IN INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 6: | (11) International Publication Number: |
| A61K 31/70, 38/00 | WO 95/15167 |

| (21) International Application Number: | (22) International Filing Date: |
| PCT/US94/13811 | 2 December 1994 (02.12.94) |

| (30) Priority Data: | (43) International Publication Date: |
| 08/161,754 | 8 June 1995 (08.06.95) |
| 3 December 1993 (03.12.93) | US |

| (81) Designated States: | Published |
| CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | With international search report. |

| (71) Applicant: | (72) Inventors: |

| (74) Agents: | |
| OLSTEIN, Elliot, M. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US). | |

| (54) Title: | (57) Abstract |
| IN UTERO GENE THERAPY FOR FETUSES | A process for effecting gene therapy in utero in a fetus, which comprises transducing fetal cells in vivo with at least one nucleic acid sequence encoding a therapeutic agent. The fetal cells may be transduced with a viral vector (such as a retroviral vector) which includes the nucleic acid sequence encoding the therapeutic agent. The viral vector may be contained in a viral supernatant which is administered to the fetus, or may be generated by a producer cell line which is administered to the fetus. |
### FOR THE PURPOSES OF INFORMATION ONLY

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

<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
<th>Code</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>Austria</td>
<td>GB</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GE</td>
<td>Georgia</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>GN</td>
<td>Guinea</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GR</td>
<td>Greece</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>HU</td>
<td>Hungary</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>IE</td>
<td>Ireland</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IT</td>
<td>Italy</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>JP</td>
<td>Japan</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
<td>KE</td>
<td>Kenya</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>KG</td>
<td>Kyrgyzstan</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>KP</td>
<td>Democratic People's Republic</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>of Korea</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>KR</td>
<td>Republic of Korea</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d'Ivoire</td>
<td>KZ</td>
<td>Kazakhstan</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>LI</td>
<td>Liechtenstein</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
<td>LK</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>CS</td>
<td>Czechoslovakia</td>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
<td>LV</td>
<td>Latvia</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>MD</td>
<td>Republic of Moldova</td>
</tr>
<tr>
<td>ES</td>
<td>Spain</td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>FI</td>
<td>Finland</td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>FR</td>
<td>France</td>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td>GA</td>
<td>Gabon</td>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td>MW</td>
<td>Malawi</td>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td>NL</td>
<td>Netherlands</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td>PT</td>
<td>Portugal</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>RU</td>
<td>Russian Federation</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>SE</td>
<td>Sweden</td>
<td>SI</td>
<td>Slovenia</td>
</tr>
<tr>
<td>SK</td>
<td>Slovakia</td>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>TD</td>
<td>Chad</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
<td>TT</td>
<td>Trinidad and Tobago</td>
</tr>
<tr>
<td>UA</td>
<td>Ukraine</td>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>UZ</td>
<td>Uzbekistan</td>
<td>VN</td>
<td>Viet Nam</td>
</tr>
</tbody>
</table>

**Note:** Codes are used for identification purposes and do not necessarily reflect the current status of States party to the PCT. The list includes traditional names only. For more information, refer to the World Intellectual Property Organization (WIPO) publications.
IN UTERO GENE THERAPY FOR FETUSES

This invention relates to gene therapy, and in particular to in utero gene therapy. More particularly, this invention relates to in vivo gene therapy for fetuses by administering to a fetus at least one nucleic acid sequence encoding a therapeutic agent.

Certain inherited metabolic diseases, such as Hurler’s syndrome, Lesch-Nyhan disease, Tay Sachs disease, and alpha-thalassemia, may produce irreversible damage to the fetus before birth. Infants born with other inherited diseases such as, for example, adenosine deaminase (ADA) deficiency, appear normal at birth; however, such diseases are manifested shortly thereafter. The above disorders, whether manifested before or after birth, could be treated if gene therapy could be accomplished safely in utero.

Kantoff, et al., Blood, Vol. 73, No. 4, pgs. 1066-1073 (March 1989), discloses the retroviral-mediated transfer of a neomycin resistance (neo<sup>8</sup>) gene into fetal sheep hematopoietic cells by exchange transfusion. In such a procedure, blood was obtained from a sheep fetus, and mononuclear cells were harvested. The cells then were transduced ex vivo with retroviral vectors including the neo<sup>8</sup> gene or a cDNA for human adenosine deaminase (ADA). The transduced cells then were reinfused into the sheep fetuses. After birth, the lambs were examined for presence
of a functioning neo<sup>k</sup> gene. Out of ten lambs analyzed, six were positive for G418 resistant hematopoietic-progenitor cells. One sheep had blood cells which expressed the neo<sup>k</sup> gene for more than two years after birth.

Such an exchange transfusion procedure, however, requires that one needs to wait until a period of time late in gestation in order for the fetus to be of a sufficient size in order to obtain sufficient blood cells to effect the gene transfer, and certain genetic diseases could be treated more successfully if gene transfer into fetal cells could be effected early in gestation. In addition, such a procedure requires multiple manipulations of the fetus, which increases the risk of damage to the fetus. Also, transduction takes place only in cells removed from the fetus.

It therefore is an object to the present invention to provide gene therapy for a fetus wherein genes encoding therapeutic agents may be transduced into fetal cells early in gestation, and to provide improved transduction of stem cells of the fetus.

In accordance with an aspect of the present invention, there is provided a process for effecting gene therapy in vivo in a fetus. The process comprises transducing fetal cells in vivo with at least one nucleic acid (DNA or RNA) sequence encoding a therapeutic agent.

The term "nucleic acid sequence" as used herein, means a DNA or RNA molecule, and includes complete and partial gene sequences, and includes polynucleotides as well. Such term also includes a linear series of deoxyribonucleotides or ribonucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of the adjacent pentoses.

The term "therapeutic" as used herein is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents.
The process of the present invention may be carried out during any stage of gestation, including the yolk sac stage. Such process may be applied to humans, wherein a human fetus may be injected intraperitoneally with a needle which is guided into the fetus by ultrasound, in a manner similar to that in which a fetus is given a blood transfusion for the treatment of alpha-thalassemia.

The nucleic acid sequence which encodes the therapeutic agent is contained in an appropriate expression vehicle which transduces the fetal cells. Such expression vectors include, but are not limited to, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial vectors), and viral vectors.

In one embodiment, the expression vector is a viral vector. Viral vectors which may be employed include, but are not limited to, retroviral vectors, adenovirus vectors, adeno-associated virus vectors, and Herpes virus vectors. Preferably, the viral vector is a retroviral vector.

In a preferred embodiment, a packaging cell line is transduced with a viral vector containing the nucleic acid sequence encoding the therapeutic agent to form a producer cell line which includes the viral vector. The producer cells then are administered in vivo to the fetus, whereby the producer cells generate viral particles capable of transducing fetal cells. Such fetal cells may be located throughout the body and include, but are not limited to, somatic and germinal cells, including bone marrow cells, including hematopoietic stem cells; peripheral blood cells; cells of the central nervous system, including brain cells; lung cells; kidney cells; testicular cells; ovarian cells; and liver cells.

In a preferred embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors...
derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. Preferably, the retroviral vector is an infectious but non-replication competent retrovirus. However, replication competent retroviruses may also be used.

Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

These new genes have been incorporated into the proviral backbone in several general ways. The most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter.

Efforts have been directed at minimizing the viral component of the viral backbone, largely in an effort to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging
cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

In one embodiment, the retroviral vector may be one of a series of vectors described in Bender, et al., J. Virol. 61:1639-1649 (1987), based on the N2 vector (Armentano, et al., J. Virol., 61:1647-1650) containing a series of deletions and substitutions to reduce to an absolute minimum the homology between the vector and packaging systems. These changes have also reduced the likelihood that viral proteins would be expressed. In the first of these vectors, LNL-XHC, there was altered, by site-directed mutagenesis, the natural ATG start codon of gag to TAG, thereby eliminating unintended protein synthesis from that point. In Moloney murine leukemia virus (MoMuLV), 5' to the authentic gag start, an open reading frame exists which permits expression of another glycosylated protein (pPr80\textsuperscript{\textregistered}). Moloney murine sarcoma virus (MoMuSV) has alterations in this 5' region, including a frameshift and loss of glycosylation sites, which obviate potential expression of the amino terminus of pPr80\textsuperscript{\textregistered}. Therefore, the vector LNL6 was made, which incorporated both the altered ATG of LNL-XHC and the 5' portion of MoMuSV. The 5' structure of the LN vector series thus eliminates the possibility of expression of retroviral reading frames, with the subsequent production of viral antigens in genetically transduced target cells. In a final alteration to reduce overlap with packaging-defective helper virus, Miller has eliminated extra env sequences immediately preceding the 3' LTR in the LN vector (Miller, et al., Biotechniques, 7:980-990, 1989).

The paramount need that must be satisfied by any gene transfer system for its application to gene therapy is safety. Safety is derived from the combination of vector genome structure together with the packaging system that is
utilized for production of the infectious vector. Miller, et al. have developed the combination of the pPAM3 plasmid (the packaging-defective helper genome) for expression of retroviral structural proteins together with the LN vector series to make a vector packaging system where the generation of recombinant wild-type retrovirus is reduced to a minimum through the elimination of nearly all sites of recombination between the vector genome and the packaging-defective helper genome (i.e. LN with pPAM3).

In one embodiment, the retroviral vector may be a Moloney Murine Leukemia Virus of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al. (1987) and Miller, et al. (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragments or truncations thereof, are not expressed.

In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral vector includes each of these cloning sites. Such vectors are further described in U.S. Patent Application Serial No. 919,062, filed July 23, 1992, and incorporated herein by reference.

When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from
the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites herein above described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vector then is employed to transduce a packaging cell line to form a producer cell line. Examples of
packaging cells which may be transfected include, but are not limited to, the PE501, PA317, -2, -AM, PA12, T19-14X, VT-19-17-H2, CRE, CRIP, GP+E-86, GP+envAm12, PAT 2.4, and DAN cell lines. Representative examples of packaging cell lines also are described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990). The vector containing the nucleic acid sequence encoding the therapeutic agent may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO₄ precipitation.

In one embodiment, a first packaging cell line, such as PE501, is transfected with the vector, and viral particles are generated. These infectious viral particles then are used to transfect a second packaging cell line, such as PA317, which generates an increased amount of viral particles.

The producer cells are then administered in vivo to the fetus in an amount effective to produce a therapeutic effect in the fetus. In general, the producer cells are administered to the fetus in an amount of from about 1 x 10⁴ cells to about 1 x 10⁶ cells, preferably from about 1 x 10⁷ cells to about 1 x 10⁸ cells, more preferably in an amount of from about 5 x 10⁷ cells to about 1 x 10⁸ cells. The producer cells may be administered to the fetus systemically, such as by intraperitoneal administration, intravenous administration, or by direct injection into an organ or muscle. The amount of producer cells to be administered is dependent upon various factors, including the disease to be treated and the extent and severity thereof.

The producer cells are administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid
carrier such as, for example, a saline solution or a buffer solution or other isomolar aqueous solution.

Upon administration of the producer cells to the fetus, the producer cells generate viral particles. The viral particles then transduce fetal cells, such as, for example, hematopoietic stem cells, cells of the central nervous system, and cells of other tissues and organs, whereby the transduced cells express the therapeutic agent.

In another alternative, a viral supernatant containing viral particles may be administered to the fetus, whereby such viral particles transduce fetal cells as hereinabove mentioned. The viral supernatant may be administered in an amount of from about $1 \times 10^3$ CFU to about $1 \times 10^9$ CFU, preferably from about $1 \times 10^4$ CFU to about $1 \times 10^8$ CFU.

Therapeutic agents which may be encoded by the at least one nucleic acid sequence include, but are not limited to, those which treat hematopoietic system deficiencies, immune deficiencies, lysosomal storage disorders, Lesch-Nyhan disease, and leukocyte adhesion deficiency. Specific examples of therapeutic agents include, but are not limited to, Factor VIII, for treating hemophilia A; Factor IX, for treating hemophilia B; FACC, for treating Fanconi anemia; $\alpha$-globin, for treating $\alpha$-thalassemia; $\beta$-globin, for treating $\beta$-thalassemia and sickle cell anemia; adenosine deaminase (ADA), and PNP, for treating severe combined immunodeficiency; the T-cell receptor $\alpha$-chain, for treating X-linked immunodeficiency; glucocerebrosidase, for treating Gaucher’s disease; iduronate sulfatase, for treating Hunter’s syndrome; $\alpha$-L-iduronidase, for treating Hurler’s syndrome, $\alpha$-galactosidase, for treating Fabry disease; the $\alpha$-subunit of hexosaminidase A, for treating Tay-Sachs disease; HPRT, for treating Lesch-Nyhan disease; and CD18 complex, for treating leukocyte adhesion deficiency. It is to be understood, however, that the scope of the present
invention is not to be limited to the above-mentioned therapeutic agents.

In addition, the process of the present invention may be applied to fetal animals in order to supply to the fetal animal therapeutic agents such as growth hormones, and agents which confer resistance to disease. In addition, the process of the present invention may be employed to provide a fetal animal with a gene encoding a human protein or therapeutic agent. For example, a fetal animal may be given a gene for human hemoglobin, which then may be harvested from the animal after birth to provide a human blood substitute.

Also, the process of the present invention may be employed to provide animal models for gene expression and gene therapy.

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Construction of pG1NaSvAd and generation of producer cells and viral supernatant therefrom

A. Construction of pG1NaSvAd

Plasmid pG1NaSvAd was derived from plasmid PG1 (Figure 3). Plasmid pG1 was constructed from pLNSX (Palmer, et al., Blood, Vol. 73, pgs. 438-445. The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6kb EcoRI fragment, containing the 5' Moloney Murine Sarcoma Virus (MoMuSV) LTR, and the 3.0kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used
to generate the vector plasmid pG1 (Figure 3) by the insertion of the 1.6kB EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 (Figure 3) consists of a retroviral vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender, et al. 1987), a 54 base pair multiple cloning site (MCS) containing, from 5' to 3' the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindII, ApaI, and ClaI and a 3' portion of MoMuLV from base pairs 7764 to 7813 (numbered as described (Van Beveren, et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985) (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo' gene, the β-galactosidase gene, the hygromycin' gene, and the SV40 promoter.

The "backbone" vector pG1Na was constructed from pG1 and pN2 (Armentano, et al., J. Virology, Vol. 61, pgs. 1647-1650 (1987)). pG1Na was constructed by cutting pN2 (Figure 4) with EcoRI and AsuII, filling in the ends of EcoRI/AsuII fragment containing the neo® gene, and ligating the fragment into SnaBI digested pG1 to form pG1Na (Figure 5).

An SvAd fragment containing the human adenosine deaminase (ADA) cDNA sequence (Adrian, et al., Mol. Cell. Biol., Vol. 4, 1712-1717(1984); Wiggman, et al., PNAS, Vol. 80, pg. 7481(1983); Wiggman, et al., Nucl. Acids Res., Vol. 12, Pg. 2439 (1984)) and promoted by an SV40 promoter (approximately 1.6 kb), was cut from pB2SA with EcoRI and blunt ended with Klenow. (SvAd is available from other vectors that are available to those skilled in the art. Preferably, it would be obtained from the vector SAX, described in Kantoff, et al., PNAS, Vol. 83, pgs. 6563-6567 (1986)) pG1Na was linearized 3' to the neo® gene by a double enzyme cut with SalI and Hind III, and blunt ended.
with Klenow. The SvAd fragment then was ligated into pG1Na to form pG1NaSvAd (Figure 7). A schematic of the construction of pG1NaSvAd is shown in Figure 6.

B. Generation of PA317/G1NaSvAd Producer Cell Line

A producer cell line was made from vector plasmid and packaging cells. The PA317/G1NaSvAd producer cell was made by the same techniques used to make previous clinically relevant retroviral vector producer cell lines. The vector plasmid pG1NaSvAd DNA was transfected into a ecotropic packaging cell line, PE501. Supernatant from the PE501 transfected cells was then used to transinfec the amphotropic, hTK containing, packaging cell line (PA317). Clones of transinfected producer cells were then grown in G418 containing medium to select clones that contain the Neo<sup>+</sup> gene. The clones were then titered for retroviral vector production. Several clones were then selected for further testing and finally a clone was selected for clinical use.

5 x 10<sup>5</sup> PE501 cells (Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989), incorporated herein by reference) were plated in 100 mm dishes with 10 ml high glucose Dulbecco’s Modified Essential Medium (DMEM) growth medium supplemented with 10% fetal bovine serum (HGD10) per dish (3-100 mm dishes are required per transfection). The cells were incubated at 37°C, in a 5% CO<sub>2</sub> atmosphere overnight.

The plasmid pG1NaSvAd then was transfected into PE501 cells by CaPO<sub>4</sub> precipitation using 50 µg of DNA by the following procedure.

50 µg of DNA, 50 µl 10 x CaCl<sub>2</sub>, and 450 µl of sterile H<sub>2</sub>O was mixed in a 15 ml polypropylene tube to yield a 0.25M Ca Cl<sub>2</sub> solution containing 50 µg DNA, 0.5 ml 2x PBS (containing 50 mM N-N-bis- (2-hydroxyethyl)- 2-aminoethane-sulfonic acid, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, and 50 mM Hepes, pH6.95), then was added to the tube and the contents of the
tube were mixed by pipetting. The DNA solution then was left at room temperature for about 20 minutes to 1 hour. 1ml of DNA solution then was added to each culture dish, and each dish was swirled to ensure even distribution of the DNA. The dishes then were incubated at 35°C in a 3% CO₂ atmosphere overnight.

A culture dish(es) with optimum precipitate following the overnight incubation then was selected. The medium/DNA precipitate was aspirated from the dish(es), and 5 mL PBS was added to each dish. The dish(es) was allowed to sit for 2 to 3 minutes to allow salts to dissolve.

The dish(es) then was washed again with PBS to remove the salt and the salt solution. 10 ml of HGD10 medium then was added to the dish(es), and the dish(es) incubated at 37°C in a 5% CO₂ atmosphere for about 48 hrs.

A 48 hour transient supernatant then was collected from the transfected cells by removing the supernatant from the cells and placing it in a 15 ml polypropylene tube. The dish(es) then was rinsed with 5 ml PBS. The PBS then was removed, and 1 ml trypsin-EDTA was added to each dish. Three 15 ml polypropylene tubes then were labeled undiluted, 1:10, and 1:100, respectively. 9 ml of HGD10 plus 0.8 mg/ml of G418 were added to the 1:10 and the 1:100 tubes.

When the cells were no longer adherent to the dish, 9 ml of HGD10 and 0.8 mg/ml of G418 were added to the undiluted tube, and the cells transferred to the undiluted tube.

Serial dilutions of the cells then were made by adding 1 ml of undiluted cells to the 1:10 tube, and then by adding 1 ml of the 1:10 cells to the 1:100 tube. The cells then were mixed.

10 ml of HGD10 and 0.8 mg/ml G418 were added to each of six 100 mm dishes. To one dish was added 0.5 ml of undiluted cells to make a 1:20 dilution of cells; to one
dish was added 0.25 ml of undiluted cells to make a 1:40 dilution of cells; to one dish was added 1.0 ml of the 1:10 dilution to make a 1:100 dilution of cells; to one dish was added 0.2 ml of the 1:10 dilution to make a 1:500 dilution of cells; to one dish was added 1.0 ml of the 1:100 dilution to make a 1:1,000 dilution of cells; and to another dish was added 0.5 ml of the 1:100 dilution to make a 1:2,000 dilution of cells.

The six plates of cells were examined daily. The medium was changed if there was a great amount of cell death. Such medium changes were repeated until few dead cells were observed. At this point, live cells or colonies were allowed to grow to a size such that the colonies are large enough to clone out (i.e., the colonies are visible to the naked eye when looking up through the bottom of the plate). Viral supernatants from such colonies of P3501 cells were collected in amounts of from about 5 ml to about 10 ml, placed in cryotubes, and frozen in liquid nitrogen at about -70°C.

PA317 cells (ATCC Accession No. CRL 9078) (Miller et al., Mol. Cell. Biol., 6:2895-2902 (1986)) and described in U.S. Patent No. 4,861,719, then were plated at a density of 5 x 10⁴ cells per 100 mm plate on Dulbecco’s Modified Essential Medium (DMEM) including 4.5 g/l glucose, glutamine supplement, and 10% fetal bovine serum (FBS).

The viral supernatant then was thawed, and 8 µg/ml of polybrev was added to viral supernatant from PE 501 cells, and the supernatant and polybrev were mixed and loaded into a syringe with a 0.22 µm filter unit. The DMEM was suctioned off the plate of cells, and 7 to 8 ml of viral supernatant was added for overnight infection.

The viral supernatant then was removed and replaced with fresh 10% FBS. One day later, the medium was changed to 10% FBS and G418 (800 µg/ml). The plate then was monitored, and the medium was changed to fresh 10% FBS and
G418 to eliminate dying or dead cells whenever necessary. The plate also was monitored for at least 10 to 14 days for the appearance of G418 resistant colonies by scanning the bottom of the dish without a microscope. When colonies are large enough to see, they then were selected as clones.

The medium then was aspirated from the dish and replaced with 5 ml PBS. The cells then were rinsed and most of the PBS was aspirated. About 0.5 to 1.0 ml of the PBS was left on the plate to keep it moist. Cloning rings then are placed on all selected colonies. Two drops of trypsin-EDTA then 2 were placed on each cloning ring. The dish then was placed in an incubator, and tapped periodically until the cells are released from the dish. 5 ml of HGD10 plus 0.8 mg/ml was added to as many wells as needed in six well dishes.

When the cells from each colony were released from the dish, 2 drops of HGD10 are added to each cloning ring. A pipette then was set to 200 μl, and inserted into a cloning ring in order to remove all the cells. The cells then were transferred to one of the wells in the 6-well dishes. Such procedure was repeated until all desired clones were picked. The 6-well dishes were incubated at 37°C in a 5% CO₂ atmosphere.

The clones then were observed for confluent growth. When a clone was confluent or almost confluent, the clone was trypsinized and expanded in a 100 ml dish.

When the expanded clone was about 90% confluent, the old medium was removed and replaced with 10 ml of fresh HGD10 medium. The dish was returned to the incubator for 20 to 24 hours.

After the incubation, the supernatant was removed from the dish, and placed in a 15 ml polypropylene tube. The tube was centrifuged at 1,200 to 1,500 rpm for 5 minutes to pellet out any cells which may have been in the supernatant.
The supernatant then was aliquoted into six cryovials (1 ml/vial). The aliquots were stored in liquid nitrogen. 5 ml of PBS were added to the dish and the cells were rinsed.

When the cells were released from the dish, 9 ml of HGD10 was added to the cells, and the cells were transferred to a 15 ml polypropylene tube. The cells were pelleted by centrifuging at 1,200-1,500 rpm for 5 minutes.

The medium was aspirated off the cell pellet. The pellet then was resuspended in 1ml HGD 10 and 1 ml of 2xDMSO freezing medium, and 1 ml of cells was aliquoted into each of two cryovials. The cryovials were placed on dry ice, and, when frozen, were transferred to liquid nitrogen.

Through the above procedures, a clone with the highest titer, designated as producer cell line PA317/G1NaSvAd.24, was used to produce a master cell bank of producer cells.

C. Production of Viral Supernatant Which have been Cryopreserved

PA317/G1NaSvAd.24 cells are thawed at 37°C and recultured as rapidly as possible to avoid damage to the cells by the cryopreservative dimethylsulfoxide (DMSO).

The contents of one working cell bank cryovial are placed in a T75 flask containing 25 ml of medium containing high glucose (4.5g/l) DMEM with 10% FBS and 2mM glutamine. The contents of this flask then are split into 4 to 10 75 cm² flasks. The contents of these flasks then can be split into additional 75 cm² flasks. When the cells are about 90% confluent, the medium in all the flasks is changed.

Supernatant then is collected from the flasks, and pooled in a large spinner flask. A sample is taken for Mycoplasma screening. The remaining supernatant is filtered through a 0.22 micron filter, pooled (samples
taken for further lot release testing), and aliquoted into the final containers. More medium is added to each flask.

One day later, supernatant again is collected from the flasks and pooled and filtered as hereinabove described. The procedure again is repeated the next day. After the supernatant is aliquoted and labeled, it is stored in a freezer at -70°C.

**Example 2**

Construction of pGI'TkSvNa and generation of producer cell line therefrom

A. **Construction of pGI'TkSvNa**

The following describes the construction of pGI'TkSvNa, a schematic of which is shown in Figure 10. This vector contains the Thymidine Kinase (hTK) gene from Herpes Simplex Virus I regulated by the retroviral promoter and the bacterial gene, neomycin phosphotransferase (Neo<sup>®</sup>) driven by an SV40 promoter. The hTK gene confers sensitivity to the DNA analogs acyclovir and ganciclovir, while the Neo<sup>®</sup> gene product confer resistance to the neomycin analogue, G418.

To make pGI'TkSvNa, a three step cloning strategy was used. First, the herpes simplex thymidine kinase gene (Tk) was cloned into the GI plasmid backbone to produce pGI'Tk. Second, the Neo<sup>®</sup> gene (Na) was cloned into the plasmid pSVBg to make pSVNa. Finally, SvNa was excised from pSVNa and ligated into pGI'Tk to produce pGI'TkSvNa.

pGI was constructed as described in Example 1.

To construct pBg (Figure 8) the 3.0 kb BamHI/EcoRI lacZ fragment that encodes β-galactosidase was isolated from pMC1871 (Pharmacia). This fragment lacks the extreme 5' and 3' ends of the β-galactosidase open reading frame. Linkers that would restore the complete lacZ open...
reading frame and add restriction sites to each end of the lacZ gene were synthesized and ligated to the BamHI/EcoRI lacZ fragment. The structure of the 5' linker was as follows: 5' - 1/2 NdeI - SphI - NotI - SnaBI - SalI - SacII - AccI - NruI - BgIII - III 27 bp ribosomal binding signal - Kozak consensus sequence/NcoI - first 21 bp of the lacZ open reading frame - 1/2 BamHI - 3'. The structure of the 3' linker was as follows: 5' - 1/2 mutated EcoRI - last 55 bp of the lacZ open reading frame - XhoI - HindIII - Smal - 1/2 EcoRI - 3'. The restriction sites in the linkers were chosen because they are not present in the neomycin resistance gene, the β-galactosidase gene, the hygromycin resistance gene, or the SV40 promoter. The 27 bp ribosomal binding signal was included in the 5' linker because it is believed to enhance mRNA stability (Hagenbuchle, et al., Cell 13:551-563, 1978 and Lawrence and Jackson, J. Mol. Biol. 162:317-334, 1982). The Kozak consensus sequence (5'-GCCGCCACCATGG-3') has been shown to signal initiation of mRNA translation (Kozak, Nucl. Acids Res. 12:857-872, 1984). The Kozak consensus sequence includes the NcoI site that marks the ATG translation initiation codon.

pBR322 (Bolivar et al. Gene 2:95, 1977) was digested with NdeI and EcoRI and the 2.1 kb fragment that contains the ampicillin resistance gene and the bacterial origin of replication was isolated. The ligated 5' linker - lacZ - 3' linker DNA described above was ligated to the pBR322 NdeI/EcoRI vector to generate pBg. pBg has utility as a shuttle plasmid because the lacZ gene can be excised and another gene inserted into any of the restriction sites that are present at the 5' and 3' ends of the lacZ gene. Because these restriction sites are reiterated in the pG1 plasmid, the lacZ gene or genes that
replace it in the shuttle plasmid construct can easily be moved into pG1.

A 1.74 kB BglII/PvuII fragment containing the Herpes Simplex Virus Type I thymidase kinase gene (GenBank accession no. V00467, incorporated herein by reference) was excised from the px1 plasmid (Huberman, et al., Exptl. Cell Res. Vol. 153, pgs 347-362 (1984) incorporated herein by reference), blunted with the large (Klenow) fragment of DNA polymerase I, and inserted into the unique SnaBI site in the pG1 multiple cloning site, to form plasmid pG1TK. (Figure 9).

A 339 bp PvuII/HindIII SV40 early promoter fragment obtained from the plasmid pSV2Neo (Southern et al., Journal of Molecular and Applied Genetics 1:327-341(1982)) was then inserted into pBgl in the unique NruI site to generate the plasmid pSvBgl (Figure 5). The pSvBgl plasmid was digested with BglII/XhoI to remove the lacZ gene, and the ends were made blunt using the Klenow fragment. An 852 bp EcoRI/AsuII fragment containing the coding sequence of the neomycin resistance gene was removed from pN2 (Armentano, et al., J. Virol., Vol. 61, pgs. 1647-1650 (1987)), blunted with Klenow fragment and ligated into the 2.5 kb blunted BglII/XhoI fragment generated hereinafore, resulting in pSvNa. The SV40 promoter/neomycin resistance gene cassette was then removed from pSvNa as a 1191bp SalI/HindIII fragment. The pG1Tk plasmid was then digested with SalI/HindIII and ligated with the SV40/neo' fragment to generate pG1TkSvNa. (Figure 10).

B. Generation of Producer Cell Line PAT 2.4/G1TkSvNa. 90 and Generation of Viral Supernatant Therefrom.

Producer cell line PAT 2.4/G1TkSvNa.90 was prepared according to the method disclosed in Example 1 for
the preparation of producer cell line PA317/G1NaSvAd.24 except that packaging cell line PAT 2.4 was used instead of packaging cell line PA317. PAT 2.4 was made according to the method for the preparation of PA317 cells disclosed in Miller et al., Mol. Cell. Biol., 6:2895-2902 (1986) and in U.S. Patent No. 4,861,719 issued August 29, 1989 to Miller, the disclosure of which is incorporated herein by reference, except that the NIH 3T3 TK-minus cells were co-transfected with the hygromycin resistance gene instead of the Herpes Simplex Virus thymidine kinase gene to provide a selectable marker and the packaging cell line was selected from oligo-clonal populations.

The method is summarized as follows. A population of the NIH 3T3 TK-minus cells was co-transfected with two plasmid DNAs at a ratio of 20:1 using standard CaPO₄ transection methodology. The first plasmid was pPAM3 (ATCC accession number 40234), which contains the promoter, gag, pol, and env sequences of amphotropic murine leukemia virus. Seventy micrograms of DNA were used. The second plasmid was pY3, which contained the hygromycin resistance gene. (The hygromycin resistance gene also is found in other plasmids which are available to those skilled in the art.) The population of cells was selected in hygromycin, and hygromycin resistant cells were frozen as primary and secondary seed lots. The population was analyzed for viral envelope expression, and the packaging function was tested in TK vector producer cell clones to look for high titer vectors. Oligo-clonal populations were created by seeding one 24-well plate with 5-10 cells per well and another plate with 10-20 cells per well. A total of 46 populations were created. Each oligoclone was expanded and frozen. Each population was tested individually for packaging efficiency by generating a producer population with supernatant from the packaging cell PE501, which had been transfected with the plasmid pGLTkSvNa, containing the
neomycin resistance gene according to the procedure of Example 1. A subpopulation capable of producing high titer producer cell lines was identified according to the procedure of Example 1 and designated PAT 2.4/GlTkSvNa.90. Viral supernatant containing GlTkSvNa was prepared from this producer cell in accordance with Example 1.

**Example 3**

**A. Gene Transfer into Sheep Fetuses**

Ten ewes with confirmed dates of pregnancy were prepared for surgery by withholding food for 48 hours and water for 18 to 24 hours. Each ewe was sedated with intramuscular ketamine (10mg/kg), and given a 0.5 to 10% halothane-oxygen inhalation mixture via an endotracheal tube. Each animal received intravenous fluids and antibiotics during surgery. The uterus was exposed by a lower midline incision, and each fetus was accessed through a small hysterotomy and transverse incision of the myometrium and chorion. The amnion was left intact. The fetus then was visualized, and gently manipulated into an amniotic bubble. The fetus then was immobilized for injection within the bubble under gentle applied pressure. At this time, the fetus is within view fully, which insures that the injected cells or viral supernatant remain within the peritoneal cavity. The sheep were injected with one of the following: (i) 2 ml of viral supernatant from G1NaSvAd.24, having a titer of $1 \times 10^6$ CFU/ml; (ii) 1 ml of $5 \times 10^7$ PA317/GlNaSvAd.24 producer cells; (iii) 1 ml of $5 \times 10^7$ PA317/GlNaSvAd.24 producer cells that were irradiated with 3,000 rads in a cesium irradiator; (iv) 2 ml containing $1 \times 10^7$ PAT2.4/GlTkSvNa.90 producer cells; (v) 1 ml of $5 \times 10^7$ PAT2.4/GlTkSvNa.90 producer cells; (vi) 2 ml containing of $1 \times 10^7$ PAT2.4/GlTkSvNa.90 producer cells that were irradiated with 3,000 rads in a cesium
irradiator; (vii) 1 ml of 5 x 10^7 PA317 packaging cells; (viii) 1 ml of 5 x 10^7 PAT2.4 packaging cells; or (ix) 2 ml containing 1 x 10^8 PAT2.4 packaging cells. The fetuses of each ewe were injected with viral supernatant or cells as shown in Table 1 below.

**TABLE I**

<table>
<thead>
<tr>
<th>Number of mls Sheep injected</th>
<th>Experimental Group</th>
<th>Number of fetuses</th>
<th>Age of fetus (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>G1NaSvAd.24 supernatant</td>
<td>2</td>
<td>672 ml</td>
</tr>
<tr>
<td>2.</td>
<td>G1NaSvAd.24 supernatant</td>
<td>1</td>
<td>672 ml</td>
</tr>
<tr>
<td>3.</td>
<td>PAT2.4/G1TkSvNa.90 producer cells</td>
<td>2</td>
<td>672 ml</td>
</tr>
<tr>
<td>4.</td>
<td>PAT2.4/G1TkSvNa.90 producer cells</td>
<td>2</td>
<td>672 ml</td>
</tr>
<tr>
<td>5.</td>
<td>PAT2.4/G1TkSvNa.90 irradiated producer cells</td>
<td>1</td>
<td>672 ml</td>
</tr>
<tr>
<td>6.</td>
<td>PAT2.4/G1TkSvNa.90 irradiated producer cells</td>
<td>2</td>
<td>672 ml</td>
</tr>
<tr>
<td>7.</td>
<td>PAT2.4/G1TkSvNa.90</td>
<td>1</td>
<td>672 ml</td>
</tr>
</tbody>
</table>
irradiated producer cells

8. PA317/G1NaSvAd.24 1 571 ml producer cells

9. PA317/G1NaSvAd.24 2 571 ml producer cells

10. PA317/G1NaSvAd.24 3 571 ml irradiated producer cells

After the injection, each fetus was returned to the primary amniotic space, and the myometrium was closed in a double layer. Following the closing of all incisions, each ewe was observed for 48 hours and, unless scheduled for further experimentation, was returned to a large animal facility for the remainder of the gestation period. Each ewe that survived was examined one week before the expected date of delivery. (Normal gestation is 145 days.) Of the 17 fetuses injected, 3 were born alive that were injected with G1NaSvAd.24 viral supernatant; 2 were born alive that were injected with non-irradiated PAT 2.4 G1TkSvNa.90 producer cells; and 4 were born alive that were injected with irradiated producer cells (2 with PA317/G1NaSvAd.24 irradiated producer cells and 2 with PAT 2.4/G1TkSvNa.90 irradiated producer cells).

B. Assay Procedures for Determining Presence of neo<sup>*</sup> and Herpes Simplex Thymidine Kinase Genes

At various points after birth, CFU-E, BFU-E, CFU-GM, or CFU-Mix bone marrow cells (all of which are hematopoietic stem cells) were taken from various sheep and
analyzed for the expression of the neo^6 gene by culturing the cells in the presence of G418, and/or for expression of the Herpes Simplex thymidine kinase (TK) gene by culturing the cells in the presence of ganciclovir.

CFU-E cells and BFU-E cells were tested in a plasma clot culture assay as follows:

Whole bone marrow from the sheep was transferred to a 15 ml centrifuge tube, and the tube was spun at 1,600 rpm for 10 minutes. The buffy coat was removed and the marrow cells brought up to 15 ml with Iscove’s solution. The cells then were layered onto 5 ml of Ficoll buffer and spun for 30 minutes at 2,000 rpm. The mononuclear layer is removed and the remaining cells are washed twice with Iscove’s solution. The cells were resuspended in 5 ml of Iscove’s solution and counted. A medium containing the following components then was formed for each plasma clot culture:

100\(\mu\)l L-asparagine
300 \(\mu\)l Fetal calf-serum
100 \(\mu\)l alpha-thiocyanate B
30 \(\mu\)l erythropoietin
100 \(\mu\)l 4 x Thrombin

630 \(\mu\)l of this mix was pipetted into each tube. The volume of cells was calculated that is needed to get 2 x 10'^6 cells. G418 in amounts of 0 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, or 3.0 mg/ml, and/or 0 \(\mu\)M, 3\(\mu\)M or 6\(\mu\)M of ganciclovir was added to each tube. Ganciclovir interacts with Herpes Simplex thymidine kinase in order to kill cells which express the Herpes Simplex thymidine kinase gene. The amount of Iscove’s solution for each tube was calculated to bring the final volume of the tubes, including the cells, to 1 ml. The Iscove’s solution was added to the tube, followed by the G418 and/or the ganciclovir, followed by the cells.
100 μl of citrated plasma, which aids in clotting, is added to the first tube. The contents of the tube are mixed by pipetting the contents up and down with a 1 ml pipette, and 200 μl of the contents is dispensed to each of eight plasma clot wells. Four wells are in one 6 well plate, and four wells are in another 6 well plate.

The above procedure is repeated for all samples. Water is added to the spaces in between the wells on the six well plate, and the plates are placed in the incubator until the cells are ready for harvest.

In order to harvest the clots, the plates are removed from the incubator, and allowed to sit under a hood for 10 minutes. During this time, PO, buffer is placed into a petri dish.

Coated slides then are set up on a cafeteria tray and labeled. Four clots are lifted out for each group onto the same slide, and are arranged in an offset pattern so that the clots do not come in contact with each other.

A filter paper then is dipped into the PO, buffer, and a piece is laid over each slide. The filter papers and the slides are allowed to sit for 1 to 2 minutes.

A large piece of Baxter filter paper then is obtained and laid over all the slides. The paper is pressed firmly to blot out all of the excess buffer. The large filter paper is discarded, and 3% glutaraldehyde is squirted onto each slide’s PO, buffer coated filter paper. The slide and filter paper are allowed to sit for 1 minute. The slide’s filter papers are removed and discarded. The slides are set in a drying rack, and are allowed to dry for several hours before staining. Each slide then was treated with methanol for one minute, and then treated with 1% benzidine in methanol for 5 minutes. Each slide then was treated with peroxidase for 3 minutes, and then washed with distilled water for 1 minute. Each slide then was stained
with hematoxylin for 8 minutes, and then placed under cool running tap water for 10 minutes.

CFU-GM and CFU-Mix colonies were evaluated for neomycin resistance or expression of the Herpes Simplex thymidine kinase gene through a methylcellulose culture assay as follows:

Bone marrow aspirates were drawn from sheep and mononuclear cells were isolated by layering 10 ml of bone marrow (diluted 1:3 in IMDM) onto a cushion of 5 ml of Ficoll-Hypaque buffer. The tubes were subjected to centrifugation at 1,500 rpm for 30 minutes. The mononuclear fraction was removed with a sterile transfer pipette and washed twice with IMDM. The cells were pelleted and cultured at 2 x 10⁷ cells/ml. Each plate consisted of 3 wells, each containing one-third of the following mixture:

1.6 ml of methylcellulose (Gibco)
60 μl of erythropoietin
(50 units/ml final concentration)
100 μl of sheep phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM)
4 x 10⁷ cells
G418 and/or ganciclovir
IMDM to make a final volume of 2 ml

G418 was added to the plates at concentrations of 0 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, and 3.0 mg/ml, and/or ganciclovir was added at 0 μM, 3 μM, or 6 μM. These plates then were cultured for 7 days (CFU-GM), and 12 days (CFU-Mix), and colonies were counted under a dissecting scope.

Example 4
The effect of G418 on colony formation was evaluated, according to the assay procedures of Example 3, with respect to CFU-Mix, BFU-E, CFU-GM, and CFU-E cells.
taken from six newborn control sheep that were not given producer cells or viral supernatant. The total number of colonies for each type of cell taken from the six sheep was calculated. The results are given in Figure 11. These sheep also were evaluated at 7 months and 17 months after birth. The results at 7 months and 17 months after birth were the same as when the sheep were newborn.

Example 5

The effect of G418 on CFU-Mix, BFU-E, CFU-GM, and CFU-E cells of three of the sheep which were born alive, and now at 7 months of age (10 months after intraperitoneal administration of viral supernatant or producer cells), was evaluated and compared with the effect of G418 on the CFU-Mix, BFU-E, CFU-GM, and CFU-E bone marrow cells of six control sheep which were not given any producer cells or viral supernatant. In this experiment, one of the three treated sheep received G1NaSvAd.24 viral supernatant, one sheep received $5 \times 10^7$ irradiated PA317/G1NaSvAd.24 producer cells, and 1 sheep received $5 \times 10^7$ PAT2.4/G1TkSvNa.90 producer cells. For each sheep, the percentage of G418 resistant colonies of each cell type was determined according to the assay procedures of Example 3, and the average percentage of G418 resistant colonies was calculated for each sheep. An average percentage of G418 resistance then was calculated for each group (control or treated) of sheep for G418 in concentrations of 0 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, and 3.0 mg/ml. The percentage of G418 resistant colonies in the control sheep and treated sheep at varying doses of G418 is given in Figure 12.
Example 6

Four sheep at age 6 months were evaluated for the presence of G418-resistant CFU-E, BFU-E, and CFU-GM cells in the presence of 2 mg/ml G418 according to the assay procedures of Example 3. Sheep 1 received $5 \times 10^7$ PAT2.4/G1TkSvNa.90 producer cells. Sheep 2 received $1 \times 10^8$ PAT2.4/G1TkSvNa.90 producer cells. Sheep 3 received 1 ml of $1 \times 10^5$ CFU/ml of G1NaSvAd.24 viral supernatant, and Sheep 4 received $5 \times 10^7$ irradiated PA317/G1NaSvAd.24 producer cells. The percent resistance of the cells from each sheep to 2 mg/ml G418 is given in Table II below:

**TABLE II**

<table>
<thead>
<tr>
<th>Sheep GM</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>59</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>25</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>31</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>46</td>
<td>37</td>
</tr>
</tbody>
</table>

Example 7

All the sheep that were born alive, which received producer cells, which produce viral particles expressing the neo\(^*\) gene, and all sheep that were born alive which received viral supernatant, were monitored for a period of 20 months for G418-resistant colonies of bone marrow cells according to the assays hereinabove mentioned in Examples 3 and 5. The average percent of G418-resistant cells for all cell types, and then for all sheep which received viral supernatant or producer cells, was
calculated. The results of such monitoring are shown in Figure 13.

Example 8

The effect of G418 and ganciclovir (GCV) on BFU-E and CFU-GM cells was measured for Sheep 1 in Table II above according to the assay procedures mentioned above in Example 3. The test cells were contacted with G418 in a concentration of 3 mg/ml, and/or ganciclovir in an amount of 6 μM. The results are shown in Figure 14.

As can be seen from Figure 14, the difference between the amount of colonies when neither G418 nor ganciclovir is administered, and when ganciclovir is administered is essentially equal to the number of G418-resistant colonies. Thus, it is implied that ganciclovir kills all the cells that are neomycin-resistant. When G418 and ganciclovir are present in the medium, essentially no cell colonies are formed, demonstrating that the cells resistant to G418 are sensitive to ganciclovir.

Example 9

The percentage of neo<sup>r</sup> expression in all sheep which were born alive, and which were given producer cells carrying the neo<sup>r</sup> gene or viral supernatant, was monitored with respect to CFU-Mix, CFU-GM, BFU-E, and CFU-E cells for a period of 20 months. Average percentages of G418-resistance for each type of cells for all sheep were calculated, and the results are given in Figure 15.

Example 10

One sheep that received 5 x 10<sup>7</sup> irradiated PA317/G1NaSvAd.24 producer cells was sacrificed 13 months after injection of the producer cells (or 10 months after birth). PCR assays then were conducted on DNA isolated from the lung, liver, kidney, and testes for presence of the neo<sup>r</sup> gene. The PCR assays were conducted as follows:

300 nanograms of total genomic DNA was subjected to analysis by the technique of the polymerase chain
reaction (PCR) as described in Saiki, et al., *Science*, Vol. 230, pgs. 1350-1354 (1985) with the following changes to the reaction constituents: the PCR primers used were 0.5 μM of primer 1 and 0.5 μM of primer 2. dATP, dCTP, and dGTP were used at 150 μM. In order to eliminate the chance of product carry-over leading to false-positives, dUTP (600 μM) was substituted for dTTP, and reactions were treated with uracil-DNA-glycosylase (UDG) prior to amplification, according to the method of Wang, et al. *American Journal of Hematology*, Vol. 40, pgs. 146-148 (1992). MgCl₂ was used at 5 mM, and 2.5 units of AmpliTaq DNA polymerase (All PCR reagents were purchased from Perkin Elmer.) were added to each 50 μl reaction. Primer 1 has the following sequence:

5'-GGT GGA GAG GCT ATT CGG CTA TGA-3'
Primer 2 has the following sequence:

5'-ATC CTG ATC GAC AAG ACC GGC TTC-3'

These primers amplify a 440 base pair fragment of the neomycin resistance gene. The samples were overlaid with 100 μl of mineral oil, heated to 95°C for 10 minutes to inactivate the UDG, and then were subjected to 40 cycles of PCR. The reactions were run in an automated PCR temperature cycling block that allowed denaturation of the DNA at 95°C for 1 minute, annealing of the primers at 65°C for 1.5 minutes, and extension of the primers at 72°C for 1.5 minutes, and extension of the primers at 72°C for 1.5 minutes. After the 40th cycle, the reactions were held at 72°C to allow complete extension of the amplification products and to prevent damage due to residual UDG activity. Chloroform extractions were performed on the reactions, and 15 μl of each resultant aqueous phase was loaded onto a 2% agarose gel and electrophoresed in Tris-acetate-EDTA.

PCR analysis of DNA from the testes and kidney showed that both tissues contained the neo² gene; however, presence of the provirus in the kidney and testes genomes
did not appear to have any deleterious effects on the animal, as such organs showed no evidence of any pathologic condition upon examination. Such results indicate that direct injection of an engineered retrovirus into a fetus is a feasible means of delivering a foreign gene to a developing fetus and achieving long term expression without endangering the recipient.

Example 11

Another sheep that received $5 \times 10^7$ irradiated PA317/G1NaSvAd.24 producer cells was sacrificed 18 months after injection of the producer cells (or 15 months after birth). A PCR assay then was conducted on DNA isolated from the brain for presence of the neo\$^g$ gene. The PCR assay was conducted as described in Example 10.

PCR analysis of DNA from the brain showed that brain cells contained the neo\$^g$ gene. Thus, it has been shown that direct injection of an engineered retrovirus into a fetus can deliver a gene to the brain of a developing fetus.

All articles cited herein are hereby incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.
WHAT IS CLAIMED IS:
1. A process for effecting gene therapy in vivo in a fetus, comprising:
   transducing fetal cells in vivo with at least one nucleic acid sequence encoding a therapeutic agent.
2. The process of Claim 1 wherein said fetal cells are transduced in vivo with a viral vector including said at least one nucleic acid sequence encoding a therapeutic agent.
3. The process of Claim 2 wherein said viral vector is a retroviral vector.
4. The process of Claim 3 wherein said in vivo transduction of fetal cells is effected by administering to said fetus producer cells which generate said retroviral vector.
5. A process for effecting gene therapy in vivo in a fetus, comprising:
   transducing fetal cells in vivo with a retroviral vector including at least one nucleic acid sequence encoding a therapeutic agent.
6. The process of Claim 5 wherein said in vivo transduction of fetal cells is effected by administering to said fetus producer cells which generate said retroviral vector.
7. The process of Claim 3 wherein said in vivo transduction of fetal cells is effected by administering to said fetus a viral supernatant which contains said retroviral vector.
8. The process of Claim 5 wherein said in vivo transduction of fetal cells is effected by administering to said fetus a viral supernatant which contains said retroviral vector.
9. The process of Claim 4 wherein said producer cells are administered intraperitoneally.
10. The process of Claim 6 wherein said producer cells are administered intraperitoneally.
11. The process of Claim 7 wherein said viral supernatant is administered intraperitoneally.
12. The process of Claim 8 wherein said viral supernatant is administered intraperitoneally.
FIG. 1
CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE

CUT WITH EcorRI/ClaI

R1
5'LTR

ligate

ClaI
3'LTR

ligate

R1
5'LTR

R1

pG1

NotI
ShaBI
SalI
BamHI
XhoI
HindIII

ApaI
ClaI

R1

pGO

NotI
ShaBI
SalI
BamHI
XhoI
HindIII

ApaI
ClaI
SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

AATTC GCGGCCGC TACGTA GTCGTA GGATCC CTCGAG AAGCTT GGGCCC
CGCCGGCG ATGCAT ATGCAT CCTAGG GAGCTC TTCGAA CCCGGG

FIG. 2
FIG. 9
FIG. II

Effect of G418 on Colony Formation by Control Sheep Bone Marrow Cells in vitro

Total Colonies (n = 6)

G418 (mg/ml)

CFU-Mix
BFU-E
CFU-GM
CFU-E
**FIG. 12**

% G418-Resistant Colonies vs. G418 (mg/ml)

- ○ Controls
- ● Treated
FIG. 13

Persistence of Neo$^R$ Expression in Sheep

% G418-Resistant Colonies

Months After Neo$^R$ Transfer

13/15
SUBSTITUTE SHEET (RULE 26)
FIG. 14

Effect of G418 & GCV on Colony Formation by Bone Marrow Cells in vitro (Sheep 1)

BFU-E

CFU-GM

Additions to Cultures
FIG. 15

Persistence of NeoR Expression in Sheep

%G418-Resistant Colonies

Months After Neo Transfer
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 31/70, 38/00
US CL : 514/44; 424/93.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 424/93.2

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US, A, 5,075,229 (HANSON ET AL.) 24 December 1991, claims 1 and 2.</td>
<td>1-12</td>
</tr>
<tr>
<td>X</td>
<td>Blood, Volume 78, No. 4, issued 15 August 1991, D.W. Clapp et al., &quot;Fetal Liver Hematopoietic Stem Cells as a Target for In Utero Retroviral Gene Transfer&quot;, pages 1132-1139, see entire document.</td>
<td>1-12</td>
</tr>
</tbody>
</table>

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

Date of the actual completion of the international search 08 FEBRUARY 1995
Date of mailing of the international search report 27 FEB 1995

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer DEBORAH CROUCH, Ph.D.
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*
### INTERNATIONAL SEARCH REPORT

**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*