Method for the preparation of a viral vector by intermolecular homologous recombination

The present invention relates to a method for preparing a viral vector *in vitro* in a prokaryotic cell and to its application for producing an infectious viral particle intended for therapeutic use, and especially for use in gene therapy.

The possibility of treating human diseases by gene therapy has changed in a few years from the stage of theoretical considerations to that of clinical applications. The first protocol applied to man was initiated in the US in September 1990 on a patient who was genetically immunodeficient as a result of a mutation affecting the gene coding for adenine deaminase (ADA). The relative success of this first experiment encouraged the development of new gene therapy protocols for various genetic or acquired diseases (infectious diseases, and viral diseases in particular, such as AIDS, or cancers). The large majority of the protocols described hitherto employ viral vectors to transfer the therapeutic gene to the cells to be treated and to express it therein.

To date, retroviral vectors are among the ones most widely used on account of the simplicity of their genome. However, apart from their restricted capacity for cloning, they present two major drawbacks which limit their systematic use: on the one hand they chiefly infect dividing cells, and on the other hand, as a result of their integration at random in the genome of the host cell, the risk of insertional mutagenesis is not insignificant. For this reason, many scientific teams have endeavored to develop other types of vector, among which those originating from adenoviruses, adeno-associated viruses (AAV), poxviruses and herpesviruses may be mentioned. Generally speaking, their organization and their infection cycle are amply described in the literature available to a person skilled in the art.

In this connection, the use of adenoviral vectors has been seen to be a promising alternative. Adenoviruses

have been demonstrated in many animal species, have a broad host range, have little pathogenicity and do not present the drawbacks associated with retroviruses since they replicate in resting cells and are nonintegrative. As a guide, their genome consists of a linear, double-stranded DNA molecule of approximately 36 kb carrying more than about thirty genes, both early genes necessary for viral replication (E1 to E4) and late structural genes (L1 to L5) (see Figure 1).

Generally speaking, the adenoviral vectors are obtained by deletion of at least one portion of the viral genes (in particular of the E1 region essential for viral replication), which are replaced by the therapeutic genes. Consequently, they are propagated in a cell line, termed complementation line, which supplies *in trans* the deleted viral functions to generate a viral vector particle which is defective for replication but capable of infecting a host cell. The line 293, established from human embryonic kidney cells, which complements adenoviruses that are defective for the E1 function (Graham et al., 1977, J. Gen. Virol., *36*, 59-72), is commonly used.

The techniques of preparation of adenoviral vectors are amply described in the literature (see, in particular, Graham and Prevec, Methods in Molecular Biology, Vol. 7; Gene Transfer and Expression Protocols; Ed: E.J. Murray, 1991, The Human Press Inc., Clinton, NJ). One of the methods most often employed consists in generating the recombinant adenoviral vector in complementation cells transfected with a bacterial plasmid carrying the gene of interest subcloned within its adenoviral insertion region and the adenoviral genome. Generally, the latter is cleaved with a suitable restriction enzyme so as to reduce the infectivity of the parent viral DNA and to increase the efficiency of isolation of the recombinant adenoviruses. However, a substantial background of infectious viral particles of parenteral origin is nevertheless observed, which necessitates an especially arduous task of analysis of the plaques obtained (arduous from a time and cost standpoint, since

each plaque has to be amplified and analyzed individually). This is problematical when the parent virus displays a selective advantage over the recombinant adenovirus, for example when the latter replicates more slowly than the parent virus as a result of the insertion of a large-sized gene of interest (factor VIII, dystrophin), reducing proportionately the probability of obtaining it.

Ligation between two DNA fragments generated by the standard techniques of molecular biology and carrying, respectively, the 5' and 3' portions of the recombinant adenoviral vector may also be employed. Transfection of the ligation mixture into the complementation cells makes it possible in theory to encapsidate the genome of the recombinant adenovirus to form an infectious particle. This technology is of low efficiency and its application limited by the restricted number of suitable and unique restriction sites.

It has now been shown to be possible to generate a recombinant adenoviral vector in *Escherichia coli* (*E. coli*) by intermolecular homologous recombination between a plasmid containing the genome of a type 5 adenovirus and an insert carrying an exogenous DNA sequence surrounded by adenoviral sequences A and B (Figure 2). This method leads to the replacement in the adenoviral genome of the targeted region located between A and B by the exogenous sequence. Transfection of the recombinant adenoviral vector thus generated into an appropriate complementation line gives rise to an infectious viral particle which may be used without a prior purification step to infect a host cell. The background (contamination with parent viral particles) is reduced or even abolished. In addition, it was found, surprisingly, that the use of E. coli recBC sbcBC strains is especially advantageous for promoting intermolecular recombination between any two DNA fragments.

The method of the present invention is based on exploitation of the endogenous enzymatic activities of the prokaryotic cells involved in homologous

recombination. This intermolecular recombination technique had already been described for the cloning of small inserts into bacterial vectors (Bubeck et al., 1993, Nucleic Acids Research, *21*, 3601-3602) or for generating hybrid genes by intramolecular recombination (Calogero et al., 1992, FEMS Microbiology Letters, *97*, 41-44; Caramori et al., 1991, Gene, *98*, 37-44). However, this recombination technology had never been employed in prokaryotic cells to generate infectious viral vectors (capable of being encapsidated into infectious viral particles).

The method of the invention has the advantage over the previous techniques of being rapid and especially efficient, and of requiring only a small number of manipulations *in vitro*. Furthermore, it may be employed with DNA fragments generated by PCR (polymerase chain reaction), thereby avoiding the steps of subcloning of the exogenous DNA sequence into the insertion region of the viral genome. Lastly, it provides an advantageous solution to the problem of background which is clearly identified in the literature. Consequently, it proves especially efficacious for vectors originating from large-sized viruses or viruses into which the insertion of a large-sized exogenous DNA sequence is envisaged.

Accordingly, the subject of the present invention is a method for preparing, in a prokaryotic cell, a recombinant viral vector derived from a parent virus into the genome of which an exogenous DNA sequence is inserted, by intermolecular recombination between (i) a first DNA fragment comprising all or part of said genome of the parent virus and (ii) a second DNA fragment comprising said exogenous DNA sequence surrounded by flanking sequences A and B which are homologous to (i).

For the purposes of the present invention, a recombinant viral vector is obtained from a parent virus modified so as to carry at a suitable site of the viral genome and to express an exogenous DNA sequence. The parent viruses capable of being used in the context of the invention are, for example, alphaviruses,

retroviruses, poxviruses, and in particular vaccinia or canarypox virus, herpesviruses, adeno-associated viruses (AAV) and most especially adenoviruses.

In this connection, the choice of parent adenovirus is very wide. It can be an adenovirus of human, canine, avian, bovine, murine, ovine, porcine or simian origin, or alternatively a hybrid adenovirus comprising adenoviral genome fragments of different origins. Very special preference will be given to a serotype C adenovirus of human origin and preferably a type 2 or 5 adenovirus, or alternatively to an adenovirus of animal origin of the CAV-1 or CAV-2 (canine), DAV (avian) or alternatively BAd (bovine) type. These viruses and their genome are described in the literature (see, for example, Zakharchuk et al., 1993, Arch. Virol., *128*, 171-176; Spibey and Cavanagh, 1989, J. Gen. Virol., *70*, 165-172; Jouvenne et al., 1987, Gene, *60*, 21-28; Mittal et al., 1995, J. Gen. Virol., *76*, 93-102).

The objective of a method according to the invention is to prepare a recombinant viral vector for the transfer of an exogenous DNA sequence to a host cell and its expression therein. "Exogenous DNA sequence" is understood to mean a nucleic acid which comprises coding sequences and regulatory sequences permitting the expression of said coding sequences, and in which the coding sequences are sequences which are not normally present in the genome of a parent virus employed in the present invention or, if they are present, are in a different genomic context. In the context of the invention, the exogenous DNA sequence perhaps [sic] composed of one or more genes. The regulatory sequences may be of any origin.

Preferably, the exogenous DNA sequence can code for an antisense RNA and/or an mRNA which will then be translated into a protein of interest. It may be of the genomic type, of the complementary DNA (cDNA) type or of mixed type (minigene, in which at least one intron is deleted). It may code for a mature protein, a precursor of a mature protein, in particular a precursor intended

to be secreted and, as a result, comprising a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origins or a mutant of a natural protein displaying improved or modified biological properties. Such a mutant may be obtained by mutation, deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the natural protein.

In the context of the present invention, it can be advantageous to use:

- the genes coding for cytokines or lymphokines, such as interferons α, β and γ, interleukins, and in particular IL-2, IL-6 or IL-10, tumor necrosis factors (TNF) and colony stimulating factors (CSF);
- the genes coding for cell receptors, such as the receptors recognized by pathogenic organisms (viruses, bacteria or parasites), preferably by the HIV virus (human immunodeficiency virus), or ligands for cell receptors;
- the genes coding for growth hormones (HGF);
- the genes coding for coagulation factors, such as factor VIII and factor IX;
- the gene coding for dystrophin or minidystrophin;
- the gene coding for insulin;
- the genes coding for polypeptides participating directly or indirectly in cellular ion channels, such as the CFTR (cystic fibrosis transmembrane conductance regulator) protein;
- the genes coding for antisense RNAs or proteins capable of inhibiting the activity of a protein produced by a pathogenic gene present in the genome of a pathogenic organism, or by a cellular gene whose expression is deregulated, for example an oncogene;
- the genes coding for a protease inhibitor such as α_1-antitrypsin or an inhibitor of a viral protease;
- the genes coding for variants of pathogenic proteins which have been mutated in such a way as to impair their biological function, such as, for example, trans-dominant variants of the HIV virus TAT protein

which are capable of competing with the natural protein for binding to the target sequence, thereby preventing the replication of HIV;

- the genes coding for antigenic epitopes so as to increase the immunity of the host cell;
- the genes coding for polypeptides having anticancer properties, and in particular tumor suppressors such as the p53 protein;
- the genes coding for proteins of the major histocompatibility complex classes I and II, as well as the genes coding for the proteins that regulate the expression of these genes;
- the genes coding for cellular enzymes or those produced by pathogenic organisms; and
- suicide genes. The HSV-1 TK gene may be mentioned more especially. The viral TK enzyme displays a markedly greater affinity than the cellular TK enzyme for certain nucleoside analogs (such as acyclovir or ganciclovir). It converts them to monophosphated molecules, which are themselves convertible by the cellular enzymes to nucleotide precursors, which are toxic. These nucleotide analogs can be incorporated in DNA molecules in the process of synthesis, hence mainly in the DNA of cells undergoing replication. This incorporation enables dividing cells such as cancer cells to be destroyed specifically.
- the genes coding for an antibody, an antibody fragment or an immunotoxin.
- reporter genes such as the LacZ gene coding for β-galactosidase, or the luciferase gene.

This list is not limiting, and other genes may naturally be employed as well.

Moreover, an exogenous DNA sequence employed in the present invention may comprise, in addition, a nontherapeutic gene, for example a gene coding for a selectable marker enabling the transfected host cells to be selected or identified. There may be mentioned the *neo* gene (coding for neomycin phosphotransferase) conferring

resistance to the antibiotic G418, the *dhfr* (dihydrofolate reductase) gene, the CAT (chloramphenicol acetyltransferase) gene, the pac (puromycin acetyltransferase) gene or alternatively the *gpt* (xanthine:guanine phosphoribosyltransferase) gene.

Naturally, a gene employed in the present invention may be placed under the control of elements suitable for its expression in a host cell. "Suitable elements" are understood to mean the set of elements necessary for its transcription into RNA (antisense RNA or mRNA) and for the translation of an mRNA into protein. Among elements necessary for transcription, the promoter assumes special importance. The latter can be a constitutive promoter or a regulable promoter, and it may be isolated from any gene of eukaryotic or even of viral origin. Alternatively, it can be the natural promoter of the gene in question. Generally speaking, a promoter employed in the present invention may be modified so as to contain regulatory sequences. As examples of tissue-specific promoters, there may be mentioned those of the immunoglobulin genes when it is sought to target the expression into lymphocytic host cells, and of the α_1-antitrypsin gene for a liver-specific expression. The constitutive promoters of the HSV-1 TK (herpesvirus type 1 thymidine kinase) and of the murine or human PGK (phosphoglycerate kinase) gene, the SV40 (simian virus 40) early promoter, a retroviral LTR or alternatively the adenoviral MLP promoter, in particular of human adenovirus type 2, may also be mentioned.

The method according to the invention employs an intermolecular homologous recombination mechanism. Generally speaking, it consists of the exchange of homologous sequences between two DNA fragments. These sequences may be identical or substantially homologous. In other words, the degree of homology of the sequences A and B with the corresponding portion of the first DNA fragment may be variable, but must be sufficient to permit intermolecular recombination. For the purposes of the present invention, it is preferable for it to be

greater than 70%, advantageously greater than 80%, preferably greater than 90%, and as an absolute preference in the region of 100%. Furthermore, a short region of homology may be sufficient (at least 10 consecutive nucleotides in common between the sequences A and B and their homologous sequences in the first DNA fragment). In the context of the present invention, the length of the sequences A and B is preferably between 10 bp and 10 kb, advantageously 20 bp and 5 kb, preferably 30 bp and 2 kb, and as an absolute preference 40 bp and 1 kb.

According to an advantageous embodiment, a method according to the invention leads to the replacement of the genetic material localized between the sequences of the first DNA fragment which are homologous to the sequences A and B by the exogenous sequence. This intermolecular exchange enables a circular recombinant viral vector to be generated in the form of a prokaryotic vector (plasmid, cosmid or phage) permitting its manipulation and/or its propagation in the prokaryotic cell. An embodiment of the mechanism employed in the context of the present invention is illustrated in Figure 2.

Although the insertion region of the exogenous sequence may be located in any position of the viral genome, it is preferable to avoid the regions acting *in cis* necessary for replication. These regions comprise, in particular, the 5' and 3' LTRs as well as the encapsidation signal in the case of retroviruses, and the 5' and 3' ITRs and the encapsidation region as regards adenoviruses. It would appear that the insertion region may be directed into a variety of positions in accordance with the chosen homologous sequences A and B.

As stated above, the first DNA fragment comprises all or part of the genome of the parent virus employed in the context of the invention. This may be the genome of a wild-type virus or the genome of a virus derived therefrom, modified by deletion, addition and/or substitution of one or more nucleotides. In particular, one or more genes may be wholly or partially deleted from the genome in question.

The first DNA fragment is preferably included in a conventional vector. The choice of the latter is very wide, but pBR322, p polyII or alternatively p polyIII*I (Lathe et al., 1987, Gene, 57, 193-201) may be mentioned more especially. According to an advantageous embodiment of the method according to the invention, the first DNA fragment is preferably linearized in the region in which the targeting of the insertion is envisaged. In particular, it may be cleaved with one or more restriction enzymes whose sites are localized between the sequences of the first DNA fragment which are homologous to the sequences A and B, but also within these latter, although this embodiment is not preferred. The choice of restriction sites to be used according to the parent virus adopted is within the capacity of a person skilled in the art. Advantageously, it will be preferable to use sites which are not strongly represented in the first DNA fragment, and especially unique sites. Moreover, such sites may also be created by the standard techniques of directed mutagenesis.

The second DNA fragment employed in the present invention carries, in particular, the exogenous DNA sequence surrounded by the sequences A and B involved in the intermolecular recombination. It is preferable to employ a linear DNA fragment. It may be generated by PCR, excised from any vector or produced synthetically or by any conventional method in the field of the art. As an example, a second DNA fragment employed in the context of the present invention may be obtained by amplification of the exogenous DNA sequence from a vector of the prior art or from a genomic library or from an appropriate cDNA using suitable primers equipped at their 5' ends with the sequences A and B. In addition, a second DNA fragment may also comprise plasmid sequences at its 5' and 3' ends, which may optionally be used by way of sequences A and B provided they are homologous to the plasmid sequences contained in the first DNA fragment.

In accordance with the objectives pursued by the present invention, a method according to the invention is

carried out for the preparation of a recombinant viral vector which is defective for replication. This term denotes a vector incapable of completing an autonomous infectious cycle in a host cell. Generally, the genome of these defective vectors lacks one or more genes essential for replication, early genes and/or late genes. They may be wholly or partially deleted or rendered nonfunctional by mutation (addition, deletion and/or substitution of one or more nucleotides). In this connection, a method according to the invention can enable a recombinant adenoviral vector lacking all or part of the E1, E2, E3 and/or E4 regions to be prepared.

By way of illustration, the following embodiments may be mentioned. When the intention is to prepare, in a prokaryotic cell, a recombinant adenoviral vector derived from a type 5 human adenovirus (Ad5) in which the insertion of an exogenous DNA sequence in place of the E1 region is envisaged, it is possible to employ (i) a vector containing the Ad5 genome cleaved with the enzyme *Cla*I (restriction site located at position 918 of the Ad5 genome, as disclosed in the Genebank databank under the reference M73260), and (ii) a DNA fragment comprising a sequence A homologous to the adenoviral encapsidation region, the exogenous DNA sequence followed by a sequence B homologous to the sequence coding for the pIX protein. Moreover, the use of a vector carrying the adenoviral genome cleaved with the enzyme *Spe*I (position 27082 of the Ad5 genome) and of a DNA fragment comprising a sequence A homologous to the 5′ end of the E2 region, the exogenous DNA sequence and a sequence B homologous to the 3′ end of the E4 region will enable a recombinant adenoviral vector to be prepared in which the exogenous DNA sequence is inserted in place of the E3 region. Naturally, these particular embodiments are mentioned only as examples. Lastly, the method according to the invention may be used to introduce deletions, mutations and/or substitutions into a particular region of a viral genome.

The present invention proves especially advantageous when the intention is to prepare a recombinant

viral vector of at least 20 kb, and as an absolute preference of at least 30 kb, and more especially in the case of a vector having a genome whose length is from 80 to 120% of that of the genome of the corresponding wild-type virus, in particular from 90 to 110% and preferably from 95 to 105%.

According to another embodiment, a method according to the invention may also be employed to insert at least two DNA fragments within the viral genome, by intermolecular recombination between (i) a first DNA fragment comprising all or part of said genome of the parent virus, (ii) a second DNA fragment comprising a first portion of said exogenous DNA sequence equipped at its 5′ end with said flanking sequences [sic] A and (iii) a third DNA fragment comprising a second portion of said exogenous DNA sequence equipped at its 3′ end with said flanking sequences [sic] B; said second and third DNA fragments containing a homologous sequence overlapping at their respective 3′ and 5′ ends. As a guide, these sequences which are homologous between the second and third DNA fragments satisfy the same criteria of homology and of length as the sequences A and B. This specific embodiment is especially advantageous for the cloning of large-sized exogenous sequences.

A method according to the invention may be carried out in any prokaryotic cell, and in particular in a bacterium derived from an *Escherichia coli* strain. However, it is most especially preferable to employ a recBC sbcBC strain such as, for example, the strains CES200, CES201, W5449 and BJ5183 (Hanahan, 1983, J. Mol. Biol., *166*, 557-580).

A procedure typical of a method according to the invention comprises the following steps:

(a) a first DNA fragment and (ii) a second DNA fragment, as are defined above, are cointroduced or introduced separately into a prokaryotic cell,

(b) the prokaryotic cell obtained in step (a) is cultured under suitable conditions to permit the generation of the recombinant viral vector by

intermolecular recombination, and

(c) the recombinant viral vector is recovered.

In the context of a method according to the invention, the amounts of first and second DNA fragments may vary. It is preferable to employ a concentration of second fragment which is 10 times as large as that of the first fragment. The introduction of the DNA fragments into a prokaryotic cell and the recovery of the vector from these same cells are carried out according to the general techniques of genetic engineering and of molecular cloning detailed in Maniatis et al. (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY).

The present invention also relates to a method for preparing an infectious viral particle containing a recombinant viral vector obtained by carrying out a method according to the invention, according to which:

(a) said recombinant viral vector is introduced into a mammalian cell to generate a transfected mammalian cell,

(b) said transfected mammalian cell is cultured under suitable conditions to permit the production of said viral particle, and

(c) said viral particle is recovered from the cell culture obtained in step (b).

The cells may be transfected according to the standard techniques well known to a person skilled in the art. The calcium phosphate technique, the DEAE-dextran technique, electroporation, methods based on osmotic shock, microinjection or methods based on the use of liposomes may be mentioned in particular. According to a preferred embodiment, the mammalian cell is advantageously a complementation cell, and in particular the 293 cell in the context of a vector derived from an adenovirus. The viral particles may be recovered from the culture supernatant, but also from the cells according to conventional protocols.

The present invention also covers the use of an infectious viral particle or of a recombinant viral

vector prepared according to a method according to the invention, for the therapeutic or surgical treatment of the human body, and in particular by gene therapy. A method according to the invention is intended more especially for the preventive or curative treatment of diseases such as genetic diseases (hemophilia; thalassemias, emphysema, Gaucher's disease, cystic fibrosis, Duchêne's [sic] or Becker's myopathy, etc.), cancers and viral diseases (AIDS, herpes infections or infections caused by cytomegalovirus or by papillomavirus). For the purposes of the present invention, the vectors and viral particles prepared by a method according to the invention may be introduced either *in vitro* into a host cell removed from the patient, or directly *in vivo* into the body to be treated. Preferably, the host cell is a human cell, and preferably a lung, fibroblast, muscle, liver or lymphocytic cell or a cell of the hematopoietic line.

The present invention also relates to a pharmaceutical composition comprising a therapeutically effective amount of an infectious viral particle or of a recombinant viral vector prepared according to a method according to the invention, in combination with a vehicle which is acceptable from a pharmaceutical standpoint. Such a pharmaceutical composition may be prepared according to the techniques commonly employed and administered by any known administration route, for example systemically (in particular intravenously, intratracheally, intraperitoneally, intramuscularly, subcutaneously, intratumorally or intracranially) or by aerosolization or intrapulmonary instillation.

Lastly, the subject of the present invention is also the use of an infectious viral particle or of a recombinant viral vector prepared according to a method according to the invention, for the expression of a DNA sequence of interest in a cell system.

The present invention is described more completely by reference to the figures which follow and by means of the examples which follow.

Figure 1 is a diagrammatic representation of the human adenovirus type 5 genome (shown in arbitrary units from 0 to 100), indicating the location of the different genes.

Figure 2 illustrates a method of intermolecular recombination between two DNA fragments. The plasmid sequences are represented by a thin line, the viral sequences by a thick line and the exogenous sequence by a shaded box.

Figure 3 is a diagrammatic representation of the vector pTG3614, corresponding to p polyII into which is cloned the genome of a recombinant adenovirus modified in the E1 region by insertion of a cassette for the expression of the human FIX gene.

Figure 4 is a diagrammatic representation of the vector pTG4652, corresponding to p polyII into which is cloned the genome of a recombinant adenovirus modified in the E1, E3 and E4 regions by insertion of a cassette for the expression of the human CF gene and partial deletions.

EXAMPLES

The examples which follow illustrate just one embodiment of the present invention.

The constructions described below are carried out according to the general techniques of genetic engineering and molecular biology detailed in Maniatis et al. (1989, *supra*). 5' protruding restriction sites may be converted to blunt sites by treatment with the large Klenow fragment of *E. coli* DNA polymerase, whereas 3' protruding sites are treated with T4 polymerase. As regards the steps of PCR amplification, the protocol as described in PCR Protocols - A guide to methods and applications (1990, ed. Innis, Gelfand, Sninsky and White, Academic Press Inc.) is applied. Cells are transfected by conventional methods and are cultured according to the supplier's recommendations. The fragments inserted into the different constructions described below are indicated precisely according to their position in the Ad5 genome as disclosed in the Genebank databank under

the reference M73260.

EXAMPLE 1: Construction of a recombinant viral vector derived from a type 5 human adenovirus in the form of a bacterial plasmid

A. *Cloning of the adenovirus 5 (Ad5) genome into p polyII*

The left-hand end of the Ad5 genome (nucleotides 1 to 935) is synthesized by PCR using the oligonucleotide primers oTG6597 (SEQ ID NO: 1) and oTG6598 (SEQ ID NO: 2). The first primer hybridizes with the first 21 nucleotides of the 5' ITR, and possesses a *Pac*I site immediately upstream of the viral sequences and an *Eco*RI site used for the cloning. The second primer enables *Sal*I and then *Bgl*II sites to be introduced downstream of the viral sequences. The template utilized in this reaction is Ad5 genomic DNA prepared according to conventional methods. The fragment thus amplified is digested with *Eco*RI and *Bgl*II [sic] and cloned into the plasmid p polyII (Lathe et al., 1987, *supra*) previously cleaved with these same two restriction enzymes, to give pTG1692.

The DNA fragment corresponding to the right-hand end of the Ad5 genome (nucleotides 35103 to 35935) is prepared according to the same principle, using the oligonucleotide pair oTG6599 (SEQ ID NO: 3) and oTG6597 (SEQ ID NO: 1). pTG1693 is constructed by inserting the amplified fragment, digested with *Eco*RI and *Bgl*II, into the same sites of p polyII. A *Bgl*II-*Bam*HI fragment carrying the amplified sequences is excised from pTG1693 so as to introduce it into the *Bgl*II site of pTG1692 downstream of the adenoviral 5' sequences. pTG3601 thereby obtained is linearized between the left-hand and right-hand ends of the Ad5 genome by digestion with restriction enzymes *Bgl*II or *Sal*I. The ends thus generated are blunted by the action of the Klenow fragment of *E. coli* DNA polymerase I. BJ5183 bacteria (Hanahan, 1983 *supra*), made competent by calcium chloride treatment, are cotransformed with the above preparation and the Ad5 genomic DNA. The colonies obtained are analyzed by restriction mapping. One clone is selected, designated

pTG3602, generated by intermolecular homologous recombination, in which the adenoviral sequences (nucleotides 936 to 35102) have been inserted between the two fragments produced by PCR, so as to generate a plasmid vector comprising the complete Ad5 genome - pTG3602 is produced in C600 bacteria (Huynh et al., 1985 DNA cloning, Volume 1, ed. Glover, IRL Press Limited: Oxford, England pp. 56-110).

B. Evaluation of the infectious power of pTG3602

The viral genome is liberated by the action of the restriction enzyme *Pac*I. 293 cells cultured in a monolayer are transfected with the product of this digestion by the calcium phosphate precipitation technique. It is also possible to use other sensitive cells, for example A549 cells (ATCC CCL185). The cells are grown under agar for 9 to 14 days, during which period the appearance of lytic plaques on the cell lawn testifies to the production of infectious viral particles, and hence to the capacity of the Ad5 genome originating from pTG3602 to replicate.

C. Construction of a defective recombinant adenoviral vector in which the exogenous gene replaces the E1 early region.

A plasmid is used in which the LacZ reporter gene coding for β-galactosidase is placed in a viral context; for example a plasmid derived from pMLP-Adk7 (Stratford-Perricaudet and Perricaudet, 1991, Human Gene Transfer, 219, 51-61) comprising the Ad5 5′ sequences (nucleotides 1 to 458), the Ad2 MLP promoter, the LacZ gene, the SV40 virus polyadenylation signal and the Ad5 sequences lying between nucleotides 3329 and 6241.

The LacZ gene expression cassette surrounded by adenoviral sequences is excised by the action of the restriction enzymes *Bsr*GI (position 192 on the Ad5 genome) and *Pst*I (position 3788) and then purified. pTG3602 is linearized at the *Cla*I site (position 918) and then treated with Klenow. The two fragments obtained are cotransformed into competent BJ5183 bacteria. Recombination at the homologous adenoviral sequences brings about

the replacement of the Ad5 E1 region (nucleotides 459 to 3328) by the expression cassette for the reporter gene in the plasmid pTG3603.

Its infectious power is verified by transfection of a lawn of 293 cells transfected with pTG3603 previously digested with *Pac*I. The lytic plaques formed are then picked out and the viral particles resuspended and used to infect a monolayer of 293 cells. The blue coloration of the cells after addition of X-Gal testifies to the expression of the LacZ gene.

D. Construction of a recombinant adenoviral vector in which the exogenous gene replaces the E3 early region.

A cassette for the expression of the gene coding for the gp19 protein of the Ad5 E3 region (nucleotides 28731 to 29217) is assembled in the bacteriophage M13mp18 (Gibco BRL) by cloning two PCR fragments, one corresponding to the RSV (Rous sarcoma virus) 3' LTR (oligonucleotide primers oTG5892-SEQ ID NO: 4 and 5893-SEQ ID NO:5) and the other to the sequence coding for gp19 (oTG5455-SEQ ID NO:6 and 5456-SEQ ID NO:7). The vector M13TG1683 is obtained, from which the expression cassette is excised by an *Xba*I digestion. After treatment with Klenow, it is introduced into the *Bsm*I site (blunted by the action of phage T4 DNA polymerase) of pTG8519. The latter is derived from the plasmid puc19 [sic] (Gibco BRL), into which the adenoviral sequences lying between the *Spe*I site and the right-hand end of the genome (nucleotides 27082 to 35935) but lacking the majority of the E3 region (nucleotides 27871 to 30758) have been inserted. pTG1695 is obtained, the *Sca*I-*Spe*I fragment of which, carrying the plasmid sequences, is replaced by a purified equivalent fragment of pTG1659. The latter corresponds to puc19 [sic] comprising the Ad5 sequences extending from nucleotides 21562 to 35826. pTG1697 thereby obtained possesses adenoviral sequences which extend from the *Bam*HI (position 21562) site to the 3' ITR (position 35935), in which sequences the E3 region is replaced by a gp19 expression cassette under the control

of the RSV constitutive promoter. the *Dra*I fragment (position 22444 and 35142 on the Ad5 genome) is purified, and cointroduced into competent BJ-5183 bacteria with pTG3602 linearized with *Spe*I and treated with Klenow. The recombinants carrying a plasmid generated by recombination are screened for the presence of the RSV promoter. pTG3605, a plasmid vector carrying the Ad-gp19+ genome, is thus demonstrated.

The infectious power [lacuna] viral genome excised from plasmid pTG3605 is tested according to the protocol already described above. The production of a functional gp19 protein is monitored by co-immunoprecipitation of the antigens of the major histocompatibility complex class I and of the protein (Burgert and Kvist, 1985, Cell, *41*, 987-997).

E. Construction of a defective recombinant adenoviral vector in which both early regions E1 and E3 are replaced by exogenous genes

BJ-5183 cells are cotransformed with the *Dra*I fragment isolated from pTG1697 mentioned above, and the *Spe*I fragment (Klenow) isolated from pTG3603. The resulting plasmid is tested under the same conditions as above.

F. Construction of a recombinant adenoviral vector expressing the FIX gene

The cDNA coding for human FIX was cloned in the form of a *Bam*HI fragment into the plasmid pTG381. In the latter, the 5' non-coding end of the FIX gene was modified so as to comply with Kozak's rules. Plasmid pTG381 is described in Patent Publication FR 2,600,334. The FIX gene is recloned into the *Bam*HI site of pTG6512 to give pTG4375. pTG6512 is derived from the vector pTG6511 after deletion of the Ad2 major late promoter (MLP) and replacement by a polylinker. As a guide, the construction of pTG6511 is described in detail in Example 1 of International Application WO 94/28152. The murine PGK promoter is isolated by conventional methods from mouse genomic DNA according to Adra et al. (1987, Gene *60*, 65-74), or by means of appropriate primers created from the data of the sequence disclosed in the Genebank databank under the

reference X15339. *Pst*I restriction sites are included at the 5' ends of the primers in order to facilitate the subsequent cloning steps. The fragment carrying the PGK promoter is treated with T4 polymerase and then introduced upstream of the human FIX gene into the *Hpa*I site of pTG4375 from which a so-called "replacement" fragment, containing the FIX cassette surrounded by E1 adenoviral sequences (185 bp at the 5' end and 2045 bp at the 3' end), is excised by *Msc*I digestion.

The latter fragment is cotransformed into BJ5183 bacteria in the presence of the vector pTG3602 digested with *Cla*I (position 918). The transformants are selected on ampicillin and analyzed by restriction mapping. The clone designated pTG3614, corresponding to an adenoviral vector containing the FIX therapeutic gene in place of the E1 region (Figure 3), is selected. A viral stock is constituted in a conventional manner, by transfection of the adenoviral genome liberated by *Pac*I digestion into the 293 line. After an amplification step, the cells are lysed and the AdTG3614 viral particles purified by centrifugation on cesium chloride.

The capacity to transfer and express the FIX gene is evaluated in a mouse animal model. For this purpose, 1.5×10^9 pfu are injected into the caudal vein of 5- to 6-week-old female C57 Black 6 mice. Blood samples are drawn regularly, on which the amount of human FIX is assayed by ELISA (Diagnostica Stago Kit, Asnières, France; Asserachirom^R IX: Ag 00564). FIX is detected in the serum of mice for several weeks with a maximum at 5 days.

G. Construction of an adenoviral vector modified in the E2 region

During the normal viral cycle, the expression of the genes of the E2 region is induced by the early products of E1 and E4, and a copious production of the proteins encoded by E2, and especially of the DBP protein (for DNA binding protein), is observed. In point of fact, this high expression can be problematical *in vivo* (induction of inflammatory responses or interference with the

expression of the transgene). For this reason, adenoviral vectors defective for the E2A function were constructed, either by introduction of a temperature-sensitive mutation into E2A (position 22795; C to T resulting in a change from Pro to Ser) or by a partial deletion of the DBP gene.

The vector pTG9551 carrying the temperature-sensitive mutation is generated by homologous recombination in BJ cells between pTG3601 digested with *Bgl*II and the purified genomic DNA of the Ad5Hts 125 virus (Ensinger and Ginsberg, 1972, J. Virol. *10*, 328-339). The genome thus reconstituted is liberated by *Pac*I digestion and transfected into 293 cells, which are incubated at 32°C for the production of the viral particles (the non-permissive temperature is 39°C).

The defective E2A character may also be generated by partial deletion of the coding regions of the DBP gene, avoiding the regions covering other reading frames. The construction is carried out by homologous recombination between pTG3601 *Bgl*II and the viral DNA prepared from the H5d1802 virus (Rice and Klessig, 1985, J. Virol. *56*, 767-778). The viruses may be produced in a suitable complementation line transcomplementing the DBP function, for example line 293 transfected with a vector permitting the constitutive or regulated expression of the DBP gene.

H. Construction of an adenoviral vector modified in the E4 region

In a first stage, a so-called "replacement" vector is constructed, comprising a cassette for the expression of the gene coding for the CFTR protein placed within the E1 adenoviral region. This vector, designated pTG8585, contains the following elements, all described in the literature:

- the Ad5 5' ITR (positions 1 to 458),
- the Ad2 MLP promoter,
- human CFTR cDNA,
- the polyadenylation signal of the rabbit β-globin gene,
- the RSV virus (Rous sarcoma virus) 3' LTR,

- the adenoviral sequences coding for the gp19 protein (positions 28731 to 29217 of Ad5), and
- the 3′ portion of the E1B region (position 3329 to 6241).

In parallel, the adenoviral sequences covering the E3 and E4 regions are subcloned into the vector p polyII and deleted by the standard techniques of molecular biology. The E3 sequences (positions 28592 to 30470) are removed by *Xba*I digestion and the E4 sequences (positions 32994 to 34998) are removed. The adenoviral genome thus modified is reconstituted by homologous recombination between a replacement fragment carrying these modifications, isolated from the above vector, and pTG3603 digested with *Spe*I. pTG8595 is obtained, carrying a recombinant adenoviral genome (LacZ gene in place of the E1 region) which is defective for the E3 and E4 functions.

The cassettes for the expression of the CFTR and gp19 genes, described above, are introduced into pTG8595 as a replacement for the LacZ gene, by cotransformation of BJ5183 cells with the *Pac*I-*Bst*EII fragment isolated from pTG8585 and the vector pTG8595 linearized with *Cla*I. pTG4652 (Figure 4) is generated, which may be propagated in a cell line complementing the defective E1 and E4 functions as are described in International Application WO94/28152.

The viral particles are amplified, the cells are lysed and the total cell extracts are analyzed for the expression of the CFTR gene by Western blotting (8% SDS-PAGE gel). The filters are incubated in the presence of a 1/5000 dilution of the monoclonal antibody MAB 1104 directed against a synthetic peptide corresponding to amino acids 722 to 734 of the human CFTR protein. The detection is carried out by the ECL (enhanced chemiluminescence; Amersham kit) method. A band of strong intensity and rather diffuse, corresponding to a high molecular weight product comigrating with a CFTR control, is observed in the extracts of cells infected with AdTG4652. This band is not present in the extracts

originating from the parent virus pTG8595 which does not contain the CFTR expression cassette.

EXAMPLE 2: Construction of a recombinant viral vector derived from canine adenovirus 2 (CAV2) in the form of a bacterial plasmid

A. Cloning of the CAV2 genome into p polyII

The CAV2 genome is cloned into p polyII SfiI-NotI 14 (Lathe et al., 1987, *supra*) and modified to generate recombinant vectors using the appropriate sites, according to the same methods as are used in the case of the manipulation of Ad5. The left-hand end (nucleotides 1 to 810 according to the numbering of the Genebank sequence D04368) and right-hand end (nucleotides ~ 27600 to the end) are synthesized by PCR between the oligonucleotide primer pairs oTG6974 (SEQ ID NO: 8) and oTG6975 (SEQ ID NO: 9) and oTG6976 (SEQ ID NO: 10) and oTG6974, respectively. They are assembled via the *Pst*I site introduced during the PCR following the viral sequences in the case of the left-hand end, and mapped at approximately 1200 bp from the end of the genome in the case of the right-hand end. Competent BJ5183 bacteria are cotransformed with the plasmid thereby obtained, linearized with *Pst*I, and CAV2 DNA. Introduction of the missing viral sequences (nucleotides 811 to 27600) is carried out by homologous recombination. The CAV2 genome carried by the resulting plasmid is excised via the *Not*I sites introduced during the PCR, and then transfected into a monolayer of MDCK cells (ATCC CCL34). The following steps are described in Example 1.

B. Construction of a recombinant adenoviral vector of canine origin

CAV2 virus (strain Toronto A 26/61; ATCC VR-800) genomic DNA is prepared by the standard technique (amplification on a dog kidney line MDCK GHK, etc., lysis of the cells, purification of the viruses by centrifugation on cesium chloride, treatment with proteinase k [sic] and lastly phenol/chloroform extraction). The CAV2 genome, which is 31 kbp in length, is introduced into a plasmid vector by homologous recombination. For this purpose, the

left-hand and right-hand ends of the CAV2 genome are isolated by PCR and enzymatic digestion, incorporating a *Not*I site immediately beyond the 5′ and 3′ ITRs. The vector pTG4457 is obtained by introducing into p polyII the 5′ portion of the viral genome as far as the *Bst*BI site (position 870) followed by the 3′ portion starting from the *Sal*1 site (position 27800). The complete genome may be reconstituted by cotransformation between the genomic DNA (100 to 500 ng) and pTG4457 digested with *Bst*BI (10 to 100 ng). The viral genome may be excised from the above vector pTG5406 by *Not*I digestion. It is verified that the DNA is infectious by transfection of 1 to 5 µg into dog MDCK or GHK cells. The production of plaques is observed.

The recombinant vector is obtained in the following manner:

The left-hand end of CAV2 is subcloned and modified so as to delete the E1A coding sequences in their entirety and the E1B coding sequences partially. This is performed by *Nar*I digestion, followed by reintroduction of a fragment amplified by PCR covering the encapsidation region, the E1A promoter region and the transcription startsite. The primers are designed so as to integrate a unique *Xba*I restriction site as well. pTG5407 is obtained, into which the CAT gene or LacZ gene (pTG5408) is introduced at the *Xba*I site. The latter plasmid is digested with *Xho*I, and the 3′ portion of the viral genome is inserted in the form of a *Sal*I fragment (position 27800 to the end of the 3′ ITR). The vector pTG5409 thereby obtained is linearized with *Sal*I and cotransformed into *E. coli* BJ5183 with the CAV-2 genome digested with *Swa*I. An adenoviral vector of canine origin carrying the CAT reporter gene in place of the E1 region is obtained.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Transgene S.A.
(B) STREET: 11 rue de Molsheim
(C) CITY: Strasbourg
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(ii) TITLE OF INVENTION: New method for the preparation of a viral vector

(iii) NUMBER OF SEQUENCES: 10

(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 #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human adenovirus
(B) STRAIN: serotype 5
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6597

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

GCCGAATTCT TAATTAACAT CATCAATAAT ATACCTTA 38

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human adenovirus
(B) STRAIN: serotype 5
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6598

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GACAGATCTG TCGACGTGGC AGGTAAGATC GATCA 35

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human adenovirus
(B) STRAIN: serotype 5
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6599

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGAGATCTG TCGACTCTCA AACATGTCTG CGGGT 35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rous sarcoma virus
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG5892

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTCGTAGGAT CCAGCTGCTC CCTGCTTGTG TGTTGGAGGT CGCTGAG 47

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rous sarcoma virus
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG5893

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTAGCTGACG TCCCAGGTGC ACACCAATGT GGTGAATGGT CAAATGG 47

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human adenovirus
(B) STRAIN: serotype 5
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG5455

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATCGGAATTC AAGATGATTA GGTAC 25

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human adenovirus
(B) STRAIN: serotype 5
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG5456

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATCGTCTAGA TTAAGGCATT TTCTTTTC 28

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: canine adenovirus
(B) STRAIN: CAV-2
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6974

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAGGGATCCG CGGCCGCATC ATCAATAATA TACAGG 36

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: canine adenovirus
(B) STRAIN: CAV-2
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6975

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTGCTGCAGT CAGAAATGCT AGCAGGAGA 29

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: canine adenovirus
(B) STRAIN: CAV-2
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide OTG6976

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGCGGATCCA CAGACTAAGC GGAGGTA 27

Claims

1. Method for preparing, in a prokaryotic cell, a recombinant viral vector derived from a parent virus into the genome of which an exogenous DNA sequence is inserted, by intermolecular recombination between (i) a first DNA fragment comprising all or part of said genome of the parent virus and (ii) a second DNA fragment comprising said exogenous DNA sequence surrounded by flanking sequences A and B which are homologous to (i).

2. Method according to claim 1, characterized in that the parent virus is selected from the group consisting of adenoviruses, retroviruses, adeno-associated viruses, poxviruses and herpesviruses.

3. Method according to claim 2, characterized in that the parent virus is an adenovirus of human, canine, avian, bovine, murine, ovine, porcine or simian origin, or alternatively a hybrid adenovirus.

4. Method according to claim 3, characterized in that the parent virus is a type CAV-2 adenovirus of canine origin.

5. Method according to claim 3, characterized in that the parent virus is a serotype C adenovirus of human origin.

6. Method according to claim 5, characterized in that the parent virus is a type 5 adenovirus of human origin.

7. Method according to one of claims 1 to 6, characterized in that said exogenous DNA sequence codes for a polypeptide of therapeutic interest selected from the group consisting of coagulation factors, growth hormones, cytokines, lymphokines, tumor-suppressing polypeptides, cell receptors, ligands for cell receptors, protease inhibitors, antibodies, toxins, immunotoxins, dystrophin and polypeptides participating in cellular ion channels, such as CFTR protein.

8. Method according to one of claims 1 to 7, characterized in that the homologous flanking sequences A and B are from 10 bp to 10 kb, advantageously from 20 bp to

* C L M S *

5 kb, preferably from 30 bp to 2 kb, and as an absolute preference from 40 bp to 1 kb, in length.

9. Method according to one of claims 1 to 8, characterized in that the first DNA fragment is linearized in the insertion region of the exogenous sequence.

10. Method according to one of claims 1 to 9, for the preparation of a recombinant viral vector which is defective for replication.

11. Method according to claim 10, for the preparation of a recombinant adenoviral vector lacking all or part of at least one region essential for replication, selected from the E1, E2 and E4 regions.

12. Method according to claim 11, characterized in that the recombinant adenoviral vector lacks, in addition, all or part of the E3 region.

13. Method according to one of claims 1 to 12, for the preparation of a recombinant viral vector of at least 20 kb.

14. Method according to claim 13, for the preparation of a recombinant viral vector of at least 30 kb.

15. Method according to claims 1 to 14, by intermolecular recombination between (i) a first DNA fragment comprising all or part of said genome of the parent virus, (ii) a second DNA fragment comprising a first portion of said DNA sequence of interest equipped at its 5′ end with said flanking sequences [sic] A and (iii) a third DNA fragment comprising a second portion of said DNA sequence of interest equipped at its 3′ end with said flanking sequences [sic] B; said second and third DNA fragments containing a homologous sequence at their respective 3′ and 5′ ends.

16. Method according to one of claims 1 to 15, for introducing a modification by deletion, mutation and/or substitution of one or more nucleotides or an exogenous DNA sequence into a viral genome.

17. Method according to one of claims 1 to 16, characterized in that said prokaryotic cell is derived from a recBC sbcBC strain of Escherichia coli.

18. Method for preparing an infectious viral particle

containing a recombinant viral vector obtained by carrying out a method according to one of claims 1 to 17, according to which:

(a) said recombinant viral vector is introduced into a mammalian cell to generate a transfected mammalian cell,

(b) said transfected mammalian cell is cultured under suitable conditions to permit the production of said viral particle, and

(c) said viral particle is recovered from the cell culture obtained in step (b).

19. Use of an infectious viral particle prepared according to claim 18 or of a recombinant viral vector prepared according to one of claims 1 to 17, for the therapeutic or surgical treatment of the human body.

20. Use according to claim 19, for the therapeutic or surgical treatment of the human body by gene therapy.

21. Pharmaceutical composition comprising a therapeutically effective amount of an infectious viral particle prepared according to claim 18 or of a viral vector prepared according to one of claims 1 to 17, in combination with a vehicle which is acceptable from a pharmaceutical standpoint.

22. Use of an infectious viral particle prepared according to claim 18 or of a recombinant viral vector prepared according to one of claims 1 to 17, for the expression of a DNA sequence of interest in a cell system.

23. Use of a recBC sbcBC strain of *E. coli* for the cloning of DNA fragments into a plasmid vector by intermolecular homologous recombination.