**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 15/10, C07K 14/025

A2

(11) International Publication Number: **WO 98/12339**

(43) International Publication Date: 26 March 1998 (26.03.98)

(21) International Application Number: PCT/US97/17579

(22) International Filing Date: 22 September 1997 (22.09.97)

(30) Priority Data:

| | | |
|---|---|---|
| 08/716,926 | 20 September 1996 (20.09.96) | US |
| 08/820,931 | 19 March 1997 (19.03.97) | US |

(71) Applicant *(for all designated States except US)*: COLD SPRING HARBOR LABORATORY [US/US]; One Bungtown Road, Cold Spring Harbor, NY 11724 (US).

(72) Inventors; and

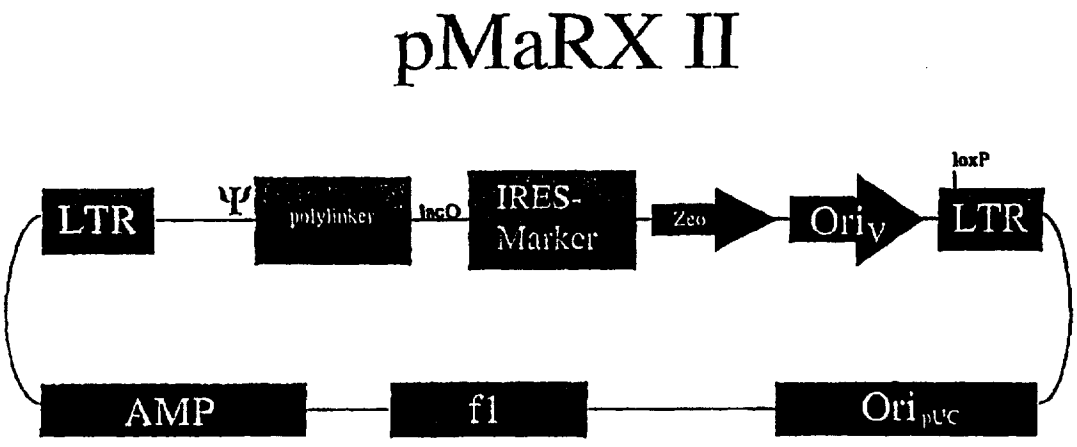
(75) Inventors/Applicants *(for US only)*: BEACH, David, H. [GB/US]; 19 Woodland Drive, Huntington Bay, NY 11742 (US). HANNON, Gregory, J. [US/US]; 92 Sammis Street, Huntington, NY 11743 (US). CONKLIN, Douglas, S. [US/US]; 414 Arthur Street, Huntington Bay, NY 11721 (US). SUN, Peiquing [CN/US]; Apartment #25, 40 Oakwood Road, Huntigton, NY 11743 (US).

(74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot LLP, One Post Office Square, Boston, MA 02109 (US).

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

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: VIRAL VECTORS AND THEIR USES




(57) Abstract

The present invention relates to methods and compositions for the elucidation of mammalian gene function. Specifically, the present invention relates to methods and compositions for improved mammalian complementation screening, functional inactivation of specific essential or non-essential mammalian genes, and identification of mammalian genes which are modulated in response to specific stimuli. In particular, the compositions of the present invention include, but are not limited to, replication-deficient retroviral vectors, libraries comprising such vectors, retroviral particles produced by such vectors in conjunction with retroviral packaging cell lines, integrated provirus sequences derived from the retroviral particles of the invention and circularized provirus sequences which have been excised from the integrated provirus sequences of the invention. The compositions of the present invention further include novel retroviral packaging cell lines.

*FOR THE PURPOSES OF INFORMATION ONLY*

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

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# *Viral Vectors and Their Uses*

**Funding**

Work described herein was supported by funding from the National Institutes of Health. The United States Government has certain rights in the invention.

**Introduction**

The present invention relates to methods and compositions for the elucidation of mammalian gene function. Specifically, the present invention relates to methods and compositions for improved mammalian complementation screening, functional inactivation of specific essential or non-essential mammalian genes, identification of mammalian genes which are modulated in response to specific stimuli, identification of secreted proteins and improved cell packaging.

**Background of the Invention**

In yeast genetic systems, many options are available for delivery of gene sequences for the purpose of conferring a phenotype onto the host cell. For example, one common delivery system is a high copy plasmid system based on the endogenous yeast 2-micron plasmid. Plasmids from this origin achieve copy numbers of roughly 100 per cell and are randomly segregated to daughter cells upon division. In another system, the CEN system. CEN plasmids are maintained at low copy number (approximately 1 to 2 per cell) are segregated to daughter cells by the same mechanism used for segregation of the host chromosomes.

Further, methods have been devised in yeast by which the problems of gene isolation and discovery of gene function can be addressed efficiently. For example, in yeast it is possible to isolate genes via their ability to complement specific phenotypes. Further, in yeast, targeted insertional mutagenesis techniques can be used in yeast to inactivate or "knock out" a gene's activity. In mammalian systems, however, such methods are, in practical terms, lacking, which has made the elucidation of mammalian gene function a very difficult task.

For example, with respect to gene inactivation techniques in mammalian cells, the fact that mammalian cells are diploid and have complex genomes cause insertional

mutagenesis techniques in mammalian systems to be a laborious, time-consuming and lengthy process.

Further, a major barrier to the development of such capabilities as complementation screening in mammalian cells has been that conventional techniques yield gene transfer efficiencies in most cells (0.01%-0.1%) that make screening of high complexity libraries impractical. While reports indicate that recombinant, replication deficient retroviruses can make possible increased gene transfer efficiencies in mammalian cells (Rayner & Gonda, 1994, Mol. Cell. Biol. 14:880-887; Whitehead et al., 1995, Mol. Cell. Biol. 15:704-710), retroviral-based functional mammalian cloning systems are inconvenient and have, thus far, failed to achieve widespread use.

The lack of convenience and impracticality of current retroviral-based cloning systems include, for example, the fact that the production of high complexity libraries has been limited by the low transfection efficiency of known retroviral packaging cell lines. Furthermore, no system has provided for routine, easy recovery of integrated retroviral proviruses from the genomes of positive clones. For example, in currently used systems the recovery of retrovirus inserts may be accomplished by polymerase chain reaction (PCR) techniques, however this is quite time consuming and variable for different inserts. Furthermore, with the use of PCR, additional cloning steps are still required to generate viral vectors for subsequent screening. Additionally, no mechanism has been available for distinguishing revertants from provirus-dependent rescues, a major source of false positives.

Further, it would be advantageous if an episomal system such as those found in yeast existed for efficient, broad spectrum use in mammalian systems. While bovine papillomaviruses (BPV), for example, replicate as extrachromosomal episomes, their use in developing episomal vectors has been limited.

Specifically, the ability of BPV replicate as episomes has been exploited in the past to create episomal vectors, using the so-called 69% fragment (T69). Vectors based upon T69 replicate in certain murine cell lines to give copy numbers that range from 15 to 500 copies per haploid genome, depending on the cell line. T69 vectors, however, exhibit a narrow host range. Further, the T69 fragment, like SV40, is oncogenic. Indeed, one method for identifying cells carrying T69 vectors specifically involves screening for transformed C127 cells.

## Summary of the Invention

The present invention relates to methods and compositions for the elucidation of mammalian gene function. Such methods can utilize novel integrating and/or episomal genetic delivery systems, thereby providing flexible, alternate genetic platforms for use in a wide spectrum of mammalian cells, including human cells. Specifically, the present invention relates to methods and compositions for improved mammalian complementation screening, functional inactivation of specific essential or non-essential mammalian genes, identification of mammalian genes which are modulated in response to specific stimuli, identification of mammalian genes that encode secreted products, and production and selection of novel retroviral packaging cell lines.

In particular, the compositions of the present invention include, but are not limited to, replication-deficient retroviral vectors, libraries comprising such vectors, retroviral particles produced by such vectors in conjunction with retroviral packaging cell lines, integrated provirus sequences derived from the retroviral particles of the invention and circularized provirus sequences which have been excised from the integrated provirus sequences of the invention.

The compositions of the present invention further include ones relating to improved mammalian episomal vectors. In particular, these compositions include, but are not limited to, expanded host range vectors (pEHRE), and libraries, cells and animals containing such vectors. The pEHRE vectors of the invention provide a consistent, stable, high-level episomal expression of gene sequences within a broad spectrum of mammalian cells. The pEHRE vectors of the invention comprise, first, replication cassettes in which papillomavirus (PV) E1 and E2 proteins are expressed from a constitutive transcriptional regulatory sequence or sequences, and, second, minimal cis-acting elements for replication and stable episomal maintenance.

The pEHRE vectors of the invention include, but are not limited to, vectors for delivery of sense and antisense expression cassettes, regulated expression cassettes, large chromosomal segments, and cDNA libraries, to a wide range of mammalian cells. Among the pEHRE vectors presented are ones which, additionally, can be utilized for the large scale production of recombinant proteins, and ones which can be utilized in the construction of cell lines that stably produce high titer viruses.

The compositions of the present invention further include novel viral packaging cell lines. In particualr, described herein are novel, stable retroviral packaging cell lines which efficiently package retroviral-derived nucleic acid into replication-deficient retroviral particles capable of infecting appropriate mammalian cells. Such packaging cell lines are produced by a novel method which directly links the expression of desirable viral

proteins with expression of a selectable marker.

The retroviral packaging cell lines of the invention provide retroviral packaging functions as part of a polycistronic message which allowing direct selection for the expression of such viral functions and, further, makes possible a quantitative selection for the highest expression of desirable sequences.

In particular, the methods of the present invention include, but are not limited to, methods for the identification and isolation of nucleic acid molecules based upon their ability to complement a mammalian cellular phenotype, antisense-based methods for the identification and isolation of nucleic acid sequences which inhibit the function of a mammalian gene, gene trapping methods for the identification and isolation of mammalian genes which are modulated in response to specific stimuli, methods for efficient large scale recombinant protein expression and methods for modulating the expression of known genes.

**Brief Description of the Figures**

FIGURE 1. The arrangement of DNA elements that comprise the replication-defective retroviral vector, MaRXII. psi denotes the packaging signal.

FIGURE 2. Diagrammatic representation of the cleavage of the loxP sites with Cre recombinase enzyme, yielding an excised provirus which upon excision, becomes circularized.

FIGURE 3. The arrangement of DNA elements that comprise the retroviral vector for expression/sense complementation screening, p.hygro.MaRXII-LI.

FIGURE 4. The arrangement of DNA elements that comprise a retroviral vector for peptide display, pMODis-I.

FIGURE 5. The arrangement of DNA elements that comprise a retroviral vector for peptide display, pMODis-II.

FIGURE 6. The arrangement of DNA elements that comprise the retroviral vector for gene trapping, pTRAPII.

FIGURE 7. The arrangement of DNA elements that comprise a retroviral vector for antisense complementation screening, pMaRXIIg.

FIGURE 8. The arrangement of DNA elements that comprise a retroviral vector for antisense complementation screening, pMaRXIIg-demV.

FIGURE 9. The arrangement of DNA elements that comprise a retroviral vector for antisense complementation screening, pMaRXIIg-va.

FIGURE 10. The arrangement of DNA elements that comprise a pEHRE vector for expression/sense complementation screening, pEHRE-E-H.

FIGURE 11. The arrangement of DNA elements that comprise a pEHRE vector for large scale protein production, pEHRE-H.

FIGURE 12. The arrangement of DNA elements that comprise a pEHRE vector for use in production of pEHRE/BAC hybrid constructs, pBPV-BacDonor.

FIGURE 13. The arrangement of DNA elements that comprise a pEHRE vector for use as a BAC cloning vector.

FIGURE 14. The arrangement of DNA elements that comprise a pEHRE antisense GSE vector, pEHRE-GSE-H.

FIGURE 15. The arrangement of DNA elements that comprise a pEHRE antisense GSE vector, pEHRE-GSEVA-H.

FIGURE 16. The arrangement of DNA elements that comprise a pEHRE antisense GSE vector, pEHRE-GSEU6-H.

FIGURE 17. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, $\psi_c$IH.

FIGURE 18. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, pEHRE-$\psi_c$IH.

FIGURE 19. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, $\psi_{env}$IH.

FIGURE 20. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, pEHRE-$\psi_{env}$IH.

FIGURE 21. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, $\psi_{g/p}$IH.

FIGURE 22. The arrangement of DNA elements that comprise a pEHRE vector for packaging cell line use, pEHRE-$\psi_{g/p}$IH.

FIGURE 23. The arrangement of DNA elements that comprise a representative retroviral secretion trapping vector.

FIGURE 24. A graph showing the relative stability of the linX packaging cell line as compared to the Phoenix and bosc23 cell lines.

FIGURE 25. An exemplary use of the reunification plasmid to restore LTR elements to excised proviral vectors.

**Detailed Description of the Invention**

Expression cloning of cDNAs using mammalian cells has been a long sought after goal in molecular biology. It is a potentially a powerful tool with which to isolate a nucleic acid of interest, such as a cDNA, under circumstances wherein a phenotypic function of a protein is known but its amino acid sequence is not known. For instance, many growth factor and cytokine genes were cloned by scoring for a growth-promoting activity of culture supernatant of COS cells transiently transfected with expression vectors engineered with cDNA libraries.

Many expression cloning systems of the prior art work on the principle of amplifying expression vectors carrying the SV40 replication origin (SV40 ori) in mammalian cells stably expressing T antigen (i.e., a transformed African green monkey kidney cell line, COS ). The presence of the SV40 large T antigen in COS cells allows replication of SV40 ori containing plasmids, thus amplifying expression of the cDNA on the plasmid.

Despite many successful applications, conventional expression cloning systems still suffer from the need for transient amplification of plasmids in particular cell lines expressing the SV40 (or polyoma) large T antigen. First of all, the function of the target gene has to be suited to transient detection. Moreover, target cells are restricted to those which allow SV40 large T antigen-based amplification and to those cell types in which the transfection efficiency is high (e.g., greater than 10%). Approaches using transient expression system in COS cells or fibroblasts have obvious limitations in searches for proteins with various functions in various types of cells.

I. Overview

To overcome these limitations, one aspect of the present invention relates to high efficiency viral expression cloning systems. In one embodiment, the subject expression constructs are generated using viral vectors which can be stably integrated into the genome of a metazoan host cell, particularly a mammalian host cell. To illustrate, in one embodiment a preferred viral expression construct is derived from a retroviral vector which, in addition to being capable of expressing a heterologous gene when integrated in the host cell, also includes one or more various other features including, e.g., means for

excising the retroviral vector from the genome of the host cell, means for recovering the excised vector, and/or means for amplifying or otherwise manipulating the vector in prokaryotic cells. Other variations are described more fully below.

To further illustrate, the subject viral vectors can be engineered with a nucleic acid library of interest, and as appropriate, infectious particles produced. For packaging into viral particles, viral packaging system known in the art can be used, or, more preferably, the viral vectors can be packaged with the novel transient packaging system described herein. The engineered virus is than used to to infect a selected host cell. The infected cells can subsequently be screened for expression of nucleic acid of interest, e.g., based on a change in phenotype of the cell.

According to the present invention, expression cloning systems based on high complexity viral libraries can allow investigatory access to many important cell types and cell signaling systems not previously accessible by prior techniques. The subject viral cDNA library transfer approaches offer numerous advantages to those interested in complementation cloning in, for example, mammalian cells. For instance, in contrast to transient transfection of plasmids, gene transfer with such viral vectors as, for example, the exemplary retroviruses and adeno-associated viruses, can deliver genes stably into a wide range of target cells. This feature helps to overcome a disadvantage of conventional transient gene expression for phenotypic selection by extending the amount of time over which the phenotypic change can be observed.

Moreover, the use the subject viral vectors can also overcome another major limitation in the art, that of generally low transfection rates which otherwise makes adequate representation of genes in complex nucleic acid libraries difficult. In contrast to transfection, the subject virus can efficiently infect and transfer genes to a wide range of cells.

Thus, the power of complementation cloning, long appreciated in bacterial and yeast genetic systems, may now be more fully accessed for mammalian and other metazoan cells by the viral-based approches we describe herein.

As described with greater detail below, such compositions of the present invention include, but are not limited to, replication-deficient retroviral vectors, libraries comprising such vectors, retroviral particles produced by such vectors in conjunction with retroviral packaging cell lines, integrated provirus sequences derived from the retroviral particles of the invention and circularized provirus sequences which have been excised from the integrated provirus sequences of the invention. Similar compositions derived using viral sequences for other genomically-incorporated viruses are also specifically contemplated,

including vectors based on the adeno-associated virus (AAV).

Yet another aspect of the present invention relates to episomal expression vectors which also can also be used to overcome certain of the above-described deficiencies in the mammalian expression cloning systems of the art. In particular, the compositions of the invention described herein further include improved mammalian episomal vectors as well as libraries, cells and animals containing such vectors. The compositions of the present invention described herein still further include novel viral, including retroviral, packaging cell lines.

Second, the methods of the invention are described. Such methods include, but are not limited to, methods for the identification and isolation of nucleic acid molecules which complement a mammalian cellular phenotype, antisense-based methods for the identification and isolation of nucleic acid sequences which inhibit the function of a mammalian gene, gene trapping methods for the identification and isolation of mammalian genes which are modulated in response to specific stimuli, methods for the identification of mammalian genes that encode secreted proteins, methods for the selection and production of novel viral packaging cell lines and methods for efficient large scale recombinant protein expression.

The methods of the present invention also include, but are not limited to, methods for the identification and isolation of peptide sequences by complementation type screens using vectors capable of displaying random or semi-random peptide sequences which will interact with proteins important for a particular cellular or viral function. This interaction can result in, e.g., the elaboration of selectable phenotype.

## II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid which is transcribed and (optionally) translated. Thus, a recombinant gene can comprise an open reading frame encoding a polypeptide, including both exon and (optionally) intron

sequences. In other embodiments, a recombinant gene can simply provide, on transcription, an antisense transcript, a ribozyme, or other RNA molecule for which the effect of transcription on the phenotype of the cell is to be scored.

By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence. On the other hand, "expression" of an antisense sequence or ribozyme will be understood to refer to the transcription of the recombinant gene sequence.

As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, e.g., a viral expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a polypeptide or, where anti-sense expression occurs from the transferred gene, the expression of a naturally-occurring form of a protein is disrupted.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein.

A cell has been "stably transfected" with a nucleic acid construct comprising viral coding regions when the nucleic acid construct has been introduced inside the cell membrane and the viral coding regions are capable of being inherited by daughter cells.

As used herein, the term "specifically hybridizes" refers to the ability of a first nucleic acid to hybridize to at least 15 consecutive nucleotides of a second nucleic acid, such as an endogenous gene or gene transcript, such that the hybridization is accompanied by less than 15%, preferably less than 10%, and more preferably less than 5% background hybridization to other cellular or viral nucleic acid (e.g., mRNA or genomic DNA).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is an genomic integrated vector, or "integrated vector", which can become integrated into the chromsomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as

"expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of a gene with which they are operably linked.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of neuronal or hematopoietic origin. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but can cause at least low level expression in other tissues as well.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule.

The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, livestock, avian species, amphibians, reptiles, etc. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be

precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

A "packaging cell" refers to a host cell which, by way of stable or transient transfection with heterologous nucleotide sequences, harbors a nucleic acid molecule comprising an viral helper construct, wherein the construct is capable of providing transient expression of packaging functions, e.g., proteins necessary for replication and encapsidation, that can be provided in trans for production of infectious viral particles. Expression of the viral helper functions can be either constitutive, or inducible, such as when the helper functions are under the control of an inducible promoter.

A "chimeric protein" or "fusion protein" is a fusion of two amino acid sequences of heterologous origin, by generating a chimeric coding sequence in which the coding sequences for the first and second polypeptide are fused in frame so as to produce, upon initial translation, a single polypeptide chain.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

A "coding sequence" or a sequence which "encodes" a particular polypeptide, is a

nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

By a "DNA binding domain" or "DBD" is meant a polypeptide sequence which is capable of directing specific polypeptide binding to a particular DNA sequence (i.e., to a DBD recognition element). The term "domain" in this context is not intended to be limited to a discrete folding domain. Rather, consideration of a polypeptide as a DBD for use in the bait fusion protein can be made simply by the observation that the polypeptide has a specific DNA binding activity. DNA binding domains, like activation tags, can be derived from proteins ranging from naturally occurring proteins to completely artificial sequences.

Throughout the application, there may be reference to particular transcriptional regulatory sequences, origins of replication, secretion signal sequences, viral vectors, etc. However, it will be appreciated that, unless clearly contrary from the context, many of these specific recitations are intended merely to be illustrative of broader classes of elements which can be used as equivalents.

III. Complementation screening and expression vectors

A principle goal of our work leading up to the present invention was to address many of the shortcomings of conventional cloning and other genetic manipulation systems utilized in mammalian and other metazoan cells. In this regard, the subject viral expression vectors are designed to possess such features as: highly efficient gene transfer; predictable expression levels; coincidence of gene transfer and expression; the ability to identify revertants; relatively easy recovery of the expressed nucleic acid; convenient secondary screens; and facile addition of heterologous DNA, e.g., in library construction. Relative to many other customary mammalian cloning vectors, the subject viral expression vectors also exhibit broad host range specificity for transduction, e.g., so that loss-of-function and/or gain-of-function type constructs can be investigated in biologically relevant cell-types.

In one aspect, the expression cloning systems of the present invention are based on

the use vectors which can be integrated into the genome of a host cell, particularly a mammalian host cell. Exemplary vectors of this sort are derived from e.g., retroviruses. adeno-associated viruses or other virally-derived vectors with appropriate transposition elements for chromosomal integration. Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the subject vectors, particularly for use with mammalian cells. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. In addition, the subject vectors also include one or more other features including, for example, a proviral excision element for excising the retroviral vector from the genome of the host cell, a proviral recovery element for recovering the excised vector, and/or an origin of replication for amplifying or otherwise manipulating the vector in prokaryotic cells. Preferably, the resulting viral vectors are replication-deficient, and, although the virus can have any tropism, they are also preferably amphotrophic with respect to humans. These and other aspects of the subject vectors are described more fully below.

In other embodiments, the expression cloning systems of the present invention are based on episomal vectors which can be maintained at high, but stable, copy numbers in the host cells, and which can deliver uniformly high levels of transcription of a heterologous nucleic acid. In the prior art system, such as the COS cell system discussed above, episomal replication can proceed in a runaway fashion, e.g., resulting in up to $10^4$ episomal copies by 48 hours after transfection. Despite efficient episomal replication in such transient transfectants, low stable transfection efficiencies have been noted (e.g., Chittenden et al, (1991) *J Virol* 65:5944), presumably because most transfectants die as a result of episome-mediated toxicity. However, the episomal vectors of the present invention provide a strategy for controlling runaway replication to yield episomal copy numbers which can persist through many generations of progeny cells. In preferred embodiments, the episomal vectors of the present invention will include a viral origin of replication, along with other necessary replication control regions, and one or more viral genes that transactivate the viral origin so as to facilitate replication of the vector to a stable copy number. It will be appreciated, however, that the viral transactivating gene(s) can be provided on separate vectors in the cell. Exemplary episomal espression vectors of the present invention include papillomavirus (PV)-derived vectors, Epstein Barr virus (EBV)-derived vectors and BK virus (BKV)-derived vectors.

Expression cloning takes on various forms depending on the mode of detection utilized to identify the nucleic acid of interest (see discussion, *infra*). However, irrespective of whether the integrating vectors or episomal vectors are utilized, the initial

step consists of generating the nucleic acid library, such as by isolating mRNA and synthesizing double-stranded deoxyribonucleic acid copies of the mRNA population (cDNAs). The vareigated population of nucleic acids must then be efficiently ligated to a vector of the present invention and transferred to the appropriate host cell prior to library screening and analysis. The subject vectors contain sets of restriction sites, making themamenable to the "adaptor" linker procedure of ligating cDNAs and other nucleic acids into the vector sequences. Also described below are various transcriptional regulatory sequences which can be used to facilitate transcription of the nucleic acid sequence of interest.

*A) Retroviral complementation screening and expression vectors*

Retroviruses are RNA viruses; that is, the viral genome is RNA. This genomic RNA is, however, reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. The integrated DNA intermediate is referred to as a provirus. The retroviral genome and the proviral DNA include three genes important to the life cycle of the vrius: the gag, the pol and the env genes. The genome of the virus is flanked at each end by long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase); and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs.

Adjacent (downstream) to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann et al. (1983) *Cell* 33:153-159; Cone et al. (1984) *PNAS*, 81:6349-6353.

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant, a "replication-deficient" retrovirus, is still capable of directing the synthesis of all virion proteins.

In choosing retroviral vectors, it is also important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the subject expression constructs, is that the target cells must be dividing. However, while most retroviral vectors require cell division, those based upon lentiviruses, such as HIV or EIAV, do not.

Replication-deficient retroviral vectors compositions are described herein which comprise a combination of features that make possible, for the first time, practical, efficient complementation screening in mammalian cells. Such vectors can also act as efficient expression vectors.

Such retroviral vectors comprise a replication-deficient retroviral genome containing one or more feactures such as a polycistronic message cassette, a proviral excision element for excising retroviral provirus from the genome of a recipient cell and a proviral recovery element for recovering excised provirus from a complex mixture of nucleic acid. The vectors are designed to facilitate expression of, for example, cDNA or genomic DNA (gDNA) sequences in mammalian cells.

In an illustrative embodiment, the retroviral vectors may include the following elements: (a) a 5' retroviral long terminal repeat (5' LTR); (b) a 3' retroviral long terminal repeat (3' LTR); (c) a packaging signal; (d) a bacterial origin of replication; and (e) a bacterial selectable marker. The polycistronic message cassette, proviral recovery element, packaging signal, bacterial origin of replication and bacterial selectable marker are located within the retroviral vector at positions between the 5' LTR and the 3' LTR. The proviral excision element, as discussed below, is preferably located within the 3' LTR. In the alternative, the proviral excision element may also be located within the retroviral vector. However, this is not preferred, since, as elaborated below, one goal of the present invention is to provide a construct wherein the recovered plasmid can be used to directly generate a virus for subsequent rounds of infection.

A variety of different retroviruses are known in the art and can be readily adapted for use in the subject invention. By selection of appropriate amphotropic or ecotropic packaging cell lines, the subject vectors can be packaged as viral particles with suitable specificity for infecting the desired host cell(s). Furthermore, it is also possible to control the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to convert an ecotropic

vector in to an amphotropic vector, can also be used to limit or expand the specificity of the infectious particle for different cell-types of an animal.

Examples of suitable retroviruses which can be used to generate the subject viral vectors include pBABE, pLJ, pZIP, pWE and pEM, each of which are well known to those skilled in the art. In certain embodiments, the viral vector is derived from a lentivirus, such as a HIV or EIAV virus.

For instance, the pZip vector has been described by Cepko et al. (1984) *Cell* 37:1053. Briefly, this vector is capable of expressing two genes: the gene of interest and the Neogene as a selectable marker.

The pLJ vector have been described in Korman et al., (1987) *PNAS* 84:2150. This vector is capable of expressing two genes: the gene of interest and a dominant selectable marker, such as the Neogene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5' LTR, while, the Neogene is placed distal to an internal promoter (from SV40) which is farther 3' than is the cloning site (is located 3' of the cloning site). Transcription from PLJ is initiated at two sites: 1) the 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the Neogene.

The pWe vector has been described by Choudory et al (1986) *CSH Symposia Quantitative Biology* 1047. Briefly, this vector can drive expression of two genes: a dominant selectable marker, such as Neo, which is just downstream from the 5' LTR and a gene of interest which can be cloned into a BamHI site just downstream from an internal promoter capable of high level constitutive expression. Several different internal promoters, such as the beta-actin promoter from chicken (Choudory, P.V. et al, CSH Symposia Quantitative Biology, L.I. 1047 (1986)), and the histone H4 promoter from human (Hanly, S.M. et al., Molecular and Cellular Biology 5:380 (1985)) have been used. Expression of the Neogene is from a transcript initiated at the 5' LTR; expression of the gene of interest is from a transcript initiated at the internal promoter.

The pEm vector is a simple vector in which the entire coding sequence for gag, pol and env of the wild type virus is replaced with the gene of interest, which is the only gene expressed. The components of the pEm vector are described below. The 5' flanking sequence, 5' LTR and 400 bp of contiguous sequence (up to the BAMHI site) is from pZIP. The 3' flanking sequence and LTR are also from pZIP; however, the Cla site 150 bp upstream from the 3' LTR has been linkered with BamHI and forms the other half of the BamHI cloning site present in the vector. The HindIII/EcoR1 fragment of pBR322 forms the plasmid backbone. This vector is derived from sequences cloned from a strain of

Moloney Murine Leukemia virus. An analogous vector has been constructed from sequences derived from the myeloproliferative sarcoma virus.

The pIp vector is capable of expressing a single gene driven from an internal promoter. The construction of these vectors is summarized below. The 5' section of the vector, including the 5' flanking sequences, 5' LTR, and 1400 bp of contiguous sequence (up to the xho site in the gag region) is derived from wild type Moloney Leukemia virus sequence. Shinnick et al. (1981) *Nature* 293:543. The difference between the two is that a SacII linker is cloned into an HaeIII restriction site immediately adjacent to the ATG of the gag gene. The 3' section of the vector, including the 3' flanking sequences, 3' LTR and 3' contiguous sequence (up to the ClaI site in the env coding region) is from pZIP. However, there are two modifications: 1) the ClaI site has been linked to BamHI and 2) a small sequence in the 3' LTR spanning the enhancer (from PvuII to XbaI) has been deleted. Bridging the 5' and 3' sections of the vector is one of several promoters; each one is contained on a XhoI/BamHI fragment, and each is capable of high level constitutive expression in most tissues. These promoters include the β-actin promoter (Choudory et al, *supra*), and the thymidine kinase promoter from Herpes Simplex Virus (Hanly et al., (1985) *Mol Cell Biol* 5:380). The vector backbone is the HindIII/EcoRI fragment from pBR322.

The RO vectors represent a heterogeneous group of vectors in which the gene of interest contains all the sequences necessary for transcription (i.e., promoter/enhancer, coding sequence with and without introns, and poly adenylation signal) and is introduced into the retroviral vector in an orientation in which its transcription is in a direction opposite to that of normal retroviral transcription. This makes it possible to include more of the cis-acting elements involved in the regulation of the introduced gene. Virtually, any of the above described genes can be adapted to be a RO vector.

In still other embodiments, it is possible to change the infectivity spectrum of a virus by causing a cell to express a viral receptor (e.g., cell surface protein) which mediates infection by the virus in other species. Thus, for example, human cells can be rendered susceptible to infection with otherwise ecotropic avian virus by causing the human host cells to express an avian gene encoding a receptor for the avian virus.

For embodiments in which it is included, the polycistronic message cassette makes possible a selection scheme which directly links expression of a selectable marker to transcription of a nucleic acid sequence of interest. Such a polycistronic message cassette can comprise, in an exemplary embodiment, from 5' to 3', the following elements: a nucleotide polylinker, an (optional) internal ribosome entry site (IRES) and a mammalian

selectable marker. The polycistronic cassette is preferably situated within the retroviral vector between the 5' LTR and the 3' LTR at a position such that transcription from the 5' LTR promoter or other transcriptional regulatory sequence transcribes the polycistronic message cassette. In the instance of the latter, the transcription of the polycistronic message cassette may be under the transcriptional control of a constitutive regulatory element, e.g., driven by an internal cytomegalovirus (CMV) promoter, or an inducible regulatory element, as may be preferable depending on the expression screen used. The polycistronic message cassette can further comprise a cDNA, genomic DNA (gDNA) or other nucleic acid sequence operatively associated within the polylinker.

In the subject constructs, the IRES element permits the efficient translation of two or more open reading frames from one messenger RNA: one reading frame, for example, encoding a recombinant protein of interest (such as from a cDNA library) and another an selectable marker (e.g. hygromycin) for selecting cells which express the polycistronic message to some extent.

Bicistronic or multicistronic vectors were developed in order to avoid the problems connected with the stability of the mRNA of different transcripts. For this purpose, the individual reading frames for each transcript (e.g., encoding a protein, providing an antisense transcript, etc) are provided in a single transcription unit (expression unit). Expression of the multicistronic gene is effected using a single promoter or regulatory sequence. While the first cistron in such vectors is normally translated very efficiently, translation of the subsequent cistrons depends on the intercistronic sequences. It was subsequently possible, with the discovery and use of particular cellular and viral sequences which render possible internal initiation of translation, such as internal ribosome entry sequences or IRES, to achieve a translation ratio between the first and subsequent cistron of 3:1.

A mechanism for initiation translation internally, discovered in recent years, makes use of specific nucleic acid sequences. The sequences include the untranslated regions of individual picorna viruses, e.g. poliovirus and encephalomyocarditis virus, (Pelletier and Sonenberg, (1988) *Nature* 334:230; Jang et al., (1988) *J. Virol.* 62:2636; Jang et al., (1989) *J. Virol.* 63:1641) as well as some cellular proteins, e.g. BiP (Macejak and Sarnow (1991) *Nature* 353:90-94). In the picorna viruses, a short segment of the 5' untranslated region, the so-called IRES or internal ribosomal entry site), is responsible for the internal binding of a preinitiation complex. IRES elements can function as initiators of the efficient translation of tandemly linked reading frames. The close coupling of the expression of the selective marker with that of the gene to be expressed is particularly advantageous when selecting for a high level of expression, in particular if prior gene

amplification is required.

Internal ribosome entry site sequences are well known to those of skill in the art and can comprise, for example, internal ribosome entry sites derived from foot and mouth disease virus (FDV), encephalomyocarditis virus, poliovirus and RDV (Scheper, 1994, Biochemic 76: 801-809; Meyer, 1995, J. Virol. 69: 2819-2824; Jang, 1988, J. Virol. 62: 2636-2643; Haller, 1992, J. Virol. 66: 5075-5086). Another exemplary bicistronic transcript of the subject vectors contains the 373-nucleotide-long 5' nontranslated region (NTR) of the classical swine fever virus (CSFV) genome as an intercistronic spacer (Rijnbrand et al. (1997) *J Virol* 71:451. The 'R' regions from HTLV-1 also has properties similar to internal ribosome entry sites ( IRES) originally found in picornavirus, Attal et al.(1996) *FEBS Lett* 392: 220, and can the IRES of that virus can be used in the subject expression constructs. Translation of aphthovirus RNA is initiated at an internal ribosome entry site ( IRES) element which can also be used in the subject vectors.

The subject vectors should also include one or more selectable marker genes. Preferably, at least one of the selectable marker genes is provided in a polycistronic transcript with a gene of interest. Any mammalian selectable marker can be utilized. The marker gene is generally one which encodes a product which is necessary for the survival or growth of a host cell transformed with the vector, and/or which can be scored for by a technique which allows cells to be segrated (and retain viability) on the basis of expression of the selectable marker. The expression of this gene product ensures that any host cell which is not transformed with the vector, or which deletes the vector or otherwises losses expression of the selectable marker will not obtain an advantage in growth, etc., over cells retaining a functional vector. Typical selection genes may encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media.

Examples of suitable drug selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase and genes encoding resistance to kanamycin/G418, hygromycin, mycohenolic acid or neomycin. Such markers enable the identification of cells which were competent to take up, and to retain over time, the subject expression vector. The mammalian cell transformants can be placed under selective conditions wherein only the transformants are uniquely adapted to survive by virtue of having taken up the vector and expressing the marker gene. Selective pressure is imposed, for example, by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to selection of transformant with amplified expression of the selection gene, and, in the polycistronic

embodiments, amplified expression of other linked coding sequences.

To illustrate, DHFR$^-$ cells which have successfully been transformed with a viral vector including the DHFR selection gene can be identified by culturing the transformants in a culture medium which lacks hypoxanthine, glycine, and thymidine. Cells which can grow under such conditions presumably express the DHFR selection gene provided in the viral vector.

In other embodiments, the marker gene can encode a protein which is detectable by FACS sorting, e.g., the marker gene can be any gene that encodes a FACS detectable gene product, which may be RNA or protein. There are at least two basic designs for such marker genes. In a "direct detection system" the marker gene encodes a product which is readily detectable by flow cytometry due to its own fluorescence activity (a "direct FACS tag"). In the alternative, the marker gene is used in an "indirect detection system", e.g., wherein the marker gene product is detected by FACS upon combination with a fluorescently active agent which specifically binds to and/or is modified by the marker gene product. Thus, the marker gene may encode a "direct FACS tag", e.g., a fluorescent polypeptide or a polypeptide which may generate a fluorescent signal by enzymatic action, or an "indirect FACS tag", e.g., a polypeptide which binds and/or modifies a fluorescently active molecule to generate a fluorescent signal. Chemiluminescent reporter groups, which are for ease of reading referred to herein as fluorescent groups, are detected by allowing them to enter into a reaction, e.g., an enzymatic reaction, that results in energy in the form of light being emitted.

In one embodiment, the marker gene encodes a fluorescently active polypeptide. Examples of such marker genes include, but are not limited to firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); phycobiliproteins (especially phycoerythrin); green fluorescent protein (GFP: see Valdivia et al. (1996) Mol Microbiol 22: 367-78; Cormack et al. (1996) Gene 173 (1 Spec No): 33-8; and Fey et al. (1995) Gene 165:127-130. Both the GFPs and the phycobiliproteins have made an important contribution in FACS sorting generally because of their high extinction coefficient and high quantum yield, and are accordingly preferred products of the marker gene.

A preferred embodiment utilizes a GFP which has been engineered to have a higher quantum yield (brighter) and/or altered excitation spectra relative to wild-type GFPs. In general, the fluorescence levels of intracellular wild-type GFP are not bright enough for flow cytometry. However, a wide variety of engineered GFPs are known in

the art which show both improved brightness and signal-to-noise ratios. For instance, the subject reproter gene can encode a GFP-Bex1 (S65T, V163A) or GFP-Vex1 (S202F, T203I, V163A). See Anderson et al. (1996) Genetics 93:8508. Other modified GFPs are described, for example, in U.S. Patents 5,360,728 and 5,541,309 which describe modified forms of apoaequorin with increased bioluminescence.

In other embodiments, the marker gene encodes an enzyme which, by acting on a substrate, produces a fluorescently active product. For instance, fluoroscein-di-β-D-galactopyranoside (FDG) is a useful substrate for a marker gene encoding a β-galactosidase in detection by flow cytometry. See Plovins et al. (1994) Applied Envir Micro 60:4638; and Alvarez et al. (1993) Biotechniques 15:974.

In yet other embodiments, the marker gene product is not itself sufficiently fluorescently active for FACS purposes. Rather, the marker gene product is one which is able to bind to a molecule (or complex of molecules), referred to herein as a "secondary fluorescent tag", which provides a fluorescently active moiety for detection by FACS. A preferred criteria for the selection of the marker gene product in these embodiments is that the host cell, except for the marker gene product, does not produce any other protein, etc., which binds to the secondary fluorescent tag at any appreciable level which would confound the FACS sorting of the host cells.

In preferred embodiments of the indirect detection system, the marker gene encodes a protein which is associated with the cellular membrane and is at least partially exposed to the extracellular milieu. For instance, the indirect FACS tag can be a transmembrane protein having an extracellular domain, or an extracellular protein with some other form of membrane localization signal which keeps the tag sequestered on the surface of the host cell, e.g., such as a myristol, farnesyl or other prenyl group. The indirect FACS tag can be a protein which is native to the host cell, but not normally expressed in the cell either because of its strain or the conditions under which the selection is carried out. In other embodiments, the indirect FACS tag is a protein which includes a portion that is non-native to the host cell, e.g., it is a naturally occurring polypeptide sequence from another species or it is man-made polypeptide sequence, and it is the heterologous portion of the fusion protein which is bound by the secondary fluorescent tag.

Where the marker utilizes an indirect FACS tag, a secondary fluorescent tag must be provided in order to label the cells of FACS. The secondary fluorescent tag can be a fluorescently-labeled antibody or other binding moiety which specifically binds to the indirect FACS tag on the surface of the ITS cell. Where the indirect FACS tag is a

receptor, or at least ligand binding domain thereof, the secondary fluorescent tags can also be a fluorescently-labeled ligand of the receptor. Such ligands can be polypeptides or small molecules.

In general, for use in flow cytometry, the fluorescently active tag should preferably have the following characteristics:

(i) the molecules of the secondary fluorecent tag must be of sufficient size and chemical reactivity to be conjugated to a suitable fluorescent dye or the secondary fluorecent tag must itself be fluorescent,

(ii) after any necessary fluorescent labeling, the secondary fluorecent tag preferably does not react with water,

(iii) after any necessary fluorescent labeling, the secondary fluorecent tag preferably does not bind or degrade proteins in a non-specific way, and

(iv) the molecules of the secondary fluorecent tag must be sufficiently large that attaching a suitable dye allows enough unaltered surface area (generally at least 500Å$^2$, excluding the atom that is connected to the linker) for binding to the indirect FACS tag on the cell.

Fluorescent groups with which the process of this invention can be used include fluorescein derivatives (such as fluorescein isothiocyanate), coumarin derivatives (such as aminomethyl coumarin), rhodamine derivatives (such as tetramethyl rhodamine or Texas Red), peridinin chlorophyll complex (such as described in U.S. Pat. No. 4,876,190), and phycobiliproteins (especially phycoerythrin).

In one preferred embodiment of the process, when the marker group is fluorescein, detection of the cells by FACS is achieved by measuring light emitted at wavelengths between about 520 nm and 560 nm (especially at about 520 nm), most preferably where the excitation wavelengths is about or less than 520 nm.

Chemiluminescent groups with which the subject secondary fluorescent tags can be generated include isoluminol (or 4-aminophthalhydrazide).

In other instances, the marker gene can encode a nucleic acid which can be detected by flow cytometry upon interaction with a FACS label. In one embodiment, the marker gene can "encode" a ribozyme, and detection of fluorescently active nucleic acid fragments can be detected for flow sorting upon addition of an appropriately labeled substrate for the ribozyme. For instance, the substrate nucleic acid can include a fluorogenic donor radical, e.g., a fluorescence emitting radical, and an acceptor radical, e.g., an aromatic radical which absorbs the fluorescence energy of the fluorogenic donor

radical when the acceptor radical and the fluorogenic donor radical are covalently held in close proximity. See, for example, USSN 5,527,681, 5,506,115, 5,429,766, 5,424,186, and 5,316,691; and Capobianco et al. (1992) Anal Biochem 204:96-102. For example, the substrate nucleic acid has a fluorescence donor group such as 1-aminobenzoic acid (anthranilic acid or ABZ) or aminomethylcoumarin (AMC) located at one position on the plymer and a fluorescence quencher group, such as lucifer yellow, methyl red or nitrobenzo-2-oxo-1,3-diazole (NBD), at a different position. A cleavage site for the ribozyme will be diposed between each of the sites for the donor and acceptor groups. The intramolecular resonance energy transfer from the fluorescence donor molecule to the quencher will quench the fluorescence of the donor molecule when the two are sufficiently proximate in space, e.g., when the substrate is intact. Upon cleavage of the substrate, however, the quencher is separated from the donor group, leaving behind a fluorescent fragment. Thus, expression of the ribozyme results in cleavage of the substrate nucleic acid, and dequenching of the fluorescent group. Similar embodiments can be generated for peptide-based substrates of enzymes.

The retroviral vectors' proviral excision element allows for excision of retroviral provirus (see below) from the genome of a recipient cell. The element comprises a nucleotide sequence which is specifically recognized by a recombinase enzyme, a restriction enzyme, or other enzyme or agent capable of selectively cleaving genomic DNA in a sequence-dependent manner. The recombinase enzyme cleaves nucleic acid at its site of recognition in such a manner that excision via recombinase action leads to circularization of the excised nucleic acid molecules. In the case of restriction enzymes, the excised retroviral sequences can remain linear, or can be circularized by religation.

Enzyme-assisted site-specific integration systems are known in the art and can be applied to the vector system of the invention to excise the viral DNA.. Examples of such enzyme-assisted integration systems include the Cre recombinase -lox target system (e.g., as described in Baubonis, W. and Sauer, B. (1993) Nucl. Acids Res. 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:7905-7909) and the FLP recombinase -FRT target system (e.g., as described in Dang, D. T. and Perrimon, N. (1992) Dev. Genet. 13:367-375; and Fiering, S. et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:8469-8473); the Piv site-specific DNA recombinase from Moraxella lacunata (e.g., described by Lenich et al. (1994) *J Bacteriol* 176) 4160); Lambda integrase (e..g, Kwon et al. (1997) *Science* 276:126;

By " recombinase target site" (RTS) herein is meant a nucleic acid sequence which is recognized by a recombinase for the excision of the intervening sequence. It is to be understood that two RTSs are required for excision. Thus, when the cre recombinase is

used, each RTS comprises a loxP site; when loxP sites are used, the corresponding recombinase is the cre recombinase. That is, the recombinase must correspond to or recognize the RTSs. When the FLP recombinase is used, each RTS comprises a FLP recombination target site (FRT); when FRT sites are used, the corresponding recombinase is the FLP recombinase.

A number of different site specific recombinase systems can be used, including but not limited to the Cre/ lox system of bacteriophage P1, the FLP/ FRT system of yeast, the Gin recombinase of phage Mu, the Pin recombinase of E. coli, and the R/RS system of the pSR1 plasmid. The two preferred site specific recombinase systems are the bacteriophage P1 Cre/ lox and the yeast FLP/ FRT systems. In these systems a recombinase (Cre or FLP) will interact specifically with its respective site-specific recombination sequence (lox or FRT respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT). Currently the FLP/FRT system of yeast is the preferred site specific recombinase system since it normally functions in a eukaryotic organism (yeast), and is well characterized.

In a preferred embodiment, the recombinase recognition site is located within the 3' LTR at a position which is duplicated upon integration of the provirus. This results in a provirus that is flanked by recombinase sites.

In an exemplary embodiment, the proviral excision element comprises a loxP recombination site located in the LTR. Contacting Cre recombinase to an integrated provirus derived from the retroviral vector results in excision of the provirus nucleic acid. In the alternative, a mutant lox P recombination site any be used (e.g., lox P511 (Hoess et al., 1986, Nucleic Acids Research 14:2287-2300)) that can only recombine with an identical mutant site.

In yet another preferred embodiment, an frt recombination site, which is cleavable by a flp recombinase enzyme, is utilized in conjunction with flp recombinase enzyme, as described above for the loxP/Cre embodiment. A "Flip Recombination Target site" (FRT) refers to a nucleotide sequence that serves as a substrate in the site-specific yeast flip recombinase system. The FRT recombination region has been mapped to an approximately 65-base pair (bp) segment within the 599-bp long inverted repeats of the 2- mu m circle (a commonly occurring plasmid in Saccharomyces cerevisiae). The enzyme responsible for recombination (FLP) is encoded by the 2- mu m circle, and has been expressed at high levels in human cells. FLP catalyzes recombination within the inverted repeats of the molecule to cause intramolecular inversion. FLP can also promote efficient recombination between plasmids containing the 2- mu m circle repeat with very high efficiency and

specificity. See, e.g., Jayaram (1985) Proc. Natl. Acad. Sci. USA 82:5875-5879; and O'Gorman (1991) Science 251:1351-1355. A "minimum FRT site" (e.g., a minimal FLP substrate) has been described in the art and is defined herein as a 13-bp dyad symmetry plus an 8-bp core located within the 65-bp FRT region. Jayaram et al., supra. Both FRT sites and FLP expression plasmids are commercially available from Stratagene (San, Diego, Calif.).

In still another preferred embodiment, an R recombinase site and R recombinase from Zygosaccharomyces rouxii can be utilized, as described above, in place of the loxP/Cre embodiment. EC 2.7.7.- ( R recombinase). See also Chen et al. (1991) *PNAS* 88: 5944.

In yet an alternative embodiment, a rare-cutting restriction enzyme (e.g., Not 1) may be used in place of the recombinase site. The recovered DNA would be digested with Not 1 and then recircularized with ligase. In this embodiment, the Not 1 site is included in the vector next to loxP. In other embodiments, the restriction enzyme can be 8 or higher base cutter, e.g., requires at least 8 baspairs for specificity.

In the complementation screening system of the invention, described below, such excision systems can also serve to discriminate revertants from virus-dependent rescue events.

The retroviral vectors' proviral recovery element allows for recovery of excised provirus from a complex mixture of nucleic acid, thus allowing for the selective recovery and excision of provirus from a recipient cell genome. The proviral recovery element comprises a nucleic acid sequence which corresponds to the nucleic acid portion of a high affinity binding nucleic acid/protein pair.

The nucleic acid can include, but is not limited to, a nucleic acid which binds with high affinity to a lac repressor, tet repressor or lambda repressor protein. For example, in one embodiment, the proviral recovery element comprises a lac operator nucleic acid sequence, which binds to a lac repressor peptide sequence. Such a proviral recovery element can be affinity-purified using lac repressor bound to a matrix (e.g., magnetic beads or sepharose). An excised provirus derived from the retroviral vectors of the invention also contains the retroviral recovery element and can be affinity purified.

Those skilled in the art will appreciate that there are a wide variety of other DNA binding proteins, including polypeptides derived from naturally occurring DNA binding proteins, as well as polypeptides derived from proteins artificially engineered to interact with specific DNA sequences, which can be used in conjunction with the appropriate proviral recovery element. Basic requirements for the DNA binding protein includes the

ability to specifically bind a defined nucleotide sequence.

In one preferred embodiment, the DNA binding protein is derived using all, or a DNA binding portion of a transcriptional regulatory protein, e.g., of either a transcriptional activator or transcriptional repressor, which retains the ability to selectively bind to particular nucleotide sequences. The DNA binding domains of the bacteriophage λcI protein (hereinafter "λcI") and the *E. coli* LexA repressor (hereinafter "LexA") represent examples of such DNA binding domains.

However, any other transcriptionally inert or essentially transcriptionally-inert DNA binding domain may be used, such DNA binding domains are well known and include, but are not limited to such motifs as helix-turn-helix motifs (such as found in λcI), winged helix-turn helix motifs (such as found in certain heat shock transcription factors), and/or zinc fingers/zinc clusters. As merely illustrative, the DNA binding protein can be constructed utilizing the DNA binding portions of the LysR family of transcriptional regulators, e.g., Trp1, HvY, OccR, OxyR, CatR, NahR, MetR, CysB, NodD or SyrM (Schell et al. (1993) *Annu Rev Microbiol* 47:597), or the DNA binding portions of the PhoB/OmpR-related proteins, e.g., PhoB, OmpR, CacC, PhoM, PhoP, ToxR, VirG or SfrA (Makino et al. (1996) *J Mol Biol* 259:15), or the DNA binding portions of histones H1 or H5 (Suzuki et al. (1995) *FEBS Lett* 372:215). Other examples include DNA binding portions of the P22 Arc repressor, MetJ, CENP-B, Rap1, Xy1S/Ada/AraC, Bir5 or DtxR

Furthermore, the DNA binding domain need not be obtained from the protein of a prokaryote. For example, polypeptides with DNA binding activity can be derived from proteins of eukaryotic origin, including from yeast. For example, the DNA binding protein can include polypeptide sequences from such eukaryotic DNA binding proteins as p53, Jun, Fos, GCN4, or GAL4. Likewise, the DNA binding protein can be generated from viral proteins, such as the pappillomavirus E2 protein (c.f., PCT publication WO 96/19566).

In yet other embodiments, the DNA binding protein can be generated by combinatorial mutagenic techniques, and represent a DNA binding domain not naturally occurring in any organism. That is, a completely arbitrary proviral receogery element can be provided in the construct, and such combinatorial approaches used to derive a new protein with sufficient specificity for nucleotide sequence of the element. A variety of techniques have been described in the art for generating novel DNA binding proteins which can selectively bind to a specific DNA sequence (c.f., U.S. Patent 5,198,346 entitled "*Generation and selection of novel DNA-binding proteins and polypeptides*").

Thus, the selection of the proviral recovery element is limited only by the

availability of a DNA binding protein which recognizes the recovery element's sequence and is compatible with the vector in the host cell and any bacterial cell in which the vector is shuttled/amplified.

In general. the 5' LTR will include a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, preferably in that order. Nucleotide sequences of these LTR elements are well known to those of skill in the art.

The 3' LTR comprises a U3 region which comprises the proviral excision element, a promoter, an R region and a polyadenylation signal. Nucleotide sequences of such elements are well known to those of skill in the art.

However, it is also specifically contemplated that the endogenous promoter of the LTR can be replaced with a heterolgous transcriptional regulatory sequence, and the 3' LTR can be.replaced with a heterologous polyadenylation signal without effecting the control. For instance, as described in US Patent 5,591,624, the U3 region in a 5' LTR can be amenable to replacement by a heterologous promoter/ enhancer.

The bacterial origin of replication (Ori) utilized is preferably one which does not adversely affect viral production or gene expression in infected cells. As such, it is preferable that the bacterial Ori is a non-pUC bacterial Ori relative (e.g., pUC, colEI, pSC101, p15A and the like). Further, it is preferable that the bacterial Ori exhibit less than 90% overall nucleotide similarity to the pUC bacterial Ori. In a preferred embodiment, the bacterial origin of replication is a RK2 OriV or f1 phage Ori.

In preferred embodiments, the retroviral vectors can further comprise a single-stranded replication origin, preferably an f1 single-stranded replication origin. The single-stranded replication origin allows for the production of normalized single-stranded retroviral libraries derived from the retroviral vectors of the invention. A normalized library is one constructed in a manner that increases the relative frequency of occurrence of rare clones while decreasing simultaneously the relative frequency of the occurrence of abundant clones. For teaching regarding the production of normalized libraries, see, e.g., Soares et al. (Soares, M.B. et al., 1994, Proc. Natl. Acad. Sci. USA 91:9228-9232, which is incorporated herein by reference in its entirety). Alternative normalization procedures based upon biotinylated nucleotides may also be utilized, and are described in greater detail below.

Any bacterial selectable marker can be utilized. As above, the marker can preferably one which renders the cell resistant to drug treatment, overcomes an auxotrophic phenotype, or provides some other signal which can be directly or indirectly measured and used as a means for selecting bacterial cells which harbor the proviral

vector. Bacterial selectable markers are well known to those of skill in the art and can include, but are not limited to, kanamycin/G418, zeocin, actinomycin, ampicillin, gentamycin, tetracycline, chloramphenicol or penicillin resistance markers.

In yet other embodiments, the retroviral vectors can further comprise a lethal stuffer fragment which can be utilized to select for vectors containing cDNA or gDNA inserts during, for example, construction of libraries comprising the retroviral vectors of the invention. Lethal stuffer fragments are well known to those of skill in the art (see, e.g., Bernord et al., 1994, Gene 148:71-74, which is incorporated herein by reference in its entirety). A lethal stuffer fragment contains a gene sequence whose expression conditionally inhibits cellular growth. Thus, by disrupting the expression of the lethal stuffer, e.g., by insertion of the nucleic acid library into the coding sequence of the stuffder fragment, vectors into which the test nucleic acid have been success ligated will no longer express a cytotoxic/cytostatic form of the stuffer fragment. These cells, therefore, can be amplified in the culture by simple virtue of the fact that relief from the inhibitory effects of the stuffer fragments is accorded by the loss-of-function mutation to the stuffer fragment gene by incorporation of the heterologous nucleic acid sequence.

In one embodiment, the stuffer fragment is present in the retroviral vectors of the invention within the polycistronic message cassette polylinker such that insertion of a cDNA or gDNA sequence into the polylinker replaces the stuffer fragment. Alternatively, the polycistronic message cassette polylinker is located within the lethal stuffer fragment coding sequence such that, upon insertion of a cDNA or gDNA sequence into the polylinker, the lethal stuffer fragment coding region is disrupted. Each of these embodiments can be utilized to counter select retroviral vectors not containing polylinker insertions.

*B) Adeno-associated virus complementation screening and expression vectors*

Yet another viral vector system useful for development of the subject vectors is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Cis-acting sequences directing viral DNA replication (ori),

encapsidation/packaging (pkg) and host cell chromosome integration (int) are contained within the ITRs. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb.

Adeno-associated virus (AAV) is a defective member of the parvovirus family. The AAV genome is encapsidated as a single-stranded DNA molecule of plus or minus polarity (Berns and Rose, 1970, J. Virol. 5:693-699; Blacklow et al., 1967, J. Exp. Med. 115:755-763). Strands of both polarities are packaged, but in separate virus particles (Berns and Adler, 1972, Virology 9:394-396) and both strands are infectious (Samulski et al., 1987, J. Virol. 61:3096-3101).

AAV possesses unique features that make it attractive as a basis for designing the vectors of the present invention. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects most (if not all) mammalian cells allowing the possibility of targeting many different tissues in vivo. Kotin et al., (1992) *EMBO J.* 11:5071-5078 reports that the DNA genome of AAV undergoes targeted integration on chromosome 19 upon infection. Replication of the viral DNA is not required for integration, and thus helper virus is not required for this process. The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of recombinant genomes feasible. Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may thus be replaced with foreign DNA such as the gene cassettes described herein, e.g., containing transcriptional regulatory sequences, DNA of interest and a polyadenylation signal. Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56o to 65o C. for several hours), making cold preservation of rAAV-based vaccines less critical. Finally, AAV-infected cells are not resistant to superinfection.

The single-stranded DNA genome of the human adeno-associated virus type 2 (AAV2) is 4681 base pairs in length and is flanked by inverted terminal repeated sequences of 145 base pairs each (Lusby et al., 1982, J. Virol. 41:518-526). The first 125 nucleotides form a palindromic sequence that can fold back on itself to form a "T"-shaped hairpin structure and can exist in either of two orientations (flip or flop), leading to the suggestion (Berns and Hauswirth, 1979, Adv. Virus Res. 25:407-449) that AAV may replicate according to a model first proposed by Cavalier-Smith for linear-chromosomal DNA (1974, Nature 250:467-470) in which the terminal hairpin of AAV is used as a primer for the initiation of DNA replication. The AAV sequences that are required in cis

for packaging, integration/rescue, and replication of viral DNA appear to be located within a 284 base pair (bp) sequence that includes the terminal repeated sequence (McLaughlin et al., 1988, J. Virol. 62:1963-1973). At least three regions which, when mutated, give rise to phenotypically distinct viruses have been identified in the AAV genome (Hermonat et al., 1984, J. Virol. 51:329-339). The rep region codes for at least four proteins (Mendelson et al., 1986, J. Virol. 60:823-832) that are required for DNA replication and for rescue from the recombinant plasmid. The cap and lip regions appear to encode for AAV capsid proteins; mutants containing lesions within these regions are capable of DNA replication (Hermonat et al., 1984, J. Virol. 51:329-339). AAV contains three transcriptional promoters (Carter et al., 1983, in "The Parvoviruses" K. Berns ed., Plenum Publishing Corp., N.Y. pp. 153-207; Green and Roeder, 1980, Cell 22:231-242; Laughlin et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:5567-5571; Lusby and Berns, 1982, J. Virol. 41:518-526; Marcus et al., 1981, Eur. J. Biochem. 121:147-154). The viral DNA sequence displays two major open reading frames, one in the left half and the other in the right half of the conventional AAV map (Srivastava et al., 1985, J. Virol. 45:555-564).

AAV-2 can be propagated as a lytic virus or maintained as a provirus, integrated into host cell DNA (Cukor et al., 1984, in "The Parvoviruses," Berns ed., Plenum Publishing Corp., N.Y. pp. 33-66). Although under certain conditions AAV can replicate in the absence of helper virus (Yakobson et al., 1987, J. Virol. 61:972-981), efficient replication requires coinfection with either adenovirus (Atchinson et al., 1965, Science 194:754-756; Hoggan, 1965, Fed. Proc. Am. Soc. Exp. Biol. 24:248; Parks et al., 1967, J. Virol. 1:171-180); herpes simplex virus (Buller et al., 1981, J. Virol. 40:241-247) or cytomegalovirus, Epstein-Barr virus, or vaccinia virus. Hence the classification of AAV as a "defective" virus.

When no helper virus is available, AAV can persist in the host cell genomic DNA as an integrated provirus (Berns et al., 1975, Virology 68:556-560; Cheung et al., 1980, J. Virol. 33:739-748). Virus integration appears to have no apparent effect on cell growth or morphology (Handa et al., 1977, Virology 82:84-92; Hoggan et al , 1972, in "Proceedings of the Fourth Lepetit Colloquium," North Holland Publishing Co., Amsterdam pp. 243-249). Studies of the physical structure of integrated AAV genomes (Cheung et al., 1980, supra; Berns et al., 1982, in "Virus Persistence" Mahy et al., eds., Cambridge University Press, N.Y. pp. 249-265) suggest that viral insertion occurs at random positions in the host chromosome but at a unique position with respect to AAV DNA, occurring within the terminal repeated sequence. Integrated AAV genomes have been found to be essentially stable, persisting in tissue culture for greater than 100 passages (Cheung et al., 1980 supra).

The desirable size of inserted non-AAV or foreign DNA is limited to that which permits packaging of the rAAV vector into virions, and depends on the size of retained AAV sequences. In the generation of the subject constructs, it may be desirable to exclude portions of the AAV genome in the rAAV vector in order to maximize expression of the inserted foreign nucleic acid sequences.

In preferred embodiments, the subject vectors are derived using replication-deficient AAV, e.g., wherein all or a substantial portion of the viral sequence which is naturally flanked by the ITR's is replaced with, for example, a polycistronic expression cassette(s), a bacterial origin of replication, a proviral recovery element, etc., as described for the retroviral vectors described herein. The ITR is also preferably engineered to include a proviral excision element, as described above. All that need be retained are those AAV sequences required for efficient packaging in a helper cell line, along with sequences necessary for chromosomal integration of the viral vector and its stable maintenance.

.In this regard, the term "helper virus" refers to a virus, such as adenovirus, herpesvirus, cytomegalovirus, Epstein-Barr virus, or vaccinia virus, which when coinfected with AAV results in productive AAV infection of an appropriate eukaryotic cell. Likewise, helper AAV DNA refers to AAV DNA sequences used to provide AAV functions to a recombinant AAV virus which lacks the functions needed for replication and/or encapsulation of DNA into virus particles. Helper AAV DNA cannot by itself generate infectious virions and may be incorporated within a plasmid, bacteriophage or chromosomal DNA. Finally, helper-free virus stocks of recombinant AAV refers to stocks of recombinant AAV virions which contain no measurable quantities of wild-type AAV or undesirable recombinant AAV.

*C. Episomal complementation and expression vectors*

As set out above, another aspect of the present invention relates to episomal expression vectors which also can as mammalian expression cloning systems Mammalian episomal vectors, such as the pEHRE vectors described herein, make possible, for the first time, stable, efficient, high-level episomal expression within a wide spectrum of mammalian cells. Such vectors can also, for example, be utilized as part of the complementation screening methods of the invention. The subject episomal vectors are designed to provide high episomal copy numbers, yet not result in runaway replication which could lead to, for example, cell death.

The subject episomal expression vectors, such as the pEHRE vectors, comprise a

replication cassette, an expression cassette and minimal cis-acting elements necessary for replication and stable episomal maintenance.

The episomal vectors of the invention can further contain at least one bacterial origin of replication and/or recombination sites. The recombination sites preferably flank the replication cassette, and can include, but are not limited to, any of the recombination sites described above.

Any bacterial origin of replication (Ori) which does not adversely affect the expression of the coding sequences provided in the expression vector can be utilized. For example, the bacterial Ori can be a pUC bacterial Ori relative (e.g., pUC, colEI, pSC101, p15A and the like). The bacterial origin of replication can also, for example, be a RK2 OriV or f1 phage Ori. The pEHRE vectors can further comprise a single stranded replication origin, preferably an f1 single-stranded replication origin. The single-stranded replication origin allows for the production of normalized single-stranded libraries derived from the pEHRE vectors of the invention. A normalized library is one constructed in a manner that increases the relative frequency of occurrence of rare clones while decreasing simultaneously the relative frequency of the occurrence of abundant clones. For teaching regarding the production of normalized libraries, see, e.g., Soares et al. (Soares, M.B. et al., 1994, Proc. Natl. Acad. Sci. USA 91:9228-9232, which is incorporated herein by reference in its entirety). Alternative normalization procedures based upon biotinylated nucleotides may also be utilized.

In instances wherein an f1 origin of replication is utilized, the pEHRE vectors can additionally comprise a nucleic acid sequence which corresponds to the nucleic acid portion of a high affinity binding nucleic acid/protein pair. Such nucleic acid/protein pairs can be as described above, the nucleic acid portion of which can include, but is not limited to, a lacO site. The nucleic acid can include, but is not limited to, a nucleic acid which binds with high affinity to a lac repressor, tet repressor or lambda repressor protein. For example, in one embodiment, the proviral recovery element comprises a lac operator nucleic acid sequence, which binds to a lac repressor peptide sequence. Such a proviral recovery element can be affinity-purified using lac repressor bound to a matrix (e.g., magnetic beads or sepharose). An excised provirus derived from the retroviral vectors of the invention also contains the retroviral recovery element and can be affinity purified.

In an exemplary embodimemt, a pEHRE vector replication cassette comprises nucleic acid sequences which encode papillomaviruses (PV) E1 and E2 proteins, wherein such nucleic acid sequences are operatively attached to and transcribed by, a constitutive or inducible transcriptional regulatory sequence, though constitutive is preferred.

Representative E1 and E2 amino acid sequences are well known to those of skill in the art. See, e.g., sequences publicly available in databases such as Genbank. The E1 and E2 coding sequences can, first, include any nucleotide sequences which encode endogenous PV, including but not limited to bovine papillomavirus (BPV), such as BPV-1 E1 or E2 gene products.

As used herein, the term "E1" also refers to any protein which is capable of functioning in PV in the same manner as the endogenous E1 protein, i.e., is capable of complementing an E1 mutation. Taking BPV as an example, an E1 protein, as described herein, is one capable of complementing a BPV E1 mutation. Likewise, the term "E2", as used herein, refers to any protein which is capable of functioning in PV in the same manner as the endogenous E2 protein, i.e., is capable of complementing a E2 mutation. Taking BPV as an example, an E2 protein, as described herein, is one capable of complementing a BPV E2 mutation.

The replication cassette transcriptional regulatory sequence can include, but is not limited to, any polII promoter, such as an SV40, CMV or PGK promoter, nucleotide sequences of which are well known to those of skill in the art.

E1 and E2 coding sequences can be operatively attached to, and transcribed by, separate transcriptional regulatory sequences. However, it is preferred that at least one, and more preferably both of the E1 and E2 sequences are provided in polycistronic arrangements, alone or together, with at least one selectable marker (discussed *infra*). In one embodiment, at least one of the E1 or E2 coding sequences can be transcribed along with a selectable marker as a polycistronic message. Such a polycistronic message construction makes possible a selection scheme which directly links expression of a selectable marker, preferably a mammalian selectable marker, to transcription of a sequence necessary for episomal maintenance and replication. For example, the portion of a replication cassette encoding such a polycistronic message could comprise, from 5' to 3': a constitutive transcriptional regulatory sequence, an E2 (or E1) coding sequence, an internal ribosome entry site (IRES), and a selectable marker.

In another embodiment, both E1 and E2 coding sequences can be transcribed together as part of a polycistronic message. That is, both E1 and E2 coding sequences, separated by an internal ribosome entry site, can be transcribed by a single transcriptional regulatory sequence.

In yet another embodiment, E1, E2 and selectable marker sequences can be transcribed as a polycistronic message. For example, the replication cassette could comprise, from 5' to 3': a constitutive transcriptional regulatory sequence, an E2 (or E1)

coding sequence, an IRES, an E1 (or E2) coding sequence, an IRES and a selectable marker.

In instances wherein the E1 and E2 coding sequences are transcribed as part of a polycistronic message, it is preferred that the order, from 5' to 3', be E2 then E1. This is to ensure against possible rare, undesirable RNA splicing events.

The episomal expression constructs of the present invention are derived to yield high level expression of a cDNA, genomic DNA (gDNA) or other nucleic acid sequence. Such a pEHRE vector expression cassette comprises, from 5' to 3', a transcriptional regulatory sequence, a nucleotide polylinker, an internal ribosome entry site, a mammalian selectable marker and, preferably, either a poly-A site or a transcriptional termination sequence, depending upon the transcriptional regulatory sequence utilized (see below). A cDNA or gDNA sequence can be expressed via operative association within the polylinker. A pEHRE expression vector can contain a single or multiple expression cassettes, such that greater than one cDNA or gDNA sequence can be expressed from the same pEHRE expression vector.

The pEHRE vector expression cassette transcriptional regulatory sequence can be either constitutive or inducible, and can be derived from cellular or viral sources. For example, such transcriptional regulatory sequences can include, but are not limited to, a retroviral long terminal repeat (LTR), cytomegalovirus (CMV), Va-1 RNA or U6 snRNA promoter sequence, nucleotide sequences of which are well known to those of skill in the art. Depending upon the transcriptional regulatory sequence chosen, the expression cassette can contain either a poly-A site (pA) or a transcriptional termination sequence. One of skill in the art will readily be able to choose, without undue experimentation, the appropriate sequence to be used with any given transcriptional regulatory sequence. In general, for example, polII-type transcriptional regulatory sequences can be coupled with pA sites, and polIII-type transcriptional regulatory sequences can be coupled with transcriptional termination sequences.

Expression from the transcriptional regulatory sequence yields a polycistronic message comprising the cDNA or gDNA sequence of interest, IRES and mammalian selectable marker. Such a polycistronic message approach allows a selection scheme which ensure that the cDNA or gDNA of interest has been expressed.

The pEHRE vectors further comprise cis-acting elements which function in replication and stable episomal maintenance. Such sequences include: a PV minimal origin of replication (MO) and a PV minichromosomal maintenance element (MME). Representative MO and MME sequences are well known to those of skill in the art. See,

e.g., Piirson, M. et al., 1996, EMBO J. 15:1-11, which is incorporated herein by reference in its entirety.

As used herein, the term "MO" refers to any nucleotide sequence capable of functioning in PV in the same manner as endogenous MO, i.e., is capable of complementing an MO mutation. Taking BPV as an example, an MO sequence, as described herein, would be one capable of complementing or replacing a BPV MO mutation. Likewise, the term "MME", as used herein, refers to any nucleotide sequence capable of functioning in PV in the same manner as endogenous MME, i.e., is capable of complementing a MME mutation. For example, a MME sequence can be one containing multiple E2 binding sites. Taking BPV as an example, a MME sequence, as described herein, would be one capable of complementing or replacing a BPV MME mutation.

The pEHRE IRES and mammalian and bacterial selectable markers can be, for example, as those described above.

Depicted in FIG. 10 is an example of one pEHRE vector embodiment, termed pEHRE-E-H. In this vector, the E1 and E2 coding sequences are BPV sequences, and are in operative association with individual SV40 promoters. E1 is transcribed as part of a polycistronic message along with the selectable marker, hygro. In this embodiment, the replication cassette further comprises an SV40 pA site downstream of the IRES-marker. Further, the MO and MME sequences are BPV-derived (in the figure, both of these sequences are illustrated as "BPV origin"). The vector's expression cassette comprises a CMV promoter operatively associated with a sequence to be expressed ("product"), said sequence in operative association with an IRES-marker (the sequence to be expressed and the IRES-marker are illustrated as "marker" in the figure), which, in turn, is in operative association with a bgH poly-A site. Finally, the vector contains a pUC bacterial origin (Ori) of replication, an f1 Ori and an ampicillin bacterial selectable marker.

The episomal expression vectors of the invention, such as pEHRE, can be utilized for the production, including large scale production, of recombinant proteins. The vectors' desirable features, in fact, make them especially amenable to large scale production. Specifically, current methods of producing recombinant proteins in mammalian cells involve transfection of cells (e.g., CHO, NS/0 cells) and subsequent amplification of the transfected sequence using drugs (e.g., methotrexate or inhibitors of glutamine synthetase). Such approaches suffer for a variety of reasons, including the fact that amplicons are subject to statistical variation depending on their genomic integration loci, and from the fact that the amplicons are unstable in the absence of continued selection (which is impractical at production scale). The subject vectors, it should be pointed out, achieve

such levels equal or higher than these naturally, that is, in the absence of outside selection.

Thus, the present invention provides a meands for producing such proteins as proteins such as human serum albumin; human interferons; human antibodies; human insulin; erythropoietin, steel factor and other hematopoietic factors; blood clotting factors, particularly the rare human blood clotting factors such as Factor IX or VIII; thrombolytic factors such as tissue plasminogen activators; human growth factors; brain peptides; interleukins; endorphins; enzymes; prolactin; viral antigens; and even plant proteins.

The pEHRE vectors of the invention, in contrast, give consistently high episomal expression, making them genomic integration-independent. Further, the episomal pEHRE vectors are retained as stable nuclear plasmids even in the absence of selective pressure.

Further, pEHRE vectors can be utilized which employ an additional level of such internal, or self, selection (that is, selection which does not depend on the addition of outside selective pressures such as, e.g., drugs). For example, pEHRE vectors can be utilized which complement a defect the specific producer cell line being utilized for expression. By way of example, and not by way of limitation, such pEHRE selection elements can complement an auxotrophic mutation or can bypass a growth factor requirement (e.g., proline or insulin, respectively) from the cell media. Preferably, the coding sequence of the marker is transcribed as part of a polycistronic message along with the coding sequence of the proteins being recombinantly expressed. For example, such an expression/selection cassette can comprise, from 5' to 3': a transcriptional regulatory sequence, recombinant protein coding sequence, IRES, selection marker, poly-A site.

The vector depicted in FIG. 11, termed pEHRE-H, depicts one embodiment of a pEHRE vector that can be utilized for large scale production. The "Marker" element represents a "self-selection" marker as discussed above operatively attached to an IRES. "Product" in the figure refers to the coding sequence of the recombinant protein being expressed. The remainder of the elements of the vector are as described for the vector presented in FIG. 10, above.

The episomal pEHRE vectors of the invention can further be utilized, for example, in the delivery of large nucleic acid segments, e.g., chromosomal segments. In one such embodiment, pEHRE vectors can be utilized in connection with bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) sequences to allow delivery of large genomic segments (e.g., segments ranging from tens of kilobases to megabases in length). For clarity, the discussion that follows describes vectors that utilize BAC sequences, but it is to be understood that vectors of the sort described here can, alternatively, utilize YAC sequences.

In one embodiment, pEHRE vectors can be combined with existing BAC clones to generate pEHRE/BAC hybrid constructs, comprising BACs into which pEHRE vector sequences have been inserted. Such pEHRE/BAC hybrids represent BACs that can replicate in a wide variety of mammalian, including human cells.

In general, pEHRE vectors which can be utilized to donate elements to BACs comprise a pEHRE replication cassette, MO and MME sequences, and a bacterial selectable marker, all flanked by BAC recombination sequences. The remainder of the vector can further comprise at least one bacterial origin of replication and a second bacterial selectable marker.

BAC recombination sequences caN include any nucleotide sequence which can be cleaved and then used to recombine with BAC elements so as to incorporate the necessary pEHRE sequences described above. Any recombination site for which a compatible recombination site exists, or is engineered to exist, in the recipient BAC can be used. For example, such BAC recombination elements can include, but are not limited to, loxP, mutant loxP or frt sites as described, above, in Section 5.1.1.

Alternatively, CosN sites, whose nucleotide sequences are well known to those of skill in the art, can be utilized. Rather than a recombinase enzyme, such CosN sites are cleaved by lambda terminase enzyme. (For general BAC teaching, including CosN teaching, see, e.g., Shizuya, H. et al., 1992, Proc. Natl. Acad. Sci. USA 89:8794-8797; and Kim, U.-J. et al., 1996, Genomics 34:213-218, which are incorporated herein by reference in their entirety.)

In order to recombine pEHRE and BAC sequences, pEHRE vectors and BAC (containing a recombination site compatible with the chosen pEHRE vector) are treated together with the appropriate recombinase or terminase enzyme. When the CosN/terminase system is used, a subsequent ligation step is included.

The treatment will result in a low level of concatamerization. Concatamers representing the desired pEHRE/BAC hybrids can be selected for based upon their resistance to both the BAC selectable marker (usually chloramphenicol) and the pEHRE vector selectable marker within the pEHRE region meant to be donated. It is, therefore, desirable that the BAC and pEHRE selectable markers be different. In a preferred embodiment, the resulting constructs are further tested to ensure that the second pEHRE bacterial selectable marker is no longer present. Plasmids which have recombined the desired BAC and pEHRE elements, will be able to replicate in E. coli, as well as a wide range of mammalian cells, including human cells.

The vector depicted in FIG. 12, termed a pBPV-BacDonor vector, represents one

embodiment of a pEHRE vector designed to donate essential pEHRE sequences to recipient BAC clones. The vector's recombination elements are depicted as containing loxP and/or CosN sites. The bacterial marker to be incorporated into the pEHRE/BAC hybrid is depicted as tetracycline or kanamycin. Finally, the vector contains a pUC bacterial origin (Ori) of replication, an f1 Ori and a second bacterial selectable marker, ampicillin.

In an alternative embodiment, pEHRE/BAC cloning vectors can be produced and utilized. Such vectors contain the pEHRE replication cassette, MO and MME sequences as described above, the nucleotide sequences necessary for BAC maintenance in E. coli (such sequences are well known to those of skill in the art; see, e.g., Shizuya and Kim, above), and a polylinker site.

The vector depicted in FIG. 13, termed pBPV-BlueBAC, represents one embodiment of such a pEHRE/BAC cloning vector. In this vector, the E1 and E2 coding sequences are BPV sequences, and are in operative association with individual SV40 promoters. E1 is transcribed as part of a polycistronic message along with the selectable marker, hygro. In this embodiment, the replication cassette further comprises an SV40 pA site downstream of the IRES-marker. Further, the MO and MME sequences are BPV-derived (in the figure, both of these sequences are illustrated as "BPV origin"). The cloning site comprises a polylinker embedded within the alpha complementation fragment of lacZ, which allows blue/white selection of recombinants. T7 and SP6 promoters flank the lacZ sequence, and the vector additionally contains cosN and loxP sites for linearization. The remainder of the elements depicted are present for BAC maintenance in E. coli.

IV. Antisense-genetic suppressor element (gse) vectors

*A) Antisense-gse retroviral vectors*

Described herein are genetic suppressor element (GSE)-producing, replication-deficient retroviral vectors. Such vectors are designed to facilitate the expression of antisense GSE single-stranded nucleic acid sequences in mammalian cells, and can, for example, be utilized in conjunction with the antisense-based functional gene inactivation methods of the invention. The GSE element can also be a ribozyme, e.g., a hammerhead ribozyme or the like, which is being designed to, for example, inhibit expression of a target gene.

The GSE-producing retroviral vectors of the invention can comprise a replication-

deficient retroviral genome containing a proviral excision element, a proviral recovery element and a genetic suppressor element (GSE) cassette.

The GSE-producing retroviral vectors can further comprise, (a) a 5' LTR; (b) a 3' LTR; (c) a bacterial Ori; (d) a mammalian selectable marker; (e) a bacterial selectable marker; and (f) a packaging signal.

The proviral recovery element, GSE cassette, bacterial Ori, mammalian selectable marker and bacterial selectable marker are located between the 5'LTR and the 3' LTR. The proviral excision element is located within the 3' LTR. The proviral excision element can also flank the functional cassette without being present in the 3' LTR.

The 5' LTR, 3' LTR, proviral excision element, bacterial selectable marker, mammalian selectable marker and proviral recovery element are as described above.

Each of the GSE cassette embodiments described below can further comprise a sense or antisense cDNA or gDNA fragment or full length sequence operatively associated within the polylinker. Moreover, the GSE cassettes can be oriented to transcribe in either the same or opposite orientation with respect to the LTR driving its transcription. That LTR can also be an intact LTR, or a self-inactivating (SIN) LTR.

The GSE cassette can, for example, comprise, from 5' to 3': (a) a transcriptional regulatory sequence; (b) a polylinker; and (c) polyadenylation signal. In one embodiment, the GSE cassette polyadenylation signal is located within the 3' retroviral long terminal repeat.

Alternatively, the GSE cassette can comprise, from 5' to 3': (a) a transcriptional regulatory sequence; (b) a polylinker; (c) a cis-acting ribozyme sequence; (d) an internal ribosome entry site; (e) the mammalian selectable marker; and (f) a polyadenylation signal.

In a further alternative, a sense GSE can be constructed, in which case the GSE cassette can further comprise a polylinker containing a Kozak consensus methionine in front of the sense-orientation fragments to create a "domain library" for domain and fragment expression.

In such an embodiment, transcription from the transcriptional regulatory sequence produces a bifunctional transcript. The first half (i.e., the portion upstream of the ribozyme sequence) is likely to remain nuclear and represents the GSE. The portion downstream of the ribozyme sequence (i.e., the portion containing the selectable marker) is transported to the cytoplasm and translated. Such a bicistronic configuration, therefore, directly links selection for the selectable marker to expression of the GSE.

In another alternative, the GSE cassette can comprise, from 5' to 3': (a) an RNA polymerase III transcriptional regulatory sequence; (b) a polylinker; (c) a transcriptional termination sequence. In a particular embodiment, the transcriptional regulatory sequence and transcriptional termination sequence are adenovirus Ad2 VA RNAI transcriptional regulatory and termination sequences.

*B) pEHRE antisense-genetic suppressor element vectors*

Described herein are genetic suppressor element (GSE)-producing, pEHRE vectors. Such vectors are designed to facilitate the expression of antisense GSE single-stranded nucleic acid sequences in mammalian cells, and can, for example, be utilized in conjunction with the antisense-based functional gene inactivation methods of the invention.

The GSE-producing pEHRE vectors of the invention can comprise a replication cassette, a genetic suppressor element (GSE) cassette and minimal cis-acting elements necessary for replication and stable episomal maintenance.

The GSE-producing pEHRE vectors can further comprise at least one bacterial origin of replication and at least one bacterial selectable marker.

The replication cassette, minimal cis-acting elements, bacterial origin of replication and bacterial selectable marker are as described in Section 5.1.1, above.

Each of the GSE cassette embodiments described below can further comprise a sense or antisense cDNA or gDNA fragment or full length sequence operatively associated within the polylinker.

The GSE cassette can, for example, comprise, from 5' to 3': (a) a transcriptional regulatory sequence; (b) a polylinker; and (c) polyadenylation signal. The GSE transcriptional regulatory sequence can be a constitutive or inducible one, and can represent, for example, retroviral long terminal repeat (LTR), cytomegalovirus (CMV), Va-1 RNA or U6 snRNA promoter sequence, nucleotide sequences of which are well known to those of skill in the art.

The vector depicted in FIG. 14 represents an example of such a pEHRE GSE vector. In this vector, the E1 and E2 coding sequences are BPV sequences, and are in operative association with individual SV40 promoters. E1 is transcribed as part of a polycistronic message along with the selectable marker, hygro. In this embodiment, the replication cassette further comprises an SV40 pA site downstream of the IRES-marker. Further, the MO and MME sequences are BPV-derived (in the figure, both of these

sequences are illustrated as "BPV origin"). The vector's GSE cassette comprises a CMV promoter operatively associated with a sequence to be expressed as a GSE, which, in turn, is operatively attached to a bgH poly-A site. Finally, the vector contains a pUC bacterial origin (Ori) of replication, an f1 Ori and an ampicillin bacterial selectable marker.

Alternatively, the GSE cassette can comprise, from 5' to 3': (a) a transcriptional regulatory sequence; (b) a polylinker; (c) a cis-acting ribozyme sequence; (d) an internal ribosome entry site; (e) the mammalian selectable marker; and (f) a polyadenylation signal.

In another alternative, a sense GSE can be constructed, in which case the GSE cassette can further comprise a polylinker containing a Kozak consensus methionine in front of the sense-orientation fragments to create a "domain library" for domain and fragment expression.

In such an embodiment, transcription from the transcriptional regulatory sequence produces a bifunctional transcript. The first half (i.e., the portion upstream of the ribozyme sequence) is likely to remain nuclear and represents the GSE. The portion downstream of the ribozyme sequence (i.e., the portion containing the selectable marker) is transported to the cytoplasm and translated. Such a bicistronic configuration, therefore, directly links selection for the selectable marker to expression of the GSE.

In another alternative, the GSE cassette can comprise, from 5' to 3': (a) an RNA polymerase III transcriptional regulatory sequence; (b) a polylinker; (c) a transcriptional termination sequence.

The vectors depicted in FIGS. 15 and 16 represent examples of this type of pEHRE GSE vector. The GSE cassette of the vector depicted in FIG. 15 comprises a Va-1 promoter which is operatively attached to a sequence to be expressed as a GSE, which is, in turn, operatively attached to a Va-1 termination sequence. The GSE cassette of the vector depicted in FIG. 16 comprises a U6 promoter which is operatively attached to a sequence to be expressed as a GSE, which is, in turn, operatively attached to a U6 termination sequence. The remainder of the elements depicted in the FIG. 15 and 16 vectors are as described for the vector shown in FIG. 14.

In a particular embodiment, the transcriptional regulatory sequence and transcriptional termination sequence are adenovirus Ad2 VA RNA transcriptional regulatory and termination sequences.

*C) Linked Marker for Antisense Development*

An important use for antisense libraries comes in the refinement/optimization of the antisense sequences which can be used to effectively inhibit expression of a gene, or function of a structural RNA element. In order to provide high through screening techniques for detecting effective antisense sequences, the subject invention also provides a linked marker construct providing a convenient readout on the level of expression of the targeted gene. In particular, the linked marker is a fusion gene comprised of a coding or non-coding sequence for which an antisense construct is sought, e.g., it can include the coding sequence for a target protein. The fusion gene also includes a coding sequence for a marker protein, e.g., a protein whose expression can be detected, and preferably quantitated. A variety of marker genes are described above for selection, and many of those can be used to generate the subject linked marker. For instance, the marker can be a cell surface marker, a detectable enzyme, a gene product which complements a condition of the host cell, a transcription factor, etc. In preferred embodiments, the target sequence and linked marker encode a fusion protein including the marker protein. In the absence of antisense effective for inhibiting the expression of the target protein, the linked marker will be expressed and detected. However, antisense which can inhibit the expression of the target protein, e.g., by hybridizing to the fusion gene or a transcript thereof, will cause a reduction in the level of detectable marker. This method can also be used to screen libraries of ribozymes, e.g., hammerhead ribozymes, in order to identify ribozymes able to inhibit expression of the target gene.

According to one aspect of the invention, there is provided a library of vectors of the present invention including variegated population of transcrible gene sequences which, upon transcription, provide a population of potential antisense transcripts for a gene, e.g., a mammalian gene.

After identification in the subject method, one or more of the antisense sequences identified can be provided in a pharmaceutical preparation suitable for antisense therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a target protein. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct identified by the method of the present invention can be

prepared for *in vivo* delivery, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

V. Vectors displaying random peptide sequences

Described herein are vectors useful for the display of constrained and unconstrained random peptide sequences. Such vectors are designed to facilitate the selection and identification of random peptide sequences that bind to a protein of interest.

The integrated and episomal vectors of the present invention can be engineered to display random peptide sequences. Such vectors of the present invention can comprise, to illustrate, (a) a splice donor site or a LoxP site (e.g., LoxP511 site); (b) a bacterial promoter (e.g., pTac) and a shine-delgarno sequence; (c) a pel B or other secretion signal sequence for targeting fusion peptides to the periplasm; (d) a splice-acceptor site or another LoxP511 site (Lox P511 sites will recombine with each other, but not with the LoxP site in the 3' LTR); (e) a peptide display cassette or vehicle; (f) an amber stop codon; (g) the M13 bacteriophage gene 111 protein C-terminus (amino acids 198-406); and optionally the vector may also comprise a flexible polyglycine linker.

A peptide display cassette or vehicle consists of a vector protein, either natural or synthetic into which a polylinker has been inserted into one flexible loop of the natural or synthetic protein. A library of random oligonucleotides encoding random peptides may be inserted into the polylinker, so that the peptides are expressed on the cell surface.

The display vehicle of the vector may be, but is not limited to, thioredoxin for intracellular peptide display in mammalian cells (Colas et al., 1996, Nature 380:548-550) or may be a minibody (Tramonteno, 1994, J. Mol. Recognit. 7:9-24) for the display of peptides on the mammalian cell surface. Each of these would contain a polylinker for the

insertion of a library of random oligonucleotides encoding random peptides at the positions specified above. In an alternative embodiment, the display vehicle may be extracellular, in this case the minibody could be preceded by a secretion signal and followed by a membrane anchor, such as the one encoded by the last 37 amino acids of DAF-1 (Rice et al., 1992, Proc. Natl. Acad. Sci. 89:5467-5471). This could be flanked by recombinase sites (e.g., FRT sites) to allow the production of secreted proteins following passage of the library through a recombinase expressing host.

In one embodiment of the present invention, these cassettes would reside at the position normally occupied by the cDNA in the sense-expression vectors described above. In an amber suppressor strain of bacteria and in the presence of helper phage, these vectors would produce a relatively conventional phage display library which could be used exactly as has been previously described for conventional phage display vectors. Recovered phage that display affinity for the selected target would be used to infect bacterial hosts of the appropriate genotype (i.e., expressing the desired recombinases depending upon the cassettes that must be removed for a particular application). For example for an intracellular peptide display, any bacterial host would be appropriate (provided that splice sites are used to remove pelB in the mammalian host). For a secreted display, the minibody vector would be passed through bacterial cells that catalyze the removal of the DAF anchor sequence. Plasmids prepared from these bacterial hosts are used to produce virus for assay of specific phenotypes in mammalian cells.

In some cases, if the target is unknown the phage display step could be skipped and the vectors could be used for intracellular or extracellular random peptide display directly. The advantage of these vectors over conventional approaches is their flexibility. The ability to functionally test the peptide sequence in mammalian cells without additional cloning or sequencing steps makes possible the use of much cruder binding targets (e.g., whole fixed cells) for phage display. This is made possible by the ability to do a rapid functional selection on the enriched pool of bound phages by conversion to retroviruses that can infect mammalian cells.

VI Gene trapping vectors

Described herein are forms of the integrating viral vectors, such as replication-deficient retroviral gene, which can be engineered as gene trapping vectors. Such gene trapping vectors contain reporter sequences which, when integrated into an expressed gene, "tag" the expressed gene, allowing for the monitoring of the gene's expression, for example, in response to a stimulus of interest. The gene trapping vectors of the invention

can be used, for example, in conjunction with the gene trapping-based methods of the invention for the identification of mammalian genes which are modulated in response to specific stimuli.

The replication-deficient retroviral gene trapping vectors of the invention can comprise: (a) a 5' LTR; (b) a promoterless 3' LTR (a SIN LTR); (c) a bacterial Ori; (d) a bacterial selectable marker; (e) a selective nucleic acid recovery element for recovering nucleic acid containing a nucleic acid sequence from a complex mixture of nucleic acid; (f) a polylinker; (g) a mammalian selectable marker; and (h) a gene trapping cassette. In addition, those elements necessary to produce a high titer virus are required. Such elements are well known to those of skill in the art and contain, for example, a packaging signal.

The bacterial Ori, bacterial selectable marker, selective nucleic acid recovery element, polylinker, and mammalian selectable marker are located between the 5' LTR and the 3' LTR. The bacterial selectable marker and the bacterial Ori are located in close operative association in order to facilitate nucleic acid recovery, as described below. The gene trapping cassette element is located within the 3' LTR.

The 5' LTR, bacterial selectable marker and mammalian selectable marker are as described in Section 5.1, above. The selective nucleic acid recovery element is as the proviral recovery element described, above, in Section 5.1, above.

The 3' LTR contains the gene trapping cassette and lacks a functional LTR transcriptional promoter.

The gene trapping cassette can comprise from 5' to 3': (a) a nucleic acid sequence encoding at least one stop codon in each reading frame; (b) an internal ribosome entry site; and (c) a reporter sequence. The gene trapping cassette can further comprise, upstream of the stop codon sequences, a transcriptional splice acceptor nucleic acid sequence.

The inclusion of the IRES sequence in the gene trapping vectors of the present invention offers a key improvement over conventional gene trapping vectors. The IRES sequence allows the vector to land anywhere in the mature message to create a bicistronic transcript, this effectively increases the number of integration sites that will report promoters by a factor of at least 10.

VII. Retroviral and pEHRE vector derivatives

Described herein are derivatives of the retroviral vectors of the invention, including libraries, retroviral particles, integrated proviruses and excised proviruses. Also described

herein are derivatives of the pEHRE vectors of the invention, including libraries, cells and animals containing such episomal vectors.

The compositions of the present invention further include libraries comprising a multiplicity of the retroviral and/or pEHRE vectors of the invention, said vectors further containing cDNA or gDNA sequences. A number of libraries may be used in accordance with the present invention, including but not limited to, normalized and non-normalized libraries for sense and antisense expression; libraries selected against specific chromosomes or regions of chromosomes (e.g., as comprised in YACs or BACs), which would be possible by the inclusion of the f1 origin; and libraries derived from any tissue source; and genomic libraries constructed using the BAC/pEHRE vectors of the invention.

The compositions of the present invention still further include retrovirus particles derived from the retroviral vectors of the invention. Such retrovirus particles are produced by the transfection of the retrovirus vectors of the invention into retroviral packaging cell lines, including, but not limited to, the novel retroviral packaging cell lines of the invention.

The compositions of the invention additionally include provirus sequences derived from the retrovirus particles of the invention. The provirus sequences of the invention can be present in an integrated form within the genome of a recipient mammalian cell, or may be present in a free, circularized form.

An integrated provirus is produced upon infection of a mammalian recipient cell by a retrovirus particle of the invention, wherein the infection leads to the production and integration into the mammalian cell genome of the provirus nucleic acid sequence.

The circularized provirus sequences of the invention are generally produced upon excision of the integrated provirus from the recipient cell genome.

The compositions of the present invention still further include cells containing the retroviral or pEHRE vectors of the invention. Such cells include, but are not limited to the packaging cell lines described, below. Additionally, the compositions of the invention include transgenic animals containing the retroviral or pEHRE vectors of the invention, including, preferably, animals containing vectors from which sequences (either sense or antisense) are expressed in one or more cells of the animal.

VIII. Packaging cell lines

A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population.

Retroviral packaging functions comprise gag/pol and env packaging functions. gag and pol provide viral structural components and env functions to target virus to its receptor. Env function can comprise an envelope protein from any amphotropic, ecotrophic or xenotropic retrovirus, including but not limited to MuLV (such as, for example, an MuLV 4070A) or MoMuLV. Env can further comprise a coat protein from another virus (e.g., env can comprise a VSV G protein) or it can comprise any molecule that targets a specific cell surface receptor.

The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag, pol, env*) has been replaced by nucleic acid encoding one of the subject CCR-proteins, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. See for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT

Application WO 92/07573). Such prior art systems can be used to package the retroviral-based vectors described above.

However, we have created second-generation retrovirus producer lines for the generation of helper free ecotropic and amphotropic retroviruses. The lines are based on the use of the above-referenced episomal vectors to create a stable, episomal expression system providing the various packaging functions required for packaging replication-deficient retroviral vectors. Salient features of the resulting packaging cell lines are discussed in greater detail below, and include the long term stability of the line, the high titre production of the cell line, and the ability to use cell-lines which are also highly transfectable by such standard techniques as calcium phosphate mediated transfection or lipid-based transfection protocols, e.g., the cells can be highly amenable to transfection with the proviral vectors.

Previously, first-generation producer system were established established using 293T cells as a packaging system for helper-free retroviral production. Into 293T cells were placed defective constructs capable of producing gag-pol, and envelope protein for ecotropic and amphotropic viruses. These lines were called BOSC23, and Bing, respectively. See, for example, Pear et al. (1993) *PNAS* 90:8392. The utility of these lines was that one could produce small amounts of recombinant virus transiently for use in small-scale experimentation. The lines offered advantages over previous stable systems in that virus could be produced in days rather than months. However, two problems are apparent with these and other packaging cell lines in use.

First, these cells are often unstable and need vigilant checking for retroviral production capacity. Second the structure of the vectors used for protein production were not considered fully safe for helper virus production do not possible homologous recombination events between the expression vector of the packaging cell line and retroviral vector

To overcome these obstacle, we have made several improvements. First, we added the facility to monitor gag-pol and/or env production on a cell-by cell basis by introducing an IRES- marker gene which as part of a polycistronic construct with the gag-pol and/or env coding sequences. Thus, marker gene expression is a direct reflection of expression of the polycistron, and accordingly of the gag-pol and/or env genes. In addition to being a valuable selection tool for early passage of packaging cells, this marker system can also be used to monitor the stability of the producer cell population over time, particularly with respect to it's ability to produce virion proteins. As described below, by proper selection of the marker gene, its expression in the cells can be readily monitored, and utilized to

select cells, by flow cytometry.

Second, for the virion protein coding sequence, e.g., both the gag-pol and envelope constructs, non retroviral promoters were used to minimize recombination potential. In preferred embodiments, one could go so far as to even use different promoters for gag-pol and envelope so as to further minimize their inter-recombination potential.

By this technique, several packaging cell lines were created. As described in the appended examples, the envelope coding sequences, Gag-pol and env, were each indvidually introduced as part of tricistronic messages with a drug selection marker (such as hygromycin) and a FACS tag as the co-selectable markers. The illustrative line LinX, is capable of carrying such episomes for long-term stable production of retrovirus. These lines are readily testable by flow cytometry for stability of envelope expression by way of the FACS tags. Indeed, after more than 60 weeks, the linX line appears more stable than the first-generation line BOSC. Moreover, the subject packaging lines can also be used to transiently produce virus in a few days. Thus, these new lines are fully compatible with transient, episomal stable, and library generation for retroviral gene transfer experiments. Thus, we have provided a means to deliver large libraries of retroviruses into nearly any mammalian cell type, e.g., mouse or human. The viral titre can be to a level, e.g., infectious titers in the range of $10^5$-$10^7$/ml or greater, which permits the sampling of complex nucleic acid libraries with enough dynamic range that even relatively rare species in the library have some reasonable chance of being expressed in infected cells.

Thus, one is provided with such viral preparations as purified virus, conditioned media, and/or packaging cell lines producing infectious virus. When working with non-adherent cells, one has the choice of infecting by adding the retroviral supernatant directly to the cells or co-cultivating the non-adherent cells with the retroviral producer cells. The advantage of the latter is that there is ongoing retroviral production; however, this must be weighed against the disadvantage of harvesting producer cells together with the target cells.

Thus, in a preferred embodiment, a retroviral packaging cell line containing a tricistronic expression cassette is used as a founder line for selection of novel efficient, stable retroviral packaging cell lines. The tricistronic message cassette comprises a gene sequence important for efficient packaging of retroviral-derived nucleic acid into functional retroviral particles in operative association with a selectable marker and a quantifiable marker. The gene sequence, the selectable marker and the quantifiable marker are transcribed onto a single message whose expression is controlled by a single set of regulatory sequences. In such an embodiment, the gene sequence important for packaging

can represent, for example, a gal/pol or an env gene sequence.

In an alternative embodiment, the retroviral packaging cell line contains a polycistronic expression cassette comprising at least two gene sequences important for efficient packaging of retroviral-derived nucleic acid into functional retroviral particles in operative association with a selectable marker and a quantifiable marker. The gene sequences, the selectable marker and the quantifiable marker are transcribed onto a single message whose expression is controlled by a single set of regulatory sequences. For example, in such an embodiment the gene sequences important for packaging can represent gag/pol and env gene sequences.

The polycistronic, such as, for example, tricistronic, message approach allows for a double selection of desirable packaging cell lines. First, selection for the selectable marker ensures that only those cells expressing the gene sequence important for packaging are selected for. Second, those cells exhibiting the highest level of quantifiable marker (and, therefore, exhibiting the highest level of expression of the gene sequence important for packaging) can be selected.

In a variation of the above embodiment, cell lines containing greater than one polycistronic, e.g., tricistronic, message cassette can be utilized. For example, one message cassette comprising a first gene sequence important for retroviral packaging, a first selectable marker and a first quantifiable marker can be utilized to select for the greatest expression of the first gene sequence, while a second message cassette comprising a second gene important for efficient retroviral packaging, a second selectable marker and a second quantifiable marker can be utilized to select for the greatest expression of the second gene sequence, thereby creating a packaging cell line which is optimized for both the first and the second gene sequences important for packaging.

The quantifiable marker is, for example, any marker gene described above that can be quantified by florescence activated cell sorting (FACS) methods, e.g., a FACS tag. Such a quantifiable marker can include, but is not limited to, any cell surface marker, such as, for example, CD4, CD8 or CD20, in addition to any synthetic or foreign cell surface marker. Further, such a quantifiable marker can include an intracellular fluorescent marker, such as, for example, green fluorescent protein. Additionally, the quantifiable marker can include any other marker whose expression can be measured, such as, for example, a beta galactosidase marker.

The selectable marker chosen can include, for example, any selectable drug marker or the like described above, including, but not limited to hygromycin, blasticidin, neomycin, puromycin, histidinol, zeocin and the like.

High level expression can be achieved by a variety of means well known to those of skill in the art. For example, expression of sequences encoding viral functions can be regulated and driven by regulatory sequences comprising inducible and strong promoters including, but not limited to, CMV promoters.

Alternatively, high copy numbers of polycistronic cassettes can be achieved via a variety of methods. For example, stable genomic insertion of high copy numbers of polycistronic cassettes can be obtained. In one method, extrachromosomal cassette copy number can first be achieved, followed by selection for stable high-copy number insertion. For example, extrachromosomal copy number can be increased via use of SV40 T antigen and SV40 origin of replication in conjunction with standard techniques well known to those of skill in the art.

High stable extrachromosomal cassette copy number can also be achieved. For example, stable extrachromosomal copy number can be increased by making the polycistronic cassettes part of an extrachromosomal replicon derived from, for example, bovine papilloma virus (BPV), human papovavirus (BK) or Epstein Barr virus (EBV) which maintain stable episomal plasmids at high copy numbers (e.g., with respect to BPV, up to 1000 per cell) relative to the 5-10 copies per cell achieved via conventional transfections. In this method the cassettes remain episomal, i.e., there is no selection for integration.

The preferred embodiment for such achieving and utilizing such high level, stable extrachromosomal copy number employs the pEHRE vectors of the invention. FIGS. 17-22 depict pEHRE vectors designed for use in such packaging cell lines. In each of these vectors, the E1 and E2 coding sequences are BPV sequences, and are in operative association with individual SV40 promoters. E1 is transcribed as part of a polycistronic message along with the selectable marker, hygro. In this embodiment, the replication cassette further comprises an SV40 pA site downstream of the IRES-marker. Further, the MO and MME sequences are BPV-derived (in the figure, both of these sequences are illustrated as "BPV origin").

The pEHRE vectors depicted in FIGS. 17 and 18, termed $\psi_c$IH and pEHRE-$\psi_c$IH, respectively, represent two different embodiments of pEHRE vectors whose expression cassette expresses a polycistronic gag/pol env message. The FIG. 17 expression cassette comprises a CMV promoter which is operatively attached to gag/pol, env coding sequences, which are operatively attached to an IRES-hygro construct, which is, in turn, operatively attached to a bGH poly-A site. The FIG. 18 expression cassette is identical to that of FIG. 17, except the promoter utilized is an LTR promoter.

The pEHRE vectors depicted in FIGS. 19 and 20, termed $\psi_{env}$IH and pEHRE-$\psi_{env}$IH, respectively, represent two different embodiments of pEHRE vectors whose expression cassette expresses an env message. The FIG. 19 expression cassette comprises a CMV promoter which is operatively attached to an env coding sequence, which is operatively attached to an IRES-hygro construct, which is, in turn, operatively attached to a bGH poly-A site. The FIG. 20 expression cassette is identical to that of FIG. 19, except the promoter utilized is an LTR promoter.

The pEHRE vectors depicted in FIGS. 21 and 22, termed $\psi_{g/p}$IH and pEHRE-$\psi_{g/p}$IH, respectively, represent two different embodiments of pEHRE vectors whose expression cassette expresses a polycistronic gag/pol message. The FIG. 21 expression cassette comprises a CMV promoter which is operatively attached to an gag/pol coding sequence, which is operatively attached to an IRES-hygro construct, which is, in turn, operatively attached to a bGH poly-A site. The FIG. 22 expression cassette is identical to that of FIG. 21, except the promoter utilized is an LTR promoter.

Among the cell lines which can be used in connection with pEHRE vectors to produce packaging cell lines are cells that express replication-competent T antigen, such as, for example, COS cells. COS cells express an SV40 T antigen that is capable of promoting replication from the SV40 origin. With respect to packaging cell lines, this can be exploited, first, to allow amplification of replication-deficient retroviral vectors. In this way, expression of retroviral RNA will be increased and higher titers should result, in that it appears that retroviral RNA abundance is the limiting factor for titers in most packaging cell lines. An alternative mechanism for increasing levels introduces a PV, preferably BPV Ori, as described for the pEHRE vectors of the invention, into the retroviral vectors described herein.

The presence of T-antigen can also be utilized to allow amplification of helper functions. This can be accomplished by including an SV40 origin of replication within the pEHRE vectors to achieve higher level expression of helper functions in replication-competent T antigen expressing cells.

Thus, the presence of T-antigen in COS cells can be exploited both to increase the levels of viral genomic RNA and to increase levels of helper functions. In the event that runaway replication of viral genomic RNA is toxic or saturates the packaging system, copy number of the retroviral vectors can be suppressed by the inclusion of BPV sequences just as are copy numbers of the vectors carrying the helper functions.

High cassette copy numbers can also be achieved via gene amplification techniques. Such techniques include, but are not limited to, gene amplification driven by

extrachromosomal replicons derived from, for example, BPV, BK, or EBV, as described above. Alternatively, the polycistronic, e.g., tricistronic, message cassettes can further comprise a gene amplification segment including, but not limited to, a DHFR or an ADA segment, which, when coupled with standard amplification techniques well known to those of skill in the art, can successfully amplify message cassette copy number.

The novel retroviral packaging cell lines of the invention can incorporate further modifications which optimize expression from retroviral LTR promoters. In one embodiment, the cell lines exhibit enforced expression of transcription factors that are known to activate retroviral LTR-driven expression in murine T cells. Such transcription factors include, but are not limited to, members of the ets family, cbf (e.g., cbf-a and cbf-b), CTF/NF-1c, glucocorticoid receptor, GRE, NF1, C/EBP, LVa, LVb, and LVc. Retroviral packaging cell lines of this embodiment are designed to more efficiently produce, for example, murine leukemia virus-derived retroviral particles, including but not limited to, Moloney murine leukemia virus (MoMuLV)-derived retroviral particles.

Packaging cell lines with a capacity for increased transcription from the MuMoLv LTR can also be selected in a genetic screen which is executed as described in section 5.7, below. A representative selection scheme begins with a precursor cell line containing a quantifiable marker whose expression is linked to a MoMuLV LTR. Preferably, such an LTR/quantifiable marker construct is excisable. As such, the construct can further comprise an excision element which is equivalent to the proviral excision element described, above, in Section 5.1.

Precursor cells are infected with a cDNA library derived from murine T-cells. Cells with increased expression, as assayed by the expression of the quantifiable marker, are then identified. Recovery of the library DNA from such cells then identifies gene sequences responsible for such increased expression rates.

The resulting packaging cell lines produced via such a selection scheme exhibit an expression pattern of genes encoding retroviral regulatory factors which closely resembles a murine T-cell pattern of expression for such factors.

Packaging cell lines can be developed which express gag, pol and/or env proteins modified in a manner that promotes an increased viral titer and/or infectivity range. For example, MuLV-based viruses are limited to the infection of proliferating cells. The block to MuLv infection is at the level of entry of the preintegration complex into the nucleus. The complex remains cytoplasmic until dissolution of the nuclear envelope during cell division. Lentiviruses escape this block by incorporating a nuclear targeting signal into the viral capsid. This signal however, must also allow targeting of capsid proteins for

assembly at the cytoplasmic face of the cell membrane during viral assembly and budding. This problem is resolved by the fact the nuclear targeting signal of lentiviral capsids is conditional.

In order to overcome the block to MuLv infection of nonproliferating cells, nuclear targeting signals can be incorporated into MuLv virons during assembly in the packaging cell lines of the invention. For example, modified gag proteins can be expressed by the packaging cell lines which can, at low levels, become incorporated into virion capsids during assembly. Nuclear targeting signal sequences are well known to those of skill in the art, and expression of such modified gag proteins can, for example, be via the pEHRE vectors of the invention.

To successfully achieve the goal of creating MuLv virions capable of infecting nonproliferating cells, the gag fusion protein bearing the target signal should be incorporated into the virion capsid as a minority species. Further, the nuclear targeting signal should be a conditional one, such that the fusion is targeted to the nucleus only in infected cells.

In one embodiment of such a modified gag fusion protein, the nuclear targeting signal is one that requires ligand binding for nuclear localization. For example, the glucocorticoid family of receptors have such a ligan-dependent nuclear targeting characteristic.

Alternatively, nuclear targeting of infected cells can be achieved by providing in the infected cell a protein which has affinity for a retroviral capsid (or a tagged retroviral capsid) and also has a nuclear targeting capability, thereby shuttling a virion to the nucleus of infected cells. For example, a single chain antibody can be expressed or introduced which recognizes capsid or capsid tag, wherein the antibody is fused to a nuclear localization signal.

It is also contemplated that similar packaging lines can be derived for adeno-associated viral vectors. For instance, the rep and cap genes are required in trans to provide functions for replication and encapsidation of AAV vectors, and AAV rep and cap coding region can accordingly be provided on the episomal vectors in a manner similar to the retroviral gag-pol and env genes.

IX Complementation screening methods

Mammalian cell complementation screening methods are described herein. Such methods can include, for example, a method for identification of a nucleic acid sequence

whose expression complements a cellular phenotype, comprising: (a) infecting a mammalian cell exhibiting the cellular phenotype with a retrovirus particle derived from a cDNA or gDNA-containing retroviral vector of the invention, or, alternatively, transfecting such a cell with a pEHRE vector of the invention wherein, depending on the vector, upon infection an integrated retroviral provirus is produced or upon transfection an episomal sequence is established, and the cDNA or gDNA sequence is expressed; and (b) analyzing the cell for the phenotype, so that suppression of the phenotype identifies a nucleic acid sequence which complements the cellular phenotype.

The term "suppression", as used herein, refers to a phenotype which is less pronounced in the presence in the cell expressing the cDNA or gDNA sequence relative to the phenotype exhibited by the cell in the absence of such expression. The suppression may be a quantitative or qualitative one, and will be apparent to those of skill in the art familiar with the specific phenotype of interest.

The present invention also includes methods for the isolation of nucleic acid molecules identified via the complementation screening methods of the invention. Such methods utilize the proviral excision and the proviral recovery elements described, e.g., in Section 5.1.1, above.

In one embodiment of such a method, the proviral excision element comprises a loxP recombination site present in two copies within the integrated provirus, and the proviral recovery element comprises a lacO site, present in the provirus between the two loxP sites. In this embodiment, the loxP sites are cleaved by a Cre recombinase enzyme, yielding an excised provirus which, upon excision, becomes circularized. The excised, circular provirus, which contains the lacO site is recovered from the complex mixture of recipient cell genomic nucleic acid by lac repressor affinity purification. Such an affinity purification is made possible by the fact that the lacO nucleic acid specifically binds to the lac repressor protein.

In an alternative embodiment, the excised provirus is amplified in order to increase its rescue efficiency. For example, the excised provirus can further comprise an SV40 origin of replication such that in vivo amplification of the excised provirus can be accomplished via delivery of large T antigen. The delivery can be made at the time of recombinase administration, for example.

In another alternative embodiment, the excised provirus may be recovered by use of a Cre recombinase. For example, the isolated DNA is fragmented to a controlled size. The provirus containing fragments are isolated via LacO/LacI. Following IPTG elution, circularization of the provirus can be accomplished by treatment with purified

recombinase.

X. Antisense methods

Antisense genetic suppressor element (GSE)-based methods for the functional inactivation of specific essential or non-essential mammalian genes are described herein. Such methods include methods for the identification and isolation of nucleic acid sequences which inhibit the function of a mammalian gene. The methods include ones which directly assess a gene's function, and, importantly, also include methods which do not rely on direct selection of a gene's function. These latter methods can successfully be utilized to identify sequences which affect gene function even in the absence of knowledge regarding such function, e.g., in instances where the phenotype of a loss-of-function mutation within the gene is unknown.

An inhibition of gene function, as referred to herein, refers to an inhibition of a gene's expression in the presence of a GSE, relative to the gene's expression in the absence of such a GSE. Preferably, the inhibition abolishes the gene's activity, but can be either a qualitative or a quantitative inhibition. While not wishing to be bound by a particular mechanism, it is thought that GSE inhibition occurs via an inhibition of translation of transcript produced by the gene of interest.

The nucleic acid sequences identified via such methods can be utilized to produce a functional knockout of the mammalian gene. A "functional knock-out", as used herein, refers to a situation in which the GSE acts to inhibit the function of the gene of interest, and can be used to refer to functional knockout cell or transgenic animal.

In one embodiment, a method for identifying a nucleic acid sequence which inhibits the function of a mammalian gene of interest can comprise, for example, (a) infecting a mammalian cell with a retrovirus derived from a GSE-producing retroviral vector containing a nucleic acid sequence from the gene of interest, or, alternatively, transfecting such a cell with a pEHRE-GSE vector of the invention containing a nucleic acid sequence from the gene of interest, wherein the cell expresses a fusion protein comprising an N-terminal portion derived from an amino acid sequence encoded by the gene and a C-terminal portion containing a selectable marker, preferably a quantifiable marker, and wherein an integrated retroviral provirus is produced, or, depending on the vector, an episomal established, that expresses the cDNA or gDNA sequence; (b) selecting for the selectable marker; and (c) assaying for the quantifiable or selectable marker, so that if the selectable marker is inhibited, a nucleic acid sequence which inhibits the function of the mammalian gene is identified.

In one preferred embodiment of this identification method, the fusion protein is encoded by a nucleic acid whose transcription is controlled by an inducible regulatory sequence so that expression of the fusion protein is conditional. In another preferred embodiment of the identification method, the mammalian cell is derived from a first mammalian species and the gene is derived from a second species, a different species as distantly related as is practical.

In a fusion protein-independent embodiment, the nucleic acid encoding the selectable marker can be inserted into the gene of interest at the site of the gene's initiation codon, so that the selectable marker is translated instead of the gene of interest. This embodiment is useful, for example, in instances in which a fusion protein may be deleterious to the cell in which it is to be expressed, or when a fusion protein cannot be made.

The method for identifying a nucleic acid sequence which inhibits the function of a mammalian gene, in this instance, comprises: (a) infecting a mammalian cell expressing a selectable marker in such a fashion with a retrovirus derived from a GSE-producing retroviral vector containing a nucleic acid sequence derived from the gene of interest, or, alternatively, transfecting such a cell with a pEHRE-GSE vector of the invention containing a nucleic acid sequence derived from the gene of interest, wherein, upon infection, an integrated provirus is formed, or, depending on the vector, an episomal sequence is established, and the nucleic acid sequence is expressed; (b) selecting for the selectable marker; and (c) assaying for the selectable marker, so that if the selectable marker is inhibited, a nucleic acid sequence which inhibits the function of the mammalian gene is identified. Selection for the marker should be quantitative, e.g., by FACS.

In an additional embodiment, the gene of interest and the selectable marker can be placed in operative association with each other within a bicistronic message cassette, separated by an internal ribosome entry site, whereby a single transcript is produced encoding, from 5' to 3', the gene product of interest and then the selectable marker. Preferably, the sequence within the bicistronic message derived from the gene of interest includes not only coding, but also 5' and 3' untranslated sequences.

The method for identifying a nucleic acid sequence which inhibits the function of a mammalian gene, in this instance, comprises: (a) infecting a mammalian cell expressing a selectable marker as part of such a bicistronic message with a retrovirus derived from a GSE-producing retroviral vector containing a nucleic acid sequence derived from the gene of interest, or, alternatively, transfecting such a cell with a pEHRE-GSE vector of the invention containing a nucleic acid sequence derived from the gene of interest, wherein,

depending on the vector, upon infection, an integrated provirus is formed, or an episomal sequence is established, and the nucleic acid sequence is expressed; (b) selecting for the selectable marker; and (c) assaying for the selectable marker, so that if the selectable marker is inhibited, a nucleic acid sequence which inhibits the function of the mammalian gene is identified.

In an alternative embodiment, such a method can include a method for identifying a nucleic acid which influences a mammalian cellular function, and can comprise, for example, (a) infecting a cell exhibiting a phenotype dependent upon the function of interest with a retrovirus derived from a GSE-producing retroviral vector containing a test nucleic acid sequence, or, alternatively, transfecting such a cell with a pEHRE-GSE vector of the invention containing a test nucleic acid sequence, wherein, upon infection the an integrated provirus is formed, or, depending on the vector, an episomal sequence is established, and the test nucleic acid is expressed; and (b) assaying the infected cell for the phenotype, so that if the phenotype is suppressed, the test nucleic acid represents a nucleic acid which influences the mammalian cellular function. Such an assay is the same as a sense expression complementation screen except that the phenotype, in this case, is presented only upon loss of function.

The above methods are independent of the function of the gene of interest. The present invention also includes antisense methods for gene cloning which are based on function of the gene to be cloned. Such a method can include a method for identifying new nucleic acid sequences based upon the observation that loss of an unknown gene produces a particular phenotype, and can comprise, for example, (a) infecting a cell with a retrovirus derived from a GSE-producing retroviral vector containing a test nucleic acid sequence, or, alternatively, transfecting such a cell with a pEHRE-GSE vector of the invention containing a test nucleic acid sequence, wherein, upon infection, an integrated provirus is formed, or, depending on the vector, an episomal sequence is established, and the test nucleic acid is expressed; and (b) assaying the infected cell for a change in the phenotype, so that new nucleic acid sequences may be isolated based upon the observation that loss of an unknown gene produces a particular phenotype. Such an assay is the same as a sense expression complementation screen except that the phenotype, in this case, is presented only upon loss of function.

The present invention also includes novel methods for the construction of unidirectional, randomly primed cDNA libraries which can be utilized as part of the function-based methods described above. Such cDNA construction methods can comprise: (a) first strand cDNA synthesis comprising priming the first strand using a nuclease resistant oligonucleotide primer that encodes a restriction site; and (b) second

strand cDNA synthesis comprising synthesizing the second strand an exonuclease deficient polymerase. The nuclease resistant oligonucleotide avoids the removal of a restriction site that marks orientation, thereby allowing for the construction of a unidirectional cDNA random primed cDNA library.

For example, a nuclease resistant chimeric oligonucleotide may be of the general structure: 5'-GCG GCG gga tcc gaa ttc nnn nnn nnn-3'. The modified backbone nucleotides are shown in upper-case, and is generally 4-6 bases, which is followed by one or two restriction sites comprised of normal DNA and nine degenerate nucleotides. A nuclease-deficient polymerase, such as the polymerase from bacteriophage phi-29, can be used.

The present invention also includes methods for the isolation of nucleic acid molecules identified via the antisense screening methods of the invention. Such methods utilize the proviral excision and the proviral recovery elements, as described above.

In one embodiment of such a method, the proviral excision element comprises a loxP recombination site present in two copies within the integrated provirus, and the proviral recovery element comprises a lacO site, present in the provirus between the two loxP sites. In this embodiment, the loxP sites are cleaved by a Cre recombinase enzyme, yielding an excised provirus which, upon excision, becomes circularized. The excised, circular provirus, which contains the lacO site is recovered from the complex mixture of recipient cell genomic nucleic acid by lac repressor affinity purification. Such an affinity purification is made possible by the fact that the lacO nucleic acid specifically binds to the lac repressor protein.

In an alternative embodiment, the excised provirus is amplified in order to increase its rescue efficiency. For example, the excised provirus can further comprise an SV40 origin of replication such that in vivo amplification of the excised provirus can be accomplished via delivery of large T antigen. The delivery can be made at the time of recombinase administration, for example.

XI. Gene trapping methods

The present invention further relates to gene trapping-based methods for the identification and isolation of mammalian genes which are modulated in response to specific stimuli. These methods utilize retroviral particles of the invention to infect cells, which leads to the production of provirus sequences which are randomly integrated within the recipient mammalian cell genome. In instances in which the integration event occurs

within a gene, the gene is "tagged" by the provirus reporter sequence, whose expression is controlled by the gene's regulatory sequences. By assaying reporter sequence expression, then, the expression of the gene itself can be monitored.

The gene trapping-based methods of the present invention have several key advantages, including, but not limited to, (1) the presence in the 3' LTR of a gene trapping cassette that is duplicated upon integration of the provirus into the host genome. This duplication results in the placement of the gene trapping cassette adjacent to genomic DNA such that polymerase entering the virus from an adjacent gene would transcribe the gene trapping cassette before encountering the polyadenylation signal that is present in the LTR. The inclusion of an IRES sequence in the gene trapping cassette allows the fusion between cellular and viral sequence to occur at any point within the mature mRNA, effectively increasing the number of possible integration sites that result in a functionally "tagged" transcript; and (2) the use of a quantifiable selectable marker that can be assessed by live sorting in the FACS, allowing for the isolation of clones that are induced, but also, of clones that tag genes that are repressed.

The term "modulation", as used herein, refers to an up- or down-regulation of gene expression in response to a specific stimulus in a cell. The modulation can be either a quantitative or a qualitative one.

Gene trapping methods of the invention can include, for example, a method which comprises: (a) infecting a mammalian cell with a retrovirus derived from a gene trapping vector of the invention, wherein, upon infection, an integrated provirus is formed; (b) subjecting the cell to the stimulus of interest; assaying the cell for the expression of the reporter sequence so that if the reporter sequence is expressed, it is integrated within, and thereby identifies, a gene that is expressed in the presence of the stimulus.

In instances wherein the gene is not expressed, or, alternatively, is expressed at a different level, in the absence of the stimulus, such a method identifies a gene which is expressed in response to a specific stimulus.

The present invention also includes methods for the isolation of nucleic acid sequence expressed in the presence of, or in response to, a specific stimulus. Such methods can comprise, for example, digesting the genomic nucleic of a cell which contains a provirus integrated into a gene which is expressed in the presence of, or in response to, the stimulus of interest; and recovering nucleic acid containing a sequence of the gene by utilizing the means for recovering nucleic acid sequences from a complex mixture of nucleic acid.

In one embodiment, the means for recovery is a lacO site, present in the integrated

provirus. The digest fragment which contains the lacO site is recovered from the complex mixture of recipient cell genomic nucleic acid by lac repressor affinity purification. Such an affinity purification is made possible by the fact that the lacO nucleic acid specifically binds to the lac repressor protein.

Such methods serve to recover proviral nucleic acid sequence along with flanking genomic sequence (i.e., sequence contained within the gene of interest). The isolated sequence can be circularized, yielding a plasmid capable of replication in bacteria. This is made possible by the presence of a bacterial origin of replication and a bacterial selectable marker within the isolated sequence.

Upon isolation of flanking gene sequence, the sequence can be used in connection with standard cloning techniques to isolate nucleic acid sequences corresponding to the full length gene of interest.

XII. Embodiments of the screening assay

As stated above, the methods of the present invention include methods for the identification and isolation of nucleic acid molecules based upon their ability to complement a mammalian cellular phenotype, antisense-based methods for the identification and isolation of nucleic acid sequences which inhibit the function of a mammalian gene, and gene trapping methods for the identification and isolation of mammalian genes which are modulated in response to specific stimuli.

The compositions of the present invention include replication-deficient retroviral vectors, such as complementation screening retroviral vectors, antisense-genetic suppressor element (GSE) vectors, vectors displaying random peptide sequences, gene trapping vectors, libraries comprising such vectors, retroviral particles produced by such vectors and novel packaging cell lines. The following provides specific embodiments for the utilization of such methods, vectors and compositions for the elucidation of mammalian gene function.

The compositions of the present invention further include pEHRE vectors, such as complementation screening retroviral vectors, antisense-genetic suppressor element (GSE) vectors, vectors displaying random peptide sequences, libraries, cells and animals comprising such vectors, and novel packaging cell lines. The following provides specific embodiments for the utilization of such methods, vectors and compositions for the elucidation of mammalian gene function.

*A) Bypass of conditional phenotypes*

Many phenotypes can be conferred upon mammalian cells in culture by conditional overexpression of known genes (e.g., growth arrest, differentiation). The interference with such phenotypes can be examined by overexpression of sense orientation genes or by functional knock-out (via GSE expression). Examples of this type of screening are given below.

i. Bypass of p53-mediated growth arrest and apoptosis.

Increases in the level of p53 can cause either growth arrest (generally by cell cycle arrest in G1) or programmed cell death. Cells lines that conditionally overexpressing p53 and contain a p53 functional knock-out will allow for the dissection of both of these processes. In the first case, mouse embryo fibroblasts (MEF) which lack endogenous p53 genes (from p53 knock-out mice) are engineered to conditionally express a fluorescently tagged p53 protein. When activated the fluorescent p53 is localized to the nucleus and enforces cell cycle arrest. Bypass of the arrest can be accomplished by overexpression of sense cDNAs or by expression of GSE fragments. Such a screen might identify components of the p53-degradative pathway, genes that do not affect p53 but allow cell cycle progression even in the presence of p53 and genes that affect p53 localization (p53 is not mutated but is mislocalized in a significant percentage of breast tumors and neuroblastomas). Therefore, use of a fluorescent p53 protein provides information as to the mechanism of bypass.

A very similar cell line can be used to dissect p53-mediated cell death. While p53 alone induces growth arrest in most fibroblasts, combination with certain oncogenes (myc, in particular) causes cell death. MEF cells that conditionally overexpress both myc and p53 are engineered. When activated in combination these genes induce cell death in a substantial fraction of cells. Rescue from this cell death via overexpression of sense oriented cDNAs can be used to identify anti-apoptotic genes (and possible p53-regulators as above). Rescue by GSE expression might identify components of the pathways by which myc and p53 induce cell death (downstream targets) or cellular genes that are required for the apoptotic program.

ii. Bypass of the M1 component of cellular immortalization.

Immortalization of mammalian cells can be divided into two functional steps, M1

and M2. M1 (senescence) can be overcome in fibroblasts by viral oncoproteins that inactivate tumor suppressors, p53 and pRB. SV40 large T antigen is one such protein. Conditionally immortal cells have been derived using temperature sensitive or inducible versions of T-antigen. Upon T inactivation these cells senesce and cease proliferation. The growth of such cells may be rescued by introduction of sense and antisense libraries.

Similar screens can be undertaken with any gene that confers a phenotype upon overexpression. Essentially identical growth-rescue screens can also be undertaken using cytokines that induce growth arrest or apoptosis (e.g., TGF-beta in HMEC or Hep3B cells, respectively).

*B) Identification of cytokines in cis and trans.*

Historically, several cytokines have been identified functionally by production in mammalian systems. Specifically, COS cells that express pools of transfected cDNAs have been used to prepare conditioned media that was then tested for the ability to induce growth of factor-sensitive cells. Growth regulatory cytokines may be identified (or survival factors that suppress cell death) by expression of cDNA libraries directly in the target cells. Such an approach has been hampered in the past by the low transfection efficiencies of the target cell types. For example, survival of hematopoietic stem cells is promoted by a variety of known and unknown factors. Therefore, upon infection of such cells with cDNA libraries derived from stromal cells that promote the growth and survival of stem cell populations, selection for surviving infected cells may identify those that carry cDNAs encoding necessary factors. Such factors would be produced in an autocrine mode. While this approach will identify trans-acting factors, cDNA that also act in cis (e.g., by short-circuiting growth-regulatory signal transduction pathways) will also be identified. These can be eliminated by searching for secreted growth regulatory factors using a two-cell system. In this case, one cell type is infected with a library and used as a factory to produce cDNA products, some of which will be secreted proteins. A second cell type that is factor-responsive is then plated over the cDNA expressing cells in a medium (e.g., soft-agar) that restricts diffusion. Responsive cells plated over the producing cells that elaborate the required factor will grow and the appearance of a colony of responsive cells will mark the underlying cells that elaborate the specific factor. The advantage of a two-cell system is more evident in the case where extracellular factors induce growth arrest or terminal differentiation. In such cases, expression in cis would be impractical since selection would be against the population expressing the desired gene. In trans, however, changes in recipient cells can be scored visually and the underlying expressing

cells can be rescued for isolation of the desired gene. Similar two cell screens could be developed using the methods of the present invention to screen for factors that promote cell migration or cell-adhesion.

*C) Identification of synthetic peptides that can affect cellular processes*

The present invention provides methods for the identification and isolation of peptides sequences by complementation type screens using vectors capable of displaying random synthetic peptide sequences that interact with a protein of interest in mammalian cells. Conventional screening methods of identifying proteins of interest have been conducted using phage systems and two hybrid screens in yeast. The present invention provides a novel screening method to extend this paradigm to mammalian cells.

i. Intracellular peptide display.

As set out above, in another aspect of the present invention the subject vectors can be used for generating peptide display libraries. For embodiments featuring an intracellular peptide library, particular where the peptides are relatively to short, e.g., 5-30 amino acid residues, the peptide can be provided as part of a fusion protein with a conformation-constrained protein (i.e., a protein that decreases the flexibility of the amino and carboxy termini of the protein). In general, conformation-constraining proteins act as scaffolds or platforms, which limit the number of possible three dimensional configurations the peptide or protein of interest is free to adopt. Preferred examples of conformation-constraining proteins are thioredoxin or other thioredoxin-like sequences, but many other proteins are also useful for this purpose. Preferably, conformation-constraining proteins are small in size (generally, less than or equal to 200 amino acids), rigid in structure, of known three dimensional configuration, and are able to accommodate insertions of proteins of interest without undue disruption of their structures. A key feature of such proteins is the availability, on their solvent exposed surfaces, of locations where peptide insertions can be made (e.g., the thioredoxin active-site loop).

As mentioned above, one preferred conformation-constraining protein according to the invention is thioredoxin or other thioredoxin-like proteins. The three dimensional structure of *E. coli* thioredoxin is known and contains several surface loops, including a distinctive Cys-Cys active-site loop between residues Cys33 and Cys36 which protrudes from the body of the protein. This Cys-Cys active-site loop is an identifiable, accessible surface loop region and is not involved in interactions with the rest of the protein which

contribute to overall structural stability. It is therefore a good candidate as a site for prey protein insertions. Both the amino- and carboxyl-termini of *E. coli* thioredoxin are on the surface of the protein and are also readily accessible for fusion construction.

It may be preferred for a variety of reasons that test peptide be fused within the active-site loop of thioredoxin or thioredoxin-like molecules. The face of thioredoxin surrounding the active-site loop has evolved, in keeping with the protein's major function as a nonspecific protein disulfide oxido-reductase, to be able to interact with a wide variety of protein surfaces. The active-site loop region is found between segments of strong secondary structure and this provides a rigid platform to which one may tether prey proteins. A small heterologous peptide inserted into the active-site loop of a thioredoxin-like protein is present in a region of the protein which is not involved in maintaining tertiary structure. Therefore the structure of such a fusion protein is stable.

Such libraries of random peptide sequences can be expressed from the subject vectors in mammalian cells. Expressed peptides that confer particular phenotypes can be isolated in genetic screens similar to those described above. The cellular targets of these peptides can then be isolated based upon peptide binding in vitro or in vivo.

ii. Extracellular peptide display.

It is well established that the interaction between extracellular signaling molecules (e.g., growth factors) and their receptors occurred over large protein surfaces. The present invention provides a novel screen that allows for rapid identification of peptides in mammalian cells by expressing constrained peptides on the surface of receptor-bearing cells and selecting directly for biological function. A synthetic peptide can be displayed in a mammalian system by replacing one flexible loop of a synthetic peptide display vehicle or cassette, the minibody, with a polylinker into which a library of random oligonucleotides encoding random peptides may be inserted. The resulting synthetic chimera can be tethered to the membrane so that it appears on the cell surface by providing a heterologous membrane anchor, such as that derived from the *c. clegans* decay accelerating factor (DAF). This chimeric protein could then serve as an extracellular peptide display vehicle. Peptide libraries in a retroviral vector could be screened directly for the ability to activate receptors, or screening in vivo could follow a pre-selection of a mini-library by phage display.

To further elaborate, the membrane anchor domain may be any moiety capable of causing attachment to the cell surface. A variety of such moieties are known in the art and

include, but are not limited to, transmembrane domains derived from known proteins, a span of hydrophobic amino acid residues sufficient to effect transmembrane spanning, an amino acid sequence that is targeted for post-translational modification by the covalent attachment of lipid molecules and polypeptides having sufficient affinity for a transmembrane protein to effect binding of the molecule to the surface of the cell membrane. Transmembrane domains, both natural and artificial, are known in the art and may be present in multiple copies separated by a sufficient number of amino acid residues to allow multiple membrane spanning by the domains. Typically, a transmembrane domain contains a number of hydrophobic amino acid residues sufficient to span a membrane, and includes at least one and usually several positively charged amino acid residues C-terminal to the hydrophobic amino acids. The positively charged amino acids prevent further transfer of the nascent protein through the membrane. Suitable membrane anchoring domains that function by lipid modification include, but are not limited to, the decay accelerating factor (DAF) which is modified by covalent linkage to glycosyl phosphatidyl inositol (GPI). Such are preferred embodiments and allow for subsequent specific cleavage of the protein from the cell surface.

*D) Resistance to parasite and viral infection*

Viruses and a number of parasitic organisms require intracellular environments for reproduction. The screens of the present invention may be utilized (e.g., sense overexpression, GSE expression, intracellular peptide display, extracellular peptide display) to identify routes to viral and parasite resistance.

For example, it has recently been demonstrated that resistance to HIV infection can be conferred by expression of a specific mutant gene. The methods of present invention may also be applied to develop a screen for other genes (natural, mutant or synthetic) that confer resistance to HIV infection or that interfere with the viral life cycle.

The methods of the present invention may also be applied to develop a screen for genes that interfere with the viral life cycle of an intracellular parasite, e.g., plasmodium.

*E) Identification of drug-screening targets for tumor cells that lack specific tumor suppressors*

A number of studies have identified two major tumor suppression pathways which are lost in a high percentage of human tumors. The p53 protein is functionally inactivated in approximately 50% of all tumors and the p16/Rb pathway is affected at an even higher

frequency. Loss of these pathways for growth control is one of the most obvious distinctions between normal and tumor cells. Many chemotherapeutic drugs act by inducing cell death, and their selectivity is based upon the fact that tumor cells are proliferating while most of the normal cells in the body are quiescent. The methods of the present invention may also be applied to develop screens to identify gene products whose inactivation induces cell death specifically in cells lacking one or both of the two major tumor suppression pathways. This should provide drug screening targets that could lead to compounds that distinguish cells not based upon their proliferation index but based on their genotype.

Identification of such drug screening targets will depend upon that isolation of GSE sequences that can induce apoptosis specifically in the absence of p53 or in the absence of the p16/Rb pathway or both. Cells which conditionally lack either p53, p16/Rb or both can be prepared using conditional viral oncoproteins. For example, p53 can be conditionally inactivated using an inducible E6 protein or using a temperature sensitive T-antigen that has also lost the ability to bind Rb. Conditional loss of p16/Rb can be accomplished using conditionally expressed E7 or again with a ts-T antigen that is mutant for p53 binding. Such cells will be infected with a GSE library and passaged under conditions where p53 or p16/Rb regulation is intact. Those sequences that induce death in normal cells will be naturally counter-selected. The desired tumor suppression pathway will then be specifically inactivated and apoptotic cells will be purified by magnetic separation techniques that rely on the ability of annexing V to bind to the membrane of apoptotic cells. DNA prepared from apoptotic populations will then be used to rescue viral libraries. Several rounds of such screening should enrich for populations of GSE sequences that induce cell death in response to loss of tumor suppressor function.

*F. Identification of genes involved in metastasis (in vivo selections)*

The methods of the present invention may also be applied to develop screens to identify genes involved in metastasis. There are a number of well-characterized systems in which the ability of tumor cells to metastasize can be studied in vivo. The most common is the mouse footpad microinjection assay. Populations of non-metastatic cells can be infected with sense and antisense libraries. These can be injected into the mouse footpad and metastatic cells can be isolated after outgrowth of remote tumors. Rescue of viruses from such cells can be used to identify genes that regulate the ability of tumor cells to metastasize.

**EXAMPLE 1:** CONSTRUCTION OF THE RETROVIRAL MaRXII VECTOR

The following example provides the methods for the construction of replication-defective retrovirus, pMaRXII. The starting vector is pBABE puro (Morgenstern, 1990, Nucleic Acids Res. 18: 3587-3596), which is modified as follows:

The insertion of a synthetic linker comprising a loxP site was into the NheI site. The sequence of the linker containing the loxP site is as follows:

5'-
CTAGCATAACTTCGTATAATGTATGCTATACGAAGTTATGTATTGAAGC-
ATATTACATACGATATGCTTCAATAGATC-3'

The insertion of this synthetic linker creates a loxP site while simultaneously destroying the 3' NheI site, leaving a unique NheI site.

The insertion of a polylinker between the BamHI and SalI sites of pBABE puro which contains a primer binding site for the universal (-20) sequencing primer and the lac operator sequence. The sequence of the upper strand of the polylinker is as follows:

5'GGATCCGTAAAACGACGGCCAGTTTAATTAAGAATTCGTTAACGCATGCCTC
GAGTGTGGAATTGTGAGCGGATAACAATTTGTCGAC3'

The insertion of a PCR fragment comprised of the bacterial EM7 promoter and the zeocin resistance gene was amplified from pZEO SV (Invitrogen) such that the SalI and StuI sites were included at the 5' end of the fragment and the BspEI and ClaI sites were included at the 3' end of the fragment. The modified pBABE puro vector was digested with SalI and ClaI and ligated with the PCR fragment. The sequence of the upper strand of the PCR fragment is as follows:

5'gtcgacaggcctCGGACCTGCAGCACGTGTTGACAATTAATCATCGGCATAGTATA
TCGGCATAGTATAATACGACTCACTATAGGAGGGCCACCATGGCCAAGTTGAC
CAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCT
GGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGT
GTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGT
GCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTAC
GCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGC
CATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGAC
CCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGAttccggatttatcg

at3'

The insertion of a PCR fragment comprised of the RK2 OriV which was amplified from the plasmid pMYC3 (Shah et al., 1995, J. Mol. Biol. 254: 608-622). The minimal oriV was chosen as defined in Shah et al. This PCR fragment contained a BspEI site at its 5' end and BglII and ClaI sites at its 3' end. The modified pBABE puro vector and the PCR fragment were both digested with BspEI and ClaI and ligated together. The sequence of the top strand of the PCR fragment is as follows:

5'TCCGGAcgagtttcccacagatgatgtggacaagcctggggataagtgccctgcggtattgacacttgaggggcgcgact
actgacagatgaggggcgcgatccttgacacttgaggggcagagtgatgacagatgaggggcgcacctattgacatttgagggg
ctgtccacaggcagaaaatccagcatttgcaagggtttccgcccgtttttcggccaccgctaacctgtcttttaacctgcttttaaacca
atatttataaaccttgtttttaaccagggctgcgccctggcgcgtgaccgcgcacgccgaaggggggtgccccccccttctcgaacc
ctcccggAGATCTatcgat3'

The inclusion of a pUC origin of replication in an equivalent position to the RK2 OriV in either orientation was found to reduce both viral titer and expression levels in infected cells.

The F1 origin of replication was also inserted in the modified pBABE puro vector. The F1 origin of replication was amplified from pBluescript SK+ (Stratagene) and Not1 restriction sites were added to the 5' and 3' ends. This fragment was inserted into the modified vector following digestion of both the modified pBABE puro vector and the fragment with Not1. An orientation of the F1 origin was chosen that would yield, upon helper rescue, the sense strand of the cDNA. The sequence of the amplified F1 fragment is as follows:

5'gcggccgcGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT
TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCG
CTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAA
TCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAA
AAAACTTGATTAGGGTGATGGTTCaCGTAGTGGGCCATCGCCCTGATAGACGG
TTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCA
AACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGAT
TTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTA
ACGCGAATTTTAACAAAATATTAACGTTTACAAgcggccgc3'

The vector was further modified by the insertion of a PacI site between the BglII and ClaI sites of the modified pBABE puro vector using the following synthetic fragment :

5'-GATCTTTAATTAAAT-3'

AAATTAATTTAGC

The vector was still further modified by the insertion of a PmeI site into the BspEI of the modified pBABE puro vector site using the following synthetic fragment :

5'-CCGGGTTTAAACT-3'

CAAATTTGAGGCC

The insertion of this fragment destroys one BspEI site, leaving the second site intact.

The vector was further modified by the insertion of a fragment comprising an IRES(EMCV)-Hygromycin resistance marker. The IRES hygromycin resistance cassette was created by amplification of the Hygromycin sequence from pBabe-Hygro (Morgenstern et al., 1990, Nucl. Acids Res. 18: 3587-3596) such that it lacked the first methionine of the hygromycin coding sequence and such that ClaI and SalI sites were added following the stop codon. This was inserted into the IRES-containing vector, pCITE (digested MscI-SalI) such that the first methionine of the hygromycin protein was donated by the vector. Methionine placement is critical for efficient function of the IRES. This cassette was amplified by PCR such that a SalI site was added upstream of the functional IRES and was re-inserted into the pBabe-Hygro following digestion of both with SalI and ClaI. This fragment was excised and inserted into the SalI site of the modified vector such that SalI sites were reformed on both sides.

The resulting vector is the MaRXII backbone (FIGURE 1). The derivation of the specific purpose vectors from the MaRXII backbone is described below.

In the illustrated MaRXII vector, excision of the provirus by recombinase treatment or the like, because of the location of the recombinase sites in the LTR sequences, results in a closed, circular vector with only one LTR. In the illustrated embodiment, the defective LTR cannot be used to make virus. However, another aspect of the present invention provides a convenient means for adding back LTR elements necessary for generating an infectious (though still replication-deficient) retroviral vector. As illustrated in Figure 25, we derived the so-called reunification vector to provide, by recombinase mediated ligation, a vector in which the LTR sequences have been restored

and the resulting vector can be used, e.g., upon isolation from bacterial cells in which it may be amplified, in the transient transfection of the packaging cell lines and the generation of a second round of infectious viral particles. In its simplest of embodiments, the subject method provides an second construct having an LTR with a recombination site which can bring about cross-ligation of the second construct with the retroviral vector so as to recapitulate a vector which includes the original retroviral sequences now being flanked by LTR sequences at both the 5' and 3' ends.

**EXAMPLE 2:** CONSTRUCTION OF THE RETROVIRAL VECTOR FOR SENSE COMPLEMENTATION SCREENING

This example provides the methods for constructing the sense-expression complementation screening vector, a pMaRXII derivative vector, pHygro MaRXII-LI (FIGURE 3). The starting point for the construction of this vector begins with the MaRXII vector, as described above.

The vector is further modified by the insertion of a synthetic NotI linker which was ligated into the NheI site such that only one NheI site was left intact. The sequence of the NotI linker is as follows:

5'CTAGATGCGGCCGCTAG3'

TACGCCGGCGATCGATC

A PCR fragment comprising the SV40 origin (below) was ligated into the PmeI site (in either orientation) to allow for replicative excision. The sequence of the fragment is as follows:

5'GGGGTTTAAACGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTG
CCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCCC3'

The NsiI-NsiI fragment was deleted from pZero (Invitrogen) and this served as a template for the amplification of the lethal insert with primers that recognized the 5' end of the pTac promoter and the 3' end of the ccdB coding sequence. These primers added EcoRI and XhoI sites, respectively. The fragment was inserted following digestion of both the plasmid and the PCR product with EcoRI and XhoI.

This forms the basic sense expression vector. Other markers can replace the IRES-Hygromycin resistance cassette (e.g. IRES-Puromycin resistance, IRES-neomycin resistance, IRES-blasticidin resistance etc.). This vector has been used to produce virus population with titers exceeding $10^6$ particles/ml (as measured on NIH 3T3 cells). This is equivalent to titers obtained from the original pBabe vector. Thus, modifications have not compromised the ability of the vector to produce virus. Furthermore, expression levels obtained from the p.Hygro.MaRXII vectors approximate those obtained with other retroviral vectors (e.g. pBabe). This vector infects with high efficiency a wide variety of tissue culture cells including but not limited to : NIH-3T3, Mv1Lu, IMR-90, WI38, Hep3B, normal human mammary epithelial cells (primary culture), HT1080, HS578t. This vector has been used to test reversion/excision with the result that following infection with a Cre-encoding virus, >99% of cells lose the phenotype conferred by the MaRX II provirus. Following recovery protocols detailed below, $>1x10^3$ independent colonies can be routinely recovered from 100 μg of genomic DNA containing the provirus (without T-antigen driven amplification).

**EXAMPLE 3:** CONSTRUCTION OF THE RETROVIRAL VECTOR FOR ANTISENSE COMPLEMENTATION SCREENING

This example provides the methods for constructing the antisense screening vectors, the MaRXIIg series, a pMaRXII derivative vector.

Construction of the MaRXIIg series began with a MaRXII vector as described above, except that it lacked the PacI site. A marker, in most cases hygromycin-resistance, is inserted into the unique SalI site created.

**MaRXIIg**

The pMARXII vector was modified by the following steps:

A synthetic polylinker of the following sequence was added between the BamHI and SalI sites of MaRXII.

```
5'-GATCGTTAATTAACAATTGG-3=
3'-     CAATTAATTGTTAACCAGCT-5=
```

A synthetic NotI linker of the following sequence was ligated into the NheI site such that only one NheI site was left intact.

5'-CTAGATGCGGCCGCTAG3'

TACGCCGGCGATCGATC

The CMV promoter was inserted into the modified pMARXII vectors as follows. The CMV promoter sequence was amplified from pcDNA3 (Invitrogen) and this served as a template for amplification of the lethal insert with primers using the following oligonucleotides:

5'-GGGAGATCTACGGTAAATGGCCCGCC-3'

5'-CCCATCGATTTAATTAAGTTTAAACGGGCCCTCTAGGCTCGAG-3'

The amplification product was digested with BglII and ClaI and inserted into a similarly digested MaRXII derivative. The polylinker was then altered by the insertion of the EcoRI-XhoI fragment of the MaRXII polylinker between the EcoRI and XhoI sites of the modified vector. This formed the MaRXIIg vector where the CMV promoter drives GSE expression using the 3'LTR polyadenylation signal to terminate the transcript (FIGURE 7).

**MaRXIIg-dccmv**

The MaRXII derivative from above was digested with NheI. A CMV promoter fragment was prepared by amplification of pHM.3-CMV with the following oligonucleotides :

5'-GGGGCTAGCACGGTAAATGGCCCGCC-'3

5'-CCCTCTAGATTAATTAAGTTTAAACGGGCCCTCTAGGCTCGAG-3'

The CMV fragment was digested with NheI and XbaI and ligated to the MaRXII derivative. An orientation was chosen such that transcription proceeded in the same direction as does transcription from the LTR promoter (FIGURE 8).

**MaRXIIg-VA**

The MaRXII derivative from above (MaRXIIg section) was digested with NheI. An adenovirus VA RNA cassette was prepared by amplification of a modified VA RNA

gene (see Gunnery, 1995 Mol Cell Biol 15, 3597-3607 (1995)) with the following oligonucleotides:

A.GGGGCTAGCCTAGGACCGTGCAAAATGAGAGCC-3'

B.5'-GGGTCTAGATTAATTAAGTTTAAACGGCCAAAAAAGCTTGCGC-3'

This fragment was digested with NheI and XbaI and ligated into the digested MaRX II derivative. An orientation was chosen such that transcription proceeded in the same direction as does transcription from the LTR promoter (FIGURE 9).

All three types of antisense vectors have been used to generate high-titer retroviruses which perform equivalently to p.hygro.MaRXII.

**EXAMPLE 4:** CONSTRUCTION OF THE RETROVIRAL VECTOR FOR GENE TRAPPING

This example provides the methods for the construction of the gene trapping vectors -- pTRAP II, a pMaRXII derivative vector (FIGURE 6).

The pTRAPII vectors are prepared in a MaRXII backbone, as described above.

The pMaRXII vector was modified by the following steps:

A synthetic polylinker was added between the BamHI and SalI sites of MaRXII, of the following sequence:

5'-GATCGTTAATTAACAATTGG-3'

3'-CAATTAATTGTTAACCAGCT-5'

A second synthetic polylinker was added between the BglII and ClaI sites. The top strand of this linker is as follows:

5'agatctTGTGGAATTGTGAGCGGATAACAATTTGGATCCGTAAAACGACGGCCA GTTTAATTAAGAATTCGTTAACGCATGCCTCGAGGTCGACatcgat3'

This incorporates restriction sites for excision from the genome as well as sequencing primer binding sites and the lacO recovery element.

The 3' LTR and accompanying sequences were removed from the pBabe-Puro using ClaI and NotI. These were inserted into a ClaI and NotI digested pBluescript SK+. Site directed mutagenesis was used to delete a segment of the 3' LTR. This was accompanied by a small insertion. The sequences that surround and thus define the

deletion are as follows:

5'-TAACTGAGAA TAGAGAAGTT CAGATCAAGG TCAGGAGATC CCTGAGCCCA CAACCCCTCA CTCGGGGCGC-3'

This fragment was re-inserted into ClaI-NotI digested pBabe-puro to create pBabe-puroSIN. This plasmid was the source for the self-inactivating LTR that was inserted into the gene trapping vector using the unique NheI and SapI restriction sites.

The plasmid pPNT (see Brugarolas et al., 1995) was modified by replacement of the neomycin coding sequence with that of hygromycin (from pBabe-Hygro). This created a hygromycin resistance gene flanked by the PGK promoter and the PGK polyadenylation signals. This cassette was amplified by PCR and inserted into the ClaI site of the gene trapping vector such that transcription from the PGK promoter opposed transcription from the 5' LTR.

A gene trapping cassette was inserted in the NheI site in the 3' LTR. This gene trapping cassette consists of a quantifiable marker whose expression is promoted by an IRES sequence. In most cases the IRES sequence is derived from EMCV although IRES sequences from other sources are equally suitable. Thus far, IRES linked beta-galactosidase and IRES linked green fluorescent protein markers have been incorporated.

**EXAMPLE 5**: CONSTRUCTION OF THE RETROVIRAL VECTOR FOR MULTIPLE ORGANISM DISPLAY VECTORS

This example provides the methods for constructing the Multiple Organism Display or peptide display vectors -- pMODisI and pMODisII, pMaRXII derivative vectors (FIGURE 4 and 5).

The pMODis vectors are designed to act as dual purpose vectors that allow the combination of phage display approaches with functional screening in mammalian systems. These are designed to allow the display of random peptide segments on the surface of filamentous bacteriophage. The displayed peptides can be screened via an affinity approach with a known ligand or a complex mixture of ligands (e.g. fixed cells). The pool of phages which bind to the desired substrate can then be used to generate retroviruses that can be used to infect mammalian cells. A large pool of phage can then be tested individually for the ability to elicit a phenotype. pMODisI is designed to allow display on the surface of phage and of mammalian cells. Additionally by passage through a specific host strain pMODisI can be used to direct secretion of displayed peptides from

mammalian cells. pMODisII is an intracellular display vector. Both are created by the insertion of cassettes between the EcoRI and XhoI sites (destroying these sites) of p.Hygro.MaRXII. The design of the individual cassettes is as follows.

**pMODisI cassette**

The pMODisI cassette contains the following elements in order

1. the beta-globin minimal splice donor site
2. the pTAC promoter
3. a synthetic ribosome binding site
4. the pelB secretion signal
5. the beta globin minimal splice acceptor site
6. a mammalian secretion signal (e.g. from the V-J2-C region of the mouse Ig kappa-chain)
7. the minibody 61 residue peptide display vehicle sequence (Tramontano, J. Mol. Recognit. 7: 9-24 (1994))
8. an FRT recombinase site
9. the 37 amino acid DAF-1 GPI anchor (see Rice et al., PNAS 89: 5467-5471 (1992))
10. an FRT recombinase site
11. an amber stop codon
12. the C-terminus of the geneIII protein, amino acids 198-406
13. non-amber stop codons

In an amber suppressor strain and in the presence of helper phage, a geneIII fusion protein is produced and displayed on the surface of the M13-type phage. This allows display of random peptide sequence cloned into one or both of the two constrained loops of the minibody to be displayed on the phage surface. Expression in packaging cells of MODisI genomic retroviral RNA allows removal of the bacterial promoter and secretion sequences by pre-mRNA splicing and causes translation in the mammalian cell to begin at the first methionine of the minibody sequence. Furthermore, in a mammalian cell, the amber codon would terminate translation prior to the geneIII sequence creating a membrane-bound extracellular minibody that displays a random peptide sequence. The

minibody could be converted to a secreted protein by passage through a FLP-expressing strain of bacteria. This would cause site-specific recombination at the FRT sites and deletion of the membrane anchor sequence.

**pMODisII cassette**

The pMODisII contains the following elements in order.

1. the beta-globin minimal splice donor site
2. the pTAC promoter
3. a synthetic ribosome binding site
4. the pelB secretion signal
5. the beta globin minimal splice acceptor site
7. the thioredoxin peptide display vehicle sequence (Colas et al., Nature 380: 548-550 (1996))
11. an amber stop codon
12. the c-terminus of the geneIII protein, amino acids 198-406
13. non-amber stop codons

This vector is designed for intracellular peptide display. As with pMODis1, the bacterial promoter and signal sequences are removed upon retrovirus production by pre-mRNA splicing.

Both of the pMODis vectors can also be used directly for peptide display in mammalian systems.

**EXAMPLE 6:** PREPARATION OF LIBRARIES

The following example provides the methods for the construction of the libraries of the present invention.

CONSTRUCTION OF SENSE EXPRESSION LIBRARIES IN p.Hygro.MaRX II-LI

*Preparation of the library vector as follows.*

For preparation of the library vector, 10-20 μg of twice CsCl purified vector are digested with 5U/μg of EcoR1 and XhoI for 90 min at 37°C. This digestion is directly loaded onto a 1% agarose gel (SeaKem GTG), and cut vector is separated by electrophoresis in TAE buffer. The vector band is excised following visualization by long-wave UV light. The cut vector is eluted from the agarose by electrophoresis in dialysis tubing. The vector is further purified by phenol/chloroform extraction and ethanol precipitation. It is expected that a vector which is suitable for library preparation can generate >$5x10^6$/0.5μg colonies with <10% background (insert-less) upon ligation with an EcoR1/XhoI digested test insert.

Preparation of cDNA libraries

cDNA synthesis begins with an RNA population that is >10-20 fold enriched (as compared to total RNA) for mRNA. First strand cDNA synthesis is accomplished by standard protocols using SuperscriptII reverse transcriptase. 5-me-dCTP replaces dCTP in the first strand synthesis reaction to block digestion of the newly-synthesized cDNA with XhoI. The first strand cDNA primer is as follows :

5'-GAG AGA GAG AGT CTC GAG TTT TTT TTT TTT TTT TTT-3'

The first nine nucleotides are modified backbone (phosphorthioate) to prevent nuclease degradation of the XhoI site (CTCGAG). Other modifications to the backbone (e.g., p-ethoxy, Peptide-nucleic acid -- PNA) would also serve. Synthesis is initiated by addition of reverse transcriptase in the presence of a saturating amount of the primer and following a controlled hybridization at 37°C to prevent synthesis of long oligo dT tails.

Second strand synthesis is accomplished by E. Coli DNA polymerase I in the presence of RNAse H and E. Coli DNA ligase. Termini generated by second strand synthesis are made blunt by the action of T4 DNA polymerase.

Double stranded cDNAs are size fractionated by gel filtration chromatography on Biogel A50M as described by Soares (Soares et al., 1994, Proc. Natl. Acad. Sci. 91:9228-9232).

Size fractionated cDNAs are ligated to commercial EcoRI adapters (Stratagene), and then treated with XhoI to create cDNA fragments with EcoR1 (5') and XhoI (3') ends. Unligated adapters are removed by chromatography on Sepharose CL4B (Pharmacia). The adapter-bearing cDNA is phosphorylated using polynucleotide kinase and is ligated using T4 DNA ligase to the EcoRI-XhoI digested library vector at 16°C for up to two days (600 ng. vector plus 250 ng insert in a volume of 10-20 μl). The library is amplified by

electroporation into ElectroMax DH12S (Gibco-BRL) which are plated on 100 150mm LB+ampicillin+IPTG plates. Alternatively, the library may be amplified in liquid media containing ampicillin and IPTG (to select against non-recombinant clones). At a minimum a library of $>5x10^6$ clones is required. This is routinely achieved using our protocols.

Normalization of cDNA libraries

We use two protocols for the normalization of cDNA libraries. Both are based upon those reported by Soares et al., 1994. This precise procedure has been used, but we have also developed a modified and streamlined using biotinylated oligonucleotides to reduce the number of steps.

Rescue of single stranded DNA

The retroviral library in E. coli DH12S is grown in 100 ml of culture volume to mid-log phase and is then infected at a m.o.i of 10 with a helper phage (e.g. M13K07 or VCS-M13+). The culture is incubated for from 2 two 4 hours at 37°C after which single stranded DNA is purified from the supernatant using standard protocols.

Purification of the single stranded library DNA

The DNA prepared as described above is a mixture containing single stranded library DNA, ssDNA from the helper phage and double stranded DNA from lysed bacteria in the culture. The DNA mixture is first digested with XbaI that cuts only double-stranded DNA within the retroviral LTR. This mixture is then treated with Klenow DNA polymerase in the presence of dATP, dGTP, dCTP and Bio-16-dUTP. This treatment will incorporate a biotin residue on both ends of each fragment. The DNA population is then annealed to an excess of a 40-mer oligonucleotide that is complementary to the helper phage. This oligonucleotide carries a biotin residue at its 5' terminus (C16-biotin, Peninsula Labs). The unincorporated nucleotides and single stranded, biotinylated oligonucleotides are removed by chromatography on sepharose CL-4B. The biotinylated DNA fragments and the oligo-bound helper phage DNA is removed from the population by incubation with magnetic-streptavidin beads (Dynal). This yields a cDNA population that is comprised essentially of the single stranded library.

Normalization of the library

Normalization of the cDNA library is accomplished by reassociation kinetics (C0t). The purified single stranded DNA is first annealed to a common primer. In our protocol this is a biotinylated oligo $dT_{18}$ primer while in the Soares protocol the primer is not biotinylated. This primer is extended by Klenow polymerase in the presence of a mixture of dNTPs and di-deoxyNTPs to synthesize fragments (average ~200 nt. in size) complementary to the 3' end of our cDNA population. Again unincorporated primers and nucleotides are removed by chromatography on CL4B. The purified DNA is concentrated by ethanol precipitation.

For the reassociation kinetics reaction, 100-200 ng. of purified, partly duplex DNA is resuspended in 2.5µl of formamide and heated at 80°c for several minutes. An excess (~5µg) of oligo dT25 is added to block interaction of the extension products (see above) with single stranded library though the oligo dT stretches that are present at the end of each clone. 0.5µl of 0.5M NaCl is added along with 0.5 µl of 100 mM Tris-HCl, 100 mM EDTA, pH 8.0 and 0.5 µl water. The mixture is incubated at 42°c for 12-24 hours to produce a C0t of 5-20.

Re-annealed duplexes represent abundant clones which are removed from the mixture (following dilution in binding buffer) by incubation with magnetic streptavidin beads. The non-bound fraction represents the normalized library and is enriched for unique sequences. This single stranded library is concentrated by precipitation and is annealed to an excess of a vector primer that lies downstream of the XhoI cloning site (lacO primer). Extension of this primer with T4 DNA polymerase (or the like) creates partially double stranded circles which are used to transform electrocompetent DH12S bacteria to produce the normalized library.

The transformed population is used for preparation of high-quality DNA by standard protocols.

Selection of retroviral sub-libraries

Specific to a given location within a genome

Sublibraries that contain sequences derived from specific loci in a given genome can be selected from the single-stranded DNA prepared as above. Loci-specific DNA sequences that contain mapped, yet unknown genes can be obtained as sorted chromosomes or as fragments born on YAC or BAC vectors. These sequences are obtained in pure form or are purified by standard methods. Purified DNA is digested with

a restriction enzyme with a four-based recognition sequence. A double stranded oligonucleotide is ligated to the ends of these fragments. Excess double stranded oligonucleotide is removed by column chromatography and the fragments are amplified by PCR with a biotinylated primer that corresponds to one strand of the double stranded oligonucleotide. This results in the production of a population of biotinylated DNA fragments that are derived from a specific genomic locus. This population is then annealed in the presence of appropriate competitive DNA sequences (e.g., yeast genomic DNA, highly repetitive human DNA) to single-stranded retroviral cDNA libraries prepared as above. cDNAs that are derived from the region of interest can then be purified using magnetic streptavidin beads and rescued in bacteria as described above. The resulting retroviral sub-library is greatly enriched for sequences that are contained on the original sorted chromosome, YAC, or BAC. The ability of sequences in this sub-library to give rise to a known phenotype can then be tested following packaging and infection of the appropriate cell type.

*Preparation of unidirectional antisense libraries*

Unidirectional antisense libraries are prepared essentially as described for the sense orientation libraries (see above). Exceptions are as follows:

First strand synthesis is accomplished using a modified backbone random primer that incorporates a restriction site. For our purposes we use the oligonucleotide:

5'-GCG GCG gga tcc gaa ttc nnn nnn nnn-3'

As with sense orientation libraries, the first six nucleotides contain a modified backbone structure that makes them nuclease resistant.

Following second strand synthesis, the library DNA is blunt-ended and ligated to XhoI linkers. These have the following structure :

5'-TCTCTAGCTCGAGCAGTCAGTCAGGATG-3'

5'-ATAAGAGATCGAGCTCGTCAGTCAGTCCTAC-3'

Ligation of these linkers permits amplification of the library by PCR. In this case, the purified cDNA must be digested with both EcoRI and XhoI. Alternatively, commercially available XhoI adapters are ligated to the cDNA. In this case, the library cannot be amplified by PCR, and digestion of the linker-ligated cDNA is with EcoRI. Size selection of the cDNAs is accomplished by gel electrophoresis since the goal is to

isolate fragments with an average size of 200-500 nucleotides. This isolated DNA is then ligated into the MaRXIIg (or IIg-VA or IIg-dccmv) ad described above. Normalization is also accomplished as described for the sense expression libraries except that the primer used for extension of the library circles is derived from a combination of the vector (lacO site) and the polylinker since these clones have no oligo dT sequences. This also necessitated the addition during the re-annealing (C0t) step of an excess of the non-biotinylated primer to suppress hybridization via primer sequences.

*Single gene unidirectional antisense libraries*

Single-gene antisense libraries (for use in targeted functional knockouts) are prepared essentially as described above except that the template for first strand synthesis is a transcript produced from a cloned cDNA using a bacteriophage RNA polymerase (typically T3, T7 or SP6 polymerase). The second deviation is that is type of library is not normalized.

**EXAMPLE 7:** PREPARATION OF VIRUS AND INFECTION AND RECOVERY

The following example provides the necessary protocols for the preparation of the virus and infection of cells with the virus, in addition to recovery of the provirus.

*Transfection of packaging cells and infection with virus*

1. Plate 6 x $10^6$ packaging cells/10 cm plate. 37 c for O/N. Cells should be about 70-80% confluent.

2. Replace medium (10 ml). 37 C for 1-4 hours.

3. Prepare 2 ml of DNA ppt solution for each transfection in two eppendorf tubes.

15 ug DNA + X ul water = 450 ul total volume add 50 ul 2.5 M $CaCl_2$/0.01 M HEPES (pH5.5). Mix dropwisely add 500 ul 2xBBS (50mM BES, 280mM NaCl, 1.5mM $Na_2HPO_4$, pH 6.95) to DNA/$CaCl_2$ mix while gently bubbling in DNA/$CaCl_2$ mix with a pasture pipette immediately and dropwisely add DNA ppt solution to cells while gently swirling the plate (2 ml DNA ppt solution/10 cm plate)

4. 37°C for O/N.

5. Replace medium. (Option: at this step dexoamethasone and sodium butyrate can be

added to medium at final concentrations of 1 μM and 500 μM, respectively. This increases the viral titer by 2-10 fold)

6. 32°C incubation for 48 hours.

7. Collect virus supernatant and filter it through a 0.45 uM syringe filter unit. (Optionally, packaging cells can be eliminated by spinning the virus supernatant at 1K for 5 minutes.)

8. Dilute virus supernatant in fresh growth medium and add polybrene to a final concentration of 8 ug/ml. Add the mixture to cells.

9. Spin the plates at 1.8K for I hour at RT.

10. 32°C incubation for O/N.

At this point, multiple infection cycles can be done by replacing the media on the producer cells and repeating steps 7-10 at 6 hour intervals.

11. Replace medium. 37°C incubation.

12. Cells are analyzed or drug selection applied after 2 days.

*Proviral excision and recovery*

Structure of the Cre and CreT viruses

Excision of viral plasmids for reversion of phenotypes is accomplished using a virus which directs the expression of Cre recombinase from the LTR promoter. This virus was prepared by excision of the Cre sequence from pMM23 (see Qin et al., 1994, PNAS 91: 1706-1710) and insertion of that fragment into pBabe-Puro. Derivatives with other markers have also been constructed. For replicative excision, a cassette that consists of the coding sequence of large T antigen (from pAT.-t (a T antigen clone that can encode large T but not small t) fused to the IRES sequence from EMCV (derived from pCITE) was inserted downstream of the Cre sequence.

Excision in vivo

Infect (as described above) MaRX virus-containing cells with pBABE-puro-Cre virus when cells are at 40-80% confluence in 10 cm using 8 ml virus (generated as described above) + 2 ml medium + 10 μl 8 mg/ml polybrene

For reversion, the cells are maintained at 32° C overnight and then transferred to

37°C. These cells are then selected for the presence of the Cre virus by incubation in selective media (e.g. containing puromycin). After one or two passages, the cells may be analyzed for loss of the phenotype.

For in vivo excision for recovery of the viral plasmid, cells are infected with either the Cre or the Cre-T virus and then incubated overnight at 32°C. Cells are subsequently transferred to 37°C for an additional 6-24 hours. DNA is prepared and the proviral plasmid is recovered by one of the methods described below.

Preparation of DNA for affinity recovery

For recovery of provirus by affinity purification, a 10 cm dish at confluence is lysed as described below. For provirus that has been excised in vivo, cells will have been treated as described above. For recovery of provirus following purification, infected cells at 80-100% confluence are used.

lysis buffer in 10 mM Tris, pH 8.0, 150mM NaCl 10mM EDTA, 1% SDS, 500μg/ml prot K, 120 μg/ml RNese A.

1. lyse cells in 10ml of lysis buffer/10 cm dish
2. incubate at 55°C for 3 hours
3. add an equal volume of phenol/chloroform, rotate 10 minutes, spin
4. add 1/5 vol 8M Kac and 1 vol chloroform, rotate 10 minutes, spin
5. add 2 volumes of ethanol and spool onto a glass rod
6. Wash genomic 3X in 70% ethanol
7. AIR dry pellet and resuspend in TE

Preparation of lacI affinity beads

LacI beads for affinity purification are prepared in one of two ways. A procedure has been published for the preparation of magnetic beads bearing a lacI-Protein A fusion. These have been prepared exactly as described by Lundeberg et al. Genet. Anal. Tech. Appl 7: 47-52 (1990).

Recovery of DNA on lacI beads

Proviral DNA can be recovered on LacI beads prepared as described above. For

recovery of provirus that is excised in vivo or for recovery of provirus for excision in vitro, DNA preparations must be slightly sheared to reduce viscosity. This can be accomplished by brief sonication, repeated passage though a narrow gauge needle or by nebulization.

1. 1-50 μg of DNA is diluted to 58 μl ddH2O
2. add 15 μl of 5X binding buffer
3. pellet 60 μl lacI beads on magnetic concentrator
4. remove the supernatant and resuspend in DNA solution
5. rotate at 37°C for 60 minutes
6. Pellet beads and wash 1X with 250 μl 1X binding buffer
7. Resuspend in 75 μl IPTG elution buffer plus 5 μl 25 mg/ml IPTG
8. rotate at 37°C for 30 minutes
9. Add 30 μg of glycogen and ethanol precipitate

For provirus that has been excised in vivo, electroporate the recovered DNA into DH12S/trfA.

For excision/recircularization in vitro:

Excision/recircularization in vitro is accomplished in one of several ways. The DNA can be treated with commercially available Cre recombinase according to the manufactures instructions. The recircularized plasmids can then be used to transform E. coli by electroporation. Alternatively, most of the MaRX derived vectors have unique rare-cutting restriction enzyme sites adjacent to the loxP sites. These enzymes (e.g. NotI in p.Hygro.MaRX II) can be used for digestion of the proviral DNA followed by recircularization using T4 DNA ligase to create a plasmid that can be both propagated in bacteria and used for the production of subsequent generations of retroviruses.

Alternative recovery method : Hirt extraction

Following in vivo excision, proviral plasmids can be recovered by the Hirt procedure (Hirt, B., J. Mol. Biol. 26: 365-369 (1967)). This can be used for the recovery of single clones but it is relatively inefficient and thus cannot be used for high-efficiency recovery of enriched sub-libraries.

1. Following in vivo excision, wash cells twice with 10 ml of PBS.

2. Add 3 ml of 0.6% SDS/10 mM EDTA (pH7.5)/10 cm plate. Incubate at RT for 15 minutes to lyse cells.

3. Transfer lysate to a 15 ml tube with a scraper and a blue tip cut wide at end (to avoid shearing genomic DNA).

4. Add 750 ul of 5 M NaCl. Mix by gently inverting the tube.

5. Incubate at 4°C for more than 8 hours.

6. Spin at 15K for 20 minutes in JA20 at 4°C and save supernatant.

7. Extract with 1 vol of phenol/chloroform and then with chloroform.

8. ppt DNA by adding 20 ug of glycogen and 2.5 vol of EtOH.

9. Dissolve DNA in 200 ul of water. Extract with 1 vol. of phenol/chloroform and then with chloroform.

10. Dissolve DNA in 10 ul of water.

11. Electroporate DNA into DH12S/trfA (see below).

5 ul of recovered DNA + 50 ul of cells on ice

1.8 kV x 25 uFD x 200 Ω in 0.1 cm cuvette (BioRAD)

add 1 ml of 2XYT

37°C recover for 1 hour

Plate 200 ul on LB(1/2NaCl, pH7.5)-zeocine (25 ug/ml)

37°C for O/N

This procedure generally yields several hundred proviral colonies.

*Proviral Host Strain : DH12S/trfA*

The RK2 replication origin (oriV) requires a replication protein, trfA for function. Otherwise it is a silent DNA element thus allowing it to co-exist with a pUC replication origin on the same plasmid. The excised provirus depends on the RK2 origin for replication and thus for propagation of this plasmid, trfA must be provided in trans. Thus, a trfA-helper strain has been constructed using DH12S as a founder strain. Several characteristics of DH12S prompted its choice for construction of the helper strain. Firstly, it is defective in the restriction system that causes degradation of methylated DNA. Secondly, it is recA, recBC and will thus more stably maintain plasmids. Thirdly, it can

be used for the production of single-stranded DNA. Finally, DH12S can give rise to high-efficiency electrocompetent cells.

Since oriV-based plasmids are generally maintained at low copy number, a copy-up mutant of the replication protein (trfA-267L; Blasina, 1996. Copy-up mutants of the plasmid RK2 replication initiation protein are defective in coupling RK2 replication origins. Proc. Natl. Acad. Sci. U.S.A. 93: 3559-3564 (1996)) was used for the preparation of the strain. This mutant was first cloned into pJEH118 (Fabry et al., 1988, FEBS Letters 237: 213-217) to place it under the control of the pTac promoter. This allows inducible, high level expression which helps to offset the loss in expression levels that occur as trfA integrated into the chromosome at single. A kanamycin resistance marker was then cloned downstream of the trfA cassette. The entire cassette was excised and inserted into a lambda phage vector (lambda-NM540) which was packaged in vitro and used for the preparation of a DH12S lysogen. Several lysogens were tested for the ability to propagate oriV plasmids and one was chosen as DH12S/trfA.

**EXAMPLE 8:** PRODUCTION OF PACKAGING CELL LINES

*Creation of cassettes that provide viral functions*

Three viral functions are provided in trans by packaging cell lines. These are gag, pol and env. In general, either all three are provided by a single cassette or the gag/pol and env functions are separated onto two cassettes. To create directly selectable cassettes that can provide viral functions in trans, genes encoding viral proteins have been transferred from a helper plasmid that consists of a defective provirus (psi⁻e; Mann et al., Cell 33: 153-9 (1983)) to pBluescript in two formats.

*Single gene helper cassettes*

To produce an ecotropic single gene helper cassette, the XhoI-ClaI fragment was purified from psi⁻e and transferred to a similarly digested pBS-SK+ to create pBS+psixc. The end of the envelope gene was reformed by adding a ~100 nt PCR product which spanned the sequences from the ClaI site to the stop codon of the envelope protein. This procedure also added a unique EcoRI site to the 3' end of the helper cassette. The PCR product was inserted into pBS-psiXC following digestion of both DNAs with EcoRI and ClaI. The resultant plasmid was pBS-psi-XE. The 5' end of the helper cassette was created by insertion of a PCR product which spanned from the retroviral splice donor site at the 5' end of the packaging signal to the unique XhoI site of MoMuLv. This PCR

product was inserted into an XhoI digested pBS-psiXE in such a way that a unique SspI site was present at the 5' end of the cassette. This formed pBS-psiCOMP. This helper cassette could encode gag, pol and env, but lacked the LTR elements and tRNA primer binding sequences necessary to produce a replication competent virus. To allow direct selections for viral functions, a tri-cistronic message cassette was created by inserting two tandem IRES-linked markers downstream from the end of the envelope sequence. In this case the cassette contained an EMCV IRES linked to human CD8 protein (a cell surface marker) linked to another EMCV IRES linked to the hygromycin resistance gene. This was inserted from EcoRI to NotI in pBS-psiCOMP to form pBS-psiCD8H. The cassette from this plasmid can be inserted into any expression vehicle following excision by SspI and NotI.

Separation of helper functions onto two cassettes was accomplished by creating deletions of pBS-psiCOMP. The env function was isolated by digestion of pBS-psiXE with XhoI and XbaI followed by insertion of a linker sequence that reformed both restriction sites. Removal of env from pBS-psiCOMP was accomplished by digestion with HpaI and EcoRI followed by ligation with a synthetic fragment that repaired the 3' end of pol and that reformed both the HpaI and EcoRI restriction sites. The single cassette amphotropic envelope (Ott, D.E. et al., J. Virol. 64, 757-766 (1990)) was formed by PCR followed by insertion into pBS. Each of these plasmids was used to generate a tri-cistronic helper cassette. Each envelope plasmid received the CD8-hygromycin cassette described above. The gag/pol plasmid received either of two cassettes. One consisted of an EMCV IRES linked to the gene encoding a cytoplasmic domain defective CD4 (another cell surface marker) linked to an EMCV IRES linked to the gene for histidinol resistance. The second cassette consisted of an EMCV IRES linked to the gene encoding green fluorescent protein linked to and FDV IRES linked to the gene encoding puromycin resistance.

Since all of these tricistronic cassettes are used similarly to introduce packaging functions into cells, introduction of the single gene helper cassette will be described. Introduction of the separated helper functions simply requires additional quantitative and qualitative selection steps.

*Expression Vehicles.*

The helper cassettes described above must be functionally linked to sequences that promote expression in mammalian cells. These constructs can then be introduced into cell lines to create a functional packaging system. In general two options are available. The

single helper cassette can be cloned in functional association with a strong promoter (e.g. CMV) in a plasmid that can replicate in the presence of SV40 T antigen. This allows amplification of the plasmid episomally. In some cases this is followed by high copy integration into the genome. Such a plasmid can also be used in the absence of SV40 T-antigen to achieve somewhat lower copy numbers. For this purpose the single helper cassette has be inserted into pcDNA3 (Invitrogen). Alternatively, the helper cassette can be placed in association with a strong promoter on a vector that replicates as a stable episome. Two such systems are in common use. The first is based upon Epstein Barr Virus. EBV-based vectors replicate via oriP which requires EBNA for function. A particularly useful vector has been produced by Invitrogen (pCEP-4). This vector has been modified to remove the hygromycin resistance cassette and the helper cassette has been inserted downstream of the CMV promoter. Upon transfection into our chosen host cell line, this vector can achieve stable copy numbers of >20/cell. The final choice is a set of vectors based upon bovine papilloma virus. Unfortunately, these vectors will not replicate in our host cell of choice and we must therefore obtain modified BPV vectors in which viral functions are expressed from a constitutive promoter that functions in our chosen cell type. These modified BPV vectors can achieve copy numbers that range from 100-1000/cell.

*Cell for the generation of packaging cell lines*

Human 293 cells have been chosen for the generation of packaging cell lines. These cells can support replication from SV40-based systems and EBV based systems. These can also be used for the high copy number, modified BPV systems. In particular, a subline of human 293 cells (293T) shows extremely high transfection efficiencies (this is critical for the production of high-complexity libraries) and contains a temperature sensitive SV40 large T antigen that can support conditional replication of SV40-based vectors.

*Selection of packaging cell clones*

Human 293T cells will be transfected with either the single helper plasmid or the two separate helper plasmids in the vectors described above. Transfected cells will be placed in selective media containing standard concentrations of hygromycin (75 μg/ml) or hygromycin plus puromycin (1.5 μg/ml). Following successful selection of stably transfected clones, high-expressing cells will be selected by FACS analysis following staining with antibodies directed against the cell surface markers or by direct detection of

gfp. The 5% of clones which display the highest expression levels will be recovered and plated again in selective media. Cells will be passed into a media containing a 50% higher concentration of each drug and the 5% of surviving cells which display the highest marker expression will be passed through another round of this procedure. At each round, levels of elaborated reverse transcriptase and transfection rates are assessed. After several rounds, at a time at which subsequent rounds fail to increase reverse transcriptase expression or at which high drug concentrations result in a reduced transfection rate, single cell clones will be chosen and analyzed for the ability to produce high titer virus. The ability to enforce direct selection for the viral helper cassettes should allow not only selection of the most efficient packaging cells but should also allow for continuous selection for maintenance of high efficiency packaging function.

It is recommended that during initial set up, the user also optimize the system by using a retroviral vector expressing an easily assayable marker such as lacZ or a cell surface protein. During optimization, one should check for transfection frequency of the producer clone and test infection rate of target cells. Tests for transfection and infection frequencies using a βgal-based system or the like can be readily measured by βgal staining or FACS staining for βgal activity. Only when the user is satisfied with the transfection conditions and infection rates should s/he proceed to using vectors with no readily assayable marker. It should be possible to scale up the protocols.

Moreover, in certain instances the initial plating of the cells may be the most important step in successfully obtaining high retroviral titers. It is extremely important that the cells are not overly clumped and are at the correct density. Unlike NIH3T3-derived cell lines, the 293-derived packaging cell lines and the like do not readily form well-spread monolayers. Instead, they tend to clump before confluence, and if the clumping is excessive, the cells will never reach confluence during the 48-72 hour period following transfection. In order to prevent clumping, it is essential that the cells are extremely healthy prior to plating. If they are overconfluent, it may be necessary to split them 1:2 or 1:3 for several passages prior to plating for transfection. In addition, the cells are much less adherent than murine fibroblasts and should be handled very gently when washing and changing medium. For consistency, it is important to count the cells rather than estimating the split. The above cell number is optimized for MFG-lacZ. Expression of other inserts may be detrimental to the growth of the cells. This effect may be noted by failure of the packaging cell line to reach confluence by 48-72 hours post-transfection. If this occurs, it may be necessary to plate more cells prior to transfection.

Further more, the addition of chloroquine to the medium appears can increase retroviral titer. This effect is presumably due to the lysosomal neutralizing activity of the

chloroquine. In many instances, it is important that the length of chloroquine treatment does not exceed about 12 hours. Longer periods of chloroquine treatment have a toxic effect on the cells causing a decrease in retroviral titers. For purposes where achieving maximal retroviral titer is not necessary, such as when comparing the relative titers of different constructs, it may be preferable to omit chloroquine treatment. If chloroquine is not used, it is unnecessary to change the medium prior to transfection.

To futher illustrate an exemplary embodiment, when the retroviral supernatant is ready for harvesting, gently remove the supernatant and either filter through a 45 μM filter or centrifuge x 5 min at 500 x g at 4°C to remove living cells. If the retroviral supernatant is to be used within several hours, keep on ice until it is used..

**EXAMPLE 9:** pEHRE-BASED PACKAGING CELL LINES

Utilizing techniques as described in the Example presented in Section 13, above, the pEHRE family of vectors has been used to successfully create packaging cell lines for the production of retroviruses following either transient or stable transfection with replication-deficient retroviral vectors.

Specifically, two ecotropic 293T based packaging lines, referred to herein as LinX I and LinX II have been created.

In LinX I, helper functions are supplied on a pEHRE vector containing a single expression cassette that encodes gag, pol and env. In LinX 11, the gag/pol and env functions are supplied on separate pEHRE vectors. Both cell lines produce virus with a titer in of $10^6$ pfu/ml as measured on NIH3T3 cells. In this respect LinX I and LinX II are equivalent to the best available packaging lines. However LinX I and LinX II do have two additional unusual and beneficial characteristics.

First, the initial, drug-selected pool from which the packaging cell lines were derived was able to package virus with an efficiency that is nearly equivalent to the clone that was finally selected as the packaging cell line. This is in contrast to cell lines constructed by standard procedures in which the efficiency of the transfected pool is 2-3 logs lower than that of a cell line that is eventually derived from the analysis of hundreds of cell clones. The ability of the pEHRE multi-copy episomal system to deliver viral helper functions, therefore makes it ideal for the rapid construction of special-purpose packaging lines (e.g. cell lines with alternative or mutant gag or envelope proteins).

The second unusual characteristic of the LinX I and LinX II cell lines is that the cells exhibit a remarkably stable ability to produce high-titer virus. The ability of standard

packaging cell lines (e.g. Bosc) to produce high titer virus decays very rapidly. For example, viral titers can decrease by more than one log per month. In contrast, LinX cells have been maintained for more than six months in culture without a detectable loss in viral titers.

This stability may result from a combination of two factors. First, the pEHRE episome is highly stable both in structure and in copy number. Second, the viral helper functions are present on these episomes as one segment of a polycistronic mRNA comprising the helper function and a drug resistance marker. Selection for the drug marker, therefore, allows direct selection for the mRNA encoding the helper function.

**EXAMPLE 9:** TARGET ANTISENSE EXPRESSION -DERIVATION OF A FUNCTIONAL KNOCKOUT

Single gene antisense libraries in the MaRXIIg vectors can be used to created targeted functional knockouts of individual genes. This can be accomplished irrespective of prior knowledge of the phenotype of the knockout by creating an indirect selection for loss of gene function. This is accomplished by creating a quantifiable marker that serves to report the levels of expression of a particular gene. This can be created in any of a number of ways as described in the text of the application. The most straightforward is to create a fusion protein and this will be the example given.

The coding sequence of the protein of interest is fused to a reporter, in this case, the green fluorescent protein. This fusion should be prepared so that the 5' and 3' untranslated sequences are present in the construct. The entire cassette, including untranslated sequences is placed within a retroviral vector that promotes constitutive expression. Inducible vectors can also be used if expression of the fusion protein is deleterious. This vector is inserted into cells of a species distinct from the species from which the knock-out target is derived. For example, mink cells would make a reasonable screening host for human proteins. A population of cells showing uniform fluorescence is selected by single-cell cloning or by FACS. A single-gene, unidirectional antisense library is constructed from the transcript encoding the target gene (see above) in one of the MaRXIIg vectors. This library is used to infect cells that express the fluorescent fusion. By FACS sorting, cells which no longer express the fusion are identified. These are cloned as single cells. A subset of these will express antisense transcripts which effectively inhibit expression of the fluorescent fusion protein, and a subset will simply have lost fusion protein expression independent of an introduced antisense (revertants). Effective antisense can be distinguished from revertants by the ability of Cre recombinase

to rescue fluorescent protein expression. Cell clones in which fluorescence is rescued by Cre will serve as a source for the recovery of viruses carrying antisense fragments which can be used to create functional knockouts in any desired cell line. It should be noted that this procedure is quantitative and qualitative; by FACS sorting, the most effective fragments can be identified as those able to quantitatively reduce fluorescence to the greatest extent. Furthermore, by replacing the CMV promoter in the MarxIIg and MaRXIIg-dccmv with an inducible promoter (in combination with a self-inactivating LTR), conditional knockouts can be created.

**EXAMPLE 10:** ACTIVATION OF THE TELOMERASE ENZYME

Telomerase is an almost universal marker for tumorigenesis. Activity is, however, absent in normal cells. Activity can be induced in a subset of normal cells (e.g., epithelial cells and keratinocytes) by introduction of the E6 protein from HPV-16. This induction is independent of the ability of E6 to direct degradation of p53 . In order to investigate the processed that lead to the induction of telomerase in tumors, we have devised an in vitro screen for genes that can induce telomerase activity in normal human mammary epithelial cells (HMEC).

Pools of cDNAs comprising from 100-100 clones each (either in the sense orientation or in the antisense orientation in the MaRXIIg vector series) are introduced into HMEC cells. These are selected for expression of cDNA and then used to prepare lysates for the assay of telomerase activity. Cell lysates are tested using a highly sensitive telomerase assay which is capable of detecting two telomerase-positive cells among 20,000 telomerase-negative cells. Those pools which upon infection cause the induction of telomerase activity in HMEC cells are subdivided into smaller pools. Sub-pools are again used for the infection of HMEC cells which are subsequently assayed for telomerase activity. Successive rounds of this procedure can identify an individual clone that acts as an inducer of the telomerase enzyme.

Such a clone could represent a direct regulator of the enzyme itself or of the expression of a component of the enzyme. Alternatively, such a clone could act as a regulator of cell mortality. Changes induced by the expression of such a clone could induce the telomerase enzyme as only one aspect of a more global change in cellular behavior.

**EXAMPLE 11:** SECRETION SCREENING

The retroviral and pEHRE vectors of the invention can be utilized in conjunction with secretion trapping constructs to identity nucleotide sequences which encode secreted proteins. Such identification schemes can serve a variety of purposes. For example, because secreted proteins are often useful as therapeutics, their identification can then be followed by additional biological screens as part of a method for identifying novel therapeutic agents. Additionally, identification of secreted proteins differentially expressed in a disorder such as, for example, cancer, can serve as convenient blood borne marker for diagnosing the presence of the disorder. Still further, identification of secreted proteins can act as a subfractionation which may make possible detection of an extremely rare sequence or event, which would go undetected if a sequence was not first enriched from a library in such a fashion.

Nucleotide sequences to be tested are introduced into the cloning site of a secretion trapping retroviral or pEHRE vector.

A plurality of secretion screening vectors containing nucleotide inserts, making up a secretion screening library, can be produced and screened simultaneously. Unidirectional random priming strategies, as described above for the production of unidirectional sense and antisense libraries can be used to produce such libraries.

In one embodiment, a secretion trapping cassette comprises from 5' to 3': a transcriptional regulatory sequence, a polylinker, a protease coding sequence, flanked by protease recognition sites, a cell surface marker coding sequence (lacking a signal sequence) and a cell surface membrane anchoring sequence (preferably one whose anchoring activity is dependent upon the presence of a signal sequence, such that background is reduced, as described below), an IRES and a selectable marker. A representative retroviral secretion screening vector is depicted in FIG. 23.

Cell surface markers can include, but are not limited to, CD4, CD8 or CD20 marker, in addition to any synthetic or foreign cell surface marker. Protease and protease recognition sequences can include, but are not limited to any retroviral protease sequences, HIV, MuLv, RSV or ASV protease sequences.

Nucleotide sequences to be tested are introduced into the polylinker. The vectors containing such sequences are transfected or transformed, depending on the vector used, into cells. The vectors' selectable markers are used to select for cells which has taken up vectors.

Sequences coding for secreted proteins (i.e., sequences which code for signal sequences) are then identified by determining which of these cells exhibit the fusion protein cell surface marker. This is because the marker will only end up transported to and anchored on the cell surface if the fusion protein it becomes a part of contains a signal sequence.

In order to reduce extraneous background cell surface targeting, the membrane targeting portion of the fusion protein should, preferably, be one whose targeting activity is dependent on the presence of a signal sequence. For example, the GPI membrane anchoring/targeting sequence only becomes tethered on the cell membrane if it first goes through the cell's endoplasmic reticulum (ER). The presence of a signaling sequence, targets a protein to the ER, then serves to "activate" GPI's membrane tethering capability.

The protease element of the fusion protein can, in general, be used to create multiple functional units from one polypeptide translational unit. The protease element of the fusion protein is, in this specific instance, used to ease the identification of those cells which exhibit the cell surface marker. Specifically, by placing the protease and protease recognition sequence at the appropriate position along the fusion protein, the protease's activation and self cleavage serve to make the cell surface marker readily available to cell surface antibodies. Standard antibody-related isolation techniques such as FACS or magnetic bead isolation techniques can be utilized.

Utilizing the FIG. 23 vector, a single positive cell in one million was successfully purified to approximately 40% purity in only 4 rounds of screening.

## DEPOSIT OF MICROORGANISMS

*E. coli* strain XL-1 carrying plasmid pMaRXII, was deposited on September 20, 1996 with the Agricultural Research Service Culture Collection (NRRL), under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures and assigned accession number B-21625.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by

reference in their entireties.

5

10

15

20

25

WHAT IS CLAIMED IS:

1. A retroviral vector comprising a

(i) polycistronic message cassette comprising, preferably 5' to 3', a polylinker or coding sequence for a first polypeptide, an internal ribosome entry site and a coding sequence selectable marker; and

(ii) enzyme-assisted site-specific integration sequences flanking the polycistronic message cassette.

2. A replication-deficient retroviral vector, comprising:

(i) a polycistronic message cassette comprising, 5' to 3', a polylinker, an internal ribosome entry site and a mammalian selectable marker;

(ii) a proviral excision element;

(iii) a proviral recovery element; and

(iv) a bacterial replication/selection cassette.

3. A genetic suppressor element-producing retroviral vector comprising:

(i) a genetic suppressor element cassette;

(ii) a proviral excision element;

(iii) a proviral recovery element; and

(iv) a bacterial replication/selection cassette.

4. A gene trapping retroviral vector comprising:

(i) a gene trapping cassette comprising a reporter sequence linked to an internal ribosome entry site;

(ii) a selective nucleic acid recovery element; and

(iii) a bacterial replication/selection cassette.

5. A peptide display retroviral vector comprising:

(i) a polycistronic message cassette comprising 5' to 3', a peptide display cassette, an internal ribosome entry site and a mammalian selectable marker;

(ii) a proviral excision element;

(iii) a proviral recovery element; and

(iv) a bacterial replication/selection cassette.

6. The peptide display vector of Claim 5, further comprising:

(i) a mammalian secretion signal; and

(ii) a membrane anchor.

7. The peptide display vector of Claim 6, in which the membrane anchor is excisable.

8. The peptide display vector of Claim 5, further comprising nucleotide sequences encoding a splice donor and a splice acceptor site flanking a bacterial promoter, a ribosome binding site, a bacterial secretion signal and all or a portion of the M13 bacteriophage gene III protein carboxy terminus.

9. A method for identifying a nucleic acid sequence whose expression complements a mammalian cellular phenotype, comprising:

(i) infecting a mammalian cell exhibiting the cellular phenotype with a retrovirus derived from the retroviral vector of Claim 1 further comprising a cDNA or gDNA sequence, linked to a quantifiable or selectable marker, wherein, upon infection, an integrated retroviral provirus is produced and the cDNA or gDNA sequence is expressed; and

(ii) analyzing the cell for the phenotype, so that alteration of the phenotype identifies a nucleic acid sequence which complements the cellular phenotype.

10. A method for identifying a nucleic acid sequence whose expression inhibits the function of a known mammalian gene, comprising:

(i) infecting a mammalian cell with a cDNA or gDNA sequence linked to a quantifiable or selectable marker;

(ii) infecting the mammalian cell from (a) with a retrovirus derived from the genetic suppressor-producing retroviral vector of Claim 2 further comprising a cDNA or gDNA sequence for the human gene, or fragments thereof, linked to a quantifiable, selectable marker, wherein, upon infection, an integrated retroviral provirus is produced and the cDNA or gDNA sequence is expressed; and

(iii) analyzing the cell for expression of the linked quantifiable or selectable maker, so that suppression of the linked marker expression identifies a nucleic acid sequence which inhibits the function of the mammalian gene.

11. A method for identifying a nucleic acid sequence whose expression influences a cellular phenotype, comprising:

(i) infecting a mammalian cell with a retrovirus derived from the genetic suppressor-producing retroviral vector of Claim 2 further comprising a cDNA or

gDNA sequence of, linked to a quantifiable or selectable marker, wherein, upon infection, an integrated retroviral provirus is produced and the cDNA or gDNA sequence is expressed; and

(ii) analyzing the cell for suppression of a phenotype, so that suppression of expression of the unknown mammalian gene identifies that nucleic acid sequence whose expression influences the cellular phenotype.

12. A method for identifying a nucleic acid sequence encoding a peptide whose expression influences a cellular phenotype, comprising:

(i) infecting a mammalian cell with a retrovirus derived from a peptide displaying the vector of Claims 4, 5, 6 or 7 further comprising a random peptide sequence, wherein upon infection, an integrated retroviral provirus is produced and the random peptide sequence is expressed; and

(ii) analyzing the cell for suppression of a phenotype, such that the nucleic acids encoding the protein influencing the phenotype may be identified by the interaction of a random peptide sequence with the protein.

13. A method for identifying a mammalian gene whose expression is modulated in response to a specific stimulus, comprising:

(i) infecting a mammalian cell exhibiting the cellular phenotype with a retrovirus derived from the gene trapping retroviral vector of Claim 3 an integrated retroviral provirus is produced;

(ii) subjecting the cell to the stimulus; and

(iii) assaying the cell for expression of the gene trapping cassette reporter sequence, so that if expression of the reporter sequence changes, it is integrated within, and identifies a gene that is induced or modulated in response to the stimulus.

14. The retroviral vector of Claim 1 or 2 wherein the retroviral vector further comprises a cDNA or gDNA insert sequence.

15. A retroviral library comprising a multiplicity of the retroviral vector of any of claims 1-8.

16. A retrovirus derived from the retroviral vector of any of claims 1-8.

17. An integrated provirus derived from the retrovirus of any of claims 1-8.

18. An excised provirus derived from the integrated provirus of any of claims 1-8.

19. An episomal expression vector, comprising:

(i) a replication cassette, comprising an E1 coding sequence and an E2 coding sequence;

(ii) an expression cassette;

(iii) an MO sequence; and

(iv) a MME sequence.

20. An episomal genetic suppressor vector comprising:

(i) a replication cassette, comprising an E1 coding sequence and an E2 coding sequence;

(ii) an genetic suppressor cassette;

(iii) an MO sequence; and

(iv) a MME sequence.

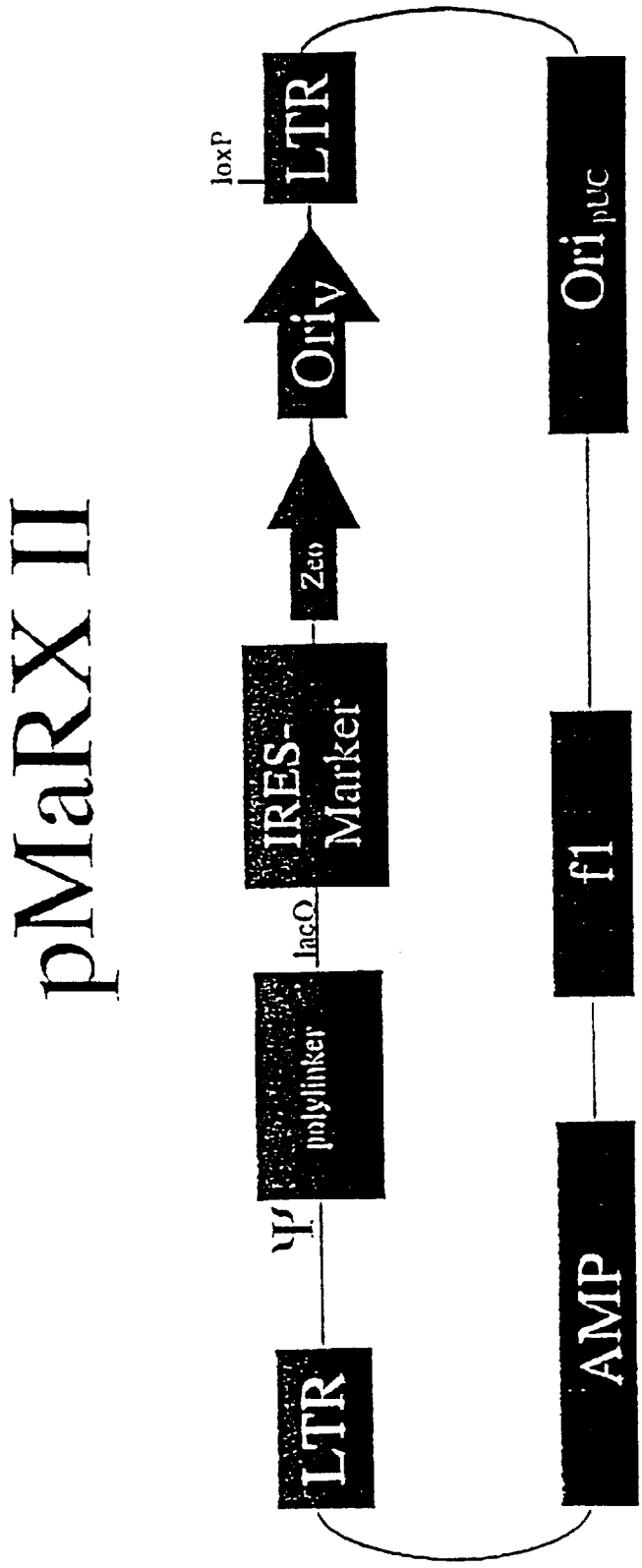

pMaRX II
loxP
LTR
Ori v
Zeo
IRES-
Marker
lacO
polylinker
Ψ
LTR
Ori pUC
f1
AMP

Fig. 1

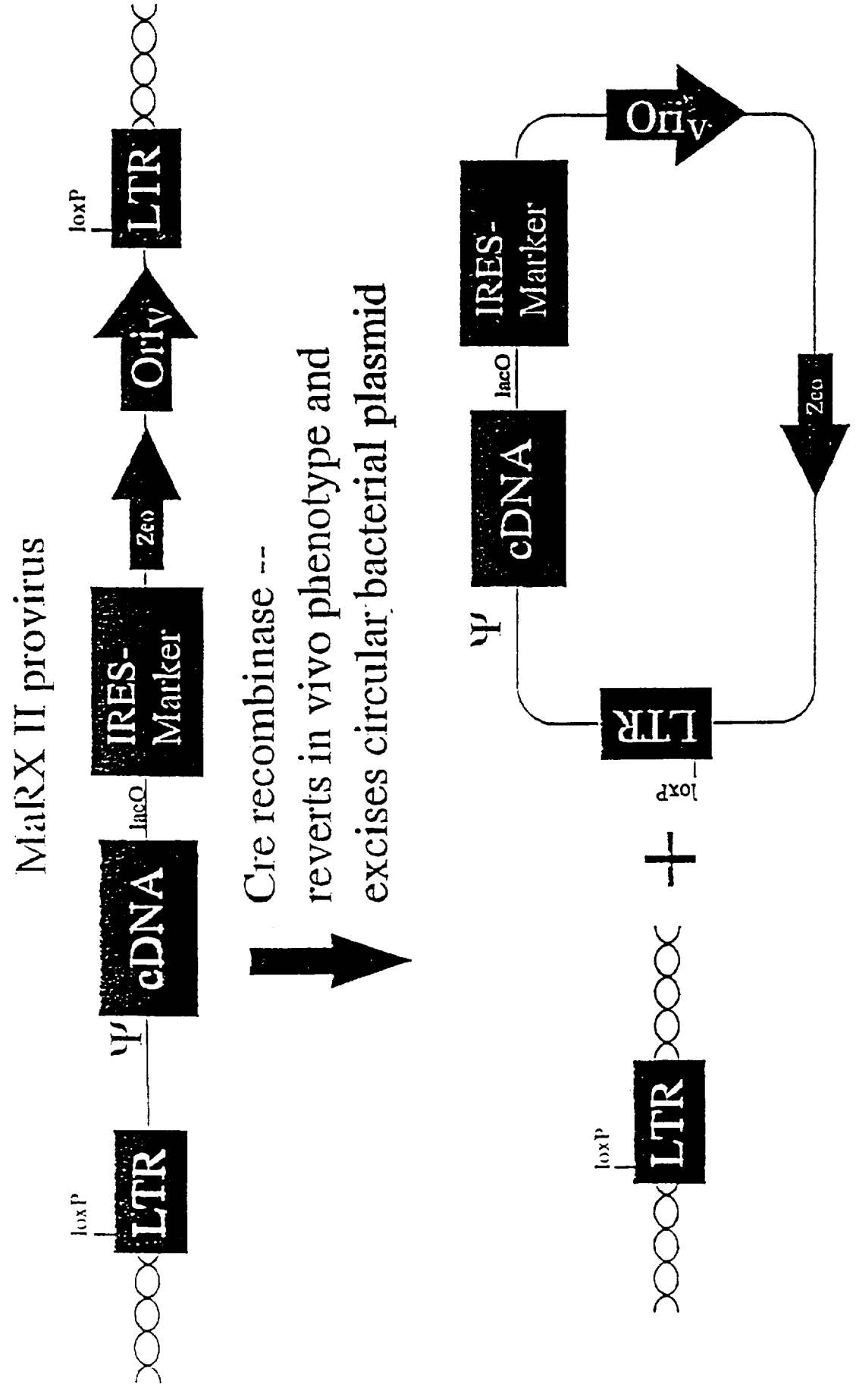

MaRX II provirus
loxP
LTR
Ψ
cDNA
lacO
IRES-
Marker
Zeo
Ori
V
LTR
loxP
Cre recombinase --
reverts in vivo phenotype and
excises circular bacterial plasmid
Ori
V
IRES-
Marker
lacO
cDNA
Ψ
Zeo
LTR
loxP
loxP
LTR

FIG. 2

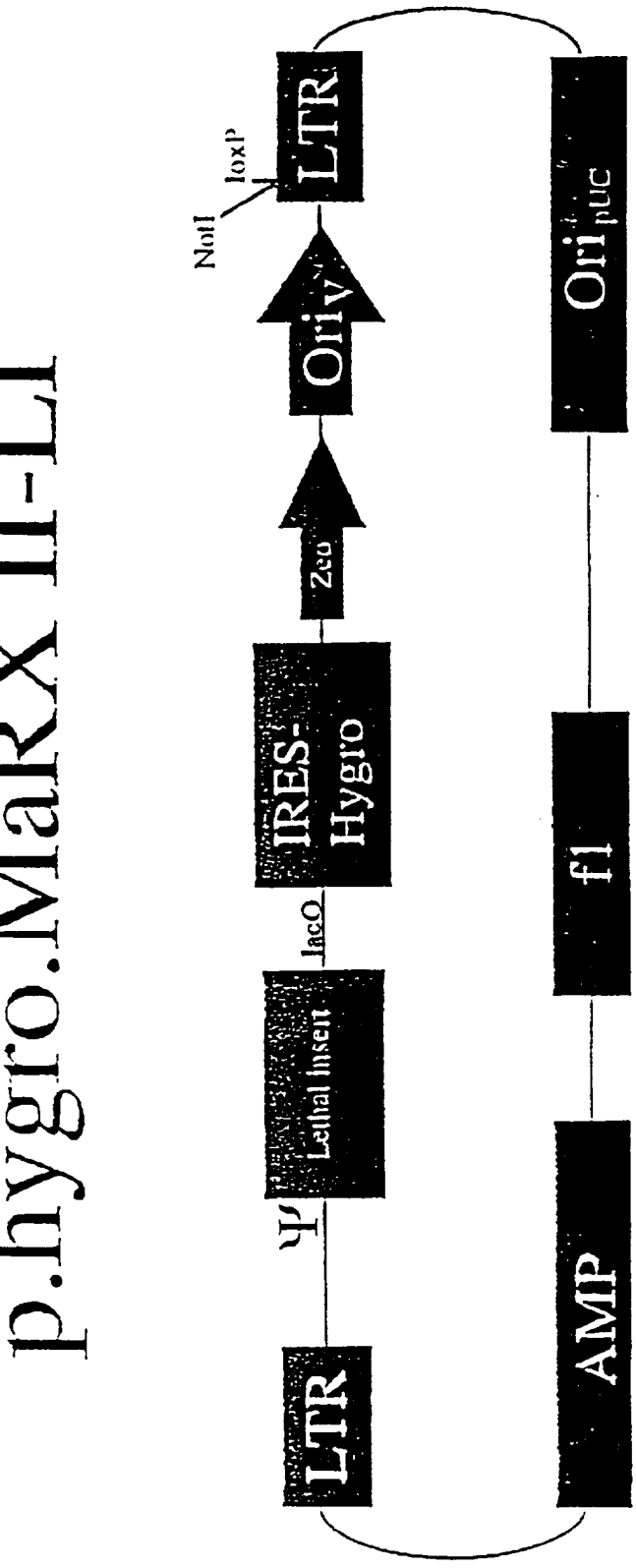

p.hygro.MaRX II-LI
LTR
NotI
loxP
Ori V
Zeo
IRES-
Hygro
lacO
Lethal insert
Ψ
LTR
Ori pUC
f1
AMP

FIG. 3

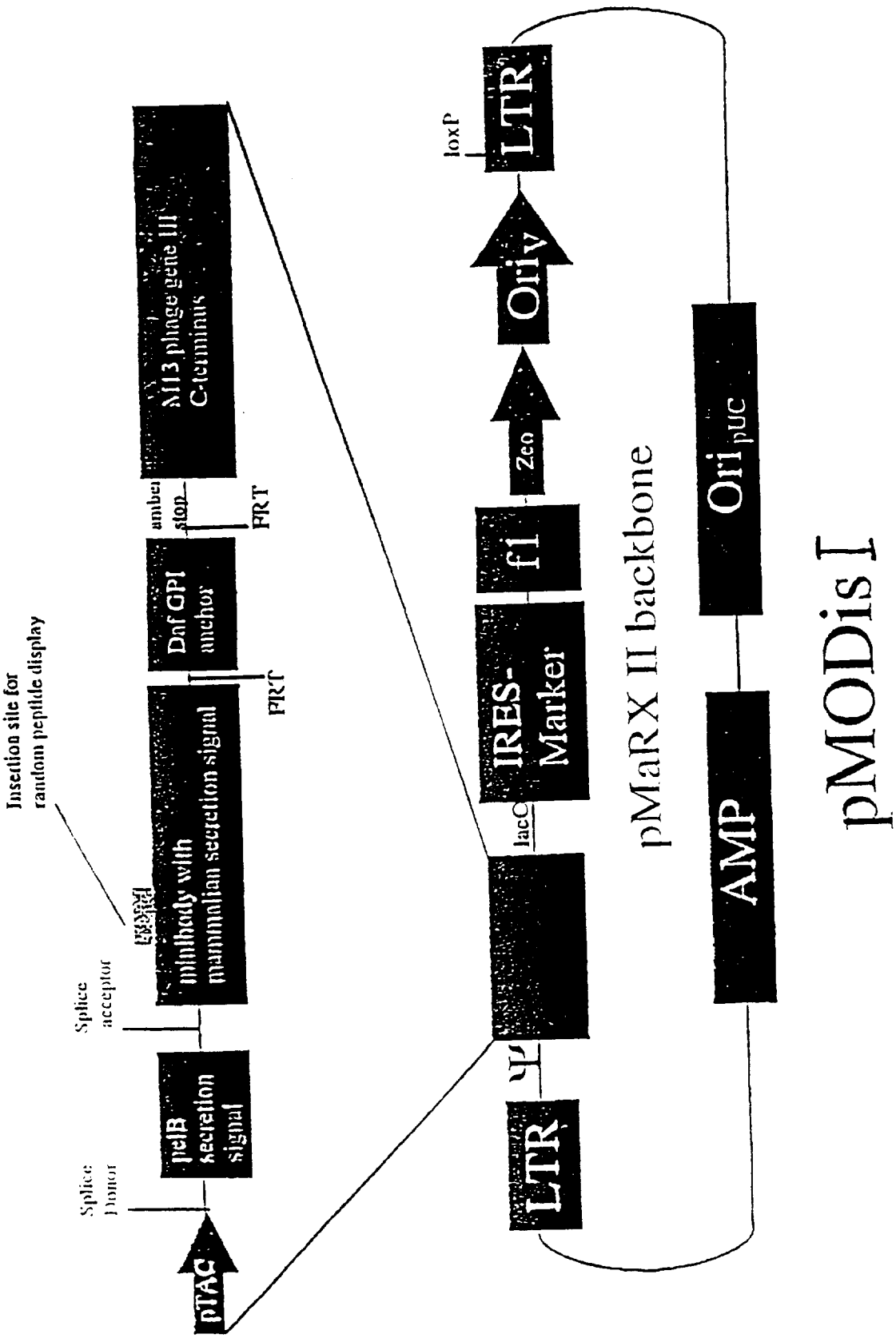

pMODis I
pMaRX II backbone
pTAC
Splice Donor
pelB secretion signal
Splice acceptor
Insertion site for random peptide display
mAb with mammalian secretion signal
Daf GPI anchor
FRT
amber stop
M13 phage gene III C-terminus
LTR
IRES-Marker
lacO
f1
Zeo
Ori$_{pUC}$
AMP
loxP
Ori$_V$
LTR

FIG. 1

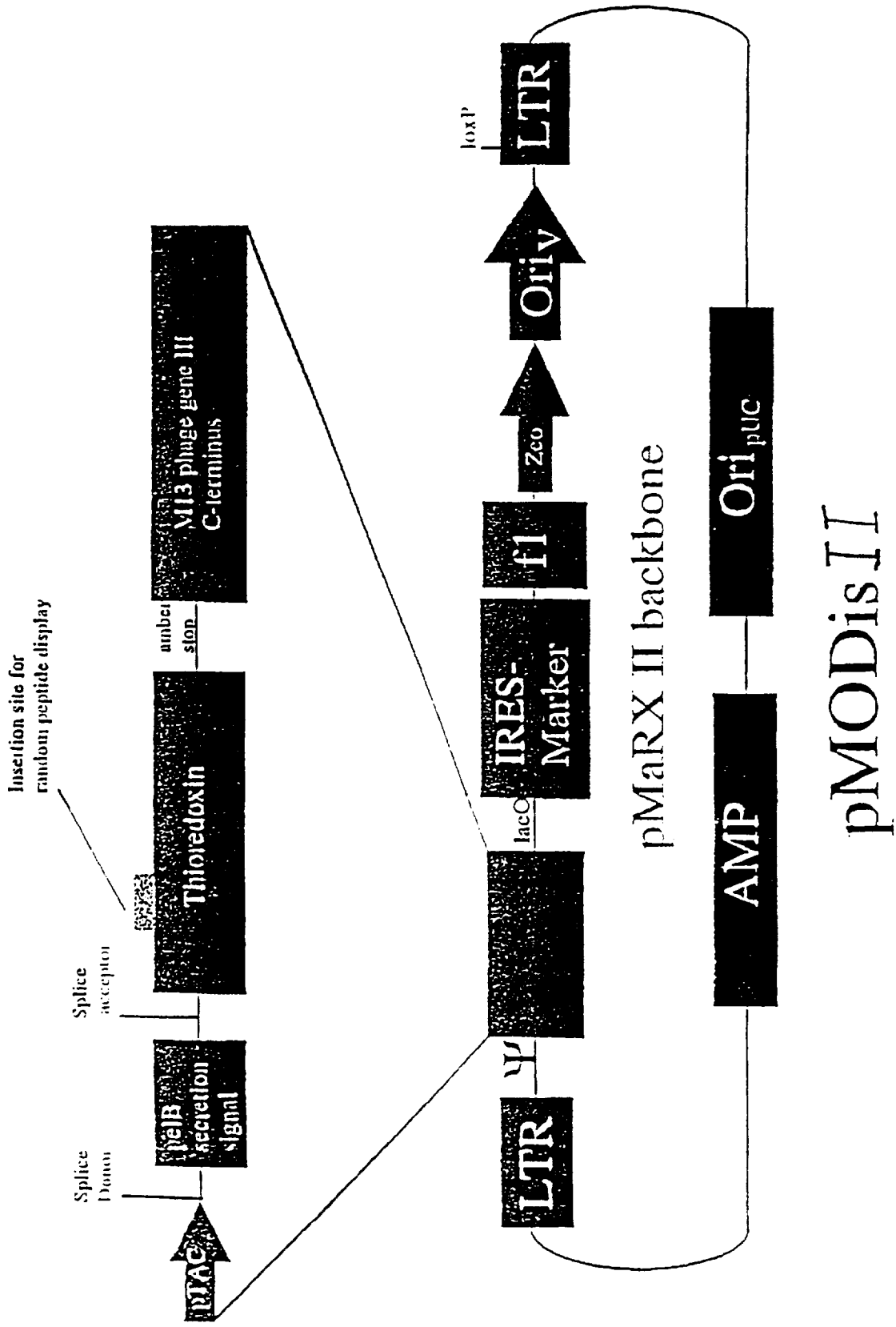

pMODis II
pMaRX II backbone
LTR
Ψ
lacO
IRES-Marker
f1
Zeo
Ori
loxP
LTR
AMP
Ori pUC
M13 phage gene III C-terminus
amber stop
Thioredoxin
Insertion site for random peptide display
Splice acceptor
pelB secretion signal
Splice Donor
tac

FIG. 5

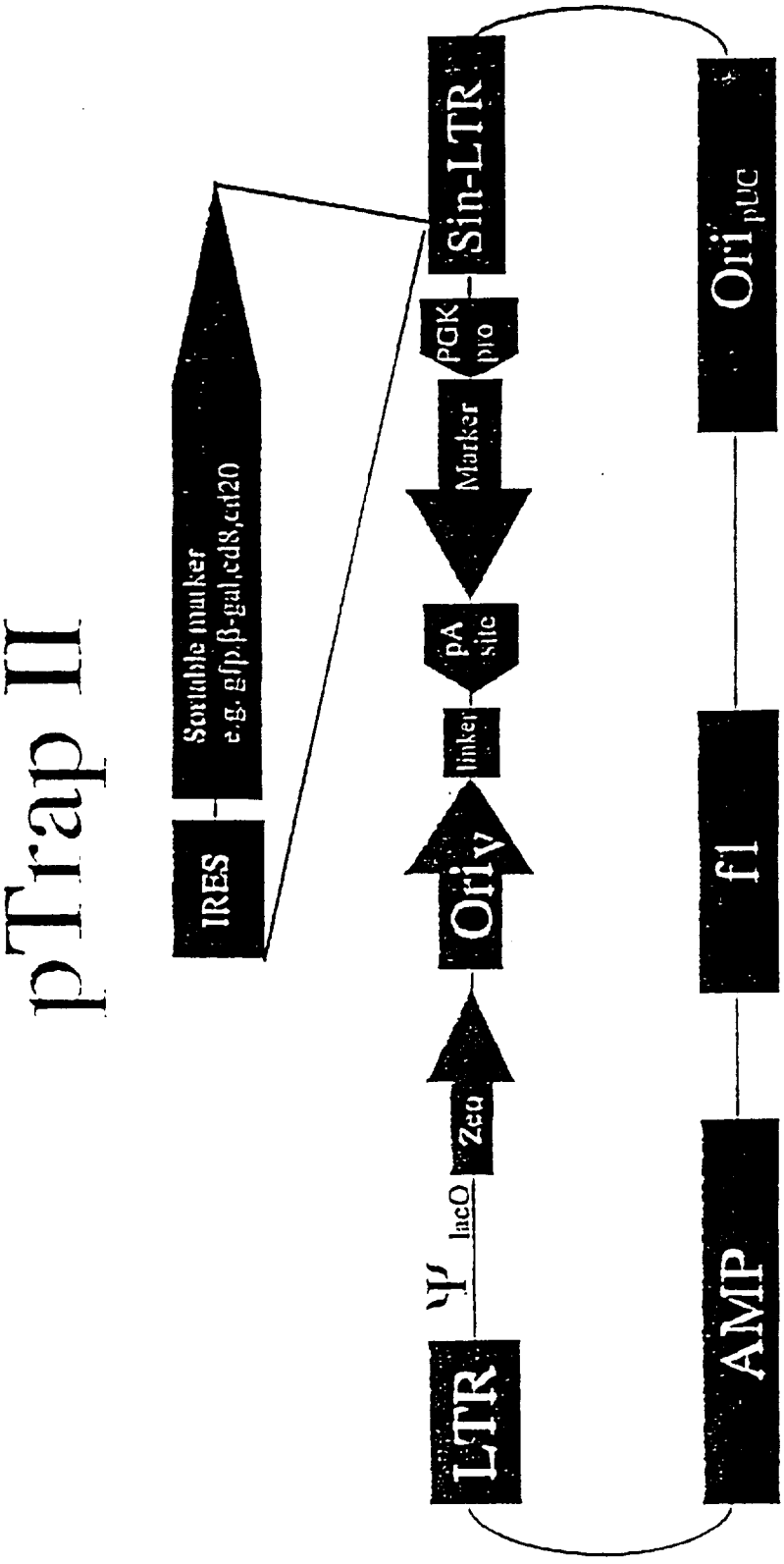

pTrap II
Sin-LTR
PGK pro
Marker
pA site
linker
Oriv
Zeo
lacO
LTR
IRES
Sortable marker
e.g. gfp,β-gal,cd8,cd20
Ori pUC
f1
AMP

Fig. 6

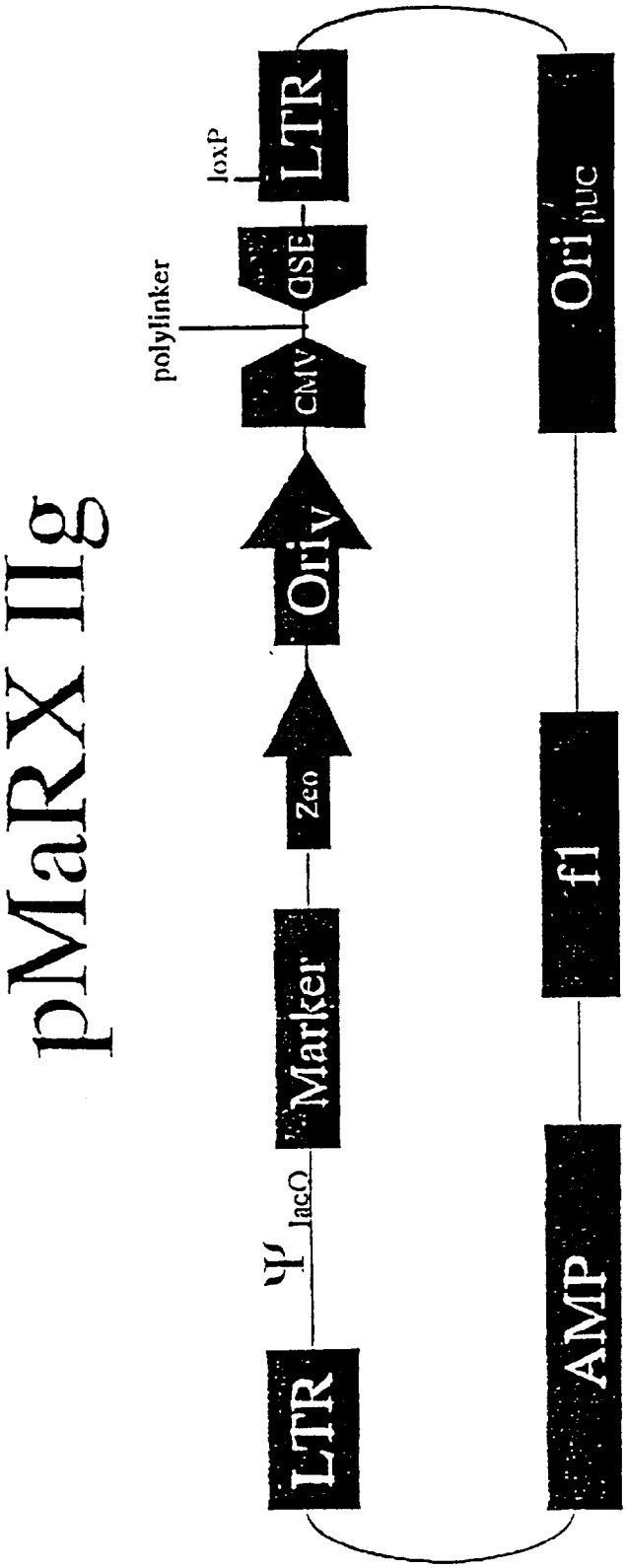

pMaRX IIg
LTR
Ψ lacO
Marker
Zeo
Ori V
CMV
polylinker
GSE
loxP
LTR
AMP
f1
Ori pUC

FIG. 7

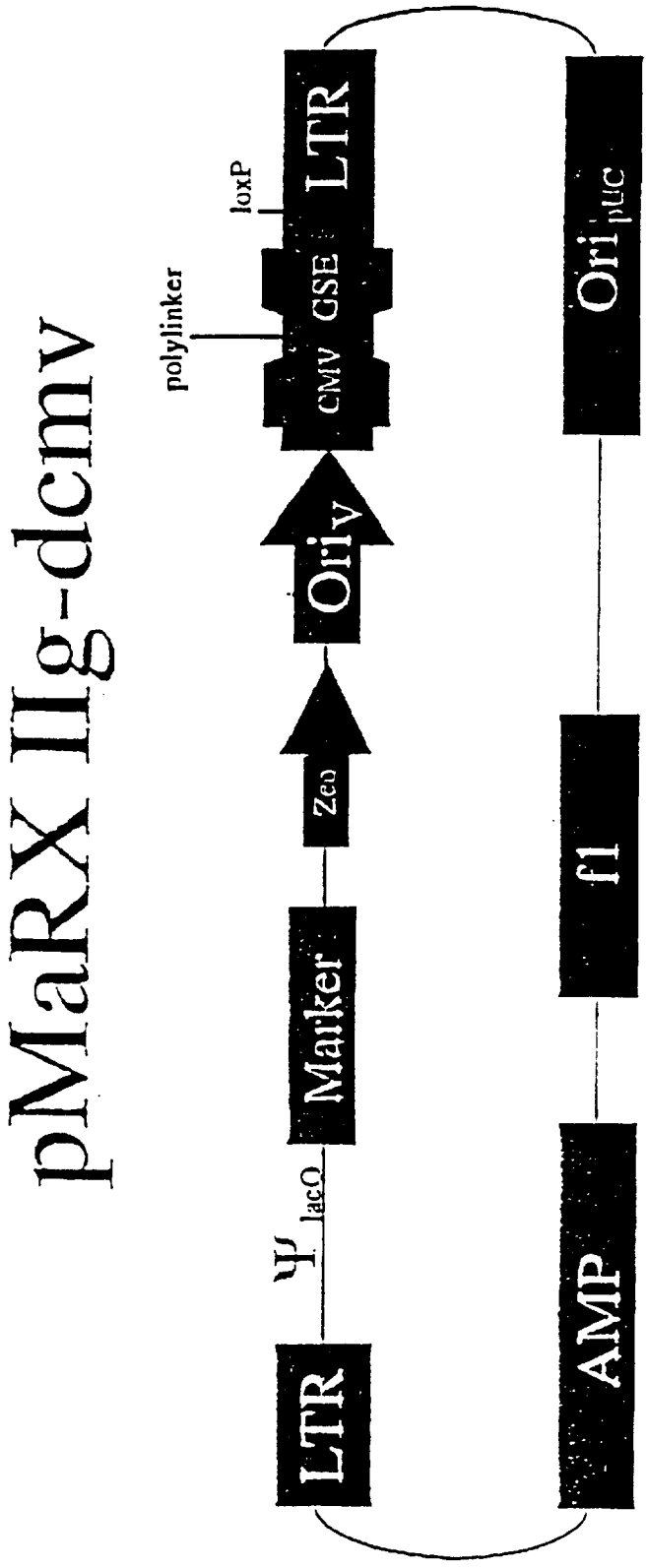

pMaRX IIg-dcmv
LTR
loxP
polylinker
CMV GSE
Ori V
Zeo
Marker
Ψ lacO
LTR
Ori pUC
f1
AMP

FIG. 8

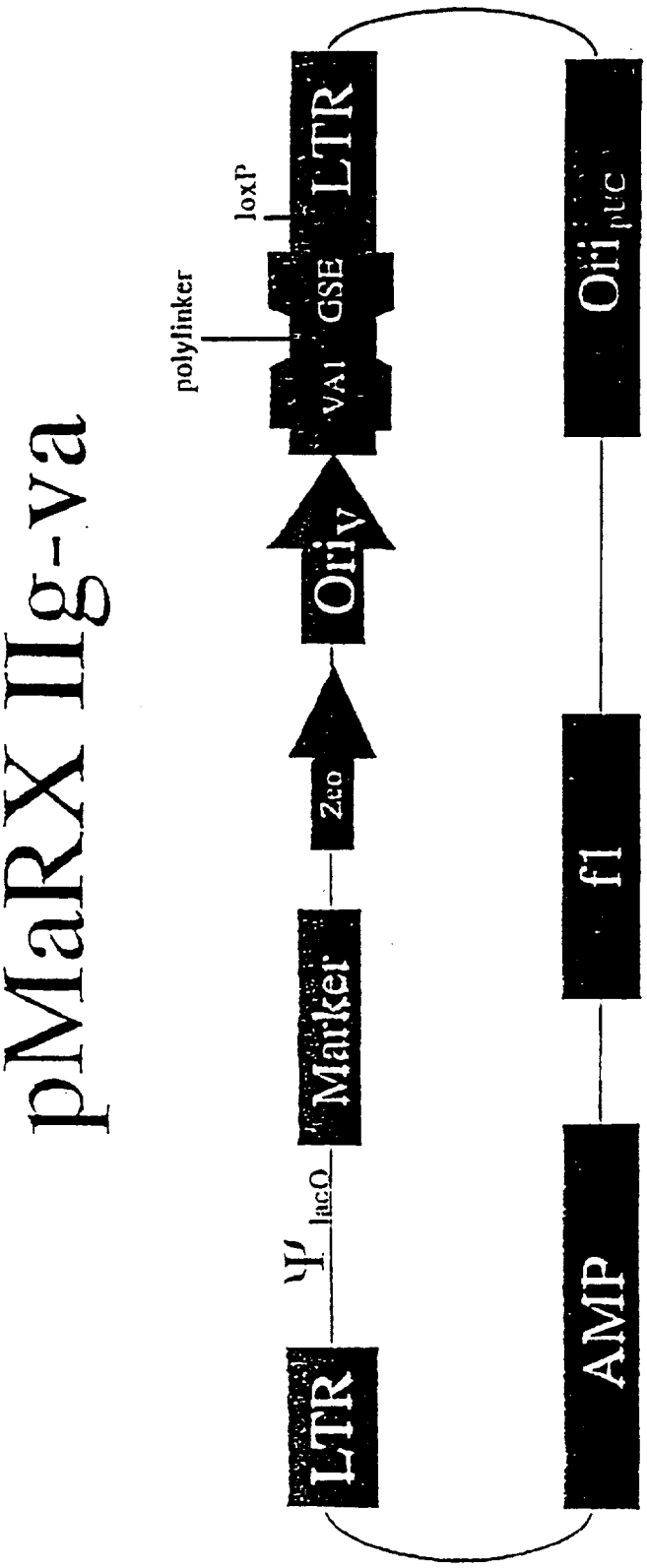

pMaRX IIg-va
LTR
AMP
Ψ lacO
Marker
Zeo
Ori V
VA1
GSE
polylinker
loxP
LTR
f1
Ori pUC

FIG. 9

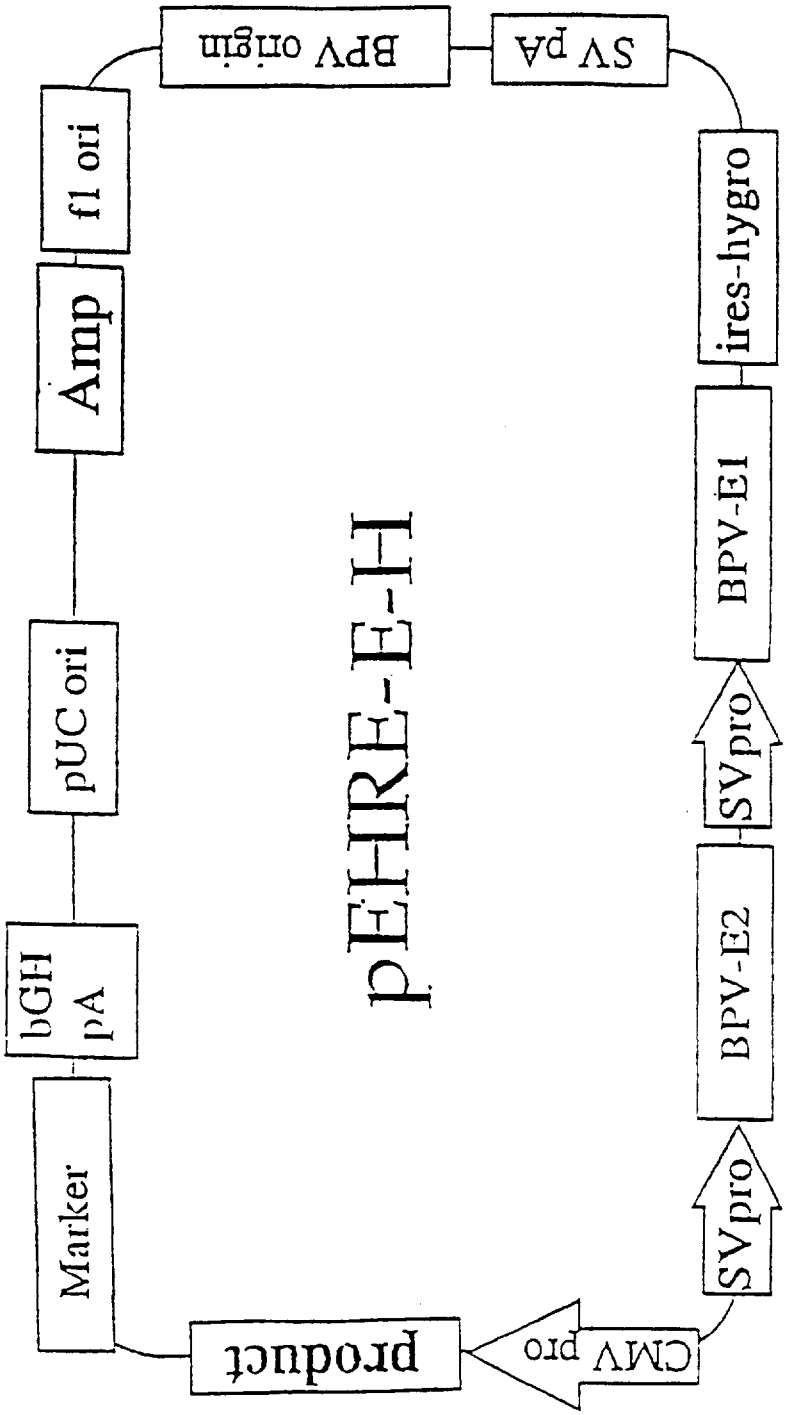

pEHRE-E-H
BPV origin
SV pA
f1 ori
ires-hygro
Amp
BPV-E1
pUC ori
SVpro
bGH pA
BPV-E2
Marker
SVpro
product
CMV pro

Fig. 10

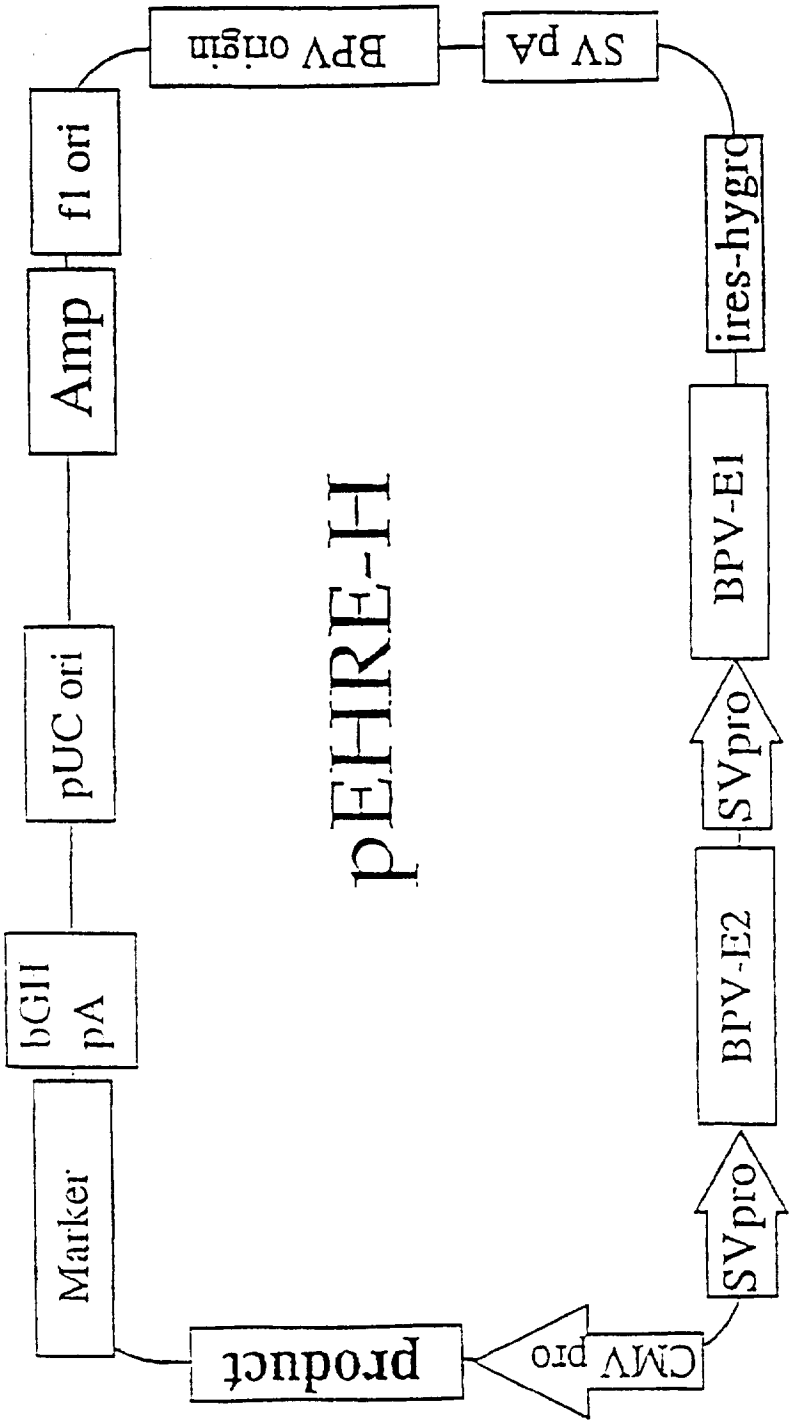

pEHRE-H
BPV origin
SV pA
f1 ori
ires-hygro
Amp
BPV-E1
pUC ori
SVpro
bGH pA
BPV-E2
Marker
SVpro
product
CMV pro

FIG. 11

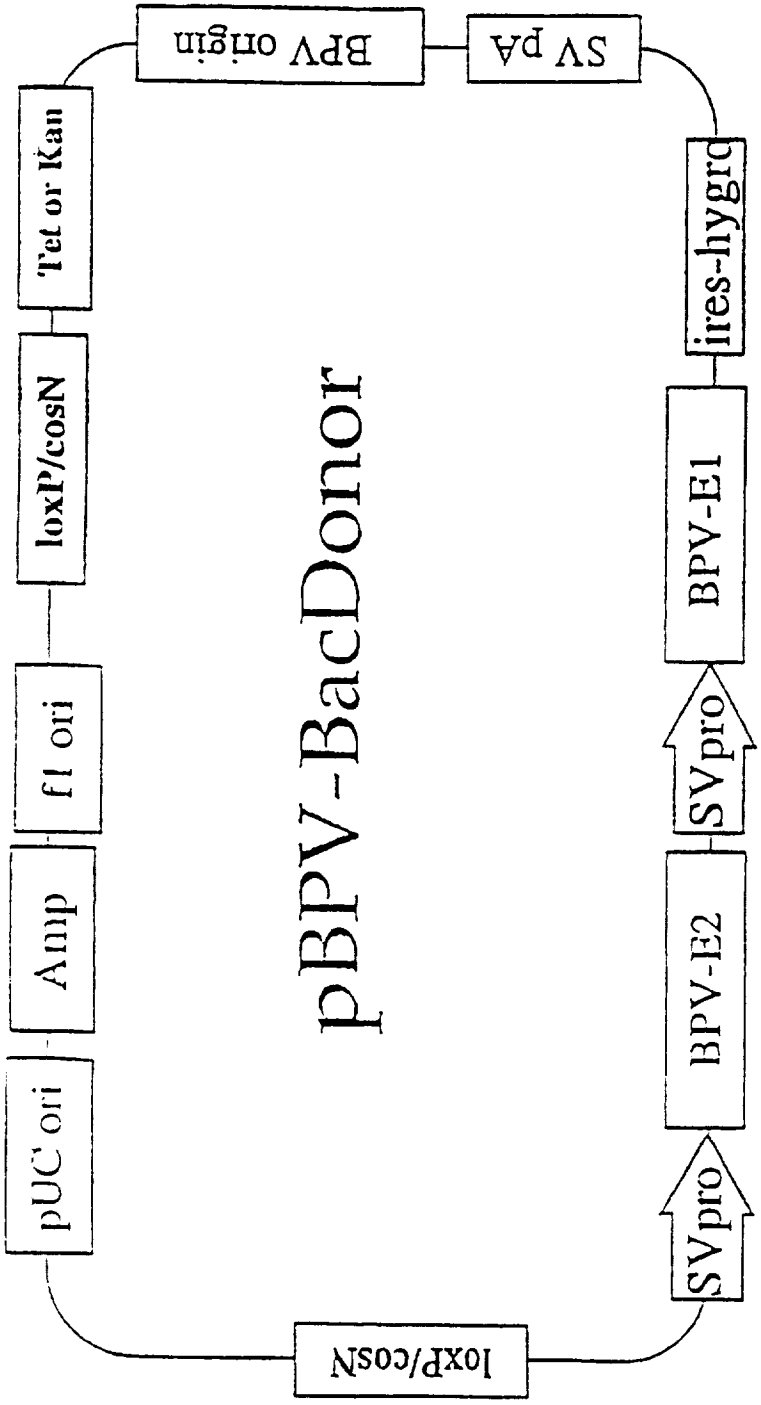

pBPV-BacDonor
BPV origin
SV pA
Tet or Kan
ires-hygro
loxP/cosN
BPV-E1
f1 ori
SVpro
Amp
BPV-E2
pUC ori
SVpro
loxP/cosN

FIG. 12

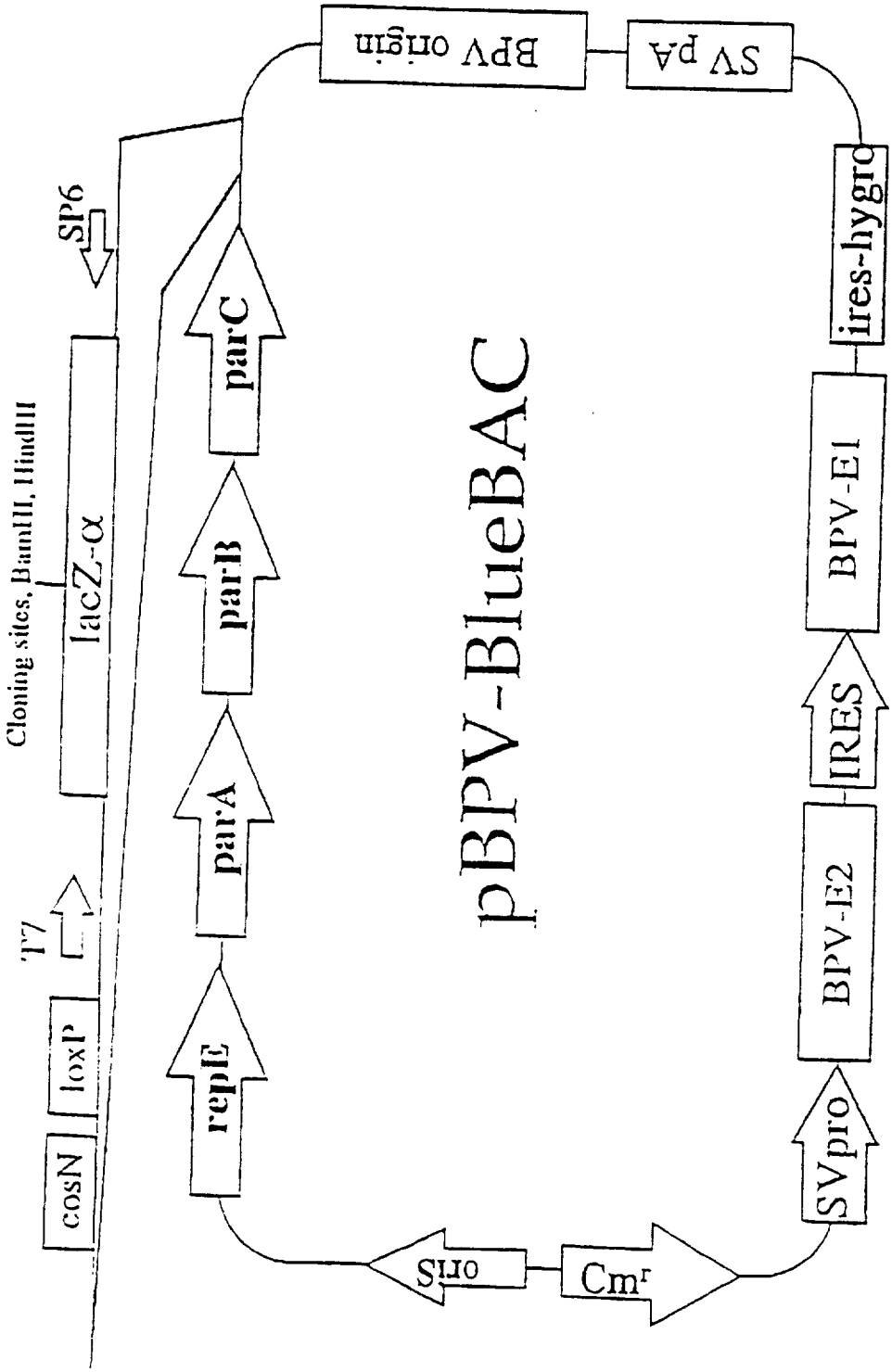

pBPV-BlueBAC
BPV origin
SV pA
ires-hygro
BPV-E1
IRES
BPV-E2
SVpro
Cm$^r$
ori$S$
repE
parA
parB
parC
SP6
lacZ-α
Cloning sites, BamHI, HindIII
T7
loxP
cosN

FIG. 13

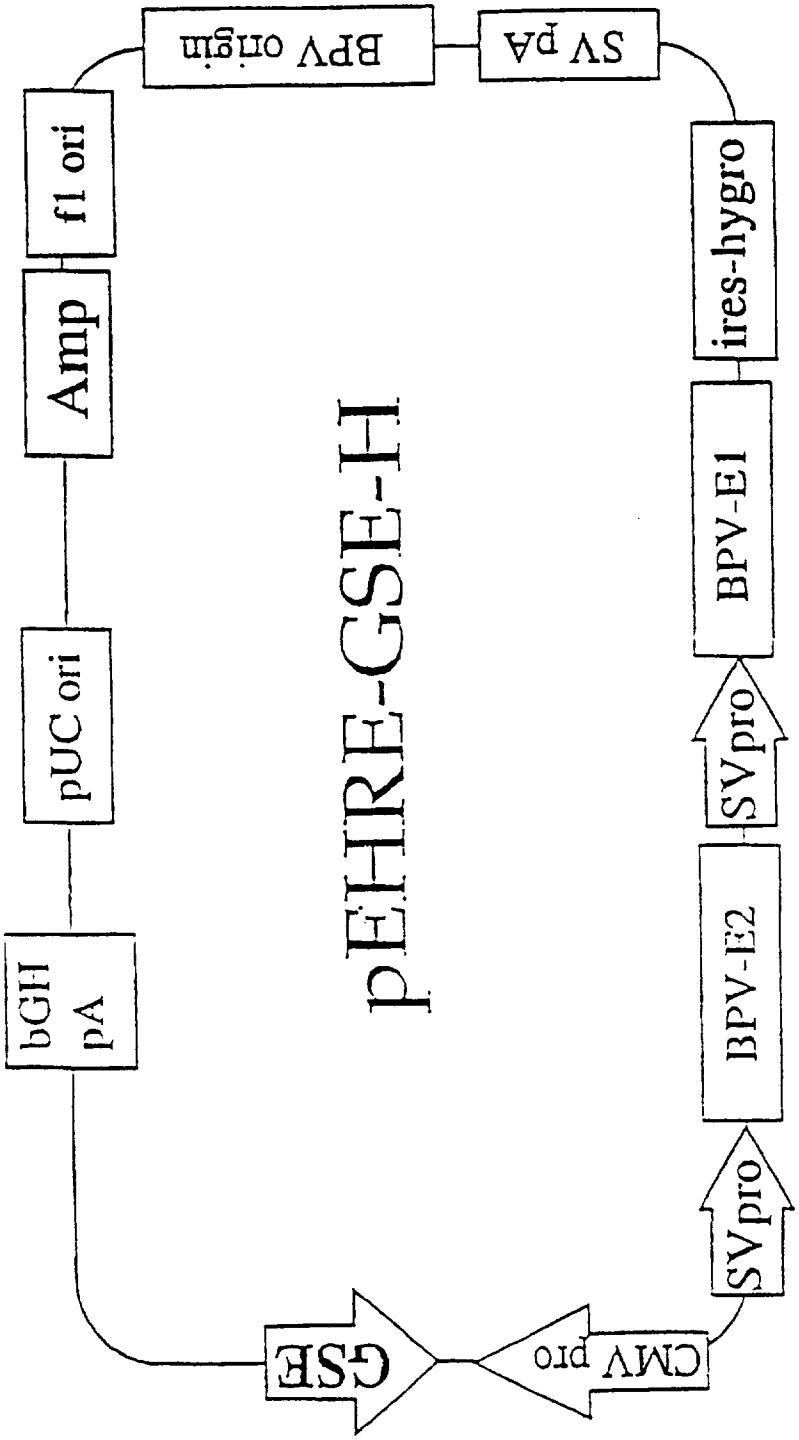

pEHRE-GSE-H
BPV origin
SV pA
f1 ori
ires-hygro
Amp
BPV-E1
pUC ori
SV pro
bGH pA
BPV-E2
SV pro
GSE
CMV pro

FIG. 14

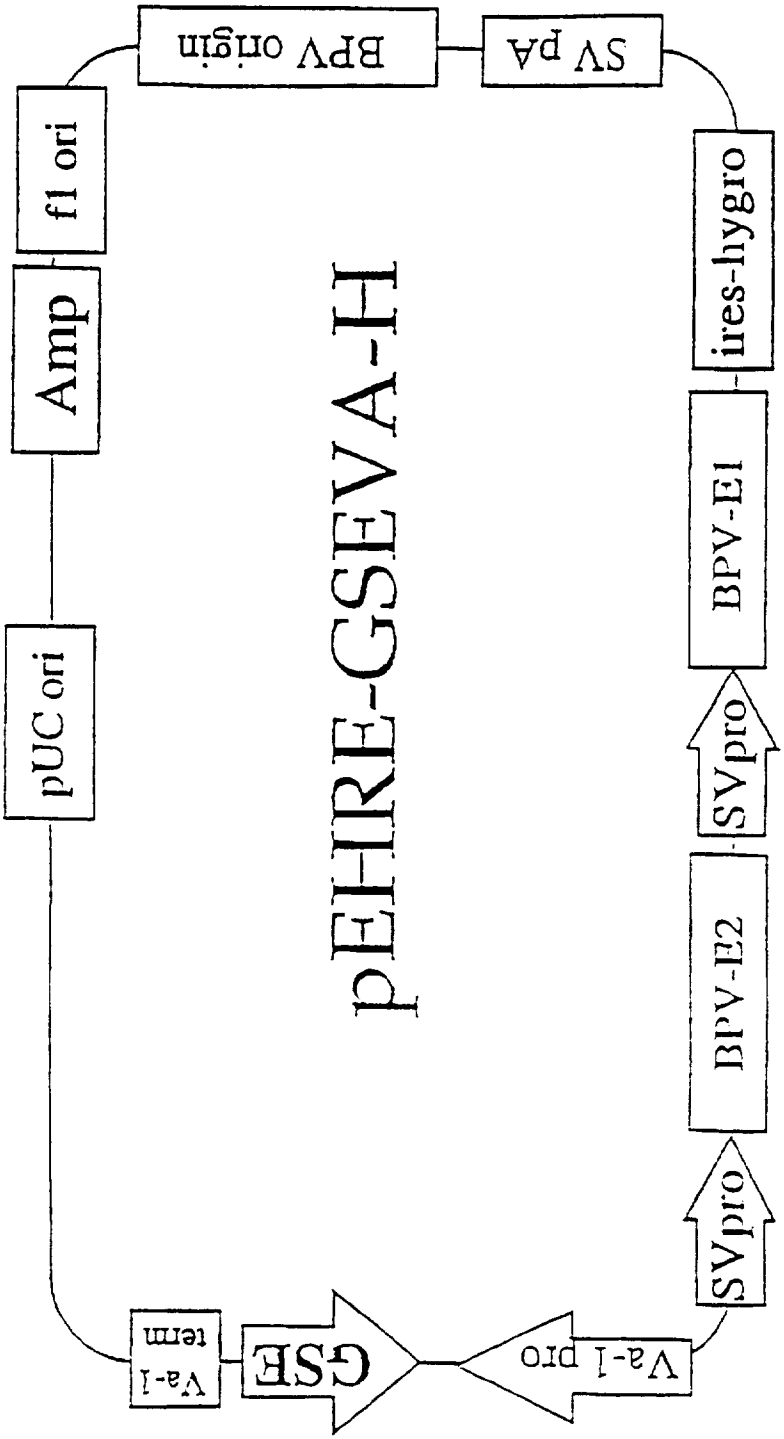

pEHRE-GSEVA-H
BPV origin
SV pA
f1 ori
ires-hygro
Amp
BPV-E1
pUC ori
SVpro
BPV-E2
SVpro
Va-1 pro
GSE
Va-1 term

FIG. 15

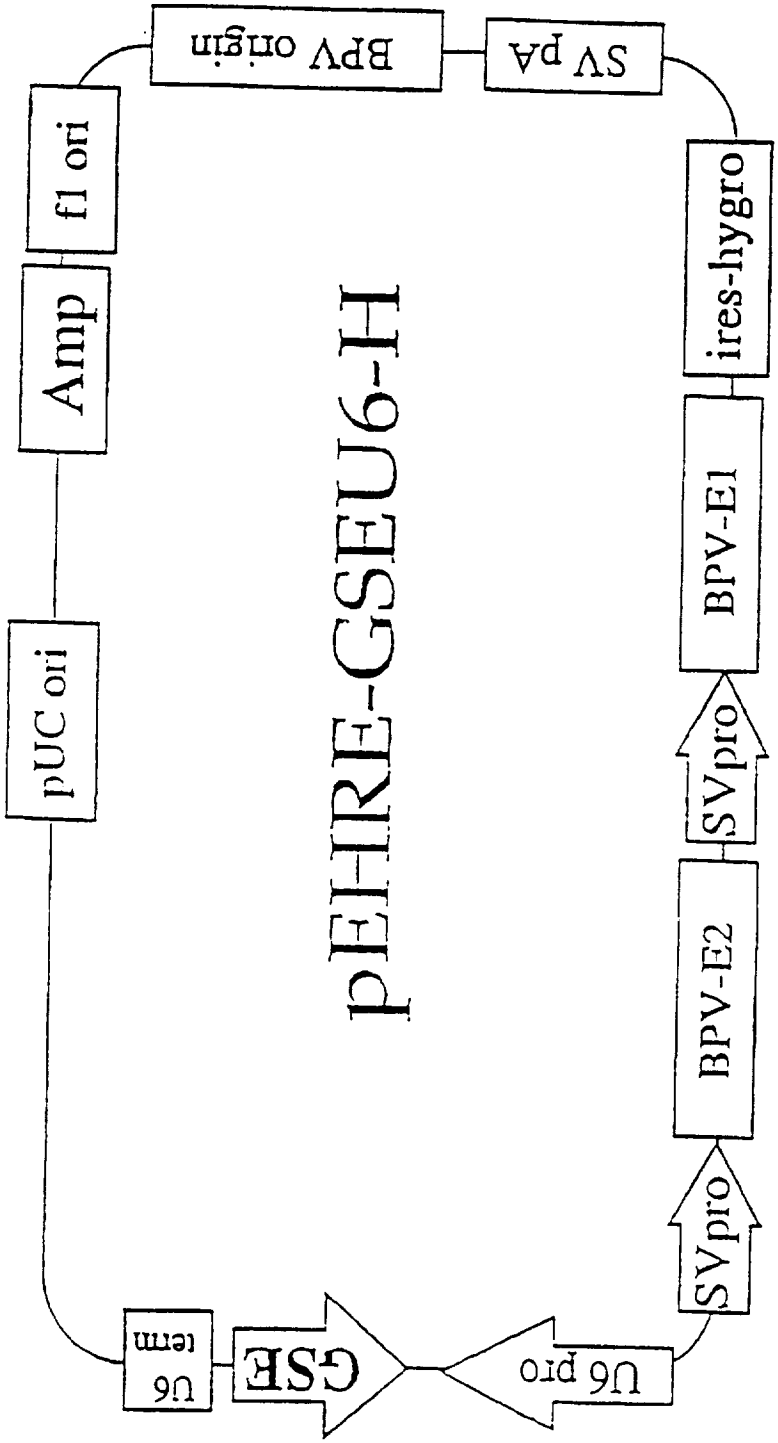

pEHRE-GSEU6-H
BPV origin
SV pA
f1 ori
ires-hygro
Amp
BPV-E1
pUC ori
SVpro
BPV-E2
SVpro
U6 term
GSE
U6 pro

FIG. 16

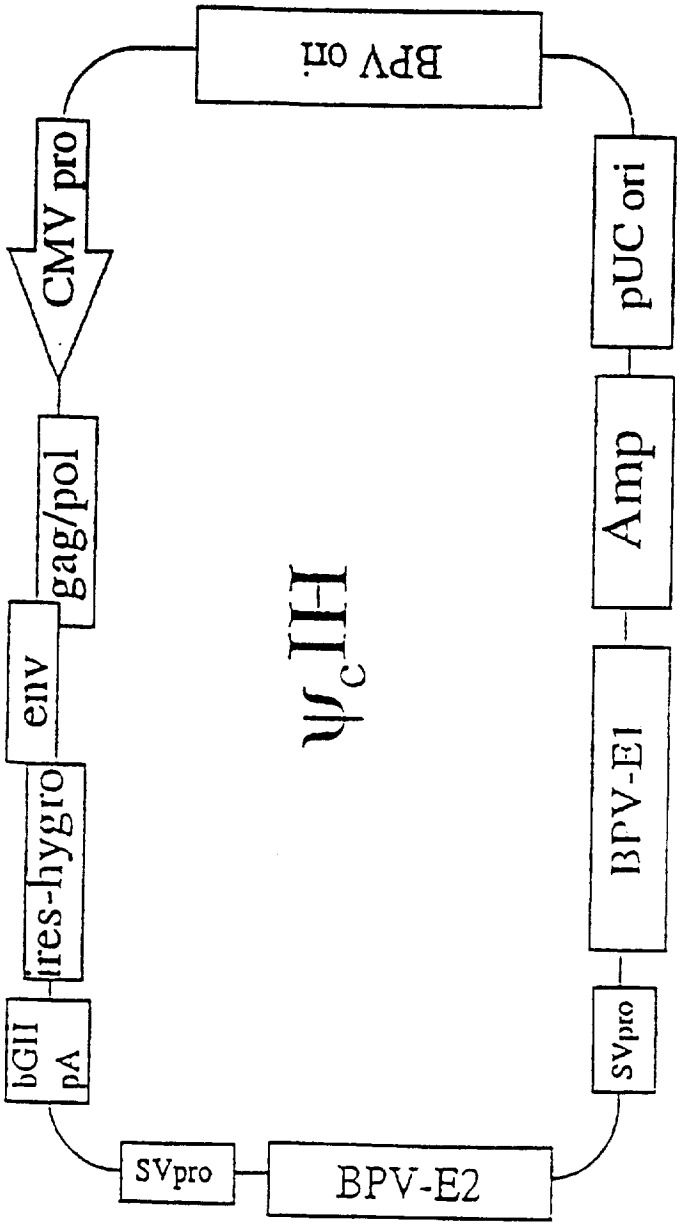

BPV ori
pUC ori
Amp
BPV-E1
SVpro
BPV-E2
SVpro
bGH pA
ires-hygro
env
gag/pol
CMV pro
ψc HIV

FIG. 17

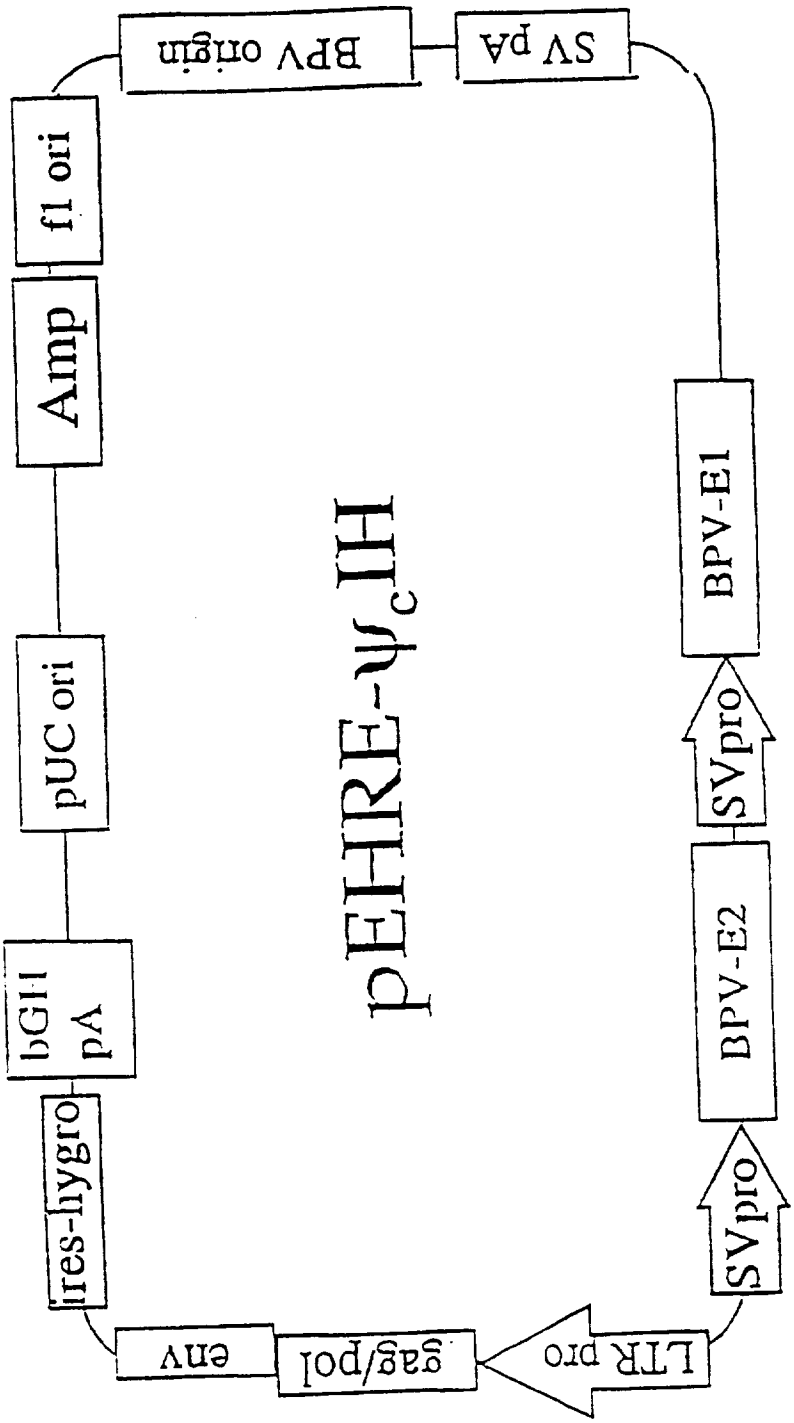

pEHRE-ψcIH
BPV origin
SV pA
f1 ori
Amp
pUC ori
bGH pA
ires-hygro
env
gag/pol
LTR pro
SVpro
BPV-E2
SVpro
BPV-E1

FIG. 18

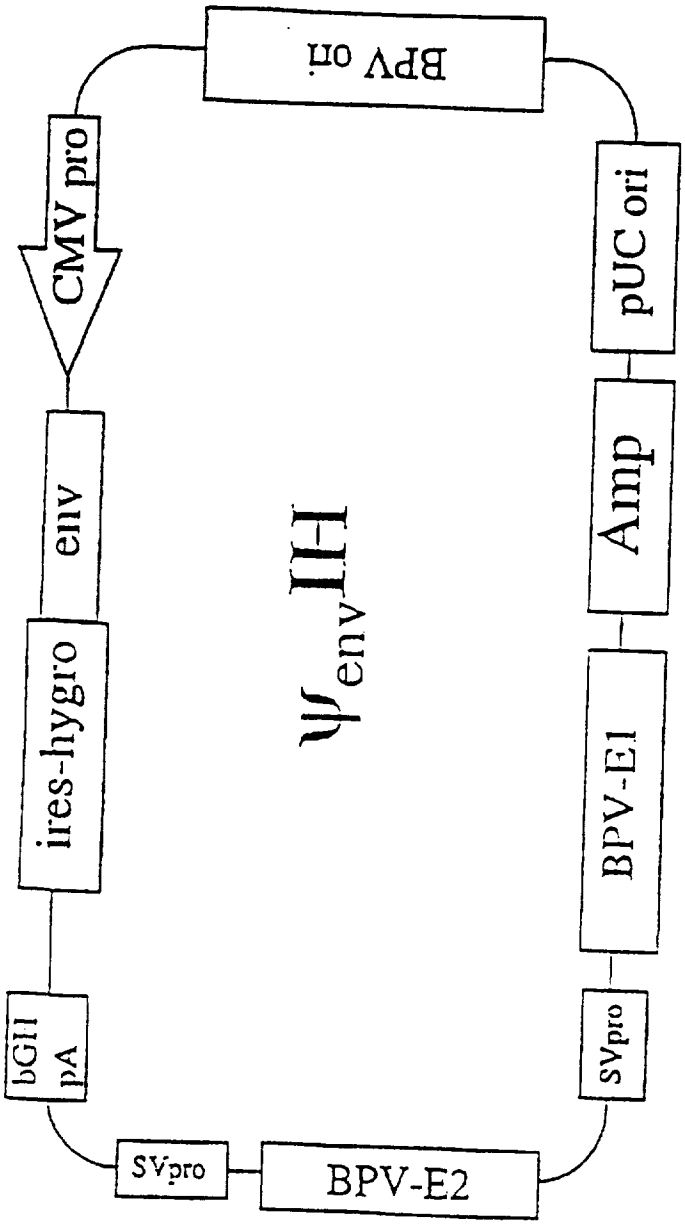

BPV ori
CMV pro
pUC ori
env
Amp
$\psi_{env}$ III-H
ires-hygro
BPV-E1
bGH pA
SVpro
SVpro
BPV-E2

FIG. 19

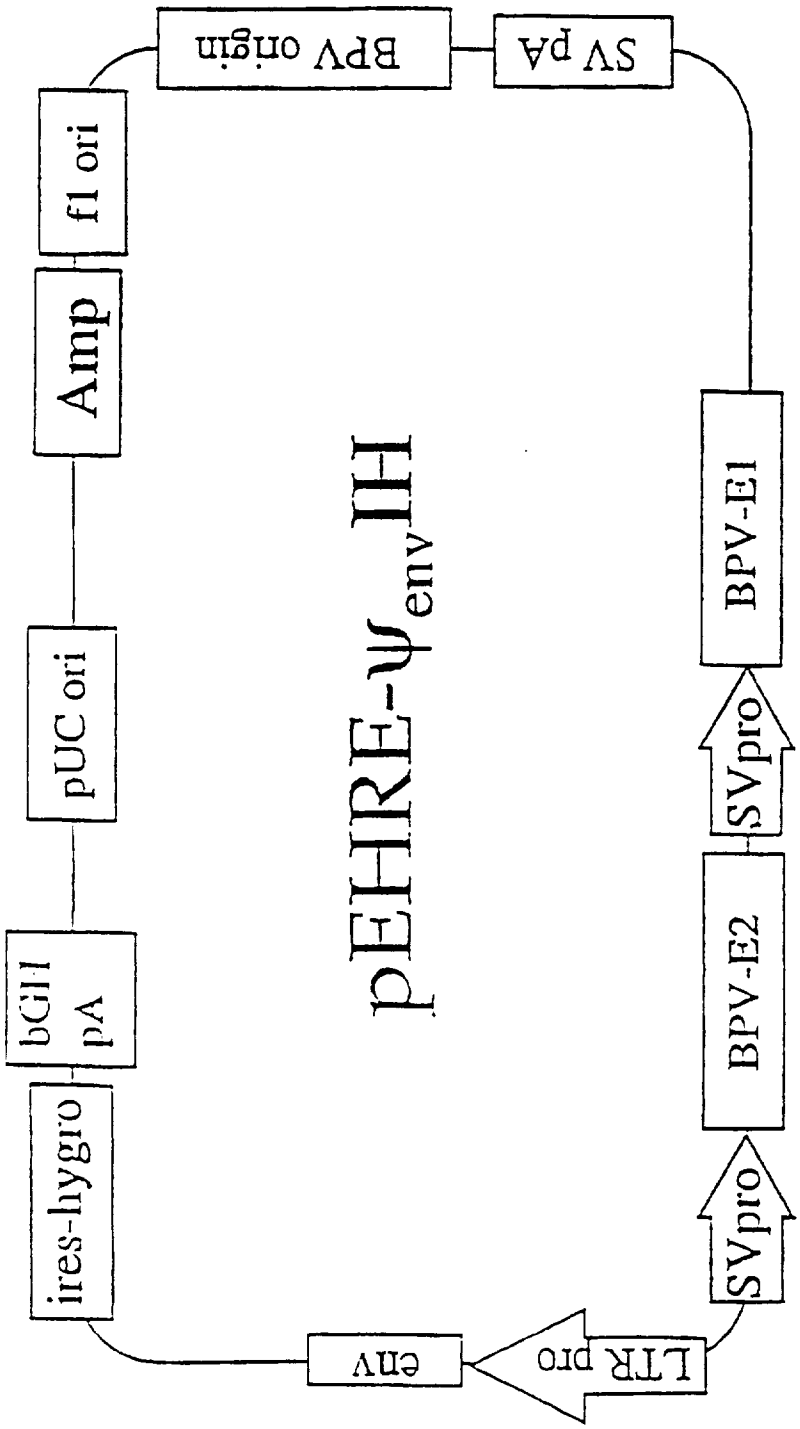

pEHRE-ψenvIH
BPV origin
SV pA
f1 ori
Amp
pUC ori
bGH pA
ires-hygro
env
LTR pro
SVpro
BPV-E2
SVpro
BPV-E1

FIG. 20

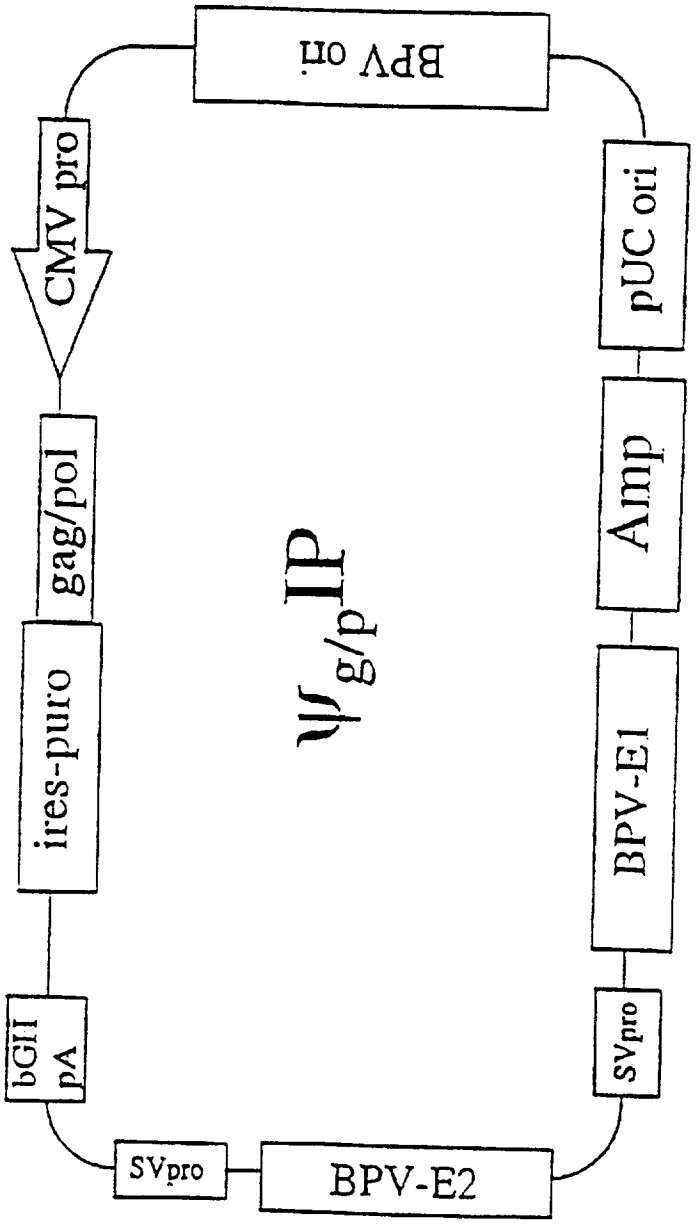

BPV ori
pUC ori
Amp
BPV-E1
SVpro
BPV-E2
SVpro
bGH pA
ires-puro
gag/pol
CMV pro
ψg/pIP

FIG. 21

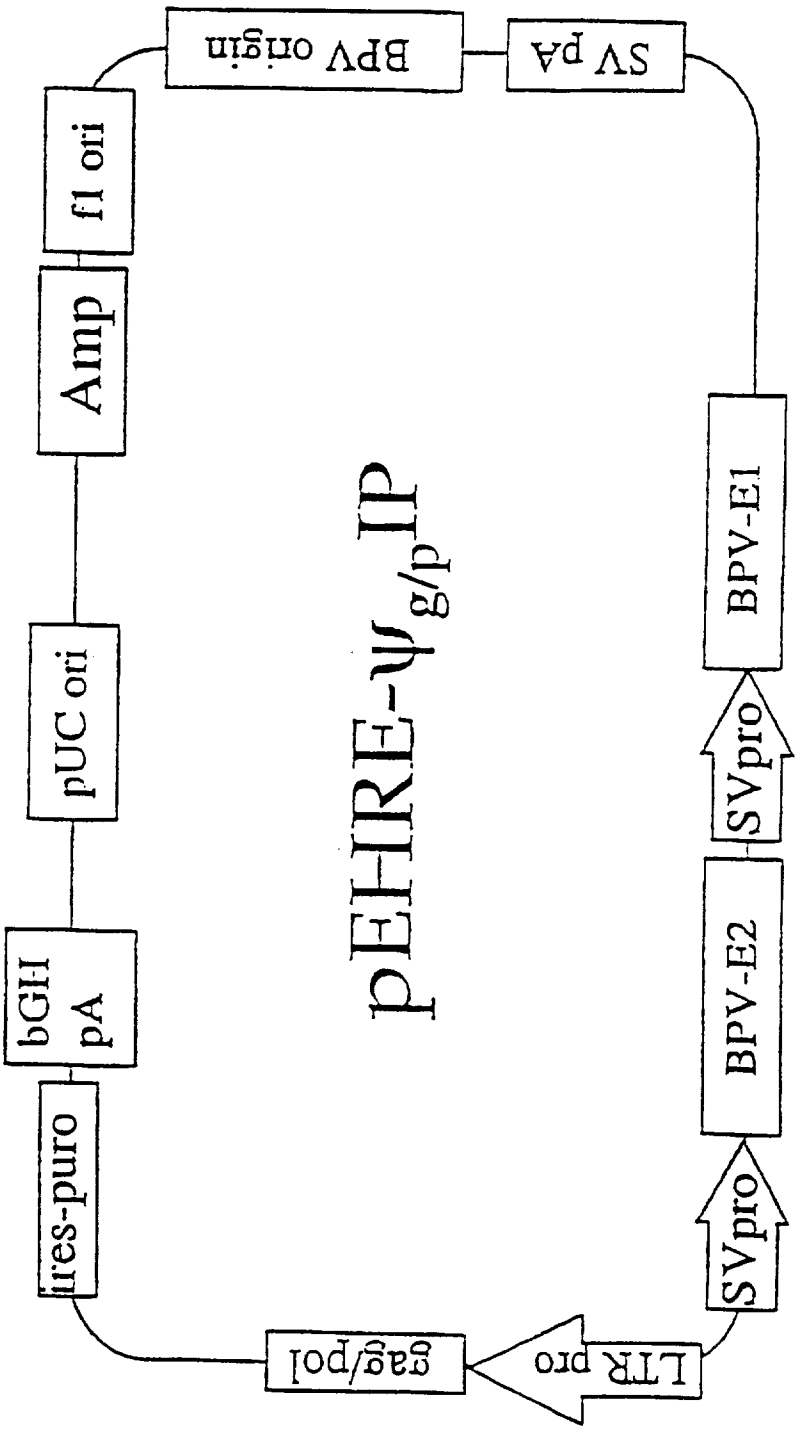

pEHRE-ψg/pIP
BPV origin
SV pA
f1 ori
Amp
pUC ori
bGH pA
ires-puro
gag/pol
LTR pro
SVpro
BPV-E2
SVpro
BPV-E1

Fig. 22

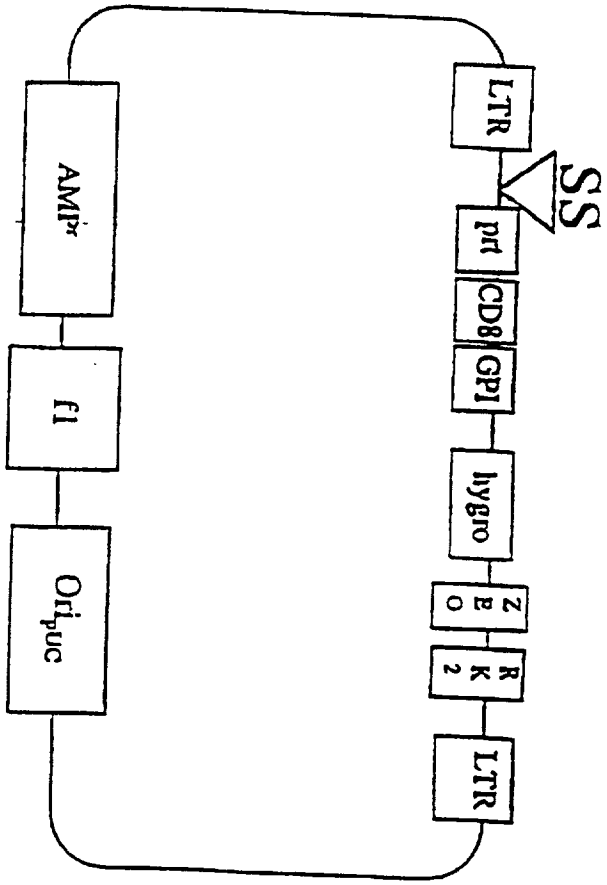



FIG. 23

Figure 24

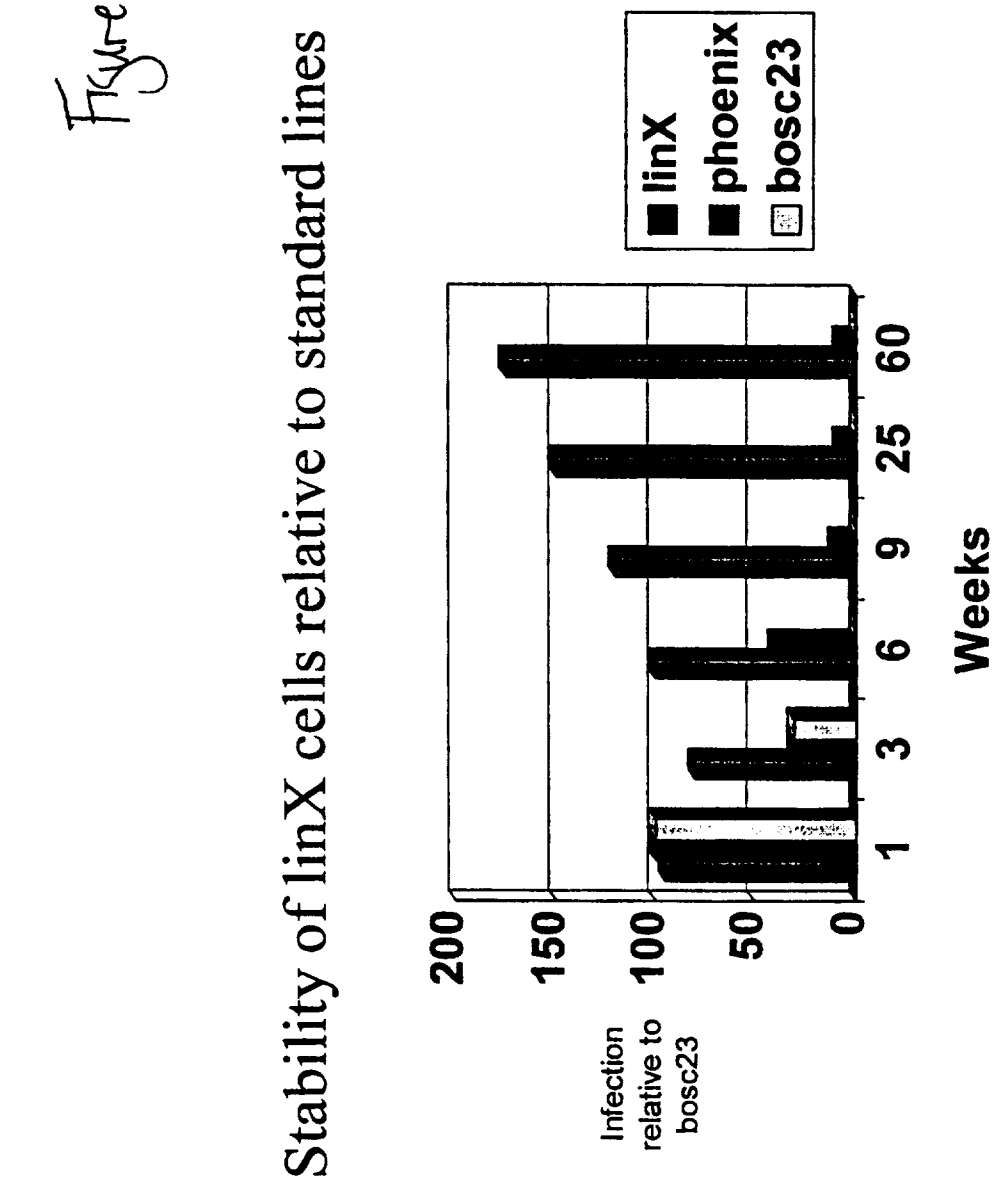
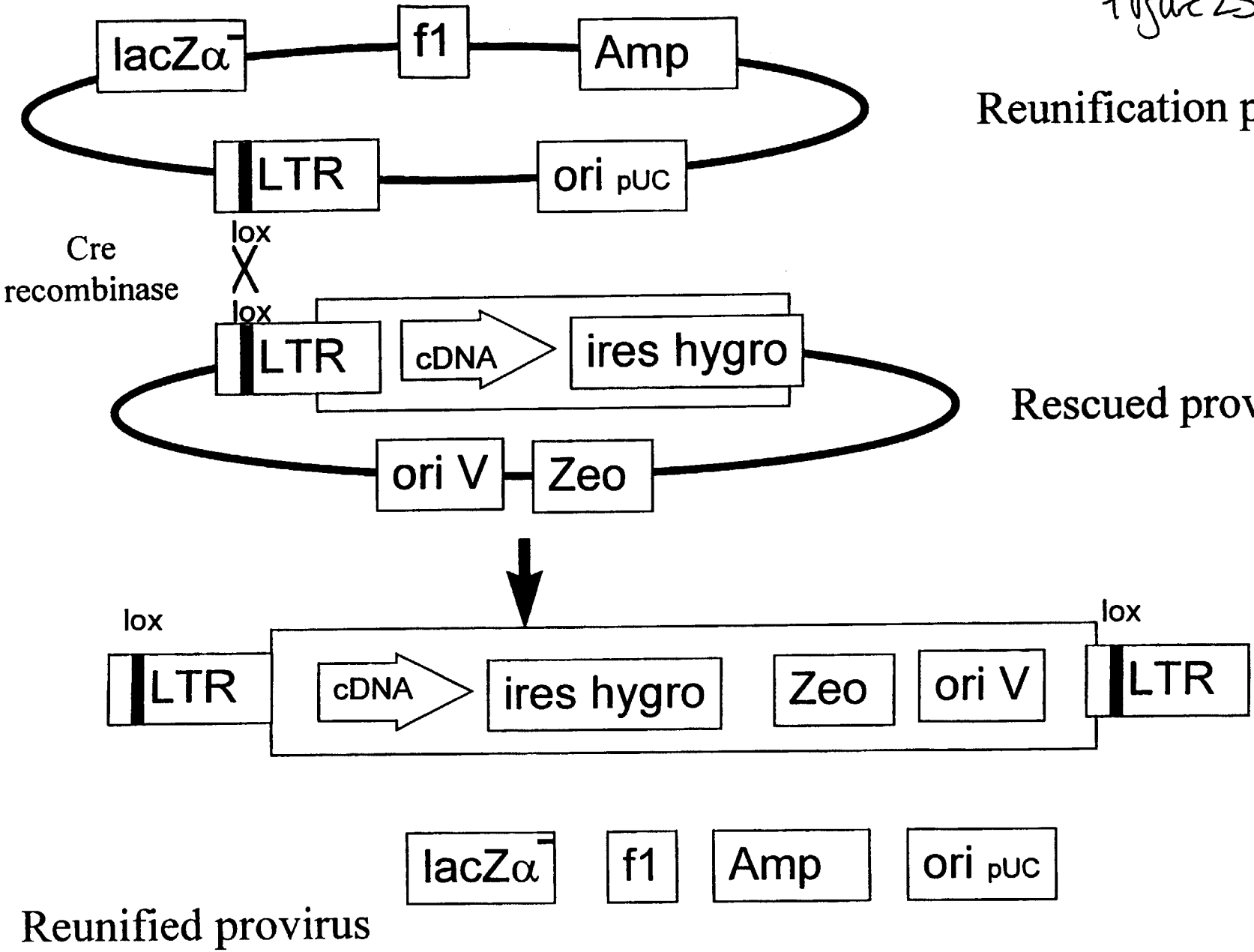

Stability of linX cells relative to standard lines
linX
phoenix
bosc23
Infection relative to bosc23
200
150
100
50
0
1
3
6
9
25
60
Weeks

Figure 25






PCT/US97/17579