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(54) Title: GENE TRANSDUCTION SYSTEM

(57) Abstract

A gene transduction system is provided using a replication-deficient SV40 virus and a selected foreign gene. Methods of producing and using the gene transduction system are also provided.

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Gene Transduction System

Background of the Invention

Gene transduction, the introduction of foreign genetic materials into cells or organisms is a requisite technology involved in approaches to correcting genetic abnormalities, i.e., gene therapy. Genes can be transfected into cells by physical means such as scrape loading or ballistic penetration; by chemical means such as coprecipitation of DNA with calcium phosphate or liposomal encapsulation; or by electrophysiological means such as electroporation. However, these methods are relatively inefficient and the cells are significantly perturbed from their normal environment. In contrast, transduction of genes by means of recombinant viruses into a cell that is held in a physiologic environment takes advantage of the relative efficiency of viral infection processes.

Current gene therapy involves infection of organisms or cells with replication-deficient recombinant viruses containing the desired gene or genes. These viruses introduce the desired gene or genes into target cells by relatively efficient infection processes. However, these viruses are rendered deficient in some later replication step so that the primary infection does not produce progeny virus thereby circumventing the problem of propagating a deleterious full lytic viral infection cycle. Ideally, this virus only infects target cells, functionally expresses the gene it carries, and perpetuates the expression of this gene in the target cell and its progeny. Practically, however, this objective has been

difficult to achieve. The reasons for these difficulties lie in the nature of the viral agents used to introduce the foreign gene(s) in question.

There are a number of replication-deficient viruses which are currently being used or have been proposed as gene transduction vectors. Examples include retroviruses, adenoviruses, adeno-associated viruses and herpesviruses. Although it has been demonstrated that gene therapy is possible using such viruses, all involve significant problems that limit or preclude their applicability to gene therapy in a clinically relevant setting.

Retroviruses, which are now approved by the Food and Drug Administration (FDA) for use in the delivery of a gene, have been shown to integrate into host cellular genomes resulting in gene expression in the cells without causing an immunologic response. However, these viral vectors only infect dividing cells. Thus, while they may be effective as transduction vectors, they cannot be used to transduce gene expression in resting cells. This presents a significant limitation for their utility in the treatment of many genetic disorders. In addition, insertion points of the virus into the genome are not random and, thus, there is a potential for cellular gene activation. Further, retroviruses become inactive at concentrations higher than 10^{6-7}/ml. An organ such as the liver may contain greater than 10^{14} cells. Therefore, to infect a sufficient amount of cells, enormous volumes of fluid containing the recombinant retroviruses would be required. In addition, transduction efficiency with retroviruses is relatively low (<10% or less usually). Finally, retroviruses currently being used have been found to be inactivated by human complement thus making them unsuitable for direct inoculation *in vivo*.

Delivery of genes using recombinant adenoviruses is also currently being investigated. However, adenovirus DNA does not integrate into the host genome, but rather is carried episomally. Accordingly, when an infected cell divides, the viral DNA which is not replicated is then present in only one-

half of the daughter cells. A second division dilutes still further the transduced gene until it is eventually lost to the cell progeny. Therefore, adenovirus mediated gene transduction allows expression in resting cells but does not permit transmission of gene expression to daughter cells. Further, adenovirus elicits a destructive immune response on the part of the host immune system that leads to the elimination of the desired transduced cells, thus making long-term gene therapy almost impossible. Co-administration with immunosuppressive agents has not been shown to be especially effective for periods of time of more than one month. Finally, production of these recombinant viral vectors is very cumbersome due to the large size (~110 kb) of the virus.

Adeno-associated virus has also been suggested for use as a vector in gene delivery. This virus integrates into the host genome, but has a preference for an integration site that is at or near an important gene translocation site for some acute leukemias. Furthermore, like retroviruses, adeno-associated virus is not generated at high virus concentrations, thereby necessitating administration of enormous volumes of fluid to be effective.

Herpes viruses have only recently been proposed as vectors for gene transduction and their use has not been fully evaluated at this time. However, they also appear to incite strong host immune responses similar to adenovirus. The resulting immunologic destruction and clearance of virus-infected cells precludes lasting gene expression in infected cell populations.

It has now been found that a DNA virus, SV40 (Simian Virus-40), provides a unique system for gene transduction which has several advantages over any of the currently available systems.

Summary of the Invention

An object of the present invention is to provide a method of introducing a foreign gene into an animal comprising administering to an animal an SV40 or modified SV40 viral

particle containing a circular, double-stranded DNA species comprising SV40 viral DNA and a selected foreign gene.

Another object of the present invention is to provide a viral vector comprising a selected portion of an SV40 genome and a selected foreign gene capable of effectively transducing a cell in an animal with the selected foreign gene.

Yet another object of the present invention is to provide a composition comprising a viral vector containing a selected portion of an SV40 genome and a selected foreign gene capable of effectively transducing a cell in an animal with the selected foreign gene and a pharmaceutically acceptable carrier.

Brief Description of the Figures

Figure 1 shows a graph of luciferase activity measured in tissue homogenates of heart (filled square), kidney (filled circle), lung (filled triangle), liver (filled diamond) and spleen (open square) following inoculation of mice with SVluc. Each time point represents at least two independent determinations. Luciferase activity is reported as light emission (arbitrary units)/100 μg wet wt.

Figure 2 shows a Northern Blot depicting levels of mRNA of α-1-anti-trypsin expressed in a human hepatocellular carcinoma cell line following administration of SV40 viral vectors of the present invention. BSV(Δ)AT204 refers to a virus comprising the AT204 ribozyme cloned into pT7SV(Δ) containing the SV40 early promoter. BSVP.AT589 refers to a virus comprising the AT589 ribozyme cloned into pT7SV(Δ) containing the SV40 early promoter. T7SV(Δ).tAT589T refers to a virus comprising the AT589 ribozyme cloned into pT7SV(Δ)t containing the tRNA early promoter.

Detailed Description of Invention

SV40 is relatively small (5.3 kilobases (kb)), double-stranded DNA virus that normally infects monkeys, but also infects other mammals. Its relative safety in humans was demonstrated unintentionally as SV40 was a contaminant of the

Salk polio virus vaccine, and so the virus was inadvertently administered to many humans with no demonstrable ill effects. SV40 has a relatively high infectivity to virus particle ratio, can integrate into the host genome, carries it own strong promoter sequences, propagates to high titer, and can be concentrated to yield high virus numbers per unit volume.

Portions of this virus have been used in the development of recombinant vaccine virus vectors. For example, Cole et al. (WO9420607) describe a recombinant retrovirus carrying the *Tag* gene of SV40 which was constructed by inserting a *Bp*II-*Hpa*I fragment of SV40 DNA into the *Bam*HI site of the pZipBeoSVX vector. A recombinant virus comprising a carcinoembryonic antigen (CEA) and a number of different viruses including SV40 for use in therapeutic formulations for the treatment of carcinomas expressing CEA was described by Kanton et al. (WO 9219266). However, use of the SV40 viral particle as a means for transducing cells in an animal with foreign DNA has not been taught.

It has now been found that the replication deficient SV40 viral particle is effective as a gene transduction system for the introduction of foreign genes into cells and organisms. Replication of SV40 is dependent upon expression of large T-antigen (*Tag*), which is encoded by the virus. This *Tag* is also a large component of the immunogenicity of SV40. Accordingly, removal of the *Tag* encoding DNA renders the virus replication-deficient and relatively non-immunogenic, thus providing an innocuous viral vector for gene transduction. The *Tag* deficient, and hence, replication deficient, SV40 can be propagated by infection of stable cell lines that have been transformed to express this antigen. Such cell lines are well known to those of skill in the art and can be routinely selected upon this disclosure. There are many examples of *Tag* expressing cell lines presently known in the art and more are reported each month. Any of these cell lines could be used in the present invention. A few examples of such cell lines include, but are not limited to, the COS-7 cell line, MIN-6 cell line, PKSV-PCT cell line, and HMEC-1 cell line.

A system has been devised for cloning foreign genes into a plasmid thereby permitting incorporation of foreign genes into the SV40 genome. A number of different plasmids have been generated by this system.

In a first embodiment, referred to as pBSV(ΔT'), the entire 5.3 kb SV40 genome is cloned into the carrier plasmid pBR322 which contains a *Bam*HI site to produce a plasmid referred to as pBSV-1. Other plasmids containing a *Bam*HI site such as pGEM3, pGEM7 and pUC19 can also be used. Tag was then excised from the pBSV-1 plasmid prepared from pBR322 with *Bcl*I followed by *Avr*II. Digestion with *Avr*II is only a partial digestion as this plasmid has two *Avr*II sites. A cloning cassette re-engineered from the cloning cassette of PGEM7 was then inserted. Other suitable cloning cassettes which could be used are well known by those of skill in the art. Examples include, but are not limited to cloning cassettes from pUC19 and pGEM9. The cloning cassette from pGEM7 was prepared from an oligonucleotide having *Bcl*I and *Avr*II sites on its flanks. The oligonucleotide was amplified by PCR, cut with *Bcl*I and *Avr*II, and cloned into the pBSV-1 from which the 2.2 kb *Bcl*I-*Avr*II fragment had been removed. The resulting plasmid was referred to as pBSV(ΔT). The *Bam*HI site in the pGEM7 cloning cassettes was removed with two other enzymes whose unique sites flank this site. The two sections were then religated to produce pBSV(ΔT').

A number of alternative plasmids useful in the present invention have also been produced. For example, the need to excise the virus genome from a carrier plasmid with *Bam*HI enzyme can impose restrictions on the nature of the foreign genetic material inserted in the plasmid, since the *Bam*HI enzyme cuts with relative frequency. Accordingly, the *Bam*HI sites in the carrier plasmids can be destroyed and replaced with restriction sites of which the corresponding enzyme cuts less frequently. Examples of such restriction sites include, but are not limited to, 8 base recognition sequences such as *Pme*I and *Not*I. Alternatively, the SV40 genome can be cloned into other carrier plasmids. For example, a plasmid referred

to as pT7SV(Δ) was derived from pBSV(ΔT'), however, both the carrier plasmid and the cloning sites were changed. To produce pT7SV(Δ), the *Bam*HI sites of pBSV(ΔT') were destroyed and replaced by *Pme*I sites. In addition, the SV40 genome was cloned into pT7 plasmid which replicates to much higher copy numbers than does pBR322.

Plasmids can be also constructed with additional or different promoters and termination sequences. For example, a plasmid referred to as pBSV(P) was derived from pBSV(ΔT') by addition of a second SV40 early promoter just downstream from the *Bst*XI site of pBSV(ΔT'). pBSV(P) contains a slightly expanded cloning cassette with a *Sma*I site added. The remainder of pBSV(ΔT') is unchanged. Another plasmid derived from pBSV(ΔT'), referred to as pBSVCMV, was constructed by excision of the second SV40 early promoter and replacement with the CMV-IE promoter. A plasmid, referred to as pT7SV(Δ)t was derived from pTSV(Δ) by adding a human tRNA promoter and a polIII termination site.

Thus, as will be obvious to one of skill in the art upon this disclosure, the vast array of transfer vectors which can be made to be used in the SV40 based gene transfer system of the present invention provides one of skill with the flexibility required to deliver genes effectively to their target.

A desired foreign gene or genetic material is routinely cloned into the cloning cassette of any of these plasmids by growth in an appropriate cell line according to compatible ends or by blunted cloning. The resulting plasmid may be amplified to high copy number. The engineered recombinant SV40 viral DNA can be excised from the cloning vector with the appropriate restriction enzymes that flank the SV40 and inserted sequences, purified away from carrier plasmid sequences, and religated to itself. This produces SV40 or modified SV40 viral particles containing a circular double-stranded DNA species of SV40 viral DNA with the inserted gene, that can be transfected into cells. By "SV40 or SV40 modified viral particle" it is meant a viral particle which is capable of infecting cells in an animal

similarly to wild-type SV40 virus but which does not replicate. By "SV40 viral DNA" which is contained in these viral particles it is meant a selected portion of SV40 viral DNA which is capable of producing a vector which effectively transduces a cell in an animal with an inserted foreign gene.

Production of replication-deficient SV40 virus particles that can infect target cells, but not replicate in them, is well known to those of skill in the art. For years virologists have produced such SV40 viruses by using cell lines that express the component of the SV40 replication complex that is missing in the transduced cells. For example, COS-7 cells, monkey cells that have been modified to contain and express high levels of the Tag, have been used to develop replication-deficient SV40 viruses. Since the replication block of the replication-deficient virus is removed by providing the missing Tag in trans, transfection of the engineered virus into these Tag expressing cells results in a full productive viral replication cycle yielding high titer virus stocks that can be used for gene transduction and gene therapy. These recombinant viruses infect cells with the relatively high efficiency of normal SV40 virus and, therefore, efficiently introduce the desired gene into cells for further stable integration and expression. However, these recombinant SV40 viruses do not lead to productive replication of virus, cell lysis and subsequent loss of the desired gene or cytopathic effects on target tissues because they lack *Tag*, which is necessary for these effects.

The ability of mammalian cells to express a foreign gene using a viral vector of the present invention was demonstrated. The *E. coli* gene, *lacZ*, which encodes a small protein that has no mammalian homolog was inserted into the pBSV(Δ*T'*) plasmid to produce pBSV(*lacZ'*). This pBSV(*lacZ'*) plasmid was transfected into cultured cells from two different species, the nontransformed rabbit cell line RK-13 and the large T antigen transformed monkey cell line COS-7. LacZ was expressed in both of these cultured cells following transfection with pBSV(*lacZ'*).

The replication-deficient SV40 virus particles have also been shown to infect target cells, while not resulting in replication of the virus. COS-7 cells which express the large T-antigen were used in these experiments. The SV40 genome lacking the gene encoding the large T-antigen but containing *lacZ* was excised from pBSV(*lacZ'*) with the *Bam*HI restriction endonuclease. The viral DNA was purified away from the pBR322 sequences and religated to itself to yield the recombinant SV40 DNA species, SV*lacZ*. The SV*lacZ* was transfected into COS-7 cells with resultant cytopathic effects indicating that SV40 virus particles were being produced. Two weeks later the SV40 virus particles were isolated, purified, titered and concentrated by standard virological methods. Their inability to replicate in cells that do not supply Tag was ascertained. These recombinant virus particles did not produce plaques in TC7 cells. The recombinant viruses were then used to infect cells and express the contained *lacZ in vitro* in tissue culture and *in vivo* in whole animals or tissue explants. Since lacZ is a bacterial protein with no vertebrate homologs and is not detectable in the uninfected cells, this gene serves as a good candidate for determining whether the recombinant SV40 construct can be used to generate an expression system in which a foreign gene could be expressed in mammalian cells. The expression of *lacZ* is conveniently monitored with standard enzyme assays on cellular tissue homogenates or *in situ* colorimetric methods established for *lacZ* which are well known in the art.

These experiments were also repeated using the gene which expresses luciferase. Luciferase is a firefly enzyme that produces light when it is expressed in cells. The light may be measured by a luminometer or by a scintillation counter. Unlike the studies with lacZ, the production of luciferase measures the ability of the recombinant virus to elicit production of a functional protein rather than simply an RNA transcript. Using the luciferase-containing SV40 recombinant, referred to as SVluc, it was shown that functional luciferase

enzyme was produced in cells transduced with the luciferase cDNA using SVluc.

The ability of the gene transduction vector of the present invention to transduce gene expression *in vivo* has also been demonstrated. SVluc administered intravenously (IV) has been demonstrated to transfer luciferase to multiple organs for at least 105 days. BALB/c mice received 10^7 pfu SVluc IV. Animals were sacrificed approximately weekly from 3 to 7 weeks and monthly thereafter. Frozen sections of colon, heart, liver, lungs, spleen, brain, stomach, tail skin (at the inoculation site) and diaphragm from SVluc recipients and control mice were assayed for luc immunochemically. The most strongly positive tissue was skin at the inoculation site. Luc was detected consistently in >75% of local basal keratinocytes and also in suprabasal keratinocytes and occasional dermal fibroblasts, through 105 days post-inoculation (dpi). Luc was detected in other tissues as well as demonstrated in the following Table 1.

TABLE 1

Summary of tissue reactivity for luc in mice receiving SVluc IV

Tissue	Cellular Localization	Degree of Positivity	Days Positive
Liver	Hepatocyte, Kupffer cells	+	20-105
Lung	Bronchial cells	+++	35-105
	Alveolar lining cells	-	
Colon	Crypt epithelium	++	20-105
Spleen	Lymphocytes	++	20-47
	Megakaryocytes	+++	27-105
Brain	Neurons, glia	+	35-105
Skin	Basal and superbasal keratinocytes	++++	20-105
Kidney	Glomerular and proximal tubular cells	+	20-105
Heart	none	-	
Diaphragm	none	-	

Stomach	none	-	

Luc expression showed tissue and cellular tropisms. For example, epithelium of conducting airways (trachea, bronchi) was positive, but alveolar cells were consistently negative. Colon crypt cells were often positive, but stroma was not.

Percentages of positive cells did not change greatly for most tissues during these studies. See Figure 1. Thus, in the liver, e.g., 1-2% of hepatocytes stained positively for luc from 20-105 dpi. In some organs, however, the population of positive cells changed over the course of the study. Splenic lymphocytes contained luc when assayed soon after inoculation ($\leq$ 35 dpi), but beyond 47 dpi, only megakaryocytes stained positively for luc in the spleen. In some tissue, very few positive cells were noted; after IV inoculation luc was detected in the brain, but <0.1% of cortical neurons and glia stained positively for luc. No evidence of inflammation was observed in any of the tissue specimens.

SVluc was also demonstrated to transfer gene expression to hematopoietic cells. Bone marrow from BALB/c mice was treated with SVluc *ex vivo* at moi of approximately 0.3, and infused into sublethally irradiated syngeneic mice. Effective transfer of *luc* to bone marrow stem cells was assayed in peripheral blood cells as luc production by immunochemistry, and as luc enzyme activity by luminometry.

Both mononuclear and polymorphonuclear leukocytes produced luc, $\geq$ 105 dpi. Percentages of each cell type bound by anti-luc antibody averaged 20% to 25% of peripheral blood nucleated cells. Platelets also reacted with anti-luc antiserum, while erythrocytes were generally negative.

Luc enzymatic activity was measured by luminometry in these blood samples. Levels of luc function detected varied with time. However, luc activity was significantly ($P<0.05$) and substantially greater (>10x) than control values obtained at most time points, including 76 and 105 dpi.

The efficacy of the gene transfer system of the present invention to effectively transduce cells to express a gene of

therapeutic interest was also demonstrated in human cells. Two different ribozyme constructs, namely AT204 and AT589, were cloned into two different SV40 promoter systems. Ribozymes are synthetic mRNAs that bind to and, in this case, destroy target mRNAs. Both ribozymes recognized mutant α-1-anti-trypsin mRNA. This mutant mRNA produces a non-functional mutant protein that interferes with the ability of wild-type α-1-anti-trypsin protein to function resulting in a disease referred to as α-1-antitrypsin deficiency. For these experiments, both AT204 and AT589 were cloned into pT7SV(Δ), and in addition AT589 was cloned into pTSV(Δ)t. Mutant, replication-deficient SV40 was produced for each of these constructs by excising the viral genome containing the ribozymes and transfecting it into the packaging cell line, COS-7. Infectious, but replication deficient virus was recovered in each case. Each of these mutant viruses was used to infect a human hepatocellular carcinoma cell line expressing the α-1-anti-trypsin gene. Northern blot analysis demonstrated that in each case, transduction of the cells with a viral vector of the present invention resulted in decreased levels of α-1-anti-trypsin mRNA which were significantly less than for controls. Infection with the gene transduction system of the present invention reduced the levels of the targeted α-1-anti-trypsin mRNA to less than 10% of control levels. See Figure 3.

These studies establish that *in vitro* studies with the SV40 gene transduction system of the present invention correlate well with *in vivo* results. The SV40 gene transduction system of the present invention overcomes many of the problems associated with viral vectors currently being tested for gene transduction. As demonstrated herein, the SV40 or modified SV40 viral particles can be concentrated to high titer of approximately 10^{12-13}/ml. The SV40 or modified SV40 viral particles are capable of infecting both resting and dividing cells. The SV40 or modified SV40 viral particles may integrate into the host genome in a totally random fashion. Further, elimination of *Tag*, renders the vector relatively non-immunogenic. Finally, even the wild-type virus has been

demonstrated to be very safe in humans. Accordingly, these studies demonstrate that the SV40 gene transduction system of the present invention effectively transduces a cell in an animal with a selected foreign gene and thus is useful in gene therapy.

The term "gene therapy" includes the processes of gene knockout and gene targeting. The purpose of gene therapy is several fold. In some cases, the aim is to introduce a gene from another species into an animal or cells. With the gene transduction system of the present invention, it is possible to, for example, introduce a herpesvirus gene such as thymidine kinase (TK) into target cells to render the cells sensitive to certain drugs. In this situation the drug could be a DNA based analog which is metabolized by the thymidine kinase to a toxic metabolite whose purpose is to kill cells bearing the TK gene. New drugs with high specificity for cells bearing this gene, such as tumor cells, can be easily identified with such a test system. In addition, the transduction system can be used to introduce a foreign gene capable of expressing a desired protein into a cell for production of that protein. The expressed protein can then be isolated and purified for a variety of uses well known to those of skill in the art.

The gene transduction system can also be used to supplement a deficient or mutant gene by the addition of a normal gene thereby correcting a defect caused by the deficient or mutant gene. For example, using the gene transduction system of the present invention, red blood cell precursor cells that contain the sickle hemoglobin gene, which is mutant in the DNA encoding a particular amino acid, can be transduced to express the normal hemoglobin A gene, rectifying the effect of the sickle hemoglobin gene. Another example of this use is if a particular oncogene is being over-expressed thus causing the cell to exhibit a cancerous phenotype. A new sequence can be inserted and incorporated into the genome using the gene transduction system of the present invention which reduces or eliminates expression of the oncogene.

The gene transduction system of the present invention can also be used to supplement insufficient expression of a structurally normal gene. For example, the pancreatic islet cells from insulin deficient diabetic patients could be transduced with a normal insulin gene having a normal insulin promoter. While individuals suffering from this disease sometimes bear a structurally normal insulin gene, it is not expressed normally. The addition of another copy of the normal gene with its normal promoter could be used to treat the diabetes in these patients.

This gene transduction system is also useful in blocking the expression of a particular gene, the expressed protein of which is either abnormal or undesirable. An example of such a disease is α-1 antitrypsin disease. In this instance, a transduced gene or cDNA could either be antisense to the transcript for the undesired gene or could be a catalytic RNA or ribozyme designed to destroy the mRNA for the particular gene. In this embodiment, the target mRNA may be either a normal cellular protein expressed aberrantly, a mutant cellular protein, or even a normal protein, the production of which interferes with a desired effect, for example, an enzyme that metabolizes a drug and prevents it from attaining adequate levels or from maintaining such levels.

The SV40 or SV40 modified viral particles of the present invention are also useful in promoting the expression of foreign proteins in cells for the purpose of immunization. For example, a hepatitis B surface antigen can be introduced with a viral vector of the present invention into a naive individual thereby resulting in protection against infection by hepatitis B virus. Alternatively, the gene transduction system of the present invention can be used to alter the course of viral infection. Transduction of cells with a viral vector promoting expression of an antiviral ribozyme or intracellular antibody (e.g. single chain variable fragment) may be used to inhibit virus replication or gene expression by recognizing certain viral transcripts or proteins and inactivating them.

Linear or circular DNA vectors presently in clinical trials, such as adenovirus and retrovirus, are introduced into cells by a variety of techniques and are transported to the nucleus where the gene target resides. The vector and target undergo recombination events so that the cellular gene is modified or rendered inoperative. The efficiency with which these DNA vectors work, however, is very low.

The gene transduction system of the present invention, however, has a relatively high infectivity to virus particle ratio and can integrate very efficiently into the host genome. This system carries its own strong promoter sequences. Accordingly, this gene transduction system provides an efficient means for the introduction of foreign genes into a cell or animal. For the purposes of this invention, "foreign gene" is meant to include, but is not limited to, genetic material from a species different to the cell or animal in which it is transduced, normal genetic material used to replace a deficient or mutant gene in the same cell or animal in which it is transduced, a normal gene used to supplement insufficient expression of structurally normal gene in the same cell or animal in which it is transduced, or a gene or cDNA which interferes with expression of a gene in a cell or animal. Such foreign genes can be routinely selected by those of skill in the art upon this disclosure. Foreign genes ranging in size from approximately 0.4 kb to approximately 2.2 kb have been transduced into cells using the present invention. However, foreign genes which are less than 0.4 kb or larger than 2.2 kb may also be used. For larger genes to be inserted, plasmids can be prepared having modified cloning cassettes or, in the alternative, by excising a larger portion of the SV40 late genome. For example, the plasmid pSV5 was derived from pBSV(P). The polylinker of pSV5, however, was expanded to include several additional unique restriction sites. In particular, unique sites for *Spe*I, *Sal*I, *Sac*II and *Not*I were added. Further, the viral genome was recloned into the pGEM13 plasmid as a *Not*I fragment. The plasmid pSV5(Δ) has the capacity for an even larger gene as a large portion of the SV40

late genome (0.8 kb) is excised as an *AflI-Bam*HI fragment from pSV5. It is believed that only an approximate 0.5 kb region of the SV40 genome must remain to produce a viral vector of the present invention which is capable of effectively transducing a cell in an animal. Accordingly, it is believed that foreign genes as large as 4.8 kb can be inserted into the viral vectors of the present invention and be effectively transduced in cells of an animal. By "animal" it is meant to include, but is not limited to, mammals, fish, amphibians, reptiles, birds, marsupials, and most preferably, humans.

The gene transduction system of the present invention can be administered to animals by a number of different routes routinely determined by those of skill in the art based upon the selected foreign gene and target site. In one embodiment, selected cells can be removed from an animal, treated *ex vivo* with a viral vector of the present invention, and administered to the animals following treatment. For example, in the case of supplementing a sickle hemoglobin gene with a normal gene in red blood cell precursor cells the target site is the bone marrow. In this case, the mode of administration could be via autologous bone marrow transplantation wherein bone marrow cells from a patient are transduced with the gene transduction system of the present invention and then infused back into the bone marrow of the patient. *Ex vivo* transfer is also used routinely by those of skill in the art in the treatment of certain types of cancers.

Other modes of administration which may be appropriate for the gene transduction system of the present invention include, but are not limited to, inhalation, intravenous, subcutaneous, intramuscular, intrathecal, intraperitoneal, or transurethral administration, intranasal or intratracheal installation, or local or topical administration to a mucosal surface either orally, nasally or via suppository.

Drug formulations, dosing regimens and administration protocols used with the present invention can be determined easily by one of skill based upon general principles of drug chemistry and testing. Compositions to be administered

comprise a viral vector of the present invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include, but are not limited to saline solutions and buffered solutions. Suitable pharmaceutically acceptable carriers are well known in the art and are described for example in Gennaro, Alfonso, Ed., *Remington's Pharmaceutical Sciences*, 18th Edition 1990. Mack Publishing Co., Easton, PA, a standard reference text in this field. Pharmaceutical carriers may be selected in accordance with the intended route of administration and the standard pharmaceutical practice.

The gene transduction system of the present invention is also useful in the development of transgenic animals by gene targeting. Thomas, K.R. and Capecchi, M.R., *Cell* **1987**, 51, 503-512, mutated the endogenous hypoxanthine phosphoribosyl transferase (HPRT) gene in male mouse embryo-derived stem (ES) cells by gene targeting. Specialized vector constructs containing the neomycin resistant gene (neo^r) inserted into a cloned exon fragment of the HPRT gene were used to transfect ES cells. The HPRT-homologies in the vector direct relatively efficient (1 in 1000 of transfectants) replacement or insertion of the exogenous neo^r gene into the endogenous HPRT locus resulting in neomycin-resistant cell colonies with disrupted HPRT genes (neo^rHPRT^-). The HPRT gene was selected primarily for two reasons. First, the HPRT gene lies on the X-chromosome so only one copy needs to be inactivated in cells derived from male embryos. Second, selection procedures exist for isolating $HPRT^-$ mutants. Most cultured cells become resistant to the base analog G-thioguanine (G-TG) through spontaneously acquired mutations in the HPRT gene. Therefore, disruption of the HPRT is not lethal and this locus can accept heterologous genes. This system allows independent assessment of transfection (neo^r) and gene targeting efficiencies ($HPRT^-$ via $G\text{-}TG^r$) efficiencies.

ES cells provide a means for generating transgenic animals having a desired mutation, following mutation or replacement of a chosen gene by gene targeting *ex vivo*. ES

cells have been shown to be pluripotent *in vitro* and *in vivo*. When introduced into mouse blastocytes these cells contribute efficiently to the formation of chimeras, including contribution to a functional germ line. Furthermore, these cells can be manipulated *in vitro* without losing their capacity to generate germ-line chimeras.

In similar fashion, the present invention can be used to introduce mutations into stem cells. The ES system disclosed by Thomas and Capecchi provides the HPRT⁻ selection for scoring. Other selection systems can be routinely engineered by one of skill in the art so that homologous recombination and transfection efficiencies can be scored. The ES system provides a means of gene targeting which is free of any limitation concerning toxicities resulting from introduction of a foreign protein or vector. Manipulations are performed *ex vivo*. Viable recombinants are then reintroduced into blastocytes on these pluripotent cells. Any lethal mutations can be maintained in heterozygous breeding populations, while homozygous gene populations on non-lethal genes can be obtained in second cross breedings.

Transgenic animals are used in the assessment of new therapeutic compositions and in carcinogenicity testing as exemplified by U.S. Patent 5,223,610. These animals are also used in the development of predictive animal models for human disease states as exemplified in U.S. Patent 5,221,778. Transgenic animals have now been developed for assessing Alzheimer's disease (WO 9307280), multi-drug resistance to anticancer agents (WO 9004632), and carcinogenic substances (U.S. Patent 4,736,866). Therefore, the methods of the present invention which enhance gene targeting would be useful in making such animals.

The following nonlimiting examples are provided for illustrative purposes only.

EXAMPLES

Example 1: Plasmids

pBSV-1, containing the wt SV40 genome was cloned as a *Bam*HI fragment into pBR322. To make pBSV(luc), *Tag* was excised from BSV-1 with *Bcl*I and *Avr*II and replaced by a polylinker containing unique *Xba*I, *Xho*I and *Bst*XI sites, as well as Sp6 and T7 promoters (pBSV(ΔT')). This construct retains SV-40 EP just upstream of the polylinker. Firefly luciferase cDNA (luc), plus a second copy of SV40-EP were cloned into pBSV(ΔT'), to yield pBSV(luc).

Example 2: SVluc

Luc-containing virus, SVluc, was made from pBSV(luc) by excising the viral genome with *Bam*HI. It was gel purified, recirculized, and transfected into COS-7 cells. Virus was harvested, purified and titered according to standard methods well known in the art. The inability of SVluc to replicate in cells that do not supply *Tag* was tested by adding SVluc to cultures of COS-7 cells, TC7 cells or medium only. Virus recovery from these cultures was measured 10 days after infection (dpi).

Example 3: Ability of mammalian cells to express a foreign gene

Rabbit kidney cells, TC7 cells and COS-7 cells were infected with recombinant SV40 virus containing the *E. coli lacZ* gene at a virus to cell ratio of approximately 1:1000. Four days after the infection, RNA was extracted from these cells. This RNA was subjected to reverse transcriptase PCR to detect the lacZ transcript. RT-PCR demonstrated that SVlacZ successfully transduced the lacZ transcript.

This series of experiments was also repeated using luciferase. The cells were infected with recombinant SV40 virus containing the gene encoding the firefly enzyme luciferase, referred to as SVluc. Luciferase activity was measured in the infected cells with a scintillation counter. It was determined that functional luciferase enzyme was produced in cells transduced with the luciferase cDNA using SVluc.

Example 4: Inoculation of Mice with SVluc

SVluc was given to BALB/cJ mice via one of two routes. Either 10^7 plaque-forming units (pfu) were administered intravenously (IV) in 0.1 ml saline (control mice received vehicle alone) or femoral bone marrow from BALB/cJ mice was treated with SVluc (multiplicity of infection (moi) was approximately 0.3) for 6 hours before transfer. Bone marrow recipients received 450R external beam radiation from a Cs source, followed by the SVluc-treated bone marrow, 3×10^7 nucleated cell/mouse, IV. Control mice received untreated bone marrow.

Example 5: Tissue Preparation in SVluc-treated mice

Peripheral blood was sampled from bone marrow recipients at intervals. It was kept frozen at -70°C for luciferase assays. Smears were air-dried, and fixed in acetone. Recipients of SVluc IV were sacrificed from 20 to 105 dpi. Selected tissues including stomach, lung, liver, spleen, kidney, heart, brain, colon, skin at the inoculation site, and diaphragm were excised and frozen for sectioning.

Example 6: Luciferase analysis

Luc was detected enzymatically in whole peripheral blood using a Luciferase detection kit (Promega Corp., Madison, WI) in accordance with the manufacturer's instructions. Luc-producing cells were visualized in peripheral blood smears or frozen tissue sections by immunostaining. Affinity-purified anti-luc antibody (Promega), diluted 1:1600, or normal IgG, was applied, followed by avidin-goat anti-rabbit IgG, then avidin-peroxidase for tissue sections or avidin-alkaline phosphatase for peripheral blood, both with biotin bridges, then substrate. As negative controls, identical serial sections, stained with normal IgG, and tissues from control mice were stained using the anti-luc antibody. Luc production was evaluated semiquantitatively, - to ++++, based on numbers of positive cells and strength of staining, relative to negative controls.

What is claimed is:

1. A method of introducing a foreign gene into an animal comprising administering to an animal an SV40 or modified SV40 viral particle containing a circular double-stranded DNA species comprising viral SV40 DNA and a selected foreign gene.

2. A method of introducing a foreign gene into an animal comprising:

(a) removing selected cells from an animal;

(b) treating the selected cells with an SV40 or modified SV40 viral particle containing a circular double-stranded DNA species comprising viral SV40 DNA and a selected foreign gene; and

(c) administering the treated cells to the animal.

3. An SV40 gene transduction vector comprising a selected portion of an SV40 genome and a selected foreign gene, said vector being capable of effectively transducing cells in an animal with the selected foreign gene.

4. A composition comprising an SV40 gene transduction vector containing a selected portion of an SV40 genome and a selected foreign gene, said vector being capable of effectively transducing cells in an animal with the selected foreign gene, and a pharmaceutically acceptable carrier.

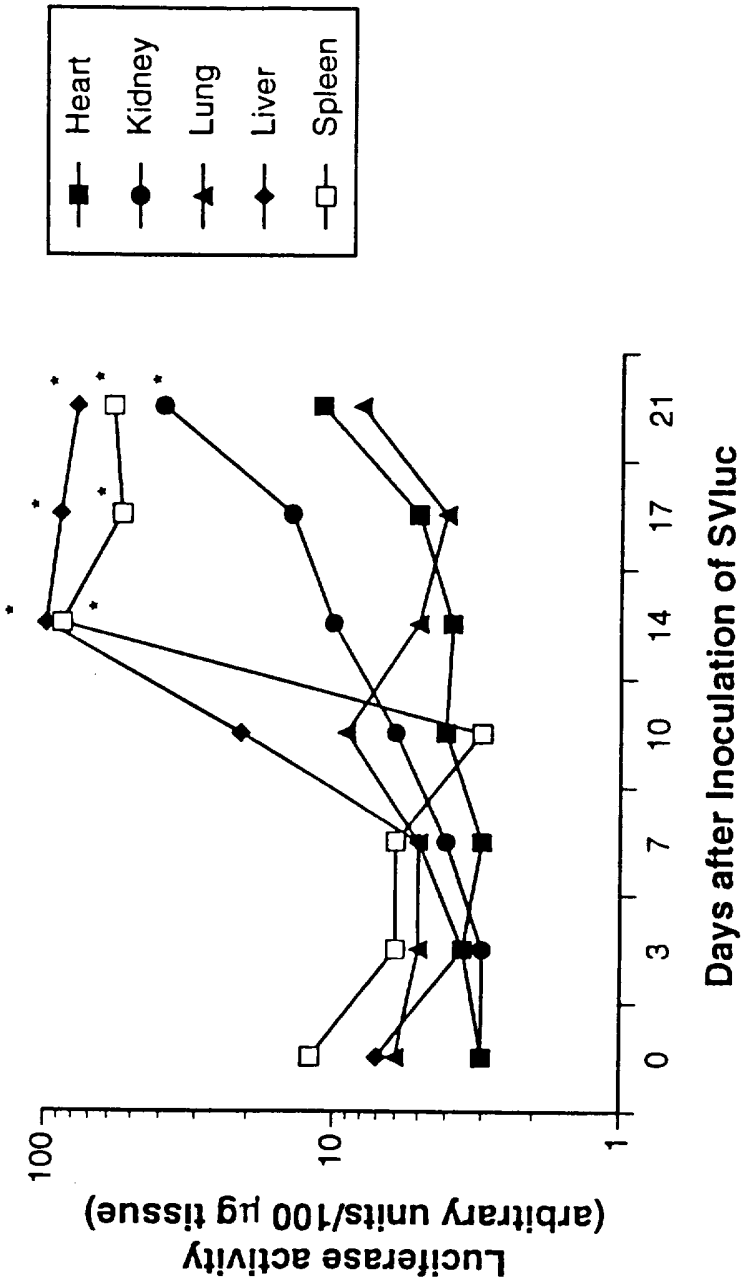
Heart
Kidney
Lung
Liver
Spleen
Luciferase activity
(arbitrary units/100 µg tissue)
100
10
1
0
3
7
10
14
17
21
Days after Inoculation of SVluc

FIGURE 1

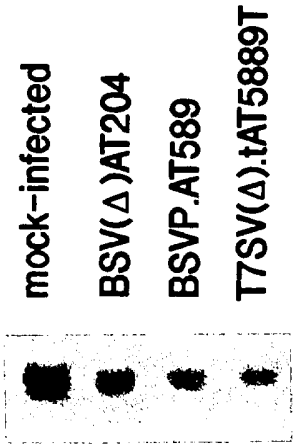

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/17065

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; A61K 31/715; C12N 15/00, 15/09; C12P 21/00
US CL :435/69.1, 172.3, 320.1; 514/44
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. : 435/69.1, 172.3, 320.1; 514/44

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)

Databases searched: APS, MEDLINE, BIOSIS, CA, EMBASE
Search Terms: sv40; virus?; vector?; transfect?; transduc?; vir?; gene#; therap?; vivo; administ?; replicat?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ANALYTICAL BIOCHEMISTRY, Volume 135, issued 1983, S. Subramani et al., "Analysis of Gene Expression Using Simian Virus 40 Vectors", pages 1-15, see entire document.	1, 3, and 4 --- 2
X --- Y	ANNUAL REVIEW OF GENETICS, Volume 15, issued 1981, J.T. Elder et al., "Simian Virus 40 as a Eukaryotic Cloning Vehicle", pages 295-340, see entire document.	1, 3, and 4 --- 2
X --- Y	GENE, Volume 66, issued 1988, D. Huyrebroeck et al., "High-level transient expression of influenza virus proteins from a series of SV40 late and early replacement vectors", pages 163-181, see entire document.	1, 3, and 4 --- 2
Y,P	US, A, 5,399,346 (ANDERSON ET AL.) 21 MARCH 1995, see entire document.	2

[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 24 APRIL 1996	Date of mailing of the international search report 28 MAY 1996
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/17065

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF REPRODUCTIVE MEDICINE, Volume 37, Number 6, issued June 1992, E.M. Karson et al., "Prospects for Human Gene Therapy", pages 508-514, see entire document.	1, 3, and 4