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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
A61K 48/00, C12N 15/86, 5/22, 5/16, 15/52, 15/38	**A1**	**WO 95/09654**
		(43) International Publication Date: 13 April 1995 (13.04.95)

(21) International Application Number: PCT/US94/10977

(22) International Filing Date: 28 September 1994 (28.09.94)

(30) Priority Data:
08/131,085 1 October 1993 (01.10.93) US

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

Published
With international search report.

(54) Title: GENE THERAPY OF THE NERVOUS SYSTEM

(57) Abstract

A method of treating an adverse condition of the nervous system in a chordate host (preferably a mammalian host) comprising administering to the cerebrospinal fluid of the host an expression vehicle capable of transducing a cell in order to express a therapeutic agent in the nervous system. The expression vehicle includes a nucleic acid sequence encoding a therapeutic agent for treating an adverse condition of the nervous system, and is administered in an amount effective in treating the adverse condition of the nervous system. The method of the present invention is particularly applicable to the treatment of meningeal carcinomatosis, wherein producer cells containing a retroviral vector including a gene encoding a negative selective marker (such as Herpes Simplex thymidine kinase) are administered to the cerebrospinal fluid of a patient. Such producer cells generate viral particles which transduce the meningeal carcinomatosis cells. The transduced meningeal carcinomatosis cells then are killed upon administration of an interaction agent (such as ganciclovir) which interacts with the negative selective marker.

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GENE THERAPY OF THE NERVOUS SYSTEM

This invention relates to the treatment of adverse conditions of the nervous system, including, but not limited to tumors of the nervous system such as, for example, meningeal carcinomatosis. More particularly, this invention relates to the treatment of adverse conditions of the nervous system, preferably the central nervous system by administering, to the cerebrospinal fluid of a host, an expression vehicle such as a viral vector contained in a viral producer cell line which produces modified viral particles which include a nucleic acid sequence encoding a therapeutic agent for treatment of adverse conditions of the central nervous system, whereby the modified viruses deliver the nucleic acid sequence encoding the therapeutic agent to cells in the central nervous system.

Meningeal carcinomatosis, also known as leptomeningeal carcinomatosis, occurs in from about 5% to about 20% of all cancer patients and results most commonly from the metastatic spread of cancers such as lung cancer, breast cancer, and melanoma to the leptomeningeal coverings of the brain and spinal cord. (Clamon, et al., Breast Cancer Res. Treat., Vol. 9, pgs. 213-217 (1987); Liaw, et al., Taiwan I Hsueh Hui Tsa Chih, Vol. 91, pgs. 299-303 (1992); Nakagawa, et al., J. Neurooncol., Vol. 13, pgs. 81-89 (1992);

Neetens, et al., Bull. Soc. Belg. Opthamol., Vol. 215, pgs. 109-115 (1985); Shapiro, West. J. Med., Vol. 154, pgs. 350-351 (1991)). Standard therapy for meningeal carcinomatosis is comprised of radiation therapy and/or intrathecal administration of chemotherapeutic agents. The need to irradiate the entire neuroaxis may cause profound bone marrow suppression,which limits the amount of systemic or intrathecal chemotherapy that the patient can tolerate. (Shapiro, 1991).

A novel approach to treat solid brain tumors by in vivo retroviral-mediated transfer of the Herpes Simplex thymidine kinase gene into tumor cells, which confers sensitivity to the antiviral drug ganciclovir, has been described (Culver, et al., Science, Vol. 256, pgs. 1550-1552 (1992); Ram, et al., Cancer Res., Vol. 53, pgs. 83-88 (1993)). Ganciclovir is phosphorylated preferentially by transduced tumor cells and interferes with DNA synthesis. Gene transfer is achieved by infection of tumor cells with murine retroviral vectors carrying the Herpes Simplex thymidine kinase gene and integration of this gene into the genome of the host cell. These vectors are produced continuously by murine vector producer cells that are injected into the tumor mass. Because retroviruses can infect only cells that are synthesizing DNA actively (i.e., replicating cells), a preferential transduction of tumor cells is achieved. This approach now is being evaluated in a clinical trial. (Oldfield, et al., Human Gene Therapy, Vol. 4, pgs. 39-69 (1993)).

Meningeal carcinomatosis and other adverse conditions of the nervous system, may provide another application of this approach. Vector producer cells, which are injected into the cerebrospinal fluid, such as, for example, by injection into the ventricular system or lumbar or subarachnoid space, will circulate in the cerebrospinal fluid, and release viral vectors (such as, for example,

retroviral vector particles) continuously. Such viral vectors will contact tumor-infiltrated leptomeninges, transduce the replicating tumor cells, and enable the selective eradication of the tumor cells with the systemic administration of ganciclovir.

In accordance with an aspect of the present invention, there is provided a method of treating an adverse condition of the nervous system in a chordate host. The method comprises administering to the cerebrospinal fluid of the chordate host an expression vehicle capable of transducing a cell in order to express a therapeutic agent in the central nervous system. The expression vehicle includes a nucleic acid sequence encoding a therapeutic agent for treating the adverse condition of the central nervous system. The expression vehicle is administered in an amount effective in treating the adverse condition of the central nervous system in the host. The cells which may be transduced with the expression vehicle include tumor cells and normal (i.e, non-tumor) cells, such as epithelial or endothelial cells of the choroid plexus.

The term "chordate" as used herein means any animal of the phylum Chordata; i.e., any animal which includes a notochord or analogous structure, such as a spinal cord. Such animals include, but are not limited to, mammals (including human and non-human mammals), reptiles, amphibians, birds, and fish.

The term "nervous system" as used herein, means any part of the animal which has a neurological and/or neuromotor function. Such parts include, but are not limited to, the brain, spinal cord, cranial nerves, and other nerves which are essential for neuromotor function.

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.

Although the scope of the present invention is not limited by any theoretical reasoning, the expression vehicle, upon administration to the cerebrospinal fluid to a host, travels throughout the cerebrospinal fluid to cells in the central nervous system, whereby a nucleic acid sequence encoding a therapeutic agent is delivered to cells in the central nervous system, and whereby the therapeutic agent is expressed by such cells. Cells which may be transduced include, but are not limited to, tumor cells of the central nervous system, brain cells, cells of the cranial nerves and other nerves essential for neuromotor function, and spinal cord cells. The type of cell to be transduced by the expression vehicle and the therapeutic agent is dependent upon the type of adverse condition of the nervous system to be treated.

The term "adverse condition of the nervous system" as used herein includes any disease of the nervous system which may be treated by gene therapy in which an expression vehicle including a nucleic acid sequence is transduced into a cell of the nervous system. Such conditions include, but are not limited to, tumors of the central nervous system, Alzheimer's disease, Parkinson's disease, Huntington's disease, degenerative disorders, mental disorders, and a variety of disorders that can be affected by introducing a new compound or modifying the levels of existing proteins in the nervous system.

The expression vehicle may be any expression vehicle which is capable of transfecting cells and expressing the therapeutic agent for treating an adverse condition of the central nervous system in vivo. Suitable expression vehicles which may be employed include, but are not limited to, eukaryotic vectors, prokaryotic vectors (such as, for

example, bacterial plasmids), and viral vectors, DNA-protein complexes, such as DNA-monoclonal antibody complexes, and receptor-mediated vectors. The vector may be contained within a liposome.

In one embodiment, the expression vehicle 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. In one embodiment, 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 for treating an adverse condition of the nervous system to form a producer cell line including the viral vector. The producer cells then are administered to the cerebrospinal fluid of the host, whereby the producer cells generate viral particles which circulate throughout the cranio-spinal subarachnoid space in the cerebrospinal fluid and are capable of transducing cells, whereby such viral vectors express the therapeutic agent in such cells.

In one embodiment, the adverse condition of the nervous system is a tumor of the nervous system, such as, for example a tumor which infiltrates the leptomeningeal coverings of the central nervous system. Preferably, such tumor of the central nervous system is treated by administering to the cerebrospinal fluid of the host producer cells which are transformed with a viral vector, and which produce a virus including a nucleic acid sequence encoding a therapeutic agent capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells. Upon administration of the producer cells to the cerebrospinal fluid, the viruses produced by the producer cells circulate through the cerebrospinal fluid and transduce the tumor cells, whereby the agent which is capable of providing for the inhibition, prevention, or

destruction of the growth of the tumor cells is expressed by the tumor cells.

Tumors of the nervous system which may be treated include, but are not limited to, meningeal carcinomatosis, resulting from a peripheral or a primary brain tumor such as a medulloblastoma; ependynoma; and tumors of the lumbosacral region of the spinal column. It is to be understood, however, that the scope of the present invention is not to be limited to the treatment of tumors of the nervous system.

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 are 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 ($Ppr80^{gag}$). 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 $pPr80^{gag}$. 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 hereinabove 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 ß-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The promoter also may be a tissue-specific or tumor-specific promoter. 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, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψ CRE, ψ CRIP, GP+E-86, GP+envAm12, and DAN cell lines, as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990). The vector containing the nucleic acid sequence encoding the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence encoding the 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 $CaPO_4$ precipitation.

The producer cells then are administered to the cerebrospinal fluid in an amount effective to inhibit, prevent, or destroy the growth of the tumor. The producer cells may be administered in an amount of from about $1x10^9$ to about $1x10^{10}$ cells per dose, preferably from about $6x10^9$ to about $1x10^{10}$ cells per dose. The exact amount of producer cells to be administered is dependent upon various factors, including,

but not limited to, the type of the tumor and the size of the tumor. In some cases, repeat administration of the producer cells may be required.

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. The producer cells may be administered to the cerebrospinal fluid intraventricularly, intrathecally, such as through the spinal subarachnoid space, or the producer cells may be administered to the choroid plexus, which may provide for continuous generation of viral vector particles into the cerebrospinal fluid.

Upon administration of the producer cells to the cerebrospinal fluid, the producer cells generate viral vector particles. The viral particles circulate throughout the cerebrospinal fluid, and transduce the tumor cells. Because tumor cells, and in particular cancerous tumor cells, in general are actively replicating cells, the retroviral particles would be integrated into and expressed preferentially or exclusively in the tumor cells as opposed to normal cells.

Tumors of the nervous system which may be treated include malignant and non-malignant tumors.

In accordance with the present invention, the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of such agent is a negative selective marker; i.e., a material which in combination with a chemotherapeutic or interaction agent inhibits, prevents or destroys the growth of the tumor cells.

Thus, upon transduction of the tumor cells with the negative selective marker, an interaction agent is administered to the human host. The interaction agent

interacts with the negative selective marker in order to prevent, inhibit, or destroy the growth of the tumor cells.

Negative selective markers which may be employed include, but are not limited to, thymidine kinase, such as Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase; and cytosine deaminase.

In one embodiment, the negative selective marker is a viral thymidine kinase selected from the group consisting of Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase. When such viral thymidine kinases are employed, the interaction or chemotherapeutic agent preferably is a nucleoside analogue, for example, one selected from the group consisting of ganciclovir, acyclovir, 1-2-deoxy-2-fluoro-β-D-arabinofuranosil-5-iodouracil (FIAU), and 6-methoxypurine-arabinonucleoside (araM). Such interaction agents are utilized efficiently by the viral thymidine kinases as substrates, and such interaction agents thus are incorporated lethally into the DNA of the tumor cells expressing the viral thymidine kinases, thereby resulting in the death of the tumor cells.

In another embodiment, the negative selective marker is cytosine deaminase. When cytosine deaminase is the negative selective marker, a preferred interaction agent is 5-fluorocytosine. Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is highly cytotoxic. Thus, the tumor cells which express the cytosine deaminase gene convert the 5-fluorocytosine to 5-fluorouracil and are killed.

The interaction agent is administered in an amount effective to inhibit, prevent, or destroy the growth of the transduced tumor cells. For example, the interaction agent may be administered in an amount from about 5 mg/kg to about 10 mg/kg of host weight, depending on overall

toxicity to a patient. In one embodiment, such dose may be administered twice per day. The interaction agent preferably is administered systemically, such as, for example, by intravenous administration, by parenteral administration, by intraperitoneal administration, or by intramuscular administration.

When producer cells or other expression media including a negative selective marker are administered to a tumor in vivo, a "bystander effect" may result, i.e., tumor cells which were not originally transduced with the nucleic acid sequence encoding the negative selective marker may be killed upon administration of the interaction agent. Although the scope of the present invention is not intended to be limited by any theoretical reasoning, the transformed tumor cells may be producing a diffusible form of the negative selective marker that either acts extracellularly upon the interaction agent, or is taken up by adjacent, non-transformed tumor cells, which then become susceptible to the action of the interaction agent. It also is possible that one or both of the negative selective marker and the interaction agent are communicated between tumor cells. Such bystander effect is described further in U.S. patent application Serial No. 07/877,519 filed May 1, 1992, which is incorporated herein by reference.

In a preferred embodiment, a packaging cell line is transduced with a retroviral vector, such as those hereinabove described, which includes the Herpes Simplex Virus thymidine kinase gene. The transduced packaging cells (producer cells) are administered in vivo to the cerebrospinal fluid in an acceptable pharmaceutical carrier and in an amount effective to inhibit, prevent, or destroy the growth of the tumor. Upon administration of the producer cells to the cerebrospinal fluid, the producer cells generate viral vector particles including a gene encoding the negative selective marker. Such viral

particles circulate throughout the cerebrospinal fluid and transduce the tumor cells. The human host then is given an agent such as ganciclovir, acyclovir, or 1-2-deoxy-2-fluoro-β- D-arabinofuranosil-5-iodouracil (FIAU), which interacts with the Herpes Simplex Virus thymidine kinase to kill the transduced tumor cells. As hereinabove mentioned, a "bystander effect" may also occur, whereby non-transduced tumor cells also may be killed as well.

In another embodiment, the expression vehicle is an adenoviral vector.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome. Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In one embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR'; an adenoviral encapsidation signal; at least one DNA sequence encoding a therapeutic agent; and a promoter controlling the at least one DNA sequence encoding the therapeutic agent. The vector is free of the adenoviral E1, E2, E3 and E4 DNA sequences, and the vector is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter; i.e., the vector is free of DNA encoding adenoviral structural proteins.

Such vectors may be constructed by removing the adenoviral 5' ITR, the adenoviral 3' ITR, and the adenoviral encapsidation signal, from an adenoviral genome by standard techniques. Such components, as well as a promoter (which may be an adenoviral promoter or a non-adenoviral promoter), tripartite leader sequence, poly A signal, and selectable marker, may, by standard techniques, be ligated into a base plasmid or "starter" plasmid such as, for example, pBluescript II KS-(Strategene), to form an

appropriate cloning vector. The cloning vector may include a multiple cloning site to facilitate the insertion of the at least one DNA sequence encoding a therapeutic agent into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector in accordance with the present invention is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning site, and then ligating the at least one DNA sequence encoding a therapeutic agent into the cloning vector.

The vector may then be packaged into infectious viral particles using a helper adenovirus which provides the necessary encapsidation materials. Preferably the helper virus has a defective encapsidation signal in order that the helper virus will not encapsidate itself. An example of an encapsidation defective helper virus which may be employed is described in Grable, et al., J. Virol., Vol. 66, pgs. 723-731 (1992).

The vector and the encapsidation defective helper virus are transfected into an appropriate cell line for the generation of infectious viral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes. Examples of appropriate cell lines include, but are not limited to, HeLa cells or 293 (embryonic kidney epithelial) cells. The infectious viral vector particles may then be transduced into cells in the central nervous system, whereby the at least one DNA sequence encoding a therapeutic agent is expressed by the cells in a host.

In another embodiment, the vector comprises an adenoviral 5′ ITR; an adenoviral 3′ ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a

therapeutic agent; and a promoter controlling the at least one DNA sequence encoding the therapeutic agent. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter. In one embodiment, the vector is also free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences. In another embodiment, the vector is free of at least the majority of the adenoviral E1 and E3 DNA sequences, and is free of one of the E2 and E4 DNA sequences, and is free of a portion of the other of the E2 and E4 DNA sequences.

In yet another embodiment, the vector is free of at least the majority of the E1 and E3 DNA sequences, is free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences, and is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a tripartite leader sequence, a multiple cloning site (which may be as hereinabove described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. Such DNA segment serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin

of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAVS6, shown in Figure 14. A desired DNA sequence encoding a therapeutic agent may then be inserted into the multiple cloning site. Homologous recombination then is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-transfection of the shuttle plasmid and the modified adenovirus into a helper cell line, such as 293 cells, by $CaPO_4$ precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed which includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the adenovirus 5 genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter; a tripartite leader sequence; at least one DNA sequence encoding the therapeutic agent; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. This vector may then be transfected into a helper cell line, such as the 293 helper cell line, which will include the E1a and E1b DNA sequences, which are necessary for viral replication, and to generate infectious viral particles.

The vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA sequence encoding a therapeutic agent is administered in an amount effective to treat the adverse condition of the

central nervous system in a host. In one embodiment, the vector particles may be administered in an amount of from 1 plaque forming unit to about 10^{14} plaque forming units, preferably from about $1x10^{6}$ plaque forming units to about $1x10^{13}$ plaque forming units. The host may be a human or non-human animal host.

The vector particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, micro- carrier beads.

Although the present invention has been described with respect to the treatment of tumors of the nervous system, it is to be understood that the method of the present invention may be employed to deliver other therapeutic gene products to the nervous system by circulation through the cerebrospinal fluid after intrathecal, intraventricular, intraspinal injection, injection into the subarachnoid space, or injection into the choroid plexus. Such gene products include proteins and peptides that are neurotransmitters, neuromodulators, neurohormones, and neurotrophic factors. Thus, the method of the present invention may be employed to treat other adverse conditions of the nervous system, including, but not limited to, traumatic injury, stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, degenerative disorders, mental diseases, and a variety of disorders that can be affected by introducing a new compound or modifying the levels of existing proteins in the cerebrospinal fluid. Thus, the method of the present invention may be employed to transduce normal cells or tissue in the nervous system in order to treat non-cancerous adverse conditions of the nervous system.

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

A. Construction of pG1TkSvNa

The following describes the construction of pG1TkSvNa, a schematic of which is shown in Figure 6. 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^R) driven by an SV40 promoter. The HTK gene confers sensitivity to the DNA analogs acyclovir and ganciclovir, while the Neo^R gene product confer resistance to the neomycin analogue, G418.

To make pGITkSvNa, a three step cloning strategy was used. First, the herpes simplex thymidine kinase gene (Tk) was cloned into the G1 plasmid backbone to produce pG1Tk. Second, the Neo^R gene (Na) was cloned into the plasmid pSvBg to make pSvNa. Finally, SvNa was excised from Psvna and ligated into pG1Tk to produce pG1TkSvNa.

Plasmid pG1TkSvNa 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^r gene, the ß-galactosidase gene, the hygromycin^r gene, and the SV40 promoter.

To construct pBg (Figure 4) 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 - BglII - 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 - SmaI - 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 Bg1II/PvuII fragment containing the Herpes Simplex Virus Type I thymidise 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 5).

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 Pbg in the unique NruI site to generate the plasmid pSvBg (Figure 5). The pSvBg plasmid was digested with Bg1II/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 hereinabove, 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^r fragment to generate pG1TkSvNa. (Figure 6).

B. Generation of Producer Cell Line

A producer cell line was made from vector plasmid and packaging cells. The PA317/G1TkSvNa producer cell was made by the same general techniques used to make previous clinically relevant retroviral vector producer cell lines. The vector plasmid pG1TkSvNa DNA was transfected into a ecotropic packaging cell line, PE501. Supernatant from the PE501 transfected cells was then used to transinfect the amphotropic packaging cell line (PA317). Clones of transinfected producer cells were then grown in G418 containing medium to select clones that contain the Neo^R 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 10^5 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. The cells were incubated at 37°C, in 5% CO_2/air overnight.

The plasmid pG1TKSvNa then was transfected into PE501 cells by $CaPO_4$ precipitation using 50 μg of DNA by the following procedure.

50 μg of DNA, 50 μl 10 x $CaCl_2$, and 450μl of sterile H_2O was mixed in a 15 ml polypropylene tube to yield a

0.25M Ca Cl_2 solution containing 50 μg DNA, 0.5 ml 2x BBS (containing 50 mM N-N-bis- (2-hydroxyethyl)- 2-aminoethane-sulfonic acid, 280 mM Na Cl, 1.5 mM Na_2 HPO_4, and 50 mM Hepes, pH6.95). The DNA solution then was left at room temperature for about 20 minutes to 1 hour. The dishes then were incubated at 35°C in a 3% CO_2 atmosphere overnight.

A culture dish(es) with optimum precipitate following the overnight incubation then was (were) selected. The dish(es) then was (were) 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% CO_2 atmosphere for about 48 hrs.

After 48 hours, supernatant was collected from the transfected cells. The dish(es) then was (were) rinsed with 5 ml PBS. The PBS then was removed, and cells were removed with trypsin-EDTA. Serial dilutions of the cells were then inoculated into six 100 mm dishes in medium containing HGD10 and 0.8 mg/ml G418.

The six plates of cells were examined daily. The medium was changed as needed to remove dead cells. Live cells or colonies were allowed to grow to a size such that the colonies are large enough to clone (i.e., the colonies are visible to the naked eye). PE501 ecotropic containing supernatants from such colonies of PE501 cells were collected in volumes of from about 5 to 10 ml, placed in cryotubes, and frozen in liquid nitrogen at -70°C.

PA317 cells (Miller et al. Mol. Cell. Biol. 6:2895-2902 (1986)) then were plated at a density of 5 x 10^4 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 PE501 supernatant then was thawed, and 8 μg/ml of polybrene was added to the supernatant. The medium was

aspirated from the plates of PA317 cells, and 7 to 8 ml of viral supernatant was added and incubated overnight.

The PE501 supernatant then was removed and the cells refed approximately 18-20 hours 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 as necessary. The plate was monitored for at least 10 to 14 days for the appearance of G418 resistant colonies.

Cloning rings were placed around all selected colonies. The cells were tyrpsinized and incubated into wells in a six well dish in 5 ml of HGD10 plus 1x hypoxanthine aminopterin thymidine (HAT).

If the clones grew to confluency, they were trypsidized and incubated in a 100 ml dish. As a clone in the 100 ml dish approached confluency, its amphotropic vector-containing supernatant was removed and centrifuged at 1,200 to 1,500 rpm for 5 minutes to pellet out cells.

Supernatants were aliquoted into six cryovials (1 ml/vial) and stored in liquid nitrogen. 5 ml of PBS were added to the dish, and the cells were rinsed, and refed with HGD-10 and frozen in 1 ml aliquots with 10% DMSO in liquid nitrogen. The different clones were monitored to determine the one with the highest titer of retroviral vector.

The clone with the highest titer, designated as producer cell line PA317/G1TkSvNa.53, was used to produce a master cell bank.

C. Preparation of pG1TK1SvNa

The plasmid pG1TK1SvNa (Figure 8), was prepared according to the schematic representation shown in Figure 7. It was prepared to remove the partial open reading frame from pG1TKSvNa (Figure 6).

Generation of pSPTK5':

DNA from the plasmid pG1NaSvTk was digested with restriction enzymes BgIII and SmaI and the 1163 base pair (bp) Herpes thymidine kinase (TK) fragment was fractionated by agarose gel electrophoresis and isolated. This fragment contains 56 bp of the TK 5'-untranslated region and 1107 bp of the TK translation open reading frame. The 1163 bp TK fragment was ligated to the plasmid vector pSP73 (Promega Corporation, Madison, WI) that had been digested with restriction enzymes BgIII and SmaI. The resulting ligated plasmid construct was named PSPTK5' because it contains the 5' portion of the TK open reading frame but lacks the last 21 bp of the open reading frame and the translation termination codon.

PCR of the TK open reading frame:

pG1NaSvTK plasmid DNA was linearized by digesting it with BgIII. The linearized pG1NaSvTK was used as a template for polymerase chain reaction (PCR) using a forward primer that contains the first 17 bases of the TK open reading frame (5'-GCACCATGGCTTCGTACCCCTGC-3') and a reverse primer that contains complementary sequence for an XhoI site, the TK translation termination codon, and the last 19 bp of the TK open reading frame (5'-CCTGCATCGATTCTCGAGTCAGTTAGCCTCCCCCATCTCC-3'). 30 cycles of PCR were performed as follows: 1 minute at 94°C and 2 minutes at 60°C with a final 7 minute extension cycle at 72°C. PCR products were fractionated on an agarose gel and the expected 1215 bp fragment that includes the full-length TK open reading frame was isolated. The isolated fragment was digested with restriction enzymes PstI and XhoI, digestion products were fractionated on an agarose gel, and the 420 bp fragment was isolated. This fragment extends from the PstI site at the nucleotides encoding amino acids 249-250 of the TK open reading frame through the XhoI site immediately downstream of the TGA translation termination codon.

Generation of pSPTK1:

PSPTK5' was digested with PstI and the 3993 bp fragment that contains the PSP73 vector and the 5' portion of the TK open reading frame was isolated following agarose gel electophoresis. This 3993 bp fragment was ligated to the PCR-generated 420 bp PstI/XhoI fragment that contains the 3' end of the TK open reading frame (above). Ligated plasmid DNA was transformed into E. coli DK5α competent cells (Gibco/BRL, Gaithersburg, MD) and DNA from ampicillin-resistant colonies was screened by restriction enzyme digestion. Plasmids that appeared to contain the full-length TK open reading frame were termed PSPTK1. The DNA from several PSPTK1 clones was dideoxy sequenced in the region from the PstI site through the XhoI site (the region that was generated by PCR). PSPTK1 clone #4 was found to match the expected TK sequence in this region and was used for construction of pG1TK1SvNa.

Generation of pG1TK1SvNa:

PSPTK1 DNA was digested with BglII and the 5' overhanging ends were repaired by incubation of the digested DNA with deoxy nucleotides and Klenow fragment of E. coli DNA polymerase I. The DNA was then digested with XhoI to generate a 1225 bp fragment that contains 56 bp of TK 5'-untranslated region and the full-length TK open reading frame. This blunt/XhoI fragment was ligated to pG1XSvNa DNA that had been digested with SnaBI and SalI.

To construct pG1XSvNa, the 1.2 kb SvNa fragment was excised from Psvna (Part A above) with SalI and HindIII. This fragment was ligated to pG1 that had been digested with SalI and HindIII. The ligated plasmid was termed pG1XSvNa where the "X" denotes a multiple cloning region.

The product DNA from the pG1XSvNa and TK ligation was transformed in DH5α and DNA from ampicillin-resistant colonies was screened as previously described. Plasmids that appeared to contain the TK fragment by diagnostic

restriction enzyme digestion were termed pG1TK1SvNa. (Figure 8). Clone #2 was dideoxy sequenced from the beginning of the 5'-LTR through the end of the 3'-LTR and was found to contain the intact TK open reading frame.

pG1TK1SvNa was used to produce a producer cell by combination with PA 317 by the hereinabove described method (Part B above). Such producer cell line was designated as producer cell line PA317/G1TK1SvNa.7.

D. Effect of cerebrospinal fluid on Herpes Simplex thymidine kinase producer cells and vectors; assessment of retroviral vector titer.

Each of the producer cell clones PA317/G1TkSvNa.53 and PA317/G1Tk1SvNa.7 was inoculated into two 6-well dishes (12 wells/clone) at 4-5x10^5 cells/well (37°C, 5%CO_2, for 24 hours or until nearly confluent).

Duplicate wells were exposed to increasing concentrations of cerebrospinal fluid (CSF) in growth medium (0%, 5%, 10%, 25%, 50%, and 75%). The cultures were observed microscopically over a 48-hour period (10 min., 1 hour, 4 hours, 24 hours, and 48 hours) for cytopathic effect (CPE). Supernatant samples then were collected for titer assay. Independently, supernatant samples of known titer (G1TkSvNa.53 and G1Tk1SvNa.7) were mixed with CSF and titered. Serial dilutions of the tested supernatant (10^0, 10^{-1}, 10^{-2}, 10^{-3}) were made in growth medium containing 8 μg/ml polybrene and then incubated for 60 min. at room temperature. Three ml of the diluted material were added to each well containing NIH 3T3 cells. The dishes were incubated at 32°C and 5% CO_2 and after 16 to 20 hours, the medium was changed to growth medium containing 0.8 mg/ml G418 (a neomycin analogue) and incubated at 37°C. The cells were grown in this medium until individual colonies (originating from cells that were transduced with the neomycin-resistant gene and thus protected from the toxic effect of G418) were visible microscopically. The cells

were then stained with methylene blue and the colonies counted. The titer per ml at each dilution was determined by calculating the average number of colonies in the wells, multiplying by the dilution factor, and dividing by 3 ml. The average titer was determined by calculating the average of the titers for all dilutions. The supernatants used as positive control (undiluted with CSF) had a neomycin-resistant gene titer between 1 x 10^5 and 4 x 10^6 particles/ml (PA317/G1Tk1SvNa.7) and 10^3 to 10^4 (PA317/G1TkSvNa.53).

No cytopathic effects were seen in the producer cells at 10 minutes, 1 hour, 4 hours, 24 hours, and 48 hours after exposure to human cerebrospinal fluid. Vector production for each clone exposed to the varying concentrations of CSF is given in Table I below.

Table I

Cell Line	% CSF	G418-Resistant Titer
PA317/G1TkSvNa.53	0	$1.1x10^3$
PA317/G1TkSvNa.53	5	$1.0x10^3$
PA317/G1TkSvNa.53	10	$0.9x10^3$
PA317/G1TkSvNa.53	25	$1.9x10^3$
PA317/G1TkSvNa.53	50	$1.9x10^3$
PA317/G1TkSvNa.53	75	$1.8x10^3$
PA317/G1Tk1SvNa.7	0	$4.6x10^4$
PA317/G1Tk1SvNa.7	5	$2.2x10^4$
PA317/G1Tk1SvNa.7	10	$2.8x10^4$

PA317/G1Tk1SvNa.7 $2.0x10^4$	25
PA317/G1Tk1SvNa.7 $3.1x10^4$	50
PA317/G1Tk1SvNa.7 $3.4x10^4$	75

The above results indicate that vector production by the producer cells was not affected by exposure to human cerebrospinal fluid.

Similarly, free Herpes Simplex thymidine kinase vector titer (from supernatants of known titer) was not affected by cerebrospinal fluid during exposure for 60 minutes prior to overnight incubation over NIH 3T3 cells.

E. Toxicity Studies of injection of Herpes Simplex thymidine kinase vector producer cells and ganciclovir.

PA317/G1TkSvNa.53 producer cells were injected (10^6 cells/10 μl) into each of four Spraque-Dawley rats (wt. from 230 to 350 g) via cisternal catheter under brief inhalation anesthesia. The rats were sacrificed on days 5 and 10 after cell injection for histologic examination of the brain and spinal cord with hematoxylin and eosin staining. None of the rats showed any signs of neurologic or systemic toxicity, and there was no histologic evidence of meningeal reaction, or parenchymal brain or spinal cord injury.

In another experiment, PA317/G1TkSvNa.53 producer cells were injected (10^6 cells/10 μl) via cistermal intrathecal catheter into each of four Sprague-Dawley rats (each weighing from 230 to 350 g), and ganciclovir was administered 7 days later (30 mg/kg/day intraperitoneally). The animals were sacrificed on days 3, 6, 10, and 14 of ganciclovir treatment for histologic examination of the brain and spinal cord with hematoxylin and eosin stain. There was no evidence of inflammation of the leptomeninges or parenchymal injury throughout the brain and spinal cord,

and the rats showed no signs of neurologic or systemic toxicity throughout the study.

In yet another experiment, this one involving non-human primates, six female Rhesus monkeys weighing from 6 to 8 kg each received an intraventricular mixture of $1x10^7$ PA317/G1TkSvNa-53 producer cells and $1x10^7$ β-galactosidase producer cells (for detection with X-Gal staining) suspended in 1 ml PBS. The B-galactosidase producer cell line, known as G1BgSvN.29, was formed by transfecting the PA317 cell line with pG1BgSvNa, a vector in which the lacZ gene replaces the Herpes Simplex thymidine kinase gene. One week after the cell injections, ganciclovir was given intravenously to two monkeys (10 mg 1 kg/day for 14 days), and was given intrathecally to two monkeys (200 μg/day for 14 days). Two monkeys received only the producer cell injections. The monkeys receiving the intravenous ganciclovir treatment underwent placement of an intracardiac catheter via the right external jugular vein with a subcutaneous access port placed in the interscapular region. The animals were placed in a stereotactic frame and a midline incision was made to expose sagittal suture and bregma. Using stereotactic coordinates, a ventricular catheter was placed in the right lateral ventricle and its location confirmed by pressure tracing. The producer cells were injected over a period of time of one minute. In the two monkeys which received the intrathecal ganciclovir, the ventricular catheter was left in place and connected to a subcutaneous access port in the interscapular region.

At the completion of the ganciclovir treatment, three monkeys, one from each group, were sacrificed for histologic evaluation of the brains, spinal cords, and peripheral organs. The three remaining monkeys were kept for long-term observation and subsequent repeat intrathecal cell injections.

Blood and cerebrospinal fluid samples were collected before cell injection, 7 days after cell injection, after 7 days of ganciclovir treatment, and at the completion of the ganciclovir treatment. Cerebrospinal fluid was analyzed for cytology, protein and glucose content. Blood samples were analyzed for routine chemistry and hematologic profiles.

Gadolinium-enhanced MRI scans of the brain were obtained before cell injection, 7 days after cell injection, after 7 days of ganciclovir treatment, and at the completion of the ganciclovir treatment. The three monkeys which were not sacrificed each underwent an additional MRI scan 45 days after cell injection. Scans were evaluated for evidence of ventriculomegaly, ependymal enhancement, leptomeningeal enhancement, mass lesions, or hemorrhage.

Cell injections and ganciclovir treatment were tolerated well by all monkeys. No neurological deficits or signs of systemic toxicity occurred in any of the animals up to 3 months after treatment.

Peripheral blood count and serum chemistry profiles remained normal in all animals throughout the study. Cerebrospinal fluid protein and glucose levels remained normal, although the two monkeys treated with ganciclovir intraventricularly developed mild cerebrospinal fluid pleocytosis which was asymptomatic and did not progress to meningitis.

No changes were observed in the MRI scans of the brain in any of the monkeys. There was no evidence of ventricular enlargement, leptomeningeal enhancement, ependymal enhancement, mass lesions or hemorrhage throughout the study.

Peripheral organs of each of the three monkeys which were sacrificed appeared normal to gross anatomic examination. Histologic examination of the brain and

spinal cord showed no evidence of structural damage, inflammation, vasculitis, or demyelination.

Examination of the choroid plexus of the lateral ventricles of the ganciclovir-treated animals (both intrathecal and intravenous administration) showed destruction of the normal papillary architecture and capillary networks. The choroid plexus from the control animal appeared normal. X-Gal staining showed diffuse staining of choroid plexus cells, but no evidence of transduction with the β-galactosidase vector was detected in the brain parenchymal or spinal cord.

Three months after the initial injection of producer cells, the monkeys which were not sacrificed received a repeat intraventricular injection of a higher dose of producer cells ($2x10^8$ PA317/G1TKSvNa 53 producer cells mixed with $1x10^7$ β-galactosidase producer cells in a total volume of 2 ml Pbs). Seven days after cell injection, two of the monkeys received intravenous ganciclovir (10 mg/kg/day) for 14 days. Toxicity was assessed by daily clinical examination, analysis of blood and cerebrospinal fluid samples, and gadolinium-enhanced MRI scans of the brain before cell injection, at the completion of the ganciclovir therapy, and at 9 weeks after cell injection. Also, cisternal and lumbar cerebrospinal fluid samples were obtained by percutaneous aspiration on days 1, 3, and 5 after intraventricular cell injection and evaluated for vector titer and cytology to assess the dynamics of producer cell and vector particle distribution in the subarachnoid space.

Retroviral vector titer was assessed as follows:

Cultured NIH 3T3 cells were grown in DMEM medium containing 10% fetal bovine serum at a density of $1x10^5$ cells/well in 6-well dishes. Serial dilutions of the tested cerebrospinal fluid (0 through $2x10^{-2}$) were made in growth medium containing 8 μg/ml polybrene. 2 ml of the

diluted solution were added to each well. Dilutions of a retroviral supernatant of known titer were used as a positive control. The dishes were incubated at 32°C and 5% CO_2. After 16-20 hours, the medium was changed to growth medium containing 0.8 mg/ml G418 (a neomycin analogue) and incubated at 37°C. The cells were grown in this medium until individual colonies (originating from cells that were transduced with the neomycin resistance gene and thus protected from the toxic effect of G418) were visible microscopically. The cells then were stained with methylene blue and the colonies counted. The titer per ml at each dilution was determined by calculating the average number of colonies in the wells, multiplying by the dilution factor, and dividing by 2 ml. The average titer was determined by calculating the average of the titers for all dilutions. The supernatant used as positive control has a neomycin-resistant gene titer between $1x10^5$ and $4x10^6$ particles/ml.

No neurologic compromise or systemic toxicity occurred during the course of repeat treatment and follow-up of 3 months. Blood and cerebrospinal fluid profiles remained normal throughout the study. MRI scans of the brain did not show any changes in the leptomeningeal signal characteristics, and there was no evidence of ventriculomegaly or parenchymal injury. Vector titers were detected in the cerebrospinal fluid samples 24 hours after repeat cell injections. Comparable titers were found in the cisternal and lumbar cerebrospinal fluid samples.

In another experiment, a mixture of 10^6 PA317/G1TkSvNa.53 producer cells and 10^6 G1BgSvN.29 producer cells in a total volume of 10μl PBS was injected into both lateral ventricles of each of 4 Sprague-Dawley rats (weighing from 230g to 350g) using a stereotactic frame (coordinates AP-.92, L-1.4, Dorso-ventral-3.6, with respect to bregma and dura over a period of 5 minutes using

a 23 gauge needle and 50μl Hamilton syringe. Seven days after the cell injections, two rats received ganciclovir (30 mg/kg intraperitoneally daily for 14 days), while 2 rats served as controls. At the completion of the ganciclovir treatment, the rats were sacrificed by intracardiac perfusion with 4% formaldehyde, and the brains were removed for histologic examination. The choroid plexus was dissected from the lateral and 4th ventricles and stained with X-gal after pre-incubation with EGTA to block endogenous β-galactosidase activity. The choroid plexus from both non-ganciclovir and ganciclovir-treated rats showed significant X-gal staining of both the lateral and 4th ventricle choroid plexus compared to control animals that received no cell injections. There was minimal difference in X-gal staining with same disruption of the choroid plexus in ganciclovir-treated rats and the control rats.

In yet another experiment, each of 6 rats (Sprague-Dawley, 230g to 350g) were injected intrathecally with a low concentration of ganciclovir (5μg/10μl PBS daily for 14 days) or a high concentration of ganciclovir (200μg/10μl PBS daily for 14 days) via an indwelling cisternal catheter. The rats were examined daily for evidence of neurological toxicity, and were sacrificed after the 14 days of ganciclovir treatment for histologic examination of the brain and spinal cord with hematoxylin and eosin staining. There was no evidence of neurologic sequelae in any of the treatment animals, and histologic appearance of the brain and spinal cord in both the high and low-concentration intrathecal ganciclovir treated rats showed no indication of meningeal reaction or parenchymal injury.

F. Administration of producer cells to rats having leptomeningeal gliosarcoma.

In this experiment, the 9L syngeneic glioma model of meningeal carcinomatosis in Fischer rats was employed. (Kooistra, et al., Cancer Res., Vol. 46, pgs. 317-323 (1986)).

The producer cell line PA317/G1TkSvNa.53 was maintained in culture in Dulbecco Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), 2 Mm L-glutamine (Gibco BRL, Gaithersburg, MD), 50 units/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), and 2.5 μg/ml Fungizone (ICN Biomedicals Inc., Costa Mesa, California). The producer cells were grown in T-175 flasks. Producer cells were harvested prior to intrathecal injection by incubation in 0.05% Trypsin-EDTA (Gibco) for 5 to 10 minutes at 37°C. The cells were collected in Hanks Balanced Salt Solution (HBSS) (Biofluids, Inc., Rockville, MD), washed twice, and resuspended at $8x10^6$ cells/ml for injection.

Some of the producer cells also were infected with the replication-competent retrovirus 4070A. In order to infect the cells with such retrovirus, 4070A virus-containing supernatant was filtered through a 0.22 μm filter onto a monolayer of the producer cells. Two passages of the culture were allowed to achieve uniform infection of the producer cells with the 4070A retrovirus.

Fischer 344 rats weighing 230-300 grams were anesthetized using intraperitoneal (i.p.) Ketamine (90 mg/Kg, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and Xylazine (10 mg/Kg, Mobay Corporation, Shawnee, Kansas). A sterile PE-10 tube was inserted into the upper thoracic subarachnoid space via the cisterna magna, secured in the subcutaneous soft tissue, and pierced through the skin on the back of the neck. The tube then was obliterated with a sterile removable steel rod. The rats were then observed

for 5 to 7 days during which any rat that developed neurological deficits was excluded from the study. Rats were housed one per cage to protect the catheters and received oral Amoxicillin (5 mg/kg in water, calculated for an average water consumption of 20 ml/rat/day) and dexamethasone (TechAmerica, Kansas City, MO) in an amount of 0.5 mg/Kg/20 ml for the duration of the study. 159 rats then were reanesthetized, using inhalation anesthesia (N_2O:O_2:Halothane mixture), and $8x10^4$ syngeneic 9L gliosarcoma cells in 10 μL Hamilton syringe. 100 rats were injected with $8x10^4$ PA317/G1TkSvNa.53 producer cells (not co-infected with replication competent virus); 28 rats were injected with $8x10^4$ PA317/G1TkSvNa.53 producer cells which were co-infected with the 4070A replication competent retrovirus; 25 rats were injected with $8x10^4$ PA317/G1TK1SVN.7 producer cells; and 6 rats were injected with $8x10^4$ G1BgSvN.29 producer cells, which generate vector particles including a ß-galactosidase gene. (Such producer cells are formed by transfecting the PA317 cell line with pG1BgSvNa, a vector in which the lac Z (ß-galactosidase) gene replaces the Herpes Simplex thymidine kinase gene.) The catheter then was flushed with 10 μl of phosphate buffered saline (PBS), and sealed with a steel rod. Rats injected with the ß-galactosidase producer cell line were sacrificed on days 3, 6, and 10 after cell injection. The brain and spinal cord were removed and histological sections were stained with X-gal histochemical technique to identify cells expressing ß-galactosidase. (Ram, et al., 1993.)

Rats that had received intrathecal injections of the PA317/G1TkSvNa.53 producer cells, or PA317/G1TkSvNa.53 producer cells co-infected with 4070A virus, began treatment with ganciclovir 7 days later, or the rats were given an intraperitoneal dose of saline. The rats which received ganciclovir received the drug daily by

intraperitoneal injections (30 mg/kg/ml PBS) or intrathecal injections (25 μg/kg or 600 μg/kg; 10 μl PBS) for 14 days. The low intrathecal dose was chosen to achieve a cerebrospinal fluid concentration of 5 to 10 μg/ml, which had been shown to be the effective antitumor concentration in vitro studies. (Culver, et al., 1992; Ram, et al., 1993). The higher concentration was chosen to deliver a great excess of the drug into the subarachnoid space.

The rats were observed daily for development of neurological deficits, which invariably almost manifested as rapidly progressing paraparesis and paraplegia leading to death within 12 to 24 hours. The Mantel-Haenzel test (Mantel, Cancer Chemother. Rep., Vol. 50. pgs. 163-170 (1966)) was used to compare survival between ganciclovir and saline-treated rats in the survival experiments. Because independent experiments were performed, statistical analyses and determinations of p-values were performed only within the same experimental group. The mean period of survival (in days) is given in Table II below. It also is to be noted that an additional 4 rats in each of Groups 1 through 4 (2 treated with ganciclovir, 2 treated with saline) were sacrificed after 7 days of treatment and the spinal cords were examined histologically. These rats were not included in the survival statistics.

Table II

oup	Number of Rats	Intrathecal Cells Injection ($8x10^4$ cells/10μl)	Ganciclovir Dose-Mode of Administration	Survival Days (Mean)	p-value
1	13	PA317/G1TkSvNa.53 producer cells	30 mg/kg intraperitoneally	21.8	0.000
	10	PA317/G1TkSvNa.53 producer cells	Saline-intraperitoneally	14.3	0.000
2	12	PA317/G1TkSvNa.53 producer cells with 4070A retrovirus	30 mg/kg intraperitoneally	19	0.153
	12	PA317/G1TkSvNa.53 producer cells with 4070A retrovirus	Saline-intraperitoneally	15	0.153
3	11	PA317/G1TkSvNa.53 producer cells	25 μg/kg intrathecal	20.2	0.434
	9	PA317/G1TkSvNa.53 producer cells	Saline-intrathecal	19.1	0.434
4	13	PA317/G1TkSvNa.53 producer cells	600 μg/kg intrathecal	19.6	0.608
	10	PA317/G1TkSvNa.53 producer cells	Saline-intrathecal	18.7	0.608
5	13	PA317/G1TKSvNa.53 producer cells	30 mg/kg intraperitoneally	17.9	0.017
	9	PA317/G1TKSvNa.53 producer cells	Saline - intraperitoneally	15.8	0.017
6	15	PA317/G1TK1SvN.7 producer cells	30 mg/kg intraperitoneally	19.5	0.003
	10	PA317/G1TK1SvN.7 producer cells	Saline intraperitoneally	15.6	0.003

With respect to Group 1, the above results show that survival was extended significantly when ganciclovir was administered

intraperitoneally to rats 7 days after injection of PA317/G1TkSvNa.53 producer cells as compared with the saline-treated rats. A survival curve for the rats of Group 1 is shown in Figure 9.

Regarding Group 2, survival of the ganciclovir-treated rats was extended as compared with the saline-treated rats. The mean survival time of the ganciclovir-treated rats in Group 2 was not significantly different, however, from rats treated with producer cells which were not infected with the 4070A replication competent retrovirus. The histological appearance of the spinal cord was similar in both groups. A survival curve for the rats in Group 2 is shown in Figure 10.

With respect to Groups 3 and 4, histological examination of the spinal cords demonstrated that the thin infiltrating layers of tumor, which invaded the leptomeninges in the saline-treated rats, were eradicated completely in the ganciclovir-treated animals.

Regarding Groups 5 and 6, survival in both groups was extended with ganciclovir treatment as compared to saline-treated rats. Although treatment with the PA317/G1TK1SvN.7 cells appeared superior to treatment with PA317/G1TKSvNa.53 cells, it was not statistically significant.

Tumor infiltration of the leptomeningeal coverings of the brain and spinal cord presents a unique therapeutic challenge. The diffuse narture of this disease requires an aggressive approach, including irradiation and chemotherapy, which results, however, in only a limited tumor response, marginal extension of survival, and significant morbidity.

Retroviral-mediated gene therapy provides an attractive treatment option in the setting of meningeal carcinomatosis. Since the retroviral vectors are replication-incompetent, and thus unable to propagate infection and gene transfer from one tumor cell to another, efficient distribution of the vector is crucial to maximize gene transfer into as many tumor cells as possible. Efficient delivery of the vector is achieved by injection of the vector-producer cells into the cerebrospinal fluid. The retroviral

vector particles containg the Herpes Simplex thymidine kinase gene are released continuously from the circulating vector producer cells and thereby reach the whole surface of the tumor-infiltrated meninges. Selective transfer of the suicide gene into the proliferating tumor then sensitizes it to the effects of ganciclovir.

In vitro measurements of retroviral titers in supernatants from the producer cell cultures revealed no significant decrease in vector production after 48 hour exposure of the cultured cells to various concentration of human DSF.

The antitumor efficacy studies with in vivo tumor transduction with the HStk gene reported herein showed that significant tumoricidal effect can be achieved with systemic (intraperitoneal) ganciclovir therapy. Histological examination of spinal cords from rats with meningeal carcinomatosis that had received intrathecal ganiclovir demonstrated almost complete eradication of the thin layers of tumor cells, which characteristically infiltrate the circumference of the spinal cord in this model of meningeal carcinomatosis.

Bystander effect, a process through which non-transduced tumor cells in the vicinity of cells transduced with a vector including the Herpes Simplex thymidine kinase gene can be killed by ganciclovir, has been previously described. One of the major components of such bystander effect was linked to transfer of phosphorylated ganciclovir from transduced to non-transduced cells via gap junctions. When topical ganciclovir interacts with the superficial layers of tumor cells in the leptomeninges, such bystander effect may be generated and account for the efficient tumor eradication of the infiltrating tumor in the circumference of the spinal cord.

The aggressive nature and rapid doubling time of the 9L tumor cells used in the model of meningeal carcinoma described in Example 1 indicate that the extension of survival observed in rats treated with intraperitoneal ganciclovir represents a significant kill of tumor cells.

Intrathecal delivery of vector-producer cells and transfer of a suicide gene to diffusely infiltrating leptomeningeal cancer cells resulted in significant prolongation of survival after systemic therapy with ganciclovir. The data indicate that such an approach may provide another therapeutic option for the treatment of this devastating complication in cancer patients.

Example 2

Human Gene Therapy for Meningeal Carcinomatosis

20 human patients suffering from meningeal carcinomatosis are grouped into four groups, termed Phase A, Phase B, Phase C, and Phase D. Phase A includes 3 patients; Phase B includes 3 patients; Phase C includes 4 patients; and Phase D includes 10 patients.

Each patient receives an Ommaya reservoir connected to an intraventricular catheter for access to the cerebrospinal fluid. The Ommaya reservoir consists of a small bubble-type reservoir and a ventricular catheter. The placement of the Ommaya reservoir requires shaving the hair over the right frontal portion of the head, just in front of the coronal suture. The area then is prepped and draped in a sterile fashion. The skin is infiltrated with local anesthesia and a small incision is made just in front of the coronal suture and 3 cm to the right of the midline. A burr hole opening in the skull is made and a catheter then is placed into the lateral ventricle on that side. The catheter then is attached to the reservoir and the skin then is closed.

After placement of the reservoir, patients are allowed to recover for 48 hours, during which time the patients receive antibiotics. At the end of 48 hours, and prior to the administration of the murine producer cells, a cerebrospinal fluid sample is taken from the reservoir and tested for the presence of malignant cells and various tumor markers.

The patients in Phase A receive an intraventricular injection of $2x10^9$ PA317/G1Tk1SvN.7 producer cells via the Ommaya reservoir. 7 days after the injection of the producer cells, the patients are

given ganciclovir by intravenous infusion over one hour at a dose of 5 mg/kg of body weight twice daily for 14 days.

The patients of Phase B receive an injection of $2x10^9$ PA317/G1Tk1SvN.7 producer cells into the right lateral ventricle via the Ommaya reservoir, and an injection of $2x10^9$ PA317/G1Tk1SvN.7 producer cells into the lumbar subarachnoid spaces via a spinal catheter. 7 days after the patients are injected with the cells, the patients each are given ganciclovir by intravenous infusion over one hour at a dose of 5 mg/kg of body weight twice daily for 14 days.

The patients of Phase C are given two injections of $2x10^9$ PA317/G1Tk1SvN.7 producer cells into the right lateral ventricle via the Ommaya reservoir for a total of $4x10^9$ cells injected into the right lateral ventricle, and two injections of $2x10^9$ PA317/G1Tk1SvN.7 producer cells into the lumbar subarachnoid space via a spinal catheter, for a total of $4x10^9$ cells injected into the lumbar subarachnoid space. 7 days after the patients are injected with the producer cells, the patients are given ganciclovir by intravenous infusion over one hour at a dose of 5 mg/kg of body weight twice daily for 14 days.

Cerebrospinal fluid samples of the patients in Phases A, B, and C are tested for vector titers twice a day for the first 7 days after the patients are given the producer cells, and at 14, 21, 35, and 48 days after the patients are given the producer cells, followed by monthly checks of vector titer. Cerebrospinal fluid samples also are analyzed for tumor markers. Upon evaluation of the cerebrospinal fluid, samples and the finding that no toxicity in the patients is encountered, the 10 patients of Phase D are treated according to the same protocol as the patients of Phase C.

Example 3

Adenoviral Gene Transfer into Meningeal Carcinomatosis

A. Construction of pAvS6.

The adenoviral construction shuttle plasmid pAvS6 was constructed in several steps using standard cloning techniques

including polymerase chain reaction based cloning techniques. First, the 2913 bp Bg1II, HindIII fragment was removed from Ad-d1327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS-(Stratagene, La Jolla, CA) (Figure 11). Ad-d1327 (Thimmappaya, et al., Cell, Vol. 31, pg. 543 (1983)) is identical to adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the adenovirus 5 genome, and which is located in the E3 region, has been deleted. The orientation of this fragment was such that the Bg1II site was nearest the T7 RNA polymerase site of pBluescript II KS$^-$ and the HindIII site was nearest the T3 RNA polymerase site of Pbluescript II KS$^-$. This plasmid was designated pHR. (Figure 11).

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification (Figure 12). The ITR and encapsidation signal (sequences 1-392 of Ad-d1327 [identical to sequences from Ad5, Genbank accession #M73260]) were amplified (amplication 1) together from Ad-d1327 using primers containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego, CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) with only the NotI primer and the SfiI primer. Complementarity between the AscI containing end of each initial DNA amplication product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-d1327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from MRNA isolated from 293 cells infected for 16 hrs. with Ad-d1327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved pHR plasmid. This plasmid was designated pAvS6A and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site (Figure 13).

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAvS6A-(Figure 13) to create pAvS6 (Figures 13 and 14.)

B. Construction of Av1LacZ4.

The recombinant, replication-deficient adenoviral vector Av1Lac Z4, which expresses a nuclear-targetable B-galactosidase enzyme, was constructed in two steps. First, a transcriptional unit consisting of DNA encoding amino acids 1 through 4 of the SV40 T-antigen followed by DNA encoding amino acids 127 through 147 of the SV40 T-antigen (containing the nuclear targeting peptide Pro-Lys-Lys-Lys-Arg-Lys-Val), followed by DNA encoding amino acids 6 through 1021 of E. coli B-galactosidase, was constructed using routine cloning and PCR techniques and placed into the EcoRV site of pAvS6 to yield pAvS6-nlacZ (Figure 15).

The infectious, replication-deficient, Av1LacZ4 was assembled in 293 cells by homologous recombination. To accomplish this, plasmid pAvS6-nLacZ was linearized by cleavage with KpnI. Genomic adenoviral DNA was isolated from purified Ad-dl327 viruses by Hirt extraction, cleaved with ClaI, and the large (approximately 35 kb) fragment was isolated by agarose gel electrophoresis and purified. The ClaI fragment was used as the backbone for all first generation adenoviral vectors, and the vectors derived from it are known as Av1.

Five micrograms of linearized plasmid DNA (pAvS6n-LacZ) and 2.5 μg of the large ClaI fragment od Ad-d1327 then were mixed and co-transfected into a dish of 293 cells by the calcium phosphate precipitation method. After 16 hours, the cells were overlaid with a 1:1 mixture of 2% Sea Plaque agar and 2x medium and incubated in a humidified, 37°C, 5% CO_2/air environment until plaques appeared

(approximately one to two weeks). Plaques were selected and intracellular vector was released into the medium by three cycles of freezing and thawing. The lysate was cleared of cellular debris by centrifugation. The plaque (in 300 μl) was used for a first round of infection of 293 cells, vector release, and clarification as follows:

One 35 mm dish of 293 cell was infected with 100 μl of plaque lysate plus 400 μl of IMEM-2 (IMEM plus 2% FBS, 2mM glutamine (Bio Whittaker 046764)) plus 1.5 ml of IMEM-10 (Improved minimal essential medium (Eagle's) with 2x glutamine plus 10% vol./vol. fetal bovine serum plus 2mM supplemental glutamine (Bio Whittaker 08063A) and incubated at 37°C for approximately three days until the cytopathic effect, a rounded appearance and "grapelike" clusters, was observed. Cells and supernatant were collected and designated as CVL-A. AvlLacZ4 vector (a schematic of the construction of which is shown in Figure 16) was released by three cycles of freezing and thawing of the CVL-A. Then, a 60 mm dish of 293 cells was infected with 0.5 ml of the CVL-A plus 3 ml of IMEM-10 and incubated for approximately three days as above. Cells and supernatant from this infection then were processed by three freeze/thaw cycles in the same manner and termed CVL-B. A 2.25ml aliquot of a mixture of 3 ml of CVL-B plus 42 ml of IMEM-2 was used to infect each of 20 15 cm dishes of 293 cells. After a 90 minute incubation, IMEM-10 was added and the incubations were continued for approximately three days until the cytopathic effect was observed. The cells and supernatant from CVL-B were harvested, collected into a 50 ml conical tube, and centrifuges at 1,500 rgm for 10 minutes. The cell pellet was resuspended in a reserved portion of the supernatant (5 ml) and stored in a 20°C freezer.

C. Administration of AvlLacZ4 virus to rats having gliosarcoma.

Batches of AvlLacZ4 virus were produced by infecting 293 cells (a human kidney epithelial cell line containing the left 11% of the Adenovirus 5 genome) with AvlLacZ4 at a multiplicity of infection of 10 plaque forming units (pfu)/cell. Growth of the replication-deficient vector in this cell line is possible because the E1A-

consituitively active region, which is necessary for viral particle production, is available as a trans activating element in these cells. Purification of the AvlLacZ4 virus yielded concentrations of $2-3x10^{11}$ pfu/ml.

Syngeneic Fischer rat 9L gliosarcoma cells were propagated in T-175 tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), 2mM L-glutamine (Gibco BRL, Gaithersburg, Md.), 50 units/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), and 2.5 μg/ml Fungizone (ICN Biomedicals, Inc., Costa Mesa, California).

The 9L syngeneic glioma model of meningeal carcinomatosis was used. (Kooistra, 1986). 12 Fischer 344 rats weighing 230 to 350g were anesthetized and a sterile PE-10 tube was inserted into the upper thoracic subarachnoid space via the cisterna magna, secured in the subcutaneous soft tissue, and pierced through the skin on the back of the neck. The tube then was obliterated with a sterile removable steel rod. The rats were observed for 5 to 7 days during which any rat that demonstrated neurological deficits was excluded from the study. Rats were housed one per cage to protect the catheters and received oral amoxicillin (5 mg/kg/day, Beecham Laboratories, Bristol, Tennessee, calculated for an average water consumption of 20 ml/rat/day) and dexamethasone (Tech America, Kansas City, Missouri) 0.5 mg/kg/20 ml for the duration of the study. The rats then were reanesthetized, using inhalational anesthesia (N_2O: O_2: halothane mixture), and $8x10^4$syngeneic 9L gliosarcoma cells in 10μl Hank's balanced salt solution were injected into the subarachnoid space using a 10 μl Hamilton syringe. AvlLacZ4 viral particles ($1x10^9$ or $2x10^8$ in 10 μl PBS) then were injected into 6 rats on the day of tumor inoculation, and to 6 rats 7 days after inoculation. The catheter then was flushed with additional 10 μl PBS and sealed with a steel rod. The rats were sacrificed on days 3, 7, and 10 after vector injection. The brains and spinal cords were removed, sectioned, and stained with X-Gal to identify cells expressing β-galactosidase.

β-galactosidase expression was detected using the X-Gal histochemical stain. (Bondi, et al., Histochemistry, Vol. 76, pgs. 153-158 (1982)). Staining with X-Gal turns β-galactosidase-expressing cells blue when an indolyl is liberated from X-Gal by the action of β-galactosidase and subsequent oxidation and self-coupling, which form a blue reaction product. In vitro staining of cultured cells was performed on cell suspensions. Counterstaining was performed with nuclear fast red (Schmid-GmbH and Co., Kongen, Germany) on all tissue specimens.

Three days after simultaneous intrathecal injection of 9L cells and adenoviral vector a homogenous transduction of the tumor mass was detected in the immediate vicinity of the catheter. Rare positive-staining arachnoid cells were also identified. Seven days after the co-injection of tumor cells and the adenoviral vector, many transduced tumor cells were seen near the catheter tract, but could also be detected within the tumor mass itself. The subarachnoid tumor was significantly larger 10 days after inoculation and subarachnoid tumor infiltration was noted. At that time, transduced tumor cells were detected in remote tumor deposits as well as along the catheter tract itself. As expected, the percentage of transduced cells was lower than that observed earlier as a result of the expanding tumor mass and dilution of the transduced tumor cells.

Three days after injection of the Av1LacZ4 vector into an established leptomeningeal tumor, β-galactosidase activity was detected in the superficial layers of the extensively infiltrating tumor, both around the catheter and along the surface of the tumor mass, including tumor infiltrates surrounding nerve roots. The depth of transduction was limited to several cell layers from the subarachnoid surface of the tumor while the center of the tumor mass showed no X-Gal-positive cells. Seven days after vector injection, transduced cells were limited to the area of the implanted catheter although some X-Gal positive tumor cells could be identified in tumor infiltrates in the upper thoracic cord and the cauda equina. Ten days after injection of the vector,

significant transduction still was detected adjacent to the implanted catheter, but at a much smaller proportion than that observed at earlier time points.

A similar pattern was observed when a lower concentration of the AvlLacZ4 vector ($2x10^8$ pfu/10μl) was injected, either simultaneously with tumor inoculation or into established meningeal tumor. Transduction rates were, however, significantly lower than those observed with injection of the high-concentration vector.

The above results indicate that injection of the AvlLacZ4 vector into the intrathecal compartment of rats with meningeal carcinomatosis resulted in the transduction and expression of the transferred gene in the infiltrating tumor in the subarachnoid space. Thus, the vector was shown to circulate in the cerebrospinal fluid and transduce meningeal tumor cells exposed to the cerebrospinal fluid at remote areas from the injection site.

Example 4

Expression of human GM-CSF in the choroid plexus

The choroid plexus, a specialized intraventricular organ that develops during embryogenesis from infolding of the ependymal cell layer, is comprised of ciliated epithelial cells surrounding a mesh of capillaries. It actively secretes cerebrospinal fluid (CSF) into the cerebral ventricles. The CSF circulates in the subarachnoid space to bathe the surface of the brain and spinal cord and to penetrate deeply into the Vircho-Robin spaces of the brain and spinal cord. (Rennels, *et al.*, Brain Research, Vol. 326, pgs. 47-63 (1985)). In contrast to the ependymal cells from which they are formed, choroid plexus epithelium and endothelial cells are the most mitotically-active cells in the normal adult brain (Johnson, *et al.*, Cancer, Vol. 13, pgs. 336-342 (1960); Kaplan, The Anatomical Record, Vol. 197, pgs. 496-502 (1980)), and, thus, are preferentially susceptible to transduction by retroviral vectors. Targeted transduction of these choroid plexus cells may allow continuous secretion of therapeutic proteins, such as nerve growth factors, neurotransmitters, enzymes, and hormones into the CSF for therapy or research.

To assess whether *in vivo* transduction of the choroid plexus and secretion of a gene product into the CSF can be achieved, the human granulocyte macrophage colony stimulating factor (hGM-CSF) gene was chosen as the model secretable gene for this approach. PA317 retroviral vector-producer cells (Miller, *et al.*, Mol. Cell Biol., Vol. 6, pgs. 2895-2902 (1986)) were engineered to produce a hGM-CSF retroviral vector known as G1GmSvNa, which is derived from the Moloney Murine Leukemia Virus (McLachlin, *et al.*, Virology, Vol. 195, pgs. 1-5 (1993)), and contains the human GM-CSF gene downstream of the 5' long terminal repeat (LTR) promoter and retroviral packaging signal. The vector also contains the neomycin resistance gene internally promoted by the SV40 promoter. The G1GmSvNa vector is packaged by the amphotropic retroviral vector producer cell line PA317 which is derived from NIH 3T3 cells. Cloned, G418-selected, human GM-CSF vector producer cells (PA317/G1GmSvNa.9) were maintained in culture and harvested by trypsinization just prior to intraventricular injection.

Fifteen Fischer 344 rats (250-300 gm) received bilateral injections of 2 x 10^6 PA317/G1GmSvNa.9 producer cells suspended in 20 μl PBS into the lateral ventricles using a stereotactic frame (coordinates for injection: AP -1.0 mm, L 1.5 mm, and DV 3.5 mm, from the bregma, and dura, respectively). The rats were sacrificed on days 1, 2, 3, 4, and 7 after cell injections to obtain cerebrospinal fluid and choroid plexus specimens (3 animals were sacrificed each day to provide triplicate specimens for each time point). The brains were removed and choroid plexus of the lateral and 4th ventricle was dissected using an operating microscope.

DNA was extracted from the choroid plexus specimens and amplified using a polymerase chain reaction (PCR) with:
1. primers that anneal specifically to the envelope gene in the pPAM3 helper plasmid in the vector-producer cells (Miller, *et al.*, Somat. Cell. Mol. Genet., Vol. 12, pgs. 175-183 (1985)) and 2. primers that anneal specifically to the hGM-CSF open reading frame. Thus, amplification of human GM-CSF sequences in the absence of pPAM3 sequence amplification provides evidence of choroid plexus

transduction with the human GM-CSF gene. Transduction of the choroid plexus was documented by Southern analysis of the PCR products in one rat 3 days after vector-producer cell injection and in three rats 7 days after vector-producer cell injection. The remaining specimens of the choroid plexus showed evidence of pPAM3 sequence amplification in addition to human GM-CSF sequence amplification, indicating that these specimens contained surviving vector-producer cells that were adherent to the choroid plexus and could not allow estimation of transduction efficiency. Quantitative evaluation of the PCR bands from the choroid plexus specimens indicated that at 7 days approximately 0.4% of the choroid plexus cells were transduced with the hGM-CSF gene. Immunohistochemistry using a mouse monoclonal antibody against human GM-CSF (Makata, *et al.*, J. Immunol., Vol. 147, pgs. 1266-1272 (1991)) confirmed *in situ* expression of human GM-CSF in choroid plexus epithelial cells after intraventricular injection of human GM-CSF vector-producer cells.

Cerebrospinal fluid specimens were collected by exposing the cisterna magna surgically and siphoning the CSF-using a capillary tube. Samples were evaluated by enzyme-linked immunoassay for human GM-CSF. Levels of human GM-CSF greater than the lowest ELISA standard curve value (10 pg/ml) were detected in all CSF samples. A significant proportion of the human GM-CSF found in the CSF was contributed by the human GM-CSF vector-producer cells themselves. However, human GM-CSF levels as high as 110 pg/ml were detectable at late time points when no vector-producer cells were present in the choroid plexus samples. If human GM-CSF is cleared from the cerebrospinal fluid by bulk-flow mechanisms, the measured levels of the cytokine could have resulted from a combination of secretion from transduced choroid plexus epithelium and residual, decaying human GM-CSF that had been previously secreted by the producer cells.

These results represent a proof of principle for the feasibility of targeting a normal component of the central nervous system with retroviral vectors. Access and delivery of the genetic

vector is facilitated by the exposed choroid plexus within the cerebral ventricles and the distribution of the gene product along the cranio-spinal axis by the constant flow of CSF in the subarachnoid space. The duration and levels of expression of gene products secreted into the CSF after choroid plexus transduction may vary, but can be modulated by repeat injection and through the use of vectors that contain tissue-specific promoters. This approach may be applied for therapy as a drug delivery system for proteins into the CSF or used as a research tool to study the effects of continuous delivery of specific gene products in to the CSF.

The disclosure of all patents, publications (including published patent applications), and database entries referenced in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be 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 method of treating an adverse condition of the nervous system in a mammalian host, comprising:

administering to the cerebrospinal fluid of said mammalian host a retroviral vector including a nucleic acid sequence encoding a therapeutic agent for treating said adverse condition of the nervous system, wherein said retroviral vector is contained in a producer cell line, whereby said producer cell line produces a retroviral vector particle from said retroviral vector, and said retroviral vector particle is capable of transducing cells present in the nervous system, in order to express said therapeutic agent in said cells, wherein said producer cells are administered in an amount effective to treat said adverse condition of the nervous system in said host.

2. The method of Claim 1 wherein said adverse condition of the nervous system is a tumor of the nervous system, and wherein viruses produced by said producer cells upon administration of said producer cells to said cerebrospinal fluid transduce cells of said tumor and said therapeutic agent is an agent capable of providing for the inhibition, prevention, or destruction of the growth of said tumor cells.

3. The method of Claim 2 wherein said agent capable of providing for the inhibition, prevention, or destruction of the growth of said tumor cells is an agent which renders said tumor cells sensitive to an interaction agent, and said method further comprises administering said interaction agent to said host.

4. The method of Claim 3 wherein said agent which renders said tumor cells sensitive to an interaction agent is a negative selective marker selected from the group consisting of Herpes Simplex thymidine kinase; cytomegalovirus thymidine kinase; varicella-zoster virus thymidine kinase; and cytosine deaminase.

5. The method of Claim 4 wherein said negative selective marker is Herpes Simplex thymidine kinase.

6. The method of Claim 4 wherein said interaction agent is selected from the group consisting of ganciclovir, acyclovir, and 1-2-deoxy-2-fluoro-ß-D-arabinofuranosil-5-iodouracil.

7. The method of Claim 6 wherein said interaction agent is ganciclovir.

8. The method of Claim 1 wherein said producer cells are administered intraventricularly.

9. The method of Claim 1 wherein said producer cells are administered intrathecally.

10. The method of Claim 1 wherein said producer cells are administered intraspinally.

11. The method of Claim 1 wherein said producer cells are administered to the choroid plexus.

12. The method of Claim 2 wherein said tumor of the nervous system is meningeal carcinomatosis.

13. The method of Claim 1 wherein said producer cells are administered in an amount of from about $1x10^9$ to about $1x10^{10}$ cells per dose.

14. The method of Claim 6 wherein said interaction agent is administered in an amount of from about 5 mg/kg to about 10mg/kg of host weight.

15. A method of treating a tumor of the nervous system in a mammalian host, comprising:

administering to the cerebrospinal fluid of said mammalian host an adenoviral vector including a nucleic acid sequence encoding an agent capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein said adenoviral vector is administered in an amount effective to inhibit, prevent, or destroy the growth of said tumor of the nervous system in said host.

16. The method of Claim 15 wherein said adenoviral vector is administered to the choroid plexus of said host, whereby said adenoviral vector is delivered to the cerebrospinal fluid of said host.

17. The method of Claim 15 wherein said tumor is meningeal carcinomatosis.

18. The method of Claim 15 wherein said agent capable of providing for the inhibition, prevention, or destruction of the growth of said tumor cells is an agent which renders said tumor cells sensitive to an interaction agent and said method further comprises administering said interaction agent to said host.

19. The method of Claim 18 wherein said agent which renders said tumor cells sensitive to an interaction agent is Herpes Simplex thymidine kinase.

20. The method of Claim 19 wherein said interaction agent is ganciclovir.

FIG. 1

CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE

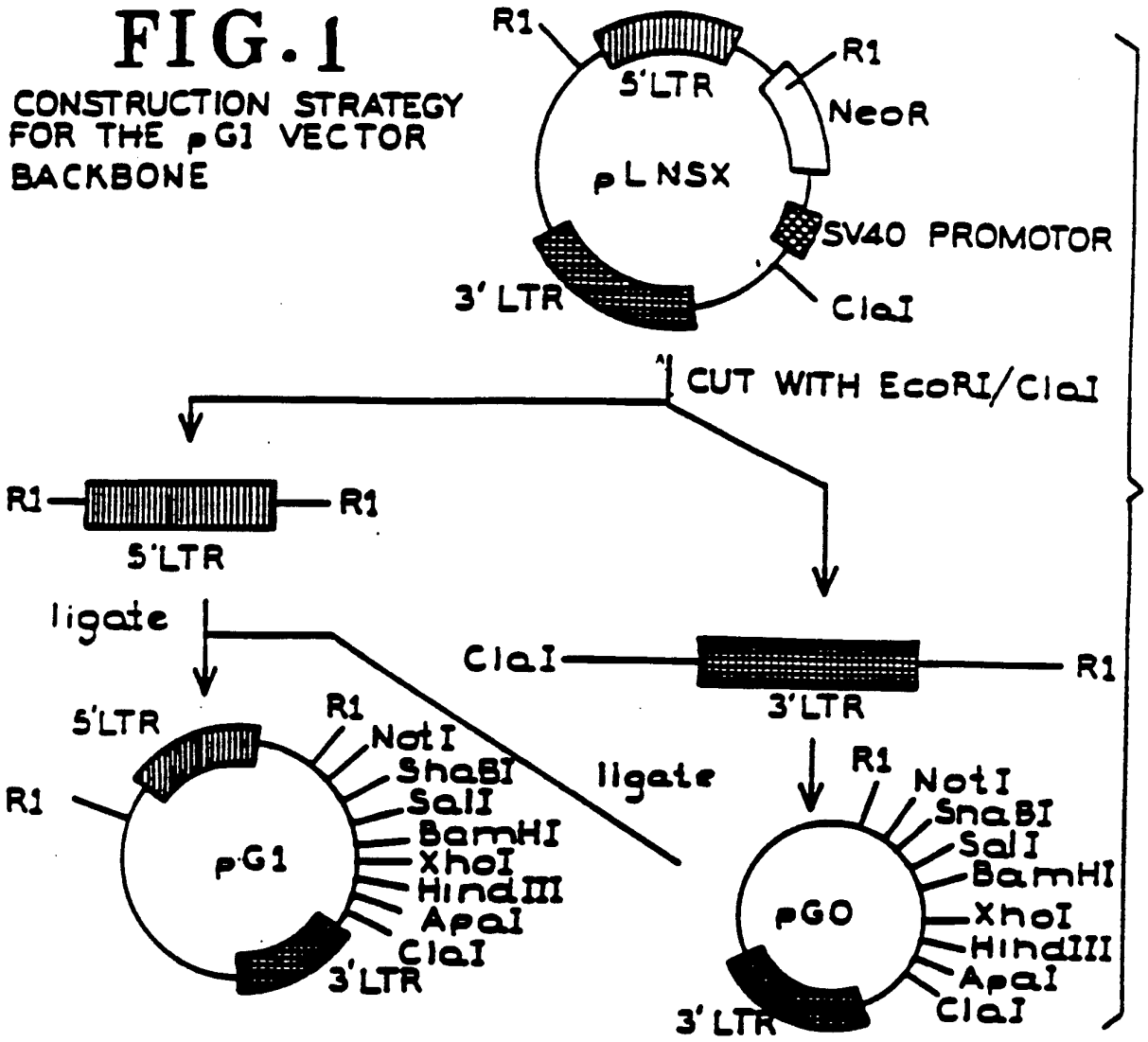


FIG. 2

SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

1/2 EcoRI	Not I	SnaBI	Sal I	BamHI	Xho I	HindIII	Apa I
AATTC	GCGGCCGC	TACGTA	GTCGAC	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	CAGCTG	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2 Cla I
AT
TAGC

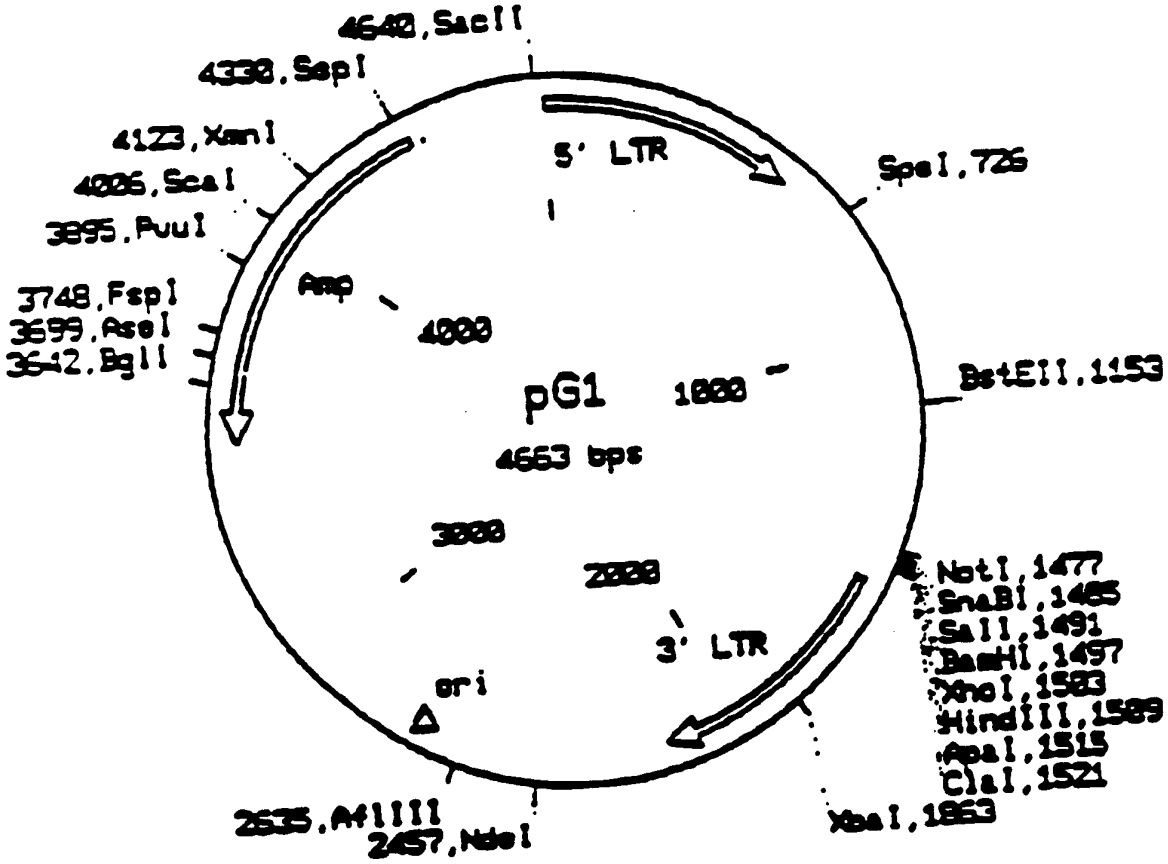

4640,SacII
4330,SapI
4123,XmnI
4006,ScaI
3895,PuuI
3748,FspI
3699,AseI
3642,BglII
Amp
4000
pG1
1000
4663 bps
3000
2000
5' LTR
1
Spe1,726
BstEII,1153
NotI,1477
SnaBI,1485
SalI,1491
BamHI,1497
XhoI,1503
HindIII,1509
ApaI,1515
ClaI,1521
XbaI,1863
3' LTR
ori
2635,AflIII
2457,NdeI

FIGURE 3

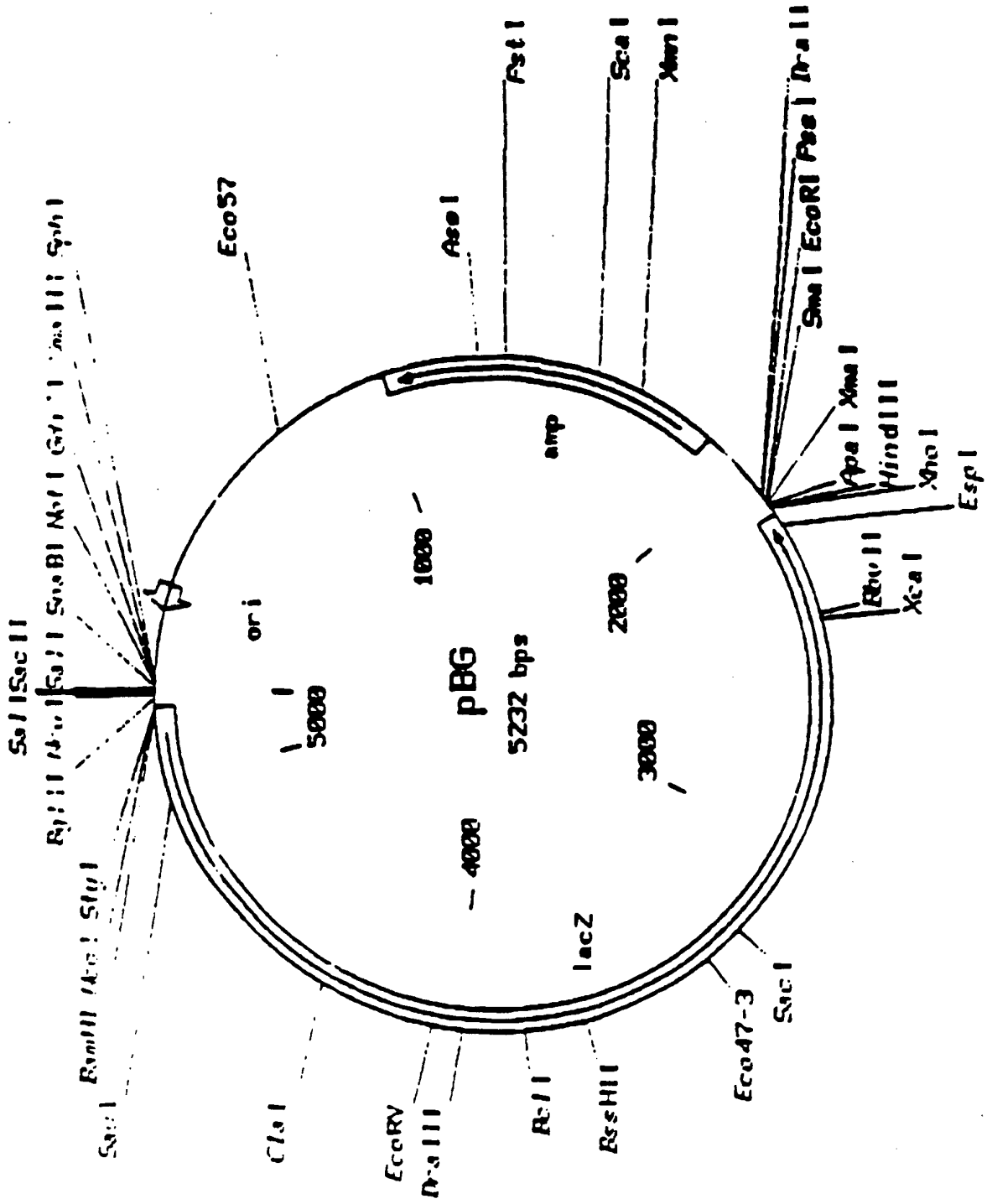

pBG
5232 bps
ori
amp
lacZ
1000
2000
3000
4000
5000
Eco57
Ase I
Pst I
Sca I
Xmn I
Sma I EcoRI Pst I Dra II
Xba I Xma I
Hind III
Xho I
Esp I
Xca I
Sac I
Eco47-3
BsrBI
BssHII
EcoRV
ClaI
SacII
SalI

FIGURE 4

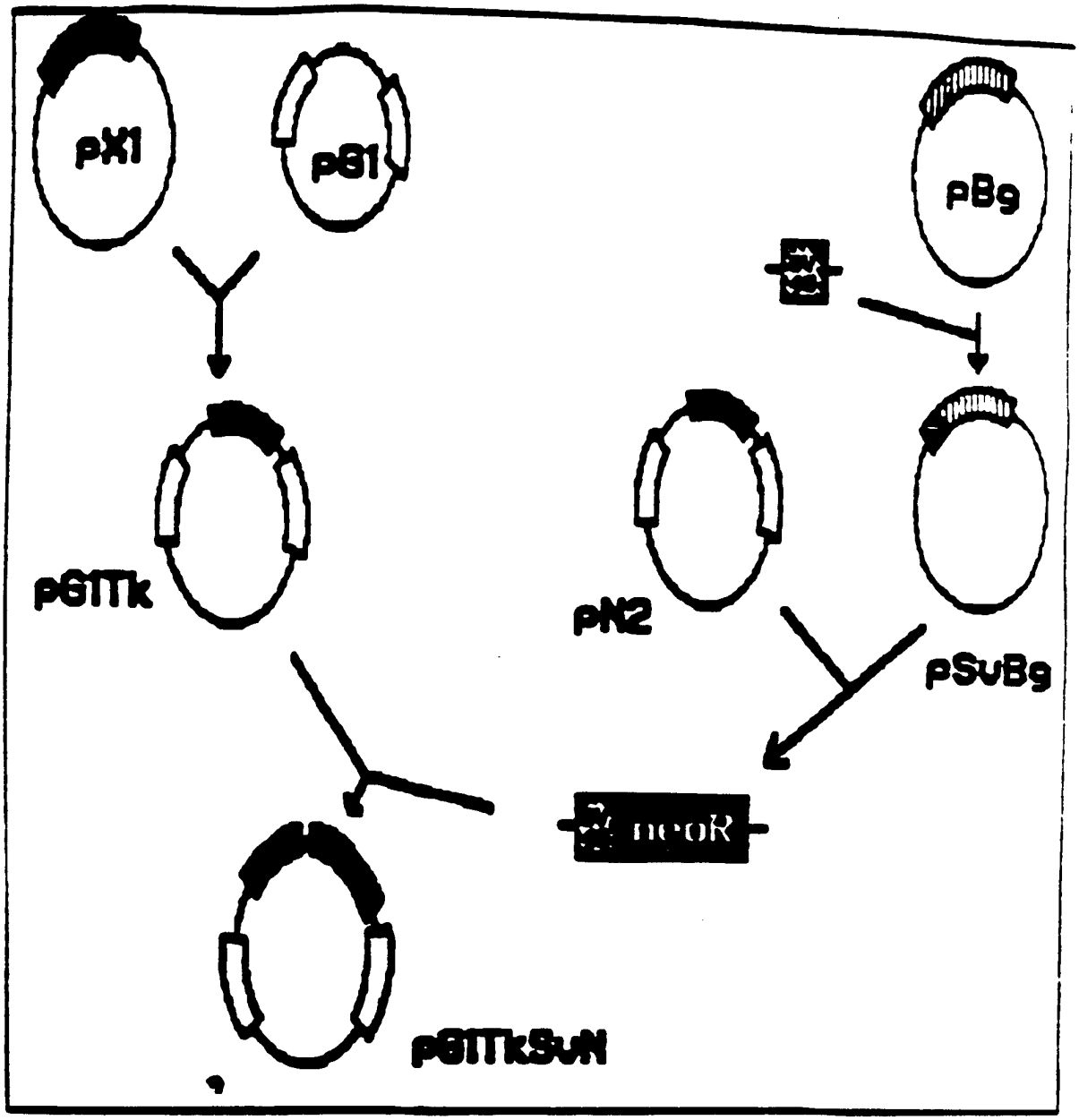


FIGURE 5

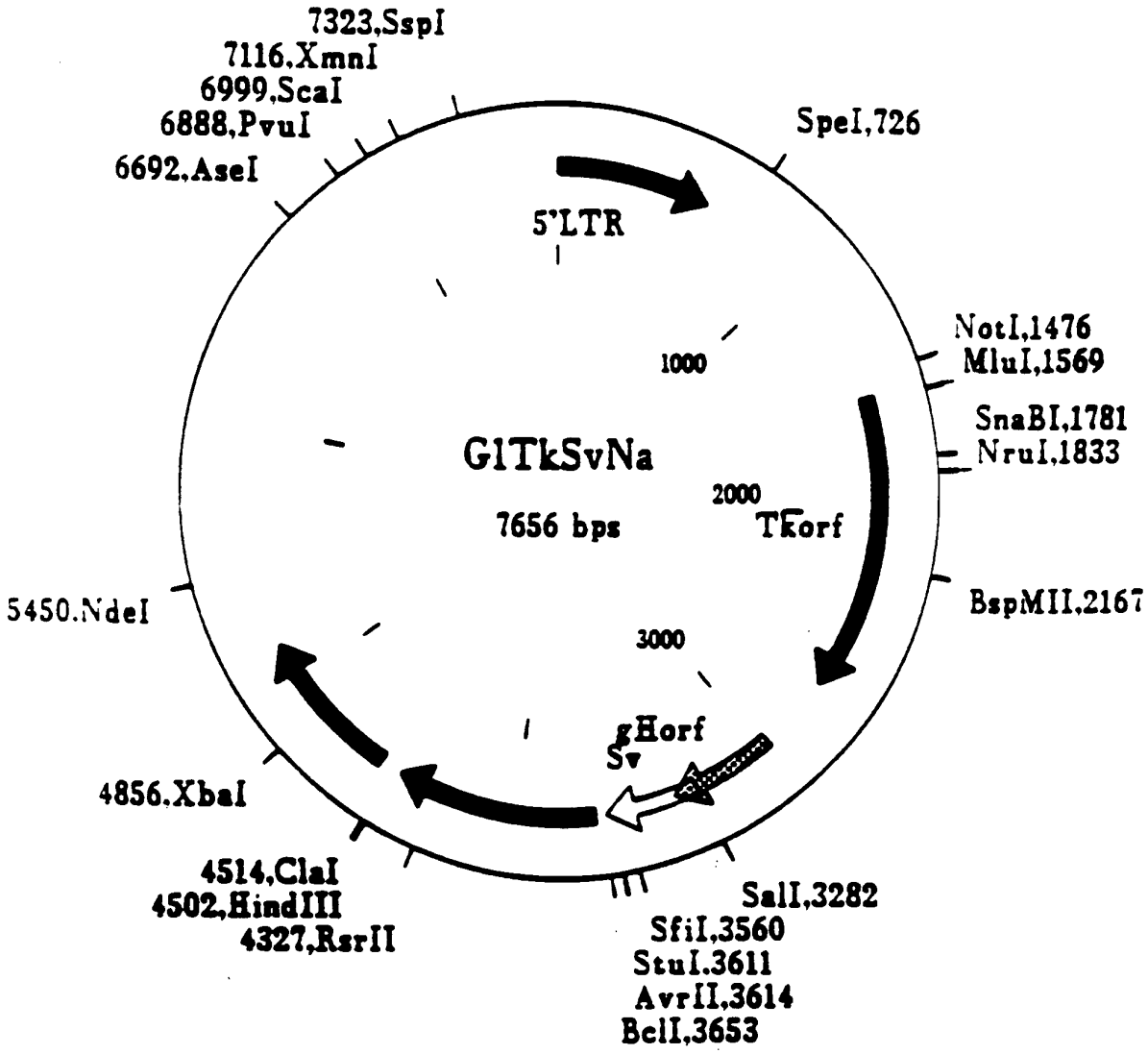

7323,SspI
7116,XmnI
6999,ScaI
6888,PvuI
6692,AseI
SpeI,726
5'LTR
NotI,1476
MluI,1569
SnaBI,1781
NruI,1833
BspMII,2167
SalI,3282
SfiI,3560
StuI,3611
AvrII,3614
BclI,3653
4514,ClaI
4502,HindIII
4327,RsrII
4856,XbaI
5450,NdeI
1000
2000
3000
G1TkSvNa
7656 bps
Tkorf
gHorf
Sv

FIGURE 6

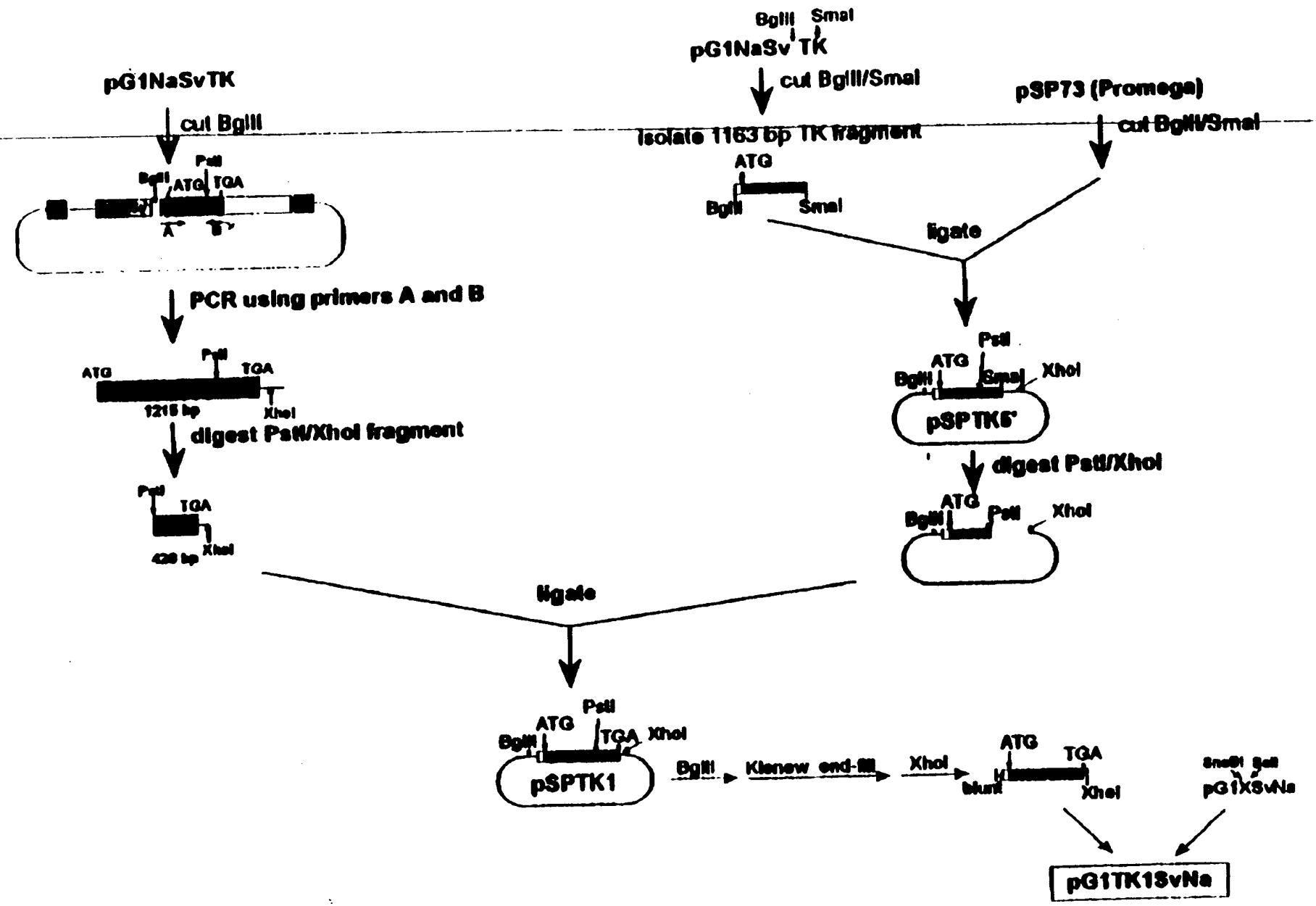

Generation of TK1 Vectors
pG1NaSvTK
cut BglII
PstI
BglII
ATG
TGA
A
B
PCR using primers A and B
ATG
PstI
TGA
1215 bp
XhoI
digest PstI/XhoI fragment
PstI
TGA
420 bp
XhoI
BglII
SmaI
pG1NaSv TK
cut BglII/SmaI
Isolate 1163 bp TK fragment
ATG
BglII
SmaI
pSP73 (Promega)
cut BglII/SmaI
ligate
PstI
ATG
BglII
SmaI
XhoI
pSPTK5'
digest PstI/XhoI
ATG
BglII
PstI
XhoI
ligate
PstI
ATG
BglII
TGA
XhoI
pSPTK1
BglII
Klenow end-fill
XhoI
ATG
TGA
blunt
XhoI
SmaBI SalI
pG1XSvNa
pG1TK1SvNa

FIGURE 7

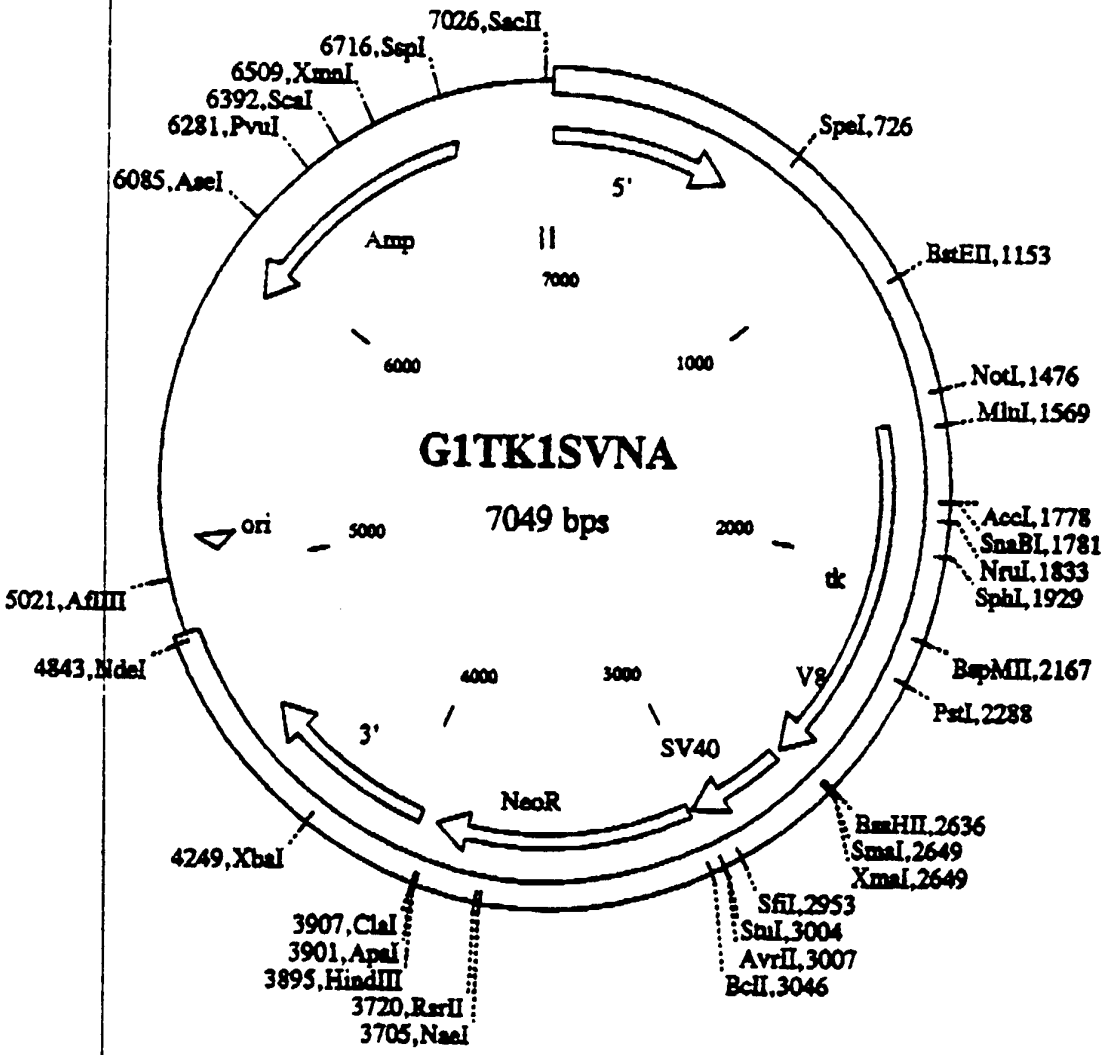

7026,SacII
6716,SspI
6509,XmnI
6392,ScaI
6281,PvuI
6085,AseI
SpeI,726
5'
Amp
7000
6000
1000
BstEII,1153
NotI,1476
MluI,1569
G1TK1SVNA
7049 bps
AccI,1778
SnaBI,1781
NruI,1833
SphI,1929
ori
5000
2000
tk
5021,AflIII
4843,NdeI
4000
3000
Vs
BspMII,2167
PstI,2288
3'
SV40
NeoR
BssHII,2636
SmaI,2649
XmaI,2649
4249,XbaI
SfiI,2953
StuI,3004
AvrII,3007
BclI,3046
3907,ClaI
3901,ApaI
3895,HindIII
3720,RsrII
3705,NaeI

Fig. 8

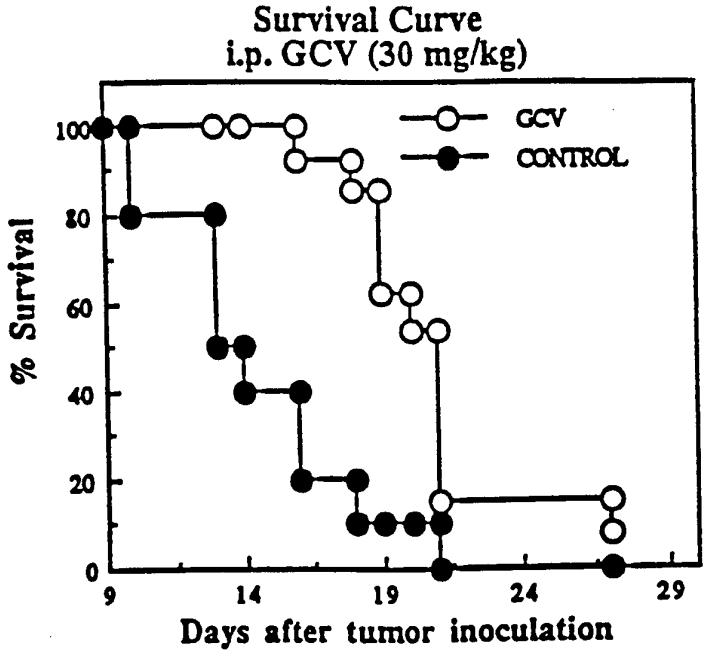

Survival Curve
i.p. GCV (30 mg/kg)
GCV
CONTROL
% Survival
100
80
60
40
20
0
9
14
19
24
29
Days after tumor inoculation

Fig. 9

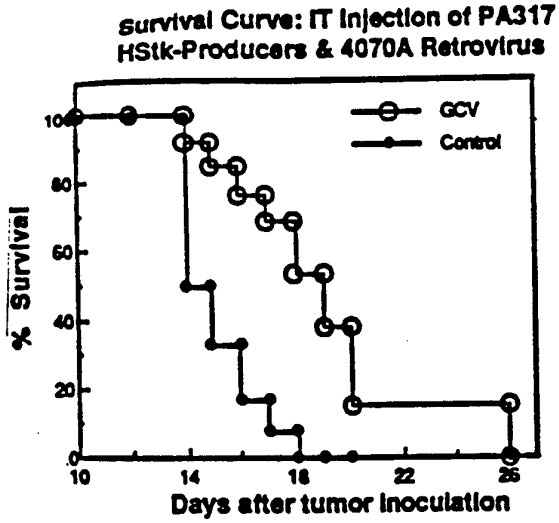

Survival Curve: IT Injection of PA317
HStk-Producers & 4070A Retrovirus
GCV
Control
% Survival
100
80
60
40
20
0
10
14
18
22
26
Days after tumor inoculation

Fig. 10

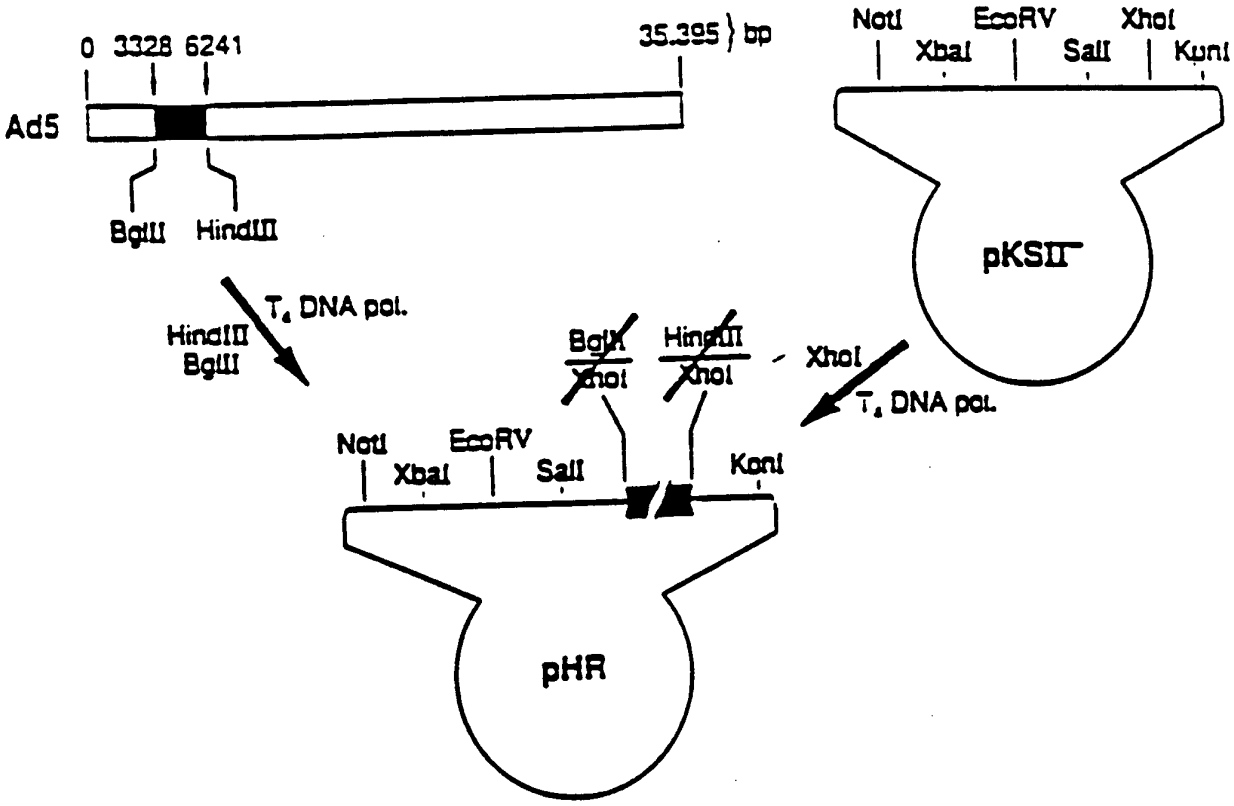

0 3328 6241
35.395 } bp
Ad5
BglII HindIII
HindIII BglII
T4 DNA pol.
NotI EcoRV XhoI
XbaI SalI KpnI
pKSII-
XhoI
T4 DNA pol.
BglII XhoI
HindIII XhoI
NotI EcoRV
XbaI SalI
KpnI
pHR

Fig. 11

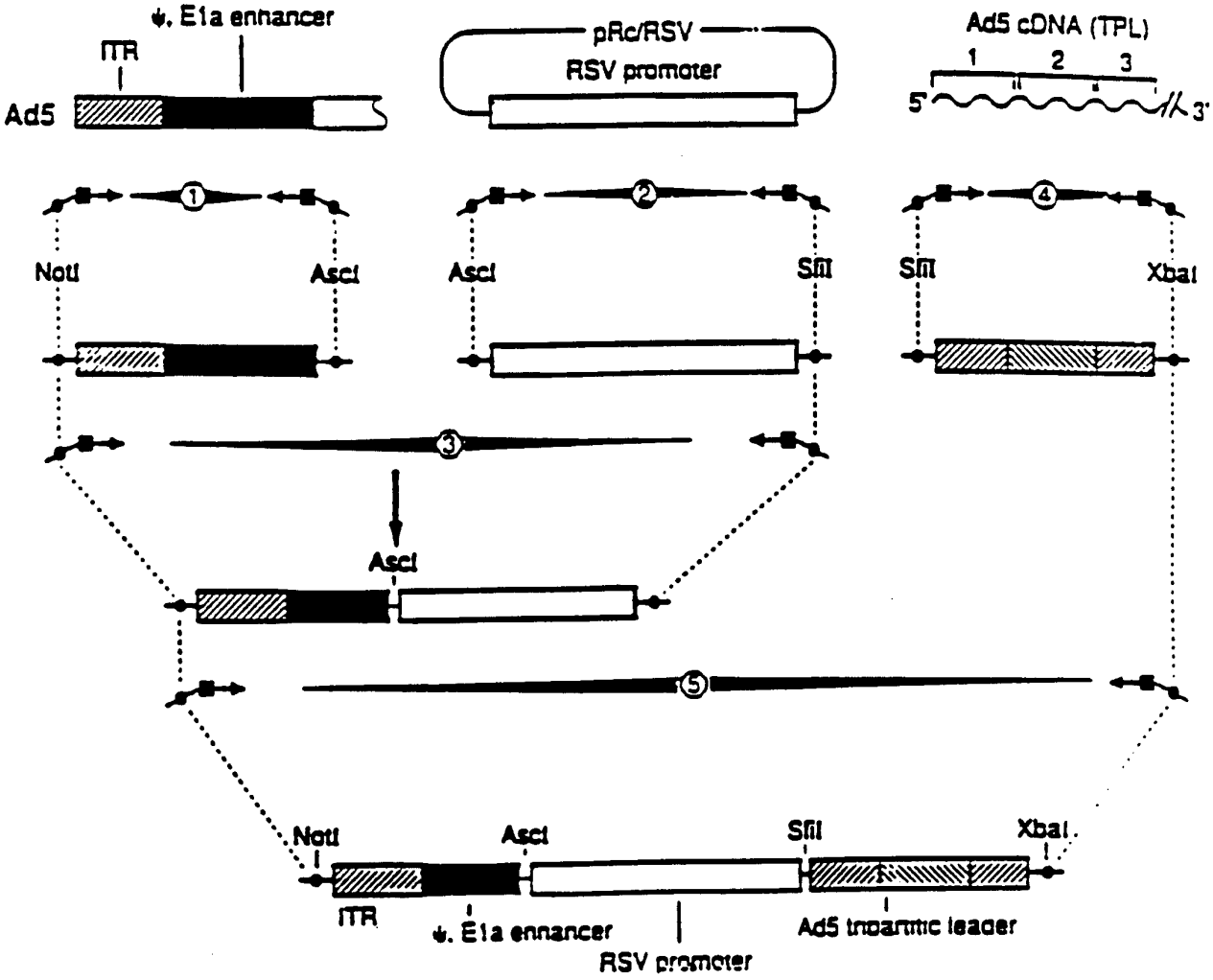

ψ, E1a ennancer
ITR
Ad5
pRc/RSV
RSV promoter
Ad5 cDNA (TPL)
1
2
3
5'
3'
NotI
AscI
AscI
SfiI
SfiI
XbaI
AscI
NotI
AscI
SfiI
XbaI
ITR
ψ, E1a ennancer
RSV promoter
Ad5 tripartite leader

Fig. 12

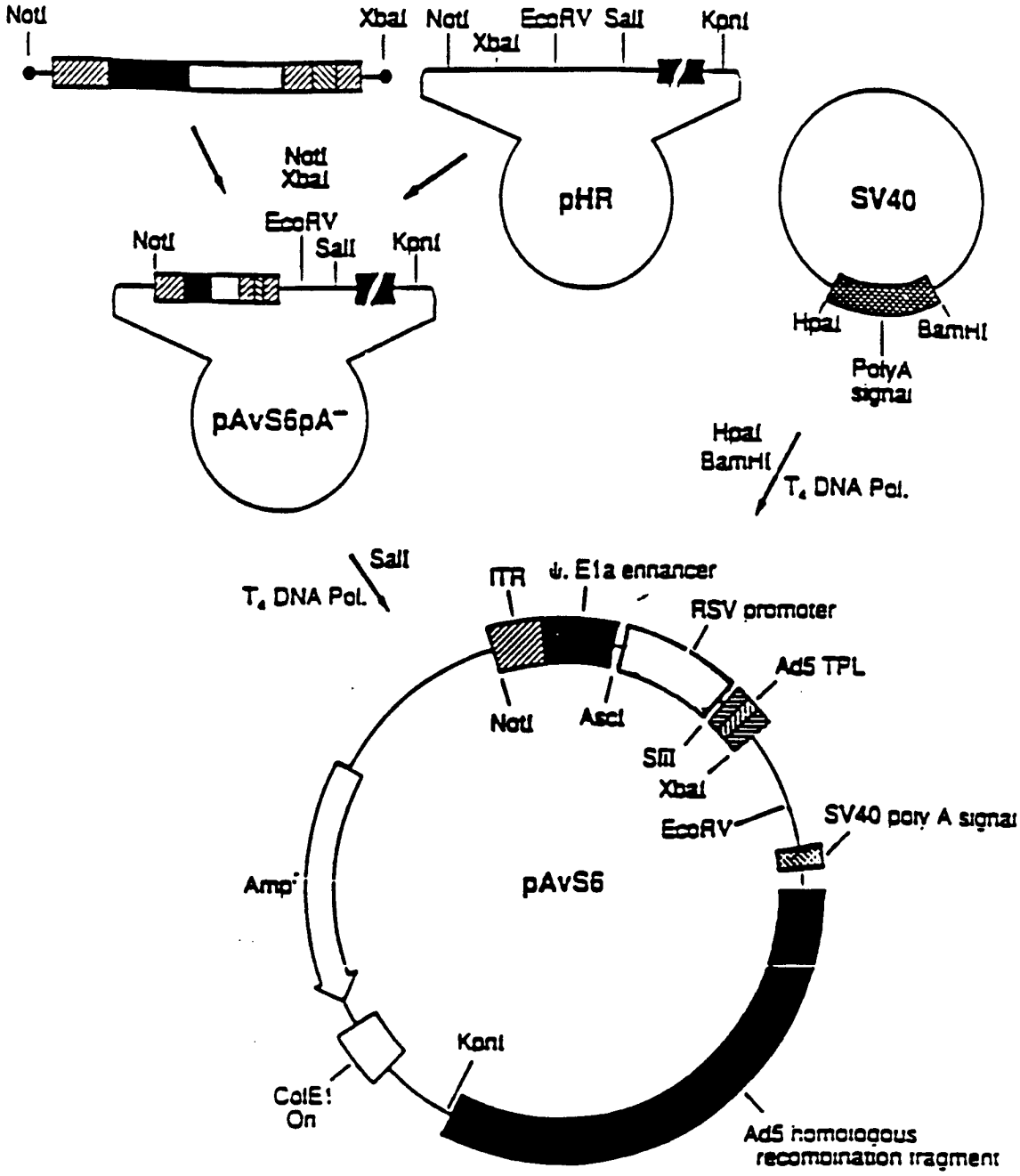

NotI
XbaI
NotI
XbaI
EcoRV
SalI
KpnI
pHR
SV40
HpaI
BamHI
PolyA signal
NotI
XbaI
EcoRV
SalI
KpnI
pAvS6pA⁻
HpaI
BamHI
T4 DNA Pol.
SalI
T4 DNA Pol.
ITR
ψ. E1a ennancer
RSV promoter
Ad5 TPL
NotI
AscI
SfiI
XbaI
EcoRV
SV40 poly A signal
pAvS6
Amp
KpnI
ColE1 On
Ad5 homologous recombination fragment

Fig. 13

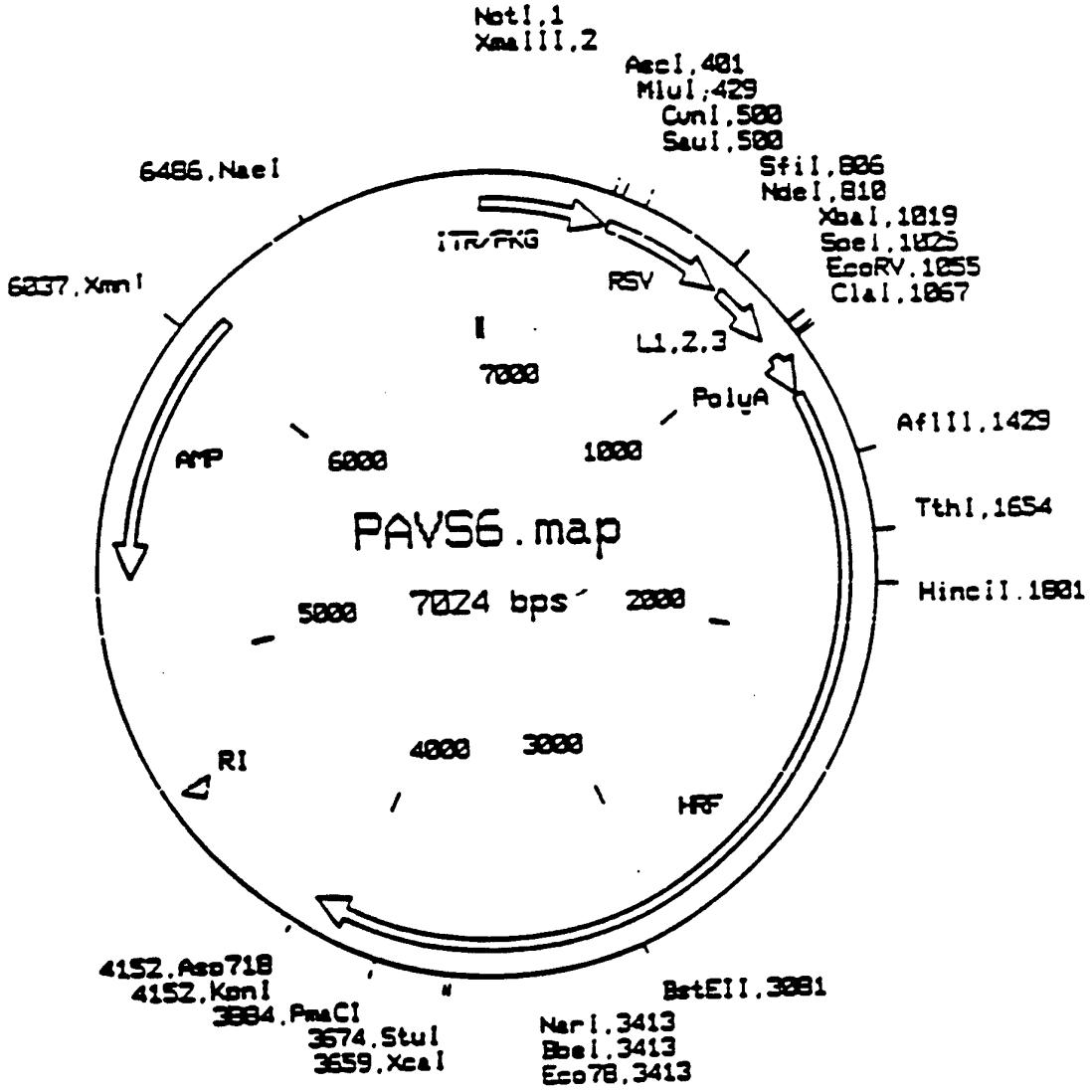

NotI.1
XmaIII.2
AscI.401
MluI.429
CunI.500
SauI.500
SfiI.806
NdeI.810
XbaI.1819
SpeI.1825
EcoRV.1855
ClaI.1867
AflII.1429
TthI.1654
HincII.1801
BstEII.3081
NarI.3413
BbeI.3413
Eco78.3413
PmaCI
3574.StuI
3659.XcaI
4152.Asp718
4152.KpnI
6486.NaeI
6237.XmnI
ITR/PKG
RSV
L1.2.3
PolyA
AMP
RI
HRF
PAVS6.map
7024 bps
1000
2000
3000
4000
5000
6000
7000

Fig. 14

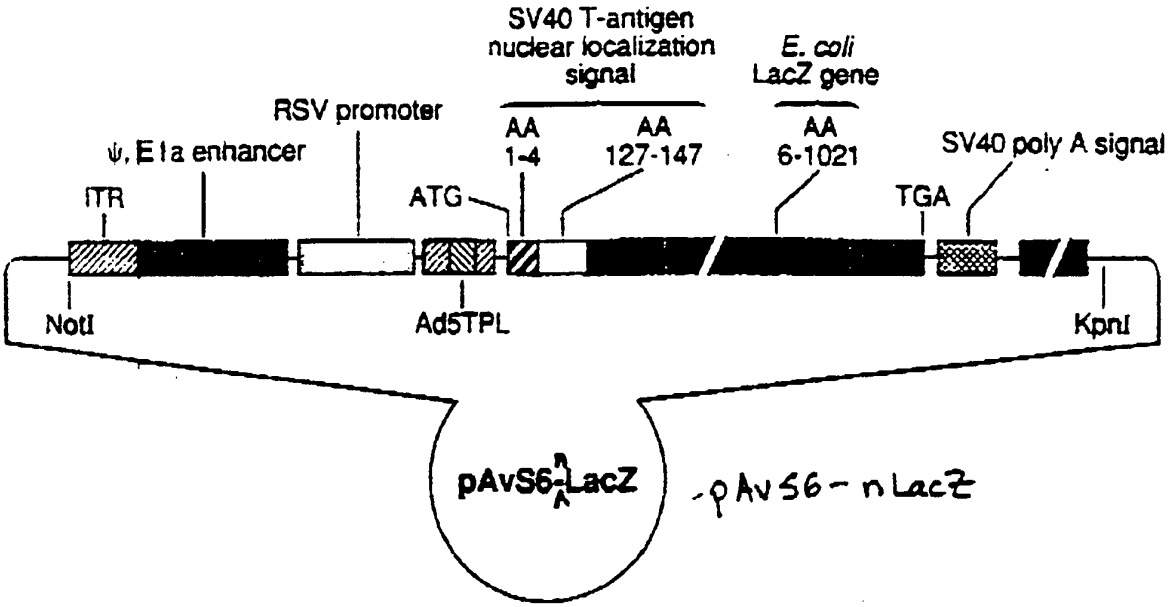

SV40 T-antigen
nuclear localization
signal
E. coli
LacZ gene
RSV promoter
AA
1-4
AA
127-147
AA
6-1021
ψ, E1a enhancer
SV40 poly A signal
ITR
ATG
TGA
NotI
Ad5TPL
KpnI
pAvS6-nLacZ
-pAvS6-nLacZ

Fig. 15

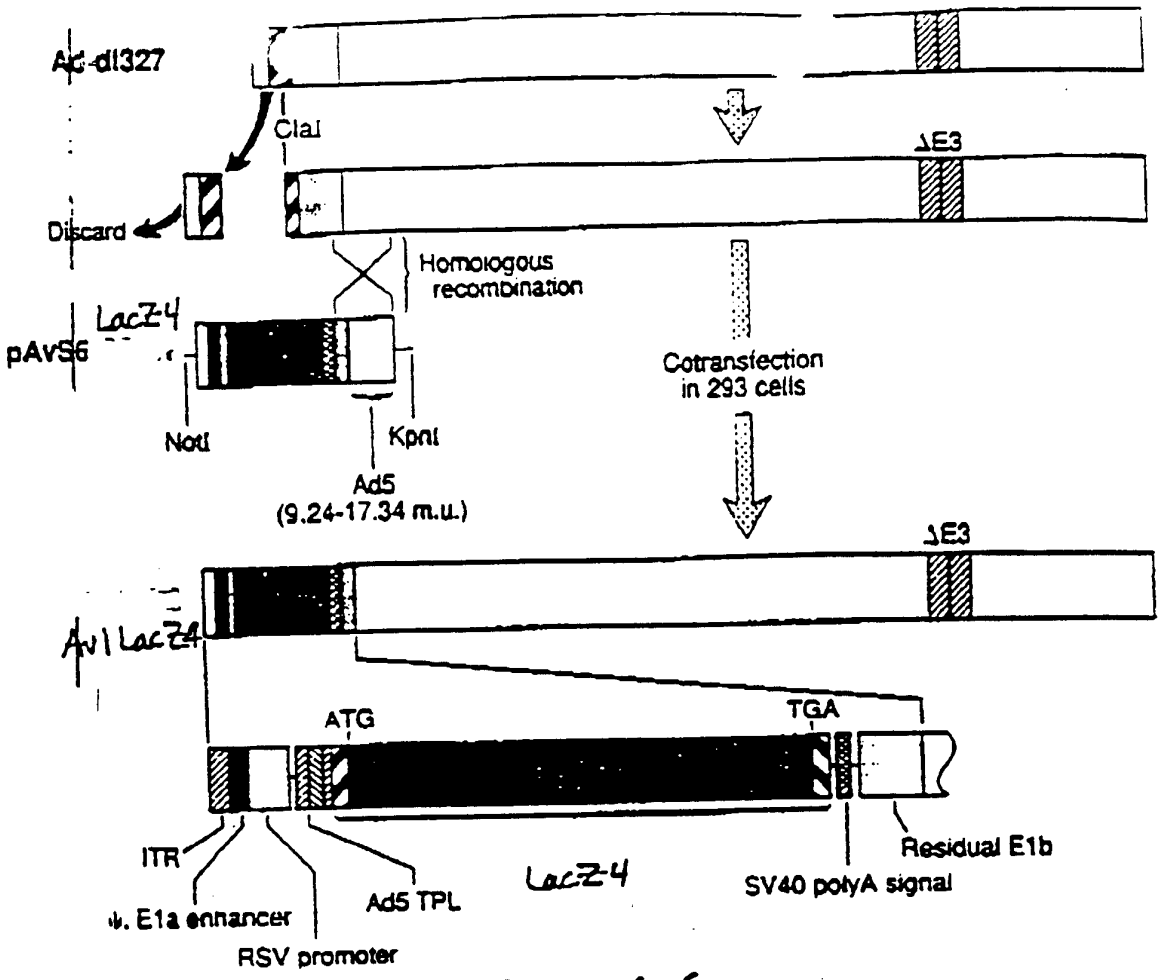

Ad dl327
ClaI
ΔE3
Discard
Homologous recombination
LacZ4
pAVS6
Cotransfection in 293 cells
NotI
KpnI
Ad5 (9.24-17.34 m.u.)
ΔE3
Av1 LacZ4
ATG
TGA
ITR
Residual E1b
LacZ4
SV40 polyA signal
Ad5 TPL
E1a enhancer
RSV promoter

Fig. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00, C12N 15/86, 5/22, 5/16, 15/52, 15/38
US CL : 424/93.21; 514/44; 435/172.3, 240.2, 320.1
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. : 424/93.21; 514/44; 435/172.3, 240.2, 320.1; 935/62

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, CAS, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 53, issued 01 January 1993, Ram et al, "*In Situ* Retroviral-Mediated Gene Transfer for the Treatment of Brain Tumors in Rats", pages 83-88, see entire document.	1-20
Y	Science, Volume 256, issued 12 June 1992, Culver et al, "*In Vivo* Gene Transfer with Retroviral Vector-Producer Cells for Treatment of Experimental Brain Tumors", pages 1550-1552, see entire document.	1-20
Y	Proc. Natl. Acad. Sci. USA, Volume 85, issued October 1988, Borrelli et al, "Targeting of an Inducible Toxic Phenotype in Animal Cells", pages 7572-7576, see entire document.	1-20

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
29 DECEMBER 1994

Date of mailing of the international search report
JAN 19 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10977

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 93/04167 (BARBA ET AL) 04 March 1993, see entire document.	1-20
Y	EP, A, 0,476,953 (MARTUZA) 25 March 1992, see entire document.	1-20
Y	Nature Genetics, issued March 1993, Bajocchi et al., "Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors", pages 229-234, see entire document.	15-20