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(54) Title: RETROVIRAL VECTOR PARTICLES PRODUCED IN A BACULOVIRUS EXPRESSION SYSTEM

(57) Abstract

A composition is described. The composition comprises at least one baculoviral component and at least one retroviral component, wherein the retroviral component is capable of being packaged into a retroviral particle.

RETROVIRAL VECTOR PARTICLES PRODUCED IN A BACULOVIRUS EXPRESSION SYSTEM

The present invention relates to a composition.

In particular, the present invention relates to a novel system for producing retroviral particles.

More in particular, the present invention relates to a composition that is capable of expressing a retroviral particle that is capable of delivering a nucleotide sequence of interest (hereinafter abbreviated to "NOI") - or even a plurality of NOIs - to a site of interest.

More in particular, the present invention relates to a composition useful in gene therapy.

Gene therapy includes any one or more of: the addition, the replacement, the deletion, the supplementation, the manipulation etc. of one or more nucleotide sequences in, for example, one or more targeted sites - such as targeted cells. If the targeted sites are targeted cells, then the cells may be part of a tissue or an organ. General teachings on gene therapy may be found in Molecular Biology (Ed Robert Meyers, Pub VCH, such as pages 556-558).

By way of further example, gene therapy also provides a means by which any one or more of: a nucleotide sequence, such as a gene, can be applied to replace or supplement a defective gene; a pathogenic gene or gene product can be eliminated; a new gene can be added in order, for example, to create a more favourable phenotype; cells can be manipulated at the molecular level to treat cancer (Schmidt-Wolf and Schmidt-Wolf, 1994, Annals of Hematology 69:273-279) or other conditions - such as immune, cardiovascular, neurological, inflammatory or infectious disorders; antigens can be manipulated and/or introduced to elicit an immune response - such as genetic vaccination.

In recent years, retroviruses have been proposed for use in gene therapy. Essentially, retroviruses are RNA viruses with a life cycle different to that of lytic viruses. In this regard, when a retrovirus infects a cell, its genome is converted to a DNA form. In otherwords, a retrovirus is an infectious entity that replicates through a DNA intermediate.

More details on retroviral infection etc. are presented later on.

There are many retroviruses and examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV may be found from the NCBI Genbank (i.e. Genome Accession No. AF033819).

All retroviruses contain three major coding domains, *gag, pol, env*, which code for essential virion proteins. Nevertheless, retroviruses may be broadly divided into two categories: namely, "simple" and "complex". These categories are distinguishable by the organisation of their genomes. Simple retroviruses usually carry only this elementary information. In contrast, complex retroviruses also code for additional regulatory proteins derived from multiple spliced messages.

Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 1-25).

All oncogenic members except the human T-cell leukemia virus-bovine leukemia virus group (HTLV-BLV) are simple retroviruses. HTLV, BLV and the lentiviruses and spumaviruses are complex. Some of the best studied oncogenic retroviruses are Rous

sarcoma virus (RSV), mouse mammary tumour virus (MMTV) and murine leukemia virus (MLV) and the human T-cell leukemia virus (HTLV).

The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiencey virus (FIV) and bovine immunodeficiencey virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11; 3053-3058, Lewis and Emerman 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

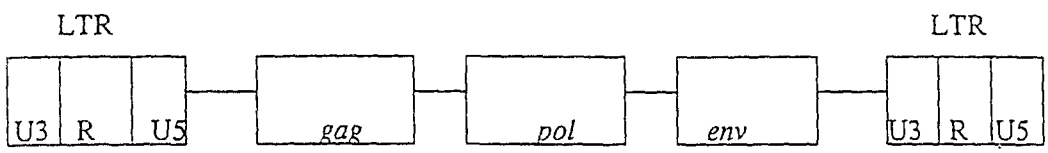
During the process of infection, a retrovirus initially attaches to a specific cell surface receptor. On entry into the susceptible host cell, the retroviral RNA genome is then copied to DNA by the virally encoded reverse transcriptase which is carried inside the parent virus. This DNA is transported to the host cell nucleus where it subsequently integrates into the host genome. At this stage, it is typically referred to as the provirus. The provirus is stable in the host chromosome during cell division and is transcribed like other cellular proteins. The provirus encodes the proteins and packaging machinery required to make more virus, which can leave the cell by a process sometimes called "budding".

As already indicated, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. These genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral gene. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral

genome.

The LTRs themselves are indentical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5'end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

For ease of understanding, a simple, generic diagram (not to scale) of a retroviral genome showing the elementary features of the LTRs, *gag*, *pol* and *env* is presented below.



For the viral genome, the site of transcription initiation is at the boundary between U3 and R in the left hand side LTR (as shown above) and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR (as shown above). U3 contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins. Some retroviruses have any one or more of the following genes that code for proteins that are involved in the regulation of gene expression: *tat*, *rev*, *tax* and *rex*.

As shown in the diagram above, the basic molecular organisation of a retroviral RNA genome is (5') R - U5 - *gag*, *pol*, *env* - U3-R (3'). In a retroviral vector genome *gag*, *pol* and *env* are absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent sequences unique, respectively, to the 5' and 3' ends of the RNA genome. These three sets of end sequences go to form the long terminal repeats (LTRs) in the proviral DNA, which is the form of the genome which integrates into the genome of the infected cell. The LTRs in a wild type retrovirus consist of (5')U3 - R - U5 (3'), and thus U3 and U5 both contain sequences which are important for proviral

integration. Other essential sequences required in the genome for proper functioning include a primer binding site for first strand reverse transcription, a primer binding site for second strand reverse transcription and a packaging signal.

With regard to the structural genes *gag*, *pol* and *env* themselves and in slightly more detail, *gag* encodes the internal structural protein of the virus. Gag is proteolytically processed into the mature proteins MA (matrix), CA (capsid), NC (nucleocapsid). The gene *pol* encodes the reverse transcriptase (RT), which contains both DNA polymerase, and associated RNase H activities and integrase (IN), which mediates replication of the genome. The gene *env* encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction leads ultimately to fusion of the viral membrane with the cell membrane.

The envelope glycoprotein complex of retroviruses includes two polypeptides: an external, glycosylated hydrophilic polypeptide (SU) and a membrane-spanning protein (TM). Together, these form an oligomeric "knob" or "knobbed spike" on the surface of a virion. Both polypeptides are encoded by the *env* gene and are synthesised in the form of a polyprotein precursor that is proteolytically cleaved during its transport to the cell surface. Although uncleaved Env proteins are able to bind to the receptor, the cleavage event itself is necessary to activate the fusion potential of the protein, which is necessary for entry of the virus into the host cell. Typically, both SU and TM proteins are glycosylated at multiple sites. However, in some viruses, exemplified by MLV, TM is not glycosylated.

Although the SU and TM proteins are not always required for the assembly of enveloped virion particles as such, they do play an essential role in the entry process. In this regard, the SU domain binds to a receptor molecule - often a specific receptor molecule - on the target cell. It is believed that this binding event activates the membrane fusion-inducing potential of the TM protein after which the viral and cell membranes fuse. In some viruses, notably MLV, a cleavage event - resulting in the removal of a short portion of the cytoplasmic tail of TM - is thought to play a key role in uncovering the full fusion activity of the protein (Brody *et al* 1994 J. Virol. 68: 4620-4627, Rein *et al* 1994 J. Virol. 68:

1773-1781). This cytoplasmic "tail", distal to the membrane-spanning segment of TM remains on the internal side of the viral membrane and it varies considerably in length in different retroviruses.

Thus, the specificity of the SU/receptor interaction can define the host range and tissue tropism of a retrovirus. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types. In some cases however, it may be beneficial, especially from a safety point of view, to specifically target restricted cells. To this end, several groups have engineered a mouse ecotropic retrovirus, which unlike its amphotropic relative normally only infects mouse cells, to specifically infect particular human cells. Replacement of a fragment of an envelope protein with an erythropoietin segement produced a recombinant retrovirus which then bound specifically to human cells that expressed the erythropoietin receptor on their surface, such as red blood cell precursors (Maulik and Patel 1997 "Molecular Biotechnology: Therapeutic Applications and Strategies" 1997. Wiley-Liss Inc. pp 45.).

In addition to *gag*, *pol* and *env*, the complex retroviruses also contain "accessory" genes which code for accessory or auxillary proteins. Accessory or auxillary proteins are defined as those proteins encoded by the accessory genes in addition to those encoded by the usual replicative or structural genes, *gag*, *pol* and *env*. These accessory proteins are distinct from those involved in the regulation of gene expression, like those encoded by *tat*, *rev*, *tax* and *rex*. Examples of accessory genes include one or more of *vif*, *vpr*, *vpx*, *vpu* and *nef*. These accessory genes can be found in, for example, HIV (see, for example pages 802 and 803 of "Retroviruses" Ed. Coffin *et al* Pub. CSHL 1997). Non-essential accessory proteins may function in specialised cell types, providing functions that are at least in part duplicative of a function provided by a cellular protein. Typically, the accessory genes are located between *pol* and *env*, just downstream from *env* including the U3 region of the LTR or overlapping portions of the *env* and each other.

The complex retroviruses have evolved regulatory mechanisms that employ virally encoded transcriptional activators as well as cellular transcriptional factors. These *trans*-acting viral proteins serve as activators of RNA transcription directed by the LTRs. The transcriptional *trans*-activators of the lentiviruses are encoded by the viral *tat* genes. Tat binds to a stable, stem-loop, RNA secondary structure, referred to as TAR, one function of which is to apparently optimally position Tat to *trans*-activate transcription.

As mentioned earlier, retroviruses have been proposed as a delivery system (other wise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a NOI, or a plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro*, *ex vivo*, *in vivo*, or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have even been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992 Curr Top Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag*, *pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

In some instances, propagation and isolation may entail isolation of the retroviral *gag*, *pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This can be used to infect cells to introduce the NOI into the genome of the cells. The recombinant virus whose genome lacks all genes required to make viral proteins can infect only once and cannot propagate. Hence, the NOI is introduced into the host cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449). However, this technique can be problematic in the sense that the titre levels are not always at a satisfactory level. Nevertheless, the design of retroviral packaging cell lines has evolved to address the problem of *inter alia* the spontaneous production of helper virus that was frequently encountered with early designs. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper has reduced the problem of helper virus production.

More recently, packaging cells have been developed in which the *gag*, *pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line so that three recombinant events are required for wild type viral production. This strategy is sometimes referred to as the three plasmid transfection method (Soneoka *et al* 1995 Nucl. Acids Res. 23: 628-633).

Transient transfection can also be used to measure vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and is used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the Gag/Pol proteins, a plasmid encoding the Env protein and a plasmid containing a NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector

encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apotosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient infection that produce vector titre levels that are comparable to the levels obtained from stable vector-producing cell lines (Pear *et al* 1993, PNAS 90:8392-8396).

In view of the toxicity of some HIV proteins - which can make it difficult to generate stable HIV-based packaging cells - HIV vectors are usually made by transient transfection of vector and helper virus. Some workers have even replaced the HIV Env protein with that of vesicular stomatis virus (VSV). Insertion of the Env protein of VSV facilitates vector concentration as HIV/VSV-G vectors with titres of 5 x 10^5 (10^8 after concentration) were generated by transient transfection (Naldini *et al* 1996 Science 272: 263-267). Thus, transient transfection of HIV vectors may provide a useful strategy for the generation of high titre vectors (Yee *et al* 1994 PNAS. 91: 9564-9568). A drawback, however, with this approach is that the VSV-G protein is quite toxic to cells.

Thus, and as indicated, retroviral vectors are used extensively in biomedical research and for gene therapy. Current methods for the production of retroviral vectors make use of the fact that the two roles of the wild-type retrovirus genome, that is protein encoding and as a template for new genome copies, can be de-coupled (e.g. Soneoka *et al* 1995 Nucl. Acids Res. 23, 628 and references therein). Protein that is required for the assembly of new virus particles and for enzyme and regulatory functions can be produced by non-genome sequences in, for example, a mammalian packaging cell line (e.g. Miller 1990 Hum. Gene Therapy 1, 5). A genome sequence lacking the protein encoding functions is provided, so that the resulting retroviral vector particles are capable of infecting but not of replicating in a target cell. The genome sequence can also be designed for delivery and integration of a therapeutic gene (Vile and Russel 1995 Brit. Med. Bull 51, 12). Standard methods for producing murine leukaemia virus (MLV)-based vectors, for example, include use of cell lines expressing the *gag-pol* and *env* genes (the packaging components) of MLV. These will package a compatible retroviral vector genome introduced by transduction or by transfection with an appropriate plasmid. An alternative method involves simultaneous

transfection of *gag-pol*, *env*, and vector genome plasmids into suitable cells.

Although the principles of these systems are well understood, in practice the re-constructed virus assembly system often fails to generate the quantity of vector particles which will be required in practice for use in gene therapy. Retroviral vector particles are generally harvested by removing supernatant from a culture of particle-producing cells. The resulting suspension may be concentrated with respect to the vector particles, using physical methods, but only to a limited degree as problems such as aggregation and damage tend to arise. Thus, it may only be possible to concentrate a suspension of vector particles by up to 100-fold.

The present invention seeks to provide an improved system for preparing viral particles that may be of subsequent use in medicine.

In particular, the present invention seeks to provide an improved system for preparing a high titre of viral particles that may be of subsequent use in medicine.

According to a first aspect of the present invention there is provided a composition comprising at least one baculoviral component and at least one retroviral component, wherein the retroviral component is capable of being packaged into a retroviral particle.

According to a second aspect of the present invention there is provided a composition wherein the composition is a baculovirus expression system comprising at least one retroviral component, wherein the retroviral component is capable of being packaged into a retroviral particle.

According to a third aspect of the present invention there is provided a retroviral particle obtainable from expression of the composition according to the present invention.

According to a fourth aspect of the present invention there is provided a process for preparing a retroviral particle comprising expressing the composition according to the present invention.

According to a fifth aspect of the present invention there is provided an insect cell comprising the composition according to the present invention.

According to a sixth aspect of the present invention there is provided a retroviral vector particle production system comprising the composition according to the present invention in an insect cell.

According to a seventh aspect of the present invention there is provided a retroviral vector particle produced by the retroviral vector particle production system according to the present invention.

According to an eighth aspect of the present invention there is provided an expression vector comprising a polynucleotide sequence which encodes a retroviral vector genome having a 5' and a 3' end, which retroviral vector genome is capable of being expressed and packaged into a retroviral vector particle in a baculovirus expression system.

Preferably the retroviral component corresponds to a retroviral genome.

Preferably the composition comprises an RNA transcription start site for the retroviral vector genome, and wherein the nucleotide sequence encoding the retroviral component is operably linked to a promoter comprising an upstream promoter component located upstream of the RNA transcription start site and a downstream promoter component located downstream of the RNA transcription start site.

Preferably the downstream promoter component is upstream of the polynucleotide sequence encoding the retroviral vector genome.

In one embodiment, preferably the promoter is a baculovirus promoter.

Preferably the promoter is the polyhedrin promoter, the *p10* promoter and/or the *polh* promoter.

In one embodiment, preferably the promoter is a non-baculovirus promoter.

Preferably the promoter is the T7 promoter or the *sp6 Salmonella* phage promoter.

Preferably the composition comprises at least one RNA cleavage component.

Preferably manipultaion of at least one of the RNA cleavage component(s) would yield a retroviral genome free of any baculoviral components.

Preferably at least one of the RNA cleavage component(s) is located between the promoter and the sequence encoding the retroviral component.

Preferably at least one of the RNA cleavage component(s) is located immediately adjacent the sequence encoding the retroviral vector component for subsequent cleavage at the 5' end of the vector component.

Preferably at least one of the RNA cleavage component(s) is located downstream of the sequence encoding the retroviral component.

Preferably the RNA cleavage component(s) has a cleavage site immediately adjacent the sequence encoding the retroviral vector component for subsequent cleavage at the 3' end of the vector component.

Preferably at least one of the RNA cleavage component(s) is a ribozyme cleavage site for subsequent cleavage thereof.

Preferably each RNA cleavage component is a ribozyme cleavage site for subsequent cleavage thereof.

The or each ribozyme cleavage site may be cleaved by a ribozyme which is independently derived from the composition. Preferably, however, any one or more of the ribozyme

cleavage sites is cleaved by a ribozyme derived from the second viral component.

Ribozymes are RNA molecules that can function to catalyse specific chemical reactions within cells without the obligatory participation of proteins. For example, group I ribozymes take the form of introns which can mediate their own excision from self-splicing precursor RNA. Other ribozymes are derived from self-cleaving RNA structures which are essential for the replication of viral RNA molecules. Like protein enzymes, ribozymes can fold into secondary and tertiary structures that provide specific binding sites for substrates as well as cofactors, such as metal ions. Examples of such structures include hammerhead, hairpin or stem-loop, pseudoknot and hepatitis delta antigenomic ribozymes have been described.

Each individual ribozyme has a motif which recognises and binds to a recognition site in a target RNA. This motif takes the form of one or more "binding arms" but generally two binding arms. The binding arms in hammerhead ribozymes are the flanking sequences Helix I and Helix III which flank Helix II. These can be of variable length, usually between 6 to 10 nucleotides each, but can be shorter or longer. The length of the flanking sequences can affect the rate of cleavage. For example, it has been found that reducing the total number of nucleotides in the flanking sequences from 20 to 12 can increase the turnover rate of the ribozyme cleaving a HIV sequence, by 10-fold (Goodchild, JVK, 1991 Arch Biochem Biophys 284: 386-391). A catalytic motif in the ribozyme Helix II in hammerhead ribozymes cleaves the target RNA at a site which is referred to as the cleavage site. Whether or not a ribozyme will cleave any given RNA is determined by the presence or absence of a recognition site for the ribozyme containing an appropriate cleavage site.

Each type of ribozyme recognizes its own cleavage site. The hammerhead ribozyme cleavage site has the nucleotide base triplet GUX directly upstream where G is guanine, U is uracil and X is any nucleotide base. Hairpin ribozymes have a cleavage site of BCUGNYR, where B is any nucleotide base other than adenine, N is any nucleotide, Y is cytosine or thymine and R is guanine or adenine. Cleavage by hairpin ribozymes takes places between the G and the N in the cleavage site.

More details on ribozymes may be found in "Molecular Biology and Biotechnology" (Ed. RA Meyers 1995 VCH Publishers Inc p831-8320 and in "Retroviruses" (Ed. JM Coffin et al 1997 Cold Spring Harbour Laboratory Press pp 683).

A broad aspect of this embodiment of the present invention relates to a composition comprising at least a first viral component obtainable from a first virus and a second viral component component obtainable from a second virus; wherein the first virus is different from the second virus; wherein the second viral component is flanked by at least two cleavage sites (which may be the same or different); wherein at least a part of the second viral component is capable of being packaged into a viral particle; which viral particle is substantially free of any first viral component.

Here, preferably at least one of the cleavage sites is a ribozyme cleavage site.

Here, preferably each of the cleavage sites is a ribozyme cleavage site.

In accordance with this aspect of the present invention, the ribozyme cleavage site may be cleaved by a ribozyme which is independently derived from the composition. Preferably, however, any one or more of the ribozyme cleavage sites is cleaved by a ribozyme derived from the second viral component.

Here, preferably the first virus is a baculovirus.

Here, preferably the second virus is a retrovirus.

Preferably the downstream promoter component is located within the sequence encoding the retroviral component.

Preferably the downstream promoter component is located within the sequence encoding the retroviral vector.

Preferably the retroviral component comprises a retroviral R region at either end of a

sequence encoding a retroviral vector genome, wherein the downstream promoter component is located in the 5' R region and has a counterpart sequence in the 3' R region.

In one embodiment, preferably the composition comprises in a downstream direction: an upstream baculovirus promoter component, a downstream baculovirus promoter component, a ribozyme sequence, a retroviral 5' R region, a retroviral U5 region, a retroviral vector region for insertion of one or more genes to be delivered by the vector, a retroviral U3 region, a retroviral 3' R region, and optionally a second ribozyme sequence.

In one embodiment, preferably the composition comprises in a downstream direction: an upstream baculovirus promoter component, a retroviral 5' R region comprising a downstream promoter component, a retroviral U5 region, a retroviral vector region for insertion of one or more genes to be delivered by the retroviral vector, a retroviral U3 region, a retroviral 3' R region, and optionally a ribozyme sequence.

Preferably the composition comprises one or more nucleotide sequences encoding one or more packaging components for producing retroviral vector particles which particles comprise the retroviral component.

Preferably the composition further comprises at least one nucleotide sequence of interest (NOI).

Preferably the NOI is useful in medicine.

Preferably the NOI is part of the retroviral component.

The term "composition" as used herein with reference to the present invention may include one entity (such as a composition of matter) or two or more entities. For example, the composition of the first or second aspect may be one entity - such as a modified baculoviral genome wherein at least a part of the baculoviral genome has been replaced with the retroviral component of the present invention. The term "modified" includes actual modification of a wild type genome or *ab initio* construction of an entity that could have

been modified from a wild type genome (such as by ligating two or more fragments so as to form an entity corresponding to the modified genome). By way of further example, the packaging components of the present invention may be contained in an entity different to that just described. By way of further example, the composition of the present invention can be a retroviral particle producer and/or vector.

The composition may even be a kit comprising a first part which includes the baculoviral component and a second part which includes the retroviral component. Optionally, the kit may include one or more other parts, such as one or more suitable restriction enzymes etc.

The term "retroviral vector genome" includes a retroviral nucleic acid which is capable of infection, but which is not capable, by itself, of replication. Thus it is replication defective. A retroviral vector genome carries or is capable of carrying polynucleotide sequences of non-retroviral origin, for delivery to target cells. A retroviral vector genome may comprise further non-retroviral sequences, such as non-retroviral control sequences in the U3 region which influence expression of the genome once it is integrated as a provirus into a target cell. The retroviral vector genome need not contain elements from only a single retrovirus. WO 96/37623, for example, describes retroviral vectors having hybrid LTRs derived from two different retroviruses.

The term "retroviral vector particle" refers to the packaged retroviral vector genome, that is preferably capable of binding to and entering target cells. The components of the particle, as already discussed for the vector genome, may be modified with respect to the wild type retrovirus. For example, the envelope proteins in the proteinaceous coat of the particle may be genetically modified in order to alter their targeting specificity or achieve some other desired function.

If the retroviral component includes an *env* nucleotide sequence, then all or part of that sequence can be optionally replaced with all or part of another *env* nucleotide sequence. Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy called pseudotyping. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-

91/00047 and Mebatsion *et al* 1997 Cell 90, 841-847.

Pseudotyping can confer one or more advantages. For example, with the lentiviral vectors, the *env* gene product of the HIV based vectors would restrict these vectors to infecting only cells that express a protein called CD4. But if the *env* gene in these vectors has been substituted with *env* sequences from other RNA viruses, then they may have a broader infectious spectrum (Verma and Somia 1997 Nature 389:239-242). By way of example - workers have pseudotyped an HIV based vector with the glycoprotein from VSV (Verma and Somia 1997 *ibid*).

In accordance with the present invention we have surprisingly found that functional retroviral vector particles can be produced in a baculovirus expression system and those particles can successfully deliver one or more NOI(s) to target cells.

Another important advantage of the present invention is that higher titres of vector particles may be produced than in the mammalian systems. Again, this is an unexpected finding.

Moreover, there is the added advantage that baculovirus expression systems may be free from endogenous mammalian genetic material, so reducing the risk of mammalian contaminants being present in the resulting retroviral vector preparation. In this regard, insect contaminants are very unlikely to be biologically active in a mammalian system and are therefore expected to present less of a problem than mammalian contaminants.

As indicated, the composition of the present invention comprises a baculoviral component.

In brief, baculoviruses are a diverse group of viruses found mainly in insects with no supposedly known arthropod hosts.(O' Reilly *et al* in Baculovirus Expression Vectors, A Laboratory Manual 1994, Oxford University Press). They can accommodate relatively large insertions of heterologous DNA - such as an NOI according to the present invention - (O'Reilly *et al.* in Baculovirus Expression Vectors, A Laboratory Manual 1994, Oxford University Press). They have the potential for very strong expression of heterologous genes DNA - such as an NOI according to the present invention - for example expression

levels of 25 to 50% of the total cellular protein have been reported.

In more detail, baculoviruses have large double-stranded, circular DNA genomes within a rod-shaped capsid. Within the capsid, the DNA is condensed into a nucleoprotein stucture known as the core. The capsid plus the core are collectively referred to as the nucleocapsid.

The length of a baculoviral DNA is between 80 and 120 kilobasepairs (kbp). The DNAs of the baculoviruses commonly exploited as expression vectors, *Autographa california* multiple nuclear polyhedrosis virus (AcMNPV) (Miller *et al* 1987 In Genetic Engineering vol 8, eds Setler, JK and Hollaender A Plenum Press, New York) and the silkworm virus, *Bombyx mori* nuclear polyhedrosis virus (BmNPV) (Maeda *et al* 1985 Nature 315: 592-594) are both approximately 130kbp.

Nucleocapsids are made in the nucleus of the infected cells and are subsequently enveloped (that is, they acquire a membrane) by one of two processes. Nuclecapsids can bud through the plasma membrane of the infected cell or they can acquire an envelope within the nucleus where they are produced. Membrane-enveloped nucleocapsids are referred to as virus particles or virions. The plasma-membrane budded form of the virus is referred to as the budded virus (BV).

During normal infection, the viruses produce nuclear occlusion bodies which comprise enveloped nucleocapsid(s) embedded in a crystalline protein matrix, the major component of which is a virus-encoded protein called polyhedrin. Polyhedrin is the product of a single gene in the baculovirus genome and is produced, late in infection, accounting for 50% or more of all proteins being made by infected insect cells. Transcription of the polyhedrin (*polh*) gene is driven by an extremely active promoter, which is therefore ideally suited as a promoter for driving expression of foreign genes. Similiar levels of protein production can occur if the polyhedrin gene is replaced by a foreign gene. Construction of expression vectors has therefore consisted of inserting a foreign coding sequence just downstream of the polyhedrin promotor. However, this cannot be achieved directly because the large size (about 130kbp) fo the viral DNA precludes simple in vitro manipulation (Old and Primose

1989 "Principles of Genetic Manipulation" 4th Ed Blackwell Scientific Publications pp290-291).

The insect baculovirus expression system provides a eukaryotic environment that is generally conducive to the proper folding, disulfide bond formation, oligomerisation and/or other posttranslational modifications required for the biological activity of some eukaryotic proteins. Post-translational modifications that have been reported to occur in baculovirus-infected insect cells include signal cleavage, proteolytic cleavage, N-glycosylation, O-glycosylation, acylation, amidation, phosphorylation, prenylation, and carboxylation. The sites of such modifications are usually at identical positions on the proteins produced in insect and mammalian cells (O'Reilly *et al ibid*).

An advantageous feature of baculovirus expression vectors is the potential for very strong expression of a heterologous gene DNA - such as an NOI according to the present invention. The highest expression levels reported using baculovirus expression vectors is 25%-50% of the total cellular protein. Such levels are equivalent to *polh* gene (Polyhedrin) expression and correspond to approximately 1 gram of protein product per 10^9 cells (eg a litre culture).

All heterologous proteins, however, are not produced at the same level as polyhedrin and levels approaching 25% of the total cellular protein have been achieved in a few cases. Most of these cases involved expression of structural genes of other virus families, the products of which are quite stable. Most heterologous proteins are produced at levels ranging from 10 mg to 100 mg per 10^9 cells. Nevertheless, in the cases where different eukaryotic expression systems have been compared, the baculovirus system has usually out-performed other expression systems in overall protein production.

Thus, in one broad aspect, the present invention provides a production facility (e.g. a vat, or even an organism or cell thereof) for producing a quantity of an NOI or the expression product thereof; wherein the facility contains a medium comprising a baculovirus composition which comprises the NOI. Preferably, the baculoviral composition is a composition according to the first aspect of the present invention.

The use of very late promoters (eg the *polh* and *p10* promoters), which are activated and strongly transcribed during then unique occlusion phase of virus replication, provides a clear advantage for baculovirus-based expression systems. Because the occlusion phase is distinct from the late phase encompassing BV formation, expression of the heterologous gene DNA - such as an NOI according to the present invention - interferes minimally with BV production and there is very little selective pressure on the virus to mutate toward heterologous gene deletion or inactivation.

Very late expression may be particularly advantageous for the expression of heterologous genes DNA - such as an NOI according to the present invention - with a deleterious effect on essential cell functions such as cytotoxic gene products. The negative aspect of expressing genes during the very late phase of virus infection is that the post-translational modification capacity of cells appears to decline during the late phase.

The rod-shaped nucleocapsids of baculoviruses can extend to accomodate larger viral DNA genomes and it is likely that a baculovirus vector can accomodate an additional 100 kbp of DNA or more. The number of genes that might be expressed simultaneously using baculovirus vector systems is also likely to be high. To make very large insertions, however. it may be necessary to construct a series of transfer plasmids that allow the building of the insert in successive increments. This limitation has more to do with the fragility of large DNAs *in vitro* rather than the vector system *per se*. Also, if more than three genes are to be expressed, additional transfer plasmids are required.

All characterised very-late genes of baculoviruses are unspliced but efficiently expressed. Thus, the baculovirus system is particularly useful for expressing unspliced cDNA genes. However, the baculovirus expression system does carry out at least some splicing and its usefulness in identifying and obtaining specific gene products from multigene families has been noted.

Thus. in one broad aspect, the present invention provides the use of a baculoviral composition to express an NOI comprising at least one intron. Preferably, the baculoviral

composition is a composition according to the first aspect of the present invention.

Baculovirus vectors are helper-virus independent and therefore relatively simple to use. Constructing a recombinant baculovirus is considerably faster and simpler than constructing a cloned, high-expressing, recombinant eukaryotic cell line.

Although the basic technology for using AcMNPV- and BmNPV- based vectors is similiar, AcNPV- based systems have a number of advantages for most common applications. The cell lines sypporting AcMNPV replicaton are superior in growth characteristics and expression leves than cell lines supporting BmNPV replication. In addition, the range of transfer plasmids and parent viruses available for the AcMNPV-based system is much greater than that for the BmNPV-based system. Furthermore, the use of a genetically modified AcMNPV-based vector is advantageous because it replicates only in insect cells and is able to carry large (greater than 15kb inserts) (Boyce and Butcher1996 PNAS 93: 2348-2352).

The cell lines that are most commonly used with AcNPV-based vectors are the *S podoptera frugiperda* (SF) cell lines because they are well-tested and have excellent growth and handling properties. These cells have been reported to produce some proteins at levels approaching 20% or more of the total cell protein, and it is unlikely that any other generally available cell line will provide more than threefold higher yields of protein than these. In particular, use of the Sf cell lines, such as the SF cell line IPBL-SF-21 AE (Vaughn *et al.*, (1977) In Vitro, 13, 213-217) is preferred. The derivative cell line Sf9 or Sf8 may be preferred. However, other cell lines, such as Tricoplusia ni 368 (Kurstack and Marmorosch, (1976) Invertebrate Tissue Culture Applications in Medicine, Biology and Agriculture. Academic Press, New York, USA) may be employed. These cell lines, as well as other insect cell lines suitable for use in the invention, such as pBAC4x-1are commercially available (e.g. from Stratagene, La Jolla, CA, USA and Novagen).

Although the host speficificity of the AcMNPV-based vectors is supposed to be restricted to arthropods, it has been demonstrated that recombinant AcMNPV virus can infect a variety of mammalian cell lines (Boyce and Butcher 1996 PNAS 93: 2348-2352). Unlike retroviral

and adenoviral vectors which do not allow for exclusive infection of the liver, the genetically modified AcMNPV baculovirus has been shown to demonstrate a strong preference for hepatocytes (Sandig *et al* 1996 Human Gene Ther 7: 1937-1945; Hofmann *et al* 1995 PNAS 92: 10099-10103).

NOIs are generally introduced into baculovirus genomes by allelic replacement. In allelic replacement strategies, the foreign gene is inserted into a transfer plasmid so that it is downstream of the required viral promoter and flanked on both sides by viral sequences that will target the gene and promoter to a particular region in the viral genome. The plasmid and the parental viral DNA are cotransfected into host cells and enzymes in the cells recombine the DNAs. This primarily involves homologous recombination between the regions of the plasmid DNA flanking the foreign gene and their homologous counterparts in the viral genome.

"Late" and "early" promoters are used in baculovirus expression systems. The *polh* and *p10* promoters are examples of strong promoters which are expressed late in infection. The regulation of transciption from these promoters is presently not well understood even though a number of viral gene products that may be important for transcription have been identified (Hasnain et al 1997 Gene 190: 113-118). Promoters that drive gene expression earlier in the infection process are also being considered for nontoxic proteins that are slowly processed in baculovirus-infected cells. The baculovirus expression system also has an excellent track record for expressing genes as nonfusion proteins (that is, for expressing genes using their natural, translation initiation codon and N-terminal sequence) but some proteins are expressed to higher levels as fusions.

It is known that insect baculovirus expression systems have been used for the production of retroviral virus-like particles (VLPs) (for example, see Gheysen *et al.* 1989 Cell 59, 103; Morikawa *et al.*, 1991 Virol. 183, 288; Griffiths *et al.*, 1993 J.Virol. 67, 3191; Sommerfelt *et al.*, 1993 Virol. 192, 298). Such insect baculovirus systems have been used extensively for the analysis of the Gag assembly reaction and a number of the observations made are relevant to the production of gene therapy vectors. Co-expression of the protease and polymerase genes (using a *gag-pol* construct) leads to maturation of the virus particles

to provide all the functional Gag and Pol antigens in the final virus particle (Konvalinka *et al.*, 1995 Eur. J. Biochem. 228, 191; Wagner *et al.*, 1992 Arch. Virol. 127, 117). Also envelope antigens present on the cell surface of VLP-expressing cells are incorporated into VLPs (Yamshchikov *et al.*, 1995 Virol. 214, 50; Garnier et al., 1995 J.Virol. 69, 4060).

However, it is to be noted that VLPs are proteinaceous particles without a viral genome. In this regard, VLPs are non-infectious and lack virus (or other DNA/RNA) required for replicaton. VLPs do not replicate in host cells. Sheep trials have shown that VLPs are more immunogenic than subunit vaccines (viral proteins) or viruses killed by chemical inactivation. In addition, they are more effective at eliciting humoral, cell-mediated and mucosal immunities. VLPs are also safe to produce and handle. The baculovirus vector and host cells used to make VLPs are not derived from a mammalian source. Hence they do not contain mammalian-derived pathogens (Roy, P 1996 Intervirology 39: 62-71). See OBM 10 spec text page 2, lines 20-30 and page 3, lines 1-10).

Also, expression of the single *gag* gene has proven to be all that is required for the formation of retroviral VLPs and the amount of retroviral VLP produced appears directly proportional to the level of expression of the *gag* encoded precursor protein. There are, therefore, no secondary modifications that limit the budding process of the retroviral VLPs and yields of VLP outweigh the levels of baculovirus produced.

WO 95/22617 suggests using a baculovirus system for retroviral vector production but, importantly, it does not indicate any manner in which this might be achieved.

Thus, despite the observations relating to retroviral proteins, and despite of the availability of the data in the literature for several years, a baculovirus expression system has never before now been used for the production of retroviral vectors. One reason for this is that while the protein components of retroviruses had been successfully produced in insect baculovirus systems, it was not expected that the requirements for a functional retroviral vector particle could be met. These requirements include the correct packaging of the vector genome, and an ability of the resulting vector particle to undergo infection of and proviral integration into the target cell. A particular consideration is the need for a vector

genome capable of undergoing accurate reverse transcription from RNA into proviral DNA in the target cell, and capable of producing proviral DNA which can successfully integrate into the target cell genome. This requires that the RNA contains the appropriate priming and other recognition sites required for reverse transcription (Reviewed in Luciw and Leung: The Retroviridae. Ed.J.A.Levy 1992 Plenum) and that the DNA product has the necessary terminal sequences for achieving integration (Reviewed in Luciw and Leung. Op.cit.; Cannon *et al.*, 1996 J. Virol. 70, 8234). In addition the integrated provirus must contain the necessary control elements for gene expression upon subsequent integration.

The results of the present invention are highly surprising because baculovirus expression systems have certain unusual features which make the design of baculovirus vectors for producing retroviral vectors far from straightforward. The elements of a baculovirus promoter that are, in part, required for its efficiency are downstream of the RNA transcription start sites (Posse and Howard, 1987 Nucl. Acids Res. 15, 10233; Rankin *et al*, 1988 Gene 70, 39; Ooi *et al.*, 1989 J.Mol.Biol. 210, 721). This means that for the efficient transcription that is required to provide the increase in vector titre the retroviral vector genome will necessarily have baculovirus sequences at its 5' end. The presence of such non-retroviral sequences is in principle undesirable, given the need for specific retrovirus terminal sequences for reverse transcription and integration, and can be expected to interfere with normal retroviral vector genome function.

As indicated, the present invention overcomes the prior art problems by providing a baculovirus expression system useful for the production of retroviral vectors and/or retroviral particle (which may be capable of acting as a vector).

The retroviral particles produced by or from the composition of the present invention (which may otherwise be expressed as being "retroviral particle vectors") are useful for the delivery of one or more NOIs to cells *in vivo* and *in vitro*, in particular the delivery of therapeutically active NOI(s). One or more selected NOI(s) may be incorporated in the vector genome for expression in the target cell. The NOI(s) may have one or more expression control sequences of their own, or their expression may be controlled by the vector LTRs. For appropriate expression of the NOI(s), a promoter may be included in or

between the LTRs which is preferentially active under certain conditions or in certain cell types. The NOI may be a sense sequence or an antisense sequence. Furthermore, if there is a plurality of NOIs then those NOIs may be sense sequences or antisense sequences or combinations thereof.

In a prefered aspect, the retroviral vector genome of the present invention may generally comprise LTRs at the 5' and 3' ends, one or more NOI(s) (including therapeutically active genes and/or marker genes), or suitable insertion sites for inserting one or more NOI(s). In some cases, there may be present a packaging signal to enable the genome to be packaged into a vector particle in a producer cell. There may even be suitable primer binding sites and integration sites to allow reverse transcription of the vector RNA to DNA, and integration of the proviral DNA into the target cell genome. In a preferred embodiment, the retroviral vector particle has a reverse transcription system (compatible reverse transcription and primer binding sites) and an integration system (compatible integrase and integration sites).

In accordance with the present invention, it is possible to manipulate the viral genome or the retroviral vector nucleotide sequence, so that viral genes are replaced or supplemented with one or more NOIs. The NOI(s) may be any one or more of selection gene(s), marker gene(s) and therapeutic gene(s). Many different selectable markers have been used successfully in retroviral vectors. These are reviewed in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 444) and include, but are not limited to, the bacterial neomycin and hygromycin phosphotransferase genes which confer resistance to G418 and hygromycin respectively; a mutant mouse dihydrofolate reductase gene which confers resistance to methotrexate; the bacterial *gpt* gene which allows cells to grow in medium containing mycophenolic acid, xanthine and aminopterin; the bacterial *hisD* gene which allows cells to grow in medium without histidine but containing histidinol; the multidrug resistance gene (*mdr*) which confers resistance to a variety of drugs; and the bacterial genes which confer resistance to puromycin or phleomycin. All of these markers are dominant selectable and allow chemical selection of most cells expressing these genes.

The composition of the present invention may be useful for *inter alia* medical applications - such as diagnostic or therapeutic applications.

Thus, in accordance with the present invention, the NOI can be a therapeutic gene - in the sense that the gene itself may be capable of eliciting a therapeutic effect or it may code for a product that is capable of eliciting a therapeutic effect.

Non-limiting examples of therapeutic NOIs include genes encoding tumour supressor proteins, cytokines, anti-viral proteins, immunomodulatory molecules, antibodies, engineered immunoglobulin-like molecules, fusion proteins, hormones, membrane proteins, vasoactive proteins or peptides, growth factors, ribozymes, antisense RNA, enzymes, pro-drugs, such as pro-drug activating enzymes, cytotoxic agents, and enzyme inhibitors.

Examples of prodrugs include but are not limited to etoposide phosphate (used with alkaline phosphatase; 5-fluorocytosine (with cytosine deaminase); Doxorubin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase); Para-N-bis (2-chloroethyl)aminobenzoyl glutamate (with Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with B-lactamase); SR4233 (with p450 reductase); Ganciclovir (with HSV thymidine kinase); mustard pro-drugs with nitroreductase and cyclophosphamide or ifosfamide (with cytochrome p450).

Diseases which may be treated include, but are not limited to cancer, heart disease, stroke, neurodegenerative disease, arthritis, viral infection, bacterial infection, parasitic infection, diseases of the immune system, viral infection, tumours, blood clotting disorders, and genetic diseases - such as chronic granulomatosis, Lesch-Nyhan sysndrome, Parkinson's disease, empysema, phenylketonuria, sickle cell anaemia, α-thalasemia, β-thalasemia, Gaucher disease.

Target cells for gene therapy using retroviral vectors include but are not limited to haematopoietic cells, (including monocytes, macrophages, lymphocytes, granulocytes, or progenitor cells of any of these); endothelial cells, tumour cells, stromal cells, astrocytes,

or glial cells, muscle cells, epithelial cells, neurons, fibroblasts, hepatocyte. astrocyte, and lung cells.

Within the retroviral vector of the present invention, the one or more NOIs can be under the transcriptional control of the viral LTRs. Alternatively, a combination of enhancer-promoter elements can be present in order to achieve higher levels of expression. The promoter-enhancer elements are preferably strongly active or capable of being strongly induced in the target cells. An example of a strongly active promoter-enhancer combination is a human cytomegalovirus (HCMV) major intermediate early (MIE) promoter/enhancer combination. The promoter-enhancer combination may be tissue or temporally restricted in their activity. Examples of a suitable tissue restricted promoter-enhancer combinations are those which are highly active in tumour cells such as a promoter-enhancer combination from a MUC1 gene or a CEA gene.

Hypoxia or ischaemia regulatable expression may also be particularly useful under certain circumstances. Hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1) (Wang and Semenza 1993 PNAS. (USA) 90: 430) which bind to cognate DNA recognition sites, the hypoxia responsive elements (HREs) on various gene promoters. A multimeric form of HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene has been used to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Firth *et al* 1994, PNAS 91(14): 6496-6500; Dachs *et al* 1997 Nature Med. 5: 515). Alternatively, the fact that glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene DNA - such as an NOI according to the present invention - expression especially in tumours. A truncated 632 base pair sequence of the grp 78 gene promoter, known to be activated specifically by glucose deprivation, has been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit *et al* 1995 Cancer Res. 55: 1660.).

The retroviral vector genomes of the present invention for subsequent use in gene therapy

preferably contain the minimum retroviral material necessary to function efficiently as vectors. The purpose of this is to allow space for the incorporation of the NOI(s), and for safety reasons. Retroviral vector genomes are preferably replication defective due to the absence of functional genes encoding one or more of the structural (or packaging) components encoded by the *gag-pol* and *env* genes. The absent components required for particle production are provided in *trans* in the producer cell. The absence of virus structural components in the genome also means that undesirable immune responses generated against virus proteins expressed in the target cell are reduced or avoided. Furthermore, possible reconstruction of infectious viral particles is preferably avoided where *in vivo* use is contemplated. Therefore, the viral structural components are preferably excluded from the genome as far as possible, in order to reduce the chance of any successful recombination.

The retroviral vector particles of the present invention are typically generated in a suitable producer cell.

Thus, the present invention also relates to a producer cell comprising the composition of the present invention.

A producer cell may be a packaging cell containing the virus structural genes, normally integrated into its genome. The packaging cell is then transfected with a nucleic acid encoding the vector genome, for the production of infective, replication defective vector particles. Alternatively the producer cell may be co-transfected with nucleic acid sequences encoding the vector genome and the structural components, and/or with the nucleic acid sequences present on one or more expression vectors such as plasmids, adenovirus vectors, herpes viral vectors, vaccinia viral vectors, or any method known to deliver functional DNA into target cells.

Suitable producer cells include insect cells and mammalian cells, but can be other suitable cells. Preferably, the producer cells are insect cells.

The present invention also provides a pharmaceutical composition for treating an individual

by gene therapy, wherein the composition comprises a therapeutically effective amount of the composition according to the present invention or a viral particle produced by or from same. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The present invention provides in one aspect an expression vector comprising a polynucleotide sequence which encodes a retroviral vector genome having a 5' and a 3' end, which retroviral vector genome is capable of being expressed and packaged into a retroviral vector particle in a baculovirus expression system. Hence, the 5' and 3' ends function in reverse transcription and integration.

Three strategies are described herein for overcoming the problem of the baculovirus promoter having components upstream and downstream of the RNA transcription start site. These strategies are illustrated in Figures 1 to 3.

In a first strategy (see Figure 1), the downstream component of the promoter is incorporated into the R region at the upstream end of the sequence encoding the retroviral vector genome (referred to as the 5' R region). This places the RNA transcription start site directly in front of the sequence encoding the retroviral vector genome. Provided that a counterpart to the downstream component of the promoter is also incorporated into the R region at the downstream end of the sequence encoding the retroviral vector genome (referred to as the 3' R region), so that the two R regions are identical or sufficiently similar for conversion of the vector RNA genome to the DNA provirus, the vector genome will function correctly.

In a second strategy (see Figure 2), a sequence capable of being cleaved in the RNA transcript is included in the vector between the baculovirus promoter and the sequence encoding the retroviral vector genome. The downstream component of the baculovirus promoter can thus be cleaved off once the primary RNA transcript has been produced. Preferably, a cleavage site is present immediately adjacent the sequence encoding the retroviral vector genome, so that suitable vector genome ends are produced. Ribozymes are RNA enzymes which can perform such a function and can be engineered into DNA constructs. The structure and function of suitable ribozymes has been well-studied (e.g. Cech 1992 Curr. Op. Struct. Biol. 2, 605). Examples include hammerhead, hairpin and hepatitis delta antigenomic ribozymes. Inclusion of a ribozyme sequence as the RNA cleavage sequence, with a cleavage site at the end of the sequence encoding the retroviral vector genome, can be used to give end-perfect RNA for all transcripts. Each transcribed molecule will contain the ribozyme sequence, and a single cleavage event in *cis* will be all that is required for each ribozyme for correct template production.

In a third strategy (see Figure 3), a non-baculovirus promoter is employed. Examples of suitable promoters include the T7 phage promoter and the sp6 salmonella phage promoter.

For the T7 promoter, the terminal G residue of the 5' R region could be positioned precisely at the transcription start site of the T7 promoter, to give an authentic 5' end residue in the retroviral vector genome. A source of T7 polymerase would need to be provided, for example by a recombinant baculovirus expressing T7 polymerase (Polkinghorn and Roy 1995 Nucl. Acids Res. 23, 188).

Preferably, the expression vector encoding the retroviral vector genome is a baculovirus expression vector, that is, a vector based on a recombinant baculovirus genome. Virus vectors are in general easier to handle than non-viral vectors. For example, the transfer of a virus vector into a cell is more reliably performed than transfection of a cell with a DNA plasmid. However, the expression vector may alternatively be a non-baculovirus expression vector, or a non-viral expression vector, such as a DNA plasmid.

In an expression vector according to any of the three strategies described above, there may be a RNA cleavage sequence located downstream of the sequence encoding the retroviral vector, to ensure correct termination of the RNA transcript and a correct 3' end for the vector genome. Preferably, the cleavage sequence has a cleavage site immediately adjacent the sequence encoding the retroviral vector genome, for cleaving at the 3' end of the genome. Ribozymes can provide suitable RNA cleavage sequences, as already discussed herein. Ribozymes may have a cleavage site either 5' or 3' to the ribozyme sequence. Thus, where there is a ribozyme for each end of the vector genome, these will usually be different. For example, the hammerhead ribozyme cleaves to the right of itself and will thus be suitable for the 5' end of the vector genome, while the hairpin ribozyme cleaves to the left and will be suitable for the 3' end of the vector genome.

The invention provides in further aspects, a retroviral vector particle production system comprising an expression vector as described herein, in an insect cell, and retroviral vector particles produced by such systems.

For the production of retroviral vector particles, packaging components will need to be provided. These will usually be *gag-pol* and *env*, which may be provided on one or more suitable expression vectors. The expression vector or vectors will need to be capable of

expressing the packaging components in a baculovirus expression system. Thus, the packaging components will be under the expression control of one or more baculovirus promoters. The expression vector or vectors carrying the packaging components are preferably baculovirus expression vectors. Alternatively they may be non-baculovirus or non-viral expression vectors, such as plasmids. Some or all of the packaging components may be provided on the same expression vector as the retroviral vector genome.

Suitable baculovirus systems for use in the invention are easily available and well known in the art. Baculovirus expression vectors are described in detail in O'Reilly *et al.* 1994 (Op. cit). One suitable baculovirus for use in a recombinant baculovirus vector is the well-studied *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A commercially available recombinant baculovirus vector such as pBAC4 x- 1 (Novagen) may be used. pBAC4x-1 contains four insertion sites, and could be engineered to accommodate nucleic acid sequences encoding the retroviral vector genome and the packaging components. Similarly, suitable baculovirus promoters for use in the invention are well known in the art. Commonly-used baculovirus promoters are the polyhedrin and *p10* promoters. The DNA sequence for a polyhedrin promoter is shown in Figure 18. The present invention also includes mutants, variants, homologues or fragments of that sequence.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the preferred enzyme of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for or is capable of coding for a promoter, preferably being at least as biologically active as the sequence shown as in Figure 18. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for or is capable of coding for a promoter. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 1. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown in Figure 18.

In particular, the term "homology" as used herein may be equated with the term "identity".

Relative sequence homology (i.e. sequence identity) can be determined by commercially available computer programs that can calculate % homology between two or more sequences. A typical example of such a computer program is CLUSTAL.

The terms "variant", "homologue" or "fragment" are synonymous with allelic variations of the sequences.

The term "variant" also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequence presented herein. Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (e.g. 65°C and 0.1xSSC {1x SSC = 0.15 M NaCl, 0.015 Na_3 citrate pH 7.0}) to the nucleotide sequence presented herein.

The present invention also covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention (including complementary sequences of those presented herein). In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC) to the nucleotide sequence presented herein (including complementary sequences of those presented herein).

In accordance with the present invention, other baculovirus promoters - as well as mutants, variants, homologues or fragments of those promoters - may also be used.

Insect cell lines for use with the expression vectors according to the invention are also readily available. One example is the sf8 insect cell line. The insect cells used need to be compatible with the particular expression vector chosen. The insect cells need to support replication of the expression vector encoding the retroviral vector genome, and to support expression of the retroviral vector genome. Certain glycosylation-deficient cell lines may be particularly suitable, because of differences in glycosylation between mammalian and insect systems. The glycosylation differences are not expected to present a problem; examples of clinical use such as clinical trials with HIV gp120 produced in insect cells, indicate that any differences in secondary modifications are not significant.

The types of retrovirus which can be used in the invention are not limited to any specific retrovirus. Oncoretroviruses such as the murine type-C virus MLV, or lentiviruses such as HIV, or other well-known retroviruses such as ASLV, SNV and RSV could be used. The invention is particularly useful for retroviral vectors based on lentiviruses, as these have been very difficult to produce in high titres. Titres of lentiviral vectors are commonly two orders of magnitude lower than the murine vectors such as MLV (e.g. Naldini *et al.* 1996 Science 272, 263). In addition, because the retroviral RNA can be so efficiently produced in baculovirus expression systems, it may be possible to omit from the retroviral vector genome lentiviral elements such as HIV Rev and RRE (Gheysen *et al.* 1989 Op. cit.). It is advantageous to avoid unnecessary retroviral elements in retroviral vectors, particularly when using HIV, or other lentiviruses, because of the possible adverse effects of these elements.

The present invention will now be described by way of example only, and with reference to the following Figures - in which:

Figure 1 is a schematic diagram;
Figure 2 is a schematic diagram;
Figure 3 is a schematic diagram;
Figure 4 is a schematic diagram;
Figure 5 is a schematic diagram;
Figure 6 is a schematic diagram;
Figure 7 is a schematic diagram;
Figure 8 is a schematic diagram;
Figure 9 is a schematic diagram;
Figure 10 is a schematic diagram;
Figure 11 is a schematic diagram;
Figure 12 is a schematic diagram;
Figure 13 is a schematic diagram;
Figure 14 is a schematic diagram;
Figure 15 is a schematic diagram;

Figure 16 is a schematic diagram;

Figure 17 is a schematic diagram;

Figure 18 is a schematic diagram;

Figure 19 is a schematic diagram;

Figure 20 is a schematic diagram;

Figure 21 is a schematic diagram;

Figure 22 is a schematic diagram;

Figure 23 is a schematic diagram;

Figure 24 is a schematic diagram;

Figure 25 is a schematic diagram;

Figure 26 is a schematic diagram;

Figure 27 is a schematic diagram;

Figure 28 is a schematic diagram; and

Figure 29 is a schematic diagram.

Figures 1, 2, 3 and 18 have been referred to in the above text.

EXAMPLE 1

EXPRESSION OF MLV gag-pol AND ENVELOPE GENES IN A BACULOVIRUS EXPRESSION SYSTEM

The strategy is to first insert the envelope sequences into pBAC4x-1 (Novagen), followed by insertion of the MLV gag-pol sequences, which will first be reconstructed in pBluescript (Stratagene).

a) Env expression (MLV ecotropic or VSV G)

For the MLV ecotropic envelope sequence, the EcoRI fragment which contains the entire env sequence from pHIT123 (Soneoka *et al* Op.Cit) is isolated. For the VSV-G sequence, an EcoRI fragment from pHCMV-G (Yee *et al.*, 1994 PNAS 91, 9564) is also isolated. These fragments are then inserted into the EcoRI site of pBAC4x-1 (Novagen) (Figure 4) to

produce pBAC4-env-e and pBAC4-env-v respectively. Any viral envelope gene could be inserted into this vector in similar ways using technology known in the field of recombinant DNA. Envelope genes might include those from any retrovirus or any other virus capable of pseudotyping or otherwise being incorporated into particles produced from retroviral *gag-pol* genes.

b) Reconstruction of MLV gag-pol in pBluescript (Figure 5)

5' and 3' fragments from pgagpolgpt (Markowitz *et al.*, 1988 J.Virol. 62, 1120) are produced by PCR (see primer list). The 5' fragment is amplified up to the *Xho*I site and it creates *Kpn*I and *Not*I sites at the beginning of the coding sequence, to generate a 940bp fragment. The 3' fragment is amplified from the *Sph*I site of the *gag-pol* sequence to the end of the *pol* coding sequence, generating a 700bp fragment. *EcoR*I sites are created at both ends of this fragment and a NotI site only at the 3' end, so that when the entire *gag-pol* sequence is reconstructed, it can be excised by digestion with *Not*I and inserted into pBAC4x-1 (Novagen) at the *Not*I site.

The 5' fragment is inserted into pBluescript at the KpnI/XhoI site, and the 3' fragment into the EcoRI site.

The *Xho*I-*Sph*I (30bp) fragment is then excised from the above plasmid and replaced with an *Xho*I-*Sph*I fragment (3580bp) from pgagpolgpt to reconstruct the entire gag-pol sequence in pBluescript to create pBluescript-gagpol.

c) **Insertion of MLV gag-pol sequences into pBAC4-env to create pBAC4-gagpolenv (Figure 6)**

The *Not*I fragment containing the gag-pol sequences from pBluescript-gagpol is isolated and inserted into the *Not*I site of pBAC4-env-e and -v to create pBAC4mgagpol-env-e and pBAC4mgagpol-env-v for the MLV envelope version and the VSV-G version respectively.

These plasmids are used as baculovirus transfer vectors using standard methods as

described in O'Reilly *et al.* (Op.Cit.). The resulting virus preparations are designated bBAC4mgagpol-env-e and bBAC4mgagpol-env-v respectively. When these viruses are used to infect insect cells such as sf8 cells large numbers of VLPs are produced.

EXAMPLE 2

EXPRESSION OF AN MLV-BASED VECTOR GENOME USING THE T7 STRATEGY (Figures 3 and 7)

In this example the vector genome is expressed from a plasmid that will be used in a transient expression system. The genome could be expressed from the same recombinant baculovirus as the gag-pol and env genes.

The MLV genome derived from pLZSN (Adams *et al.*, 1991 J.Virol. 65, 4985) is inserted into pEc-Hd (Polkinghorn 1996 D.Phil. Thesis, University of Oxford) at the *EagI*/*SmaI* sites. The genome that is placed into this vector has the following structure:

The 5' and 3' ends of the genome are amplified by PCR so that the T7 promoter sequence is placed immediately upstream of the 5' R region. The 3' end is amplified up to the end of the 3' R sequence, generating a blunt-ended product so that it will be precisely fused to the hepatitis delta antigenomic ribozyme motif. A proof-reading polymerase is used for PCR to generate the blunt-ended product.

The 5' end of the genome is amplified up to the *EagI* site in the packaging signal by PCR. An additional EagI site is created at the very 5' end for insertion into the EagI site of pEc-Hd. The 3' end of the genome is amplified from the *SmaI* site in the 3' R sequence, up to the very end of the R sequence.

The 3' fragment is inserted first into pEc-Hd at the *SmaI* site (a SmaI site will not be regenerated at the very 3' end), then the 5' fragment is inserted into the *EagI* site.

The *SmaI* fragment from above is excised and replaced with the SmaI fragment (5850bp) from pLZSN to create pEc-Hd-LZSN. The same expression cassette could be assembled

into a baculovirus transfer vector with the gag-pol and env sequences by standard procedures.

Plasmid pEc-Hd-LZSN can be used to transfect sf21 cells that are coinfected with bBAC4mgagpol-env-e or bBAC4mgagpol-env-v and Bac-T7 which expresses the T7 polymerase. In these cells all the components of a retroviral vector are expressed and so the production of functional vectors can be tested. High titres of functional vectors are produced that transfer the lacZ gene to target cells.

Primers for MLV gag-pol, env and VSV-G constructions:

To amplify the MLV gag-pol sequences:

5' fragment

Forward (35MER NOTFOR)

5'-CGG *GGTACC GCGGCCGC* ATGGGCCAGACTGTTACC-3

Kpn NotI

Reverse (22MER XHOREV)

5'-GGGCG *CTCGAG* GGGAAAAGCGG-3'

XhoI

3' fragment

Forward (27MER SPHFOR)

5'-CCG *GAATTC GCATGC* CTCAGGTATTGG-3

EcoRI SphI

Reverse (33MER NOTREV)

5'-TTT *GAATTC GCGGCCGC* TTAGGGGGCCTCGCGG-3'

EcoRI NotI

To amplify the genome (pLZSN):

5' fragment

Forward (44MER EAGFOR)

5'-GCG *CGGCCG* TAATACGACTCACTATA GCGCCAGTCTTCCGATAG-3'

EagI T7 promoter

Reverse (18MER EAGREV)

5'-TTG *CGGCCG* GGTGTTCAG-3'

EagI

3' fragment

Forward (20MER SMAFOR)

5'-TCG *CCCGGG* TACCCGTGTAT-3'

SmaI

Reverse (22MER SMAREV)

5'-TGCAACTGCAAGAGGGTTTATT-3'

EXAMPLE 3

EXPRESSION OF HIV gag-pol and VSV-G IN A BACULOVIRUS EXPRESSION SYSTEM (Figure 8).

The source of the HIV gag-pol sequences is plasmid pRV664. This is a pWI3 (Kim *et al.*, 1989 J.Virol. 63, 3708) derived gagpol vif expression plasmid. The RRE of pWI3 (Genbank Accession number: U26942) is inserted by blunt-ending the *Sty* I/*Sty* I fragment (7720-8050) into Sma I cut pBluescript KS+ (Stratagene) thereby creating pBSRRE. The *Nar* I/*Eco RI* fragment of pWI3 (637-5743) is inserted into pBSRRE cut with *Cla* I and *Eco RI* to create pBSGPRRE1. The *Xho* I/*Not* I fragment (containing gagpol and RRE) is inserted into the expression plasmid pCI-Neo to create pGR-PRE1.

A 5.6kb *Xho*I-*Not*I fragment containing the HIV-1 gag-pol sequences from pRV664 is isolated and blunt ended with the Klenow fragment of DNA polymerase I. This is then inserted into the SmaI site of pBAC4-env-v to produce pBAC4hgagpol-env-v. This is used in the same way as the plasmids described in Example 1 were used.

EXAMPLE 4

EXPRESSION OF AN HIV-BASED VECTOR GENOME USING THE T7 STRATEGY (Figures 3 and 9 - 12)

The genome from pH4nZ has the structure as shown in Figure 9. It is produced as follows. HIVdge is made from HIVgpt (Page *et al.*, 1990 J.Virol. 64, 5270) by blunt-ending the *Cla I* site (829) to create a frameshift mutation. HIVdge is then cut with *Bgl* II and *Pst* I (473-1414) and inserted into pTIN406 (Cannon et al. Op. Cit). pTIN406 has an LTR structure of CMV, R (HIV) and U5 (MLV). This creates a hybrid LTR containing CMV, and R, U5 from HIV called pBS5'. To provide the plasmid with Rev and RRE the *Eco* RI/*Xho* I fragment (5743 ...97) is cut from HIVdge1.2 which is a HIVdge derivative containing a deletion from *Nde* I to *Bgl* II (6403-7621) and is inserting into pBS5' to create pBS5'R. The 3' LTR is provided by inserting the *Not* I/*Xho I* fragment of pBS3' into pBS5'R creating pH2. pBS3' is created by a three way ligation of the *Xho* I/*Hind* III fragment of

pWI3, the *Hind* III/*Kpn* I fragment of pTIN408 (Cannon *et al.* Op. Cit.) and pBluescript KS+ cut with *Xho* I/*Kpn* I. A CMV promoter fragment (*Sal* I/*Xho* I) from pSPCMV is inserted into the unique *Xho* I site of pH2 making pH2CMV. pSPCMV is created by inserting the *Pst* I/*Hind* III fragment from pLNCX (Genbank Accession number: M28246) into pSP72 (Promega). The β-galactosidase gene is inserted from pTIN414 (Cannon *et al.* Op.Cit.) into pSP72 (*Xho* I/*Sph* I) to make pSPlacZ. A *Xho* I/*Sal* I digest of pSPlacZ gives the β-galactosidase coding region which is inserted into pH2-CMV to give pH3Z. pH4Z is constructed to create tat-deficient vector. The first 50 bp of the tat-coding region is removed by replacing *EcoR*I (5743)I-*Spe*I fragment in pH3 with *EcoR*I (5881)-*Spe*I PCR product amplified using PCR primers

DELT5 (5'-CGTGAATTCGCCTAAAACTGCTTGTACCA-3') and

DELT3 (5'-GAACTAATGACCCCGTAATTG-3')

to create pH4.

The *Nsi* I/*Spe* I fragment from pH4 is inserted into pH3Z to generate pH4Z.

Two PCR reactions are performed to amplify the 5' and 3' ends of the genome for baculovirus expression. Subsequently, the entire genome is reconstructed by inserting the missing intervening sequences. A PCR reaction is performed to amplify the 3' sequences of the genome. The *Sca*I site in the 3' U3 sequence is changed to a SmaI site and amplified to the very end of the R sequence. This change creates a 3 base pair mutation but should not affect integration. A proof-reading polymerase is used to create the 900bp blunt-ended product. This product is then inserted into the *Sma*I site of pEc-Hd (Figure 10). This destroys the *Sma*I site at the junction of the R sequence and pEc-Hd.

The 5' sequences are first amplified from the beginning of the R sequence up to the first *EcoR*I sequence located in the packaging signal. This produces a 900bp fragment. An EagI site is placed at both ends of the product and a *Xho*I site at the very 5' end of the fragment. This product is inserted into the *Xho*I-*EcoR*I site of pBluescript KS (Stratagene) (Figure 11).

The sequences spanning the *EcoR*I site and the *Spe*I site (in the CMV promoter) are isolated from pH4nZ and inserted into the *EcoR*I-*Spe*I site of the above plasmid (Figure 11).

The EagI-SpeI fragment containing the genome sequences is then isolated from pBluescript KS and inserted into the *Eag*I-*Spe*I sites of pEc-Hd (Figure 12).

The entire genome is finally reconstituted by insertion of the *Spe*I-*Sca*I fragment (3.9kb) isolated from pH4nZ into the *Spe*I-*Sma*I site of pEc-Hd (Figure 12) to produce pEc-Hd-

H4nZ. This plasmid can be used in a similar way to pEc-Hd-LZSN but with a baculovirus vector derived from pBAC4hgagpol-env-v to produce HIV-based retroviral vectors at very high titre.

EXAMPLE 5

EXPRESSION OF EIAV gag-pol AND VSV-G IN A BACULOVIRUS SYSTEM (Figure 13)

The starting molecule is pSPEIAV19 (AC:U01866) which is a proviral clone of EIAV. The envelope encoding region is disrupted by cutting pSPEIAV19 with *Hind* III (5835/6571) within the envelope region and self ligating to produce pSPEIAV19dH. This creates an envelope minus proviral clone. pSPEIAV19dH is then cut with *Mlu* I (216/8124) and the resulting fragment inserted into pCI-Neo (Promega) cut with *Mlu* I (216) to make pONY3.

The *MluI* fragment from pONY3 containing the EIAV gag-pol, tat and rev coding sequences is isolated and blunt ended with the Klenow fragment of DNA polymerase I. This is then inserted into pBAC4-VSVenv at the *SmaI* site (blunt-ended) to produce pBAC4egagpol-env-v. This plasmid is used in a similar way to that described in Example 1 to produce a recombinant baculovirus that will produce high levels of EIAV VLPs.

EXAMPLE 6

EXPRESSION OF AN EIAV-BASED VECTOR GENOME USING THE T7 STRATEGY (Figures 3 and 14-17)

The genome of plasmid pONY2.1nlslacZ has the structure as shown in Figure 14. This is constructed as follows. The 5' LTR of EIAV clone pSPEIAV19 is PCR amplified using *pfu* polymerase with the following primers:

5'GCATGGACCTGTGGGGTTTTTATGAGG

3' GCATGAGCTCTGTAGGATCTCGAACAGAC

The amplified fragment is blunt ended by 5' overhang fill-in and is inserted into pBluescript II KS+ which has been cut with *Bss* HII and blunt ended by 3' overhang removal. This construct is called pONY1 and the orientation is 5' to 3' in relation to β-galactosidase of pBluescript II KS+. Sequencing of pONY1 revealed no mutations. Plasmid pSPEIAV19dH is cut with *Mlu* I (216/8124) and the fragment is inserted into pONY1 *Mlu* I cut (216) to make pONY2. A *Bss* HII digest (619/792) of pBluescript II KS+ is carried out to obtain the multiple cloning site. This is blunt ended by 5' overhang fill-in and ligated to pONY2 cut with *Bgl* II and *Nco* I (1901/4949) and blunt ended by 5' overhang fill-in. The orientation is 3' to 5' in relation to the EIAV sequence. This is called pONY2.1 Plasmid pSPCMV is created by inserting the PstI/HindIII fragment from pLNCX (Genbank Accession number: M28246) into pSP72 (Promega). The β-galactosidase gene is inserted from pTIN414 (Cannon *et al.* Op.Cit.) into pSP72 cut with *Xho* I and *Sph* I to make pSPlacZ. The 5' end to the β-galactosidase gene is replaced by the SV40 T antigen nuclear localisation signal from pAD.RSVBgal (Bloggs *et al.*, 1992 J.Clin.Invest. 90, 626). pAD.RSVbgal is cut with *Xho* I/ *Cla* I and the fragment inserted into *Xho* I/ *Cla* I cut pSPlacZ to make pSPnlslacZ. The *Pst* I fragment containing the CMV promoter driving the lacZ gene from pSPnlslacZ is inserted into the *Pst* I site of pONY2.1 in the 5' to 3' orientation of EIAV. This is designated pONY2.1nlslacZ .

Two PCR reactions are then performed using pONY2.1nlslacZ as template to amplify the 5' and 3' ends of the genome for the baculovirus expression cassette. Subsequently, the entire genome is reconstructed by inserting the missing intervening sequences.

The 3' PCR product is amplified from the SmaI site in the env sequence to the very end of the R sequence using a proof-reading polymerase to produce blunt-ended products. The product is then inserted into the *Sma*I site of pEc-Hd. The very 3' end loses the *Sma*I site. (Figure 15).

The *Sma*I fragment containing the CMVnlslacZ sequences is isolated from pONY2.1nlslacZ and inserted into the *Sma*I site (Figure 16).

The 5' PCR product is amplified up to the *Eag*I site in the MCS and an *Eag*I site is added at the 5' end. This product is then inserted into the *Eag*I site to reconstitute the pONY genome in pEc-Hd (Figure 17) thereby creating pEc-Hd-ONY2.1nlslacZ. This can be used in conjunction with pBAC4egagpol-env-v to produce high titres of EIAV-based vectors in a similar way to that described in Examples 2 and 4.

EXAMPLE 7

EXPRESSION OF AN MLV-BASED VECTOR GENOME USING THE R-REGION REPLACEMENT STRATEGY (FIGURES 1, 18 and 19).

The plasmid containing the LZSN retroviral vector genome expressed from the polyhedrin promoter (O'Reilly *et al.* Op.Cit.) is called pBHLZSN (Figure 19). This plasmid is constructed as follows: The starting template for a series of PCR reactions is pHIT111 (Soneoka *et al.*, 1996 Op.Cit.). The 5'LTR containing the polyhedrin promoter is created using PCR primers in reaction A to produce a PCR product shown as PCR:A (Figure 19). A second reaction (B) creates a fragment that overlaps with PCR:A. Reaction C then produces a combined fragment that is cleaved with SalI and *Hind*III. The resulting fragment is then inserted into pBluescriptKS+ to produce plasmid pBHR (Figure 18). In order to construct the 3' LTR with the appropriate component of the polyhedrin promoter within the newly constructed R region PCR reactions D and E (Figure 19) are carried out. The products of these reactions are then used to produce a combination PCR product (PCR:F) which is cut with *Xba*I and *Not*I and the resulting fragment is inserted into pBHR to produce pBHU3HR (Figure 19). The final stage is to then insert the internal region of LZSN from pHIT111 by cutting pHIT111 with *Spe*I and *Xba*I and inserting the resulting fragment into pBHU3HR cut with the same enzymes (Figure 19).

Primers used for PCR reactions for construction of pBHLZSN

A.5' Primer (polyhA5)-TACT GTCGAC ATA ACC ATC TCG CAA ATA AAT (Underline = Sal I); 3' Primer (PolyhA3)-CAG TCT ATC GGA AGA CTG GCG CT ATT TAT AGG TTT TTT TAT

B. 5' Primer (polyhB5)- ATA AAA AAA CCT ATA AAT AGC GC CAG TCT TCC GAT AGA CTG;3' Primer (polyhB3)- GCTA AAGCTT TCC GCC AGA TAC AGA GCT (Underline = Hind III)

C.Use primer polyhA5 and PolyhB3 with PCR products A and B to obtain the 5' LTR hybrid (polyderin.R)

D. 5' Primer (polyhD5)- AGTT TCTAGA GAA CCA TCA GAT GTT TCC AGG (Underline = Xba I); 3' Primer (PolyhD3)- TAC AAA ACT GTT ACG AAA ACA GTA AAA TAC TT C CCG AGT GAG GGG TTG TGG

E. 5' Primer (polyhE5)- TTC GTA ACA GTT TTG TAA TAA AAA AAC CTA TAA ATA GCG CCA GTC CTC CGA TTG; 3' Primer (polyhE3)- GATC GCGGCCGC AAT GAA AGA CCC CCG CTG (Underline = Not I)

F. Use primer polyhD5 and PolyhE3 with PCR products D and E to obtain the 3' LTR hybrid (U3.Polyherin.R)

EXAMPLE 8.

EXPRESSION OF AN EIAV-BASED VECTOR GENOME USING THE R-REGION REPLACEMENT STRATEGY.

The plasmid containing the pONY2.1nlslacZ retroviral vector genome expressed from the polyhedrin promoter with appropriate R=-region replacements is called pBONY2.1nlslacZ. This plasmid is constructed as follows: The starting template for a series of PCR reactions is pONY2.1nlslacZ (see Example 6 and GB patent application number 9727135.7 and GB patent application number 9711578.6). The 5' LTR containing the polyhedrin promoter is created using PCR primers in reaction AE to produce a PCR product shown as PCR:AE. A second reaction (BE) creates a fragment that overlaps with PCR:AE. Reaction CE then produces a combined fragment that is cleaved with Sal I and Hind III. The resulting fragment is then inserted into pBluescript KS+ to produce plasmid pBEHR. In order to

constuct the 3'LTR with the appropriate component of the polyhedrin promoter within the newly constructed R region PCR reactions DE and EE are carried out. The products of these reactions are then used to produce a combination PCR product (PCR:FE) which is cut with *Xba* I and *Not* I and the resulting fragment is inserted into pBEHR to produce pBEHU3HR. The final stage is to then insert the internal region of pONY2.1nlslacZ by cutting it with *Nar* I and *Nsp* V and inserting the resulting fragment into pBEHU3HR cut with the same enzymes (see the commentary below and Figure 20). Plasmid pBONY2.1nlslacZ can be used in conjunction with pBAC4egagpol-env-v to produce high titres of EIAV-based vectors. (Reference may also be made to Figure 21).

Construction of pONY2.1nlsLacZ

A.

5' Primer (polyhAE5)

TACT GTCGAC ATA ACC ATC TCG CAA ATA AAT (Underline = Sal I)

3' Primer (PolyhAE3)

AGA CCG CAG AAT CTG AGT GCCC T ATT TAT AGG TTT TTT TAT

This PCR product will give the polyhedrin promoter with part of R

B.

5' Primer (polyhBE5)

ATA AAA AAA CCT ATA AAT AGG CCA CTC AGA TTC TGC GGT CTG

3' Primer (polyhBE3)

ACTG AAGCTT CAG GTC CCT GTT CG GGCGCC A ACT G (Bold = *Hind* III, Underline = Nar I)

This PCR will give part of polyhedrin with R.

C.

Use primer polyhAE5 and PolyhBE3 with PCR products AE and BE to obtain the 5' LTR hybrid (polyderin.R)

Insert into pBluescrip KS+ II by cutting with *Sal* I and *Hind* III called pBEHR.

3' LTR (U3.polyhedrin.R.U5).

D. Within U3 to R but including Polyhedrin promoter

5' Primer (polyhDE5)

CTG TCTAGA A GA TTCGAA GCG AAG GAGGAAA C... (Underline = *Xba* I. bold *Nsp*V)

3' Primer (PolyhDE3)

TAC AAA ACT GTT ACG AAA ACA GTA AAA TAC TT ATTGTCAGAATACAAGCACT

This PCR will give U3 with part of polyhedrin promoter.

E. R TO U5 with polyhedrin promoter

5' Primer (polyhEE5)

TTCGTA ACA GTT TTG TAA TAA AAA AAC CTA TAA ATA GGCACTCAGATTCTGCGGTC

3' Primer (polyhEE3)

GATC GCGGCCGC CTGTAGGATCTCGAACAGACAAAC (Underline = *Not* I)

This PCR will give part of polyhedrin promoter with R.

F.

Use primer polyhDE5 and PolyhEE3 with PCR products DE and EE to obtain the 3' LTR hybrid (U3.Polyhedrin.R)

Insert into pBEHR by cutting wtih *Xba* I and *Not* I called pBEHRU3HR

G.

Cut pONY2.1nlslacZ with *Nar* I and *Nsp* V to obtain a 7.8 kb fragment and insert into pBEHRU3HR via *Nar* I and *Nsp* V to create pBEHONYnlslacZ.

EXAMPLE 9.

EXPRESSION OF AN HIV-BASED VECTOR GENOME USING THE R-REGION REPLACEMENT STRATEGY

The plasmid containing the pH4Z retroviral vector genome expressed from the polyhedrin promoter is called pBH4Z. This plasmid is constructed as follows: The starting template for a series of PCR reactions is pH4Z (see PCT/GB 97/02857 and GB patent application number: 9711578.6). The 5' LTR containing the polyhedrin promoter is created using PCR primers in reaction AH to produce a PCR product shown as PCR:AH. A second reaction (BH) creates a fragment that overlaps with PCR;AH. Reaction C then produces a combined fragment that is cleaved with *Sal* I and *Hind* III. The resulting fragment is then inserted into pBluescript KS+ to produce plasmid pBHHR. In order to constuct the 3'LTR with the

appropriate component of the polyhedrin promoter within the newly constructed R region PCR reactions DH and EH are carried out. The products of these reactions are then used to produce a combination PCR product (PCR:FH) which is cut with *Xba* I and *Not* I and the resulting fragment is inserted into pBHHR to produce pBHHU3HR. The final stage is to then insert the internal region of pH4Z by cutting it with *Nar* I and *Sph* I and inserting the resulting fragment into pBHHU3HR cut with the same enzymes (see the commentary below and Figure 22). Plasmid pBH4Z can be used in conjunction with pBAC4hgagpol-env-v to produce HIV-based vectors at high titres. (Reference may also be made to Figure 23.)

Construction of pBH4Z

A.

5' Primer (polyhAH5)

TACT GTCGAC ATA ACC ATC TCG CAA ATA AAT (Underline = *Sal* I)

3' Primer (PolyhAH3)

AGA CCG CAG AAT CTG AGT GCCC T ATT TAT AGG TTT TTT TAT

This PCR product will give the polyhedrin promoter with part of R

B.

5' Primer (polyhBH5)

ATA AAA AAA CCT ATA AAT AGG GCA CTC AGA TTC TGC GGT CTG

3' Primer (polyhBH3)

ACTG AAGCTT GGT CCC TGT TCG GGCGCC AC

(Bold = *Hind* III, Underline = *Nar* I)

This PCR will give part of polyhderin with R.

C.

Use primer polyhAH5 and PolyhBH3 with PCR products AH and BH to obtain the 5' LTR hybrid (polyderin.R)

Insert into pBluescrip KS+ II by cutting with *Sal* I and *Hind* III called pBHHR.

3' LTR (U3.polyhedrin.R.U5).

D. Within U3 to R but including Polyhedrin promoter

5' Primer (polyhDH5)

GATC TCTAGA A AAGCATGCCTGCAGGTCGAGGTCGAT... (Underline = *Xba* I, bold *Sph* I)

3' Primer (PolyhDH3)

TAC AAA ACT GTT ACG AAA ACA GTA AAA TAC TT AGT ACA GGC AAA AAG CAG CTG C

This PCR will give U3 with part of polyhedrin promoter.

E. R TO U5 with polyhedrin promoter

5' Primer (polyhEH5)

TTCGTA ACA GTT TTG TAA TAA AAA AAC CTA TAA ATA

GGGTCTCTCTGGTTAGAC

3' Primer (polyhEH3)

GATC GCGGCCGC TGC TAG AGA TTT TCC ACA CTG (Underline = *Not* I)

This PCR will give part of polyhedrin promoter with R.

F.

Use primer polyhDH5 and PolyhEH3 with PCR products DH and EH to obtain the 3' LTR hybrid (U3.Polyherin.R)

Insert into pBHHR by cutting wtih *Xba* I and *Not* I called pBHHRU3HR

G.

Cut pH4Z with *Nar* I and Sph I to obtain a 6.7 kb fragment and insert into pBHHRU3HR via *Nar* I and *Nsp* V to create pBH4Z.

EXAMPLE 10

EXPRESSION OF FUNCTIONAL MLV, EIAV AND HIV VECTOR GENOMES FROM A POLYHEDRIN PROMOTER: THE DOUBLE RIBOZYME CLEAVAGE METHOD.

This strategy is achieved by the inclusion of hammerhead ribozymes downstream of the polyhedrin promoter sequence designed such that they delete both themselves and the 5' polyhedrin sequence from the transcript to which they are joined. Outlined in Figures 24, 25 and 26 are schematic descriptions of how this is achieved for MLV, EIAV and HIV vectors respectively.

The strategy is the same in all three instances with only the ribozyme helix 1 sequence differing to match the respective complementary *R* sequences of MLV, EIAV and HIV.

Upon cleavage by the ribozyme at the indicated positions the resulting RNA transcripts now contain the correct *R* sequences at their five prime end.

The cloning strategy for the construction of each of these self-cleaving polyhedrin promoter based retroviral genomic expression vectors will now be outlined in turn. Each of these can be used in conjunction with theeeir cognate gagpol and env expression systems to produce high titre retroviral vector preparations. In addition their genome expression cassettes can be inserted into baculovirus vectors by standard procedures.

(i) **Construction of the MLV based vector:**

Primers polyhAM5 and polyhAM3 (see Figure 27 - and the commentary below) are used to PCR amplify the 5'LTR of pEc-Hd-LZSN to incorporate the required changes (polyhedrin promoter/ribozyme sequence addition flush to the 5' *R* sequence) into a PCR fragment which is then cloned into pEc-Hd-LZSN by *EcoR*1- *Spe*1 digestion to produce the finished vector- pBHz-Hd-aR.

Construction of pBHz-Hδ-αR

5' Primer (polyhAM5)

actg gaattcATAACCATCTCGCAAATAAATAAGTA

TTTTACTGTTTTCGTAACAGTTTTGTAATAAAAA

AACCTATAAATA GGACTGGCGC CTGATGAGCG

GCCGAAAGCCCGCGAAACCTGCGTCGACACGC

AGGTC *GCGCCAGTCCTCCGATTGACTGAGTC*

(Underlined = *EcoR* I site)

3' Primer (polyhAM3)

GTTAGCTA ACTAGT ACAGACGCAG (Underline = *Spe* I)

This PCR will give polyhderin, hammerhead ribozyme, MSV R region, U5 and leader.

This fragment can be cloned into pEc-Hd- LZSN at the *EcoR* I and *Spe* I sites to give pBHz-Hδ-αRLZSN.

(ii) Construction of the EIAV based vector:

Primers polyhAEM5 and polyhAEM3 (see Figure 28 - and the commentary below) are used to PCR amplify the 5'LTR of pEc-Hd-ONY2.1nlslacZ to incorporate the required changes (polyhedrin promter/ribozyme sequence addition flush to the 5' *R* sequence) into a PCR fragment which is then cloned into pEc-Hd-ONY2.1nlslacZ by *Eag* 1 digestion to produce the finished vector- pBEHz-Hδ-αR.

Construction of pBEHz-Hδ-αR

5' Primer (polyhAEM5)

actg cggccg ATAACCATCTCGCAAATAAATAAGTATTTT

ACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTAT

AAATA CTGAGTGCCC CTGATGAGCGGCCGA

AAGCCCGCGAAACCTGCGTCGACACGCAGGTC

GGGCACTCAGATTCTGCGGTCTG

(Underlined = *Eag* I site)

3' Primer (polyhAEM3)

CTAGTTCTAGAGCGGCCGCCAC (Underline = *Eag* I)

This PCR will give polyhedrin, hammerhead ribozyme, EIAV R region, U5 and leader.

This fragment can be inserted into pEc-Hd-ONY2.1nlslacZ at the *Eag* I and *Eag* I sites to give pBEHz-Hδ-αR.

(iii) Construction of the HIV based vector:

Primers polyhAHM5 and polyhAHM3 (see Figure 29 - and the commentary below) are used to PCR amplify the 5'LTR of pEc-Hd-H4nZ to incorporate the required changes (polyhedrin promter/ribozyme sequence addition flush to the 5' *R* sequence) into a PCR fragment which is then cloned into pEc-Hd-H4nZ by *Eag*1-*Nar*1 digestion to produce the finished vector- pBHHz-Hδ-αR.

Construction of pBHHz-Hδ-αR

5' Primer (polyhAHM5)

ACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTAT

Hammer head Rz

AAATA AGAGAGACCC CTGATGAGCGGCCGA

AAGCCCGCGAAACCTGCGTCGACACGCAGGTC

R

GGGTCTCTCTGGTTAGACCAGATC ...

(Underlined = *Eag* I site)

3' Primer (polyhAHM3)

CCCTGTTCGGGCGCCACTGC (Underline = *Nar* I)

This PCR will give polyhderin, hammerhead ribozyme, HIV R region, U5 and leader.

This fragment can be inserted into pEc-Hd- H4nZ at the Eag I and Nar I sites to give pBHHz-Hδ-αRLZSN.

Summary

Thus, the present invention provides a novel system for producing retroviral vector particles. In a highly preferred embodiment, the novel system uses a baculovirus expression vector encoding a retroviral vector genome. In another preferred embodiment, the present invention provides a baculovirus expression vector encoding a retroviral vector genome, and to retroviral vector particles produced by the novel system of the invention.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A composition comprising at least one baculoviral component and at least one retroviral component, wherein the retroviral component is capable of being packaged into a retroviral particle.

2. A composition wherein the composition is a baculovirus expression system comprising at least one retroviral component, wherein the retroviral component is capable of being packaged into a retroviral particle.

3. A composition according to claim 1 or claim 2 wherein the retroviral component corresponds to a retroviral genome.

4. A composition according to any one of the preceding claims wherein the composition comprises an RNA transcription start site for the retroviral vector genome, and wherein the nucleotide sequence encoding the retroviral component is operably linked to a promoter comprising an upstream promoter component located upstream of the RNA transcription start site and a downstream promoter component located downstream of the RNA transcription start site.

5. A composition according to claim 4 wherein the downstream promoter component is upstream of the polynucleotide sequence encoding the retroviral vector genome.

6. A composition according to claim 4 or claim 5 wherein the promoter is a baculovirus promoter.

7. A composition according to claim 6 wherein the promoter is the polyhedrin promoter and/or the *p10* promoter and/or *polh* promoter.

8. A composition according to any one of claims 1 to 5 wherein the promoter is a non-baculovirus promoter.

9. A composition according to claim 8 wherein the promoter is the T7 promoter or the *sp6 Salmonella* phage promoter.

10. A composition according to any one of the preceding claims wherein the composition comprises at least one RNA cleavage component.

11. A composition according to claim 10 wherein at least one of the RNA cleavage component(s) would yield a retroviral genome free of any baculoviral components.

12. A composition according to claim 11 when dependent on any one of claims 4 to 9 wherein at least one of the RNA cleavage component(s) is located between the promoter and the sequence encoding the retroviral component.

13. A composition according to claim 12 wherein at least one of the RNA cleavage component(s) is located immediately adjacent the sequence encoding the retroviral vector component for subsequent cleavage at the 5' end of the vector component.

14. A composition according to any one of claims 4 to 13 wherein wherein at least one of the RNA cleavage component(s) is located downstream of the sequence encoding the retroviral component.

15. A composition according to any claim 14 wherein the RNA cleavage component(s) has a cleavage site immediately adjacent the sequence encoding the retroviral vector component for subsequent cleavage at the 3' end of the vector component.

16. A composition according to any one of claims 4 to 15 wherein at least one of the RNA cleavage component(s) is a sequence recognised by a ribozyme for subsequent cleavage thereof.

17. A composition according to any one of claims 4 to 15 wherein each RNA cleavage component is a sequence recognised by a ribozyme for subsequent cleavage thereof.

18. A composition according to any one of claims 4 to 17 wherein the downstream promoter component is located within the sequence encoding the retroviral component.

19. A composition according to claim 18 wherein the downstream promoter component is located within the sequence encoding the retroviral vector.

20. A composition according to claim 19 wherein the retroviral component comprises a retroviral R region at either end of a sequence encoding a retroviral vector genome, wherein the downstream promoter component is located in the 5' R region and has a counterpart sequence in the 3' R region.

21. A composition according to any one of the preceding claims wherein the composition comprises in a downstream direction: an upstream baculovirus promoter component, a downstream baculovirus promoter component, a ribozyme sequence, a retroviral 5' R region, a retroviral U5 region, a retroviral vector region for insertion of one or more genes to be delivered by the vector, a retroviral U3 region, a retroviral 3' R region, and optionally a second ribozyme sequence.

22. A composition according to any one of the preceding claims wherein the composition comprises in a downstream direction: an upstream baculovirus promoter component, a retroviral 5' R region comprising a downstream promoter component, a retroviral U5 region, a retroviral vector region for insertion of one or more genes to be delivered by the retroviral vector, a retroviral U3 region, a retroviral 3' R region, and optionally a ribozyme sequence.

23. A composition according to any one of the preceding claims wherein the composition comprises one or more nucleotide sequences encoding one or more packaging components for producing retroviral vector particles which particles comprise the retroviral component.

24. A composition according to any one of the preceding claims wherein the composition

further comprises at least one nucleotide sequence of interest (NOI).

25. A composition according to claim 24 wherein the NOI is useful in medicine.

26. A composition according to claim 24 or claim 25 wherein the NOI is part of the retroviral component.

27. A retroviral particle obtainable from expression of the composition according to any one of the preceding claims.

28. A process for preparing a retroviral particle comprising expressing the composition according to any one of the preceding claims.

29. An insect cell comprising the composition according to any one of the preceding claims.

30. A retroviral vector particle production system comprising comprising the composition according to any one of the preceding claims in an insect cell.

31. A retroviral vector particle produced by the retroviral vector particle production system of claim 30.

32. An expression vector comprising a polynucleotide sequence which encodes a retroviral vector genome having a 5' and a 3' end, which retroviral vector genome is capable of being expressed and packaged into a retroviral vector particle in a baculovirus expression system.

33. A composition comprising at least a first viral component obtainable from a first virus and a second viral component component obtainable from a second virus; wherein the first virus is different from the second virus; wherein the second viral component is flanked by at least two cleavage sites (which may be the same or different); wherein at least a part of the second viral component is capable of being packaged into a viral particle; which viral particle is substantially free of any first viral component.

34. A composition according to claim 33 wherein at least one of the cleavage sites is a ribozyme cleavage site.

35. A composition according to claim 34 wherein each of the cleavage sites is a ribozyme cleavage site.

36. A composition according to any one of claims 33 to 35 wherein the first virus is a baculovirus.

37. A composition according to any one of claims 33 to 36 wherein the second virus is a retrovirus.

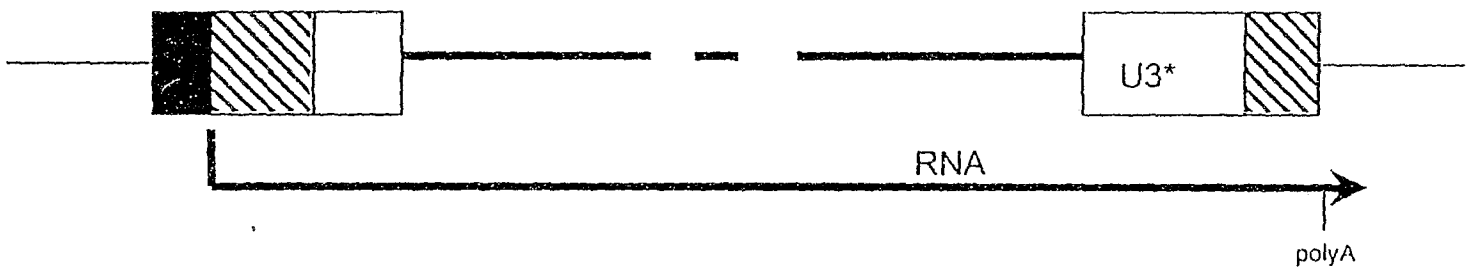
38. Use of a baculoviral composition to express an NOI comprising at least one intron.

39. A production facility for producing a quantity of an NOI or the expression product thereof; wherein the facility contains a medium comprising a baculovirus composition which comprises the NOI.

40. A composition substantially as described herein and with reference to the accompanying Figures.

SUBSTITUTE SHEET (RULE 26)

Figure 1



= Upstream baculovirus promoter components

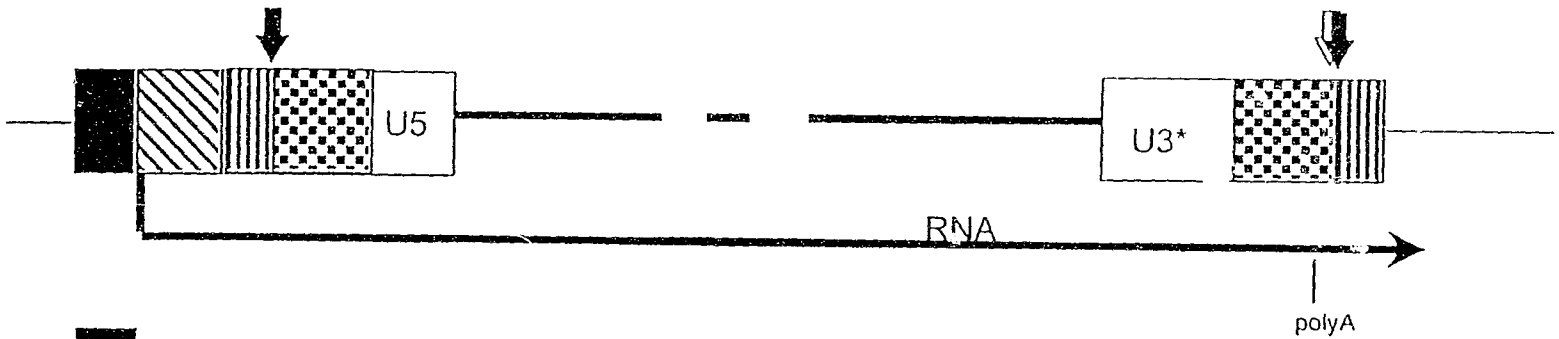
= Synthetic R region containing downstream baculovirus promoter components

= Retroviral LTR sequences

U3* = Retroviral U3 region containing non-retroviral control sequences.

SUBSTITUTE SHEET (RULE 26)

Figure 2



= Upstream baculovirus promoter components

= Downstream baculovirus promoter components

= Retroviral LTR sequences

= Retroviral R region

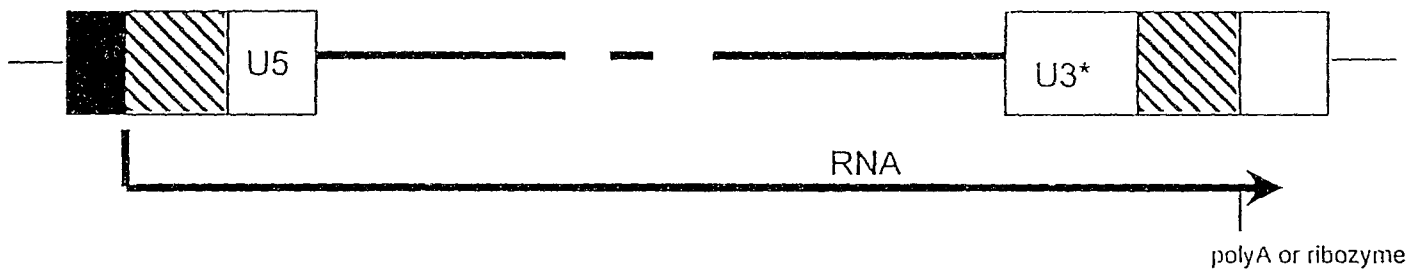
= Ribozyme

= Ribozyme cleavage point

U3* = Retroviral U3 region containing non-retroviral control sequences.

SUBSTITUTE SHEET (RULE 26)

Figure 3



=T7 promoter

= R region

= Retroviral LTR sequences

U3* = Retroviral U3 region containing non-retroviral control sequences.

Figure 4

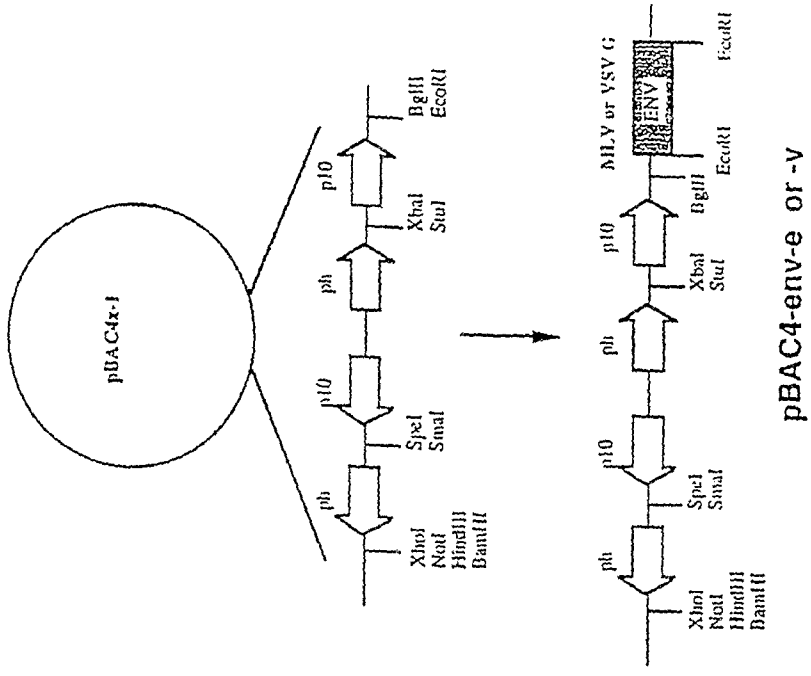

pBAC4x-1
ph
p10
XhoI
NotI
HindIII
BamHI
p10
SpeI
SmaI
ph
p10
XbaI
StuI
p10
BglII
EcoRI
ph
p10
XhoI
NotI
HindIII
BamHI
p10
SpeI
SmaI
ph
p10
XbaI
StuI
p10
BglII
EcoRI
MLV or VSV G
ENV
EcoRI
pBAC4-env-e or -v

Figure 5

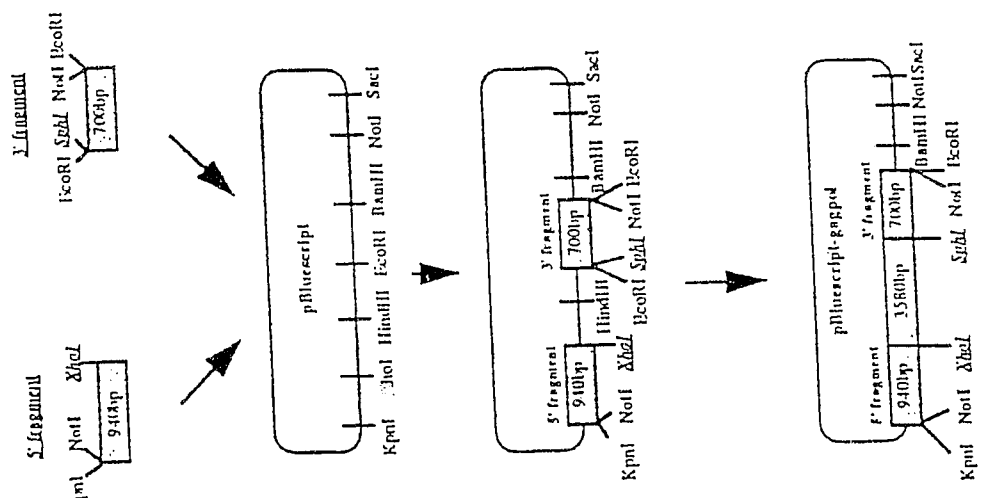

3' fragment
EcoRI SphI NotI EcoRI
700bp
5' fragment
KpnI NotI XbaI
940bp
pBluescript
KpnI SacI NotI BamHI EcoRI HindIII SmaI
5' fragment
940bp
3' fragment
700bp
BamHI NotI SacI
HindIII
KpnI NotI XbaI SphI NotI EcoRI
pBluescript-gapped
5' fragment
940bp
3' fragment
700bp
3580bp
BamHI NotISacI
SphI NotI EcoRI
KpnI NotI XbaI

Figure 6

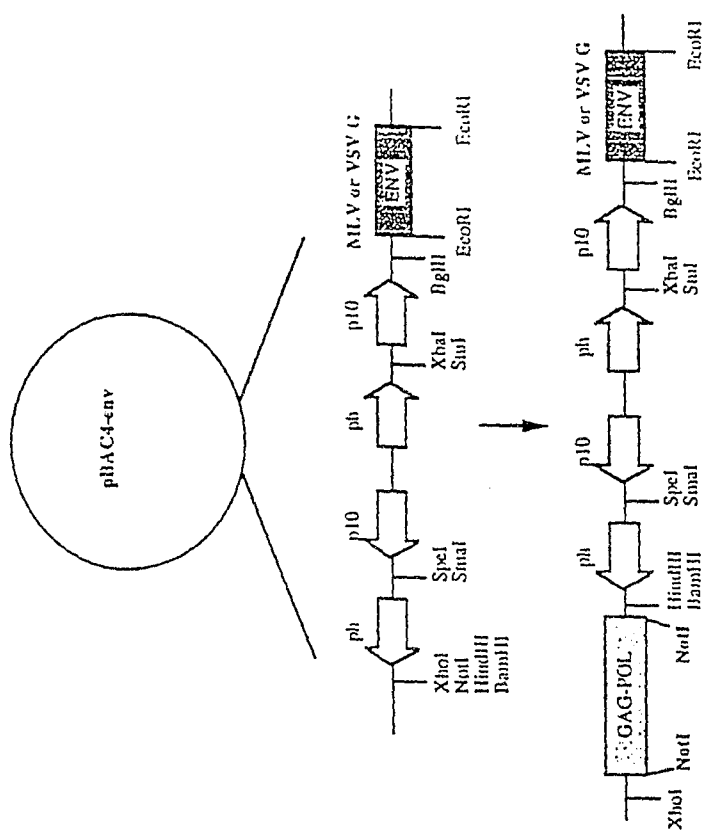

pBAC4-env
ph
p10
ph
p10
MLV or VSV G
ENV
XhoI
NotI
HindIII
BamHI
SpeI
SmaI
XbaI
SuI
BglII
EcoRI
GAG-POL
pBAC4-mgagpolenv-e or -v

Figure 7

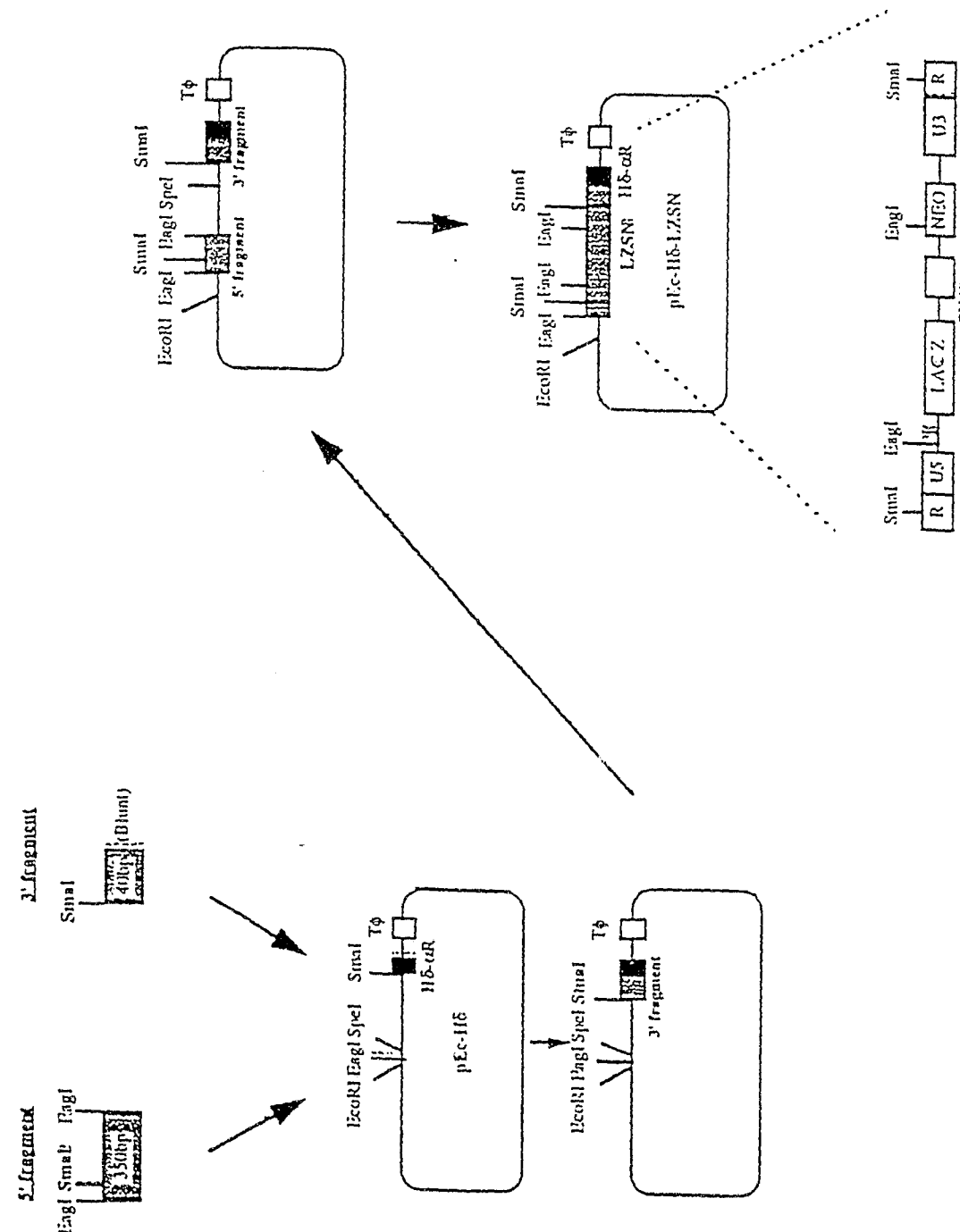

5' fragment
EagI SmaI EagI
3' fragment
SmaI
(Blunt)
EcoRI EagI SpeI SmaI
Tφ
H6-αR
pEc-H6
EcoRI EagI SpeI SmaI
3' fragment
EcoRI EagI SpeI SmaI
5' fragment
3' fragment
pEc-H6-LZSN
LZSN
SmaI EagI
R U5
LACZ
NEO
U3 R
SV40 prom.

Figure 8

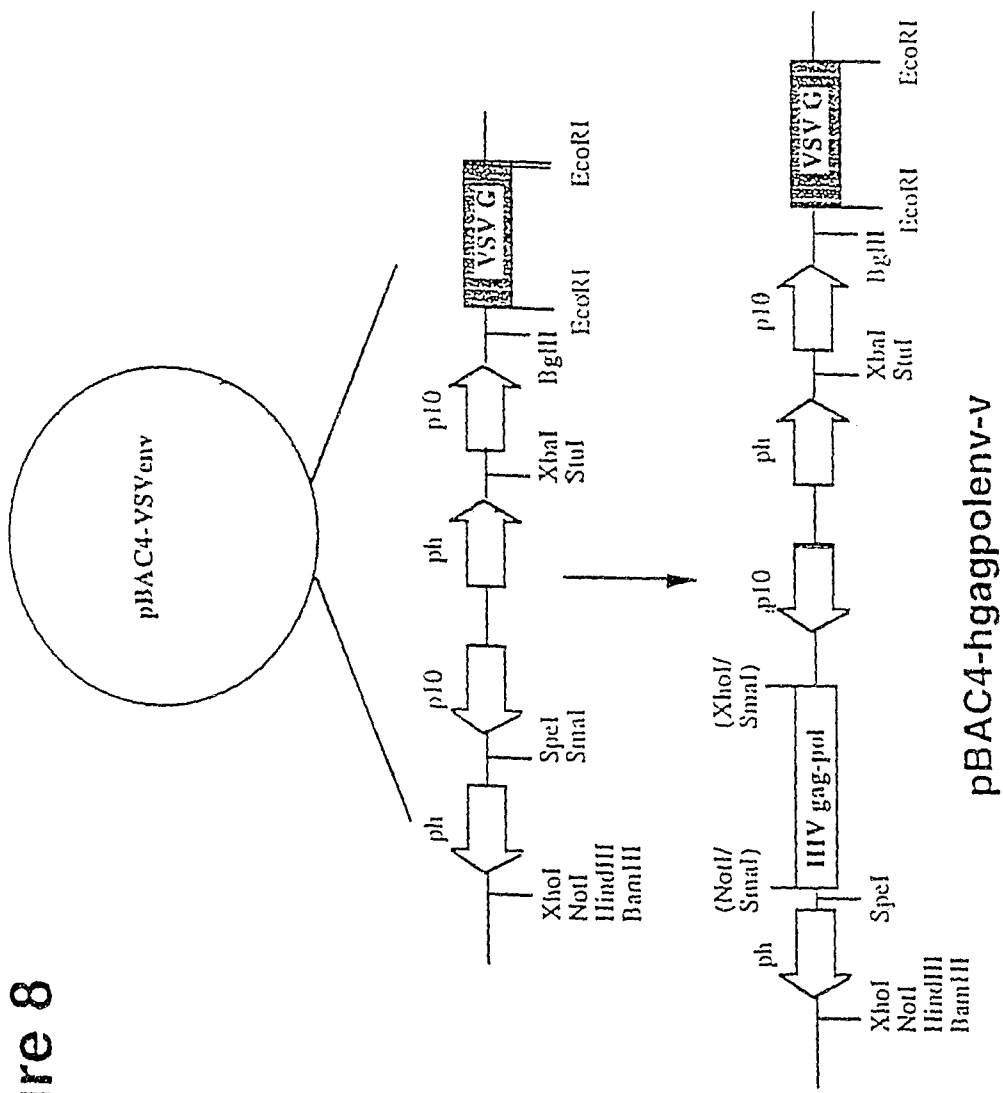

pBAC4-VSVenv
ph
p10
ph
p10
ph
XhoI
NotI
HindIII
BamHI
SpeI
SmaI
XbaI
StuI
BglII
EcoRI
VSV G
EcoRI
(NotI/
SmaI)
(XhoI/
SmaI)
HIV gag-pol
SpeI
pBAC4-hgagpolenv-v

Figure 9

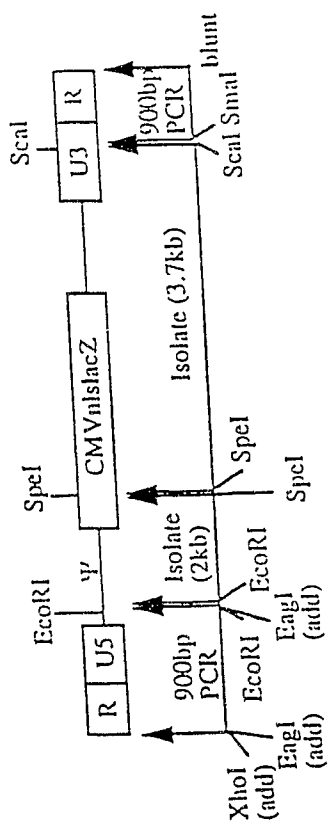

ScaI
U3
R
900bp PCR
blunt
ScaI SmaI
Isolate (3.7kb)
SpeI
CMVnlslacZ
SpeI
SpeI
Isolate (2kb)
EcoRI
Ψ
EcoRI
EagI (add)
EcoRI
R
U5
900bp PCR
EagI (add)
XhoI (add)

Figure 10

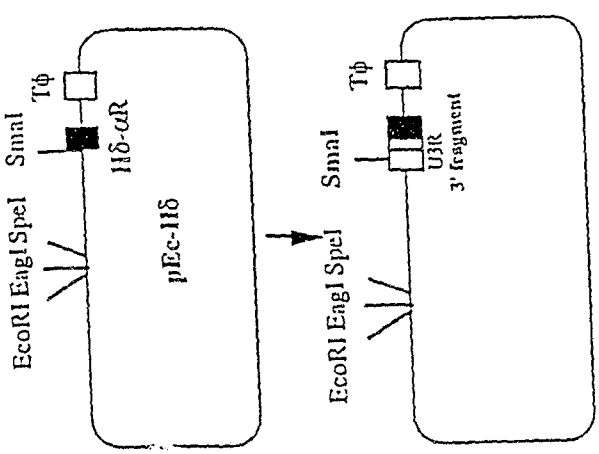

Tφ
SmaI
HB-αR
EcoRI EagI SpeI
pEc-HB
Tφ
SmaI
U3R
3' fragment
EcoRI EagI SpeI

Figure 11

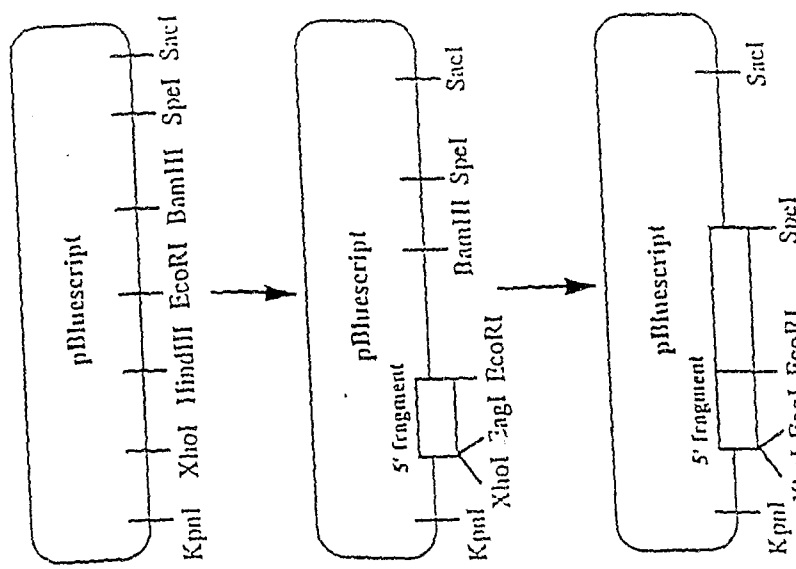

pBluescript
KpnI XhoI HindIII EcoRI BamHI SpeI SacI
pBluescript
5' fragment
KpnI XhoI EagI EcoRI
BamHI SpeI SacI
pBluescript
5' fragment
KpnI XhoI EagI EcoRI
SpeI SacI

Figure 12

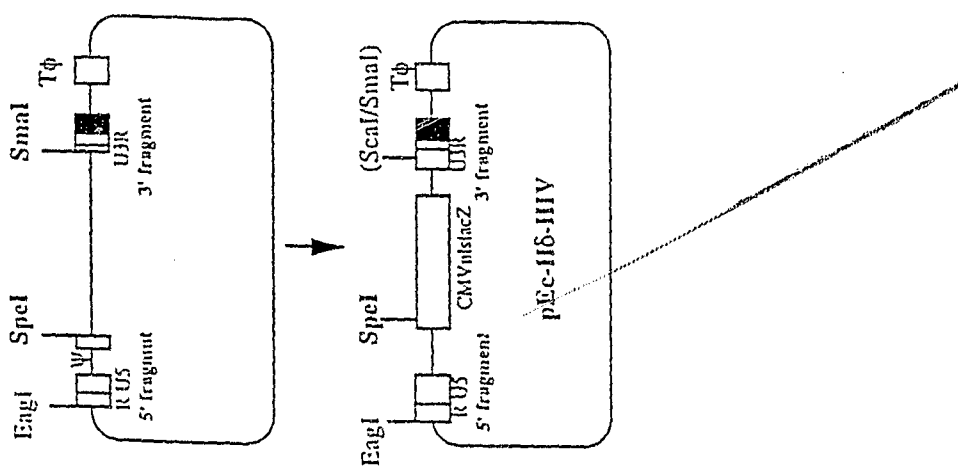

EagI
SpeI
Ψ
R U5
5' fragment
SmaI
Tφ
U3R
3' fragment
(ScaI/SmaI)
CMVinlslacZ
pEc-HB-HIV

Figure 13

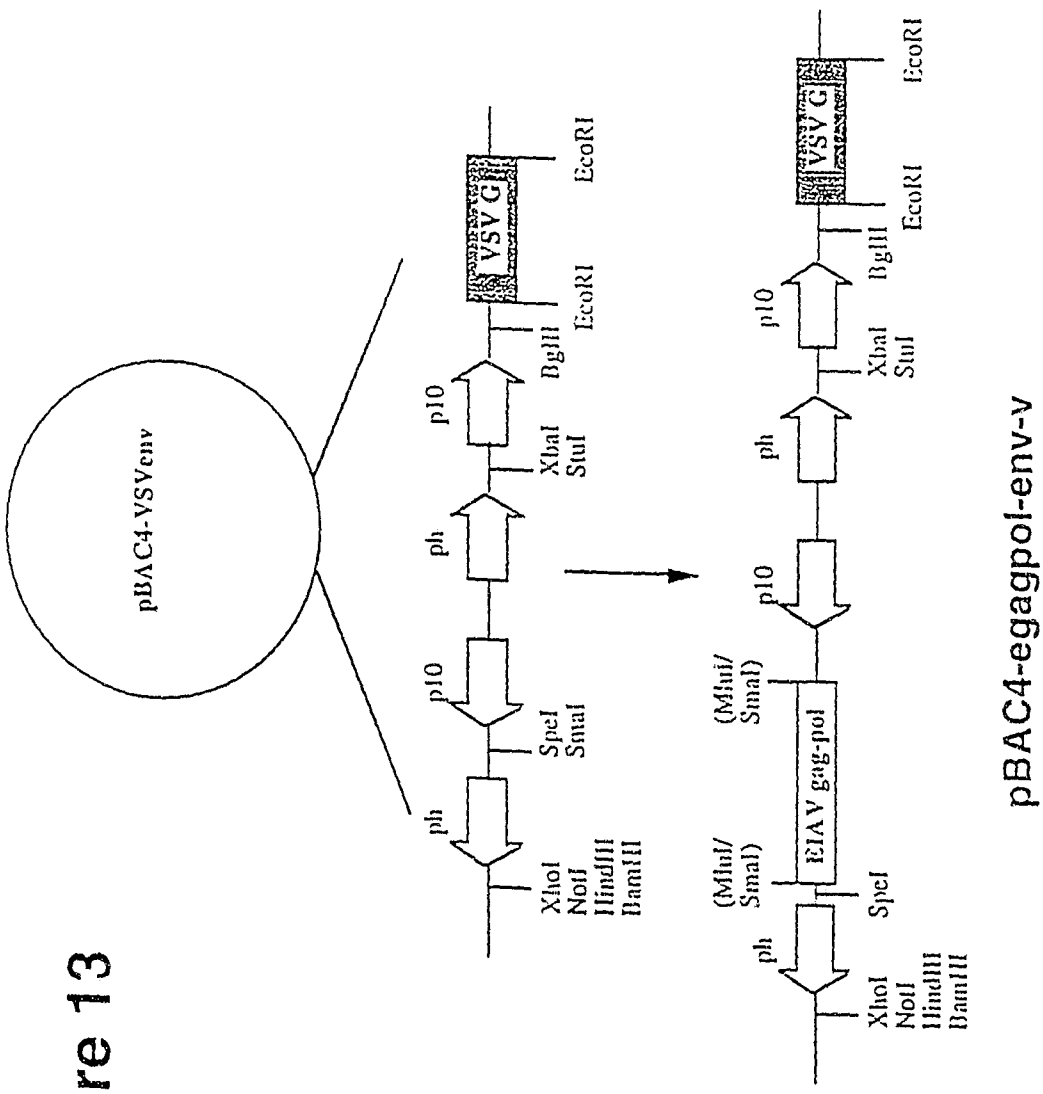

pBAC4-VSVenv
ph
p10
ph
p10
VSV G
XhoI
NotI
HindIII
BamHI
SpeI
SmaI
XbaI
StuI
BglII
EcoRI
EcoRI
(MluI/
SmaI)
EIAV gag-pol
SpeI
pBAC4-egagpol-env-v

Figure 14

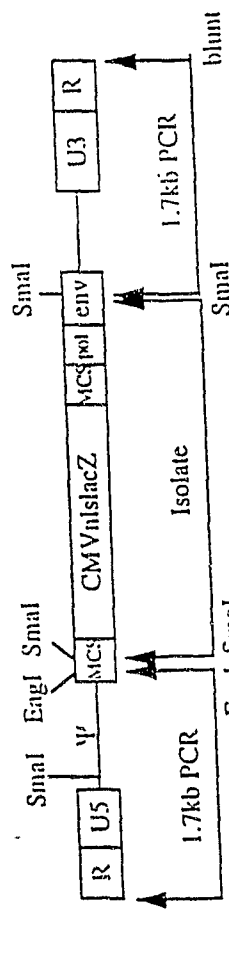

SmaI
env
pol
MCS
CMVnlslacZ
MCS
EagI SmaI
Ψ
SmaI
U5
R
1.7kb PCR
EagI (add)
Isolate
SmaI
EagI SmaI
U3
R
1.7kb PCR
blunt

Figure 15

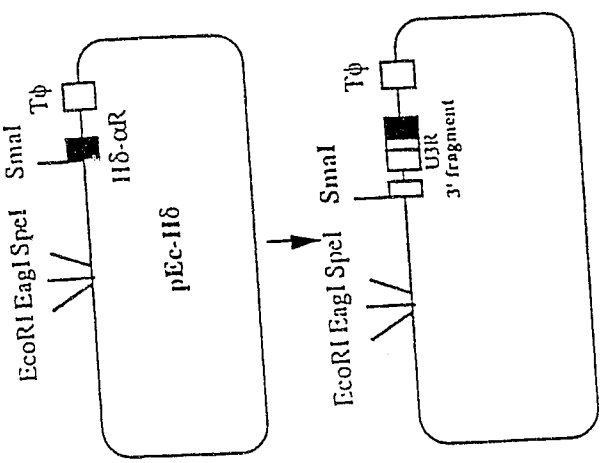

SmaI
Tφ
IIδ-αR
EcoRI EagI SpeI
pEc-IIδ
Tφ
SmaI
U3R
3' fragment
EcoRI EagI SpeI

Figure 16

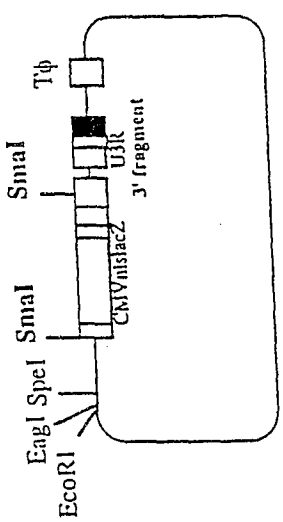


Figure 17

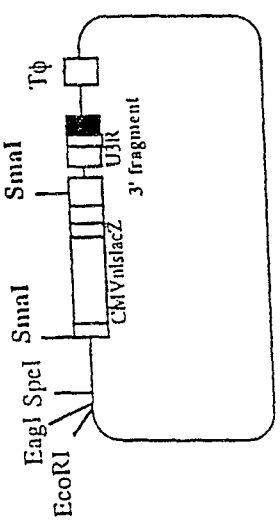


Figure 18 - Structure of pBHLZSN

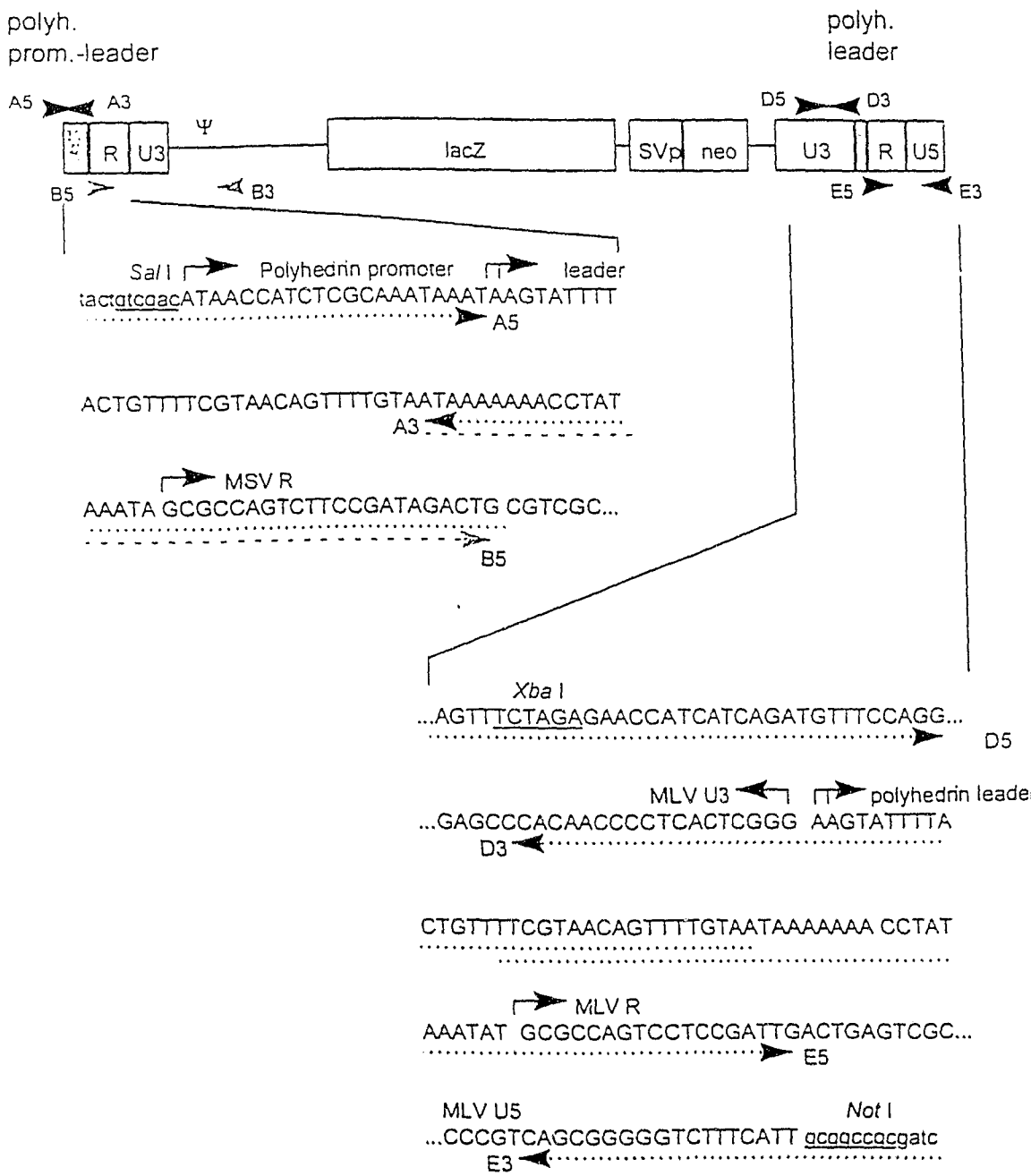


Figure 19 - Construction of pBHLZSN

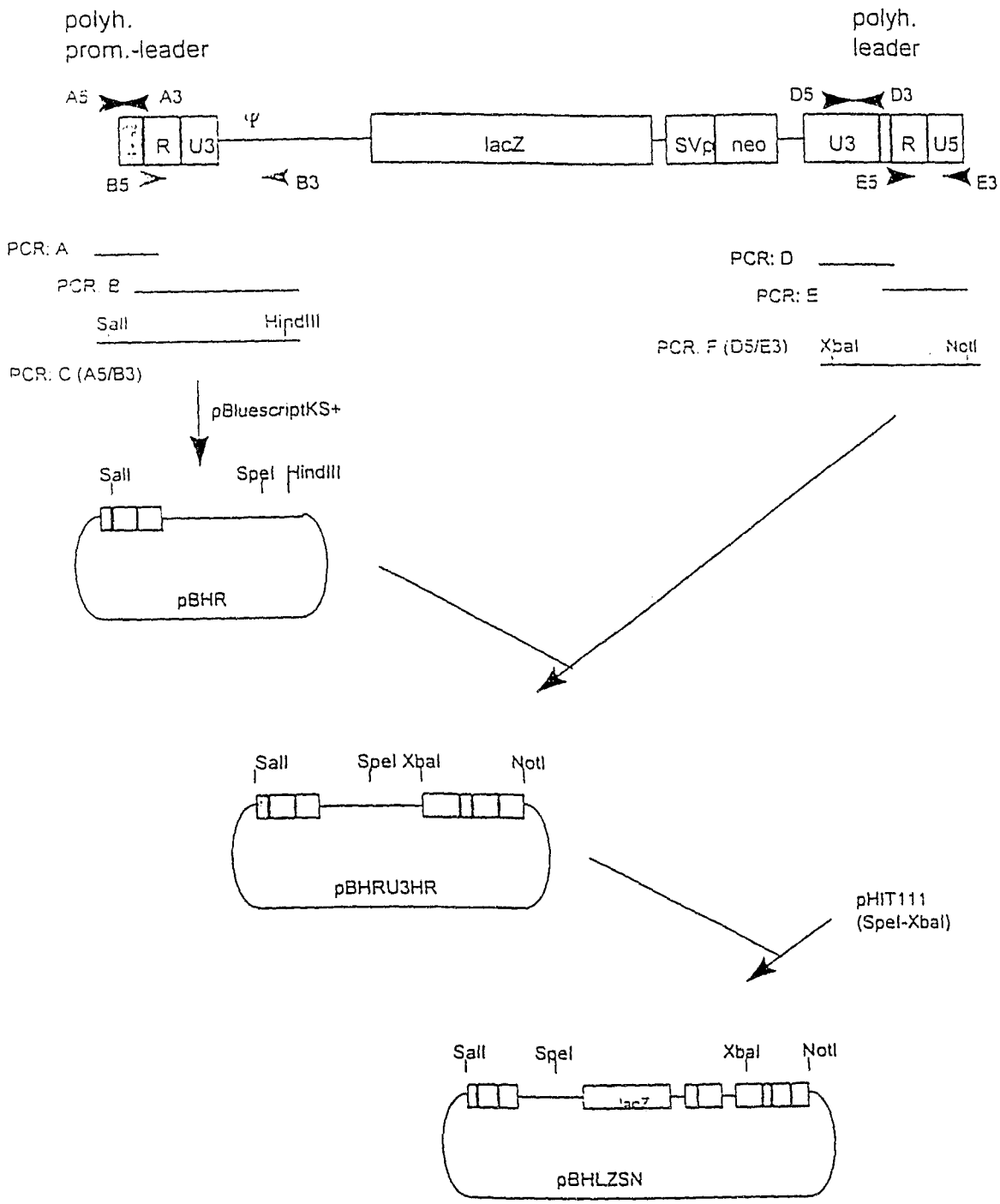


Figure 20 - Structure of pBONY2.1nlslacZ

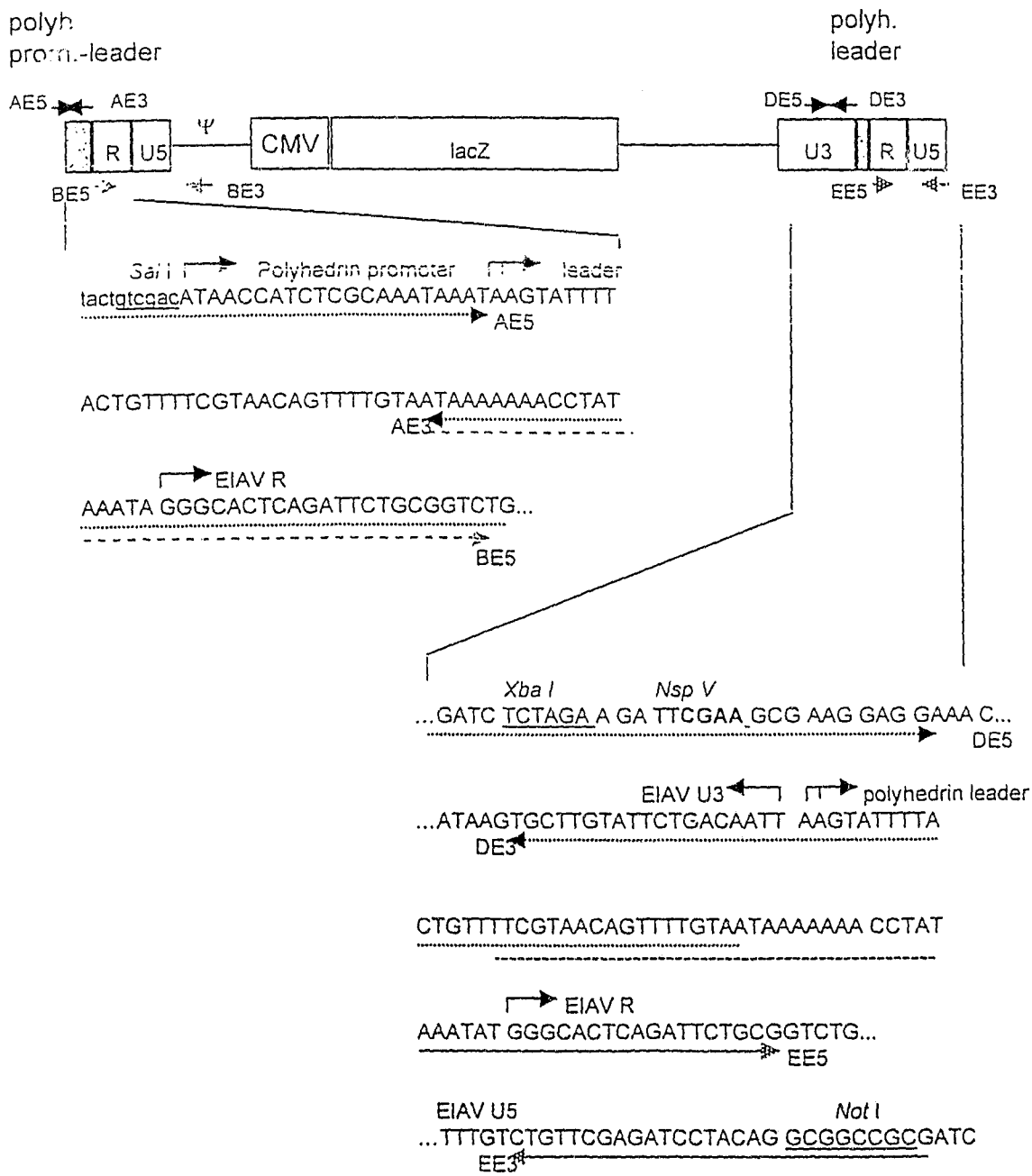


Figure 21 - Construction of pBONY2.1nlslacZ

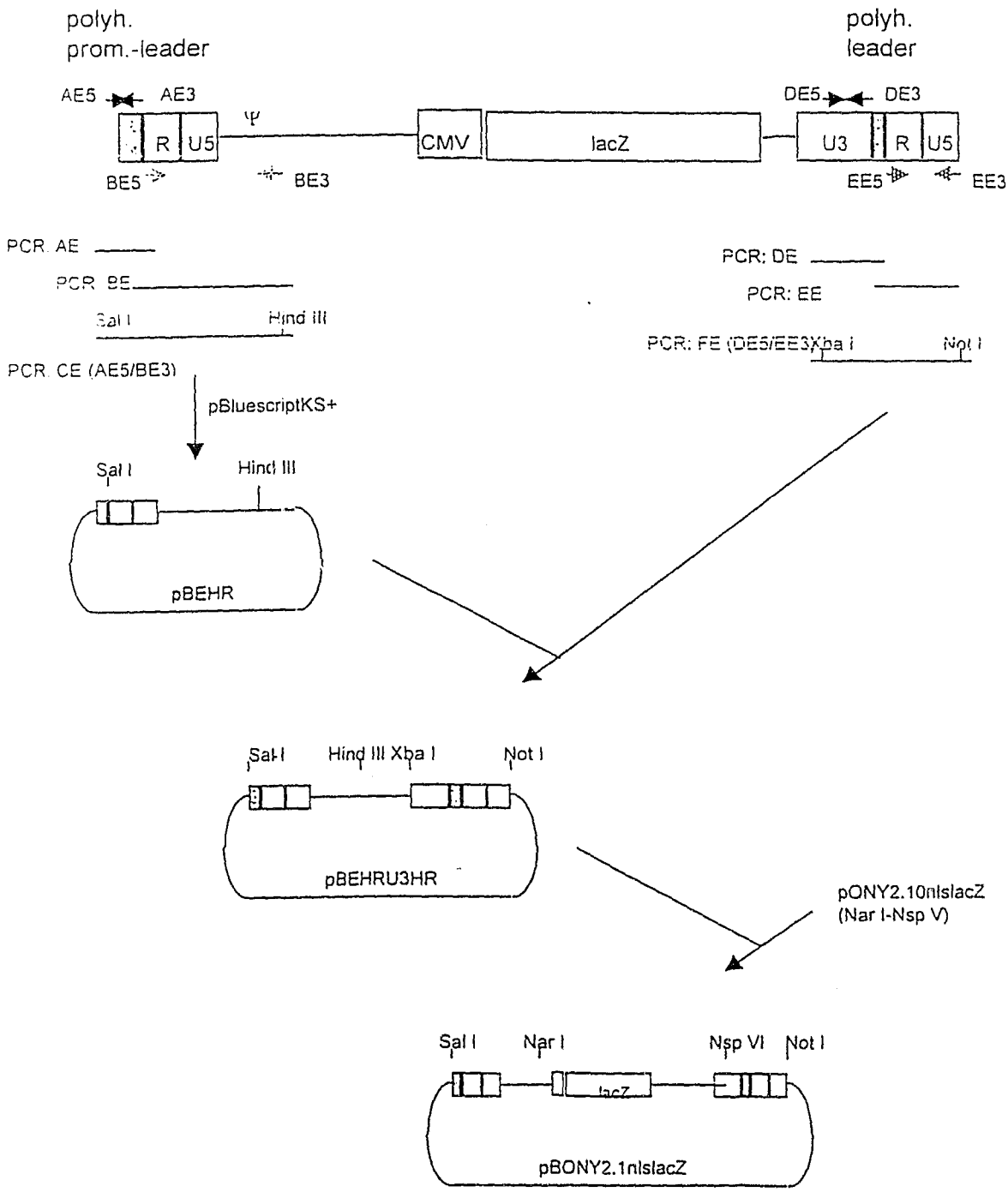


Figure 22 - Structure of pBH4Z

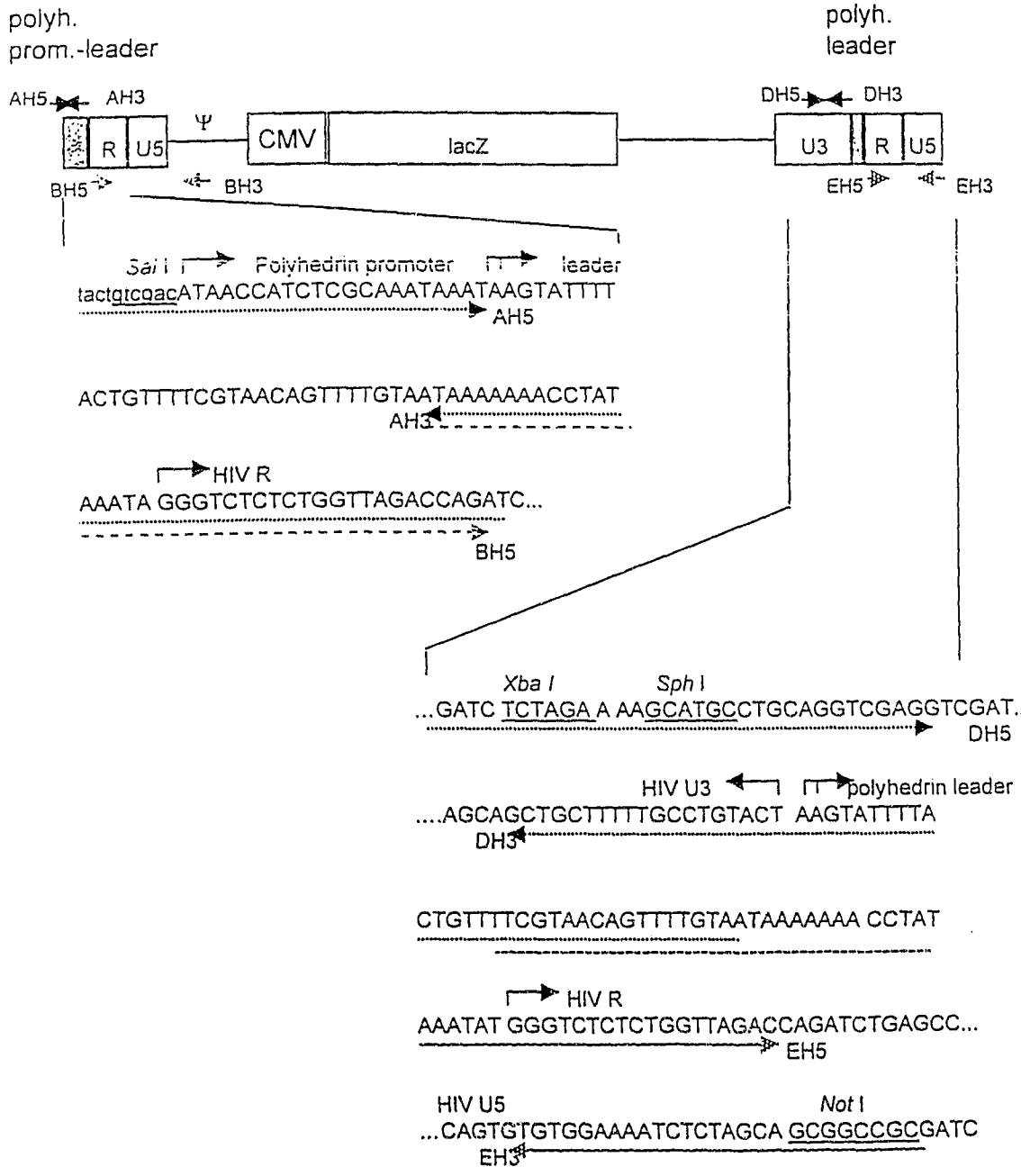


Figure 23 - Construction of pBH4Z

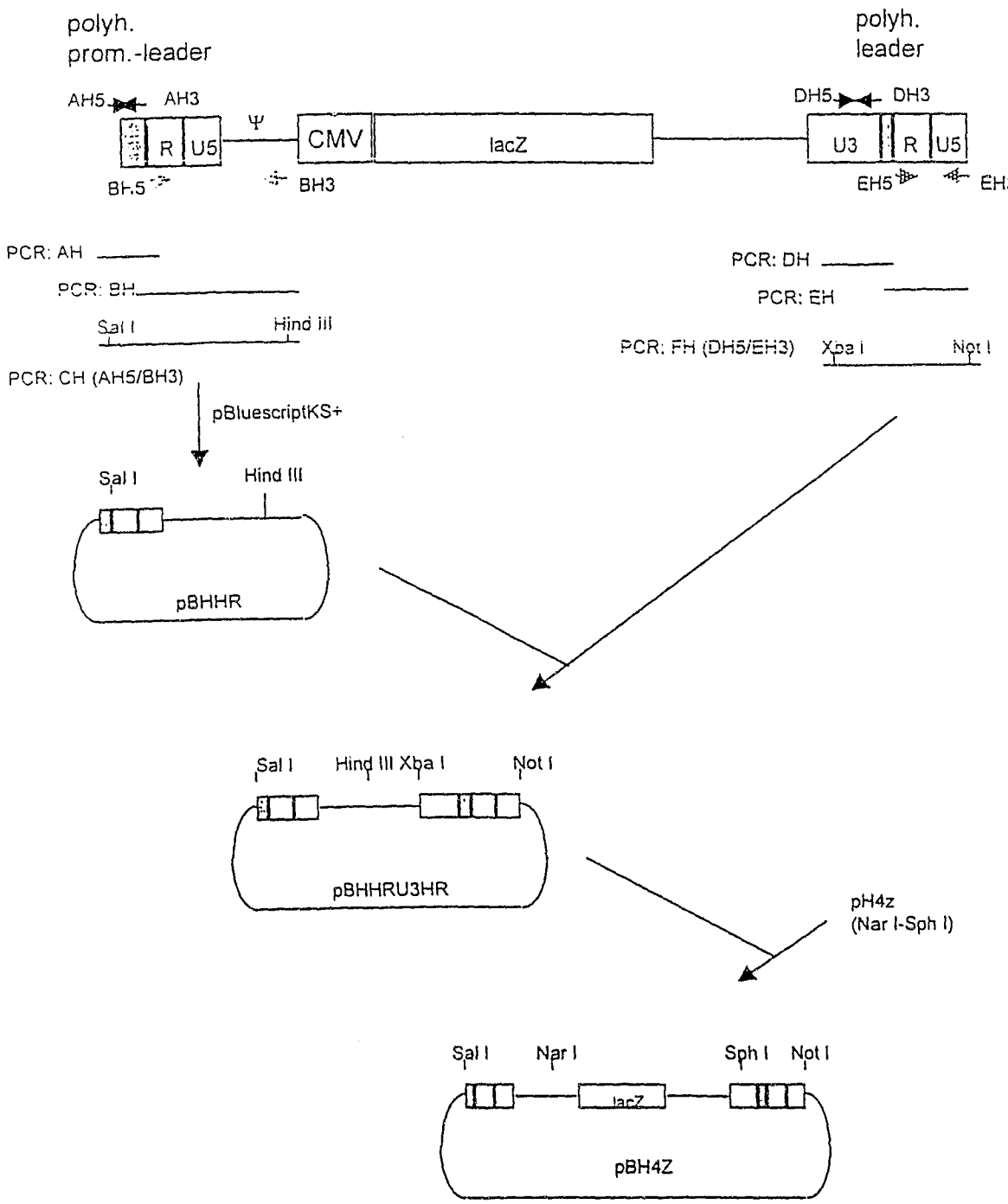
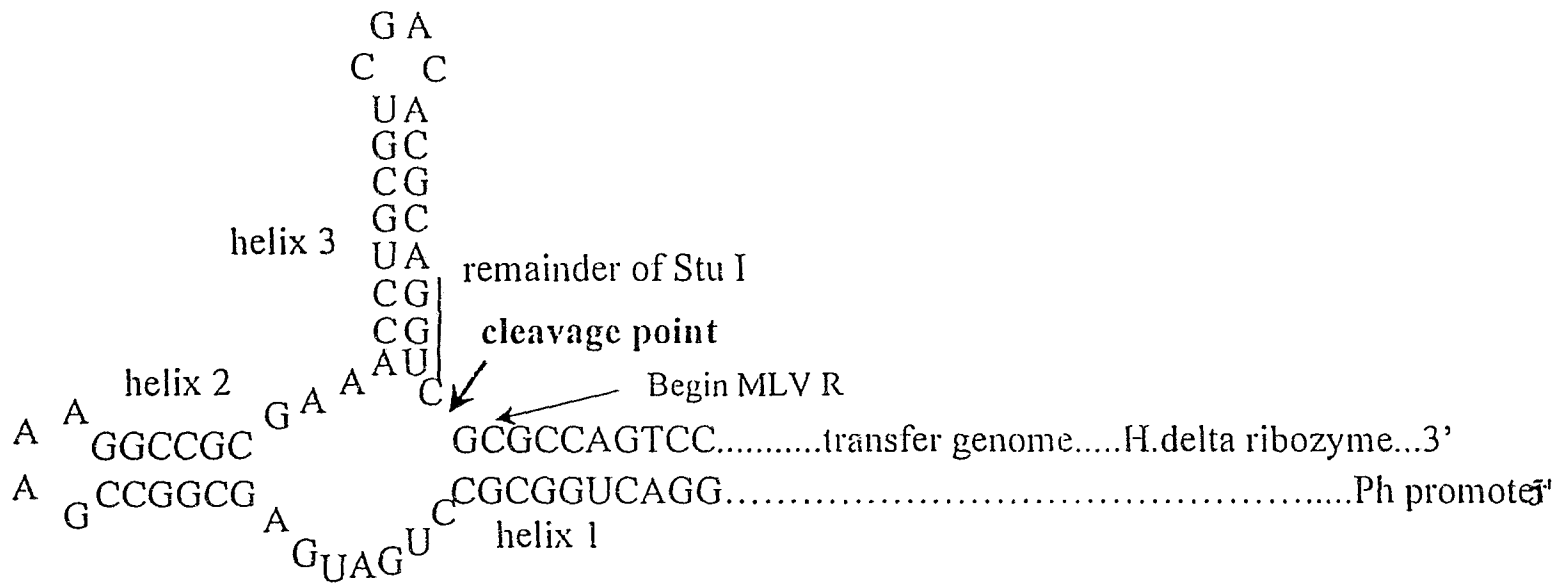


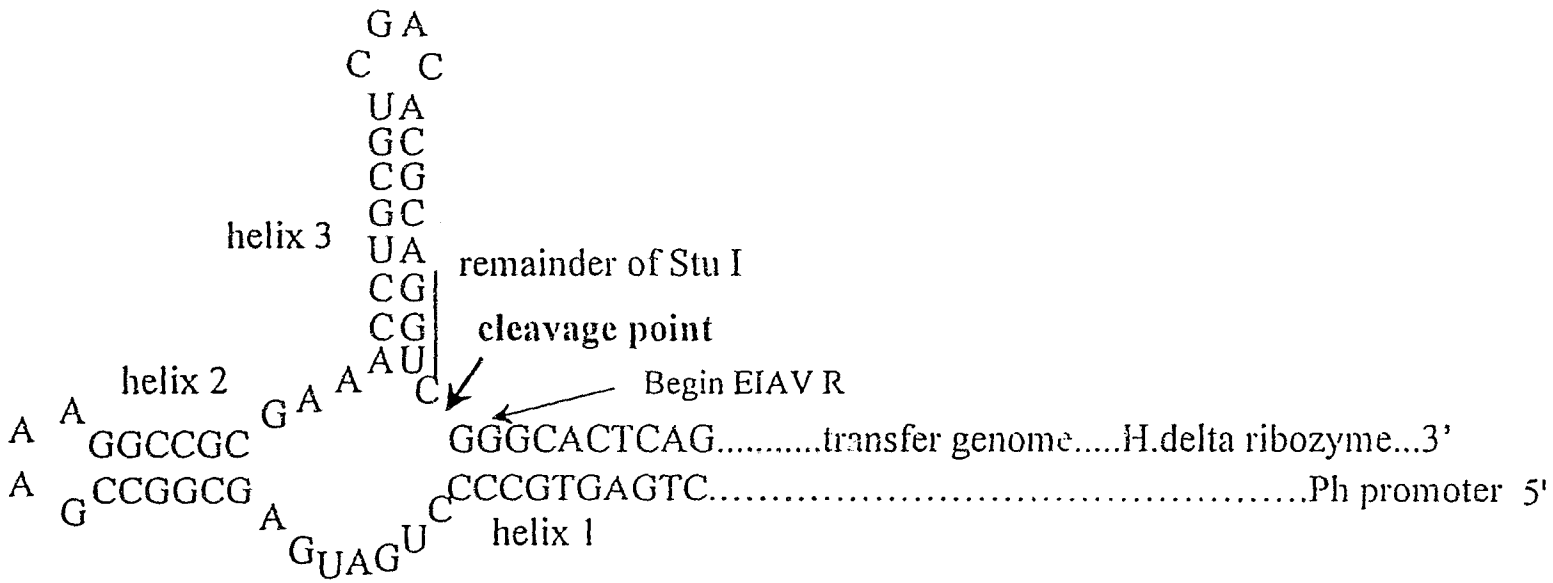
Figure 24



Example of hammerhead strategy for perfect 5' end formation. A number of designs that create stable helices 1,2 and 3 allow for cleavage at the point shown.

SUBSTITUTE SHEET (RULE 26)

Figure 25



Example of hammerhead strategy for perfect 5' end formation. A number of designs that create stable helices 1,2 and 3 allow for cleavage at the point shown.

Figure 26

SUBSTITUTE SHEET (RULE 26)

Figure 27 - Structure of pBHz-Hδ-αR

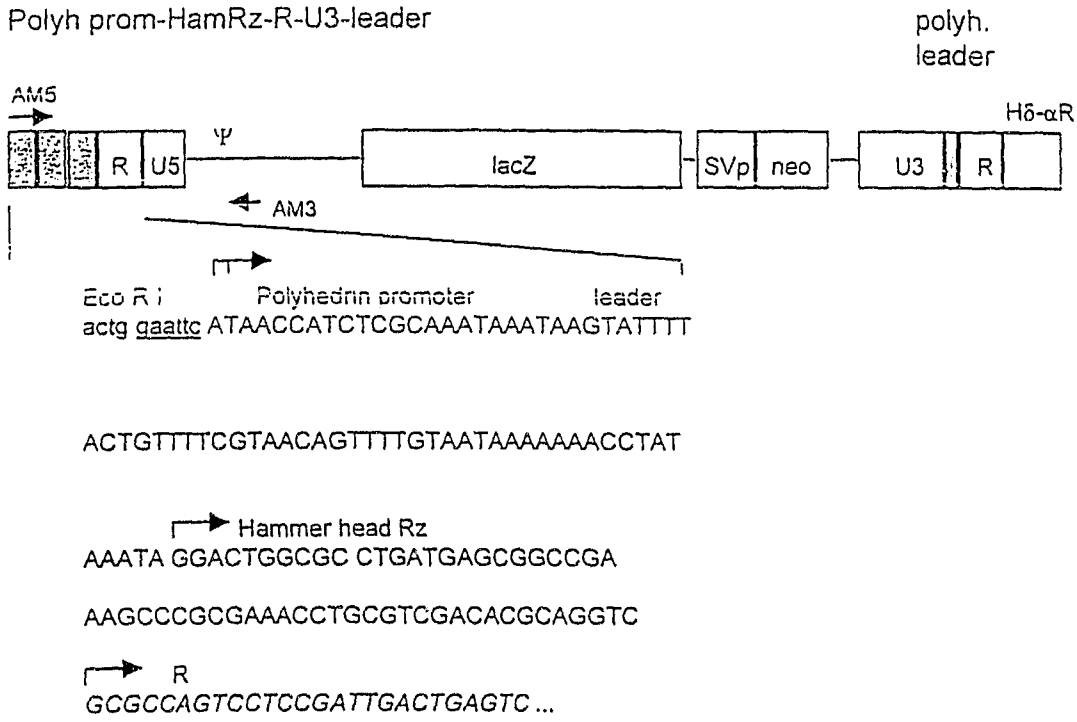


Figure 28 - Structure of pBEHz-Hδ-αR

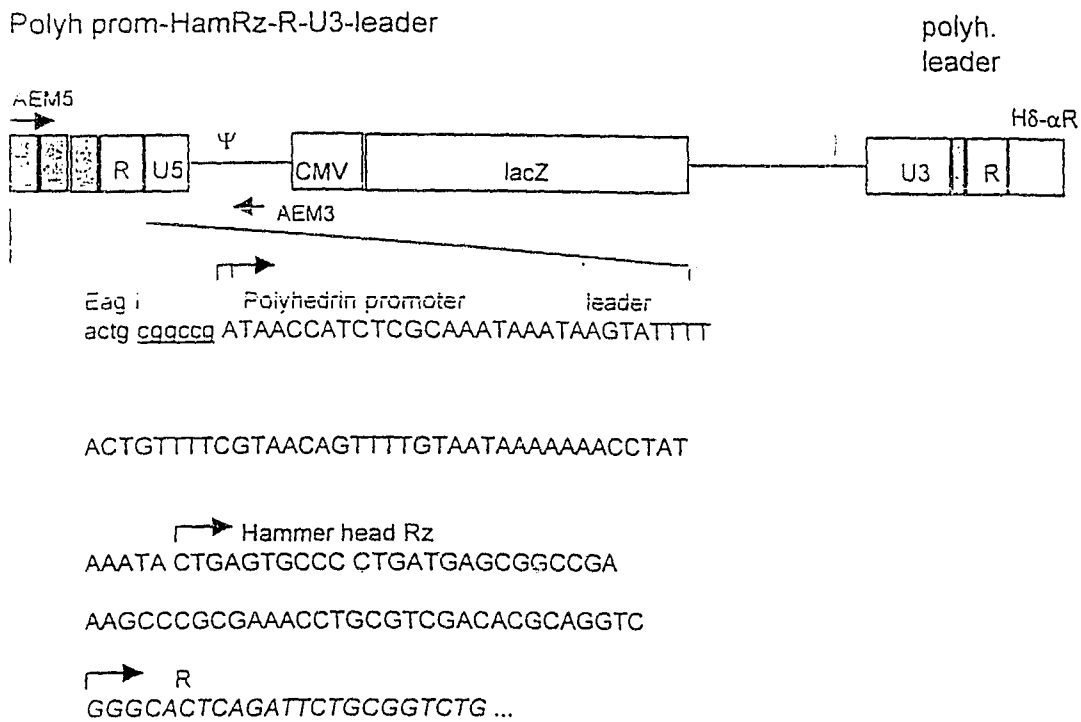
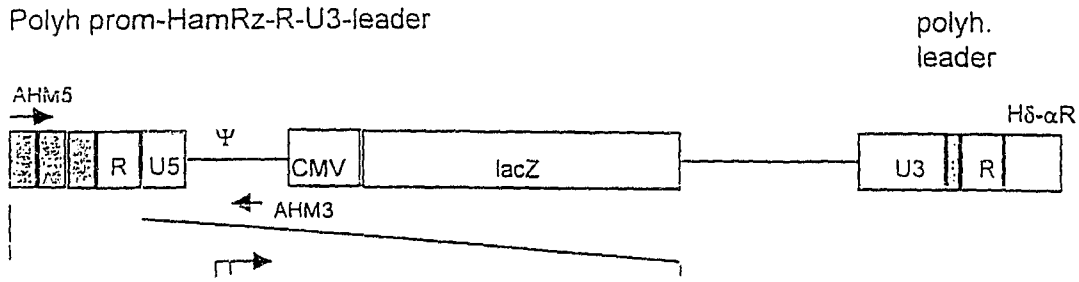


Figure 29 - Structure of pBHHz-Hδ-αR



ACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTAT

Hammer head Rz

AAATA AGAGAGACCC CTGATGAGCGGCCGA

AAGCCCGCGAAACCTGCGTCGACACGCAGGTC

R

GGGTCTCTCTGGTTAGACCAGATC ...

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/GB 98/01626

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/16 C12M3/00

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)
IPC 6 C12N

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 334 301 A (VIAGENE INC) 27 September 1989 see column 6, line 35 - column 8, line 12 see column 36, line 1 - column 37, line 30 ---	1-5,8,9, 23-32
X	SAVARD N. ET AL: "TRANSIENT RETROVIRAL PACKAGING SYSTEMS GENERATED BY HERPES SIMPLEX VIRUS DERIVED VECTORS" GENE THERAPY, vol. 2, no. SUPPL. 01, 17 November 1995, page S12 XP000617926 see abstract ---	27
X	WO 91 19803 A (APPLIED BIOTECHNOLOGY INC) 26 December 1991 see page 14, line 4 - page 16, line 8 --- -/--	27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 but 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
8 October 1998	20/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/GB 98/01626

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TOBIN G. J. ET AL.: "Synthesis and assembly of chimeric human immunodeficiency virus gag pseudovirions 'published erratum appears in Intervirology 1996;39(4):258!." INTERVIROLOGY, vol. 39, no. 1-2, - 1996 pages 40-48, XP002079915 see the whole document	1-37
A	BOULANGER P. AND JONES I.: "Use of heterologous expression systems to study retroviral morphogenesis." CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, vol. 214, 1996, pages 237-260, XP002079916 see page 241, paragraph 3 - page 242, paragraph 1	1-37
X	WO 96 37623 A (OXFORD BIOMEDICA LTD ;KINGSMAN ALAN JOHN (GB); KINGSMAN SUSAN MARY) 28 November 1996	27
A	see the whole document	1-26, 28-37
X	TAIRA K. ET AL.: "CONSTRUCTION OF SEVERAL KINDS OF RIBOZYMES THEIR REACTIVITIES AND UTILITIES" 1 January 1992 , GENE REGULATION, BIOLOGY OF ANTISENSE RNA AND DNA, PAGES 35 - 54 , EDITORS: ERICKSON R. P. AND IZANT J. G. XP002002021	33-35
A	see the whole document, especially Fig.2 and page 36	36,37
A	TAIRA K. ET AL.: "CONSTRUCTION OF A NOVEL ARTIFICIAL-RIBOZYME-RELEASING PLASMID" PROTEIN ENGINEERING, vol. 3, no. 8, 1 August 1990, pages 733-737, XP000563750 see the whole document	33-37
X	DAVRINCHE C. ET AL.: "Expression of human cytomegalovirus immediate early protein IE1 in insect cells: splicing of RNA and recognition by CD4+ T-cell clones." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 195, no. 1, 1993, pages 469-477, XP002079917 see the whole document	38
X	WO 89 01029 A (CETUS CORP) 9 February 1989 see the whole document	39

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 98/01626

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	GOLDMANN C. ET AL.: "Cell-specific and efficient gene delivery systems based on virus-like particles." JOURNAL OF MOLECULAR MEDICINE, vol. 75, no. 7, July 1997, pages b210-b211, XP002079918 see abstract	1-32

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INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/GB 98/01626

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

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

1. [X] Claims Nos.: 40
because they relate to subject matter not required to be searched by this Authority, namely:
Said claim relates to nothing specific but the whole disclosure 'with reference to the accompanying Figures'. Consequently, the scope of said claim is unclear and not sufficiently specified.

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:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] 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/ GB 98/01626

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-37,40

   A composition comprising at least one baculoviral component and at least one retroviral component that can be packaged into a retroviral vector particle; an insect cell comprising said composition; a process and a production system for preparing a retroviral vector particle employing said composition; the retroviral vector particle prepared by said production system; an expression vector comprising a retroviral vector genome that is capable of being expressed and packaged into a retroviral vector particle in a baculovirus expression system; a composition comprising a first viral component

   from a first virus and a second viral component from a second virus which is flanked by at least two cleavage sites and at least a part of which can be packaged into a viral particle which is substantially free of the first viral component.

2. Claim : 38

   Use of a baculoviral composition to express a NOI comprising at least one intron.

3. Claim : 39

   A production facility for producting a NOI or an expression product thereof, which contains a medium comprising a baculovirus composition which comprises the NOI.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter... ...nal Application No
PCT/GB 98/01626

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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