only a handful of the hundreds of known vertebrate retroviruses have been deliberately subverted for use as carriers of recombinant genetic material. Retroviruses receive their name from the fact that their genome undergoes conversion from RNA to DNA following infection of a host cell. Also characteristic of retroviruses and uncommon for most other types of viruses is that the genome of the retrovirus integrates itself permanently into the DNA of the host cell. Once integrated into the host genome, the inserted provirus acts as a factory for producing more retroviral RNA genomes and expressing retroviral packaging proteins. Both components combine to form viral particles that bud from the surface of the infected cells.

For gene transfer to mammalian cells, most recombinant retroviral vectors are derived from the Moloney murine leukemia virus (MLV), a mammalian type C retrovirus.\(^1\) Recently, recombinant human immunodeficiency virus (HIV) has also been used to transfer recombinant genetic material to mammalian cells and tissues. Both viruses have their individual strengths and weaknesses as gene transfer vectors. Moloney murine leukemia virus genome that encodes the canonical group-specific core antigen (Gag), polymerase (Pol), and envelope (Env) gene products is relatively well understood and has been genetically engineered to produce both packaging cell lines and plasmids for the production of recombinant virus. The MLV is also attractive for reasons of safety, and while there are exceptions, neither the production of recombinant MLV-based retroviruses nor cells infected with these viruses are generally considered high-risk biohazards.

Human immunodeficiency virus–based vectors are capable of infecting nondividing, postmitotic cell types that include neurons, whereas MLV-based vectors are only able to efficiently infect actively dividing cells. This characteristic, and extended high-level gene expression, makes them ideal therapeutic agents for the transfer of novel genetic material to the adult central nervous system (CNS). The weakness of HIV-based vectors, however, is that they currently require stringent biosafety procedures to produce safe recombinant virus. Furthermore, the presence of an infectious lentivirus (HIV) in the human population warns that measures need to be taken to ensure that the recombinant virus is not rescued by wild-type infection of human subjects. Research is currently directed at the production of novel recombinant retroviral vectors that will combine the efficiency and flexibility of HIV with the safety and simplicity of MLV-like retroviral vectors.

THE PRODUCTION OF RECOMBINANT RETROVIRUSES

All viral methods of gene transmission rely on separation of the means of forming an infectious particle and the infectious particle itself. A virion may be separated into 2 components: the nucleic acid genome that encodes the proteins necessary for perpetuation of the viral life cycle, and the protein package that houses and conveys the genome. Development of recombinant retroviral vectors began with plasmids encoding contiguous retroviral genomes with both noncoding and coding regions (Figure 1, A). The noncoding regions include the retroviral long terminal repeats that function as promoters for the expression of viral proteins and other packaging signals (ψ) required for encapsidation, reverse转录...
tion, and insertion into the host genome. The coding regions encode Gag, Pol, and Env polyproteins required for production of infectious virions. The regions of DNA encoding the Gag, Pol, and Env proteins were excised from the intact retroviral genome, leaving noncoding, but essential retroviral sequences (Figure 1, A).

**Figure 1.** A retroviral genome is divided into packaging and vector components (A). LTR indicates long terminal repeats; VSV-G, vesicular stomatitis virus G-protein. Two methods of producing recombinant retroviruses are also illustrated (B). Three plasmids are transiently cotransfected into cells to produce recombinant virus in the transient method (left); a packaging plasmid is stably introduced into cells to make a packaging cell line followed by a second round of stable transduction with the vector plasmid to make a stable producer cell line in the stable method (right).
The excised gag, pol, and env genes were then introduced into cultured cells to create packaging cell lines. In 1983, Mann et al. demonstrated that stable transduction of plasmids carrying the noncoding vector components (long terminal repeats, etc.) into packaging cells results in the production of viral particles that are able to infect target cells but are themselves unable to replicate or repackage into new infectious viral particles. 

Foreign genes are introduced into retroviral vectors in the vacancy left by gag, pol, and env gene deletion from the complete genome (Figure 1, A). The insertion of genes encoding selectable markers, internal promoters, and multiple cloning sites has converted currently available retroviral constructs into complex, multifunctional vectors for gene transfer. The stable introduction of a recombinant retroviral vector into a packaging cell line results in a “producer cell line.” Producer cell lines often produce replication-defective retroviruses at titers greater than 1 × 10^5 infectious particles per milliliter.

Recently, an alternative to the production of recombinant retroviruses using packaging cells and stable producer cell lines is to transiently package virus using cotransfection of plasmids encoding the packaging proteins and the retroviral vector simultaneously into easily transduced cell types (Figure 1, B). Although the transfected cells produce virus for only a few days, within this time they can produce titers exceeding the capacity of traditional producer cell lines. The transiently higher titer stems from both the very high plasmid copy number (10-50 copies) in transfected cells relative to the lower copy number characteristic of stable producer cells (<10 copies), and the enhanced ability to concentrate transiently packaged retroviruses by centrifugation. Most natural retroviral Env proteins cannot survive the rigors of centrifugal concentration; however, it has been empirically established that virions pseudotyped with some viral Env proteins such as the vesicular stomatitis virus G-protein (VSV-G) readily survive the concentration process. Production of pseudotyped recombinant virus with the VSV-G–env requires that the gag-pol-env expression cassette be subdivided into separate Gag-Pol and Env encoding plasmids. Three plasmids, the first encoding gag-pol, the second encoding VSV-G–env, and the third encoding the vector, must be cotransfected together into an efficiently transduced cell type such as the hk293 line. Unfortunately VSV-G cannot be used in the context of traditional producer cell lines because long-term expression is cytotoxic. Since transiently transfected hk293 cells produce high titers within 2 to 3 days of transfection, however, high-titer virus can be harvested before severe toxicity kills the transient producer cells. The harvest and concentration of media from transiently transfected hk293 cells that produce VSV-G–pseudotyped retrovirus can result in titers in excess of 1 × 10^8 infectious particles per milliliter.

**UTILITY OF RECOMBINANT RETROVIRUSES**

Recombinant retroviruses are most frequently used for 2 purposes: the infection of cultured cells in vitro or the direct infection of cells and tissue in vivo. The use of infected cultured cells for therapeutic transplantation is generally termed ex vivo gene transfer, whereas direct infection of animal or human tissues in a target subject is termed in vivo gene transfer. Since cultured cells are most often also mitotic cells, MLV-based retroviruses are frequently used for transfer of novel transgenes for ex vivo applications. For efficient infection of neuronal cell types in vivo, HIV-derived vectors are needed. Both approaches have either been tested or have potential for use on target subjects at all ages and stages of development.

Individual steps of both ex vivo and in vivo gene transfer methods using recombinant retroviruses are outlined in **Figure 2**. For ex vivo applications (Figure 2, right), medium containing a known titer of recombinant retrovirus is used to infect populations of cultured cells. A variety of cell lines and primary cultured cells are amenable to infection; however, to circumvent problems of immune rejection, autologous cells cultured from the host are the most desirable. Skin fibroblasts are readily obtained by biopsy, proliferate in culture, are amenable to infection by recombinant retroviruses, and enter a postmitotic, contact-inhibited growth state following reintroduction into the host body. After molecular and biochemical characterization of the infected cells in vitro, genetically modified fibroblast populations are transplanted into discrete locations within the CNS where they act as a “biological pump,” secreting physiologically and therapeutically relevant levels of neuroptides and neurochemicals into the surrounding tissue.

In vivo application of retroviral gene transfer (Figure 2, left) begins with extensive characterization of the resulting recombinant retrovirus population. In addition to determining the titer of infectious viral particles, one must also screen recombinant retroviruses for the presence of contaminating wild-type or recombinant virus that is capable of replication. These “helper” viruses are a relatively common problem when using stable producer cell lines, but they are rarely, if ever, observed when using transient packaging methods for either lentiviruses or other retroviral types. In addition to assays for helper virus, viral preparations are generally tested on cultured cells to characterize infectivity and the expression level of the transgene. After being tested in vitro, viral supernatants are concentrated by centrifugation to increase the relative titer for direct delivery into the brain or spinal cord (Figure 2, right). The concentrated high-titer virus is then stereotactically injected into discrete locations within the target tissue where viral particles enter cells directly, uncoat, reverse transcribe, and integrate themselves stably into the host cell genome. The transgene is frequently expressed at detectable levels within 24 to 48 hours following integration into the target cell. One distinct advantage of direct gene transfer over ex vivo approaches is that factors with nondiffusible therapeutic value may be used. Expression of a specific transgene may protect an infected cell from the cytotoxic effects of ischemia, target cell loss, or other pathological agents; however, such a factor may have no protective value when secreted or provided from extracellular sources to local cells.
Recombinant retroviruses have many uses both in research and in potential application to the treatment of human disease. Ex vivo retroviral gene transfer for the study or potential amelioration of neurologic disease may be divided into 3 broad categories: the production of cells that secrete therapeutic factors following transplantation, the production of cells that have novel features of growth or differentiation for introduction into the CNS, or the production of cells for the delivery of cytotoxic “suicide” viruses, viruses that either kill infected cells or make them susceptible to a specific cytotoxic agent. In vivo retroviral gene transfer by direct intracerebral injection has also been demonstrated to efficiently deliver transgenes, including nondiffusible factors such as antiapoptotic agents, to a variety of CNS cell types for extended periods of time. The limited scope of this minireview precludes the presentation of an extensive bibliography, so we have selected individual reports (as opposed to reviews) that provide detailed methods and procedures for each general application.

The Biological Pump

There have been numerous studies examining the potential of retrovirally transduced cells to produce and secrete neurotransmitters and neurotrophic or tropic proteins in animal models of CNS disease or trauma. Of neurotransmitter-replacement therapies, many studies have focused on ex vivo cell transplantation to address the loss of dopamine in Parkinson disease. Levodopa treatment, and to a lesser extent the transplantation of fetal tissue, suggests that local restoration of dopamine levels within the striatum can ameliorate many of the most severe symptoms of Parkinson disease. A variety of cell types, including primary fibroblasts, have been infected with recombinant retroviruses encoding tyrosine hydroxylase (TH), a key enzyme in the synthesis of dopamine. Cells infected with TH secrete high levels of levodopa, which is converted after transplantation to

**Applications of Recombinant Retroviruses**

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**Figure 2.** In vivo and ex vivo therapeutic uses of recombinant retroviruses are schematically illustrated. PCR indicates polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.
dopamine by endogenous dopa decarboxylase. The utility of TH-transduced cells to supplement local dopamine levels has been demonstrated in rats with dopaminergic neuron-specific hydroxydopamine lesions of the nigral-striatal pathway. Apomorphine injections of animals with unilateral lesions result in a rotational behavior that is directly proportional to the asymmetry in the dopamine level. Fisher and colleagues have reported that transplantation of TH-expressing primary fibroblasts into the dopamine-depleted striatum can reduce apomorphine-induced rotation by as much as 65% within 2 weeks of transplantation. Through the use of autologous skin fibroblasts instead of transformed cell lines, the risk of immune rejection or the formation of tumors was eliminated. Studies of methods to extend the duration of transgene expression in transplanted TH-expressing cells, and to improve the efficiency of function by providing other enzymes within the dopamine metabolic pathway, are currently under way and will refine this approach for future clinical use.

Neurotrophin-producing cells have been used as possible therapeutic agents in the adult CNS. Senut et al have described the engraftment of neurotrophin-3–secreting primary autologous fibroblasts in the rat CNS and in the spinal cord. While neurotrophin-3–secreting cells induced no dramatic sprouting response from neighboring cells in several locations within the adult brain, transplants of neurotrophin-3–secreting cells to the adult spinal cord elicited dramatic ingrowth of neurites. Studies of many neurotrophic factors that include neurotrophin-3 are currently under way to further examine their potential in the regeneration of damaged nerve fibers following spinal cord injury. Gene-based therapies are particularly attractive as most conventional therapeutic approaches have achieved very limited success in the restoration of lost spinal nerve function.

Customized Cell Types for Transplantation

A second broad application of recombinant retroviruses to ex vivo therapy for disease of the CNS is in the modification of cultured cells to better adapt them for survival or function following transplantation. Most frequently, this application of recombinant retroviruses is used to convert neuronal cell types that are normally not amenable to prolonged culture and expansion in vitro into populations of cells that may be used for therapeutic replacement of damaged or missing CNS tissue. Snyder et al have reported the infection of cells harvested from 4-day-old mouse cerebellum with a recombinant retroviral vector encoding the oncogene v-myc. The v-myc–transformed cells, unlike the original primary population, were capable of extended culture in vitro. Using a second retrovirus encoding a LacZ reporter gene, the v-myc–transformed cells were further modified to allow their identification following introduction into the CNS. When the doublly modified cells were implanted into either juvenile or adult animals, they were found to be multipotent and able to generate neurons, astrocytes, and oligodendrocytes in situ. In addition to multipotentiality, the engrafted cells took cues from the local environment to form appropriate connections and cytoarchitecture, essentially blending into the area in which they were introduced.

A similar approach has been used with a novel retroviral vector in which the transforming v-myc oncogene is capable of regulation by an exogenous factor. Hoshimaru et al have produced a line of rat neuronal progenitor cells by infecting adult FGF-2–responsive hippocampal precursors (hcn-v-myc) with a tetracycline-regulated v-myc-expressing retrovirus termed LINX–v-myc. Following LINX–v-myc infection, a clonal population of rapidly dividing cells was isolated and further characterized. When these cells were treated with analogs of the antibiotic tetracycline, v-myc expression was repressed, resulting in differentiation of the cells to adopt a neuronal morphological structure and expression of antigens characteristic of mature neurons. Derepression through the removal of tetracycline did not result in a resumption of cell proliferation or a loss of neuronal markers, suggesting that once maturity had been reached, the cell was left in a permanently differentiated post-mitotic state. Subsequent studies further defined the capacity of these cells to differentiate and participate in neuronal architecture after transplantation. Both the hcn–v-myc cells and the multipotent v-myc–transformed cerebellar progenitors clearly demonstrate the potential of retroviral growth altered cells to participate in the development of future ex vivo approaches to CNS gene therapy.

Suicide Gene Delivery

Among the most aggressive and least treatable forms of cancer are brain tumors arising from the malignant transformation of glial cells. In our general review of gene therapy for neurological disease 5 years ago, animal studies suggested that the use of recombinant retroviruses for the treatment of this form of cancer may be very effective. In theory, retroviruses carrying suicide genes, genes that either kill infected cells or make them susceptible to a specific cytotoxic agent, could be used as anticancer agents. The most common suicide gene used to date is the herpes simplex virus thymidine kinase gene that renders actively dividing and expressing cells sensitive to the antiviral drug ganciclovir. Since MLV-based retroviruses exclusively infect mitotic cells, cells of the tumor are preferentially infected and subjected to the killing effect. To enhance the titer of introduced retrovirus, the producer cells themselves are injected into the tumor where they reside for days or even weeks releasing infectious virus into the surrounding tissue. After sufficient time for infection of much of the tumor, the cytotoxic agent is injected systemically and results in the death of the infected tumor cells as well as the original producer cell population (if immune surveillance has not yet eliminated them).

Unfortunately the success of retrovirus-mediated anticancer strategies observed in animal glioma models has not translated to clinical studies in human subjects because populations of transformed cells escape infection. The extent of antitumor activity is well documented in a recent report by Ram et al, who clearly show that poor infection of the
tumor mass is the primary obstacle to large-scale destruction of targeted glomas. In most cases, only cells immediately adjacent to virus-producing cells were directly infected and subject to the killing effects of ganciclovir. In addition, killing of neighboring glioma cells by “bystander effect,” cell-to-cell transferal of thymidine kinase via gap junctions, was not observed to proceed as efficiently in the human glomas as in animal tumor models. Two methods of increasing the efficacy of thymidine kinase–ganciclovir (or other suicide gene) therapy on human glomas are to pharmacologically increase gap junction formation within the tumor, thereby increasing bystander–effect killing, or to saturate the gloma and surrounding tissue with sufficient virus to ensure infection of many more cells than can currently be infected by transplanted producer cells. Recently, Feng et al. have devised a potential approach to the latter problem by using a replication-defective adenovirus encoding retroviral packaging proteins and a second adenovirus encoding a replication-defective retroviral genome that complement each other to produce an infectious retroviral particle. Adenovirus can be grown to titers in excess of $1 \times 10^{10}$/ml and readily infects a wide variety of cells in vivo. Chimeric viral vectors such as these could conceivably be used to make a very large population of producer cells in situ (either from the gloma cells themselves or neighboring nontransformed cell types) that may surpass the retroviral production of graded producer cells. Other antiangiogenic factors such as cytokine- or antiangiogenic factors combined with improved vector titers and even genetic antitumor “vaccines” may yet prove very effective agents in future treatments of glioma.

**Gene Transfer to Neuronal Cells In Vivo**

Historically, neurons of the adult nervous system have been refractory to direct gene transfer with recombinant retroviruses. In 1996, Naldini et al. described efficient gene transfer to neurons in vivo using a replication-defective retroviral vector derived from HIV. Vesicular stomatitis virus G-protein–pseudotyped HIV-based virions carrying a LacZ reporter gene were injected bilaterally into the hippocampus or striatum of female adult rats and the distribution and level of expression were examined at 1-week and 1-month intervals. Immunolocalization of $\beta$-galactosidase with glial or neuronal markers revealed that both classes of cells were infected and were readily detectable at both the early and late time points. Subsequent reports and work in progress suggest that lentiviral-based vectors continue to express for at least 18 months after injection.

Lentiviral vectors carrying either nerve growth factor (NGF) or the antipoptotic factor Bcl-xL were recently shown to partially protect septal cholinergic neurons from axotomy-induced cell death. Fimbria fornix-lesioned rats receiving either saline or a lentiviral vector encoding the green fluorescent protein displayed a 52% loss of septal cholinergic cells, whereas animals receiving an NGF or Bcl-xL–bearing vector prior to lesion lost only a respective 27% or 41% of ChAT-positive (cholinergic) cells. While a diffusible factor such as NGF could be delivered by any number of methods, including transplantation of NGF-secreting nonneuronal cells, the retroviral introduction of neuroprotective proteins such as Bcl-xL may only be achieved through the use of the HIV-derived vectors. Because lentiviral vectors have been described only recently, studies to date have just scratched the surface with regard to their full therapeutic potential within the CNS.

**PROSPECTS OF RETROVIRAL THERAPY FOR NEUROLOGIC DISEASE**

Recombinant retroviral vector technologies to both study and potentially combat CNS disease grow increasingly sophisticated with the passage of time. Transient retroviral production permitting concentration of virus to high titers, the development of vectors from retroviruses like HIV with novel properties, chimeric viral vectors, and the recent emergence of retroviral vectors that can be regulated by exogenous ligands all suggest that we are on the verge of a renaissance in the use of these vectors to understand the healthy—and repair the damaged—CNS.

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