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(71) Applicants:  
The General Hospital Corporation [US/US];  
The McLean Hospital Corporation [US/US];  
115 Mill Street, Belmont, MA 02178 (US).  

(72) Inventors:  
Bakefield, Xandra, O.;  
127 Homer Street, Newton Center, MA 02159 (US).  
Isacson, Ole;  
58 Pinckney Street, Boston, MA 02114 (US).  
Rosenberg, William, S.;  
43 Notthinghill Road #2, Brighton, MA 02135 (US).  

(74) Agents:  
GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler,  
Goldstein & Fox, 1225 Connecticut Avenue, N.W., Suite  
300, Washington, DC 20036 (US).  

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METHOD OF GENE DELIVERY TO POST-MITOTIC CELLS  

(57) Abstract  
A method for extensive delivery and expression of a gene construct to post-mitotic cells is provided. The method involves providing the retrovirus vector utilizing a packaging cell line. In this manner, stable expression of the gene sequence of interest can be maintained in non-dividing target cells. A helper virus can also be used to promote delivery and integration.
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METHOD OF GENE DELIVERY TO POST-MITOTIC CELLS

Field of the Invention

The invention relates to the transfer and expression of genes utilizing retrovirus vectors.

BACKGROUND OF THE INVENTION

The capability to introduce and express a particular gene sequence into a cell holds great promise. Such capability provides a means for studying gene regulation, for defining the molecular basis of a disease, and for designing a therapeutic basis for the treatment of disease.

Several techniques have been developed that allow the introduction of recombinant DNA molecules into cells. These methods include DNA-mediated transformation, protoplast fusion, intracellular microinjection, and infection with viral vectors.

Retroviral vectors utilize the infectious mechanism unique to retroviruses to transfer genes with high efficiency into a wide variety of cells both in tissue culture and in living animals. Retrovirus vectors are extremely versatile tools that have been used in a wide range of systems.

Retroviruses are naturally occurring, RNA-containing viruses. The viral genes are encoded in a single-stranded RNA molecule. After entering a cell, the viral RNA is converted to double-stranded DNA. The viral DNA is subsequently found in the nucleus where it reproducibly integrates into the host genome. They can infect many cell types from many different species.

While retroviral vectors are useful for many kinds of in vitro gene transfer studies, the problem of relatively low titers limits their use for some in vitro and most in vivo studies in the brain. Furthermore, integration of retroviral vectors into the host genome was thought to be restricted to cells undergoing DNA replication. Thus, although retroviral vectors are capable of infecting a broad class of cell types, cell replication and DNA synthesis
promote provirus integration. This effectively restricts the efficient use of retrovirus vectors to replicating cells. Thus, retroviruses have not been utilized to introduce genes into post-mitotic cells.

Efforts to introduce recombinant molecules into post-mitotic cells, including neurons and other neural cells, have been limited. In fact, current therapies are generally inadequate. A need therefore exists for efficient viral vectors which are capable of mediating gene transfer into nonreplicating cells.

Related Art


SUMMARY OF THE INVENTION

The present invention is drawn to the use of retroviral vectors to express gene sequences in post-mitotic cells. The method involves providing the retrovirus vector to such cells, over an extended period of time utilizing a packaging cell line. Using the method of the present invention, stable expression of foreign genes can be maintained in nonreplicating target cells.
The invention is useful for introducing and expressing gene sequences in cells of the central nervous system, for treating neurological deficiencies of the central nervous system, and for modulating neuronal physiology by delivery of neuropeptide genes.

Brief Description Of The Figures

Figure 1. Gene delivery using retroviral packaging cell. (A) The packaging cell contains wild-type retroviral structural genes with a defective packaging (psi) sequence (Mann et al., Cell 33:153-159 (1983)), and can only produce replication-defective BAG vector, in which the RNA contains a competent psi sequence. The BAG vector can introduce its RNA into a target cell as its DNA equivalent, which following integration into the cellular genome produces β-galactosidase (B). If the packaging cell is modified by infection with wild-type virus containing a competent psi sequence, it will produce both helper virus and BAG vector. Infection of cells with these viruses can produce three possible outcomes: (I) Infection of a target cell by helper virus alone will result in the production of only helper virus, and will eventually prevent subsequent infection with either helper virus or vector. (II) Infection of the target cell by both BAG vector and helper virus will result in production of the BAG vector and helper virus. (III) Infection by BAG vector alone produces the translation of β-galactosidase by the target cell without virion production (as in A).

Figure 2. Anti-β-gal immunocytochemistry following implantation of ψ2-BAG packaging cells. There is intense staining bilaterally of large cells with neuronal morphology in the medial septum and diagonal band (A), ventral pallidum (B) and nucleus basalis of Meynert/substantia innominata (C).

Figure 3. Anti-β-gal immunocytochemistry following implantation of ψ2-BAG packaging cells. Staining was found in the substantia nigra (A) and hippocampus (B), including large cells with the morphology of
pyramidal neurons (insert). Cerebellar Purkinje cells (C) and scattered pontine neurons (D) also stained intensely.

Figure 4. X-gal histochemistry following implantation of ψ2-BAG packaging cells infected with helper virus.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Methods for utilizing retrovirus vectors to transfer and express genes of interest in post-mitotic cells is provided. Stable expression of a foreign gene can be maintained with no detectable toxicity.

The present invention concerns a method for introducing gene sequences into post-mitotic cells. By post-mitotic cells is intended non-replicating cells such as cells of the nervous system, bone marrow cells, muscle cells, liver cells, and the like. Cells of the nervous system include neurons, glial cells, etc. Such cells are collectively described herein as neural or neuronal cells. Neural cells are described, for example, by Barr, M.L., The Human Nervous System An Anatomic Viewpoint, 3rd ed., Harper & Row, New York (1979), which reference is herein incorporated by reference.

Viral vectors are genetically modified viruses, in which some or all of the structural, infectious genes of the native virus have been removed and replaced with a gene sequence to be delivered to target cells. Taking advantage of the parasitic nature of viruses at the cellular level, the vector injects its genetic message into the target cell, which then is transcribed and translated into protein products.

Retroviral vectors can integrate their genetic message into the host cell's genome. Until the present invention, retroviral vectors were thought to have an absolute requirement for cell division to achieve integration. (See, Cepko, C.L., Neuron. 1:345-353 (1988)). Thus, until the present invention retroviral vectors have been considered unsuitable for gene delivery to mature neurons or other post-mitotic cells. See, for example, Breakfield et al., Mol. Neurobiol. 1:339-371 (1987).
A viral vector contains nucleic acid molecule in which a gene sequence which is to be transferred to cells of interest, is fused to a subset of viral sequences. The viral sequences and the total genome size is selected such that the vector is capable of being encapsulated in a virus particle and thus be capable of binding to, and introducing it's gene sequences into a virus-sensitive host cell. Thus, when using retrovirus vectors, the gene of interest is first inserted into a retrovirus vector and converted into a virion before gene transfer takes place.

Methods are available in the art to insert a gene into a retrovirus vector, obtain recombinant virus, and infect target cells. See, Gilboa et al., BioTechniques 4:504-512 (1986); and Cepko, C., In Neuromethods: Molecular Neurobiological Techniques, 16, Boulton et al., eds. Clifton, New Jersey: Humana, 177-219 (1989); and Cepko, C.L., Neuron. 1:345-353 (1988) and the references cited therein.

Generally, vectors contain the cis-acting viral sequences necessary for the viral life cycle. Such sequences include the $\psi$ packaging sequence, reverse transcription signals, integration signals, viral promoter, enhancer, and polyadenylation sequences. Constructs can be made which lack some of these functions, for example, the viral promoter and/or enhancer. The vectors do not include the structural genes for production of a retrovirus particle. Therefore, these functions are supplied in trans.

These trans functions for the production of a retrovirus particle are provided by packaging cell lines. Generally, packaging cell lines are derived from fibroblasts and contain all of the structural protein information necessary for production of retrovirus particles. This information includes the viral gag, pol, and env genes. The viral sequences lack the packaging sequence $\psi$. Methods for construction of a retrovirus packaging mutant is provided in Mann et al., Cell 33:153-159 (1983) which disclosure is herein incorporated by reference. See, also, Cepko, supra, and the references cited therein.

The retroviral vector is introduced via transfection or infection into the packaging cell line. Then, the packaging cell line produces viral particles
that contain the vector genome. It is noted that most packaging cell lines will resist subsequent infection with another retrovirus except that an ecotropic vector can infect an amphotropic packaging cell line and vice versa.

Methods for transfection or infection are known in the art. See, Cepko, supra, and the references cited therein. See, also, Gilboa et al., supra (1986).

The packaging cell line may additionally contain a helper virus. This helper virus can provide essential trans functions. When the helper virus is present in the packaging lines with a retroviral vector, recombination can occur. That is, in psi' packaging lines, psi' vector sequences can recombine with psi' wild-type sequences to generate wild-type psi' helper virus. A more stable packaging cell line can be created by using multiple insertion sites for the viral structural sequences (See, Adv. Exp. Med. Biol. 271:149-160 (1980)).

Results, described below, using a helper virus indicate that infection of the packaging cell line with a helper virus (psi'), prior to grafting, provides for more extensive stable gene delivery. While the mechanism for the influence of the helper virus is not known, several theories are discussed in the Experimental Section. However, the invention is not limited or restricted to any proposed mechanism.

By the method of the present invention, retroviral vectors are capable of transferring a gene sequence into post-mitotic cells. By gene sequence is intended to refer to a nucleic acid molecule, preferably DNA. Such gene sequences may be derived from a variety of sources including DNA, cDNA, synthetic DNA, RNA, or combinations thereof. Such gene sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, introns, or poly A sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription, or other means.
The desired gene sequence is operably linked, and under the control of a promoter sequence so that the gene sequence will be expressed in the host cell. The promoter sequence may be homologous or heterologous to the desired gene sequence. A wide range of promoters may be utilized including viral or mammalian promoters. Cell or tissue specific promoters can be utilized to target expression of gene sequences in specific cell populations. Such promoters include, but are not limited to, specific promoters for neurotransmitters, neuromodulators, antrophic factors, glial specific promoters, and the like, e.g., neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), glutamate (acid) decarboxylase (GAD), tyrosine hydroxylase (TH), neural growth factor (NGF), choline acetyltransferase (ChAT), brain derived neurotrophic factor (BDNF), neurotrophin (NT), etc. Inducible promoters, such as drug inducible promoters, can also be utilized. Suitable mammalian and viral promoters for the present invention are available in the art.

The gene construct will contain the promoter, the coding sequence, and any regulatory sequences that are necessary for expression in the target cells. Once a retrovirus vector has been made which contains a gene construct of interest, it is introduced into a packaging cell line.

In addition, the retrovirus vectors may contain a marker gene or selectable gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration.

The packaging cell line containing the retroviral vector is then implanted into a host. Cells may be implanted into various regions of the host depending upon the target cells. Implantation or grafting of the packaging lines may be done in virtually any part of the body including muscle, the brain, the spinal cord, cerebral ventricles, subarachnoid space (including thecal sac), the peritoneum, into nerve endings in the skin, blood vessels, ventricular surface, etc. Methods are available in the art for the implantation or grafting of the retroviral packaging cell line. See generally, Rosenberg et al., *Science* 242:1575-1577 (1988); Wolff et al., *Proc. Nat. Acad. Sci. USA* 86:9011-9014 (1989); Shimohama et al., *Molecular Brain Res.* 5:271-

This implantation or grafting method delivers the retrovirus vector and maintains it in the presence of the target cells over an extended period. By extended period is intended that the retrovirus is maintained for a period at least several hours, generally for at least several days. This extended delivery method is capable of integration of the retrovirus vector into post-mitotic and non-replicating cells, as well as replicating cells.

It is recognized that the method of the invention can be modified to provide for wider delivery of the retrovirus vector. In this manner, grafting of packaging cell lines derived from astrocytes may be utilized. These astrocytes are capable of migration and delivery of the vector to a wider area for a prolonged period. The grafting method can be utilized in combination with DNA damaging agents, such as X-irradiation, to promote integration of the retrovirus into the cellular genome or other methods to stimulate DNA replication.

Other methods for modification concentrate on maintaining the packaging cell line containing the retroviral vector in contact with the target nonreplicating cells for extended periods of time. Such methods include immunosuppression of the host receiving the grafted packaging cells to prevent rejection of the packaging cell lines and prolong vector delivery. Moreover, related or more virulent retroviruses could be utilized to increase viral vector delivery. Furthermore, the viral vector envelope can be designed to target particular cell populations. That is, the viral envelope membrane is made so that it preferentially recognizes and attaches to particular cells. These methods could be used alone or in combination with each other. For example, the viral membrane of the vector could be designed to recognize a particular subset of cells while the vector contains a promoter which is expressed only in the same cell population.

A number of vectors are known in the art which can be modified for use in the invention, including WM-E, Cas-Br-E, MoMLV, etc. For example,

Gene transfer technology has several applications to neuroscience and neurochemistry. The most immediate applications are, perhaps, in elucidating the function and nature of neural peptides and the functional domains of proteins. Cloned cDNA or genomic sequences for neural proteins can be introduced *in vivo* in order to study cell type-specific differences in processing and cellular fate and the function of the protein. By placing the coding sequences under the control of a strong promoter, a substantial amount of the protein can be made, thus avoiding difficulties in characterizing trace amounts. Furthermore, the specific residues involved in protein processing, intracellular sorting, or biological activity can be determined by mutational change in discrete residues of the coding sequence. By using a cell-specific promoter, one can obtain atypical expression of an endogenous gene; an inducible promoter, one can control expression; and, an antisense construct, one can decrease expression of an endogenous gene.

Gene transfer technology can also be applied to provide a method to control expression of a protein and to assess its capacity to modulate cellular events in the central nervous system. Certain functions of neural proteins can be studied *in vivo*, for example, at different times in development or aging in order to monitor changes in receptor density, cell number, fiber growth, electrical activity, and other relevant properties.

Gene transfer provides a means to study the DNA sequences and cellular factors which regulate expression of neural specific genes. One approach to such a study would be to fuse the regulatory elements to be studied to a particular reporter gene and subsequently assaying for the expression of the reporter gene.

The regulation of gene expression in neuronal cells has been found to have a role in maintaining homeostasis and is believed to have a role in mediating information retention in response to external and internal signals (Black, I.B. *et al.*, *Science* 236:1263-1268 (1987).) During development, coordinated regulation of gene expression serves to produce a differentiated
phenotype, e.g., as in catecholamine metabolism and myelin biosynthesis. Regulation depends on many factors including chromatin structure, DNA methylation, and trans-acting factors, which respond to phosphorylation, hormones, and other signals. It is a complex process that allows sets of genes to be expressed together or differentially and may involve a combinatorial code of regulatory sequences.

Issues of cellular fate and interactions in the central nervous system can also be addressed by gene transfer. For example, genes which encode histological markers can be introduced into embryonic cells to determine lineage relationships during development and to elucidate neuronal pathways and to follow cell maturation and fate. In addition, genes encoding growth factors, oncogenic proteins, toxic peptides, or other physiologically important proteins, can be introduced into specific areas of the brain and spinal cord to study their effects on cell division, survival, and differentiation. For some studies, gene transfer or gene expression must be restricted to specific cells in the nervous system.


The above diseases fall into two classes: deficiency states, usually of enzymes, which are inherited in a recessive manner; and unbalanced states, at least sometimes involving structural or regulatory proteins, which are inherited in a dominant manner.

For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced state diseases, gene transfer could be used to create the disease state in a model system, which could be used in efforts to counteract the effect of the imbalance. Thus, the methods of the present invention permit the treatment of neurological diseases. As used herein, a deficiency state disease is "treated" by partially or wholly remedying the deficiency which causes the deficiency or which makes it more severe.

Animal models for diseases could be created not only by using antisense constructs but also by transferring and expressing "defective" or mutant proteins.

The methods of the invention can also be used to treat a disease condition by transferring and expressing gene sequences which encode antisense messages to block expression of a mutant protein or a dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease. Such methods are also useful for the treatment of
Huntington's disease, hereditary Parkinsonism, dystonia and other diseases. Antisense messages can also be used to block expression of proteins associated with toxicity.

The method of this invention may also be used to modulate normal physiologic processes, e.g., delivery of growth factors or other peptides or enzymes to optimize neural regeneration after injury or prolong cell survival in aging, in neurodegenerative diseases (including Alzheimer's and Huntington's diseases), or after toxic insults. In addition, it can be used to regulate transmission across certain synapses, for example, to kill neurons in the pathway, or alter neurons within phenotype by up or down regulation of normal neuropeptides or producing neuropeptide analogs as in pain transmission. It is further useful in control of blood pressure, mood, behavior, etc., by the atypical expression of peptides involved in these processes.

Having now generally described this invention, the following examples are offered by way of illustration and not to be limiting of the invention, unless specified.

EXPERIMENTAL

There are many diseases of the central nervous system (CNS) that may be amenable to gene therapy. Many neurologic disorders, like the idiopathic neurodegenerative disorders and hereditary storage diseases, presently lack efficacious treatment. One alternative to the traditional medical therapies would be the direct introduction of a gene that can alter the pathophysiology of he diseased nervous system. In animal models, implantation of cells, genetically engineered to produce trophic factors or neurotransmitter synthesizing enzymes, have been shown to protect neurons from degeneration and to attenuate functional deficits (Breakfield et al., Mol. Neurobiol. 1:339-371 (1987); Gage et al., Neurosci. 23:795-807 (1987); Morellou et al., Eur. J. Neurosci. 2:115-119 (1990); Schumacher et al., Neurosci. (in press)). However, this method of substance delivery does not change the genotype
of resident CNS cells. While there is great potential medical value in methods that allow gene delivery to the CNS, previous experimental attempts of gene transfer in vivo have had varied success (Breakfield et al., Mol. Neurobiol. 1:339-371 (1987)).

Of the potential approaches to the genetic engineering of endogenous cells of the CNS in vivo, one of the most promising utilizes viral vectors. Viral vectors are genetically modified viruses, in which the structural/infectious genes of the native virus have been removed and replaced with the gene to be delivered. Taking advantage of the parasitic nature of viruses at the cellular level, the vector "injects" its genetic message into the target cell, which then is transcribed and translated into viral protein products. The two major categories of viral vectors for delivery to the nervous system investigated to date have been constructed from either herpes simplex virus (HSV) or retroviruses, each having their distinct advantages and disadvantages (Breakfield et al., New Biol. 3:293-217 (1991); Cepko, C.L., Neuron 1:345-353 (1988)).

Vectors based on HSV have been used to deliver a specific marker gene (E. coli β-galactosidase [β-gal]) to rodent neurons by direct injection into the rat brain (Chiocca et al., New Biol. 2:739-746 (1990); Dobson 1989, Dobson 1990, Ho 1989). Although the retrograde transport of HSV makes this vector advantageous for focal gene delivery to small groups of neurons, widespread gene transfer to a large number of neurons can cause neurotoxicity, even under conditions in which the virus cannot replicate. Infection with wild-type HSV can result in either cell lysis or entry into a latent state. In latency, the viral genome exists in an episomal form within the cell nucleus, without actually integrating within the host genome (Stevens, J.G., Microbiol. Rev. 53:318-332 (1989)), although it is not yet clear whether all neurons and/or other cell types are able to harbor the virus in a latent state.

The other major class of viral vectors, retroviruses, can integrate their genetic message into the host cell's genome. They are thought, however, to have an absolute requirement for cell division to achieve integration (Cepko,
C.L., *Neuron* 1:345-353 (1988)), and have been considered unsuitable for gene delivery to mature neurons, which are postmitotic. Indeed, previous attempts at gene transfer to endogenous cells in the CNS by intracerebral injection of retroviral vector or packaging cell line were unsuccessful (Breakfield *et al.*, *Mol. Neurobiol.* 1:339-371 (1987); Short *et al.*, *J. Neurosci. Res.* 27:427-439 (1990)). The technical difficulties of delivering a large number of vector particles, combined with the limited four hour half-life of the virion, as measured *in vitro* (Cepko, C., In: *Neurometods: Molecular neurobiological techniques*, Vol. 16, Boulton, A.A., Baker, G.B., and Campagnoni, A.T. eds. (Clifton, N.J.: Humana) 177-219 (1989)), may have contributed to these negative results.

It was hypothesized that the integration of retroviral genome into the adult CNS might be achieved through extended delivery of viral vector, during DNA repair and/or DNA synthesis, which is a constant phenomenon in all cells. Under such circumstances, the prolonged delivery of vector particles may result in the integration of the desired gene into adult CNS cells. To achieve such a delivery, we stereotaxically implanted, into the adult rat brain, a retroviral packaging cell line, \(\psi 2\)-BAG (Mann *et al.*, *Cell* 33:153-159 (1983)), derived from an immortalized mouse fibroblast line, which constitutively produces the BAG vector (Price *et al.*, *Proc. natl. Acad. Sci* 84:156-60 (1987)). This retroviral vector, based on the Moloney murine leukemia virus, bears the *lacZ* marker gene. This packaging cell line contains wild-type retroviral sequences within its genome, under the control of the retroviral long terminal repeat (LTR) promoter, as well as a neomycin selection gene. The structural genes are attached to a defective packaging sequences (psi), which determines whether a given RNA transcript will be packaged into the virion (Mann *et al.*, *Cell* 33:153-159 (1983)) (Figure 1A). A vector containing the gene to be delivered is then used to transfect the packaging cell line. The resulting cell produces the necessary proteins to construct virions, but the only RNA transcript bearing a competent psi sequence is associated with the gene of interest. Therefore, this RNA is normally the only transcript capable of being packaged into a virion. If,
however, the psi sequence of the vector recombines with the wild-type retroviral sequences in such a way as to cause the packaging of replication-competent wild-type viral genome, virus particles (helper virus) would then be produced in addition to the vector itself (Figure 1B).

We stereotaxically implanted a retroviral packaging cell line into the dorsal striata of adult male rats, and then performed histological analysis of the perfused brains to assess transfer of the marker gene. By using this novel grafting method for gene delivery, we have been able to achieve stable gene transfer to neurons and other cells throughout the adult rat cortex, subcortical structures, brainstem and cerebellum in a consistent and reproducible pattern.

EXPERIMENTAL PROCEDURES

Design

To investigate gene delivery to the central nervous system by a retroviral packaging cell line, we stereotaxically implanted the mouse fibroblast packaging cell line ψ2 (Mann et al., Cell 33:153-159 (1983)) transfected with the BAG vector, which bears the Escherichia coli lacZ gene under the control of the Moloney murine leukemia virus promote-enhancer element Price et al., Proc. natl. Acad. Sci 84:156-60 (1987)), into the brains of 25 male Sprague-Dawley rats. This gene encodes a marker protein, β-gal, which can be detected using histochemical or immunocytochemical methods. To test whether the presence of helper virus could augment gene delivery, 12 of the animals were implanted with ψ2-BAG that had been pre-infected with helper virus. The rats were perfused at several time intervals post-implantation and their brains examined for the marker gene, using both a histochemical reaction specific for E. coli β-gal metabolism of 5-bromo-4-chloro-β-indolyl-β-D-galactoside (X-gal) to a blue, insoluble product, and immunocytochemical detection using antibodies specific for the bacterial enzyme.
Packaging cell line culture and harvest

The ecotropic (strains of retrovirus that preferentially infect rodent cells) retrovirus producer line ψ2-BAG 2-14 (derived from a fibroblast line, therefore positive for fibronectin) was obtained from M. Rosenbert (UCSD) and C. Cepko (Harvard Medical School) (Price et al., Proc. natl. Acad. Sci 84:156-60 (1987); Short et al., J. Neurosci. Res. 27:427-439 (1990)). This cell line was grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO), 10% fetal calf serum (source), with 100 U/ml penicillin, 100 µg/ml streptomycin (D10 P/S), and 500 µg/ml of the neomycin analogue, G418 (source). Infection with ecotropic helper virus, obtained from Dr. Richard Mulligan (Whitehead Inst.), was performed by adding 5 ml of conditioned media on three consecutive days to 10 ml of media overlying subconfluent cultures of ψ2-BAG cells. To prepare cells for engraftment, confluent cultures were washed with Dulbecco's phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and incubated briefly with 0.05% trypsin at room temperature. The cells were resuspended in DMEM with 10% fetal calf serum and pelleted by centrifugation for 5 min at 1,200 x g. This was done twice to eliminate all trypsin activity from the cell suspension. Prior to the final wash the cells were counted in a hemocytometer with trypan blue to assess viability, and the final cell suspension was made at a density of 10⁵ cells/µl in complete PBS (PBS containing 1 µg/ml MgCl₂, 1 µg/ml CaCl₂, 0.1% glucose and 5% rat serum [GIBCO]). The cell suspension was maintained at 4°C until implantation 3-6 hours after harvesting.

Viral titer determinations

Virus was harvested by replacing the overlying media of nearly confluent cultures of ψ2-BAG cells with a reduced volume of fresh media without G418. The conditioned media containing viral particles was removed 24 hours later, filtered through cellulose acetate membranes (pore size 0.45 µm, Nalgene), and stored at 70°C. The virus was titered as colony
forming units (cfu) on 3T3 cells in the presence of neomycin analog, G418. Viral titers were 1 to $3 \times 10^4$ cfu/ml.

**Graft implantation**

Male Sprague-Dawley rats were anesthetized prior to surgery with sodium pentobarbital by intraperitoneal injection (55 mg/kg). A Kopf stereotaxic frame and a 10 μl gastight Hamilton syringe were used for grafting. Following a small craniotomy anterolateral to bregma, 13 rats were implanted with ψ2-BAG, which had been previously infected with helper virus, in the dorsal right caudate. In each instance, a volume of 4 μl of cell suspension containing $4 \times 10^5$ cells was injected over 5 min. After injection, the needle was left in place for 3 min and then slowly withdrawn. Stereotaxic coordinates were calculated from begma (AP + 1.2, L -2.4, V - 5.4). All cell suspensions showed > 90% viability, as evaluated by trypan blue dye exclusion prior to implantation. In addition, 2 rats were injected intraperitoneally with the unmodified ψ2-BAG cell line ($2 \times 10^7$ cells in 500 μl complete PBS) to assess systemic dissemination of the vector. At various intervals after engraftment, the rats were deeply anesthetized with a lethal dose of chloral hydrate and quickly perfused through the heart with cold heparinized saline (10 units/ml), followed by 250 ml of cold perfusate (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4). Brains were removed and post-fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 at 4°C, followed by immersion in cryoprotectant (2% dimethylsulfoxide (DMSO), 20% glycerol in 0.1M phosphate buffer) for 24 hours. Two unoperated rats were perfused and used as controls. All brains were sectioned on a freezing microtome at 40 μm intervals and placed serially into trays and vials containing PBS, pH 7.4.
Histology

Sections were either processed for anti-β-gal activity (X-gal assay), using a modification of the method of Turner and Cepko (Turner et al., Nature 328:131-136 (1987)), as described by Short et al. (Short et al., J. Neurosci. Res. 27:427-439 (1990)). Briefly, a 2% stock solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Boehringer Mannheim) was prepared in DMSO. The free-floating sections were washed twice in PBS, pH 7.4 and incubated overnight at 37°C in a solution of PBS at exactly pH 7.4, containing 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.01% sodium deoxycholate and 0.02% NP-40; X-gal was added to a final concentration of 0.1% just prior to use. After incubation, the sections were either mounted on slides and counterstained with H&E, or stained immunocytochemically (Vectastain ABC, Vector Labs) with diaminobenzidine as the final chromagen for cellular fibronectin (monoclonal, 1:400; Sigma), GFAP (monoclonal, 1:500; Dako), Cholline acetyltransferase (ChAT)(polyclonal, 1:500; Boehringer Mannheim) or HLA-DR (monoclonal, 1:100; Dako) antigens. Other sections were immunostained for E. coli β-gal (monoclonal, 1:1000; 5 Prime - 3 Prime, Inc.) antigen. To verify the specificity of primary antibody binding to β-gal, primary antibody solution was pre-incubated at room temperature with antigen (1.2 mg/ml E. coli β-gal, grade VIII; Sigma) for 6 hours prior to staining. This was performed in parallel with normal antibody solution as a positive control. All results reported here were confirmed as specific using this antigen preabsorption method.

RESULTS

Postoperative observations. All animals survived until they were perfused for histologic analysis, up to 75 days. At the time of perfusion they had a normal, healthy appearance. Rats implanted with ψ2-BAG modified
with ecotropic helper virus also appeared to be free of clinical symptoms up to 75 days post-graft.

**Implantation of ψ2-BAG cells.** After implantation into the right striatum, animals were sacrificed at 3, 6, and 21 days post-graft. At 3 days, the graft site in the striatum demonstrated a well-defined, cellular graft, approximately 1-2 mm³ in size, that stained positive for bacterial β-galactosidase by both histochemistry and immunocytochemistry, as well as for the fibroblast marker fibronectin. No migration of fibronectin-positive cells was observed. There was a minimal inflammatory response surrounding the graft, as demonstrated by hematoxylin and eosin (H&E) histology and HLA-DR antigenicity, which is found in activated cells of the macrophage/monocyte line. There were rare reactive, glial fibrillary acidic protein (GFAP)-positive glia surrounding the graft site. By 6 days post-graft, there was a dense inflammatory reaction at the graft site, and β-gal was only faintly detectable, using either histochemistry or immunocytochemistry. There were no fibronectin-positive cells, indicating the complete rejection of the graft by this time. The remaining β-gal activity at the graft site was found predominantly in small, round, perivascular cells that were HLA-DR positive inflammatory cells. The GFAP-positive glial reaction around the graft was also more pronounced at this time. By 21 days, the inflammation at the site of engraftment had begun to subside. There were still rare, faintly β-gal-positive inflammatory cells at the depth of the injection site, but their number was decreasing. There was, however, a marked gliotic reaction surrounding the graft site throughout the striatum and overlying cortex.

β-gal reactivity in anatomic sites distant from the site of engraftment varied, depending on the assay used (Table 1). X-gal histochemistry revealed no apparent neuronal staining at 3 days post-graft; a few positive endothelial and choroidal epithelial cells were observed. After 6 days, a small number of large cells with neuronal morphology became histochemically positive in the ventral pallidum, the nucleus basalis of Meynert (NBM) and substantia innominata (SI), several thalamic nuclei, the dorsomedial hypothalamic nucleus, and scattered neurons of the central pons. In addition, the
endothelium now demonstrated rare, positive cells and the choroidal staining had increased. This pattern persisted through 21 days post-graft with the exception that the reactivity in the thalamic nuclei was no longer present.

By contrast, antiβ-gal immunocytochemistry revealed a more widespread presence of antigen than the less sensitive assay for X-gal histochemistry detected (Figures 2 and 3). Initially (3 days post-graft), there was strong staining in the cholinergic subnuclei of the medial septum, diagonal band, ventral pallidum and NBM/SI (Figure 2). These cells were characteristically large (>20 μm diameter) with several long processes and morphologically identical with cholinergic neurons (as identified by anti-ChAT immunostaining) in the same regions. Weak staining was detected in cells of the substantia nigra (Figure 3A), some hippocampal cells (Figure 3B), Purkinje cells in some regions of the cerebellar cortex (Figure 3C), and scattered pontine neurons (Figure 3D), as well as cells in the middle cortical layer and certain thalamic nuclei. The antigen was not located in the cerebrovascular endothelium at this early time point. Through the analysis of the two control rats, it was determined that the cerebrovascular endothelium displayed a falsely positive reaction on X-gal histochemistry, showing rare, faintly positive cells. By 21 days post-graft, the staining of the ventral pallidum, diagonal band, medial septum and NBM was detectable but diminished (Table 1). There was also weak staining in the substantia nigra, but the other brain regions no longer demonstrated detectable antigen. The white matter, conversely, developed a fibrillary staining pattern by 6 days post-graft that was not seen at the earlier time point and persisted through 21 days. The endothelium was now strongly, but variably stained for β-gal antigen. In all regions, specificity of primary antibody binding was confirmed using antibody preabsorbed with E. coli β-gal antigen and incubated on parallel sections. Thus, the translated product of the delivered gene was still detectable, albeit with diminished intensity, in certain neuronal subpopulations at 21 days post-graft.

**Implantation of ψ2-BAG cells modified with helper virus.** Rats were perfused 7 and 28 days following engraftment of ψ2-BAG that had been
previously infected with ecotropic Moloney murine leukemia helper virus. Histologic evaluation of the graft site revealed a mass of dense, inflammatory cells that was reduced in volume at 28 days post-graft. At both the early and late time points, there was intense histochemical reaction for β-gal surrounding the graft site, although the fibroblast packaging cell line did not appear to survive longer than 7 days.

Staining for β-gal enzymatic activity (X-gal histochemistry) in CNS sites distant from the site of engraftment demonstrated a consistent and reproducible pattern (Table 1), similar to that obtained with β-gal immunocytochemistry in the group implanted with ψ2-BAG without prior helper virus infection (Figures 3 and 4). The scattered, large (>20 μm diameter) neuron-like cells were strongly positive for β-gal in the caudate bilaterally (Figure 3G), and similarly appearing cells in the ventral pallidum (Figure 3A and B), diagonal band and medial septum (Figure 3C). There were patchy areas in the cortex containing positive cells with neuronal morphology that appeared to be confined to cortical layer III (Figure 3D). In addition, some areas displayed diffusely distributed positive cells with glial morphology that did not respect the neocortical layered architecture. In the region of the NBM/Sl (Figure 3E and F), there was diffuse, intense staining of groups of cells with both neuronal and glial morphologies. In the dentate gyrus and CA4 region of the hippocampus, there were rare, pyramidal cells positive for β-gal activity (Figure 4A). The substantia nigra displayed β-gal-positive neurons and glia, in both the pars compacta (Figure 4B) and the pars reticulata (Figure 4D). The pons showed individual neurons that were expressing the lacZ marker gene scattered diffusely throughout the white matter (Figure 4C). There were also many small (<10 μm diameter) cells with large nuclei and scant cytoplasm, possibly oligodendrocytes, in the pontine white matter expressing β-gal histochemical activity (Figure 4E). The Purkinje cell layer of the cerebellar cortex also stained intensely with X-gal histochemistry (Figure 4F). The cerebrovascular endothelium and choroidal epithelium were strongly β-gal-positive.
This histochemical pattern of staining persisted with little change through the latest time point examined (28 days post-graft), with the exception that staining in the substantia nigra appeared slightly more faint than it had at 7 days post-graft. Otherwise, the functional expression of the lacZ marker gene was stable in distribution and intensity through 28 days post-implantation. Moreover, these findings were corroborated using anti-β-gal immunocytochemistry (Table 1), which was controlled for specificity by the antigen preabsorption technique described in Experimental Procedures.

Intraperitoneal implantation of ψ2-BAG cells. Two rats were implanted with packaging cells into their peritonea and sacrificed 3 days post-engraftment. Gross autopsy, with particular attention to the abdominal contents, revealed no abnormalities or macroscopic tumors, and microscopic examination of representative sections of the peritoneal surface were unremarkable.

Histologic analysis of the brain demonstrated intense staining on X-gal histochemistry in cerebrovascular endothelium and choroidal epithelium. In addition, there was strong enzymatic activity in discrete regions of the hypothalamus, and scattered staining in pontine neurons and in the Purkinje cell layer of the cerebellar cortex. There was a noticeable absence of reactivity in the other regions described above.

Discussion

We report here the novel finding that implantation of a retroviral packaging cell line into the adult rat brain can achieve extensive gene delivery to postmitotic CNS neurons. Such implantation resulted in a reproducible anatomic distribution of gene transfer, which was stable. This first demonstration of gene transfer and stable expression in neurons of the CNS using a retroviral vector may have implications for both basic science and future clinical practice.

Infection of neurons, a postmitotic cell population, by a retroviral vector is surprising, since retroviruses are believed to require cell replication
for genomic integration. However, Sharpe et al. (Sharpe et al., Nature 346:181-183 (1990)) have recently published data suggesting that a related retrovirus, Cas-Br-E, can cause the "abortive" infection of mouse CNS neurons. In this study, viral antigens were detected predominantly within neurons, which, interestingly, did not stain using anti-env antibodies, thus designating the infection "abortive". Still, the mechanism of retroviral integration into a postmitotic cell, a process thought to require DNA replication (Cepko, C.L., Neuron 1:345-353 (1988)), remains unknown. Neuronal integration of the retroviral genome in these experiments may have occurred in association with DNA repair, since the enzymology of DNA repair is similar to that of DNA replication (Kuenzle, C.C., Brain Res. Rev. 10:2310245 (1985)).

The anatomic distribution of gene transfer to CNS sites distant from the graft followed a reproducible pattern. Implantation of the ψ2-BAG packaging cell line yielded gene delivery to the cortex, hippocampus, cholinergic system (ventral pallidum, diagonal band, medial septum, NBM/SI), certain thalamic and hypothalamic nuclei, substantia nigra, pons and cerebellar Purkinje cells. Vector dissemination via cerebrospinal fluid pathways as well as systemic dissemination by anterograde or retrograde axonal transport from the implantation site is feasible (Cserr, H.F., NY Acad. Sci. 529:9-20 (1988); Esiri et al., J. Neurol. Sci. 100:3-8 (1990)). However, it is recognized that the invention is not limited to any particular mechanism.

A finding that may be related to the regional distribution in our study is that of DesGroseillers et al. (DesGroseillers et al., Proc. Natl. Acad. Sci. 82:8818-8822 (1985)), who were able to develop a new neuropathologic entity in mice, consisting of pathologic change in the cortex, striatum and thalamus and neurologic symptoms referable to the pyramidal and extrapyramidal motor systems, using a chimeric construct of the retrovirus Cas-Br-E containing a promoter identical with the one in our vector. Ordinarily, this retrovirus produces a characteristic viral infection of the cerebellum, brainstem and anterior horns of the lumbosacral spinal cord (Kay et al., Proc. Natl. Acad. Sci. 88:1281-1285 (1991)). However, these
authors were not able to demonstrate neuronal infection in these regions. Another finding that may have relevance to our results is the demonstration of abnormal DNA repair mechanisms (Kuenzle, C.C., *Brain Res. Rev.* 10:2310245 (1985); Li *et al.*, *Biochem. Biophys. Res. Commun.* 129:733-738 (1985)) in cells from patients with Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, olivopontocerebellar degeneration and other diseases associated with these CNS regions. It is possible that neurons in these regions may therefore require an increased basal rate of DNA repair for homeostasis, as compared to other neuronal populations. This high rate of repair might increase the frequency of retroviral integrations. The regional neuroanatomic similarities among the infectivity of chimeric retroviruses, possibly altered DNA repair in certain neurodegenerative disorders, and the distribution of gene transfer in our experiments may point to common mechanisms.

Expression of the delivered gene in animals implanted with unmodified ψ2-BAG cells which were not infected with helper viruses was detectable only with immunocytochemical technique and, except for faint detection in the cholinergic system, pons and cerebellum, failed to achieve stability. These data suggest an initial low level of expression, visible with the more sensitive antibody-based assay. The instability of gene expression can be explained by two possibilities: (1) failure of the viral genetic material to integrate, an hypothesis requiring a transcribed episomal form of the viral genome, which has never been documented for retroviruses, or (2) suppression of the viral promoter following integration, a process known to occur occasionally following introduction of the promoter into cells in vivo (Breakfield *et al.*, *Mol. Neurobiol.* 1:339-371 (1987)).

Infection of the packaging cell line with helper virus prior to grafting caused more extensive, stable gene delivery to identical neuroanatomic areas and to the striatum. Helper virus may influence gene delivery in several ways. The combined presence of helper virus and viral vector can "recruit" other cells to become packaging cells by simultaneous infection or by first being infected with vector and then helper virus (Figure 1B, II). This is
supported by the large amount of β-gal activity observed at the graft site in the region of inflammatory infiltrate in these animals, even at the longest time interval. A "recruited" packaging cell line would expose the CNS to higher titers of viral vector for a longer duration than with the unmodified packaging cell line, which is rejected within 6 days. The vector would have more opportunities to integrate, particularly if this event were dependent on DNA repair. Alternatively, the helper virus may facilitate vector integration indirectly by stimulating DNA repair in the target cells, through an unknown process. Since our data indicate that the entry of vector into the target cell is independent of the presence of helper virus, stimulation of DNA repair mechanisms in those cells could increase the frequency of integration of the vector. More insertion sites would result in higher levels of expression, and increase the probability that one of the inserts would give high level and/or stable expression. Further experimentation will be necessary to distinguish among these possibilities.

The findings using the intraperitoneal administration of packaging cells demonstrate an ability of the vector to cross the blood-brain barrier in certain discrete locations, with limited delivery of its gene. Consistent with this result is the infection by Cas-B-E retrovirus of the brainstem and cerebellar region of mice following intraperitoneal administration (Kay et al., Proc. Natl. Acad. Sci. 88:1281-1285 (1991)).

The ability to deliver virtually any cloned gene to neurons and other cells throughout the CNS may have a dramatic impact on the clinical treatment of many neurologic disorders. Diseases of enzyme deficiency with neurologic manifestations, such as the lipidoses, leukodystrophies, mucopolysaccharidoses and other inborn errors of metabolism, may benefit from the ability to administer gene replacement therapy in the postnatal state. In addition, we have demonstrated that gene delivery is possible to certain specific groups of neurons that are associated with human neurodegenerative diseases (e.g., the cholinergic system in Alzheimer's disease, the substantia nigra in Parkinson's disease). Gene therapy to these neuronal subpopulations, through the neurosurgical implantation of a
retroviral packaging cell line containing therapeutic gene capable of ameliorating the degenerative process may prove effective in the treatment of these disorders.

Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in medicine, immunology, neurology, physiology, virology, pharmacology, and/or related fields are intended to be within the scope of the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.
What is claimed is:

1. A method for delivery and expression of a gene sequence to post-mitotic cells of a host, said method comprising:
   implanting a retroviral packaging cell line into said host, wherein said cell line delivers a retroviral vector over an extended period to said post-mitotic cells of said host, wherein said vector comprises said gene sequence;
   delivering said retroviral vector over an extended period to said post-mitotic cells of said host; and
   expressing said gene sequence in said post-mitotic cells of said host.

2. The method of claim 1, wherein said post-mitotic cells of said host are selected from the group consisting of cells of the central nervous system, muscle cells, bone marrow cells and liver cells.

3. The method of claim 2, wherein said post-mitotic cells of said host are cells of the central nervous system.

4. The method of claim 1, wherein said vector comprises a promoter operably linked to said gene sequence.

5. The method of claim 4, wherein said promoter is a viral promoter.

6. The method of claim 4, wherein said promoter is a mammalian promoter.

7. The method of claim 6, wherein said promoter is a cell specific promoter.

8. The method of claim 1, wherein said packaging cell line further comprises a helper virus.
9. The method of claim 1, wherein said packaging cell line is implanted in brain, spinal cord, nerve endings, blood vessels, cerebral ventricles, subarachnoid space, peritoneum, or ventricular surface.

10. The method of claim 4, wherein said gene sequence encodes an antisense message.

11. The method of claim 10, wherein said gene sequence is an antisense message for amyloid precursor protein.

12. The method of claim 10, wherein said gene sequence is an antisense for a protein associated with toxicity.

13. The method of claim 10, wherein said gene sequence is associated with Alzheimer’s, Parkinson’s or Huntington’s disease.

14. A method for treating a neurological deficiency of the central nervous system in a host, said method comprising:

   implanting into said host a packaging cell line wherein said cell line delivers a retroviral vector over an extended period to post-mitotic cells of said host, wherein said vector comprises a gene sequence operably linked to a promoter sequence so that said gene sequence will be expressed in said post-mitotic cells of said host and the expressed gene product complements the deficiency;

   delivering said retroviral vector over an extended period to said post-mitotic cells of said host; and

   expressing said gene sequence which encodes a gene product which complements said neurological deficiency.

15. The method of claim 14, wherein said packaging cell line further comprises a helper virus.
16. A method for modulating neuronal physiology in a host, said method comprising:
   implanting into said host a packaging cell line wherein said cell line delivers a retroviral vector over an extended period to post-mitotic cells of said host, wherein said vector comprises a gene sequence operably linked to a promoter sequence so that said gene sequence will be expressed in said post-mitotic cells of a host and the expressed gene product modulates neuronal physiology;
   delivering said retroviral vector over an extended period to said post-mitotic cells of said host; and
   expressing said gene sequence which encodes a gene product that modulates neuronal physiology.

17. The method of claim 16, wherein said packaging cell line further comprises a helper virus.

18. A non-human model for a disease state wherein said model is prepared by the method of claim 10.
Figure 1.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPCI(S) A61K 37/00, 31/70, 49/00; C12N 15/00
US CL 424/9, 93A; 514/44; 800/2, D1G5
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. : 424/9, 93A; 514/44; 800/2, D1G5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

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

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>Proceedings National Academy of Sciences, Volume 84, issued August 1987, F. D. Ledley et al, &quot;Retroviral Gene Transfer into Primary Hepatocytes: Implications for Genetic Therapy of Liver-Specific Functions&quot;, pages 5335-5339, see entire document, especially page 5338, col. 2, parag. 3.</td>
<td>1-18</td>
</tr>
<tr>
<td>Y</td>
<td>US, A, 4,861,719 (Miller) 29 August 1989, see entire document, especially col. 11, example 8.</td>
<td>1-18</td>
</tr>
<tr>
<td>Y</td>
<td>Journal of Virology, Volume 29, issued March 1979, E. M. Scolnick et al, &quot;Defective Retrovirus-Like 3OS RNA Species of Rat and Mouse Cells are Infectious if Packaged by Type C Helper Virus&quot;, 964-972, see entire document, especially &quot;Discussion&quot;.</td>
<td>1-18</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
'A' document defining the general state of the art which is not considered to be part of particular relevance
'E' earlier document published on or after the international filing date
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"a" document member of the same patent family

Date of the actual completion of the international search
05 November 1992

Date of mailing of the international search report
30 NOV 1992

Name and mailing address of the ISA/Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
DEBORAH CROUCH, PH.D.

Facsimile No. NOT APPLICABLE
Telephone No. (703) 300-0196

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