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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, A61K 48/00

A1

(11) International Publication Number: WO 95/33842

(43) International Publication Date: 14 December 1995 (14.12.95)

(21) International Application Number: PCT/US95/06594

(22) International Filing Date: 7 June 1995 (07.06.95)

(30) Priority Data:
08/255,771 7 June 1994 (07.06.94) US

(71) Applicant: IMMUNOMEDICS, INC. [US/US]; 300 American Road, Morris Plains, NJ 07950 (US).

(72) Inventors: LEUNG, Shui-on; 254 Kings Road, Madison, NJ 07940 (US). CHANG, Chien-Hsing; 349 French Circle, Downingtown, PA 19335 (US).

(74) Agents: SAXE, Bernhard, D. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., P.O. Box 25696, Washington, DC 20007-8696 (US).

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

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SAFE RETROVIRAL VECTORS, THEIR PRODUCTION AND USE

(57) Abstract

Safe retroviral vectors and corresponding packaging cell lines are provided for the treatment of disease caused by related retroviruses. The packaged vectors lack the enzymes to convert genomic RNA to DNA and/or to integrate into the host cell genome. Thus, the vectors are only active in cells where these enzymes are present, i.e., in cells already infected with a retrovirus. In this manner, selective targeting of previously infected cells is achieved. After superinfecting such cells, genes carried by the vectors effect killing of the host cell or inhibit replication of retrovirus production therefrom.

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SAFE RETROVIRAL VECTORS, THEIR PRODUCTION AND USE

BACKGROUND OF THE INVENTION

Retroviruses are a diverse taxonomic group of viruses that infect a wide range of mammalian and non-mammalian hosts including humans, horses, cats, mice and chickens. These infections range from asymptomatic to lethal. Some of the more devastating clinical results of retrovirus infection are tumor formation, leukemia, immunodeficiency and neurologic disfunction.

Retroviruses have single-stranded (ss) ribonucleic acid genomes (RNA). After infection of a host cell, the enzyme reverse transcriptase (*RT*), encoded by one of the retroviral *pol* genes, converts the ssRNA genome into double-stranded (ds) deoxyribonucleic acid (DNA). The protease gene (*prt*) is necessary for the generation of a functional *RT* enzyme as *prt* cleaves the polyprotein *gag-RT* precursor. A third retroviral enzyme called integrase (*int*) facilitates integration of the dsDNA into the host cell genome. Subsequently, a host-encoded RNA polymerase transcribes genes encoded by the integrated retroviral sequences. These viral messenger RNA's (mRNA) are translated by host cell enzymes and new, infectious retroviral particles are then assembled. Each new particle contains two copies of the viral RNA. The fidelity of the packaging of viral RNA is achieved by virtue of a particular sequence within the retrovirus genome, designated ψ.

One of the difficulties in treating a subject infected with a retrovirus is that the infectious agent becomes part of the genome of the infected host cells. Eradication of disease-causing retroviral sequences, and only the retroviral sequences, is not a reasonable undertaking. Rather, the more promising approach appears to be the selective destruction of retrovirally-infected cells ("RIC's"). The question then becomes how to (i) specifically target only RIC's for non-specific killing or (ii) target all cells capable of infection with a retrovirus but selectively kill only those cells actually

infected with the retrovirus. Neither of the questions have been answered adequately in the prior art.

One way to target cells capable of infection with retroviruses is to use retroviral vectors. Retroviral vectors are recombinant retroviruses that can be utilized for the introduction of genes into eukaryotic cells. To date, all known retroviral vectors carry endogenous reverse transcriptase and *int*, thereby facilitating incorporation of the retroviral vector DNA into the host genome. This permits exogenous genes that have been recombined into the retroviral vector DNA to be inserted into the host's genetic material. If the integration is stable and compatible with the host, the exogenous gene is expressed.

While retrovirus vectors allow the targeting of cells capable of infection by a particular retrovirus, the question of selective killing of RIC's remains. Moreover, a retroviral vector that can infect and integrate into the genome of a non-infected host cell presents an unacceptable risk in and of itself. Thus, a need exists for a retroviral vector that (i) is capable of infecting target cells, (ii) does not integrate into the genome of non-infected host cells and (iii) can either kill infected cells or inhibit the replication of other retroviruses therein.

SUMMARY OF THE INVENTION

Therefore, one object of the present invention is to provide a retroviral vector that (i) is capable of infecting target cells, (ii) does not integrate into the genome of, or otherwise replicate in, non-infected host cells and (iii) can either kill infected cells or inhibit the replication of retroviruses therein.

Another object of the present invention is to provide a packaging cell line that (i) will support the replication of the retroviral vector described above but (ii) does not confer on that virus the ability to integrate or replicate in non-infected host cells.

Yet another object of the present invention is to provide a method of treatment for an animal infected with a retrovirus using an integration-incompetent vector, produced in a replication-supporting cell line.

In achieving the foregoing objects, a retroviral vector is provided that lacks all sequences except the LTR, the ψ sequence, a selectable marker and one or more genes capable of effecting either (i) the inhibition of replication of retroviruses in a RIC or (ii) the selective killing of a RIC.

In one embodiment, a retroviral vector is provided, where inhibition of retroviral replication is the result of an anti-sense RNA expressed by the retroviral vector.

In another embodiment, a retroviral vector is provided, wherein selective killing is the result of a toxin expressed by the retroviral vector.

In another embodiment, a retroviral vector is provided, where selective killing is the result of a surface-expressed, non-retroviral, antigenic protein expressed by the retroviral vector.

In another embodiment, a retroviral vector is provided, where selective killing is the result of the interaction between a gene expressed by the retroviral vector and an agent administered to the patient.

In another embodiment, a packaging cell line is provided that contains a retroviral construct which lacks the ψ sequence and one or more of the *RT*, *int* and *prt* genes but contains all the structural genes necessary for assembly of virions.

In another embodiment, a method of therapy is provided wherein a therapeutic, integration-incompetent retroviral vector is used to treat subjects suffering from a retroviral infection where the vector is only active in cells infected the retrovirus.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however,

that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Like other retroviral vectors, the therapeutic retroviral vectors of the present invention exhibit normal host range functions, at least at the level of attachment and internalization. In contrast to the retroviral vectors of the prior art, the present vectors do not integrate into the genome of cells unless those cells have already been infected with a disease-causing retrovirus. Capitalizing on this unique property, therapeutic vectors of the present invention also carry genes that facilitate either (i) the killing of RIC's or (ii) the inhibition of the replication of RIC's.

The key to the selective treatment of RIC's using these therapeutic vectors is the reliance of the vectors on the presence of reverse transcriptase, integrase and protease in the cells which they infect. This reliance results from the absence of at least one of a functional *RT*, *int*, or *prt* coding sequences in both the vector and the cell line in which the vector is grown and packaged. When therapeutic vectors of the present invention infect cells that do not contain active reverse transcriptase, integrase or protease, the vector RNA rapidly will be degraded. On the other hand, if the target cells contain active forms of these three enzymes, *i.e.*, are already infected by an integration competent retrovirus, the retroviral vector RNA will be converted to DNA. The DNA copy then integrates into the host chromosome, resulting in "superinfection" of the host cell. After integration, genes encoded by the retroviral vector will be expressed.

Having thus addressed the problem of selective integration, the present invention also provides

mechanisms for killing infected cells or inhibiting their replication. The mechanisms can be direct, *i.e.*, caused by vector-based products, or they can be indirect, *i.e.*, caused by the interaction of some other factors with vector-based products. An example of a direct mechanism is where the vector carries a gene encoding a toxin. Expression of the toxin after integration of the vector would kill the host cell. Another example of the direct mechanism would be the expression of an anti-sense RNA from sequences carried by the vector. The antisense RNA would be designed to hybridize to transcripts of one of the essential genes expressed by the disease-causing retrovirus, thereby blocking translation. An indirect method might involve the expression of a surface bound, heterologous, antigenic protein encoded by the vector. If sufficient levels of circulating antibodies are not produced in response to the antigen or are not already present due to prior immunization, antibodies or antibody-conjugates specific for this antigen that effect cell killing are then administered to the patient. The antigenic protein might also elicit a cellular response capable of killing RIC's. Alternatively, the killing might be effected by the interaction of a therapeutic gene product and a pharmacologic agent administered to the patient which interacts with the therapeutic gene.

RETROVIRAL VECTORS

Retroviral vectors according to the present invention generally contain a long terminal repeat (LTR), packaging (ψ) sequence, a selectable marker and one or more therapeutic genes under the control of appropriate promoters. Preferably, all structural genes are removed since this eliminates the possibility of recombination with proviral sequences. The LTR, and any other regulatory sequences should be compatible with trans-acting factors provided by the host cell. In addition, the reverse transcriptase, protease and integrase of the disease-causing virus must be compatible with signals contained in the vector. Thus, selection of the starting

material from which the therapeutic vector is generated depends, to a large measure, on the nature of the disease-causing retrovirus of the disease to be treated.

Selectable marker genes are often drug resistance genes or genes that produce color or light reactions upon appropriate treatment. Favored drug markers are hygromycin-B-phosphotransferase, aminoglycoside phosphotransferase and xanthine-guanine phosphoribosyltransferase (*gpt*). A favored color marker is ß-galactosidase. A favored fluorescent marker is luciferase.

Therapeutic genes include, but are not limited to xenogenic major histocompatibility complex proteins, toxins, immunogenic membrane-bound proteins and appropriate antisense constructs. For xenogenic MHC molecules, these could include class I and II antigens from rat, mouse, horse, pig or other species. The presence of pre-existing antibodies against xenogenic MHC molecules, even in immunocompromised subjects, provides a mechanism, similar to xenogenic graft rejection, by which antigen bearing cells are eliminated. Appropriate toxins include ricin A chain, *Pseudomonas* exotoxin and pokeweed antiviral protein. Immunogenic membrane bound proteins include any surface bound protein from a species other than the host for which the corresponding antibody is abundantly available. Alternatively, an immunogenic membrane bound protein could be derived from a pathogen for which an individual already possesses antibodies or for which the individual could be vaccinated. Antisense constructs are derived from any viral gene that is essential to viral replication, assembly or expression (*e.g.*, *tar*, ψ). In addition, genes which render target cells susceptible to drugs, such as thymidine kinase (*tk*), also are contemplated.

Retroviral sequences are cloned into standard vectors such as pBR322 and its derivatives, the pUC plasmid series or any of a variety of commercially available vectors. Ligation is accomplished by

directional (dual site) or non-directional cloning (blunt-end or single site). The methods for manipulation of nucleic acids are well known and set forth, for example, in Sambrook *et al.*, MOLECULAR CLONING: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), incorporated by reference.

Construction and manipulation of retroviral vectors has been described in detail in the relevant literature. See generally, *J. Virol.* **54**:401-407 (1985); *Mol. Cell. Biol.* **5**:431-437 (1985); *Nature* **306**:155-160 (1983); *Proc. Nat'l Acad. Sci. USA* **79**:5986-5990 (1982), all of which are incorporated by reference. For deletion of segments of cloned retroviral sequences, appropriate restriction enzyme sites flanking the segments to be deleted are identified based on sequence analysis. The sites are cleaved with endonucleases specific for the appropriate sites, the intervening segment is removed, and the ends ligated together.

If blunt or non-matching sticky ends are generated by the cleavage, linkers preferably are employed. In cases where only an appropriate restriction site is found in the relevant region, it is possible to use a different approach. Following cleavage at the single site, the cleaved ends are treated with an exonuclease, *e.g.*, example *Bal*31, which removes base pair progressively from both cleaved ends. Blunting of the new ends with Klenow fragment and religation completes the process. This method produces a population of deletions, however, all surrounding the single restriction site. Thus, it is necessary to screen this population for a deletion with appropriate size and location. In some cases, it is desirable to replace deleted sequences with extraneous segments in order to preserve the natural spacing of non-deleted elements.

It also is possible to make mutations simply by introducing linker sequences at particular restriction enzyme sites in a given region of DNA. This is

accomplished by cleaving a single site within a target sequence and ligating a linker into that site. The introduction of even a few base pairs may result in frame-shift leading to premature termination of a polypeptide encoded by the target sequence. Alternatively, the region "downstream" of the linker insertion may still code for a polypeptide sequence, but that polypeptide may be completely different than the original polypeptide sequence. Finally, if the insertion consists of a sequence that is of the length 3n, where n is 1, and no "stop" codons are introduced, the only change will be the addition of n number of amino acids at the insertion site. Insertional mutagenesis can also be applied to non-coding regions but with less predictable results.

In a similar fashion, entire genes or genetic control regions may easily be introduced into a vector. Selectable markers like hygromycin, *tk*, neomycin and *gpt* or therapeutic genes such as MHC molecules, toxins or antisense constructs are examples of genes whose expression which might be useful in the context of this invention. Typically, when multiple genes are introduced into a single retroviral vector according to the present invention, all the genes should be next to one another and under the control of the same promoter. Preferably, the therapeutic gene should be located upstream of the marker gene to ensure that all selection-resistant clones contain sequences from both ends of the inserted segment (5'-end: promoter; 3'-end: marker gene). In such a case, it is likely that the intervening sequences encoding the therapeutic gene are present as well. In cases where the therapeutic gene will have a detrimental effect on the host packaging cell line (discussed further below), such as a toxin, a separate promoter should be employed that will be active in the target cell (*e.g.*, CD4 promoter, IL-2 promoter, *etc.*) but not in the packaging cell line.

PACKAGING CELL LINES

Packaging cell lines are mammalian cells that carry "proviruses" integrated into their chromosomes. The proviral sequences, however, are defective in their ability to be packaged and, thus, produce empty virions. These cell lines are used to support the replication of retroviral vectors that lack certain genes necessary for the assembly of the viral capsid.

Methods for the generation of packaging cell lines are well-known in the art. Briefly, cloned retroviral sequences are genetically-manipulated rendering them packaging-incompetent. These sequences are then transfected into host cells capable of supporting retroviral replication. Popular cells for this purpose include the various fibroblasts, H9 cells, MOLT-3 cells, MT4 cells and others. Transfection can be accomplished by a variety of means including electroporation, calcium phosphate coprecipitation, lipofection or protoplast fusion. Some of the transfected sequences will integrate into the host cell DNA and will be maintained in a stable fashion. To facilitate screening of potential cell lines, a selectable marker is usually covalently linked to the retroviral sequences used for transfection. Such markers are discussed above.

In the present invention, proviruses contained in packaging cells, in addition to lacking ψ sequence, also lack the sequence for at least one of a functional reverse transcriptase, a functional protease and a functional integrase. When a retroviral vector lacking one or more of these enzymes, the retrovirus produced will be incapable of integrating into the host genome of a normal cell it infects subsequently. Should this same retroviral vector infect a host cell already infected by an integration competent retrovirus, the latter will provide enzymes necessary for the integration of the former and superinfection will occur.

In selecting the appropriate provirus, it is necessary to choose a provirus closely related to the disease-causing retrovirus to be treated. Mainly, this involves selecting a provirus with a host range that encompasses the cells which are to be treated, *i.e.*, the host range of the disease-causing retrovirus. It is also desirable to optimize the similarity between the provirus and therapeutic vector DNA to be packaged in the cell line. In particular, packaging signals from the therapeutic vectors must be compatible with the packaging machinery of the cell line.

The following are examples of packaging cell lines entertained by the present invention:

TYPE I CELLS. Type I packaging cell lines contain a helper provirus wherein the packaging sequence (ψ) is deleted and the reverse transcriptase is defective. Thus, type I cells produce virions that do not contain proviral RNA and functional reverse transcriptase.

TYPE II CELLS. Type II packaging cell lines contain a helper provirus wherein the packaging sequence is deleted and the protease gene is defective. Thus, type II cell produce virions that do not contain proviral RNA and functional aspartate protease.

TYPE III CELLS. Type III packaging cell lines contain a helper provirus wherein the packaging sequence is deleted and the integrase gene is defective. Thus, type III cell produce virions that do not contain proviral RNA and functional integrase.

A suitable retroviral provirus can be constructed using virtually any retroviral genome. For example, genomic human immunodeficiency virus type 1 (HIV-1) DNA can be obtained from American Type Culture Collection (ATCC #53069-pBT-1). *See* U.S. Patent 4,784,941. Deletion of the ψ sequence can be achieved by either oligo-directed mutagenesis, restriction digestion + *Bal*31 treatment, or other standard techniques

Type I cells: In order to inactivate the gene encoding reverse transcriptase, part or all of the *RT* gene can be deleted, usually by restriction digestion and religation of the *RT* sequences. Preferably, a selectable marker replaces the deleted *RT* sequences. The presence of the marker activity indicates that the *RT* sequences have not been "rescued" by recombination with wild-type sequences.

Type II cells: The cells are constructed in a manner similar to that described for TYPE I cells with the exception that the protease gene is subjected to inactivation/deletion.

Type III cells: In these cells, the integrase gene is subjected to mutagenesis rather than either the *RT* or protease genes. Again, it is preferable to insert a marker gene in the integrase coding region that acts as a selectable surrogate for the *int*⁻ phenotype.

CULTIVATION AND HARVESTING OF THERAPEUTIC RETROVIRAL VECTORS

Once a therapeutic vector has been generated, along with an appropriate packaging cell line, the vector DNA is transfected into the packaging cells by any of the methods discussed above in the section dealing with packaging cell lines. The transfected cells, *i.e.*, the producing cells, are cultured and the packaged retroviral vectors are isolated as concentrated culture supernatant and stored at -70°C. These stocks are stable for several years. In situations where the marker genes in either or both the packaging cells and the vector are drug resistance/selection genes, the transfected cells can be subjected to single or dual antibiotic selection.

METHODS OF TREATMENT

As mentioned above, a number of different therapeutic genes can be delivered to cells infected with a disease-causing retrovirus. These genes all have the ultimate effect of (i) killing the infected cell or (ii) inhibiting the replication of the disease-causing retrovirus contained therein.

A. Killing of Cells

Two basic methods of cell killing can be effected according to therapeutic vectors of the present invention. First, a gene encoding a toxin may be included in the vector. The toxin must be under the control of a promoter that is not active in the packaging or producing cells but is active in the target cells. When the vector RNA is converted to DNA and integrates into the chromosome of the target cell, the toxin is transcribed and translated by target cell synthetic enzymes. The toxin, selected for its toxicity inside the cell, kills the target. In so doing, the production of the disease-causing retrovirus is eliminated.

Alternatively, the vector DNA encodes a surface-bound, antigenic protein not normally expressed in the patient. This antigen may elicit a protective humoral or cellular immune response from the patient that results in destruction of antigen-expressing cells. Alternatively, the antigen may be one for which the patient has already acquired immunologic sensitivity. In a variation of this method, an antibody or antibody-conjugate may be provided passively to the patient which effects cell killing. This approach may be preferable where multiple rounds of superinfection are desired.

Another version of toxin action involves the expression of a gene that interacts with a pharmaceutical substance, resulting in the formation of a toxic compound. By administering the pharmaceutical substance before or after infection with the therapeutic vector, one can regulate the number of rounds of replication of the therapeutic vector.

B. Inhibition of Replication

Instead of killing the infected cells outright, it may be sufficient to curtail the production of the disease-causing retrovirus. Antisense therapy is well-suited for such an approach. In one form of this method, the therapeutic vector encodes a gene which is the complement to a gene essential for the replication of the

disease-causing retrovirus. When the essential gene and the antisense gene are transcribed, they hybridize to each other, thereby preventing translation of the essential gene's message. Without the essential product, replication of the disease-causing retrovirus is blocked.

Similarly, a defective essential gene can be employed to thwart replication. In this version, the therapeutic vector encodes a non-functional form of some gene essential for replication of the disease-causing retrovirus. When this non-functional protein is generated, it supplants use of the normal protein. To the extent that this use can overwhelm use of the normal protein in the replication cycle, the production of infectious disease-causing retrovirus is reduced.

In addition, it may be possible to attack cellular processes which do not adversely affect the cell but specifically inhibit viral replication. The target for such treatment would be cellular enzymes that are not required for the maintenance of cells but are required at some point in the viral replication cycle.

Several methods are envisioned by which a therapeutic vector may be deployed in a clinical setting. One approach is to culture packaging and producer cells *in vitro* and purify large quantities of the therapeutic vector. The vector would then be administered in one or more doses to the patient via an intravenous or subcutaneous route.

An alternative approach involves the use of a patient's own cells in an *ex vivo/in vivo* context. Briefly, cells amenable to *in vitro* culture are isolated from an patient and transfected, as discussed above, to generate packaging and producer cell lines. The producer cells would then be reintroduced into the patient and would produce therapeutic vector for the period during which the cells remained viable. In this way, the total amount of therapeutic virus provided, as well as the period during which the patient is exposed to the vector, is increased.

An even more aggressive regimen involves the same general strategy as set out in the previous paragraph, with the exception that the patient's cells are immortalized prior to their introduction back into the patient. Because the producing cells are rendered immortal, their longevity *in vivo* is greatly enhanced, further increasing the total dose and exposure of the patient to the therapeutic vector.

Of course, there are obvious concerns associated with introducing immortalized cells into a patient. These concerns largely are obviated, however, by rendering the cells suspectable to killing once their role as therapeutic vector "factories" is fulfilled. One way in which this may be accomplished is to transform the immortalized cells with a *tk* gene. Such transformed cells are susceptible to treatment with drugs, for example, gangcyclovir. After a sufficient time period during which the cells proliferate and produce therapeutic vector *in vivo*, the patient is treated with gangcyclovir to kill the transformed cells. In a preferred embodiment, the *tk* gene also is part of the therapeutic vector and renders retrovirally-infected cells susceptible to gangcyclovir treatment. This cycle can be repeated until all retrovirally-infected cells have been eliminated.

Another variation of the foregoing strategy involves the use of a separate therapeutic gene under the control of a cell specific promoter. Thus, producer cells would be rendered immortal and tk^+, but the therapeutic vector would not encode the *tk* gene product nor would the therapeutic product be expressed in the producer cells.

In cases where the retroviral infection has resulted in formation of a tumor, the tumor cells make an ideal target for conversion into packaging and producer cells. Tumor cells transfected with a therapeutic vector construct already are immortalized and need only been rendered tk^+ before use *in vivo*. In addition,

gangcyclovir killing of the transformed tumor cells *in vivo* also may render non-transformed tumor cells susceptible to the drug therapy by virtue of the "bystander effect." See Culver *et al.*, *Science* **256**:1550-1552 (1992).

The following examples are provided by way of illustration and those of skill in the art will recognize numerous variations serving the same purpose. These examples should not be read to limit the scope of the present invention.

Example 1 - **Construction of a packaging cell line.**

Vectors containing the genomic sequences of the appropriate retroviruses can be obtained from the American Type Culture Collection, Rockville, MD. The retroviral packaging sequence ψ, approximately 350 bp near the 5'-end of genomic retroviral DNA, are deleted by standard techniques. Similar deletions are described in the literature. See for example, Mann and Baltimore, *J. Virol.* **54**:401-407 (1985); Mann *et al.*, *Cell* **33**:153-159 (1983).

In one approach, using human immunodeficiency virus (HIV) as an example, the *Bgl*II/*Cla*I fragment, containing the R and U5 sequences of the LTR as well as the 5' untranslated region and part of the N-terminal coding region of the *gag* gene, is subcloned into the corresponding site of a staging vector, *e.g.*, pSP70 (Promega, Madison, WI) to facilitate subsequent manipulation. For details on the DNA sequence of HIV, see Ratner *et al.*, *Nature* **313**:277-283 (19xx) and Muesing *et al.*, *Nature* **313**:450-458 (19xx). The DNA subclone (10 μg) is digested with the restriction enzyme *Sac*I (100 U) to remove the ψ sequence. Additional *Bal*31 treatment (1.5 U of *Bal*31 in a final volume of 50 μl of buffer containing 650 μg/ml DNA, 600 mM NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris-HCl at pH 8.0, 1 mM EDTA, at 30°C for 10 min) may be used until the whole ψ sequence is removed. *Bal* digests are repaired by T4 DNA polymerase (1-2 U in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM

dithiothreitol supplement with 170 μg/ml bovine serum albumin and dNTP's) at 37°C for 30 min, ligated (15 U of T4 DNA ligase in a final volume of 20 μl of buffer containing 50 mM Tris-HCl at pH 8.0, 10 mM $MgCl_2$, 10 μg/ml BSA 20 mM dithiothreitol, 1 mM ATP) with linkers (1 μg) containing rare restriction site *Ppu*MI to facilitate further modification when needed. The linkers are synthesized using an automated DNA oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). Deletion of the desired sequence is confirmed by dideoxy sequencing. The modified *Bgl*II/*Cla*I fragment with the ψ sequence deleted is reinserted at the corresponding site in the HIV DNA genome.

Having thus prepared a packaging-deficient provirus, the next step is to create a series of proviral constructs that are defective for additional retroviral functions. Three different proviruses, which give rise to three distinct packaging cell lines (Types I-III), are contemplated. Each of these cell lines, in addition to producing empty virions, also lacks a particular retroviral gene product.

Type I: Using the packaging-deficient retrovirus sequences, a second deletion is undertaken by a strategy similar to that described above. The reverse transcriptase gene (*RT*) with the *pol* region is destroyed by digestion with *Kpn*I and *Pvu*II restriction enzymes. Excision of the intervening sequence results in loss of 3' coding regions for reverse transcriptase. To completely eliminate the coding sequence for the RT gene, additional *Bal*31 treatment from the *Kpn*I site can be carried out as described above. Following blunting of the ends and ligation of a linker containing the restriction sites for *Pme*I and *Pac*I, the gene for the selectable marker neomycin is cloned into the site vacated by the reverse transcriptase.

Type II: Using the packaging-deficient retrovirus sequences, a second deletion is undertaken by a strategy similar to that described above. The *prt*

gene, which precedes the *RT* coding sequence, is eliminated by *Bal*31 treatment at the *Dra*I site located 180 base pairs downstream of the translation initiation site of the *prt* gene. The deletion is extended to eliminate the upstream 180 nucleotides, including the translation initiation codon. *Bal*31 digests are repaired by DNA polymerase treatment and ligated with linkers containing the restriction sites for *Pme*I and *Pac*I and a translation initiation site at the 3'-end of the linker. Sequencing is performed to confirm an in-frame fusion of the ATG site of the linker to the rest of the *prt* sequence as well as the *RT* sequence. The gene for the selectable marker neomycin is cloned into the restriction sites in the linker.

Type III: Using the packaging-deficient retrovirus, a second deletion is undertaken by a strategy similar to that described above. A 1095 base pair *Eco*RI fragment of HIV-1 containing the 3' half of the HIV-1 integrase gene is subcloned into pUC18 and deletions originating from the 5' *Eco*RI site generated by digestion with *Bal*31. *Bal* digests are repaired by T4 DNA polymerase and ligated with a linker containing the restriction sites for *Pme*I and *Pac*I and the selectable marker neomycin. The modified *Eco*RI fragment is reinserted into the corresponding site of the HIV-1 genome.

To introduce the ψ-lacking retroviral sequences into cells, ten to twenty μg of modified HIV genomic DNA is mixed with 5-10 million H9 cells in electroporation buffer (20 mM HEPES: 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 2 mM ß-mercaptoethanol - pH 7.5) in a final volume of 0.7 ml in a 0.4 cm electroporation cuvette (BIORAD, Hercules, CA). Following electroporation, cells are distributed into 96-well microtiter plates and overlayed with fresh tissue culture media. Selection media containing 0.1-0.5 mg/ml of hygromycin B (Calbiochem, San Diego CA) is added 24 to 48 h after electroporation.

An alternative method of introducing the provirus involves transfection by calcium phosphate precipitation. Prior to transfection, modified HIV genomic DNA, carried in an appropriate vector, is linearized and adjusted to a concentration of 40-60 μg/ml in 0.1 X TE (pH 8.0, 1 mM Tris-HCl, 0.1 mM EDTA). Four hundred forty μl of the DNA solution is mixed with 500 μl of 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES) in a disposable, sterile 5 ml plastic test tube. Sixty-two μl of 2 M $CaCl_2$ are added to mixture with gentle mixing over a period of thirty seconds. Precipitation proceeds to completion at room temperature for 20 to 30 min. Meanwhile, 1 X 10^7 exponentially growing H9 cells were washed and pelleted. The calcium phosphate-DNA suspension is then used to gently resuspend the cell pellet. After leaving the mixture for 20 min at room temperature, 10 ml of complete media is added. The suspension is then transferred to a 90 mm tissue culture dish. Cells are washed once with PBS after an incubation period of 6-24 hours at 37°C in the presence of 5% CO_2. Cells are resuspended in 10 ml of prewarmed complete medium and incubated at 37°C, 5% CO_2. Selection medium containing 1 mg/ml G418 (Gibco/BRL, Gaithersburg, MD) is added 24-48 h later.

Cells surviving selection indicate the presence of the selectable marker stably integrated into the chromosome of the cells and, therefore, presumably proviral DNA. Chromosomal DNA is prepared from cloned, drug resistant cell lines and subjected to southern blot analysis to confirm the presence of retroviral sequences. One of the hybridization positive clones is then selected on the basis of high level production of empty virions.

Example 2 - **Construction of a therapeutic retrovirus.**

Using HIV as an example, most or all of the sequences (except the ψ encapsidation sequence) encoding the genes *gag*, *pol* and *env* are removed by treatment with *Pst*I and *Kpn*I digestion. A polylinker containing the staggered ends of *Pst*I and *Kpn*I as well as the

restriction sites for *Bam*HI, *Eco*RI, *Eag*I, *Eco*RV, *Xho*I and *Pac*I is ligated to the corresponding sites of the HIV sequence. Different therapeutic genes can be inserted into the restriction sites within the linker. For example, the herpes simplex virus thymidine kinase (*tk*) gene can be inserted into the *Pvu*II restriction site within the linker such that transcription is controlled by the HIV LTR. A hygromycin selection marker with an SV40 promoter, which is different from the one used in the packaging vector, is obtained from the vector pSVhyg (ATCC) and is inserted downstream of the *tk* gene. The therapeutic vector should then contain, in its 5′ to 3′ orientation, the HIV LTR, a packaging sequence ψ, a promoterless *tk* gene, a hygromycin resistant gene driven by an SV40 promoter, and a downstream HIV LTR. Cells expressing the *tk* gene are sensitive to the cytotoxic effect of a gancylcovir. Other therapeutic genes or combinations of genes, such as a xenogenic MHC molecule from heterologous source, an antisense construct, a toxin such as ricin A chain or some other surface marker for which specific antibodies are available, can be used in place of or in conjunction with the *tk* gene.

Example 3 - **Cultivation of a therapeutic retrovirus in a packaging cell line.**

Packaging cells lines (Types I-III) are produced by transfecting H9 cells with one of the constructs as described in Example 1. Individual clones are isolated by further culture in cloning cylinders or by limiting dilution in microtiter plates in the presence of 0.5 mg/ml of hygromycin B for at least five days. Individual clones so selected are expanded into cell lines. Cell lines producing the highest titers of empty virions are identified by a standard ELISA assay. Briefly, supernatant samples (100 μl) containing the empty virions are added in triplicate to ELISA microtiter plates and are incubated overnight at 4°C to allow precoating of the virions onto the plates. After washing three times with wash buffer (PBS containing 0.05% polysorbate-20), anti-

*gp*120 antibody (Chemicon, Temecula, CA) is added to react with the virions bound to the plate. At a final concentration of 1.0 μg/ml, horseradish peroxidase conjugated goat-antimouse, Fc-specific antibody (Jackson ImmunoResearch, West Grove, PA) is added to the wells. Following an incubation of 1 h, the plates were washed three times.

A reaction solution (100 μl, containing 167 μg of ortho-phenylene-diamine (Sigma, St. Louis, MO), 0.025% hydrogen peroxide in PBS) is added to the wells. Color is allowed to develop in the dark for 30 min. The reaction is stopped by the addition of 50 μl of 4N HCl solution to each well before measuring absorbance at 490 nm in an automated ELISA reader (Bio-Tek Instruments, Winooski, VT).

The packaging cell line with the highest level of empty virion production is used for transfection. Approximately 10-20 μg of linearized therapeutic retroviral vector, as described in Example 2, is used to transfect 5 to 10 X 10^6 packaging cells by the method described in Example 1. Transfected cells are resuspended in 20 ml of culture media (DMEM + 10% FCS + 100 μg/ml gentamicin) and distributed into the wells of two microtiter plates. After incubating the cells at 37°C in the presence of 5% CO_2 for two days, fresh media containing 0.5 mg/ml of hygromycin B (Calbiochem, San Diego, CA) is added. Colonies usually emerge 2-3 weeks post-transfection. Supernatants from hygromycin-resistant colonies are collected for virion detection by ELISA. The presence of virion RNA is detected by a slot blot *in situ* hybridization assay using ^{32}P-labeled DNA fragment from the therapeutic gene as the probe. Details of this procedure can be found in Sambrook *et al.*, *MOLECULAR CLONING: A LABORATORY MANUAL*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). Working virus stocks are obtained from the culture supernatant of the clones producing the highest number of therapeutic retrovirions every three to five days.

Culture medium in which the infected packaging cells are grown is centrifuged at 5000 x g for 5 min to pellet cells. The supernatant is then frozen in liquid nitrogen.

Example 4 - **Treatment of a subject infected with a retrovirus using a therapeutic retrovirus.**

Skin fibroblasts from an asymptomatic HIV-positive patient are obtained and transformed *in vitro* by standard techniques to generate an immortalized cell line. The immortalized fibroblasts are transfected by the methods described above and converted into packaging and producer cells, of which those producing the highest level of viral particles are selected and expanded in culture. The HS-*tk* retroviral vector-producing fibroblasts (10^7 cells) are injected into the donor patient subcutaneously and, after about one week, gangcyclovir (5 mg/kg) is given intravenously at a constant rate over one hour, twice daily, for one week. In the third week, the patient receives a second injection of HS-*tk* retroviral vector-producing fibroblasts (10^7 cells) and, one week later, gangcyclovir is given twice daily for one week. This two-week treatment cycle is repeated a third time and the effectiveness of the therapy is monitored at the end of each treatment cycle by measuring the presence of HIV in plasma or blood cells using PCR for HIV-1 mRNA. For maintenance treatment, the patient will receive gangcyclovir (5 mg/kg), once daily.

What is claimed is:

1. A retroviral nucleic acid vector comprising

(i) the coding sequences for the LTR and packaging sequence of a first retrovirus;

(ii) at least one of

(a) a gene, the corresponding message or polypeptide of which inhibits the replication of at least one of said first retrovirus and a second retrovirus; and

(b) a gene that encodes a surface-expressed antigenic determinant; and

(iii) a selectable marker gene,

wherein said vector lacks

(iv) the coding sequence for at least one of a functional reverse transcriptase, a functional protease, and a functional integrase; and

(v) at least one gene or DNA segment necessary for the packaging of said genome.

2. A retroviral nucleic acid vector according to claim 1, wherein said at least one gene, (ii), is a gene (a) the corresponding message or polypeptide of which inhibits the replication of at least one of said retrovirus and a second retrovirus.

3. A retroviral nucleic acid vector according to claim 1, wherein said at least one gene, (ii), is a gene (b) that encodes a surface-expressed antigenic determinant.

4. A retroviral nucleic acid vector according to claim 2, wherein said gene (a) is thymidine kinase.

5. A retroviral nucleic acid vector according to claim 2, wherein said gene (a) is a toxin and is under the control of a target cell-specific promoter.

6. A retroviral nucleic acid vector according to claim 5, wherein said target cell-specific promoter is a CD4 promoter.

7. A retroviral nucleic acid vector according to claim 1, wherein at least one of said first and said second retrovirus is HIV-1.

8. A retroviral packaging cell, comprising an animal host cell wherein said host cell contains the genome of a retrovirus which lacks

(i) a functional packaging sequence and

(ii) at least one gene essential for the integration of said retrovirus.

9. A retroviral packaging cell according to claim 8, wherein said gene encodes a retroviral reverse transcriptase.

10. A retroviral packaging cell according to claim 8, wherein said gene encodes a retroviral protease.

11. A retroviral packaging cell according to claim 8, wherein said gene encodes a retroviral integrase.

12. A retroviral packaging cell according to claim 8, wherein said genome contains a gene encoding thymidine kinase.

13. A method for treating retroviral infection in an animal, comprising the step of infecting an animal with a retroviral nucleic acid vector comprising

(A) the coding sequences for the LTR and packaging sequence of a first retrovirus;

(B) at least one of (i) a gene, the corresponding message or polypeptide of which inhibits the replication of at least one of said first retrovirus and a second retrovirus, and (ii) a gene that encodes a surface-expressed antigenic determinant; and

(C) a selectable marker gene,

wherein said vector lacks

(D) the coding sequence for at least one of a functional reverse transcriptase, a

functional protease, and a functional integrase; and

(E) at least one gene or DNA segment necessary for the packaging of said genome.

14. A method according to claim 13, which further comprises the step of administering a therapeutically effective amount of an antibody-conjugate to said animal, wherein said conjugate comprises a molecule selected from the group consisting of a drug, a toxin and a radioisotope.

15. A method according to claim 13, wherein said gene, the corresponding message or polypeptide of which inhibits the replication of at least one of said first retrovirus and a second retrovirus, interacts with a drug administered to said animal, to effect the inhibition of replication.

16. A method according to claim 13, wherein said animal is infected by administering to said animal a cell line producing said retroviral nucleic acid vector and wherein said cell line is subject to elimination by treatment of said animal with a pharmaceutical or biological composition.

17. A method according to claim 13, wherein said gene (i) is a toxin and is under the control of a target cell-specific promoter.

18. A method according to claim 17, wherein said target cell-specific promoter is a CD4 promoter.

19. A method according to claim 13, wherein at least one of said first and said second retrovirus is HIV-1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06594

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/86; A61K 48/00
US CL :435/320.1; 424/93.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 424/93.2

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

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

APS; DIALOG DATABASES: CA SEARCH, MEDLINE, AIDSLINE, BIOSIS PREVIEWS, WORLD PATENT INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0 334 301 (VIAGENE INC.) 27 September 1989, see entire document.	1-19
Y	WO, A, 90/07936 (CHIRON CORPORATION) 26 July 1990, see entire document.	1-19
Y	WO, A, 90/12087 (NOVACELL CORPORATION) 18 October 1990, see entire document.	1-19
Y	WO, A, 92/15693 (THE WELLCOME FOUNDATION LIMITED) 17 September 1992, see entire document.	1-19

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 AUGUST 1995	01 NOV 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06594

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 90, issued August 1993, P. Salmon et al., "Characterization of the human CD4 gene promoter: Transcription from the CD4 gene core promoter is tissue-specific and is activated by Ets proteins," pages 7739-7743, see entire article.	6, 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06594

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06594

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-7, 13, 15 and 17-19, drawn to retroviral vectors and a method for using these vectors to treat retroviral infections in an animal.

Group II, claims 8-12, drawn to packaging cells.

Group III, claim 14, drawn to a method for treating retroviral infections in an animal, the method including a distinct step of administering antibody-conjugate to the animal.

Group IV, claim 16, drawn to a distinct method for treating retroviral infections in an animal, the method wherein a cell line is administered to the animal.

The inventions listed as Groups I through IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a first product and a method of use of that product. Group II is drawn to an unrelated product in the art, packaging cells. Groups III and IV are drawn to distinct methods in the art by virtue of the compositions used in them. PCT Rule 13 does not provide for multiple products or methods within a single application. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.