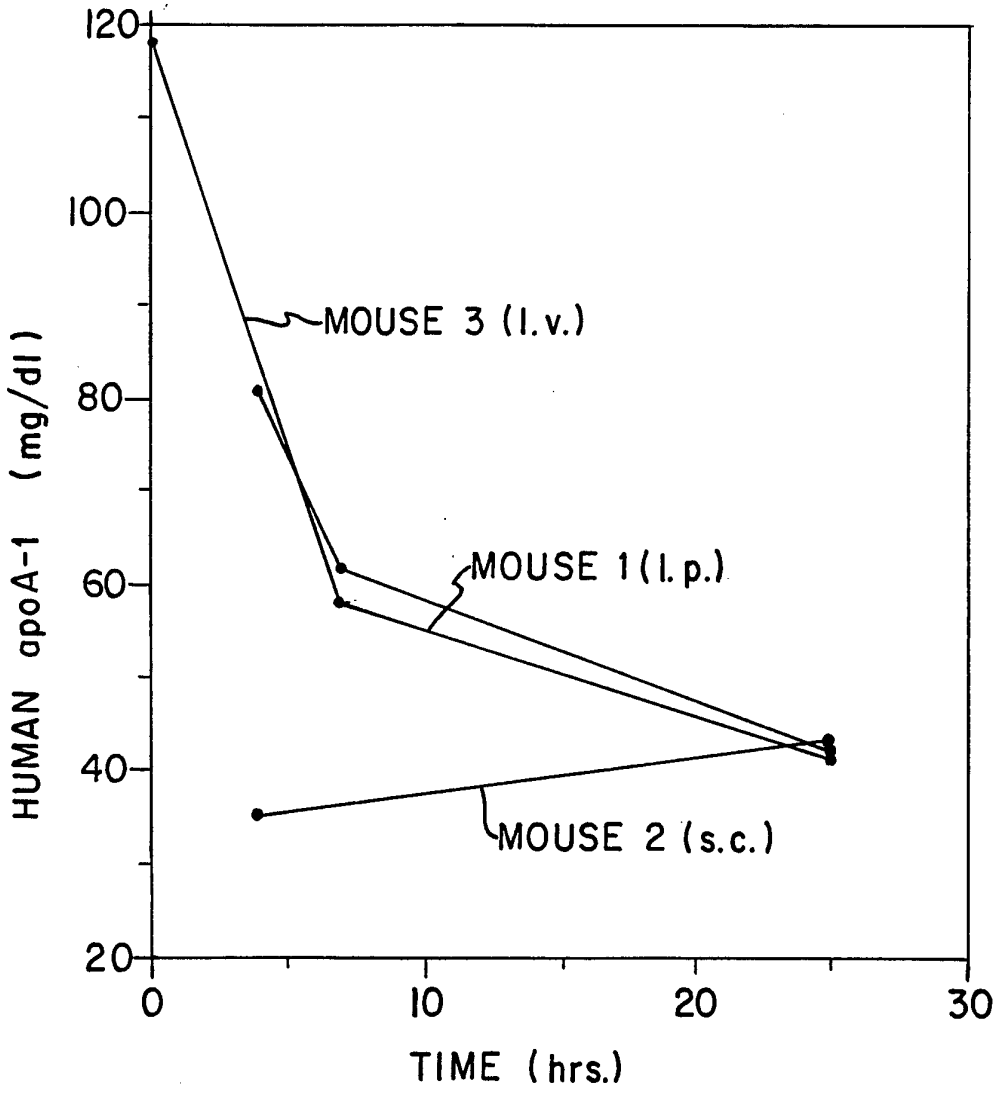
27. A method for preventing septic shock in an animal in need of such treatment, said method comprising administering to said animal an anti-septic shock effective amount of a recombinant apoA1 prtein prepared by a method as defined in claim 1.

28. A method for treating septic shock in an animal in need of such treatment, said method comprising administering to said animal an anti-septic shock effective amount of a recombinant apoA1 protein prepared by a method as defined in claim 1.

29. A method for protecting erythrocytes against the generation of procoagulant activity in an animal in need of such treatment, said method comprising administering to said animal an anti-procoagulant effective amount of a recombinant apoA1 protein prepared by a method as defined in claim 1.

30. A method for preventing thrombosis in an animal in need of such treatment, said method comprising administering to said animal an anti-thrombosis effective amount of a recombinant apoA1 protein prepared by a method as defined in claim 1.

31. A method for reversing the inhibitory effect of oxidized LDL on endothelium-dependent arterial relaxation in an animal in need of such treatment, said method comprising administering to said animal a reversal effective amount of a recombinant apoA1 protein prepared by a method as defined in claim 1.



INTERNATIONAL SEARCH REPORT

...rnational application No.
PCT/US95/03826

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/21, 15/70, 15/72; C07K 1/22, 14/775; A61K 38/17
US CL :435/69.6, 69.7, 252.33, 320.1; 514/2; 530/359, 380, 413, 415
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.6, 69.7, 252.33, 320.1; 514/2; 530/359, 380, 413, 415

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: apolipoprotein, recombinant, expression, atherosclerosis, cardiovascular restenosis, septic shock, thrombosis, arterial relaxation dilation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,943,527 (PROTTER ET AL) 24 July 1990, see entire document.	1-22
Y	US, A, 5,284,933 (DOBRELI ET AL) 08 February 1994, see entire document.	1-22
Y	NUCLEIC ACIDS RESEARCH, Volume 19, Number 22, issued 1991, Hoffmann et al, "Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies", pages 6337-6338, see entire document.	1-22
Y	JOURNAL OF MOLECULAR BIOLOGY, Volume 189, issued 1986, Studier et al, "Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes", pages 113-130, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 MAY 1995	Date of mailing of the international search report 25 MAY 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN WALSH Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

. .rnational application No.
PCT/US95/03826

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,128,318 (LEVINE ET AL) 07 July 1992, column 3, lines 50-68, and paragraph bridging columns 6-7.	23,27-29
A	US, A, 4,643,988 (SEGREST ET AL) 17 February 1987, columns 1-3.	23,29-31
A	US, A, 5,278,189 (RATH ET AL) 11 January 1994, columns 1-2.	23-26