HYBRID ENVELOPE GENES

Technical Field

The present invention relates generally to retroviruses, and more specifically, to a hybrid envelope gene useful for pre-paring recombinant retroviruses which are capable of delivering vector constructs to susceptible target cells. These vector constructs are typically designed to express desired proteins in target cells, for example, proteins which stimulate immunogenic activity or which are conditionally active in defined cellular environments.

Background of the Invention

Although bacterial diseases are, in general, easily treatable with antibiotics, very few effective treatments or prophylactic measures exist for many viral, cancerous, and other nonbacterial diseases, including genetic diseases. Traditional attempts to treat these diseases have employed the use of chemical drugs. In general, these drugs have lacked specificity, exhibited high overall toxicity, and thus have been therapeutically ineffective.

Another classic technique for treating a number of nonbacterial diseases involves the elicitation of an immune response to a pathogenic agent, such as a virus, through the administration of a noninfectious form of the agent, such as a killed virus, thereby providing antigens from the pathogenic agent which would act as an immunostimulant.

A more recent approach for treating viral diseases, such as acquired immunodeficiency syndrome (AIDS) and related disorders, involves blocking receptors on cells susceptible to infection by HIV from receiving or forming a complex with viral envelope

proteins. For example, Lifson et al. (Science 232:1123-1127, 1986) demonstrated that antibodies to CD4 (T4) receptors inhibited cell fusion (syncytia) between infected and noninfected CD4 presenting cells in vitro. A similar CD4 blocking effect using monoclonal antibodies has been suggested by McDougal et al. (Science 231:382-385, 1986). Alternatively, Pert et al. (Proc. Natl. Acad. Sci. USA 83:9254-9258, 1986) have reported the use of synthetic peptides to bind T4 receptors and block HIV infection of human T-cells, while Lifson et al. (J. Exp. Med. 164:2101, 1986) have reported blocking both syncytia and virus/T4 cell fusion by using a lectin which interacts with a viral envelope glycoprotein, thereby blocking it from being received by CD4 receptors.

A fourth, recently suggested technique for inhibiting a pathogenic agent, such as a virus, which transcribes RNA is to provide antisense RNA which complements at least a portion of the transcribed RNA, and binds thereto, so as to inhibit translation (To et al., Mol. Cell. Biol. 6:758, 1986).

However, a major shortcoming of the techniques described above is that they do not readily lend themselves to control as to the time, location or extent to which the drug, antigen, blocking agent or antisense RNA are utilized. In particular, since the above techniques require exogenous application of the treatment agent (i.e., exogenous to the sample in an in vitro situation), they are not directly responsive to the presence of the pathogenic agent. For example, it may be desirable to have an immunostimulant expressed in increased amounts immediately following infection by the pathogenic agent. In addition, in the case of antisense RNA, large amounts would be required for useful therapy in an animal, which under current techniques would be administered without regard to the location at which it

is actually needed, that is, at the cells infected by the pathogenic agent.

As an alternative to exogenous application, techniques have been suggested for producing treatment agents endogenously. More specifically, proteins expressed from viral vectors based on DNA viruses, such as adenovirus, simian virus 40, bovine papilloma, and vaccinia viruses, have been investigated. By way of example, Panicali et al. (Proc. Natl. Acad. Sci. USA 80:5364, 1983) introduced influenza virus hemagglutinin and hepatitis B surface antigens into the vaccinia genome and infected animals with the virus particles produced from such recombinant genes. Following infection, the animals acquired immunity to both the vaccinia virus and the hepatitis B antigen.

However, a number of difficulties have been experienced to date with viral vectors based on DNA viruses. These difficulties include (a) the production of other viral proteins which may lead to pathogenesis or the suppression of the desired protein; (b) the capacity of the vector to uncontrollably replicate in the host, and the pathogenic effect of such uncontrolled replication; (c) the presence of wild-type virus which may lead to viremia; and (d) the transitory nature of expression in these systems. These difficulties have virtually precluded the use of viral vectors based on DNA viruses in the treatment of viral, cancerous, and other nonbacterial diseases, including genetic diseases.

Due to the nontransitory nature of their expression in infected target cells, retroviruses have been suggested as a useful vehicle for the treatment of genetic diseases (for example, see F. Ledley, The Journal of Pediatrics 110:1, 1987). However, in view of a number of problems, the use of retroviruses in the treatment of genetic diseases has not been attempted. Such problems relate to (a) the apparent need to infect a large number of cells in inaccessible tissues (e.g.,

brain); (b) the need to cause these vectors to express in a very controlled and permanent fashion; (c) the lack of cloned genes; (d) the irreversible damage to tissue and organs due to metabolic abnormalities; and (e) the availability of other partially effective therapies in certain instances.

In addition to genetic diseases, other researchers have contemplated using retroviral vectors to treat nongenetic diseases (see, for example, EP 243,204 - Cetus Corporation; Sanford, J. Theor. Biol.130:469, 1988; Tellier et al., Nature 318:414, 1985; and Bolognesi et al., Cancer Res. 45:4700, 1985).

Tellier et al. suggested protecting T-cell clones by apparently infecting stem cells with "defective" HIV having a genome which could express antisense RNA to HIV RNA. Bolognesi et al. have suggested the concept of generating a nonvirulent HIV strain to infect stem cells so that T4 cells generated therefrom would carry interfering, nonvirulent forms of virus and thereby protect those cells from infection by virulent HIV. However, it would appear that the "attenuated" or "defective" HIV viruses used in both of the foregoing papers could reproduce (i.e., are not replication defective) such that the resulting viruses could infect other cells, with the possibility of an increased risk of recombination with previously present HIV or other sequences, leading to loss of attenuation. Non-nonreplicative forms would necessitate a defective helper or packaging line for HIV. However, since the control of HIV gene expression is complex, such cells have to date not been constructed. Furthermore, as the infecting attenuated or defective virus is not chimeric (a "nonchimeric" retrovirus being one with substantially all of its vector from the same retrovirus species), even if they were made replication defective, for example, by deletion from their genomes of an essential element, there still exists a significant possibility

for recombination within the host cells with resultant production of infectious viral particles.

Although Sanford (J. Theor. Biol. 130:469, 1988) has also proposed using a genetic cure for HIV, he notes that due to the potential that exists for creating novel virulent viruses via genetic recombination between natural AIDS virus and therapeutic retroviral vectors carrying anti-HIV genes, retroviral gene therapy for AIDS may not be practical. Similarly, while McCormick & Kriegler (EP 243,204 A2) have proposed using retroviral vectors to deliver genes for proteins, such as tumour necrosis factor (TNF), the techniques they describe suffer from a number of disadvantages.

According to the invention there is provided a hybrid env gene useful for preparing a retrovirus which can selectively carry a gene of interest to a target cell, the env gene coding for:

(a) a cytoplasmic segment of a first retroviral phenotype; and

(b) a binding segment exogenous to the first retroviral phenotype, the binding segment being capable of selectively binding to the target cell.

The technique for producing recombinant retroviruses which can be targeted for preselected cell lines utilizes recombinant retroviruses having an env gene comprised of a cytoplasmic segment of a first retroviral phenotype, and an extracelluar binding segment exogenous to the first retroviral phenotype.

The binding segment may be from a second viral phenotype or from another protein with desired binding properties which is selected to be expressed as a peptide which will bind to the desired target.

Techniques for integrating a retroviral genome at a specific site in the DNA of a target cell involve the use of a homologous recombinant, or alternatively, the use of a modified integrase enzyme which will recognise a specific site on the target cell genome. Such site-specific insertion allows genes to be inserted at sites on the target cells' DNA, which will minimise the chances of insertional metagenesis, minimise interference from other sequences on the DNA, and allow insertion of sequences at specific target sites so as to reduce or eliminate the expression of an undesirable gene (such as a viral gene) in the DNA of the target cell.

It will be appreciated that any of the above-described techniques may be used independently of the others in particular situations, or can be used in conjunction with one or more of the remainder of the techniques.

Brief Description of the Drawings

Figure 1 depicts three different families of vectors used to produce HIV env and which may or may not have the selectable SV-Neo cassette inserted.

Figure 2 illustrates the HIV env expression levels seen in

polyacrylamide gel electrophoresis of HIV env specific radioimmune precipitations of extracts of human Sup T1 cells transfected with the vectors shown. The markers are in kilodaltons, gp 160 and gp 120 mark the appropriate proteins, and 517 + tat is the positive control (HIV LTR driving env in the presence of tat).

Figure 3 depicts the protocol for testing T-cell killing induced in mice injected with syngeneic

tumor cells expressing HIV env (the vector is pAF/env-SV-Neo).

Figure 4 graphically depicts the results of the experimental protocol in Figure 3. The specific killing is seen in the top graph with BC10MEenv-29 killing vs. B/C10ME resistance to killing.

Figure 5 depicts a vector designed to express sCD4.

Figure 6 illustrates the construction of the plasmids carrying the vectors TK1 (without SV-Neo) and TK3 (plus SV-Neo).

Figure 7 illustrates the construction of the plasmid carrying the vector KTVIHAX.

Figure 8 illustrates the construction of the plasmids carrying the vectors KTVIH5 (without SV-Neo) and KTVIH Neo (with SV-Neo).

Figure 9 illustrates construction of the plasmid carrying the vector MHMTK-Neo.

Figure 10 illustrates the construction of the plasmids carrying the tat-his (tat in sense direction) or αtat (tat in antisense direction) vectors.

Figure 11 graphically depicts the preferential killing of PA317 cells infected with tathis vector (5 clones, TH1-5) compared to control PA317, upon infection with the three conditional lethal vectors shown and treatment with acyclovir (ACV).

Figure 12 depicts the construction of a viral vector carrying HIV inducible marker/reporter genes such as alkaline phosphatase (AP).

Figure 13 depicts the structure of an HIV inducible marker/reporter gene carried on a plasmid which can be transfected into cells.

Figure 14 graphically depicts a time course of HIV infection of Sup T1 cells carrying the AP marker in Figure 13 with HIV at various concentrations of AZT. The level of HIV infection was measured by taking small aliquots of supernatant.

Figure 15 graphically depicts the results of the same experiment as in Figure 14, but with ddC as the HIV inhibitor.

Figure 16 diagrammatically illustrates the number of cells surviving after phleomycin selection upon transfection of cells with a plasmid which expresses the phlemoycin resistance gene (PRG) directly from a promoter (right) and with another which expresses PRG with a coding sequence interposed between it and the promoter.

Figure 17 depicts four plasmids designed to express retroviral proteins in mammalian cells. pSVgp and pRSVenv are cotransfected with a selectable marker, while pSVgp-DHFR and pRSVenv-phleo are the equivalent plasmids with the selectable marker placed downstream of the viral protein-coding regions.

Figure 18 depicts three sites of fusion of HIV env and MLV env after site-directed metagenesis of both coding sequences to create new compatible restriction enzyme sites. The N terminal sequences are at the left in both cases. The numbering is according to nucleotide numbers. ST, SR, SE are the starts of tat, rev and env while TT, TR, and TE are the corresponding termination sites.

Figure 19 depicts the substitution of U3 in a 5' LTR by a heterologous promoter/enhancer which can be fused to either the Sac I, Bsst II or other site in the region.

Figure 20 illustrates a representative method for crossing transgenic mice expressing viral protein or vector RNA.

I. <u>Immunostimulation</u>

The ability to recognise and defend against foreign pathogens is central to the function of the

immune system. This system, through immune recognition, must be capable of distinguishing "self" from "nonself" (foreign), which is essential to ensure that defensive mechanisms are directed toward invading entities rather than against host tissues. The fundamental features of the immune system are the presence of highly polymorphic cell surface recognition structures (receptors) and effector mechanisms (antibodies and cytolytic cells) for the destruction of invading pathogens.

Cytolytic T lymphocytes (CTL) are induced normally by the display of processed pathogen specific peptides in conjunction with the MHC class I or class II cell surface proteins. Within one embodiment of the present invention, presentation of immunogenic viral determinants in the context of appropriate MHC molecules efficiently induces optimal CTL responses without exposing the patient to the pathogen. This vector approach to immunostimulation provides a more effective means of inducing protective and therapeutic CTL responses, because the type of immunity induced by the vector more closely resembles that induced by exposure to natural infection. Based on current knowledge of several viral systems, it is unlikely that exogenously supplied, nonreplicating viral antigens, such as peptides and purified recombinant proteins, will provide sufficient stimulus to induce optimal class I-restricted CTL responses. Alternatively, vector-delivered expression of selected viral proteins within target cells as described within the present invention provides such a stimulus.

By way of example, in the case of HIV-1 infections, patients develop antibodies specific for a variety of viral envelope-region determinants, some of which are capable of <u>in vitro</u> virus neutralization. Nevertheless, disease progression continues and the patients eventually succumb to the disease. Low-level CTL responses against infected patients' cells (Plata

et al., Nature 328:348-351, 1987) and against target cells infected with recombinant vaccinia vectors expressing HIV gag, pol, or env (Walker et al., Nature 328:345-348, 1987; Walker et al., Science 240:64-66, 1988) have been detected in some HIV-1 seropositive patients. In addition, it has recently been shown that murine as well as human CTL can be induced by autologous stimulator cells expressing HIV gp 120 via transfection (Langlade-Demoyan et al., J. Immunol. 141:1949, 1988). Improved CTL induction could be therapeutically advantageous to injected patients and provide effective preventive therapy to individuals under noninfectious conditions. HIV infection itself may not be producing adequate CTL response because other elements associated with HIV infection may prevent proper immune stimulation. In addition, it may be that stimulation of T-cells by infected cells is an interaction that leads to infection of the stimulated T-cells.

Example 1 describes procedures for constructing plasmids capable of generating retroviral vectors in packaging cells, which then lead to expression of HIV viral antigens.

EXAMPLE 1

Vectors Expressing HIV Antigens

A. Env Expression Vector (See Figure 1):

A 2.7 kb Kpn-Xho I DNA fragment was isolated from the HIV proviral clone BH10-R3 (for sequence, see Ratner et al., Nature 313:277, 1985) and a ≈400 bp Sal-Kpn I DNA fragment from IIIexE7deltaenv (a Bal31 deletion to nt. 5496) was ligated into the Sal I site in the plasmid SK^+. From this clone, a 3.1 kb env DNA fragment (Xho I-Cla I) which also encodes rev, essential for env expression, was purified and ligated into a retroviral vector called pAFVXM (see Kriegler et al., Cell 38:483, 1984). This vector was modified in that the Bgl II site was changed by linker insertion to a

Xho I site to facilitate cloning of the HIV env coding DNA fragment.

A dominant selectable marker gene comprised of a SV40 early promoter driving expression of neomycin phosphotransferase gene was inserted into the vector at the Cla I site to facilitate isolation of infected and transfected cell lines.

The Xho I site upstream from the ENV gene in the vector provides a convenient site to insert additional promoters into the vector construct as the RSV promoter, SV40 early or late promoter, the CMV immediate early (IE) promoter, human beta-bactin promoter, and Moloney murine MLV SL3-3 promoter.

One such promoter, the CMV Immediate Early gene promoter, a 673 bp DNA fragment Hinc II to Eag I, results in a tenfold increase in ENV expression in a human T-cell line called Sup TI when compared to the parental construct pAF ENV^r SV_2 Neo.

B. Gag Expression Vector:

A 2.5 kb Sac I-Eco RV DNA fragment was isolated from pBH10-R3 (see Ratner et al., op. cit.) and ligated into the Sac I-Sal I site of pUC31. pUC31 is derived from pUC19 with additional Xho I, Bgl II, Bsst II and Nco I sites inserted between the Eco R1 and Kpn I sites of the poly linker. However, this construct contained the major splice donor (SD) site from HIV and thus could be problematic in virus generation. The SD site was removed by subcloning a 70 bp Rsa I-Cla I fragment with a 2.1 kb Cla I-Bam H1 DNA fragment into the Hinc II-Bam H1 site of SK^+. The Bam H1 site was converted into a Cla I site by linker insertion. This construct was designated SK^+ gag protease SD delta.

The 2.5 kb Xho I-Cla I DNA fragment from SK^+ gag protease SD delta was inserted into the Xho I/Cla I sites of the vector pAFVXM just as described above.

These plasmids, when placed in a suitable packaging cell, expressed a retroviral vector construct which contains a packaging signal. The packaging signal directed packaging of the vector construct into a capsid and envelope along with all further proteins required for viable retroviral particles. The capsid, envelope, and other proteins are preferably produced from one or more plasmids containing suitable genomes placed in the packaging cell. Such genomes may be proviral constructs, which in a simple case may merely have the packaging signal deleted. As a result, only the vector will be packaged. Suitable packaging or packaging cell lines, and the genome necessary for accomplishing such packaging, are described in Miller et al. (Mol. Cell. Bio. 6:2895, 1986), which is incorporated herein by reference. As described by Miller et al., it is preferable that further changes be made to the proviral construct other than simple deletion of the packaging signal in order to reduce the chances of recombination events occurring within the packaging cell line, which may result in production of viral particles which are not replication defective.

It will be understood that Example 1 is merely illustrative of a procedure for generating an HIV envelope glycoprotein (gp) or other viral antigen. It is also possible to provide a proviral vector construct which expresses a modified HIV envelope gp on the target cells which will likewise stimulate an immune response, but with less T-cell cytopathic effects. Envelope glycoproteins can be suitably modified using techniques well known in the art, for instance through use of the disclosure of articles such as Kowalski et al. (Science 237:1351, 1987), which is herein incorporated by reference. Thus, a proviral construct may be constructed by the above technique which generates retroviral constructs expressing such a suitably modified gp. This construct is then placed in a

packaging cell as described above. The resulting recombinant retroviruses produced from the packaging cell lines may be used in vitro and in vivo to stimulate an immune response through the infection of susceptible target cells. It will be appreciated that other proteins expressed from the HIV genome, such as gag, pol, vif, nef, etc., may also elicit beneficial cellular responses in HIV-infected individuals. Proviral vectors such as those described below are designed to express such proteins so as to encourage a clinically beneficial immune response. It may be necessary for certain vectors to include rev coding sequences as well as a rev responsive element (Rosen et al., Proc. Natl. Acad. Sci. 85:2071, 1988).

The following example demonstrates the ability of this type of treatment to elicit CTL responses in mice.

EXAMPLE 2

Immune Response to Retroviral Vector-Encoded Antigens

A murine tumor cell line (B/C10ME) ($H\text{-}2^d$) was infected with the pAF env^rSV40 Neo vector construct coding for HIV env. One cloned HIV-env expressing cell line (B/C10ME-29) was then utilized to stimulate HIV-env-specific CTL in syngeneic (i.e., MHC identical) Balb/c ($H\text{-}2^d$) mice. Mice were immunized by intraperitoneal injection with B/C10ME-29 cells (4×10^7 cells) and boosted on day 7-14. Responder spleen cell suspensions were prepared from these immunized mice and the cells cultured in vitro for 4 days in the presence of either B/C10ME-29 or B/C10ME mitomycin-C-treated cells at a stimulator:responder cell ratio of 1:50 (Figure 3). The effector cells were harvested from these cultures, counted, and mixed with radiolabeled (^{51}Cr) target cells (i.e., B/C10MEenv-29 or B/C10ME) at various effector:target (E:T) cell ratios in a standard 4-5 hr ^{51}Cr-release assay. Following incubation, the

microtiter plates were centrifuged, 100 ul of culture supernate was removed, and the amount of radiolabel released from lysed cells quantitated in a Beckman gamma spectrometer. Target cell lysis was calculated as: % Target Lysis = Exp CPM - SR CPM/MR CPM - SR CPM x 100, where experimental counts per minute (Exp CPM) represents effectors plus targets; spontaneous release (SR) CPM represents targets alone; and maximum release (MR) CPM represents targets in the presence of 1 M HCL.

The results (Figure 4) illustrate that CTL effectors were induced which specifically lysed HIV-env-expressing target cells (B/C10MEenv-29) significantly more efficiently than B/C10ME targets. Primed spleen cells restimulated in vitro with non-HIV-env-expressing control cells (B/C10ME) did not show significant CTL activity on either B/C10MEenv-29 or B/C10ME targets, particularly at lower E:T cell ratios. Spleen cells obtained from naive nonimmunized Balb/c mice which were restimulated in vitro with B/C10MEenv-29 did not generate CTL, thus suggesting the importance of the in vivo priming and boosting event. This experiment has been repeated and similar results obtained.

In another experiment, effector cells obtained from Balb/c mice immunized, boosted and restimulated in vitro with a different H-2^d HIV-env-expressing tumor cell clone (L33-41) infected with the same pAF env^r SV40 Neo (HIV-env) vector construct were capable of lysing B/C10MEenv-29 target cells. This provides additional support that the CTL generated in these mice are specifically recognizing an expressed form of HIV-env rather than simply a unique tumor cell antigen on these cells. This result also suggests that the vector-delivered antigen is presented in a similar manner by the two tumor cell lines.

Implementation of this immunostimulant application in humans requires that (1) the gene coding for the antigen of interest be delivered to cells,

(2) the antigen be expressed in appropriate cells, and (3) MHC restriction requirements, i.e., class I and class II antigen interaction, are satisfied. Preparations of vector are made by growing the producer cells in normal medium, washing the cells with PBS plus human serum albumin (HSA) at 10 mg/ml, then growing the cells for 8-16 hours in PBS plus HSA. Titers obtained are typically 10^4 to 10^6/ml depending on the vector, packaging line or particular producer line clone. The vector supernatants are filtered to remove cells and are concentrated up to 100-fold by filtration through 100,000 or 300,000 pass AMICON trade mark filters (Wolff et al., Proc. Natl. Acad. Sci 84:3344, 1987). This lets globular proteins of 100,000 or 300,000pass but retains 99% of the viral vector as infectious particles. The stocks can be frozen for storage since they lose about 50% of the infectious units on freezing and thawing. The most direct delivery involves administration of the appropriate gene-carrying vector into the individual and reliance upon the ability of the vector to efficiently target to the appropriate cells, which can then initiate stimulation of the immune response. The dose would be 10^5 - 10^6 infectious units/kg body weight. However, a more practical approach may involve the extracorporeal treatment of patient peripheral blood lymphocytes (PBL), fibroblasts or other cells obtained from each individual with the vector. PBL could be maintained in culture through the use of mitogens (phytohemagglutinin) or lymphokines (e.g., IL-2). This type of approach allows for directed vector infection, monitoring of expression and expansion of the antigen presenting cell population prior to

injection, and return of vector-expressing cells to the respective patient. Other types of cells could also be explanted, vector introduced, and the cells returned to the patient. Only a moderate number of infected cells (10^5 - 10^6/kg body weight) is necessary to elicit strong immune responses.

A different form of administration is the implantation of producer lines making retroviral vector particles. These may be immunologically unmatched classical producer cell lines or the patients own cells, which have been explanted, treated and returned (see VI Alternative Viral Vector Packaging Techniques, below). Both types of implants (10^5 - 10^6/kg body weight) would have a limited life span in the patient, but would lead to the retroviral vector infecting large numbers (10^7 - 10^{10}) of cells in their vicinity in the body.

In any case, the success of the treatment can be assayed by removing a small amount of blood and measuring the CTL response using as targets the individual's own cells infected with vector leading to env expression.

When it is desired to stimulate an MHC class I or class II restricted immune response to pathogens, including pathogenic viruses other than HIV, suitable forms of envelope or other antigens associated with such retroviruses which will stimulate an immune response can be ascertained by one skilled in the art. In general, suitable forms of antigens associated with pathogenic agents can readily be selected which will stimulate an immune response to those pathogenic agents.

An alternative approach to creating a desired immune response is to deliver an antigen-specific T-cell receptor gene to an appropriate cell, such as a T-cell. Another possible recipient is NK cells where the cytotoxic immune response is not MHC limited. A specific immunoglobulin gene could similarly be useful if delivered to a B-cell.

II. Blocking Agents

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly

enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by producing, in vivo, an analogue to either of the partners in an interaction.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a retroviral vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability. Delivery of a retroviral vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env may also be constructed as described above. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., J. Virol. 62:139, 1988; Fisher et al., Science 233:655, 1986). The following example describes the construction of a

CD4 vector from which infectious vector particles were made.

EXAMPLE 3

sCD4 Vector

1. A 1.7 kb Eco R1 - Hind III DNA fragment from pMV7.T4 (Maddon et al., Cell 47:333, 1986) was blunt-end ligated to the Hinc II site of Sk^+.

2. A universal translation termination sequence containing an Xba I site was inserted into the Nhe I site of the CD4 fragment.

3. The 1.7 kb Xho I-Cla I fragment was excised and cloned into the Xho I - Cla I site of pXFVXM. These vector plasmids can be used to generate infectious vector particles, as described in Example 1.

Such infectious blocking vectors, when put into human T-cell lines in culture can inhibit the spread of HIV infections. Preparation, concentration and storage of infectious retroviral vector preparations is as for the immunostimulant. Route of administration would also be the same, with doses about 10-fold higher. Another route which may be used is the aspiration of bone marrow, infection with retroviral vector and return of this infected marrow (Gruber et al., Science 230:1057, 1985) to the patient. Since the marrow replication will amplify the vector expression through cell replication, doses in the range of the immunostimulant can be used (10^5 - 10^6/kg body weight).

In any case, the efficacy of the treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of non-specific infections.

III. Expression of Palliatives

Techniques similar to those described above can be used to produce recombinant retroviruses with vector constructs which direct the expression of an

agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (e.g., contact inhibition), and lack of production of anti-oncogene proteins.

(i) Inhibitor Palliatives

In one aspect of the present invention, the vector construct directs the expression of antisense RNA (or complementary RNA) to RNA of a pathogenic virus, such as HIV (or a pathogenic gene, such as an oncogene), to thereby inhibit its replication or pathogenesis. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated with the pathogenic condition (an "identifying agent"). Alternatively, the expression may be tissue-specific due either to targeting of vector entry or to tissue-specific control sequences in the vector.

In one embodiment, retroviral viruses which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the vector would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the retroviral RNA genome.

In a third embodiment, a retroviral vector may be introduced which expresses a protein that interferes with the pathogenic state. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu^5" mutant; see Wachsman et al., Science 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., Nature 335:452, 1988).

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected for. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not

caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" retroviral vector (i.e., one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

In a fourth embodiment, the HSVTK gene product is used to more effectively metabolize potentially antiviral nucleoside analogues, such as AZT or ddC. The HSVTK gene is expressed under the control of a constitutive macrophage or T-cell-specific promoter and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase and thus HIV replication (Furmam et al., Proc. Natl. Acad. Sci. USA 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleotide and nucleotide phosphate kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active mucleotide triphosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious. A description of this method is set forth in Example 4.

EXAMPLE 4

Vectors Designed to Potentiate the Antiviral Effect of AZT and Analogues

I. All of the following retroviral vectors are based on the "N2" vector (see Keller et al., Nature 318:149-154, 1985). Consequently, 5' and 3' Eco R1 LTR fragments (2.8 and 1.0 kb, respectively) were initially subcloned into plasmids containing polylinkers (into SK+ to give pN2R5[+/=]; into pUC31 to give p31N2R5[+/-] and p31N2R3[+/-] to facilitate vector construction. In one case, a 1.2 kb Cla I/Eco R1 5' LTR fragment was subcloned into the same sites of an SK+ vector to give pN2C. In another case, the 5' LTR containing a 6 bp deletion of the splice donor sequence was subcloned as a 1.8 kb Eco R1 fragment into pUC31 (p31N25delta[+]). The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene was isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK (3.5 kb Bam H1 fragment of HSVTK cloned into Bam H1 of pBR322) and cloned into Bgl II/Sma I-digested pUC31 (pUCTK). For constructs which require deletion of the terminator signals, pUCTK was digested with Sma I and Bam H1. The remaining coding sequences and sticky-end Bam H1 overhang were reconstituted with a double-stranded oligonucleotide made from the following oligomers:

5' GAG AGA TGG GGG AGG CTA ACT GAG 3'

and 5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3'

forming the construct pTK delta A.

For diagnostic purposes, the oligos were designed to destroy the Sma I site while keeping its Ava I site without changing the translated protein.

The 0.6 kb HIV promoter sequences were cloned as a Dra I/Hind III fragment from pCV-1 (see Arya et al., Science 229:69-73, 1985) into Hinc II/Hind III-cut SK^+ (SKHL).

A. Construction of TK-1 and TK-3 retroviral vectors (see Figure 6).

1. The 5 kb Xho I/Hind III 5′ LTR and plasmid sequences were isolated from p31N2R5(+).

2. HSVTK coding sequences lacking transcriptional termination sequences were isolated as 1.2 kb Xho I/Bam H1 fragment from pTKdeltaA.

3. 3′ LTR sequences were isolated as a 1.0 kb Bam H1/Hind III fragment from pN2R3(-).

4. The fragments from steps 1-3 were mixed, ligated, transformed into bacteria, and individual clones identified by restriction enzyme analysis (TK-1).

5. TK-3 was constructed by linearizing TK-1 with Bam H1, filling in the 5′ overhang and blunt-end ligating a 5′-filled Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, plus Tn5 Neo^r gene. Kanamycin-resistant clones were isolated and individual clones were screened for the proper orientation by restriction enzyme analysis.

These constructs were used to generate infectious recombinant vector particles in conjunction with a packaging cell line, such as PA317, as described above.

Administration of these retroviral vectors to human T-cell and macrophage/monocyte cell lines can increase their resistance to HIV in the presence of AZT and ddc compared to the same cells without retroviral vector treatment.

Preparation, concentration and storage of the retroviral vector preparations would be as described above. Treatment would be as previously described but ex corpore treatment of patients' cells would aim for uninfected potentially susceptible T-cells or monocytes. One preferred method of targeting the susceptible cell is with vectors which carry HIV env or hybrid env (see Section VIII Cell Line Specific Retroviruses, below) to direct absorption of vector particles to $CD4^+$ cells.

Normal adults have about 5 x 10^9 T4 cells in their total blood and about the same number of monocytes.

A preferred method of administration would be leukophoresis, where about 20% of an individual's PBLs can be removed at any one time and manipulated in vitro. Thus, about 2 x 10^9 cells would be treated and replaced. Since the current maximum titres are around 10^6/ml, this would require 2 to 20 liters of starting viral supernatant. Repeat treatments would be performed. Alternatively, bone marrow would be treated and allowed to amplify the effect as described above. Treatment with AZT would be at lower than normal levels to avoid toxic side effects, but still efficiently inhibit spread of HIV. The course of the treatment would be followed as described for the blocker.

A fifth embodiment for producing inhibitor palliatives involves the expression of defective interfering viral structural proteins, which inhibit viral assembly. Vectors would code for defective gag, pol, env or other viral particle proteins or peptides, and these would inhibit in a dominant fashion the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

A sixth such embodiment involves the expression of inhibiting peptides specific for viral protease. Viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Vectors to inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

A seventh embodiment involves the delivery of supressor genes which, when deleted or not expressed in

a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Examples of such cancers are retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the retroviral delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In an eighth embodiment, the retroviral construct provides a therapeutic effect by inserting itself into a virus, oncogene, or pathogenic gene, thereby inhibiting a function required for pathogenesis. This embodiment requires the direction of retroviral integration to a specific site in the genome by homologous recombination, integrase modification, or other methods (described below).

(ii) Conditional Toxic Palliatives

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the proviral vector should be limited by the presence of some intracellular signal identifying the pathogenic state in order to avoid destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection by targeting recombinant retrovirus carrying the vector to cells having or being susceptible to the pathogenic condition.

In one embodiment of this method, a recombinant retrovirus (preferably, but not necessarily, a recombinant MLV retrovirus) carries a vector construct containing a cytotoxic gene (such as ricin) expressed from an event-specific promoter, such as a cell cycle-dependent promoter (e.g., human cellular thymidine kinase or transferrin receptor promoters), which will be

transcriptionally active only in rapidly proliferating cells, such as tumors. In this manner, rapidly replicating cells, which contain factors capable of activating transcription from these promoters, are preferentially destroyed by the cytotoxic agent produced by the proviral construct.

In a second embodiment, the gene producing the cytotoxic agent is under control of a tissue-specific promoter, where the tissue specificity corresponds to the origin of the tumor. Since the viral vector preferentially integrates into the genome of replicating cells (for example, normal liver cells are not replicating, while those of a hepatocarcinoma are), these two levels of specificity (viral integration/replication and tissue-specific transcriptional regulation) lead to preferential killing of tumor cells. Additionally, event-specific and tissue-specific promoter elements may be artificially combined such that the cytotoxic gene product is expressed only in cell types satisfying both criteria (e.g., in the example above, combined promoter elements are functional only in rapidly dividing liver cells). Transcriptional control elements may also be amplified to increase the stringency of cell-type specificity.

These transcriptional promoter/enhancer elements need not necessarily be present as an internal promoter (lying between the viral LTRs) but may be added to or replace the transcriptional control elements in the viral LTRs which are themselves transcriptional promoters, such that condition-specific transcriptional expression will occur directly from the modified viral LTR. In this case, either the condition for maximal expression will need to be mimicked in retroviral packaging cell lines (e.g., by altering growth conditions, supplying necessary transregulators of expression or using the appropriate cell line as a parent for a packaging line), or the LTR modification is

limited to the 3' LTR U3 region, to obtain maximal recombinant viral titers. In the latter case, after one round of infection/integration the 3' LTR U3 is now also the 5' LTR U3 giving the desired tissue specific expression.

In a third embodiment, the proviral vector construct is similarly activated but expresses a protein which is not itself cytotoxic, and which processes within the target cells a compound or a drug with little or no cytotoxicity into one which is cytotoxic (a "conditionally lethal" gene product). Specifically, the proviral vector construct carries the herpes simplex virus thymidine kinase ("HSVTK") gene downstream and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Expression of the tat gene product in human cells infected with HIV and carrying the proviral vector construct causes increased production of HSVTK. The cells (either in vitro or in vivo) are then exposed to a drug such as acyclovir or its analogues (FIAU, FIAC, DHPG). These drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms (see, for example, Schaeffer et al., Nature 272:583, 1978). Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., Proc. Natl. Acad. Sci. USA 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs. In addition, an extra level of specificity is achieved by including in the vector the HIV rev protein, responsive CRS/CAR sequences. In the presence of the CRS sequence gene expression is suppressed, except in the presence of

the CAR sequences and the rev protein. Example 5 provides an illustration of this technique.

EXAMPLE 5

Vector to Conditionally Potentiate the Toxic Action of ACV or Its Analogues

Construction of Vectors

A. Construction of pKTVIHAX (see Figure 7).

1. A 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

2. A 0.6 kb Xho I/Bam H1 promoter fragment was isolated from plasmid pSKHL.

3. A 0.3 kb Bg lII/Acc I and a 1.5 kb Acc I/Acc I fragment were purified from pUCTK.

4. The fragments from 1, 2, and 3 were ligated, transformed into bacteria, and appropriate Amp^r clones of the given structure identified by restriction enzyme analysis.

B. Construction of pKTVIH-5 and pKTVIH5 Neo retroviral vectors (see Figure 8).

1. 4.5 kb 5′ LTR and vector was isolated as an Xho I/Bam H1 fragment from vector p31N25delta(+).

2. The 1.0 kb 3′ LTR was isolated as an Apa I/Bam H1 fragment from pN2R3(+) fragment.

3. The 0.6 kb HIV promoter element was isolated from pSKHL as an Apa I/Eco R1 fragment.

4. The HSVTK coding sequence and transcriptional termination sequences were isolated as 1.8 kb Eco R1/Sal I fragment from pUCTK.

5. The fragments from 1-4 were combined, ligated, transformed into bacteria, and clones of the given structure were identified by restriction enzyme analysis (pKTVIH-5).

6. pKTVIH5 Neo was constructed by linearizing pKTVIH5 with Cla I; mixing with a 1.8 kb Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, and Tn5 Neo^r marker,

ligating, transforming bacteria and selecting for kanamycin resistance. Clones with the insert in the indicated orientation were identified by restriction analysis.

C. Construction of MHMTK Neo retroviral vector (see Figure 9).

1. Construction of intermediate plasmid MHM-1:

a) plasmid pN2CR5 was linearized by partial digestion with Fok I, the 5′ overhang filled in with deoxynucleotide triphosphates using Klenow DNA polymerase, and Hind III linkers inserted. After transformation into bacteria, a clone with a Hind III linker inserted in the MLV LTR Fok I site was identified by restriction enzyme analysis (pN2CR5FH).

b) pN2CR5FH was linearized with Nhe I, the 5′ overhang filled in with Klenow polymerase digested with Hind III, and the 4.3 kb fragment with promoterless MLV sequences isolated.

c) 0.5 kb Eco RV/Hind III HIV promoter sequences were isolated from pSKHL.

d) b and c were mixed, ligated, used to transform bacteria, and the structure of MHM-1 was confirmed by restriction enzyme analysis.

2. A 0.7 kb Eco RV/Bal I fragment isolated from MHM-1 was subcloned into the Eco RV site of plasmid I30B (a modified IBI30 plasmid containing additional Bgl II, Bst II, Neo I and Nde I sites in the polylinker). After transformation into bacteria, clones with the appropriate orientation were identified by restriction enzyme analysis (pMHMB).

3. pMHMB was digested with Apa I and Xho I and gel purified.

4. MHM-1 was digested with Apa I/Bam H1 and the 1.8 kb MHMLTR/leader sequence gel purified.

5. A 2.8 kb Bgl II/Sal I fragment containing the HSVTK coding region upstream of the SV40 early promoter driving Neo^r taken from pTK-3 (see Figure 3).

6. 3-5 were mixed, ligated, used to transform bacteria, and appropriate clones were identified by restriction enzyme analysis.

D. Construction of tat and anti-tat expression vectors (see Figure 10).

These vectors are used as pseudo-HIV to test-activate tat-dependent HSVTK vectors.

1. The His^r expression vector pBamHis was linearized with Bam H1 and treated with calf intestinal phosphatase.

2. The Sac I site of pCV-1 was mutagenized to a Bam H1 site and the 350 bp Bam H1 coding sequence of HIV tat was isolated.

3. The fragments purified in steps 1 and 2 were mixed, ligated, used to transform bacteria, and clones with tat in both orientations were identified by restriction enzyme analysis.

These constructs were used to generate infectious recombinant vector particles in conjunction with a packaging cell line such as PA317, as described above.

The biological properties of these retroviral vectors are described hereinafter. The HIV tat gene ("tathis" vector -- see Figure 10) was transfected into mouse PA317 cells. Five individual histidinol-resistant subclones were obtained (TH 1-5) which express HIV tat. These cells are thus an experimental model for HIV infection. The vectors KTVIHAX, KTVIH5NEO, and MHMTKNEO, were subsequently introduced by infection into these tat-expressing cell lines as well as their parent cell line lacking tat. Cell viability was then determined in various concentrations of the HSVTK-specific cytotoxic drug, acyclovir. The data are reported here as LD50 (the drug concentration at which

50% toxicity is observed). The parental cell line containing the vector but lacking tat (non-HIV-infected model) showed no detectable toxicity by ACV at the concentrations tested (see Figure 11). These cells thus require 100 uM ACV or greater for cytotoxicity. This is true also for these cells lacking the vectors. Thus the vectors alone, ACV alone, or even the vector +ACV (solid boxes) is not cytotoxic. However, cell lines which express HIV tat (the experimental representation of an HIV infection) are effectively killed by ACV. This is true to varying degrees for all three vectors tested. These data indicate that HIV-infected cells will be killed in the presence of ACV and "potentiator" vectors.

Similarly, HIV infection of human T-cell lines +/- FIAU shower preferential inhibition (through cell killing) of HIV infection. Cultures were first treated with vector, then challenged with low multiplicity HIV infection for 4 days. Viral supernatants were titred using the HIV, as described in Section IV.

In the case of HIV-infected cells, expression of the conditionally lethal HSVTK gene was made even more HIV specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., Proc. Natl. Acad. Sci. USA 85:2071, 1988). More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (i.e., post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (i.e., rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) could result in even greater specificity. It should be noted that this kind of conditional activation of an inactive precursor into an active product in cells may also be achieved using

before or by ex corpore treatment of PBL and/or bone

much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

(iii) Immune Down-Regulation

Specific down-regulation of inappropriate or unwanted immune response, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using anti-MHC class I genes, such as immune-suppressive viral genes. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells and prevents terminal glycosylation and translation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with gp 19-encoding vector constructs which upon expression of the gp 19 inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with

fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythromiatis, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

IV. Expression of Markers

The above-described technique of expressing a palliative in a cell, in response to some identifying agent, can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), and carrying a promoter, which responds to the presence of the identifying protein in indicator cells, by switching expression of the reporting product between expressing and nonexpressing states. For example, HIV, when it infects suitable indicator cells, makes tat and rev. The indicator cells can thus be provided with a genome (such as by infection with an appropriate recombinant retrovirus) which codes for a marker gene, such as the alkaline phosphatase gene, β-galactosidase gene or the luciferase gene, and a promoter, such as the HIV promoter, which controls expression of the marker gene. When the indicator cells are exposed to a clinical sample to be tested, and the sample contains HIV, the indicator cells become infected with HIV, resulting in tat and/or rev expression (an identifying protein) therein. The HIV expression controls in the indicator cells would then respond to tat and/or rev proteins by switching expression of genes encoding β-

galactosidase, luciferase, or alkaline phosphatase (marker products) from normally "off" to "on." In the case of β-galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is positive for HIV. For intracellular enzymes such as β-galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making cell extracts and performing a suitable assay. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of this marker system can be used for different purposes. These include counting active virus or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and positive where there were no antibodies, this would assist in confirming the presence of HIV.

Within an analogous system for an <u>in vitro</u> assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable retroviral vector which carries the reporter gene linked to the expression controls of the virus of interest. The reporter gene, after entering the sample

cells, will express its reporting product (such as β-galactosidase or luciferase) only if the host cell expresses the appropriate viral proteins.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of reporting product than identifying agent present, which results in an amplification effect. Example 6 describes a representative technique for detecting a gene which expresses an identifying protein.

EXAMPLE 6

HIV-Specific Marker System or Assay

A. Constructs

Reporter constructs under the control of the HIV expression system are shown in Figure 12 (a recombinant retroviral vector) and in Figure 13 (a simple plasmid used by transfection). The pieces of these preferred vector and plasmid reporters were derived as follows:

The retroviral backbone was derived from the construct pAFVXM (Krieger et al., Cell 38:384, 1984), which had been linearized using Xho I and Cla I. SV_2neo was obtained from the plasmid pKoneo (Hanahen, unpubl.) by isolation of the 1.8 kb Cla I fragment.

The HIV LTR was isolated as a 0.7 kb Hind III fragment from the plasmid pC15CAT (Arya et al., Science 229:69, 1985). Beta-gal was obtained from the plasmid pSP65 β-gal (Cepko, pers. comm.) as a Hind III-Sma I fragment. A secreted form of human placental alkaline phosphatase was produced by introduction of a universal terminator sequence after amino-acid 489 of the cell surface form of alkaline phosphatase (as described by Berger et al., Gene 66:1, 1988). The secreted alkaline phosphatase gene was isolated as a 1.8 kb Hind III to Kpn I fragment. The CRS-CAR sequences from HIV env were obtained by isolating the 2.1 kb Kpn I to Bam H1 fragment from HTLVIIIB/BH10R3 (Fisher et al., Science

233:655, 1986). This fragment was inserted into pUC31 linearized by Bam HI, and Kpn I pUC31 is pUC19 (Yanisch-Perron et al., Gene 33:103, 1985) with extra Xho I, B-gl II, Bssh II and Nco I sites between the Eco R1 and Kpn I sites of pUC19. The Bam H1 site of the resulting construct was converted to a Nco I site to allow resection of the CRS-CAR sequences by Nco I digestion. The SV40 t intron was obtained from pSVOL (de Wet et al., Mol. Cell. Biol. 7:725, 1987) as a 0.8 kb Nco I to Bam H1 fragment.

B. Indicator Cells and Retroviral Vectors

Human T-cell (H-9, CEM and Sup T1) and monocyte (U-937) cell lines were obtained from ATCC, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

The nonretroviral vectors were introduced into cell lines by electroporation using a Bio-Rad Gene Pulser. The cell lines were selected in G-418 (1 mg/ml) for 2-3 weeks to obtain stable $G\text{-}418_R$ cell lines, and then dilution cloned to obtain clonal cell lines.

The pAF vectors were transfected into the PA317 packaging cell line as a calcium phosphate precipitate (Wigler et al., Cell 16:777, 1979). The virus-producing PA317 cells were co-cultivated with human monocyte cell lines for 24 hours in the presence of polybrene, after which the suspension cells were removed and selected in G-418 and subcloned as above.

C. Assay

Stable cell lines were infected with HIV ($HTLV\ III_B$) and the cells (β-gal) or media (alkaline phosphatase) assayed on a daily basis for 6 days post-infection.

β-Galactosidase Assay

Infected cells could be assayed by either: (i) In situ histochemical staining as described by

MacGregor et al. Somatic Cell and Mol. Genetics 13:253, 1987); or (ii) by using cell extracts in a solution enzymatic assay with ONPG as a substrate (Norton and Coffin, Mol. Cel. Biol. 5:281, 1985).

Soluble Alkaline Phosphatase Assay

Medium was removed from infected cells, microfuged for 10 seconds, and then heated to 68°C for 10 minutes to destroy endogenous phosphatases. The medium was then microfuged for 2 minutes and an aliquot (10-50 µl of buffer (1 M diethanolamine, pH 9.8; 0.5 Mm $MgCl_2$; 10 mM L-homoarginine) was added and then 20 µl of 120 mM p-nitrophenylphosphate (in buffers) was added. The A_{405} of the reaction mixture was monitored using an automatic plate reader.

Figures 14 and 15 depict typical results of a time course of infection of Sup T1 cells using the alkaline phosphatase assay in the presence of varying concentrations of antiviral drugs. The "+" and "-" on day 6 indicate the presence of absence of syncytia.

A number of other techniques (described below) can be used with the retroviral vector systems employed above, so as to enhance their performance. Alternatively, these techniques may be used with other gene-delivery systems.

V. **Packaging Cell Selection**

This is based, in part, upon the discovery of the major causes of low recombinant virus titres from packaging cells, and of techniques to correct those causes. Basically, at least five factors may be postulated as causes for low recombinant virus titres:

1. the limited availability of viral packaging proteins;

2. the limited availability of retroviral vector RNA genomes;

3. the limited availability of cell membrane for budding of the recombinant retroviruses;

4. the limited intrinsic packaging efficiency of the retroviral vector genome; and

5. the density of the receptor specific for the envelope of a given retrovirus.

As noted above, the limited availability of viral packaging proteins is the initial limiting factor in recombinant retrovirus production from packaging cells. When the level of packaging protein in the packaging cells is increased, titre increases to about 10^5 infectious units/millilitre, following which increasing packaging protein level has not further effect on titres. However, titres can be further augmented by also increasing the level of retroviral vector genome available for packaging. Thus, as described herein, it is advantageous to select producer cells that manufacture the maximum levels of packaging proteins and retroviral vector genomes. It has been discovered that the methods of identifying and thus selecting, packaging cells and producer cells, described earlier under the section entitled "Background of the Invention" tend to lead to selection of many producer cells which produce low titres for the reasons described below.

The present method takes advantage of the previously disadvantageous fact that the protein expression level of a gene downstream from the 5' LTR or other promoter, and spaced therefrom by an intervening gene, is substantially less than if the intervening gene were absent. In the present method, the selectable gene is placed downstream from a gene of the packaging genome or the gene of interest carried by the vector construct, but is still transcribed under the control of the viral 5' LTR or other promoter without any splice donor or splice acceptor sites. This accomplishes two things. First, since the packaging genes or genes of

interest are now upstream with no intervening gene between themselves and the promoter, their corresponding proteins (packaging protein or protein of interest) will be expressed at a higher level (5- to 20-fold) than the selectable protein. Second, the selectable protein will be expressed on average at a lower level, with the distribution of level of expression shifting toward lower levels. In the case of the phleo^r protein, this shift in distribution is illustrated by the broken curve indicated in Figure 16. However, the selection level for resistance to phleomycin remains the same, so that only the top-end expressing cells survive. The levels of the packaging protein or of the protein of interest will still be proportional, only in this case, a higher level of selectable protein corresponds to a much higher level of packaging protein or protein of interest.

Preferably, the foregoing procedure is performed using a plasmid carrying one of the proviral gag/pol or env packaging genes, along with a first selectable gene. These cells are then screened for the cells producing the highest levels of protein by reaction with an antibody against env (or possibly gag/pol), a second fluorescent antibody, and then sorted on a fluorescence-activated cell sorter (FACS). Alternatively, other tests for protein level may be used. Subsequently, the procedure and screening are repeated using those selected cells, and the other of the gag/pol or env packaging genes. In this step, a second selectable gene (different from the first) would be required downstream from the packaging gene and the cells producing the largest amount of the second viral protein selected. The procedure and screening are then repeated using the surviving cells, with a plasmid carrying the proviral vector construct bearing the gene of interest and a third selectable gene, different from the first or second selectable gene. As a result of this procedure, cells producing high titres of the

desired recombinant retrovirus will be selected, and these can be cultured as required to supply recombinant retrovirus. In addition, gag and pol can be independently introduced and selected for.

Example 7 describes the construction of gag/pol and env plasmids designed to use these procedures.

EXAMPLE 7

Plasmids Designed to Make High Levels of Packaging Proteins (Figure 7)

1. The 2.7 kb Xba I fragment from pPAM (Miller et al., Mol. Cell. Biol. 5:431, 1985), which contains the amphotropic env segment, was cloned in pUC18 at the Xba I site, then removed with Hind III and Sma I. This fragment was cloned into the vector pRSV neo (Gorman et al., Mol. Cell. Biol. 2:1044, 1982; Southern et al., J. Mol. Appl. Genet. 1:327, 1982) cut with Hind III and Pvu II, to give pRSV env. A 0.7 kb Bam H1 to BstE II fragment from the plasmid pUT507 (Mulsant et al., Somat. Cell. Mol. Genet. 14:243, 1988) with the BstE II end filled in carries the phleo resistance coding sequence. The 4.2 kb Bam H1 to Xho I fragment, the contiguous 1.6 kb Xho I to Xba I (Xba I filled in) from RSVenv, and the phleo fragment were ligated to give pRSVenv-phleo.

2. A fragment from the Pst I site at nucleotide 563 of MLY (RNA Tumor Viruses, Vol. II, 1985, Cold Spring Harbor) to the Sca I site at 5870 was derived from pMLV-K (Miller et al., 1985, op. cit.) and cloned in the Pst I to Bam H1 (Bam H1 filled-in) fragment from p4aA8 (Jolly et al., Proc. Natl. Acad. Sci. USA 80:477, 1983) that has the SV40 promoter, the pBR322 ampicillin resistance and origin of replication and the SV40 poly A site. This gives pSVgp. pSVgpDHFR was made using the following fragments: the 3.6 kb Hind III to Sal I fragment from pSVgp containing the

SV40 promoter plus MLY gag and some pol sequences; the 2.1 kb Sal I to Sca I fragment from pMLV-K with the rest of the pol gene, the 3.2 kb Xba I (Xba I filled-in) to Pst I fragment from pF400 with the DHFR gene plus poly A site, pBR322 origin and half the ampicillin resistance gene; the 0.7 kb Pst I to Hind III fragment from pBR322 with the other half of the ampicillin resistance gene. This gives pSVgp-DHFR. All these constructs are shown in Figure 7. These plasmids can be transfected into 3T3 cells or other cells and high levels of gag, pol or env obtained.

An additional method for accomplishing selection is to use a gene selection in one round and its antisense in a subsequent round. For example, gag/pol may be introduced into an HPRT-deficient cell with the HPRT gene and selected for the presence of this gene using that media which requires HPRT for the salvage of purines. In the next round, the antisense to HPRT could be delivered downstream to env and the cell selected in 6 thioguanine for the HPRT-deficient phenotype. Large amounts of antisense HPRT would be required in order to inactivate the HPRT gene transcripts, assuming no reversion occurred.

Envelope Substitutions

The ability to express gagpol and env function separately allows for manipulation of these functions independently. A cell line that expresses ample amounts of gagpol can be used, for example, to address questions of titre with regard to env. One factor resulting in low titres is the density of appropriate receptor molecules on the target cell or tissue. Given that env expression is from a separate unit, a variety of envelope genes (requiring different receptor proteins), such as xenotropic, polytropic, or amphotropic envs from a variety of sources, can be tested for highest titres on a specific target tissue. Furthermore, envelopes

from nonmurine retrovirus sources can be used for pseudotyping a vector. In addition, hybrid envelopes (as described below) can be used in this system as well, to tailor the tropism (and effectively increase titres) of a retroviral vector. Conversely, a cell line that expresses ample amounts of a given envelope gene can be employed to address questions of titre with regard to gag and pol.

VI. Alternative Viral Vector Packaging Techniques

Two additional alternative systems can be used to produce recombinant retroviruses carrying the vector construct. Each of these systems takes advantage of the fact that the insect virus, baculovirus, and the mammalian viruses, vaccinia and adenovirus, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. For example, see Smith et al. (Mol. Cell. Biol. 3:12, 1983); Piccini et al. (Meth. Enzymology, 153:545, 1987); and Mansour et al. (Proc. Natl. Acad. Sci. USA 82:1359, 1985).

These viral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and, hence, could be adapted to make retroviral vector particles.

Adenovirus vectors are derived from nuclear replicating viruses and can be defective. Genes can be inserted into vectors and used to express proteins in mammalian cells either by in vitro construction (Ballay et al., EMBO J. 4:3861, 1985) or by recombination in cells (Thummel et al., J. Mol. Appl. Genetics 1:435, 1982).

One preferred method is to construct plasmids using the adenovirus Major Late Promoter (MLP) driving: (1) gag/pol, (2) env, (3) a modified viral vector construct. A modified viral vector construct is possible because the U3 region of the 5' LTR, which

contains the viral vector promoter, can be replaced by other promoter sequences (see, for example, Hartman, Nucl. Acids Res. 16:9345, 1988). This portion will be replaced after one round of reverse transcriptase by the U3 from the 3' LTR.

These plasmids can then be used to make adenovirus genomes in vitro (Ballay et al., op. cit.), and these transfected in 293 cells (a human cell line making adenovirus E1A protein), for which the adenoviral vectors are defective, to yield pure stocks of gag, pol, env and retroviral vector carried separately in defective adenovirus vectors. Since the titres of such vectors are typically 10^7-10^{11}/ml, these stocks can be used to infect tissue culture cells simultaneously at high multiplicity. The cells will then be programmed to produce retroviral proteins and retroviral vector genomes at high levels. Since the adenovirus vectors are defective, no large amounts of direct cell lysis will occur and retroviral vectors can be harvested from the cell supernatants.

In an alternative system (which is more truly extracellular), the following components are used:

1. gag/pol and env proteins made in the baculovirus system in a similar manner as described in Smith et al. (supra) (or in other protein production systems, such as yeast or E. coli);

2. viral vector RNA made in the known T7 or SP6 or other in vitro RNA-generating system (see, for example, Flamant and Sorge, J. Virol. 62:1827, 1988);

3. tRNA made as in (2) or purified from yeast or mammalian tissue culture cells;

4. liposomes (with embedded env protein); and

5. cell extract or purified necessary components (when identified) (typically from mouse cells) to provide env processing, and any or other necessary cell-derived functions.

Within this procedure (1), (2) and (3) are mixed, and then env protein, cell extract and pre-liposome mix (lipid in a suitable solvent) added. It may, however, be necessary to earlier embed the env protein in the liposomes prior to adding the resulting liposome-embedded env to the mixture of (1), (2), and (3). The mix is treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of the nascent viral particles with lipid plus embedded env protein in a manner similar to that for liposome encapsidation of pharmaceuticals, as described in Gould-Fogerite et al., Anal. Biochem. 148:15, 1985). This procedure allows the production of high titres of replication incompetent recombinant retroviruses without contamination with pathogenic retroviruses or replication-competent retroviruses.

VII. Cell Line-Specific Retroviruses - "Hybrid Envelope"

The host cell range specificity of a retrovirus is determined in part by the env gene products. For example, Coffin, J. (RNA Tumor Viruses 2:25-27, Cold Spring Harbor, 1985) notes that the extracellular component of the proteins from murine leukemia virus (MLV) and Rous Sarcoma virus (RSV) are responsible for specific receptor binding. The cytoplasmic domain of envelope proteins, on the other hand, are understood to play a role in virion formation. While pseudotyping (i.e., the encapsidation of viral RNA from one species by viral proteins of another species) does occur at a low frequency, the envelope protein has some specificity for virion formation of a given retrovirus. The present invention recognizes that by creating a hybrid env gene product (i.e., specifically, an env protein having cytoplasmic regions and exogenous binding regions which are not in the same protein molecule in nature) the host range specificity may be changed independently from the cytoplasmic function.

Thus, recombinant retroviruses can be produced which will specifically bind to preselected target cells.

In order to make a hybrid protein in which the receptor binding component and the cytoplasmic component are from different retroviruses, a preferred location for recombination is within the membrane-spanning region of the cytoplasmic component. Example 8 describes the construction of a hybrid env gene which expresses a protein with the CD4 binding portion of the HIV envelope protein coupled to the cytoplasmic domain of the MLV envelope protein.

EXAMPLE 8

Hybrid HIV-MLV Envelopes

A hybrid envelope gene is prepared using in vitro mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492, 1985) to introduce a new restriction site at an appropriate point of junction. Alternatively, if the two envelope sequences are on the same plasmid, they can be joined directly at any desired point using in vitro mutagenesis. The end result in either case is a hybrid gene containing the 5′ end of the HIV gp 160 and the 3′ end of MLB p15E. The hybrid protein expressed by the resulting recombinant gene is illustrated in Figure 18 and contains the HIV gp120 (CD4 receptor binding protein), the extracellular portion of HIV gp 41 (the gp 120 binding and fusigenic regions), and the cytoplasmic portion of MLV p15E, with the joint occurring at any of several points within the host membrane. A hybrid gene made by fusion at the new Eco R1 site shown in Figure 18 is expressed under control of the CMVIE gene in human Sup T1 cells and leads to syncytia, as is the case with the authentic HIV env gene. Thus the hybrid is correctly transported to the cell surface and displayed there.

While Example 8 illustrates one hybrid protein produced from two different retroviruses, the

possibilities are not limited to retroviruses or even simply other viruses. For example, the beta-receptor portion of human interleukin-2 may be combined with the envelope protein of MLV. In this case, a recombination would preferably be located in the gp 70 portion of the MLV env gene, leaving an intact p15E protein. Furthermore, the foregoing technique may be used to create a recombinant retrovirus with an envelope protein which recognizes antibody Fc segments. Monoclonal antibodies which recognize only preselected target cells only could then be bound to such a recombinant retrovirus exhibiting such envelope proteins so that the retrovirus would bind to and infect only those preselected target cells.

VIII. Site-Specific Integration

Targeting a retroviral vector to a predetermined locus on a chromosome increases the benefits of gene-delivery systems. A measure of safety is gained by direct integration to a "safe" spot on a chromosome, i.e., one that is proven to have no deleterious effects from the insertion of a vector. Another potential benefit is the ability to direct a gene to an "open" region of a chromosome, where its expression would be maximized. Two techniques for integrating retroviruses at specific sites are described below.

(i) Homologous Recombination

One technique for integrating an exogenous gene of a vector construct of a recombinant retrovirus into a specific site in a target cell's DNA employs homologous recombination. Plasmids containing sequences of DNA of greater than about 300 bp that are homologous to genomic sequences have been shown to interact (either by replacement or insertion) with those genomic sequences at a rate that is greater than 10^3-fold over a

specific interaction in the absence of such homology (see Thomas and Capecchi, Cell 51:503-12, 1987; and Doetscheman et al., Nature 330:576-78, 1987). It has been shown that an insertion event, or alternatively, a replacement event, may be driven by the specific design of the vector.

In order to employ homologous recombination in site-specific retroviral integration, a vector construct should be modified such that (a) homologous sequences (to the target cell's genome) are incorporated into the construct at an appropriate location; and (b) the normal mechanism of integration does not interfere with the targeting occurring due to homologous sequences. A preferred approach in this regard is to add homologous sequences (greater than about 300 bp) in the 3' LTR, downstream from the U3 inverted repeat. In this approach, the construct is initially made with a region of homology inserted in the 3' LTR at the Nhe 1 site in U3. Reverse transcription in the host cell will result in a duplication of the region of homology in the 5' LTR within 31 bp of the end of the inverted repeat (IR). Integration into the host genome will occur in the presence or absence of the normal integration mechanism. The gene in the vector may be expressed, whether from the LTR or from an internal promoter. This approach has the effect of placing a region of homology near a potential free end of the double-stranded retrovirus vector genome. Free ends are known to increase the frequency of homologous recombination by a factor of approximately 10. In this approach, it may be necessary to defeat the normal mechanism of integration, or to at least modify it to slow down the process, allowing time for homologous DNAs to line up. Whether this latter modification is required in a particular case can be readily ascertained by one skilled in the art.

(ii) Integrase Modification

Another technique for integrating a vector construct into specific, preselected sites of a target cell's genome involves integrase modification.

The retrovirus pol gene product is generally processed into four parts: (i) a protease which processes the viral gag and pol products; (ii) the reverse transcriptase; and (iii) RNase H, which degrades RNA of an RNA/DNA duplex; and (iv) the endonuclease or "integrase."

The general integrase structure has been analyzed by Johnson et al. (Proc. Natl. Acad. Sci. USA 83:7648-7652, 1986). It has been proposed that this protein has a zinc binding finger with which it interacts with the host DNA before integrating the retroviral sequences.

In other proteins, such "fingers" allow the protein to bind to DNA at particular sequences. One illustrative example is the steroid receptors. In this case, one can make the estrogen receptor, responding to estrogens, have the effect of a glucocorticoid receptor, responding to glucocorticoids, simply by substituting the glucocorticoid receptor "finger" (i.e., DNA binding segment) in place of the estrogen receptor finger segment in the estrogen receptor gene. In this example, the position in the genome to which the proteins are targeted has been changed. Such directing sequences can also be substituted into the integrase gene in place of the present zinc finger. For instance, the segment coding for the DNA binding region of the human estrogen receptor gene may be substituted in place of the DNA binding region of the integrase in a packaging genome. Initially, specific integration would be tested by means of an in vitro integration system (Brown et al., Cell 29:347-356, 1987). To confirm that the specificity would be seen in vivo, this packaging genome is used to make infectious vector particles, and infection of and

integration into estrogen-sensitive and estrogen-nonsensitive cells compared in culture.

Through use of this technique, incoming viral vectors may be directed to integrate into preselected sites on the target cell's genome, dictated by the genome-binding properties of site-specific DNA-binding protein segments spliced into the integrase genome. It will be understood by those skilled in the art that the integration site must, in fact, be receptive to the fingers of the modified integrase. For example, most cells are sensitive to glucocorticoids and hence their chromatin has sites for glucocorticoid receptors. Thus, for most cells, a modified integrase having a glucocorticoid receptor finger would be suitable to integrate the proviral vector construct at those glucocorticoid receptor-binding sites.

IX. Production of Recombinant Retroviral Vectors in Transgenic Animals

Two problems previously described with helper line generation of retroviral vectors are: (a) difficulty in generating large quantities of vectors; and (b) the current need to use permanent instead of primary cells to make vectors. These problems can be overcome with producer or packaging lines that are generated in transgenic animals. These animals would carry the packaging genomes and retroviral vector genomes. Current technology does not allow the generation of packaging cell lines and desired vector-producing lines in primary cells due to their limited life span. The current technology is such that extensive characterization is necessary, which eliminates the use of primary cells because of senescence. However, individual lines of transgenic animals can be generated by the methods provided herein which produce the packaging functions, such as gag, pol or env. These lines of animals are then characterized

for expression in either the whole animal or targeted tissue through the selective use of housekeeping or tissue-specific promoters to transcribe the packaging functions. The vector to be delivered is also inserted into a line of transgenic animals with a tissue-specific or housekeeping promoter. As discussed above, the vector can be driven off such a promoter substituting for the U3 region of the 5' LTR (Figure 19). This transgene could be inducible or ubiquitous in its expression. This vector, however, is not packaged. These lines of animals are then mated to the gag/pol/env animal and subsequent progeny produce packaged vector. The progeny, which are essentially identical, are characterized and offer an unlimited source of primary producing cells. Alternatively, primary cells making gag/pol and env and derived from transgenic animals can be infected or transfected in bulk with retrovirus vectors to make a primary cell producer line. Many different transgenic animals or insects could produce these vectors, such as mice, rats, chickens, swine, rabbits, cows, sheep, fish and flies. The vector and packaging genomes would be tailored for species infection specificity and tissue-specific expression through the use of tissue-specific promoters and different envelope proteins. An example of such a procedure is illustrated in Figure 20.

Although the following examples of transgenic production of primary packaging lines are described only for mice, these procedures could be extended to other species by those skilled in the art. Given the homology to MLV sequences in mice genome, the final preferred animals would not be mice.

EXAMPLE 9

Production of Gag-Pol Proteins Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

An example of a well-characterized housekeeping promoter is the HPRT promoter. HPRT is a purine salvage enzyme which expresses in all tissues. (See Patel et al., Mol. Cell Biol. 6:4__-403, 1986 and Melton et al., Proc. Natl. Acad. Sci. 81:2147-2151, 1984). This promoter would be inserted in front of various gag/pol fragments (e.g., Bal I/Sca I; Aat II/Sca I; Pst I/Sca I of MoMLV; see RNA Tumor Viruses 2, 1985, Cold Spring Harbor Laboratory, 1985) that are cloned in Bluescript plasmids (Strategene, Inc.) using recombinant DNA techniques (see Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982). The resulting plasmids are purified (Maniatis et al., op. cit.) and the relevant genetic information isolated using Geneclean (Bio 101) or electroelution (see Hogan et al.(Eds.) Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, 1986).

These fully characterized DNAs would be microinjected in the pronucleus of fertilized mouse ova at a concentration of 2 ug/ml. Live born mice would be screened by tail blot analyses (see Hogan et al., op. cit.). Transgenic-positive animals would be characterized for expression levels of gag-pol proteins by immunoprecipitation of radiolabeled primary cells, such as fibroblast (see Harlow et al., eds. Antibodies: A Laboratory Manual, Cold Spring Harbor, 1988). Animals would then be bred to homozygosity for establishment of animal lines that produce characterized levels of gag-pol.

EXAMPLE 10

Production of env Proteins/Hybrid Envelope Proteins Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This example utilizes the HPRT promoter for expression of either envelope or hybrid envelope proteins. The envelope proteins could be from any retrovirus that is capable of complementing the relevant gag-pol, in this case that of MLV. Examples are ecotropic MLV, amphotropic MLV, xenotropic MLV, polytropic MLV, or hybrid envelopes. As above, the envelope gene would be cloned behind the HPRT promoter using recombinant DNA techniques (see Maniatis et al., op. cit.). The resulting "minigene" would be isolated (see Hogan et al., op. cit.), and expression of envelope protein would be determined (Harlow et al., op. cit.). The transgenic envelope animals would be bred to homozygosity to establish a well-characterized envelope animal.

EXAMPLE 11

Production of gag-pol-env Animals Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This would use the well-characterized gag-pol animals, as well as the animals for the establishment of a permanent gag-pol/envelope animal line. This would involve breeding to homozygosity and the establishment of a well-characterized line. These lines would then be used to establish primary mouse embryo lines that could be used for packaging vectors in tissue culture. Furthermore, animals containing the retroviral vector could be bred into this line.

EXAMPLE 12

Production of Tissue-Specific Expression of gag-pol-env or Hybrid Envelope in Transgenic Animals

The example given here is to direct tissue expression of the gagpol, envelope, or hybrid envelope to specific tissues, such as T-cells. This involves the use of CD2 sequences (see Lang et al., EMBO J. 7:1675-1682, 1988) that give position and copy number independence. The 1.5 kb Bam Hl/Hind III fragment from the CD2 gene would be inserted in front of gag-pol, envelope, or hybrid envelope fragments using recombinant DNA techniques. These genes would be inserted into fertilized mouse ova by microinjection. Transgenic animals would be characterized as before. Expression in T-cells would be established. Animals would be bred to homozygosity to establish well-characterized lines of transgenic animals. Gag-pol animals would be mated to envelope animals to establish gag-pol-env animals expressing only in T-cells. The T-cells of these animals would then be a source for T-cells capable of packaging retroviral vectors. Again, vector animals could be bred into these gag-pol-env animals to establish T-cells expressing the vector.

This technique allows the use of other tissue-specific promoters, such as milk-specific (whey), pancreatic (insulin or elastase), or neuronal (myelin basic protein) promoters. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the progeny.

EXAMPLE 13

Production of Either Housekeeping or Tissue-Specific Retroviral Vectors in Transgenic Animals

The insertion of retroviruses or retroviral vectors into the germ line of transgenic animals results

in little or no expression. This effect, described by Jaenisch (see Jahner et al., Nature 298:623-628, 1982), is attributed to methylation of 5' retroviral LTR sequences. This technique would overcome the methylation effect by substituting either a housekeeping or tissue-specific promoter to express the retroviral vector/retrovirus. The U3 region of the 5' LTR, which contains the enhancer elements, is replaced with regulatory sequences from housekeeping or tissue-specific promoters (see Figure 20). The 3' LTR is fully retained, as it contains sequences necessary for polyadenylation of the viral RNA and integration. As the result of unique properties of retroviral replication, the U3 region of the 5' LTR of the integrated provirus is generated by the U3 region of the 3' LTR of the infecting virus. Hence, the 3' is necessary, while the 5' U3 is dispensable. Substitution of the 5' LTR U3 sequences with promoters and insertion into the germ line of transgenic animals results in lines of animals capable of producing retroviral vector transcripts. These animals would then be mated to gag-pol-env animals to generate retroviral-producing animals (see Figure 20).

This application is a divisional application based on GB 9020586.5 (GB2236753A). Attention is also drawn to co-pending application No. 9217086.9.

* C L M S *

CLAIMS

1. A hybrid env gene useful for preparing a retrovirus which can selectively carry a gene of interest to a target cell, the env gene coding for:

(a) a cytoplasmic segment of a first retroviral phenotype; and

(b) a binding segment exogenous to the first retroviral phenotype, the binding segment being capable of selectively binding to the target cell.