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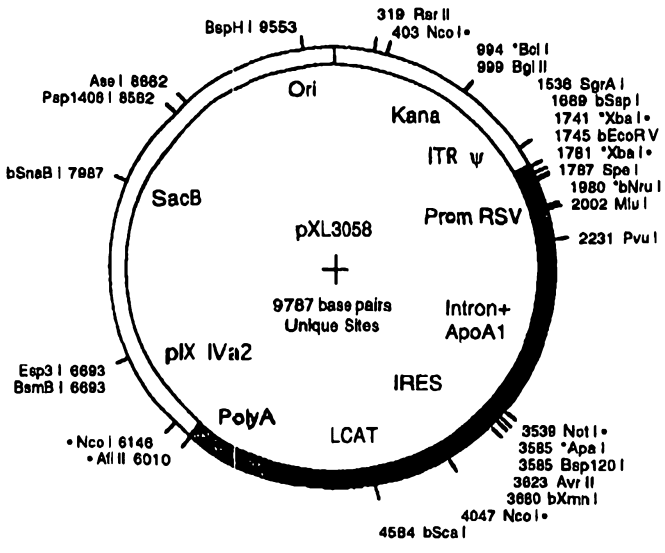
(57) Abstract

The invention concerns a defective recombinant virus and preferably an adenovirus characterised in that it comprises at least two nucleic acids coding for distinct enzymes, proteins and/or co-factors involved in the reverse transfer of cholesterol, said nucleic acids being operationally bound to a transcriptional promoter and mutually separated by a sequence coding for an internal entry site of the IRES ribosome. The invention further concerns plasmid constructs useful for preparing these adenovirus, and cells transformed by these plasmids or adenovirus and pharmaceutical compositions containing said adenovirus.



(57) Abrégé

La présente invention concerne un virus recombinant défectif et de préférence un adénovirus caractérisé en ce qu'il comprend au moins deux acides nucléiques codant pour des enzymes, protéines et/ou co-facteurs distincts et impliqués dans le transport inverse du cholestérol, lesdits acides nucléiques étant liés opérationellement à un promoteur transcriptionnel et séparés l'un de l'autre par une séquence codant pour un site d'entrée interne du ribosome IRES. Elle se rapporte en outre à des constructions plasmidiques utiles pour préparer ces adénovirus, à des cellules transformées par ces plasmides ou adénovirus et à des compositions pharmaceutiques contenant lesdits adénovirus.

BICISTRONIC RECOMBINANT VIRUSES USEFUL FOR THE TREATMENT OF DYSLIPOPROTEINEMIA-RELATED PATHOLOGIES

The present invention relates to new recombinant viruses, to their preparation and to their use in gene therapy, for the transfer and expression in vivo of desired genes. More specifically, it relates to recombinant viruses comprising at least two inserted genes whose expression products are involved in the reverse transport of cholesterol. It also relates to the shuttle plasmids useful for the production of adenoviruses in accordance with the invention. More particularly, the present invention relates to defective recombinant adenoviruses and to their use for the prevention or treatment of pathologies linked to dyslipoproteinemias which are known for their serious consequences at the cardiovascular and neurological level.

Dyslipoproteinemias are disorders of the metabolism of the lipoproteins responsible for the transport, in the blood and peripheral fluids, of lipids such as cholesterol and triglycerides. They result in major pathologies, linked respectively to hypercholesterolemia or hypertriglyceridemia, such as especially atherosclerosis.

Atherosclerosis is a polygenic complex disease which is defined from the histological point of view by deposits (lipid or fibrolipid plaques) of

lipids and of other blood derivatives in the wall of the large arteries (aorta, coronary arteries, carotid). These plaques, which are calcified to a greater or lesser extent according to the progression of the process, can be associated with lesions and are linked to the accumulation, in the arteries, of fatty deposits consisting essentially of cholesterol esters. These plaques are accompanied by a thickening of the arterial wall, with hypertrophy of the smooth muscle, the appearance of spumous cells and the accumulation of fibrous tissue. The atheromatous plaque is very clearly in relief on the wall, which confers on it a stenosing character responsible for vascular occlusions by atheroma, thrombosis or embolism which occur in the patients most affected.

Hypercholesterolemias can therefore result in very serious cardiovascular pathologies such as infarction, sudden death, cardiac decompensation, cerebrovascular accidents and the like.

It is therefore particularly important to be able to have available treatments which make it possible to reduce, in certain pathological situations, the plasma cholesterol levels or even to stimulate the efflux of cholesterol (reverse transport of the cholesterol) in the peripheral tissues in order to discharge the cells having accumulated cholesterol within the context of the formation of an atheroma

plaque. The cholesterol is carried in the blood by various lipoproteins including the low-density lipoproteins (LDL) and the high-density lipoproteins (HDL). The LDLs are synthesized in the liver and make it possible to supply the peripheral tissues with cholesterol. In contrast, the HDLs capture cholesterol in the peripheral tissues and transport it to the liver where it is stored and/or degraded.

Among the most common dyslipemias are those characterized by a high LDL (low-density lipoprotein) cholesterol level and hypoalphalipoproteinemia. The latter is characterized by an HDL (high-density lipoprotein) cholesterol level of less than 35 mg/dl and it represents 40% of dyslipemia cases (Genest et al., 1992). Hypoalphaliproproteinemia appears to be linked to a genetic deficiency in one or more proteins involved in the synthesis, maturation and breakdown of the HDL particles and its consequence is often the early appearance of cardiovascular diseases (Dammermann et al., 1995). In general, there is an inverse correlation between the incidence of the latter diseases and the levels of HDL particles (Miller et al., 1987).

The protective effect of the HDLs against cardiovascular diseases has been demonstrated by gene transfer experiments in vivo in strains of mice likely to develop atherosclerotic lesions and in which an increase in the number of HDL particles inhibits the

development of these lesions (Rubin et al., 1991; Plump et al., 1994). It has been proposed that the protective effect of the HDL particles may be due to their role in the reverse transport of cholesterol (Reiche et al., 1989). Reverse transport is the process by which excess cholesterol is transferred from the peripheral tissues to the liver for its removal (Figure 1). It is composed of a series of stages comprising the capture and esterification of cholesterol on the HDL particles as well as its transfer onto the low-density lipoprotein particles which are subsequently recaptured by the liver and this process of course involves the participation of a number of proteins such as CETP, of enzymes including cholesterol acyltransferase and hepatic lipase (and/or their cofactors such as apolipoproteins AI and AIV.

Whereas effective treatments exist for reducing the level of LDL cholesterol and of triglycerides based on hypolipidemic and antihypertensive drugs, current treatments for hypoalphalipoproteinemia offer only a limited efficacy.

The present invention relates precisely to the treatment, by gene therapy, of pathologies linked to hypoalphalipoproteinemia.

The therapeutic approach adopted in the present invention aims to increase the kinetics of the reverse transport of cholesterol in order to induce the regression of the atherosclerotic lesions or

alternatively to prevent their formation. Advantageously, this objective is achieved according to the invention via a simultaneous and effective expression, in the cells to be treated, of at least two of the proteins, enzymes or cofactors involved in the reverse transport of cholesterol.

For the purposes of the invention, a protein, enzyme and/or one of their cofactors is considered to be involved in the reverse transport of cholesterol insofar as any disruption in the level of its cellular concentration automatically affects the reverse process of cholesterol.

By way of representatives of the enzymes involved, there may be mentioned more particularly lecithin cholesterol acyltransferase and hepatic lipase.

Lecithin cholesterol acyltransferase (LCAT) is a glycoprotein of 67 kDa, synthesized in the liver, which catalyses the transfer of an acyl group from lecithin to cholesterol, thereby producing lysophosphatidylcholine and cholesterol esters (Glomset, 1968). The cofactor for this enzyme is apolipoprotein AI which is bound to the surface of the HDL particles. By maintaining the cholesterol concentration gradient between the peripheral cells and the HDLs, it plays a principal role in the first stage of the reverse transport of cholesterol.

Human hepatic lipase (HL) is a glycoprotein

of 66 kDa with triglyceride hydrolase and phospholipase activities, which is synthesized and secreted by the hepatocytes. Bound to the surface of the hepatic cells, via the proteoglycans heparin sulphate, it participates in the metabolism of the chylomicrons, of the IDLs (intermediate density lipoproteins) and of the HDLs. HL has a particular affinity for the large HDL particles whose phospholipids and triglycerides it breaks down, generating discoidal particles of HDL which have the property of accepting cellular cholesterol. HL deficiency in man is characterized by a high quantity of LDLs rich in triglycerides, large HDL particles and the early development of atherosclerosis (Hegele et al., 1993).

As regards the proteins, they are, for the purposes of the invention, more preferably cholesterol ester transfer protein (CETP), apolipoproteins AI and AIV or one of their variants.

Cholesterol ester transfer protein (CETP) is a glycoprotein of 74 kDa synthesized in the adipose tissue and the liver. In the plasma, it is mainly associated with the HDLs. This is where it catalyses the transfer of cholesterol esters from the HDLs to the low-density lipoproteins. This transfer is followed by the passage of the triglycerides from the low-density lipoproteins to the HDLs. The overexpression of CETP in transgenic hypertriglycemidic mice inhibits the development of atherosclerotic lesions (Hayek et al.,

1995). This experiment is in agreement with the observation, in individuals deficient in CETP, of an early development of cardiovascular diseases (Zhong et al., 1996).

Apolipoprotein AI is a protein consisting of 243 amino acids, which is synthesized in the form of a prepropeptide of 267 residues, having a molecular mass of 28,000 Daltons. It is synthesized in man specifically in the liver and the intestine and it constitutes the essential protein of the HDL particles (70 % of their mass in proteins). It is abundant in plasma (1.0-1.2 g/l). Its best biochemically-characterized activity is the activation of lecithin-cholesterol acyltransferase (LCAT), but numerous other activities are attributed to it, such as especially the stimulation of the efflux of cellular cholesterol. Apolipoprotein AI plays a major role in resistance to atherosclerosis, linked to the reverse transport of cholesterol. Its gene, 1863 bp in length, has been cloned and sequenced (Sharpe et al., Nucleic Acids Res. 12(9) (1984) 3917). Among the protein products with apolipoprotein AI type activity, there may be mentioned especially the natural variants described in the prior art.

Apolipoprotein AIV (apoAIV) is a protein consisting of 376 amino acids, which is specifically synthesized in the intestine in the form of a precursor of 396 residues. As regards its physiological activity,

it is known that it can activate, in vitro, lecithin-cholesterol acyltransferase (LCAT) (Steinmetz et al., 1985, J. Biol. Chem., 260: 2258-2264) and that it can, like apolipoprotein AI, interfere with the binding of the HDL particles onto bovine aortic endothelial cells (Savion et al., 1987, Eur. J. Biochem., 257: 4171-4178). These two activities indicate that apoAIV very probably acts as mediator of the reverse transport of cholesterol. The apoAIV gene has been cloned and described in the prior art (see especially WO 92/05253). Among the protein products with apolipoprotein AIV type activity, there may be mentioned especially the fragments and derivatives described in Patent Application FR 92 00806.

More precisely, the present invention is based on the use of recombinant viruses which make it possible to transfer and express at least two nucleic acids encoding enzymes, proteins and/or cofactors involved in the reverse transport of cholesterol.

Unexpectedly, the applicant has demonstrated that it is possible to effectively provide for the transfer and expression of at least two nucleic acids from the same recombinant virus, by integrating these nucleic acids into the said virus in the form of a bicistronic unit. It is clear that the use of a single virus and not of two has numerous advantages from the therapeutic point of view.

First of all, it is more advantageous to

construct a single recombinant virus incorporating the two genes than two respective recombinant viruses.

Likewise, the use, from the therapeutic point of view, of a vector as claimed makes it possible to reduce by half the quantities of recombinant virus necessary for the expression of the said genes. This is most particularly beneficial in the light of the immune response conventionally manifested towards cells infected by recombinant viruses. This immune response normally results in a destruction of the infected cells and/or a major inflammatory response. It is clear that these two manifestations are highly damaging at the level of the duration of expression of the therapeutic genes and therefore of the expected therapeutic effect.

Finally, an efficient transfer and at equal concentration of two distinct recombinant viruses is an uncertain event which therefore needs to be controlled by additional operations. In the case of the use of a recombinant virus according to the invention, this type of control can be advantageously dispensed with.

Advantageously, the claimed viruses are capable of transferring and of efficiently expressing, for a long duration and with no cytopathological effect, two nucleic acids encoding proteins, enzymes and/or cofactors involved in the reverse transport of cholesterol.

A first subject of the invention therefore consists in a defective recombinant virus comprising at

least two nucleic acids encoding enzymes, proteins and/or cofactors, which are distinct and are involved in the reverse transport of cholesterol, the said nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

The nucleic acids are preferably chosen from the genes encoding all or part of lecithin cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), hepatic lipase (HL), apolipoproteins AI and AIV or one of their variants.

The inserted nucleic acids may be complementary DNA fragments (cDNA), genomic DNA (gDNA), or hybrid constructs consisting, for example, of a cDNA into which one or more introns would be inserted. They may be synthetic or semisynthetic sequences. As indicated above, they may be a gene encoding all or part of one of the enzymes, proteins and/or cofactors involved in the reverse transport of cholesterol, or of a variant thereof. For the purposes of the invention, the term variant designates any mutant, fragment or peptide possessing at least one biological property of the protein product considered, as well as, where appropriate, their respective natural variants.

These fragments and variants may be obtained by any technique known to a person skilled in the art and in particular by genetic and/or chemical and/or

enzymatic modifications. The genetic modifications include suppressions, deletions, mutations and the like.

The inserted nucleic acids for the purposes of the invention are preferably the genes encoding all or part of the corresponding human enzymes, proteins and/or cofactors. They are more preferably cDNA or gDNA.

Each inserted nucleic acid may also comprise activating or regulatory sequences or the like. Moreover, it generally comprises, upstream of the coding sequence, a signal sequence directing the polypeptide synthesized in the secretory pathways of the target cell. This signal sequence may be its natural signal sequence, but it may also be any other functional signal sequence or an artificial signal sequence.

According to a specific embodiment of the invention, the claimed recombinant viruses comprise at least one nucleic acid encoding LCAT. More preferably, the second inserted nucleic acid encodes HL, CETP or ApoAI.

As explicitly stated above, the coexpression of the two nucleic acids considered is ensured preliminarily via the formation of a single RNA which is then translated to give the two respective enzymes. For these reasons, the recombinant virus incorporates, in addition to the two nucleic acid sequences, at least

one transcriptional promoter, a polyadenylation site and an IRES sequence.

This transcriptional promoter is operably linked to the coding nucleic acids so as to produce the bicistronic mRNA and to lead to the expression of the two respective enzymes from the said mRNA. According to a preferred embodiment of the invention, it is directly placed upstream of the first nucleic acid.

This transcriptional promoter may be chosen in particular from sequences which are naturally responsible for the expression of the said nucleic acid in relation to which to which it is placed upstream provided of course that these sequences are capable of functioning in the infected cell. They may also be sequences of a different origin (responsible for the expression of other proteins, or even synthetic). In particular, they may be sequences sequences of eukaryotic or viral nucleic acid sequences or derived sequences, stimulating or repressing the transcription of a gene specifically or otherwise and inducibly or otherwise. By way of example, they may be promoter sequences derived from the genome of the cell which it is desired to infect, or from the genome of a virus, and in particular the promoters of the adenovirus MLP and E1A genes, the CMV, RSV-LTR, MT-1 and SV40 promoter and the like. Among the eukaryotic promoters, there may also be mentioned the ubiquitous promoters (HPRT, vimentin, α-actin, tubulin and the like), the promoters

for the intermediate filaments (desmin, neurofilaments, keratin, GFAP and the like), the promoters of therapeutic genes (type MDR, CFTR, factor VIII, and the like), tissue-specific promoters (pyruvate kinase, villin, promoter for the fatty acid-binding intestinal protein, promoter for the α-actin of the smooth muscle cells, promoters specific for the liver: ApoAI, ApoAII, human albumin and the like) or alternatively promoters which respond to a stimulus (steroid hormone receptor, retinoic acid receptor and the like). In addition, these expression sequences may be modified by addition of activating and regulatory sequences and the like.

As regards the IRES sequence, it is preferably derived from a picornavirus. More precisely, this picornavirus IRES sequence is derived either from the encephalomyocarditis virus or from the poliovirus. It is more preferably the fragment derived from encaphalomyocarditis present in the vector pCITE-2a+ from NOVAGEN.

For the sake of clarity, the construct defined by the transcriptional promoter, the two nucleic acids, the polyadenylation site and the IRES sequence which exists between the two nucleic acids, will be defined below by the name bicistronic cassette.

The viruses according to the present invention are defective, that is to say are incapable of autonomously replicating in the target cell. Generally, the genome of the defective viruses is used within the

framework of the present invention and therefore lacks at least the sequences necessary for the replication of the said virus in the infected cell. These regions may be either eliminated (completely or partly), or made nonfunctional, or substituted by other sequences and especially by the bicistronic cassette defined above. Preferably, the defective virus conserves, nevertheless, the sequences of its genome which are necessary for the encapsidation of the viral particles.

The virus according to the invention may be derived from an adenovirus, an adeno-associated virus (AAV) or a retrovirus. According to a preferred embodiment, it is an adenovirus.

There are various adenovirus serotypes whose structure and properties vary somewhat. Among these serotypes, the type 2 or 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see application WO94/26914) are preferably used within the framework of the present invention. Among the adenoviruses of animal origin which can be used within the framework of the present invention, there may be mentioned the adenoviruses of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or alternatively simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus [manhattan or A26/61 strain (ATCC VR-800) for example]. Preferably, adenoviruses of human or canine or mixed

origin are used within the framework of the invention.

Preferably, in the genome of the adenovirus of the invention, at least the E1 region is made nonfunctional. The viral gene considered may be made nonfunctional by any technique known to persons skilled in the art, and in particular by total suppression, by substitution, by partial deletion or by addition of one or more bases to the gene(s) considered. Such modifications may be obtained in vitro (on the isolated DNA) or in situ, for example by means of genetic engineering techniques, or alternatively by treatment by means of mutagenic agents. Other regions may also be modified, and in particular the E3 (WO95/02697), E2 (WO94/28938), E4 (WO94/28152, WO94/12649, WO95/02697) and L5 (WO95/02697) region. According to a preferred embodiment, the adenovirus according to the invention comprises at least one deletion in the E1 region and a deletion in the E3 region. In the viruses of the invention, the deletion in the E1 region preferably extends from nucleotides 455 to 3329 on the Ad5 adenovirus sequence. According to another preferred embodiment, the bicistronic cassette is inserted at the level of the deletion in the E1 region.

A specific mode of the present invention therefore relates to a defective recombinant adenovirus which comprises at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and involved in the reverse transport of

cholesterol, the said nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

By way of preferred recombinant adenoviruses according to the invention, there may be mentioned more particularly the defective recombinant adenoviruses comprising at least one nucleic acid encoding LCAT and one nucleic acid encoding either HL, CETP or ApoAI, the said nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

By way of a representative of these adenoviruses, there may be mentioned that represented in Figure 2, containing the genes encoding LCAT and CETP, respectively, and derived from homologous recombination between pXL2974 and pXL2822.

The defective recombinant adenoviruses according to the invention can be prepared by any technique known to persons skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid carrying, inter alia, the bicistronic cassette. The homologous recombination occurs after cotransfection of the said adenoviruses and plasmid into an appropriate cell line. The cell line used should preferably (i) be

transformable by the said elements, and (ii) comprise the sequences capable of complementing the defective adenovirus genome part, preferably in an integrated form to avoid the risks of recombination. By way of example of a line, there may be mentioned the human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains in particular, integrated into its genome, the left part of the genome of an Ad5 adenovirus (12%) or lines capable of complementing the E1 and E4 functions as described especially in applications No. WO94/26914 and WO95/02697. Next, the adenoviruses which have multiplied are recovered and purified according to conventional molecular biology techniques, as illustrated in the examples.

However, according to a preferred embodiment of the invention, the claimed adenoviruses are prepared according to a novel process described in patent application WO96/25506 which uses, as shuttle plasmid, a prokaryotic plasmid comprising a recombinant adenovirus genome bordered by one or more restriction sites not present in the said genome. This protocol is in particular illustrated in Figure 2. This process is particularly advantageous since it makes it possible to dispense with the use of a second construct providing another part of the viral genome, and with the step of recombination in the transcomplementation line.

A second subject of the present invention relates precisely to specific prokaryotic plasmids used

for the production of the claimed adenoviruses.

According to a preferred embodiment of the invention, these prokaryotic plasmids integrate the bicistronic cassette described above.

More precisely, the subject of the present invention is also a prokaryotic plasmid comprising an adenovirus genome and at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, the two nucleic acids being operably linked to a transcription promoter and separated from each other by a sequence encoding an internal ribosome entry site, IRES.

Preferably, the prokaryotic plasmids according to the invention comprise a first region allowing replication in prokaryotic cells and a second region comprising the adenoviral genome bordered by one or more restriction sites not present in the said genome and in which are present at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, these two nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site, IRES.

As regards the definitions of the enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol,

the transcriptional promoter, the polyadenylation site, the IRES sequence as well as what affects their organization within the said cassette, reference may be made to what was described above.

The region allowing replication in prokaryotic cells, which is used in the claimed plasmids, may be any replication origin which is functional in the chosen cells. It may be a replication origin derived from a plasmid of the P incompatibility group (example = pRK290) which allows replication in the E. coli pol A strains. More generally, it may be any replication origin derived from a plasmid replicating in the prokaryotic cells. This plasmid may be a derivative of RK2, of pBR322 (Bolivar et al., 1977), a derivative of pUC (Viera and Messing, 1982), or of other plasmids which are derived from the same incompatibility group, that is to say from ColE1 or from pMB1 for example. These plasmids may be chosen, moreover, from other incompatibility groups replicating in Escherichia coli. They may be plasmids derived from plasmids belonging to the incompatibility groups A, B, FI, FII, FIII, FIV, H1, H11, I1, I2, J, K, L, N, OF, P, Q, T, U, W, X, Y, Z or 9 for example. Other plasmids may also be used, among which the plasmids not replicating in E. coli but in other hosts such as B. subtilis, Streptomyces, P. putida, P. aeruginosa, Rhizobium meliloti, Agrobacterium tumefaciens, Staphylococcus aureus, Streptomyces pristinaespiralis,

Enterococcus faecium or Clostridium. Preferably, the replication origins derived from plasmids replicating in E. coli are used.

As indicated above, the adenoviral genome present in the plasmids of the invention is advantageously a complete or functional genome, that is to say not requiring the provision of other regions, by recombination or ligation, for the production of viral stocks in the chosen encapsidation lines.

Preferably, the recombinant adenoviral genome comprises at least ITR sequences or a sequence allowing the encapsidation. In a preferred embodiment of the invention, this adenovirus genome used lacks all or part of the E1 region. Advantageously, the adenovirus genome used lacks part of the E1 region between nucleotides 454 to 3328 (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment).

According to a particularly advantageous embodiment, the adenovirus genome used also lacks all or part of the E3 and/or E4 region. According to a specific embodiment of the invention, it is an adenovirus genome lacking E1 and E3 regions.

More precisely, the claimed prokaryotic plasmids comprise, in the 5'-3' orientation, at least one replication origin functional in the prokaryotic cells, a first part of an adenoviral genome comprising the viral ITR and ψ sequences, a transcriptional promoter, a first nucleic acid encoding an enzyme,

protein and/or a cofactor involved in the reverse transport of cholesterol, an IRES sequence, a second nucleic acid encoding a second enzyme, protein and/or a cofactor involved in the reverse transport of cholesterol, a polyadenylation site and a second part of an adenoviral genome comprising the pIX-IVA2 region.

Advantageously, the claimed prokaryotic plasmids according to the invention also comprise a region allowing the selection of the prokaryotic cells containing the said plasmid. This region may consist in particular of any gene conferring resistance to a product, and in particular to an antibiotic. Thus, there may be mentioned the nucleic acid sequences conferring resistance to kanamycin (Kan^r), to ampicillin (Amp^r), to tetracycline (tet^r) or to spectinomycin, for example, which are commonly used in molecular biology (Maniatis et al., 1989). The selection of plasmids may be carried out with nucleic acid sequences other than genes encoding markers for resistance to an antibiotic. In general, this involves a gene which gives the bacterium a function which it no longer possesses (and this may correspond to a gene which has been deleted from the chromosome or made inactive), the gene on the plasmid restoring this function. By way of example, it may be a gene for a transfer RNA which restores a deficient chromosomal function (Somoes et al., 1991).

According to a preferred embodiment, the

claimed prokaryotic plasmid comprises at least one nucleic acid encoding LCAT, the second nucleic acid being chosen from those encoding CETP, HL or ApoAI.

By way of representatives of these prokaryotic plasmids, there may be mentioned in particular the plasmids pXL2974 and pXL3058, represented in Figures 4 and 6, respectively. The plasmid pXL2974 comprises, in the 5'-3' orientation, a replication origin, a gene for resistance to spectinomycin, the Sac B gene for sensitivity to sucrose, the viral ITR and ψ sequences, the RSV promoter, the two transgenes LCAT and CETP separated by the IRES, the polyadenylation site and the viral sequences pIX and IVA2. The plasmid pXL3058 comprises, in the 5'-3' orientation, a replication origin, a gene for resistance to kanamycin, the viral ITR and ψ sequences, the RSV promoter, the two transgenes LCAT and intron+ApoAI separated by the IRES, the polyadenylation site, the viral sequences pXI and IVA2 and the Sac B gene for sensitivity to sucrose.

The present invention also extends to the plasmid constructs used for the construction of these prokaryotic plasmids and which also comprise the bicistronic cassette defined according to the invention.

The claimed prokaryotic plasmids according to the invention may in particular be obtained by transformation of an initial shuttle plasmid comprising

the bicistronic cassette defined according to the invention. Therefore the invention further provides a shuttle plasmid which comprises two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, the two nucleic acids being operably linked to a transcriptional promoter, a polyadenylation site and a sequence encoding an internal ribosome entry site, IRES, situated between the two nucleic acids.

By way of representatives of these shuttle plasmids, there may be mentioned in particular the plasmids pXL2970 and pXL2984 described in Figures 3 and 5 respectively.

As regards the definitions of the enzymes involved in the reverse transport of cholesterol, the transcriptional promoter and the IRES sequence as well as what affects their organization within the said cassette, reference may be made to what has been described above.

Another subject of the present application relates to any prokaryotic cell containing a prokaryotic plasmid as defined above. It may be in particular any bacterium for which there is a vector system where recombinant DNA may be introduced. There may be mentioned, for example, Escherichia coli, Salmonella typhimurium, Bacillus subtilis, Pseudomonas putida, Pseudomonas aeruginosa, Agrobacterium tumefaciens, Rhizobium meliloti or bacteria of the genus Streptomyces. These cells are advantageously obtained by transformation according to techniques known to persons skilled in the art.

It also relates to the use of a defective recombinant virus, a defective recombinant adenovirus or a plasmid, as defined above, for the preparation of a pharmaceutical composition intended for the treatment or prevention of pathologies linked to hypoalphalipoproteinemia including more particularly atherosclerosis and/or restenosis.

The present invention also relates to a pharmaceutical composition comprising one or more defective recombinant viruses, defective recombinant adenoviruses or plasmids as described above and a pharmaceutically acceptable vehicle. Such compositions may be formulated for administration by the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular route and the like.

Preferably, the composition according to the invention contains pharmaceutically acceptable vehicles for an injectable formulation. They may be in particular isotonic sterile saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, in particular freeze-dried, compositions which, upon addition depending on the case of sterilized water or physiological saline, allow the reconstitution of injectable solutions.

The virus doses used for the injection can be adapted according to various parameters, and in particular according to the mode of administration used, the relevant pathology or the desired duration of treatment. In general, the recombinant viruses according

to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml. The term pfu ("plaque forming unit") corresponds to the infectivity of a suspension of virions, and is determined by infecting an appropriate cell culture and measuring, generally after 48 hours, the number of plaques of infected cells. The techniques for determining the pfu titre of a viral solution are well documented in the literature.

The present invention offers a new, very effective means for the treatment or prevention of pathologies linked to hypoalphalipoproteinemia, in particular in the field of cardiovascular conditions such as myocardial infarction, angina, sudden death, cardiac decompensation, cerebrovascular accidents, atherosclerosis or restenosis.

Accordingly the present invention provides a method of treatment or prevention of a pathology linked to hypoalphalipoproteinemia which comprises administering to a patient a pharmaceutical composition according to the invention.

In addition, this treatment can be applied both to man and to any animal such as ovines, bovines, domestic animals (dogs, cats and the like), horses, fish and the like.

The present invention is more fully described with the aid of the following examples which should be considered as illustrative and nonlimiting.

LEGEND TO THE FIGURES

Figure 1: Schematic representation of the

presumed mechanism of reverse transport of cholesterol.

Figure 2: Representation of the protocol for the production of adenoviruses by homologous recombination in E. coli.

Figure 3: Protocol for the construction of the bicistronic shuttle plasmid pXL2970 comprising RSV-LCAT-IRES-CETP.

Figure 4: Protocol for the construction of the prokaryotic plasmid pXL2974 comprising RSV-LCAT-IRES-CETP.

Figure 5: Protocol for the construction of the plasmid pXL2984 comprising RSV-LCAT-IRES-HL.

Figure 6: Representation of the prokaryotic plasmid pXL3058 comprising RSV-ApoAI-IRES-LCAT.

I MATERIALS AND METHODS

I-1 MATERIALS

1) The plasmids used for the construction of the bicistronic recombinant adenoviruses LCAT-IRES-CETP, LCAT-IRES-HL and LCAT-IRES-ApoAI are:

- pSK IRES (marketed by NOVAGEN)
- pXL2616 LCAT cDNA (Séguret-Macé et al. Circulation 1996, 94 (9): 2177-21841)
- pCRII (marketed by INVITROGEN)
- pXL2794 (WO96/25506)
- pXL2757 (WO96/25506)

2) The total RNAs obtained from hepatocytes and HepG2 cells

3) The cells 293, human kidney cells,

containing the gene encoding the adenoviral E1 protein (Graham et al., 1977)

4) The bacteria Escherichia coli: subtype DH5α of genotype EndA1,recA1,hsdR17, supE44,I-,thy-1, gyrA,rel A1,lacZD M15,deoR$^+$, F$^+$,dam$^+$,dcm$^+$ (Woodcock et al., 1989)

5) The enzymes and restriction buffers are provided by New England Biolabs.

I-2 METHODS

GENERAL MOLECULAR BIOLOGY TECHNIQUES

The methods conventionally used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in caesium chloride gradient, agarose or acrylamide gel electrophoresis, purification of DNA fragments by electroelution, phenol or phenol-chloroform extraction of proteins, ethanol or isopropanol precipitation of DNA in saline medium, transformation in Escherichia coli and the like, are well known to persons skilled in the art and are widely described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

The pBR322 and pUC type plasmids and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments can be

separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol and then incubated in the presence of phage T4 DNA ligase (Biolabs) according to the recommendations of the supplier.

The filling of the protruding 5' ends can be performed with the Klenow fragment of E. coli DNA polymerase I (Biolabs) according to the specifications of the supplier. The destruction of the protruding 3' ends is performed in the presence of phage T4 DNA polymerase (Biolabs) used according to the recommendations of the manufacturer. The destruction of the protruding 5' ends is performed by a controlled treatment with S1 nuclease.

Site-directed mutagenesis in vitro by synthetic oligodeoxynucleotides can be performed according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

The enzymatic amplification of the DNA fragments by the so-called PCR technique [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be performed using a DNA thermal cycler (Perkin Elmer Cetus) according to the specifications of the manufacturer.

The verification of the nucleotide sequences

can be performed by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467] using the kit distributed by Amersham.

CELL CULTURE TECHNIQUES

a) Culture of the cells

The cells 293 are cultured on an Eagle medium (MEM, Gibco BRL) supplemented with 10% foetal calf serum (FCS, Gibco BRL) at 37°C and at 5% CO_2.

b) Transfection of the cultured cells

The cells 293 cultured in 100 mm dishes on MEM medium and supplemented with 10% foetal calf serum are transfected with 1 mg of DNA using lipofectamine (Gibco BRL). Six hours later, the medium is removed and the cells are incubated in complete medium (MEM + 10% FCS). The supernatants of cultured cells transfected with the recombinant plasmids RSV LCAT-IRES-CETP, RSV LCAT-IRES-ApoAI and RSV LCAT-IRES-HL as well as the β-galactosidase controls and the supernatant of untransfected cells were recovered 72 hours after transfection and stored at 4°C. The tests for the activity of the enzymes LCAT, CETP, ApoAI and HL are carried out on 10 to 30 ml fractions of cell supernatant using human plasma as control.

c) Production and purification of the recombinant adenoviruses

The bicistronic adenoviral DNA is obtained by homologous recombination in E. coli. Subsequently, it is linearized with PacI and the virus produced after

transfection of the cells 293. The cells 293, transcomplementing for the E1 protein, are transfected, using lipofectamine, with 10 mg of linearized adenoviral DNA. After recovering, using agar, the cells 293 in MEM medium supplemented with 4% FCS and incubating for 8 days at 37°C, the plaques containing the recombinant virus are collected and their restriction profile analysed.

d) Infection of the cultured cells

The infection with 0.25, 0.5 and 1 ml of supernatant containing the bicistronic recombinant adenovirus is carried out on a 12-well plate containing approximately 4 x 10^5 cells 293. After incubating for 72 h in 2 ml of MEM supplemented with 2% FCS, the supernatant of infected cells is collected and the enzymatic activities are assayed.

BIOCHEMICAL VALIDATION OF THE RESULTS IN VITRO

a) Assay of the LCAT activity

The LCAT activity is estimated by measuring the conversion of free ^{14}C-cholesterol to esterified ^{14}C-cholesterol using proteoliposomes as substrate in the manner described by Chen et al. (1982). The proteoliposomes are prepared from phosphatidylcholine, cholesterol, ^{14}C-cholesterol and apolipoprotein AI and incubated with 20 ml of supernatant of transfection culture to be tested. The products (free ^{14}C-cholesterol and esterified ^{14}C-cholesterol) of the reaction are separated, by difference in their solubility in the

organic solvent, in thin-layer chromatography and detected by autoradiography on Instant Imager (Packard). The LCAT activity is expressed as percentage of ^{14}C-cholesterol esterified per hour and per 20 ml of supernatant tested.

b) Assay of the CETP activity

The CETP activity is determined by measuring the capacity of this enzyme to transfer cholesterol esters from a high-density lipoprotein particle (donor) to a low-density lipoprotein particle (acceptor). The substrates of this reaction were provided by a Wak-Chemie kit, Medical GmbH. The fluorescence of the cholesterol linoleate contained in the donor particle is quenched in the latter's native state and it is only in the presence of active CETP, which will catalyse the transfer of the fluorescent molecule to the acceptor particle, that the fluorescence can be detected at 535 nm. The CETP activity is expressed as value of intensity of fluorescence emitted at 535 nm for 30 ml of culture supernatant tested and per hour.

c) Assay of the HL activity

The hepatic lipase activity is estimated using a synthetic triglyceride substrate provided by a Progen Biotechnik GmbH kit. This substrate contains a fluorescent pyrene group which is masked by trinitrophenol in the native state of the molecule and the effect of the hydrolysis of the latter is the emission of fluorescence at 400 nm. By measuring the

intensity of fluorescence emitted at 400 nm after incubation of the substrate with the sample to be tested and using the standard plasma provided by the kit, the quantity (in pmol/min) of HL contained in 20 ml of supernatant can be estimated.

d) Assay of apolipoprotein AI

The ApoAI activity is estimated using an anti-ApoAI monoclonal antibody. For that, Immulon II plaques (Dynatech) are coated with an anti-ApoAI monoclonal antibody (10 mg/ml in carbonate buffer pH 9.6) by incubating overnight at 4°C, and then saturated with 2% BSA in PBS pH 7.4 for one hour at 37°C. The cellular supernatants are then incubated for one hour at 37°C, optionally after dilution in PBS 2% BSA. The revealing is then performed by incubating for one hour at 37°C with a mixture of anti-ApoAI monoclonal antibodies labelled with peroxidase, and diluted 1/5000. The binding of the peroxidized antibodies is finally revealed by incubating with 250 µl of TMB (KPL) and reading the plaques at 630 nm.

TECHNIQUE FOR CONSTRUCTING THE SHUTTLE PLASMIDS

The constructions of the bicistronic plasmids are detailed in Figures 3 to 5. The bicistronic plasmids obtained are, at the same time, shuttle plasmids because they contain the adenoviral sequences pIX-IVa2 necessary for the recombination with the viral genome. This recombination occurs either by cotransfection with a cleaved genome derived from a

virus β-galactosidase (conventional shuttle vector), or by double recombination in E. coli (coli shuttle vector).

The sequence conformity of various plasmid structures is examined by analysing their restriction profile. This examination makes it possible to select recombinant clones which will serve for subsequent clonings as well as to validate the results of the clonings. The restriction enzymes are chosen so as to have the most complete information on the entire cloned cDNA and the number of strategic sites for the cloning. However, this control does not make it possible to exclude the existence of certain mutations such as non-sense or substitution point mutations which may occur at any stage of the cloning. Such mutations can be detected only by complete sequencing of the cDNA in question. The final control of the validity of the constructs obtained is made by biochemical tests of enzymatic activities of LCAT, CETP and HL in vitro, or detecting the presence of ApoAI.

EXAMPLE 1:

Construction of the shuttle plasmids LCAT-IRES-CETP

1) Construction of the conventional shuttle vector pXL2970

The expression vector pXL2968 RSV LCAT polyA bGH is obtained by cleaving the plasmids pXL2616, containing the LCAT cDNA, on the one hand, and the plasmid pXL LPL, under the control of the RSV promoter

and upstream of the bovine growth hormone polyadenylation signal, on the other hand, with the restriction enzymes SalI and ClaI, and ligating the resulting fragments with T4 DNA ligase.

To do this:

The plasmids pXL RSV LPL (2 μg) and pXL2616 (2 μg) are each digested with 10 units (u) of ClaI, in buffer 4 (20 mM Tris acetate, 10 mM Mg-acetate, 50 mM K-acetate and 1 mM DTT) supplemented with 100 μg/ml of acetylated BSA, for 90′ at 37°C. 100 mM of NaCl and 10 u of SalI are added and the reaction mixture is again incubated at 37°C for a further 90′. After electrophoretic migration on a 0.7% agarose gel of the products of the digestions, the 6.5 and 1.7 Kb bands corresponding to pXLRSV and to the LCAT cDNA are cut out from the gel and extracted with a Qiaquick kit. The two bands are ligated with 400 u of T4 DNA ligase after incubating overnight at 14°C.

The recombinant plasmid pXL2969 IRES-CETP is generated from the plasmid bluescript possessing the CETP cDNA modified at its 5′ end by introducing an NcoI site using a PCR 5′ GCCTGATAAC CATGGTGGCT GCCACAG 3′ (SEQ ID No. 1). The plasmid thus modified is cleaved by NcoI and Sal and cloned downstream of the adenoviral IRES sequence included in the plasmid pSKIRES cleaved by the same restriction enzymes in the following manner:

2.5 μg of the plasmid CETP and 2.5 μg of the plasmid pSKIRES are digested with 10 u of NcoI for 90′

at 37°C in buffer 3 (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) followed by digestion with 10 u of SalI for 90' at 37°C. The products of the digestions are subjected to electrophoretic migration and the bands of 1.5 Kbp (CETP cDNA) and of 3.5 Kbp are extracted and ligated under the same conditions as are described in the paragraph above.

To obtain the plasmid pXL2970, the procedure is carried out as follows:

Four μg of plasmid pXL2968 are linearized with 10 u of SalI (buffer 3+BSA, 90' at 37°C). The DNA is then extracted using the Qiaquick kit and recovered in 50 μl of water preheated to 50°C. The cohesive ends resulting from the SalI cleavage are made blunt by incubating with 5 u of Klenow polymerase for 15' at 25°C. The DNA is reextracted with the Qiaquick kit and dephosphorylated with 2 u of calf intestinal phosphatase (CIP) for 60' at 37°C. The plasmid pXL2969 (4 μg) is first digested with 5 U of SmaI (buffer 4, 90' at 25°C) and then with 5 U of HincII (buffer 3+BSA, 100 mM NaCl, 90' at 37°C). The products of the digestions of the 2 plasmids are subjected to electrophoretic migration on a 0.7% agarose gel and the DNA bands of 8.5 Kbp (pXL2968) and of 2.2 Kbp (IRES CETP cDNA) are extracted with the Qiaquick kit and ligated with 400 u of T4 DNA ligase (Figure 3).

The LCAT and CETP activities of the plasmid pXL2970 are assayed in the 293 cell culture supernatant

three days after transfection.

The LCAT activity of this plasmid corresponds to 3.5% of cholesterol esters formed per hour and the CETP activity to 120% (Table 1 below). These activity values show that the plasmid pXL2970 indeed synthesizes LCAT and CETP which are catalytically active.

2) The coli shuttle plasmid LCAT-IRES-CETP

In this technology, the shuttle vector should comprise:

- the sequences ITR-inverted terminal repeat and ψ encapsidation sequence which are necessary for homologous recombination in E. coli, surrounding the LCAT-IRES-CETP sequence,

- a col E1 replication origin making the plasmid nonreplicative in the E. coli C2110 strain and thus allowing the clonality of the recombinant plasmid,

- a suicide gene sucrose B (lethal for the bacterium in culture on sucrose) and a gene for resistance to spectinomycin which will allow the selection of the recombinant clone.

To do this, the bicistronic shuttle plasmid pXL2970 RSV LCAT-IRES-CETP is cleaved with BstEII and SpeI so as to introduce therein ITR and ψ sequences of the adenoviral genome and thus obtain a coli shuttle plasmid. The ITR and ψ sequences are isolated by digesting the plasmid pXL2794 with BstEII and XbaI. The blunt-ended fragments containing the spectinomycin-sucrose B cassette obtained by digesting pXL2757 with

EcoRV and SmaI was introduced into the shuttle plasmid pXL2970 + 2794 linearized with bFspI (Fig. 4).

Experimentally, the procedure is carried out according to the following protocol:

Three μg of the plasmid pXL2970 are digested with 20 u of BstEII (buffer 2: 10 mM Tris HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT; 90′ at 60°C), then with 10 u of SpeI (90′ at 37°C).

The resulting DNA fragment is extracted with the Qiaquick kit and dephosphorylated with 2 u of CIP (60′ at 37°C). The plasmid pXL2794 is digested with 20 u of BstEII (buffer 2, 90′ at 60°C) followed by 20 u of XbaI (90′ at 37°C). The bands of 6.5 Kbp (pXL2970) and of 2.9 Kbp (ITR ψ and Kan^r of pXL2794), extracted after electrophoretic migration, are ligated using T4 DNA ligase (40 Ou).

The resulting plasmid pXL2970+2794 (1.5 μg) is cleaved with 5 u of FspI (buffer 4, 60′ at 37°C), extracted using the Qiaquick kit, and ligated (T4 DNA ligase, 400 u) with the 3.8 Kbp DNA fragment extracted from the gel containing the sacB-$spect^r$ cassette derived from the digestion of the plasmid pXL2757 with 10 u of SmaI (buffer 4, 90′ at 25°C) followed by 10 u of EcoRV (90′ at 37°C).

The shuttle plasmid pXL2974 LCAT-IRES-CETP is subjected to a first selection on spectinomycin medium and spectinomycin+sucrose medium. The results of the NcoI (6.2+3+2.2+2), NotI (13.5 Kbp) and EcoRV

(1+3+9.5 Kbp) digestions are in conformity with the restriction map of the said plasmid.

The LCAT activity of the plasmid pXL2974 corresponds to 2% of cholesterol esters formed per hour and that of CETP to 114% (Table 1). The LCAT and CETP activities are found at the level of the coli shuttle plasmid LCAT-IRES-CETP.

	LCAT ACTIVITY	CETP ACTIVITY
Shuttle plasmid LCAT-IRES-CETP	3.5 +/- 0.2%	120 +/- 2%
Coli shuttle plasmid LCAT-IRES-CETP	2 +/- 0.2%	114 +/- 2%

Table 1

EXAMPLE 2:

Construction of the shuttle plasmid RSV LCAT-IRES-HL

The HL cDNA is cloned behind the IRES in the vector bluescript and the IRES-HL fragment is then included in a manner similar to the vector LCAT-IRES-CETP according to the following protocol:

The plasmid pXL2971 (4 μg) is digested, so as to remove an NcoI site, with 40 I.U. of BglII (buffer 3, 90' at 37°C) and then with 40 u of SalI for 90' at 37°C. The 2.5 Kbp DNA fragment (approximate mass of 0.5 μg) derived from these digestions is subjected, after

migration and extraction on a gel, to a controlled digestion with NcoI (0.1-1 u NcoI/μg of DNA) in buffer 4 for 60' at 37°C. The products of digestion are analysed by migration on a 0.7% agarose gel and the 1.5 Kbp band containing the HL cDNA ABC, obtained with 0.5 u of NcoI, is ligated (T4 DNA ligase, 400 u) with the pSK IRES fragment (1.3 μg) resulting from the NcoI and SalI digestions (1 u of each enzyme, buffer 3, 37°C).

The final vector is presented in Figure 5. The corresponding bicistronic shuttle plasmid LCAT-IRES-HL is called pXL2984.

	LCAT ACTIVITY	HL ACTIVITY
Shuttle plasmid LCAT-IRES-HL	1.2 +/- 0.2%	46 +/- 2%

Table 2

EXAMPLE 3

Construction of the shuttle plasmid ApoAI-IRES-LCAT

The general principle for this construction is identical to the previous ones. LCAT is mutated by PCR, including an additional NcoI site allowing its ligation in the right place behind the IRES. Its sequence was completely checked. The IRES-LCAT fragment is then ligated behind ApoAI. The resulting vector is derived directly from the coli technology and is called

pXL3058 (Figure 6). After the usual double recombinations, the resulting viral vector was checked in terms of activity. The ApoAI was detected by Western blotting and the LCAT activity evaluated at 1.3% (background noise at 0.2%).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(D) COUNTRY: FRANCE

(E) POSTAL CODE: 92165

(F) TELEPHONE: 01.55.71.69.22

(G) TELEFAX: 01.55.71.72.96

(ii) TITLE OF INVENTION: BICISTRONIC RECOMBINANT VIRUSES USEFUL FOR THE TREATMENT OF DYSLIPOPROTEINEMIA-RELATED PATHOLOGIES

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Tape

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCCTGATAAC CATGGTGGCT GCCACAG

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Defective recombinant virus which comprises, at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and are involved in the reverse transport of cholesterol, the said nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

2. Defective recombinant virus according to claim 1, wherein the inserted nucleic acids are chosen from the genes encoding all or part of lecithin cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), hepatic lipase (HL), apolipoproteins AI and AIV or one of their variants.

3. Defective recombinant virus according to claim 1 or 2, wherein the nucleic acids are genes encoding all or part of the corresponding human enzymes, proteins and/or cofactors.

4. Defective recombinant virus according to any one of the preceding claims, wherein the nucleic acids are cDNA or gDNA.

5. Defective recombinant virus according to any one of the preceding claims, wherein one of the nucleic acids encodes LCAT.

6. Defective recombinant virus according to claim 5, wherein the second nucleic acid encodes CETP, HL or ApoAI.

7. Defective recombinant virus according to any one of the preceding claims, wherein the transcriptional promoter is chosen from the promoters of the adenovirus

MLP and E1A nucleic acid sequences and the CMV, RSV-LTR, MT-1 and SV40 promoters.

8. Defective recombinant virus according to any one of the preceding claims, wherein the IRES sequence is derived from a picornavirus.

9. Defective recombinant virus according to claim 8, wherein the picornavirus IRES sequence is derived either from an encephalomyocarditis virus or from the poliovirus.

10. Defective recombinant virus according to any one of the preceding claims, which lacks at least the regions of its genome which are necessary for its replication in the infected cell.

11. Defective recombinant virus according to any one of the preceding claims, which is an adenovirus.

12. Defective recombinant virus according to claim 11 which is of the human Ad5 or Ad2 type or of animal origin.

13. Defective recombinant adenovirus which comprises at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and involved in the reverse transport of cholesterol, the said nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

14. Defective recombinant adenovirus according to claim 13, which comprises at least one gene encoding LCAT and one gene encoding HL, the said genes being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome

entry site IRES.

15. Defective recombinant adenovirus according to claim 13, which comprises at least one gene encoding LCAT and one gene encoding apoA-I, the said genes being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

16. Defective recombinant adenovirus according to claim 13, which comprises at least one gene encoding LCAT and one gene encoding CETP, the said genes being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site IRES.

17. Defective recombinant adenovirus according to claim 16, which is derived from homologous recombination between pXL2974 and pXL2822.

18. Defective recombinant adenovirus according to any one of claims 13 to 17, which comprises at least one deletion in the E1 region and one deletion in the E3 region.

19. Prokaryotic plasmid comprising an adenovirus genome and at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, the two nucleic acids being operably linked to a transcription promoter and separated from each other by a sequence encoding an internal ribosome entry site, IRES.

20. Prokaryotic plasmid comprising a first region allowing replication in prokaryotic cells and a second region comprising the adenoviral genome bordered by one

or more restriction sites not present in the said genome and in which are present at least two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, these two nucleic acids being operably linked to a transcriptional promoter and separated from each other by a sequence encoding an internal ribosome entry site, IRES.

21. Prokaryotic plasmid according to claim 19 or 20, wherein the E1 and E3 regions of the adenoviral genome are deleted.

22. Prokaryotic plasmid according to claims 19 to 20 or 21, which comprises the viral ITR and ψ sequences.

23. Prokaryotic plasmid according to any one of claims 19 to 22, wherein the replication origin is derived from a bacterial plasmid chosen from RK2, pBR322 and pUC.

24. Prokaryotic plasmid comprising, in 5'-3' orientation, at least one replication origin functional in the prokaryotic cells, a first part of an adenoviral genome comprising the viral ITR and ψ sequences, a transcriptional promoter, a first nucleic acid encoding an enzyme, protein and/or cofactor involved in the reverse transport of cholesterol, an IRES sequence, a second nucleic acid encoding an enzyme, protein and/or cofactor involved in the reverse transport of cholesterol, a polyadenylation site and a second part of an adenoviral genome comprising the pIX-IVa2 region.

25. Prokaryotic plasmid according to any one of claims 19 to 24, which comprises, in addition, a region

allowing the selection of the prokaryotic cells containing the said plasmid.

26. Prokaryotic plasmid according to any one of claims 19 to 25, wherein at least one of the nucleic acids encodes LCAT and the second nucleic acid is chosen from those encoding HL, ApoAI or CETP.

27. Prokaryotic plasmid according to claim 26, which is pXL2974 containing nucleic acids encoding LCAT and CETP.

28. Prokaryotic plasmid according to claim 26, which is pXL3058 containing nucleic acids encoding LCAT and ApoAI.

29. Shuttle plasmid which comprises two nucleic acids encoding enzymes, proteins and/or cofactors which are distinct and which are involved in the reverse transport of cholesterol, the two nucleic acids being operably linked to a transcriptional promoter, a polyadenylation site and a sequence encoding an internal ribosome entry site, IRES, situated between the two nucleic acids.

30. Shuttle plasmid according to claim 29, which is the plasmid pXL2984 comprising two nucleic acids encoding HL and LCAT respectively.

31. Shuttle plasmid according to claim 29, which is the plasmid pXL2970 comprising two nucleic acids encoding LCAT and CETP respectively.

32. Prokaryotic cell transformed with a prokaryotic plasmid according to any one of claims 19 to 28.

33. Use of a defective recombinant virus according to any one of claims 1 to 12, of a defective recombinant

adenovirus according to any one of claims 13 to 18 or of a plasmid according to any one of claims 19 to 31 for the preparation of a pharmaceutical composition intended for the treatment or prevention of a pathology linked to hypoalphalipoproteinemia.

34. Use according to claim 33 for the preparation of a pharmaceutical composition intended for the treatment of atherosclerosis and/or of restenosis.

35. Pharmaceutical composition comprising one or more defective recombinant viruses according to any one of claims 1 to 12, a defective recombinant adenovirus according to any one of claims 13 to 18 or a plasmid according to any one of claims 19 to 31 and a pharmaceutically acceptable vehicle.

36. Pharmaceutical composition according to claim 35, which is in an injectable form and comprises from 10^4 to 10^{14} pfu/ml of adenovirus.

37. Method of treatment or prevention of a pathology linked to hypoalphalipoproteinemia which comprises administering to a patient a pharmaceutical composition according to claim 35 or 36.

38. Method of treatment of atherosclerosis and/or of restenosis which comprises administering to a patient a pharmaceutical composition according to claim 35 or 36.

39. Defective-recombinant virus according to claim 1 substantially as hereinbefore described in any one of the Examples.

40. Defective-recombinant adenovirus according to claim 13 substantially as hereinbefore described in any one of the Examples.

41. Prokaryotic plasmid according to claim 19, 20 or 24 substantially as hereinbefore described in any one of the Examples.

42. Shuttle plasmid according to claim 29 substantially as hereinbefore described in any one of the Examples.

43. Prokaryotic cell according to claim 32 substantially as hereinbefore described in any one of the Examples.

Dated this 26th day of April 2000.

Rhone-Poulenc Rorer S.A.

By its Patent Attorneys

Davies Collison Cave

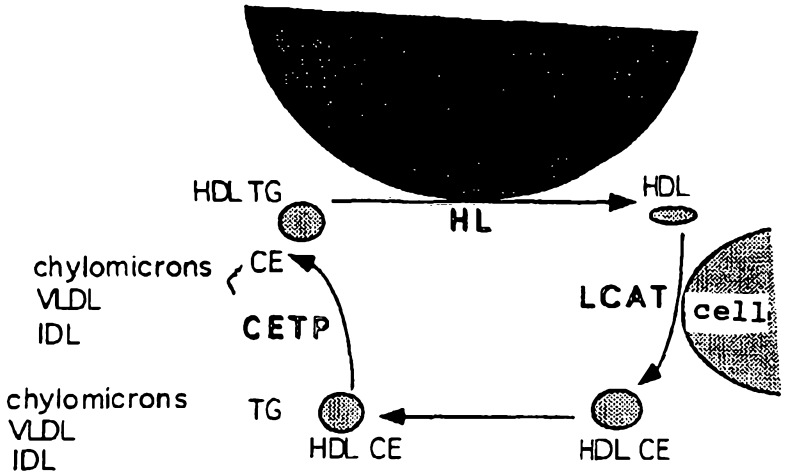
HDL TG
HDL
HL
chylomicrons
VLDL
IDL
CE
CETP
LCAT
cell
chylomicrons
VLDL
IDL
TG
HDL CE
HDL CE

Figure 1

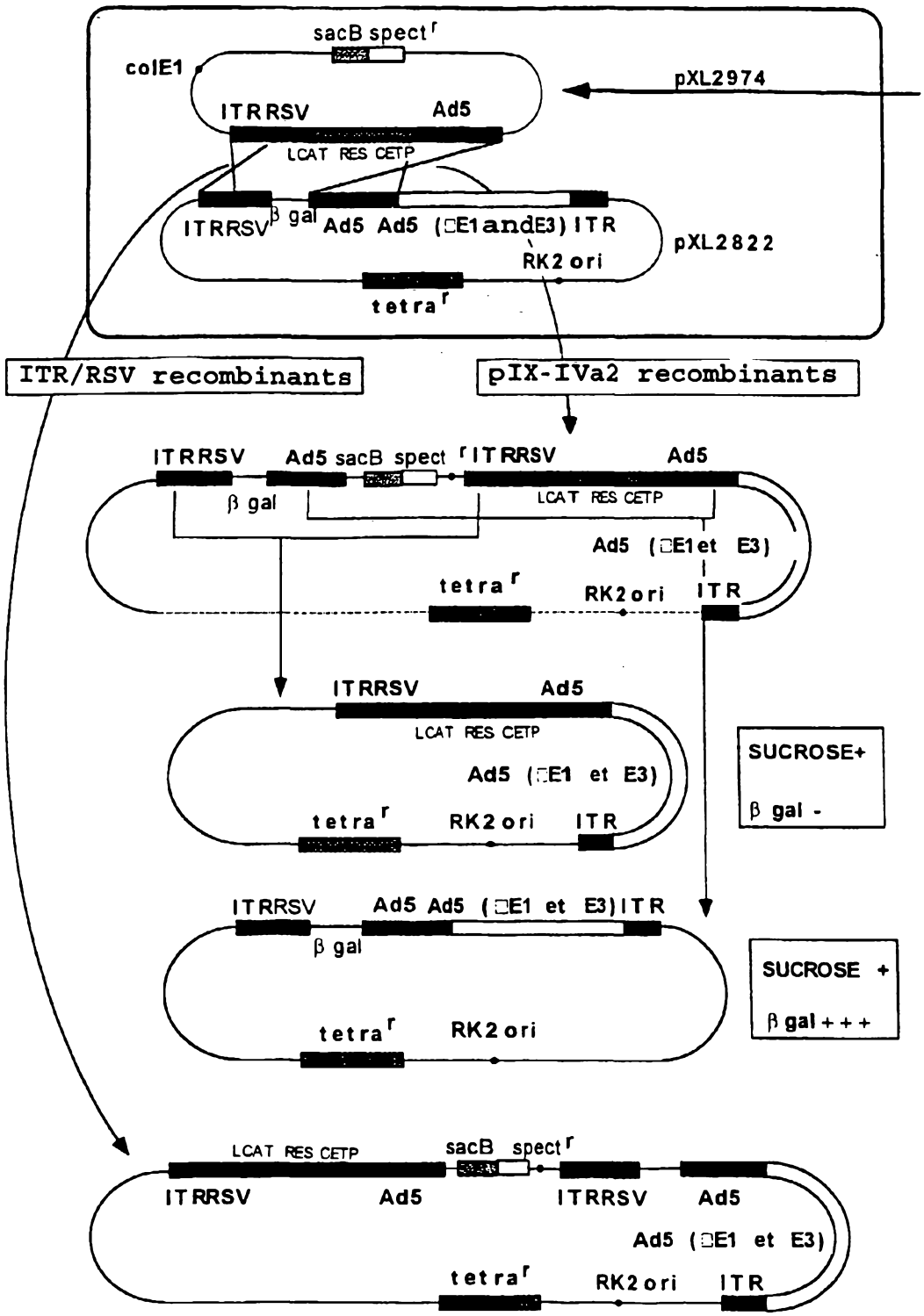
sacB spect r
colE1
pXL2974
ITRRSV
Ad5
LCAT RES CETP
ITRRSV
β gal
Ad5 Ad5 (□E1 and E3) ITR
pXL2822
RK2 ori
tetra r
ITR/RSV recombinants
pIX-IVa2 recombinants
ITRRSV
Ad5 sacB spect r
ITRRSV
Ad5
β gal
LCAT RES CETP
Ad5 (□E1 et E3)
tetra r
RK2 ori
ITR
ITRRSV
Ad5
LCAT RES CETP
Ad5 (□E1 et E3)
tetra r
RK2 ori
ITR
SUCROSE+
β gal -
ITRRSV
Ad5 Ad5 (□E1 et E3) ITR
β gal
tetra r
RK2 ori
SUCROSE +
β gal + + +
LCAT RES CETP
sacB spect r
ITRRSV
Ad5
ITRRSV
Ad5
Ad5 (□E1 et E3)
tetra r
RK2 ori
ITR

Figure 2

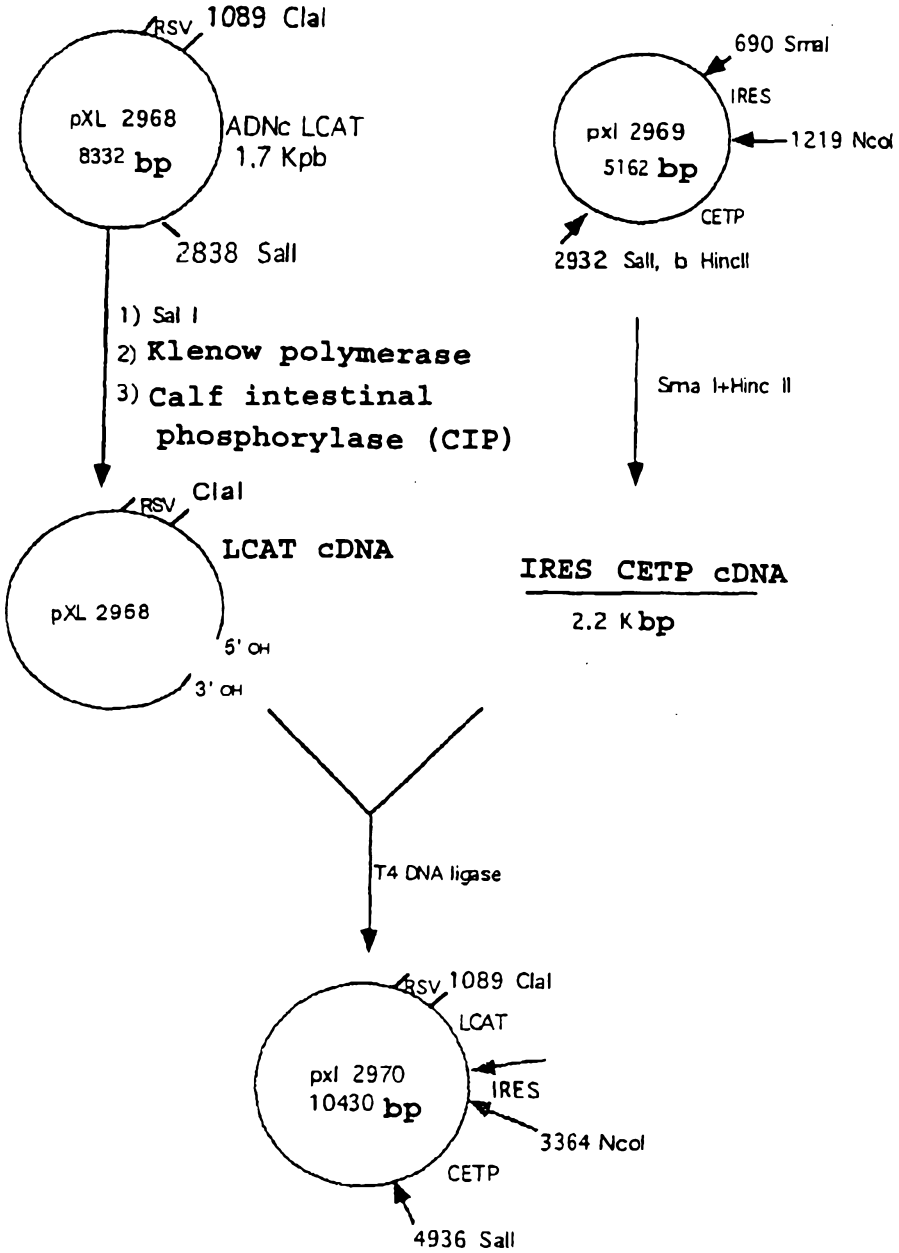
RSV
1089 ClaI
pXL 2968
8332 bp
ADNc LCAT
1.7 Kpb
2838 SalI
690 SmaI
IRES
pxl 2969
5162 bp
1219 NcoI
CETP
2932 SalI, b HincII
1) Sal I
2) Klenow polymerase
3) Calf intestinal phosphorylase (CIP)
Sma I+Hinc II
RSV
ClaI
LCAT cDNA
pXL 2968
5' OH
3' OH
IRES CETP cDNA
2.2 Kbp
T4 DNA ligase
RSV
1089 ClaI
LCAT
pxl 2970
10430 bp
IRES
3364 NcoI
CETP
4936 SalI

Figure 3

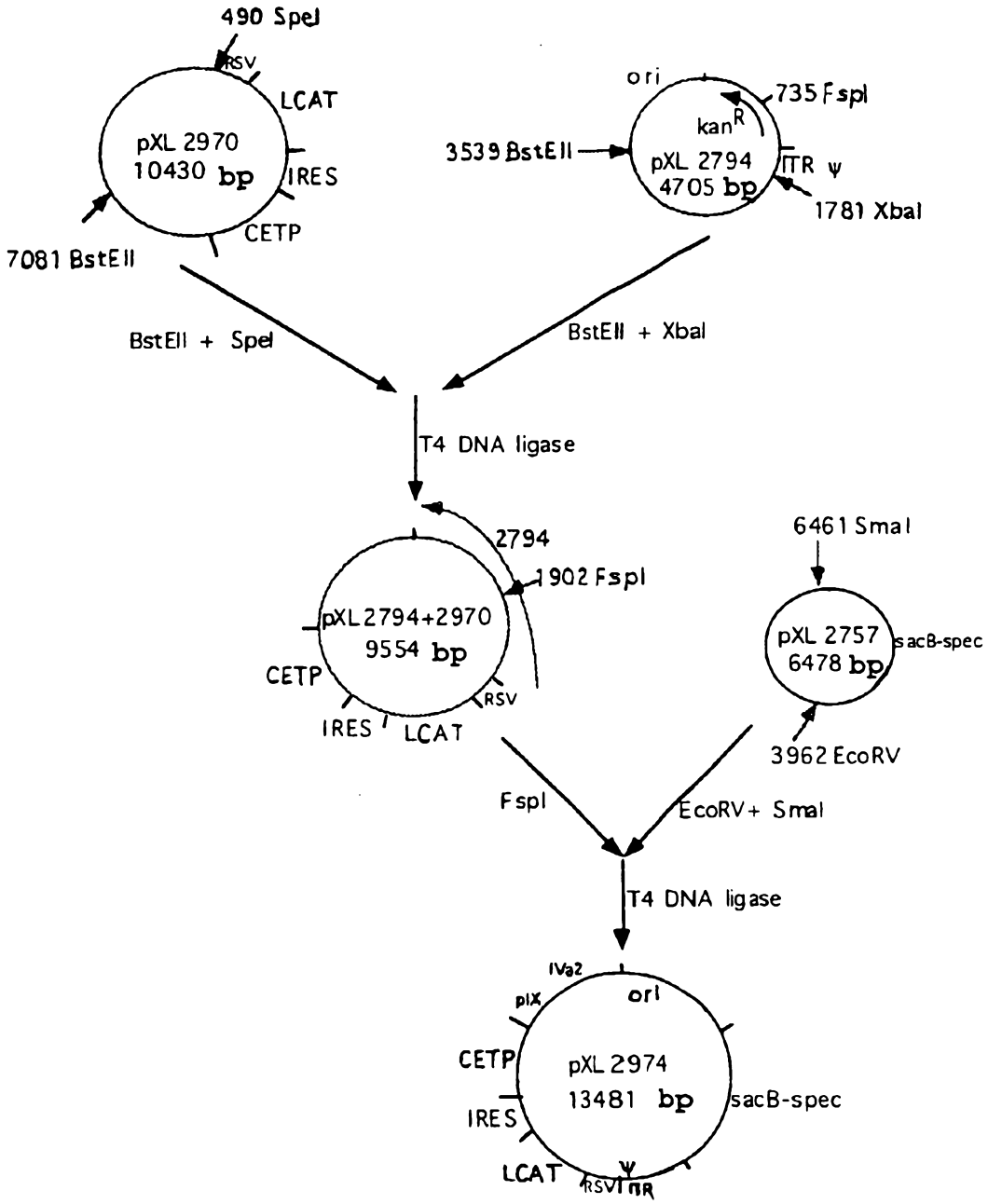
490 SpeI
RSV
LCAT
pXL 2970
10430 bp
IRES
CETP
7081 BstEII
ori
735 FspI
kanR
3539 BstEII
pXL 2794
4705 bp
ITR ψ
1781 XbaI
BstEII + SpeI
BstEII + XbaI
T4 DNA ligase
2794
1902 FspI
pXL2794+2970
9554 bp
CETP
IRES
LCAT
RSV
6461 SmaI
pXL 2757
6478 bp
sacB-spec
3962 EcoRV
FspI
EcoRV+ SmaI
T4 DNA ligase
IVa2
pIX
ori
CETP
pXL 2974
13481 bp
sacB-spec
IRES
LCAT
RSV
ITR
ψ

Figure 4

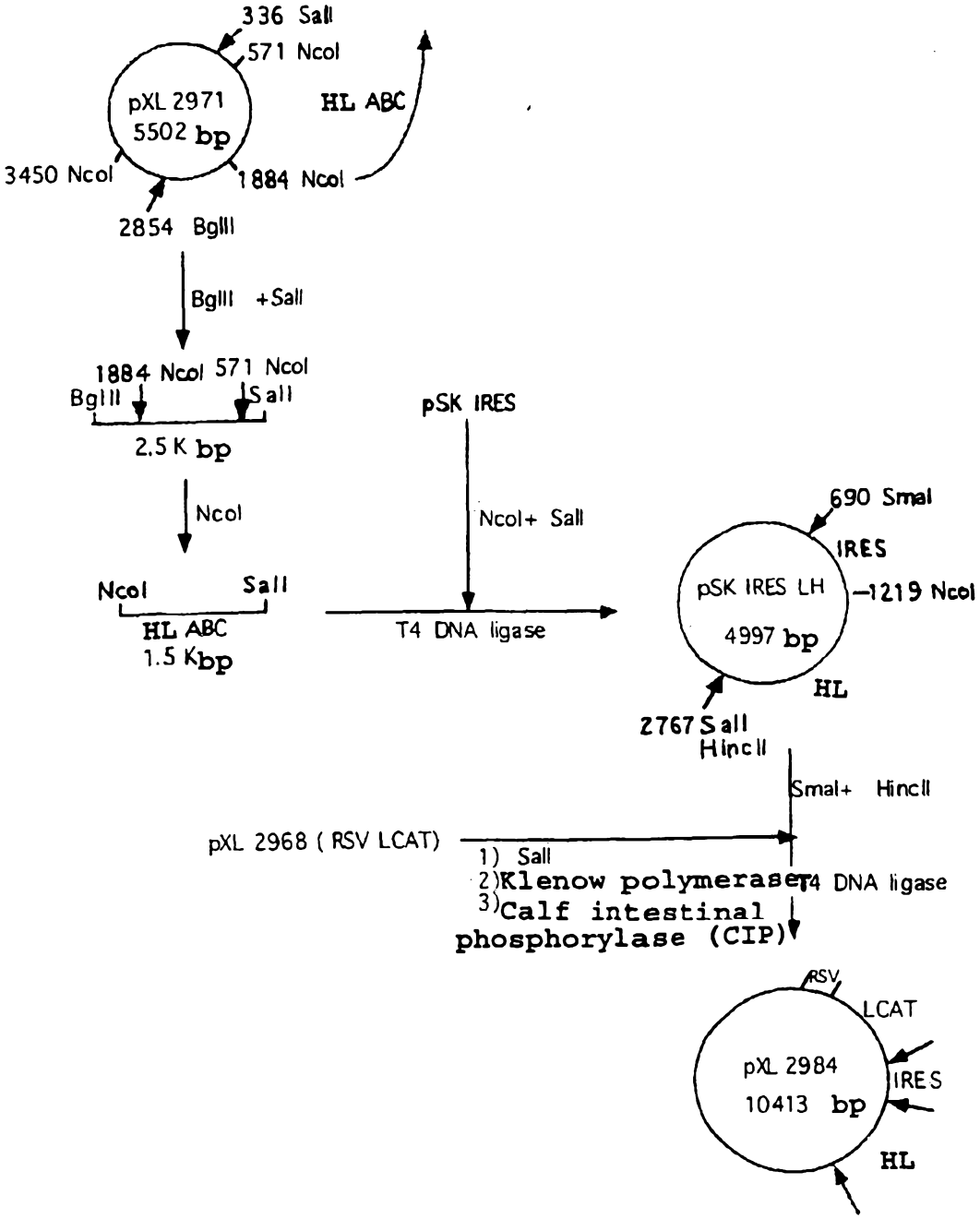
336 SalI
571 NcoI
pXL 2971
5502 bp
HL ABC
3450 NcoI
1884 NcoI
2854 BglII
BglII +SalI
1884 NcoI 571 NcoI
BglII SalI
2.5 K bp
NcoI
NcoI SalI
HL ABC
1.5 Kbp
pSK IRES
NcoI+ SalI
T4 DNA ligase
690 SmaI
IRES
pSK IRES LH
4997 bp
1219 NcoI
HL
2767 SalI
HincII
SmaI+ HincII
pXL 2968 (RSV LCAT)
1) SalI
2)Klenow polymerase
3)Calf intestinal
phosphorylase (CIP)
T4 DNA ligase
RSV
LCAT
pXL 2984
10413 bp
IRES
HL

Figure 5

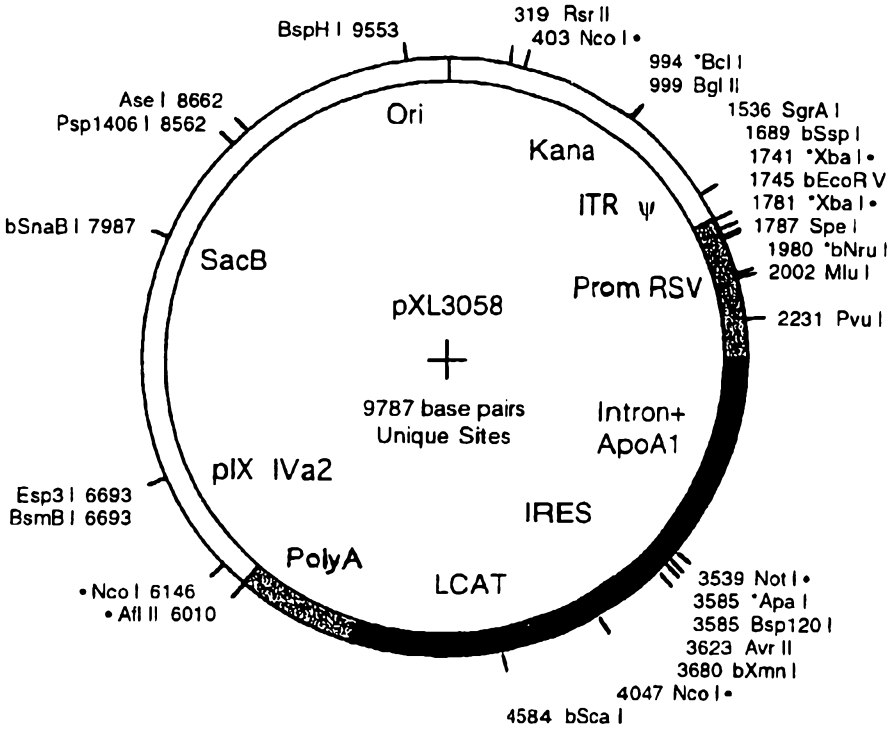
BspH I 9553
319 Rsr II
403 Nco I •
994 *Bcl I
999 Bgl II
1536 SgrA I
1689 bSsp I
1741 *Xba I •
1745 bEcoR V
1781 *Xba I •
1787 Spe I
1980 *bNru I
2002 Mlu I
2231 Pvu I
Ase I 8662
Psp1406 I 8562
bSnaB I 7987
Esp3 I 6693
BsmB I 6693
• Nco I 6146
• Afl II 6010
3539 Not I •
3585 *Apa I
3585 Bsp120 I
3623 Avr II
3680 bXmn I
4047 Nco I •
4584 bSca I
Ori
Kana
ITR ψ
Prom RSV
SacB
pXL3058
9787 base pairs
Unique Sites
Intron+
ApoA1
pIX IVa2
IRES
PolyA
LCAT

Figure 6