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(54) Title: ANTI-CANCER IMMUNOTHERAPEUTIC VECTOR CONSTRUCTS

(57) Abstract

The present invention provides a method of destroying selected tumor cells comprising administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells. Also provided are vector constructs which direct the expression of altered cellular components. Representative altered cellular components include ras*, p53*, Rb*, alter protein encoded by the Wilms' tumor gene, ubiquitin*, DCC, APC. MCC, neu, an altered receptor, and bcr/abl. Also provided are recombinant viruses carrying a vector construct, target cells infected with the recombinant virus and pharmaceutical compositions comprising the recombinant virus and a pharmaceutically acceptable carrier or diluent.

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Description

Anti-cancer immunotherapeutic vector constructs.

Technical Field

The present invention relates generally to the field of anti-cancer immunotherapy, and more specifically, to methods of killing selected t · cells by generating an immune response against the tumor cells.

Background of the Invention

Cancer accounts for one-fifth of the total mortality in the United States, and is the second leading cause of death. Cancer is typically characterized by the uncontrolled division of a population of cells. This uncontrolled division typically leads to the formation of a tumor, which may subsequently metastasize to other sites.

Primary solid tumors can generally be treated adequately by surgical resection. However, the majority of patients which present with solid tumors also possess micrometastases beyond the primary tumor site. If treated with surgery alone, approximately 70% of these patients will experience recurrence of the cancer. In addition to surgery, many cancers are now also treated with a combination of therapies involving cytotoxic chemotherapeutic drugs (*e.g.*, vincristine, vinblastine, cisplatin, etc.) and/or radiation therapy. One difficulty with this approach, however, is that radiotherapeutic and chemotherapeutic agents are toxic to normal tissues, and often create life-threatening side effects. In addition, these approaches often have extremely high failure/remission rates (up to 90% depending upon the type of cancer).

In addition to chemo- and radiation therapies, many have attempted to bolster or augment an individual's own immune system in order to eliminate the cancer cells. Several immunotherapies have utilized bacterial or viral components in order to stimulate the immune system to destroy the tumor cells. Examples of such components include immunomodulatory agents (such as BCG, endotoxin, and mixed bacterial vaccines), interferons (α, β, and γ), interferon inducers (*e.g.*, *Brucella abortus*, and various viruses), and thymic factors (*e.g.*, thymosin fraction 5, and thymosin alpha-1) (*see generally* "Principles of Cancer Biotherapy," Oldham (ed.), Raven Press, New York, 1987). Such agents

have generally been useful as adjuvants and as nonspecific stimulants in animal tumor models, but have not yet proved generally effective in humans.

Lymphokines have also been utilized in the treatment of cancer. Briefly, lymphokines are secreted by a variety of cells, and generally have an effect on specific cells in the generation of an immune response. Examples of lymphokines include Interleukins (IL)-1, -2, -3, and -4, as well as colony stimulating factors such as G-CSF, GM-CSF, and M-CSF. Recently, one group has utilized IL-2 to stimulate peripheral blood cells in order to expand and produce large quantities of cells which are cytotoxic to tumor cells (Rosenberg et al., *N. Engl. J. Med. 313*:1485-1492, 1985).

Others have suggested the use of antibody-mediated anti-cancer therapies. Briefly, antibodies may be developed which recognize certain cell surface antigens that are either unique, or more prevalent on cancer cells compared to normal cells. These antibodies, or "magic bullets," may be utilized either alone or conjugated with a toxin in order to specifically target and kill tumor cells (Dillman, "Antibody Therapy," *Principles of Cancer Biotherapy*, Oldham (ed.), Raven Press, Ltd., New York, 1987). For example, Ball et al. (*Blood 62*:1203-1210, 1983) treated several patients with acute myelogenous leukemia with one or more of several monoclonal antibodies specific for the leukemia, resulting in a marked decrease in circulating leukemia cells during treatment. Similarly, others have used toxin-conjugated antibodies therapeutically to treat a variety of tumors, including, for example, melanomas, colorectal carcinomas, prostate carcinomas, breast carcinomas, and lung carcinomas (*see* Dillman, *supra*). One difficulty however, is that most monoclonal antibodies are of murine origin, and thus hypersensitivity against the murine antibody may limit its efficacy, particularly after repeated therapies. Common side effects include fever, sweats and chills, skin rashes, arthritis, and nerve palsies.

Therefore, agents which can augment natural host defences against tumor induction or progression may increase remission rates and enhance survival of patients, without the cytotoxic side effects of prior methods. The present invention provides such agents, and further provides other related advantages.

Summary of the Invention

The present invention provides methods for destroying selected tumor cells with an altered cellular component which is normally associated with the selected tumor cells. Within one aspect, a method is provided for

destroying selected tumor cells comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells. Within another aspect of the invention, a method is provided for destroying selected tumor cells in a warm-blooded animal comprising the steps of (a) removing cells from a warm-blooded animal, (b) administering to the removed cells a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells, and (c) returning the cells to a warm-blooded animal, such that the selected tumor cells are destroyed. As will be evident to one of ordinary skill in the art, the animal from which the cells are removed need not be the same animal to which they are returned, although preferably, they should be histocompatible. In addition, it should be understood that within the context of the present invention when reference is made to a viral construct which "expresses" any substance in a cell, that this in fact refers to protein production of the resulting provirus following reverse transcription of the viral RNA into the cell. Within various embodiments of the invention, the vector construct may be carried by a recombinant retrovirus, or by other recombinant viruses such as those selected from the group consisting of adeno-associated virus, canary pox virus, adenovirus, and pox virus. Alternatively, an immunogenic form of an altered cellular component may be manufactured *in vitro*, and given to patients with an appropriate adjuvant, preferably one which leads to induction of cellular immunity.

Within another aspect of the present invention, a vector construct is provided which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component. Within various embodiments, the cellular component may be altered by a point mutation, by a deletion, or by a chromosomal translocation. Within other embodiments, the altered cellular components include, ras^*, $p53^*$, Rb^*, altered protein encoded by the Wilms' tumor gene, $ubiquitin^*$, DCC, APC, MCC, neu, an altered receptor, or polypeptides resulting from chromosomal translocations such as bcr/abl. Within another embodiment, non-tumorigenic altered cellular components are provided, including for example, $\Delta\ ras^{*12}$, $\Delta\ ras^{*13}$, and $\Delta\ ras^{*61}$. Within other aspects of the invention, the altered cellular component is $mucin^*$. Also provided are vector constructs which direct the expression of several altered cellular components, including, for example, a vector construct which

directs the expression of both ras* and p53*, or a vector construct which directs the expression or ras*, mucin*, and DCC.

Within another aspect of the invention, recombinant retroviruses as well as other recombinant viruses, such as polioviruses, rhinoviruses, pox viruses, adenoviruses, parvoviruses, herpes viruses and sindbis viruses, are provided for carrying the above-described vector constructs. Target cells infected with these recombinant viruses are also provided, including, for example, embodiments wherein the target cells are selected from the group consisting of human, macaque, dog, rat, and mouse cells.

Also provided are pharmaceutical compositions comprising the above-described recombinant viruses, in combination with a pharmaceutically acceptable carrier or diluent.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a schematic illustration which outlines the construction of the plasmid SP-Val^{12}(100).

Figure 2 is a schematic illustration which outlines the construction of the plasmid SP-Δ-Val^{12}.

Figure 3 is a schematic illustration which outlines the construction of the plasmid N2-ras-Val^{12}.

Figure 4 is a schematic illustration which outlines the construction of the plasmid N2-Δ-ras-Val^{12}.

Figure 5 is a schematic illustration of mucin cDNA cloned into the KT-3 retroviral backbone.

Figure 6 is a Western Blot which illustrates the expression of mucin from various cell types.

Figure 7 is a graph which illustrates the CTL response for several BC10ME clones.

Figure 8 is a FACS analysis of mucin expression on several different BC10ME clones.

Figure 9 is a bar graph which illustrates the tumorigenicity of two B16F10 clones.

Figure 10 is a bar graph which illustrates protection in mice injected with the B16F10-Mucin clone.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Altered Cellular Component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general but are not required or essential for rendering the cell tumorigenic. Within one aspect of the invention, before alteration of the cellular component, the cellular component may be essential to normal cell growth and regulation. Examples include proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular components no longer perform their regulatory functions, and hence the cell may experience uncontrolled growth. Representative examples of such altered cellular components include ras*, p53*, Rb*, altered protein encoded by the Wilms' tumor gene, ubiquitin*, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, and altered or mutated forms of the thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor. Within other aspects of the present invention, the cellular component may become altered by expression in a cell type that does not normally express the cellular component. For example, mucin will experience abberant glycosylation upon expression in cell types other than normal breast or pancreatic epithelium, and hence become "altered". These as well as other cellular components are described in more detail below, as well as discussed in cited references. All references which have been cited below are hereby incorporated by reference in their entirety.

"Non-tumorigenic" refers to altered cellular components which will not cause cellular transformation or induce tumor formation in nude mice. Representative assays which distinguish tumorigenic cellular components from non-tumorigenic cellular components are described in more detail below and in Example 4.

"Immunogenic" as utilized within the present invention refers to altered cellular components which are capable, under the appropriate conditions, of causing an immune response. This response must be cell-mediated and may also include a humoral response. Representative assays which may be

utilized to determine immunogenicity are described in more detail below and in Example 5.

"Vector construct" refers to an assembly which is capable of expressing the sequence(s) or gene(s) of interest. The vector construct must include promoter elements and preferably includes a signal that directs polyadenylation. In addition, the vector construct must include a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. Preferably, the vector construct may also include a selectable marker such as Neo, SV_2 Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the vector construct is placed into a retrovirus, the vector construct must include a packaging signal and long terminal repeats (LTRs) appropriate to the retrovirus (if these are not already present).

As noted above, the present invention provides methods and compositions suitable for destroying selected tumor cells. Within one aspect of the present invention, a method is provided which comprises the step of administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells. Within another aspect of the present invention, a method is provided for destroying selected tumor cells in a warm-blooded animal comprising the steps of (a) removing cells from a warm-blooded animal, (b) administering to the removed cells a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells, and (c) returning the cells to a warm-blooded animal, such that the selected tumor cells are destroyed. Within a third aspect of the present invention, a method is provided for introducing proteins manufactured elsewhere which correspond to altered cellular components with a suitable adjuvant into a warm-blooded animal such that an immune response is generated and selected tumor cells are destroyed. Utilizing these methods, an immune response may be generated which destroys tumor cells that are associated with the altered cellular component.

Briefly, the ability to recognize and defend against foreign pathogens is central to the function of the immune system. This system, through immune recognition, is capable of distinguishing "self" from "nonself" (foreign), which is essential to ensure that defensive mechanisms are directed towards

invading entities rather than against host tissues. The fundamental features of the immune system are the presence of highly polymorphic cell surface recognition structures (receptors) and effector mechanisms (antibodies and cytolytic cells) for the destruction of invading pathogens.

Cytolytic T lymphocytes (CTL) are normally induced by the display of processed pathogen-specific peptides in conjunction with MHC molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989). Other genes coding for proteins that enhance the stimulation or recognition of cell mediated responses may also be used in this context. Antigenic peptide presentation in association with MHC (major histocompatibility) Class I molecules leads to $CD8^+$ CTL production. Peptides presented in association with MHC Class II molecules leads to production of antibodies, helper cells and B-cell memory and may induce $CD4^+$ CTLs. The methods which are described in greater detail below provide an effective means of inducing potent class I-restricted protective and therapeutic CTL responses, as well as humoral responses.

As noted above, altered cellular components refers to proteins and other cellular constituents which are either associated with rendering the cell tumorigenic, or are associated with tumorigenic cells in general, but are not required or essential for rendering the cell tumorigenic. Representative examples of alterations which occur in cellular components include point mutations, deletions, and chromosomal translocations. These alterations serve to generate an altered cellular component which the host immune system may not recognize as "self," and thereby eliminate the neoplastic or pre-neoplastic cells containing the altered cellular component.

Within one embodiment of the present invention, a vector construct is provided which directs the expression of a non-tumorigenic, altered ras (ras^*) gene. Briefly, the ras^* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras^* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an

important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras* genes, on the other hand, alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science 248*:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

Ras* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas (*see* Table 1 below).

TABLE 1

Tumor type	Incidence of ras mutations
Pancreatic Adenocarcinoma	90%
Colon Adenoma	50%
Colon Adenocarcinoma	50%
Seminoma	40%
Lung Adenocarcinoma	30%
Myelodisplatic Syndrome	30%
Acute Myelogenous leukemia	30%
Keratinoacanthoma	30%
Thyroid carcinoma	25%
Melanomas	20%
Bladder carcinoma	6%

The spectrum of mutations occurring in the ras* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras* occur primarily (*in vivo*) in only 3 codons: 12, 13 and 61; with mutations at codon 12 being the most prevalent in both human and animal tumors. Table 2 below sets forth the incidence of mutations at codons 12 and 13 for various human tumors. (The normal codons for positions 12 and 13 are GGT and GGC, respectively, both of which code for the amino acid glycine.)

TABLE 2

Approximate percentage of specific mutations at codons 12 and 13 of ras*

Tumor type/Mutation	GAT Asp(12)	AGT Ser(12)	CGT Arg(12)	TGT Cys(12)	GTT Val(12)	GCT Ala(12)	GAC Asp(13)
Pancreatic Carcinoma	47%	2%	10%	12%	27%	<1%	2%
Colorectal Adenoma or Carcinoma	39%	3%	<1%	9%	23%	2%	23%
Lung Carcinoma	17%	4%	4%	40%	30%	<1%	4%

Table 3 summarizes known *in vivo* mutations (codons 12, 13 and 61) which activate human ras, as well as potential mutations which have *in vitro* transforming activity. Briefly, potential mutations with *in vitro* transforming activity may be produced by the systematic substitution of one nucleic acid of a normal codon, in order to produce upon expression another amino acid (*e.g.*, in this manner other amino acids were substituted for normal glycine a position 12). Such mutations, while not presently known to occur in humans or animals, may serve as the basis for an anti-cancer immunotherapeutic if they are eventually found to arise *in vivo*.

Table 3

Amino acid substitutions that activate human *ras* proteins

Amino Acid Mutant Codon	Gly 12	Gly 13	Ala 59	Gln 61	Glu 63	Asn 116	Lys 117	Asp 119
In vivo	Val	Asp		Arg				
	Arg	Val		His				
	Asp	Arg		Leu				
	Cys							
	Ala							
	Ser							
	Phe							
In vitro	Ala	Ser	Thr	Val	Lys	His	Glu	His
	Asn			Ala		Ile	Arg	Glu
	Gln			Cys				Ala
	Glu			Asn				Asn
	His			Ile				
	Ile			Met				
	Leu			Thr				
	Lys			Tyr				
	Met			Trp				
	Phe			Phe				
	Ser			Gly				
	Thr							
	Trp							
	Tyr							

Alterations as described above result in the production of proteins containing novel coding sequence(s). The novel proteins encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequences (ras^*).

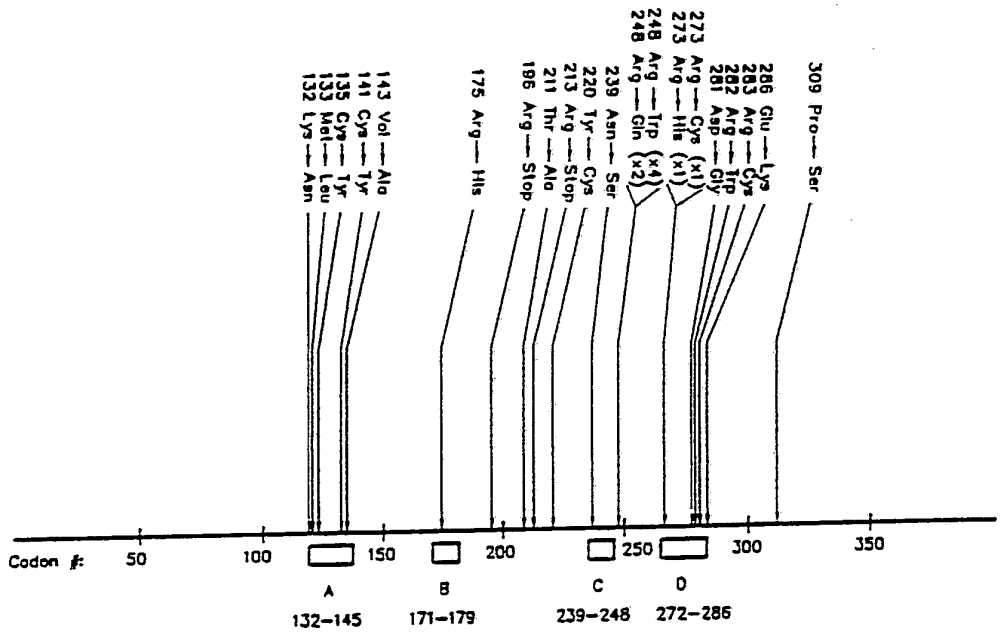
Within another embodiment of the present invention, a vector construct is provided which directs the expression of an altered p53 ($p53^*$) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells, and thus was initially classified as an oncogene (Linzer and Levine, *Cell 17*:43-52, 1979; Lane and Crawford, *Nature 278*:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol. 63*:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles,

one through deletion, and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (*e.g.*, $p53^{*1}$, $p53^{*2}$, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature 351*:453-456, 1991; *see also* the following references which describe specific mutations in more detail: Baker et al., *Science 244*:217-221, 1989; Nigro et al., *Nature 342*:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature 348*:681-682, 1990; Takahashi et al., *Science 246*:491-494, 1989; Iggo et al., *Lancet 335*:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA 86*:2858-2862, 1989; Mackay et al., *Lancet 11*:1384-1385,1988; Kelman et al., *Blood 74*:2318-2324, 1989; Malkin et al., *Science 250*:1233-1238, 1990; Baker et al., *Cancer Res. 50*:7717-7722, 1991; Chiba et al., *Oncogene 5*:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene 5*:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol. 10*:5502-5509, 1990; Bartek et al., *Oncogene 5*:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA 87*:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA 87*:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA 87*:5863-5867, 1990; and Romano et al., *Oncogene 4*:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature 350*:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B_1, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286:



Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature 351*:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence ($p53^*$).

Within another embodiment of the present invention, a vector construct is provided which directs the expression of an altered Rb (Rb^*) gene. Briefly, retinoblastoma is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned which produces a nuclear phosphoprotein of about 110kd (Friend et al., *Nature 323*:643, 1986; Lee et al., *Science 235*:1394, 1987; and Fung et al.. *Science 236*:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus. and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F

(Bagchi et al., *Cell 62*:659-669, 1990) and DRTF (Shivji and La Thangue, *Mol. Cell. Biol. 11*:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety of nuclear proteins involved in cellular proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science 247*:712-715, 1990; Horowitz et al., *Science 243*:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff. 1*:17, 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA 87*:6, 1990). Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

Within another embodiment of the present invention, a vector construct is provided which directs the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney, and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; and Haber et al., *Cell 61*:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

Mutations of the Wilms' tumor gene include the insertion of lysine, threonine, and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation results in the insertion of about 17 amino acids in the region immediately NH_2-terminal to the zinc finger domain (Madden et al., *Science 253*:1550-1553, 1991; Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; Haber et al., *Cell 61*:1257, 1990; and Buckler et al., *Mol. Cell. Biol. 11*:1707, 1991).

Alterations as described above result in the production of protein(s) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s), which cause Wilms' tumor.

Within another embodiment of the present invention, a vector construct is provided which directs the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem 27*:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature 343*:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., "Identification of a Chromosome 18q Gene That Is Altered in Colorectal Cancers," *Science 247*:49-56, 1990).

Within another embodiment of the present invention, a vector construct is provided which directs the expression of MCC or APC. Both MCC (mutated in colorectal cancer) and APC have been identified as tumor suppressor genes (Kinzler et al., *Science 251*:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis (FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science 253*:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the

colon and rectum. This proliferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two separate patients, one with a desmoid tumor, (3) codon 414, an arginine to cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science 253*:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons number 289, 332, 438, and 1338).

Alterations as described above result in the production of protein(s) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s) which cause DCC, APC, or MCC.

Within another embodiment of the present invention, a vector construct is provided which directs the expression of altered ubiquitin. Briefly, ubiquitin is a cellular protein which is involved in cell-cycle control and DNA replication. Other functions of ubiquitin include intracellular protein degradation, heat-shock response, transcriptional regulation, cell-cycle control, and cell-cell interaction. Ubiquitin is believed to be a marker molecule that targets proteins for a variety of metabolic fates, and a cDNA sequence which encodes this protein has been identified (Lund et al., "Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor," *J. Biol. Chem. 263*:4926-4931, 1985).

A mutant ubiquitin (ubiquitin*) has recently been identified in a human colon carcinoma cell line (Mafune et al., *Arch.-Surg. 126*:462-466, 1991). This tumor cell contains a novel fusion protein consisting of a hybrid ubiquitin-ribosomal protein S27a. The fusion junction of this protein results in a novel nonself protein sequence which may be immunogenic, and therefore used to eliminate tumor cells carrying this fusion protein.

Within another embodiment of the present invention, a vector construct is provided which directs the expression of altered bcr/abl. Briefly, in

tumor cells from almost all patients with chronic myelogenous leukemia, the Philadelphia chromosome, a fusion of chromosomes 9 and 22, directs the synthesis of the fused $P210^{bcr/abl}$ protein. This hybrid gene encodes a 210kD phosphoprotein with disregulated protein-kinase activity which leads to the chronic myelogenous leukemia (Daley et al., *Science 247*:824-829, 1990; Shtivelman et al., *Nature 315*:550-554, 1985; Ben-Neriah et al., *Science 233*:212-214, 1986; and Shtivelman et al., *Cell 47*:277-284, 1986). The fusion junction of these two chromosomes results in a novel nonself protein sequence which may be immunogenic, and thus used to eliminate tumor cells carrying this fusion protein.

Within other embodiments of the invention, a vector construct is provided which directs the expression of an altered receptor which is functionally locked or stuck in an "ON" or "OFF" mode. Briefly, many cellular receptors are involved in cell growth by monitoring the external environment and signaling the cell to respond appropriately. If either the monitoring or signalling mechanisms fail, the cell will no longer respond to the external environment and may exhibit uncontrolled growth. Many different receptors or receptor-like structures may function as altered cellular components, including, for example, neu and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the Interleukin receptors (*e.g.*, IL-1, -2, -3, etc. receptors), or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors.

For example, neu (also referred to as the Human Epidermal Growth Factor Receptor "HER" or the Epidermal Growth Factor "EGF" receptor) is an altered receptor which is found in at least 28% of women with breast cancer. A cDNA clone which encodes this protein has been isolated (Slamon et al., *Science 244*:707-712, 1989; Slamon et al., *Cancer Cells 7*:371-380, 1989; Shih et al., *Nature 290*:261, 1981). This clone encodes a protein that has extracellular, transmembrane, and intracellular domains (Schechter, *Nature 312*:513, 1984; Coussens et al., *Science 230*:1132, 1985) and thus is believed to encode the neu receptor.

Studies of the rat neu gene isolated from chemically induced neuroglioblastoma cells indicate that it contains a single mutation at position 664 from valine to glutamic acid (Bargmann et al., *EMBO J. 7*:2043, 1988). In other studies, baby rats which were treated with N-ethyl-N-nitrosourea developed malignant tumors of the nervous system. All 47 trigeminal schwannomas and 12 neurinomas which developed carried a T to A transversion

at position 664 of the neu gene (Nikitin et al., *Proc. Natl. Acad. Sci USA 88*:9939-9943, 1991).

Other altered receptors may also be expressed by vector constructs in order to destroy selected tumor cells. For example, a deletion in chromosome 3p21-p25 has been associated with small-cell lung carcinomas (Leduc et al., *Am. J. Hum. Genet. 44*:282-287, 1989). A deletion is believed to occur in the ERBAβ gene which otherwise codes for a DNA-binding thyroid hormone receptor (THR).

Alterations in receptors as described above result in the production of protein(s) (or receptors) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorige : cells containing the altered sequence(s) or gene(s).

As noted above, within other aspects of the present invention, a vector construct is provided which, when expressed in cell types other than normal breast or pancreatic epithelium, directs the expression of mucin which is abberantly glycosylated (mucin*). Briefly, mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J. Cancer 43*:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem. 265*(25):15286-15293, 1990; Lan et al., *J. Biol. Chem. 265*(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem. 265*:5573-5578, 1990). Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al., *Cancer Res. 51*:2908-2916, 1991). CTL lines which have been developed to breast tumors cross-react with pancreatic tumor targets, and further, appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats, or even the entire mucin cDNA, may be expressed by a vector construct of the present invention, in order to develop an immune response against tumor cells which express abberantly glycosylated mucin. A particularly preferred embodiment of the invention is set forth in more detail below in Examples 6 to 10.

Sequences which encode the above-described altered cellular components may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained

from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see, for example*, ATCC No. 41001 which contains a sequence which encodes the normal ras protein, ATCC No. 57103 which encodes abl, and ATCC Nos. 59120 or 59121 which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*see* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

In like manner, sequences which encode normal cellular components may be obtained from cells, and mutated by site-directed mutagenesis in order to obtain sequences which encode the altered cellular component. Such sequences may be readily obtained by, for example, preparing primers on either side of the sequence, and amplifying the sequence by PCR (*see* U.S. Patent Nos. 4,683,202; 4,683,195; and 4,800,159) (*see also PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989). Briefly, double-stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode altered cellular components may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California). Such sequences may be ligated to form a long single-stranded DNA molecule. Briefly, short, overlapping antisense linkers are mixed with the primary sequences, after which the primary sequences may be ligated to form a long, single-stranded DNA molecule.

Once a sequence encoding the altered cellular component has been obtained, it is necessary to ensure that the sequence encodes a non-

tumorigenic protein. Various assays are known and may easily be accomplished which assess the tumorigenicity of a particular cellular component. Representative assays include a rat fibroblast assay (which is described in more detail below in Example 4), tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of a particular cellular component. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if infected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the vector construct is administered to syngeneic murine cells, followed by injection into nude mice. The mice are visually examined for a period of 2 to 8 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst. 48*:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells, New Products and Risk*, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std. 13*:135-141, 1985).

Tumorigenicity may also be assessed by visualizing colony formation in soft agar (Macpherson and Montagnier, *Vir. 23*:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an altered cellular component. (Stewart et al., *Cell 38*:627-637, 1984; Quaife et al., *Cell 48*:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA 86*:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the animal. This dysregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

If the altered cellular component is associated with making the cell tumorigenic, then, it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment, the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced into the gene which encodes the altered cellular component, downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the ras* gene is truncated in order to render the ras* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of ras* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the ras* gene is truncated in the purine ring formation, for example around the sequence which encodes amino acid number 110. The ras* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s) are encoded by the vector construct, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the p53* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the p53 protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, p53* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both neu and bcr/abl may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above, or as described in Example 4.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or

essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb*, ubiquitin*, and mucin*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays. A particularly preferred method for determining immunogenicity is the CTL assay which is described in detail below in Example 5.

As noted above, within another aspect of the present invention, several different altered cellular components may be co-expressed in order to form a general anti-cancer therapeutic. Generally, it will be evident to one of ordinary skill in the art that a variety of combinations can be made. Within preferred embodiments, this therapeutic may be targeted to a particular type of cancer. For example, nearly all colon cancers possess mutations in ras, p53, DCC APC or MCC genes. A vector construct which co-expresses a number of these altered cellular components may be administered to a patient with colon cancer in order to treat all possible mutations. This methodology may also be utilized to treat other cancers. Thus, a vector construct which co-expresses mucin*, ras*, neu, and p53* may be utilized to treat breast cancer.

In addition, the altered cellular components of the present invention may also be co-expressed with a lymphokine and/or immune modulator. Representative examples of lymphokines include tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1, and G-CSF. Representative examples of immune modulators include CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, β-2-microglobulin, chaperones, alpha interferon and gamma interferon, and major histocompatibility complex (MHC).

Once a particular altered cellular component has been selected, it is placed into a vector construct which directs its expression. Vector constructs of the present invention may be used as an alternative to surgery, or may be

used in combination with surgical or adjuvant modalities, and may prove more effective post-surgically then chemotherapy or radiotherapy since a specific cytotoxicity against remaining tumor cells is elicited. Construction of retroviral vector constructs is described in greater detail below in Example 2. In addition, construction of additional vector constructs as well as administration of retroviral constructs by direct injection is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990). This application is incorporated by reference in its entirety.

Other viruses may also be utilized to administer vector constructs, including, for example, poliovirus (Evans et al., *Nature 339*:385-388, 1989, and Sabin, *J. of Biol. Standardization 1*:115-118, 1973); rhinovirus (Arnold, *J. Cell. Biochem.* L401-405, 1990); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112 and 4,769,330; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *The New England Journal of Medicine 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); adenovirus (Berkner, *Biotechniques 6*:616-627, 1988, and Rosenfeld et al., *Science 252*:431-434, 1991); parvovirus such as adeno-associated virus (Samulski et al., *Journal of Virology 63*:3822-3828, 1989, and Mendelson et al., *Virology 166*:154-165, 1988); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989); SV40; HIV; measles (EP 0 440,219); and Sindbis virus (Xiong et al., *Science 234*:1188-1191, 1989). Furthermore, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910, 1988), and yet induce cellular immune responses, including CTL.

Various methods may be utilized to administer the vector construct, or nucleic acids which encode the altered cellular component to patients directly, including, for example, transfection by methods utilizing various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991); liposomes (Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984); or DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989).

In addition, a CTL response may also be generated by administration of a bacteria which expresses the altered cellular component(s) on its cell surface. Representative examples include BCG (Stover, *Nature 351*:456-458, 1991) and salmonella (Newton et al., *Science 244*:70-72, 1989).

Cell mediated and humoral responses may also be induced against tumors by parenteral administration of the altered cellular components themselves. Briefly, altered cellular components (ras*, p53*, etc.) or peptides carrying relevant epitopes can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol. 31*:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16*, 1986, Applied Biosystems, Foster City California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology 131*:51-68, 1986), mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol. 63*:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature 307*:178-180), and prokaryotic systems (Burrel et al., *Nature 279*:43-47, 1979).

The proteins or peptides can be purified by conventional means and delivered by a number of methods to induce cell-mediated responses, including class I and class II responses. These methods include use of adjuvants of various types, such as ISCOMS (Morein, *Immunology Letters 25*:281-284, 1990; Takahashi et al., *Nature 344*:873-875m, 1990), liposomes (Gergoriadis et al., *Vaccine 5*:145-151, 1987), lipid conjugation (Deres et al., *Nature 342*:561-564, 1989), coating of the peptide on autologous cells (Staerz et al., *Nature 329*:449-451, 1987), pinosomes (Moore et al., *Cell 54*:777-785, 1988), alum, complete or incomplete Freund's adjuvants (Hart et al., *Proc. Natl. Acad. Sci. USA 88*:9448-9452, 1991), or various other useful adjuvants (*e.g.*, Allison and Byars, *Vaccines 87*:56-59, Cold Spring Harbor Laboratory, 1987) that allow effective parenteral administration.

Alternatively, the proteins or peptides corresponding to altered cellular components can be encapsidated for oral administration to elicit immune response in enteric capsules (Channock et al., *J. Amer. Med. Assoc. 195*:445-452, 1966) or other suitable carriers, such as poly (DL-lactide-co-glycolate) spheres (Eldridge et al. in *Proceedings of the International Conference on Advances in AIDS Vaccine Development, DAIDS, NIAID*, U.S. Dept of Health & Human Services, 1991), for gastrointestinal release.

In addition, the proteins or peptides can be manipulated to render them more immunogenic (*e.g.*, by adding amino acid sequences that correspond

to T helper epitopes), to promote cellular uptake by adding hydrophobic residues, to particulate structures, or any combination of these (Hart et al., *PNAS 88*:9448-9452, 1991; Milich et al., *Proc. Natl. Acad. Sci. USA 85*:1610-1614, 1988; Willis, *Nature 340*:323-324, 1989; Griffiths et al., *J. Virol. 65*:450-456, 1991).

Within one aspect of the invention, a method is provided for destroying selected tumor cells in a warm-blooded animal comprising the steps of (a) removing cells from a warm-blooded animal, (b) administering to the removed cells a vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component normally associated with the selected tumor cells, and (c) returning the cells to a warm-blooded animal, such that said selected tumor cells are destroyed. Within the context of the present invention it should be understood that the removed cells need not necessarily be returned to the same animal, but may be utilized to destroy selected tumor cells in another animal. In such a case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991). In addition, it should be understood that a variety of cells (target cells) may be utilized within the context of the present invention, including for example, human, macaque, dog, rat, and mouse cells.

Cells may be removed from a variety of locations, including for example from the skin (dermal fibroblasts) and the blood (peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be removed from the blood for administration of the vector construct (*e.g.*, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be administered to the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal.

Within another aspect of the present invention, a vector construct is provided which directs the expression of a tumorigenic cellular component and a prodrug activator. For example, within one embodiment, genes for an altered cellular component and a prodrug activator, such as Herpes Simplex Virus Thymidine Kinase (HSVTK), are incorporated into the vector construct. This vector construct is then administered to cells in the presence of an exogenous substance, such as acyclovir, which kills cells that express the HSVTK. As one of ordinary skill in the art will readily appreciate, this vector

construct may also be utilized to ensure that even if the delivered genes contribute to a tumorigenic event in cells which have taken up the vector, these cells can be skilled by, for example, acyclovir.

Prior to administering the vector construct, it may first be desirable to determine what altered cellular component(s) are associated with the tumor cells. This may be determined in a number of ways. For example, ELISA-based assays may be utilized to detect specific tumor markers or altered cellular components.

Alternatively, presence of an altered cellular component may also be determined on a genetic level. For example, DNA or cDNA may be obtained directly from a tumor and subjected under hybridizing conditions with a labeled probe specific for the altered cellular component. If the number of tumor cells is small, PCR (as described above) may be utilized to amplify selected nucleic acid regions, which may then similarly be subjected to hybridization with the labeled probe. The hybridization probe should be selected and utilized under conditions which allow it to specifically bind to the sequence which encodes the altered cellular component (*see* Orkin et al., *J. Clin. Invest. 71*:775-779, 1983). In addition, it should be recognized that one of ordinary skill in the art could readily apply other detection methods to the native or amplified nucleic acids, including, for example, use of the RNase A mismatch cleavage method (Lobez-Galindez et al., *Proc. Natl. Acad. Sci. USA 85*:3522-35-26, 1988).

Within preferred embodiments of the present invention, pharmaceutical compositions are provided comprising one of the above described recombinant viruses, such as a recombinant retrovirus or recombinant virus selected from the group consisting of adeno-associated virus, canary pox virus, adenovirus, and pox virus, or a recombinant DNA vector with or without attached ligands, in combination with a pharmaceutically acceptable carrier or diluent. The composition may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for either injection, oral, or rectal administration. Within certain embodiments of the invention, compositions may be prepared such that they may be directly injected into a selected tumor.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic

saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a vector or recombinant virus in 10 mg/ml mannitol, 1 mg/ml HSA, 20mM Tris pH=7.2 and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 μg of material, it may be less than 1% of high molecular weight material and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months. The composition may be injected intravenously (i.v.) or subcutaneously (s.c.), although it is generally preferable to inject it intramuscularly (i.m.). The individual doses normally used are 10^7 to 10^8 c.f.u. (colony forming units of neomycin resistance titered on HT1080 cells). These are administered at one to two week intervals for three or four doses initially. Subsequent booster shots may be given as one or two doses after 6-12 months, and thereafter annually.

Oral formulations may also be employed with carriers or diluents such as cellulose, lactose, mannitol, poly (DL-lactide-co-glycolate) spheres, and/or carbohydrates such as starch. The composition may take the form of, for example, a tablet, gel capsule, pill, solution, or suspension, and additionally may be formulated for sustained release. For rectal administration, preparation of a suppository may be accomplished with traditional carriers such as polyalkalene glucose, or a triglyceride.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Isolation of ras^{*12}

A 700 base pair Hind III fragment containing the entire T24 ras^{*12} coding region is obtained from plasmid HRAS1 (ATCC No. 41000) and ligated into the Hind III site of pSP73 (Promega, Madison, Wisconsin). This plasmid is designated SP-Val^{12}(100) (*see* Figure 1). Plasmids containing ras^{*12} may also be obtained from other sources, such as the Advanced Biotechnologies (Columbia, Maryland).

In order to determine proper orientation of ras^{*12} in pSP73, clones are subjected to Pvu II digestion, and a clone containing a 100 bp digest is selected. This clone is designated SP-Val^{12}(100).

E. coli (DH5 alpha) (Bethesda Research Labs, Gaithersburg, Maryland) is transformed with the SP-Val^{12} vector construct, and propagated to generate a quantity of plasmid DNA. The plasmid is then isolated and purified, essentially as described by Birnboim et al. (*Nuc. Acid Res. 7*:1513, 1979; *see also*, "Molecular Cloning: A Laboratory Manual," Sambrook et al. (eds.), Cold Spring Harbor Press, p. 1.25 *et seq.*, 1989).

Example 2

Preparation of a vector construct containing Δ ras^{*12}

A. PREPARATION OF Δ RAS^{*12}

A Nco I-Sma I fragment from SP-Val^{12}(100) is removed by restriction endonuclease cleavage (*see* Figure 2). A Xba I linker (New England Biolabs, Beverly, Massachusetts) containing a universal stop codon in all three reading frames is inserted 3' to the ras coding sequence. This process forms a polylinker region which can be removed by restriction endonuclease cleavage at Xba I sites followed by ligation. This mutant is designated SP-Δ-Val^{12} and expresses non-active truncated ras (ras*) protein.

B. INSERTION OF Δ RAS^{*12} INTO THE RETROVIRAL BACKBONE

N2-ras-neo and N2-ras*-neo retroviral vectors are constructed essentially as described in U.S.S.N. 07/586,603. Briefly, this engineered N2 murine recombinant retrovirus contains the SV40 early promoter and the neomycin phosphotransferase gene to facilitate isolation of the infected and transfected cell lines. The N2 Mo MLV gag ATG initiator codon is also altered to ATT by *in vitro* site-directed mutagenesis in order to increase retroviral titer and enhance the level of expression of transduced genes.

A 350 bp Xho I-Cla I fragment from SP-Δ-Val^{12}(100) is then ligated into the retroviral vector. This construct was designated N2-Δ-ras^{*}-Val^{12} (*see* Figure 4).

The full-length SP-Val^{12}(100) cDNA is similarly ligated into the retroviral vector to be used as a positive control for transformation. This construct is designated N2-ras-Val^{12} (*see* Figure 3).

Example 3

Transfection of Mammalian Cells

The murine fibroblast cell lines BC10ME (BC with MHC I type H-2d) and L33 (also H-2d) (obtained from Gunther Dennert, University of Southern California), and human fibroblast cell line HT1080 (HT) (ATCC No. CCL 121), are grown in DMEM (Irvine Scientific, Santa Ana, California), containing 10% fetal bovine serum (Gemini, Calabasas, California). BC or HT cells are transduced or transfected with the vector constructs described above. BC-ras^{*} cells are used for immunization of mice.

Recombinant retrovirus is transfected by the $CaPO_4$ method in CA cells (an amphotropic packaging line) made from the dog cell line CF2; *see* U.S.S.N. 07/586,603). Cells are G418 selected, cloned, and expanded in DMEM supplemented with 10% fetal bovine serum. Viral supernatant from the highest titer clone is filtered with a 0.45 um filter and stored at -70°C.

Alternatively, higher titers may be obtained when retroviral vectors are introduced into packaging cell lines (PCLs) by infection (Miller et al., *Somat. Cell Mol. Genet. 12*:175-183, 1986). Briefly, although amphotropic MLV vectors are known to infect PCLs, they may be blocked from infecting such cell lines due to expression of ampho env ("viral interference"). In order to overcome this problem, vectors containing other viral envelopes (such as xenotropic env or VSG G protein, which bind to cell receptors other than the

ampho receptor) may be generated. Briefly, 10 ug of the vector DNA of interest is co-transfected with 10 ug of DNA which expresses either xeno env (pCMVxeno, above), or a VSV G protein expression vector, MLP G, onto a cell line which expresses high levels of MoMLV gag/pol such as 2-3 cells. The resultant vector containing xenotropic env or VSV G protein, respectively, may then be produced transiently in the co-transfected cells, and after 2 days cell-free supernatants may be added to the potential PCLs. Vector-infected cells may be identified by selection in G418.

The mouse fibroblast cell lines BC10M and L33 may be transfected with the retroviral vector DNA using the $CaPO_4$ technique, and clones selected using 800 ug/ml G418 for 8 days. Cells may then be lysed and assayed for ras* protein expression using western blots (*see generally* Sambrook et al., 18.60 *et seq.*).

Example 4

Transformation (Tumorigenicity) Assay

Rat 2 cells (ATCC No. CRL 1764) are grown in Dulbecco-Vogt modified Eagle medium supplemented with 10% fetal bovine serum. Rat 2 cells are plated at 10^6 cells per 5 cm dish 1 day before transfection. The cells are transfected with 0.1-1.0 ug of construct DNA as previously described (Graham and Van Der Eb, 1973; Corsaro and Pearson, 1981). The next day the cells are trypsinized and seeded into three 5 cm dishes and fed every three days thereafter with medium containing 5% fetal bovine serum plus 2 x 10^{-6} M dexamethasone (this enhances the contrast between transformed and non-transformed rat 2 cell morphology). Transformed foci are visible after about 1 week. The plates are stained and foci counted after about three weeks (Miller et al., *Cell 36*:51, 1984).

Cells transfected with ras* recombinant retroviruses formed transformed foci, whereas those transfected with Δ ras* recombinant retroviruses did not.

Example 5

Cytotoxicity Assay

Six- to eight-week old female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Indiana) are injected once intraperitoneally (i.p.) with 5 x 10^6 irradiated (10,000 rads, 60°C) vector transfected cells (*e.g.*, BC-ras*). Animals are sacrificed 7 days later and the splenocytes (3 x 10^6/ml) cultured *in vitro* with irradiated syngeneic transduced cells (6 x 10^4/ml) in flasks (T-25, Corning, Corning, New York). Culture medium consists of RPMI 1640, heat-inactivated fetal bovine serum (5%, Hyclone, Logan, Utah), sodium pyruvate (1 mM), gentamicin (50 ug/ml) and 2-mercaptoethanol (10^{-5} M, Sigma Chemical, St. Louis, Missouri.). Effector cells are harvested 4-7 days later and tested using various Effector:Target cell ratios in 96 well microtiter plates (Corning, Corning, New York) in a standard 4-6 hour assay. The assay employs $Na_2{}^{51}CrO_4$-labeled (Amersham, Arlington Heights, Illinois) (100 uCi, 1 hr at 37°C) target cells (1 x 10^4 cells/well) in a final volume of 200 ul. Following incubation, 100 ul of culture medium is removed and analyzed in a Beckman gamma spectrometer. Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as: [(Effector cell + target CPM) - (SR)/(MR) - (SR)] x 100. Spontaneous release values of targets are typically 10%-20% of the MR.

Example 6

Preparation of Murine Provector DNA

1. **Cloning of Mucin Gene into KT-3**

In order to develop immunotherapeutics based upon the altered form of mucin, the following experiments may be undertaken to show that the gene for the cDNA of polymorphic epithelial mucin ("PEM") can be packaged, delivered, and expressed in various cell types. Briefly, mucin cDNA is obtained from Dr. Joyce Taylor-Papadimitriou at the Imperial Cancer Research Fund (ICRF) in the SK^+ vector (Stratagene, San Diego, Ca.). The mucin cDNA fragment containing the full coding sequence but lacking the polyadenylation site is isolated by digestion with Xmn I (nucleotide 38) and Bam HI (nucleotide 1766) (EC 3.1.21.4, Boehringer Mannheim, Indianapolis, Indiana). This numbering corresponds to the published sequence having a single hypothetical tandem repeat while the actual cDNA clone contains approximately 32 tandem repeats. The 4.0 kilobase (Kb) Xmn I-Bam HI mucin cDNA fragment is blunt

ended using Klenow (EC 2.7.7.7, Boehringer Mannheim, Indianapolis, Indiana), and cloned into a replication defective MoMuLV KT-3 retroviral backbone containing a neomycin resistance gene (see Figure 5). The retroviral backbone is digested with Xho I and Cla I, blunt ended using Klenow and the ends dephosphorylated with calf intestinal phosphatase (CIP EC 3.1.3.1, Boehringer Mannheim, Indianapolis, Indiana).

The ligated vector is transformed into bacterial cells and the orientation of the cDNA is determined using restriction enzyme as well as sequencing the 5′ and 3′ junctions. Two clones are selected, one with PEM in the sense orientation and the other with PEM in the antisense orientation.

2. Transduction of Packaging Cell Line CA

A cell line (CA) for the packaging of replication defective vectors based on the CF-2 dog cell line (ATCC CRL 6574) may be prepared essentially as described in patent application WO 92/05266. Briefly, the CA packaging cell line expresses MoMuLV amphotrophic envelope and gag-pol proteins encoded by two different plasmids which possess non-LTR promoters. In addition, a MoMuLV gag-pol expressing human cell line 293 (derived from ATCC No. CRL 1573) may also be established as described in patent application WO 92/05266. This is a versatile partial packaging cell line in which different envelope specificities could be expressed by cotransfection of an envelope expression vector, in this case the VSVG protein described in the above application, with the replication defective retroviral vector containing the mucin cDNA.

The recombinant retroviral vector (designated "PEM vector") which is prepared as described above, may then be utilized to transduce cell lines such as L33, BC10ME and CA.

Example 7

Murine Tumor Line Transduction

L33, BC10ME, and CA cell lines are transduced with the PEM vector containing the mucin cDNA at a multiplicity of infection ("M.O.I.") of between 1-10 in the presence of polybrene (4 mg/ml, 1,5-dimethyl-1,5-diazaundeca-methylene, polymethobromide, Sigma, St. Louis, Missouri). G418 selection is initiated 24 hours after infection. Cell types

which should express PEM are lysed and analyzed by Western blot using HMFG-1 (a human milk-fat globule monoclonal antibody) which recognizes the epitope expressed by both AGM and normal mucin. The HMFG-1, HMFG-2, and the SM3 antibodies were obtained from Dr. Joyce Taylor-Papadimitriou at the ICRF. Mucin expression is demonstrated in all three cell lines with a predominance toward lower molecular weight forms as compared to the human breast cancer cell line MCF-7 (ATCC HTB-22) (see Figure 6). The electrophoretic pattern resulted in a smear of various molecular weight forms presumably due to the heterogeneity of glycosylation. The data suggests that the mucin protein is predominantly underglycosylated in these cell types, and therefore most likely expresses the AGM epitope which is recognized by the SM3 antibody.

Example 8

BC10ME Assays

In order to determine whether retroviral vectors which direct the expression of the altered forms of mucin could generate a CTL response, Balb/C mice are injected with either BC10ME cells, or one of two other BC10ME clones that express AGM. These mice were injected once intraperitoneally (i.p.) with 1.0×10^7 irradiated (10,000 Rads) vector-transduced cells. Animals are sacrificed 7 to 14 days later and the harvested splenocytes (3,000,000 cells/ml) are cultured with irradiated mucin-vector transduced cells (either BC10ME, BC10ME AGM #6 or BC10ME AGM #10, respectively, at 60,000 cells/ml). Culture medium consisted of RPMI 1640 (Irvine Scientific, Calif.) supplemented with 5% heat-inactivated fetal calf serum (FBS, Hyclone Logan, Utah) 1mM sodium pyruvate, 10 mM of HEPES, pH=7.4, 50 ug/mL gentimicin (Sigma, St. Louis Missouri) and 1.0×10^{-5} 2-mercaptoethanol. After 4-7 days, these in vitro restimulated splenocytes are harvested and tested using various effector to target cell ratios in 96-well microtiter plates in a standard 4-6 hour assay. The assay employed radioactive chromium-labeled (CR^{51} target cells. (10,000 cells/well) in a final volume of 200 ul. Following incubation, 100 ul of supernatant is removed from the various wells and analyzed in a Beckman Gamma Counter (Beckman, Calif.). Percent target cell lysis is calculated as [(Effector cell + target CPM)-(SR)/(MR)-SR] x 100. Only one of

the AGM expressing clones (BC10ME #6) generated a CTL response (see Figure 7).

In order to understand why the CTL response is different for the two AGM clones, it is necessary to determine the level of expression of AGM on the surface of these respective clones. Single cell suspensions of AGM #6 and AGM #10 are incubated with mouse HMFG-2 or SM3 monoclonal antibodies, washed, and incubated with a FITC-conjugated rabbit anti-mouse antibody and then analyzed in a Fluorescence Activated Cell Sorter (FACS). The FACS analysis of AGM #10 confirmed the expression of AGM on the cell surface. Clone AGM #6 which generated the CTL response demonstrated that there was no surface expression of AGM (see Figure 8). However, previous Western blot analysis data confirmed that clone AGM #6 did express AGM intracellularly. Thus, the induction of the CTL response suggests the possibility that mouse T cells may recognize AGM in the context of self MHC molecules, and that large amounts of AGM expressed on the surface could block the presentation of peptide by MHC.

Example 9

Effect of PEM on Tumorigenesis

The following experiment may be conducted in order to test whether mucin can inhibit tumor formation. Briefly, the B16F10 cell line was transduced with replication defective virus particles containing mucin cDNA at a M.O.I of 1-10 in the presence of polybrene (4 mg/ml). G418 selection is started 24 hours later. Mice are then injected with 400,000 cells intravenously of either parent B16F10, or one of two B16F10 clones expressing mucin. One of the clones was shown to be slightly less tumorigenic than either parent B16F10 or the other mucin expressing clone. This is presumably due to the increased immunogenicity created by the surface expression of one of the forms of mucin (Figure 9).

Example 10

Vaccination of Mice for PEM-Expressing Tumors by Injection of PEM Vector

In order to test whether retroviral vectors which direct the expression of altered forms of mucin could function as a vaccine, mice were injected with mucin encoding retroviral vectors, and then challenged with B16F10 or B16F10 cells expressing mucin. Briefly, pretreated C571B1/6 mice are injected intraperitoneally with 1.8×10^6 virus particles in a 2 ml volume in a two dose regimen with 1 week between each dose. Two weeks after the second dose of vector, mice are challenged with 400,000 cells intravenously with either B16F10 or B16F10 expressing mucin. As the data in Figure 10 demonstrates, the immunization with the mucin vector protected against subsequent growth of the mucin expressing tumors.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A vector construct which directs the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component.

2. The vector construct of claim 1 wherein said cellular component is altered by a point mutation.

3. The vector construct of claim 1 wherein said cellular component is altered by a chromosomal translocation.

4. The vector construct of claim 1 wherein said cellular component is altered by a deletion.

5. The vector construct of claim 1 wherein said altered cellular component is selected from the group consisting of ras*, p53*, Rb*, alter protein encoded by the Wilms' tumor gene, ubiquitin*, DCC, APC, MCC, neu, an altered receptor, and bcr/abl ras*.

6. The vector construct of claim 1 wherein said non-tumorigenic form of an altered cellular component is selected from the group consisting of Δ ras*12, Δ ras*13, and Δ ras *61.

7. The vector construct of claim 1 wherein said construct directs the expression of both ras* and p53*.

8. The vector construct of claim 1 wherein said construct directs the expression of ras*, mucin*, and DCC.

9. A recombinant retrovirus carrying a vector construct according to any of claims 1 to 8.

10. A recombinant virus carrying a vector construct which directs the expression of mucin*.

11. A recombinant virus carrying a vector construct according to any of claims 1 to 8, wherein said virus is selected from the group consisting of adeno-associated virus, canary pox virus, adenovirus, and pox virus.

12. Target cells infected with the recombinant retrovirus of claim 10.

13. The target cells of claim 12 which are selected from the group consisting of human, macaque, dog, rat, and mouse.

14. Target cells infected with the recombinant virus of claim 11.

15. The target cells of claim 14, which are selected from the group consisting of human, macaque, dog, rat, and mouse.

16. Target cells infected with the recombinant virus of claim 10.

17. A composition comprising an immunogenic, non-tumorigenic form of an altered cellular component, for use as an active therapeutic substance.

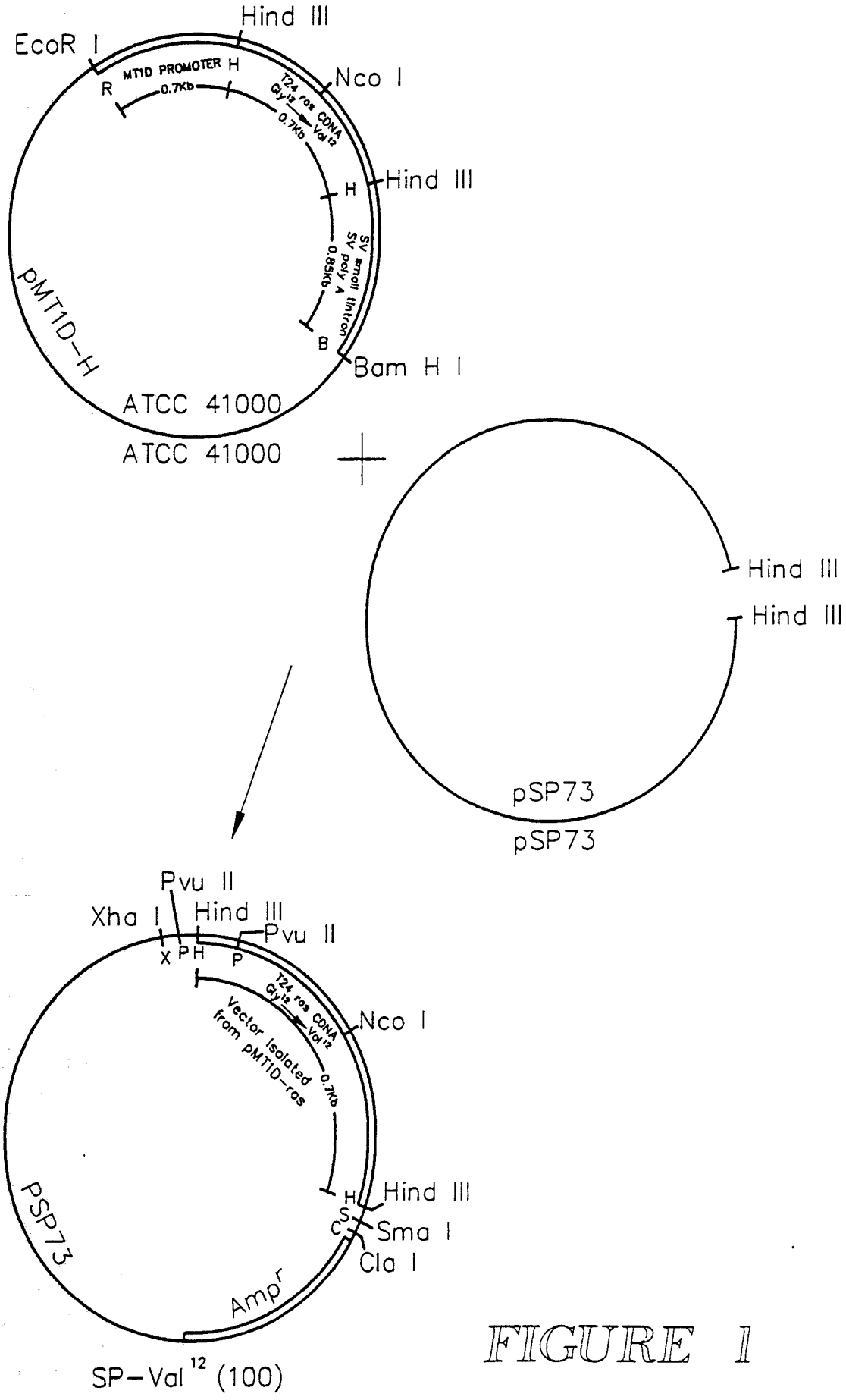
18. A composition according to claims 1 to 8 for use as an active therapeutic substance.

19. A composition according to claims 9 to 11 for use as an active therapeutic substance.

20. Use of a composition comprising an immunogenic, non-tumorigenic form of an altered cellular component, for producing a medicament for treating selected tumor cells.

21. Use of a composition according to claims 1 to 8 for producing a medicament for treating selected tumor cells.

22. Use of a composition according to claims 9 to 11 for producing a medicament for treating selected tumor cells.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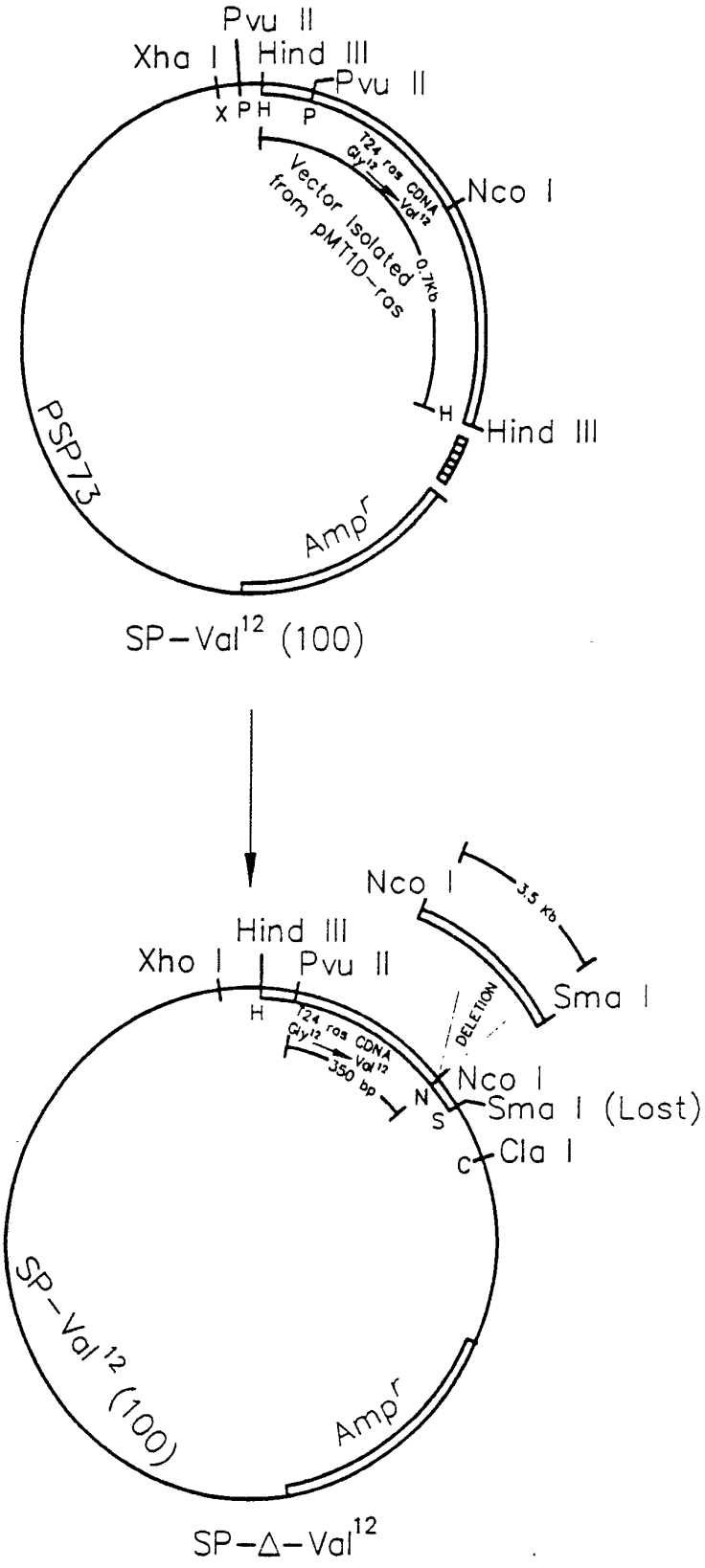
EcoR I
MT1D PROMOTER
H
R
0.7Kb
Nco I
T24 ras CDNA
Gly12 Val12
0.7Kb
Hind III
H
SV small intron
SV poly A
0.85Kb
B
Bam H I
pMT1D-H
ATCC 41000
ATCC 41000
Hind III
Hind III
pSP73
pSP73
Pvu II
Xha I
Hind III
Pvu II
X P H
P
T24 ras CDNA
Gly12 Val12
Nco I
Vector isolated from pMT1D-ros
0.7Kb
H
Hind III
S
Sma I
C
Cla I
PSP73
Ampr
SP-Val12 (100)

FIGURE 1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Xha I
Hind III
Pvu II
X P H P
T24 ras cDNA
Gly12 Val12
Vector Isolated from pMT1D-ros
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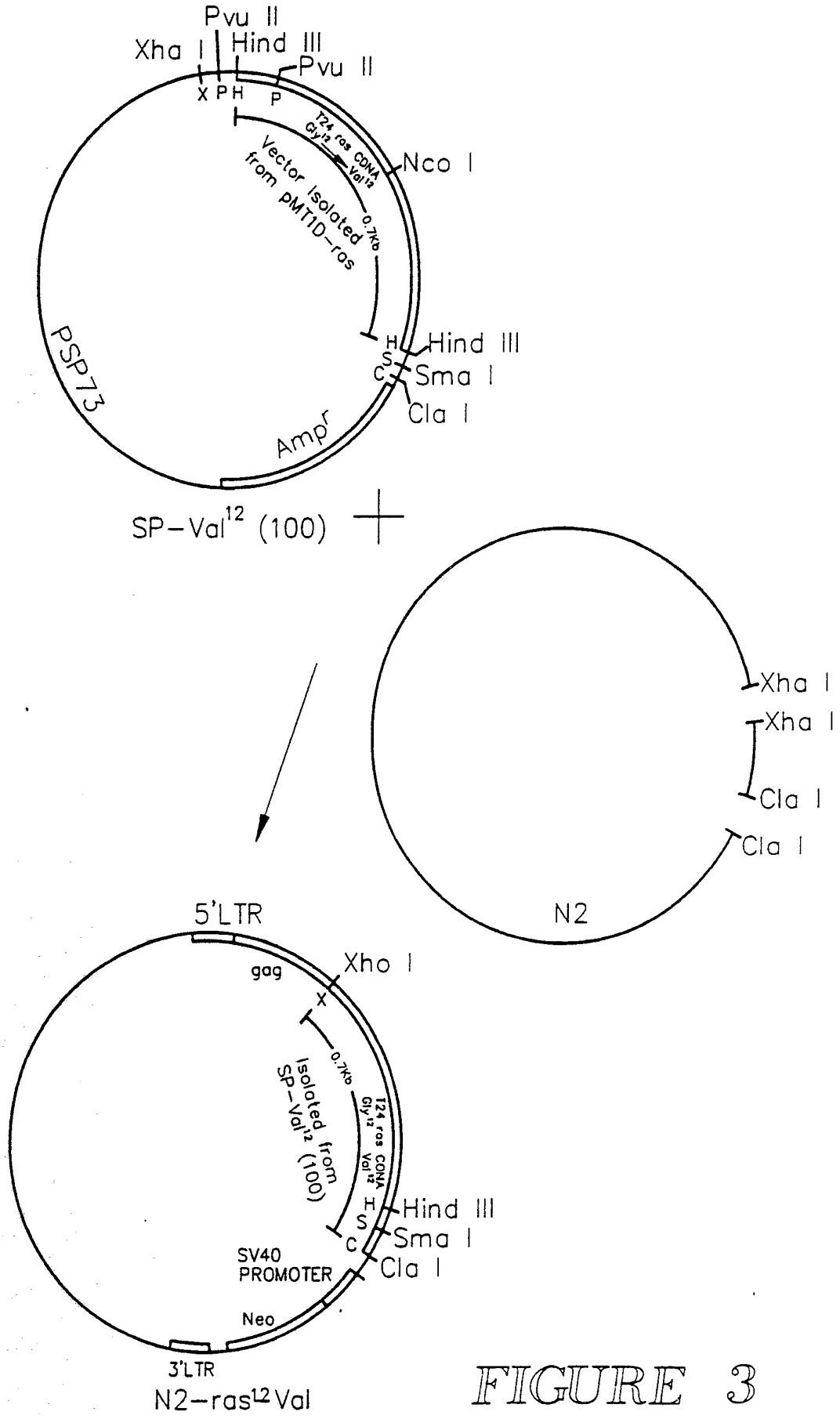
Nco I
H
Hind III
PSP73
Ampr
SP-Val12 (100)
Nco I
3.5 Kb
Hind III
Xho I
Pvu II
Sma I
H
T24 ras cDNA
Gly12 Val12
350 bp
DELETION
Nco I
N
Sma I (Lost)
S
C
Cla I
SP-Val12 (100)
Ampr
SP-Δ-Val12

FIGURE 2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Xha I
Hind III
Pvu II
X P H P
T24 ras CDNA Gly12
Vector Isolated from pMT1D-ras
0.7Kb
Nco I
PSP73
H
S
C
Hind III
Sma I
Cla I
Ampr
SP-Val12 (100)
Xha I
Xha I
Cla I
Cla I
N2
5'LTR
gag
Xho I
0.7Kb
Isolated from SP-Val12 (100)
T24 ras CDNA Val12
H
S
C
Hind III
Sma I
Cla I
SV40 PROMOTER
Neo
3'LTR
N2-ras12 Val

FIGURE 3

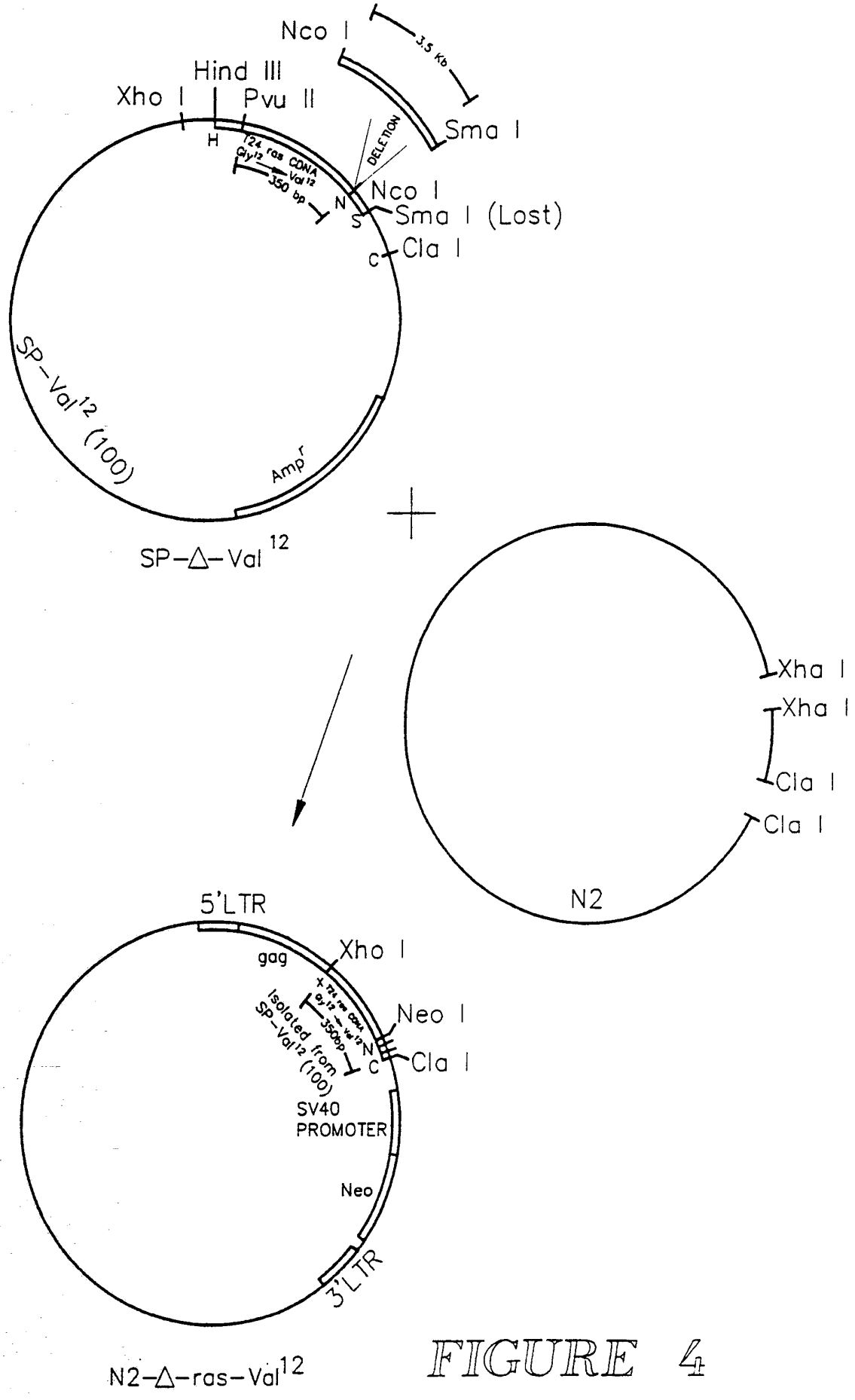
Nco I
3.5 Kb
Hind III
Xho I
Pvu II
Sma I
H
DELETION
Nco I
N
Sma I (Lost)
S
Cla I
C
SP-Val12 (100)
Ampr
SP-Δ-Val12
Xha I
Xha I
Cla I
Cla I
N2
5'LTR
gag
Xho I
Neo I
Cla I
Isolated from SP-Val12 (100)
SV40 PROMOTER
Neo
3'LTR
N2-Δ-ras-Val12

FIGURE 4

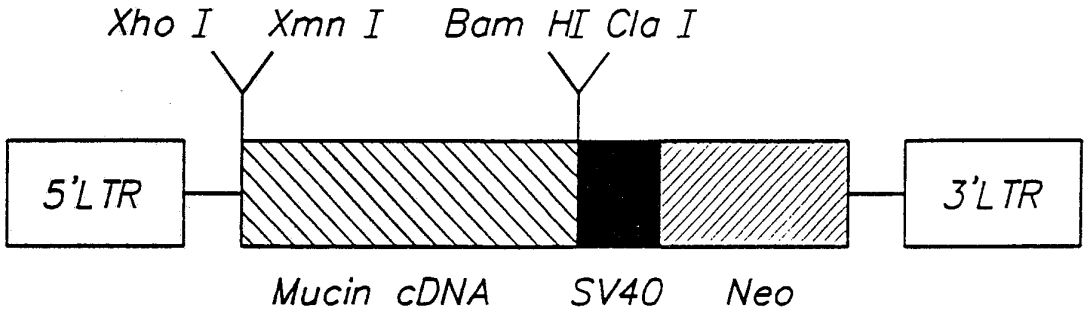

FIGURE 5

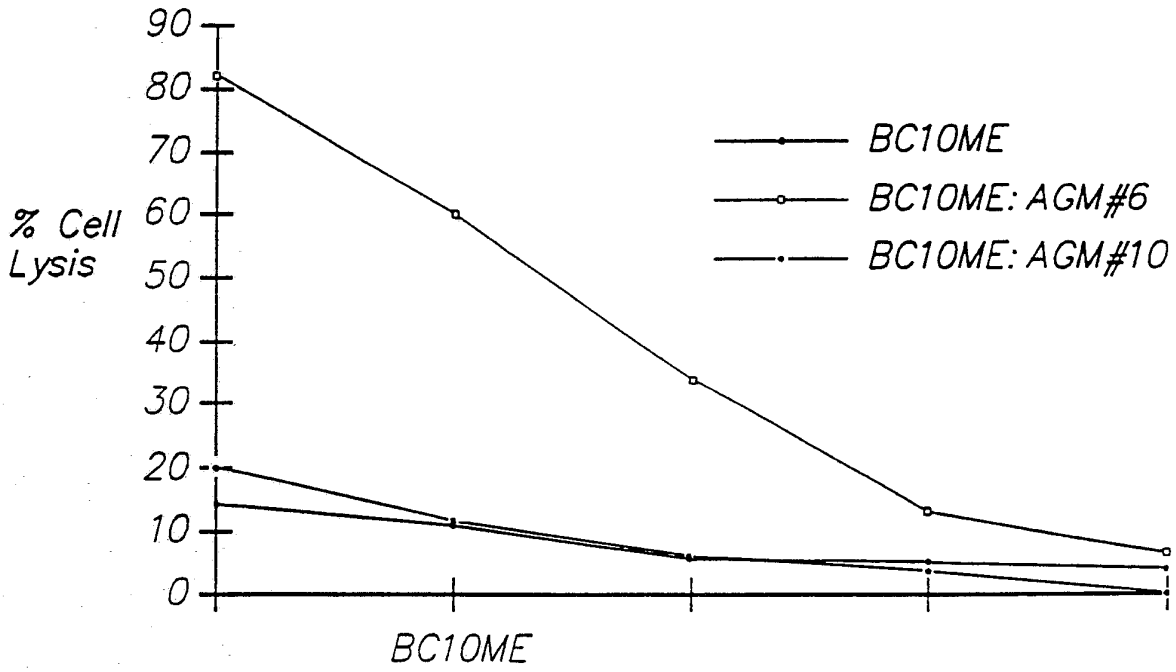

FIGURE 7

a

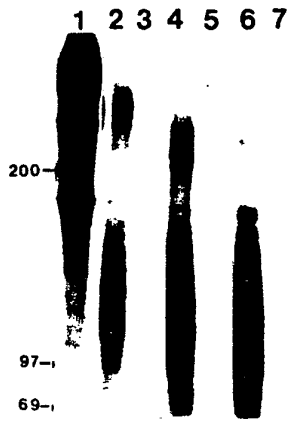

FIGURE 6

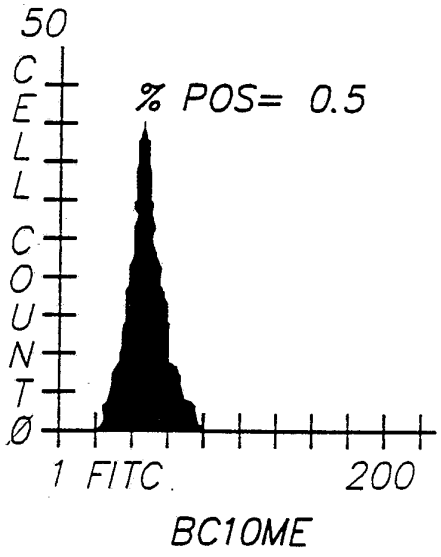

FIG. 8A

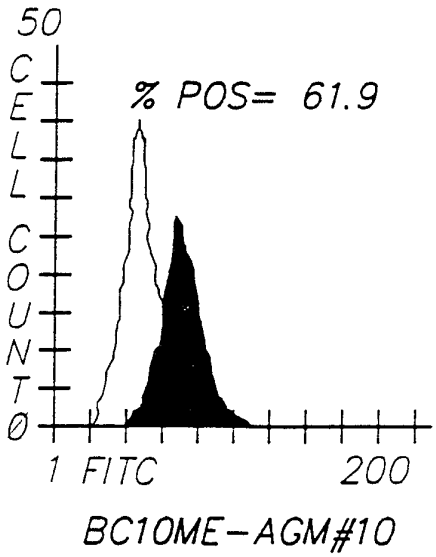

FIG. 8B

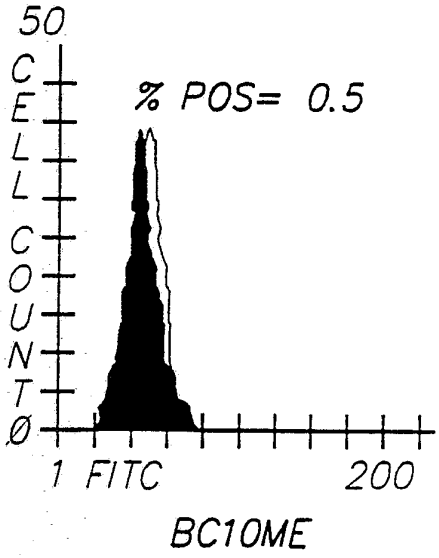

FIG. 8C

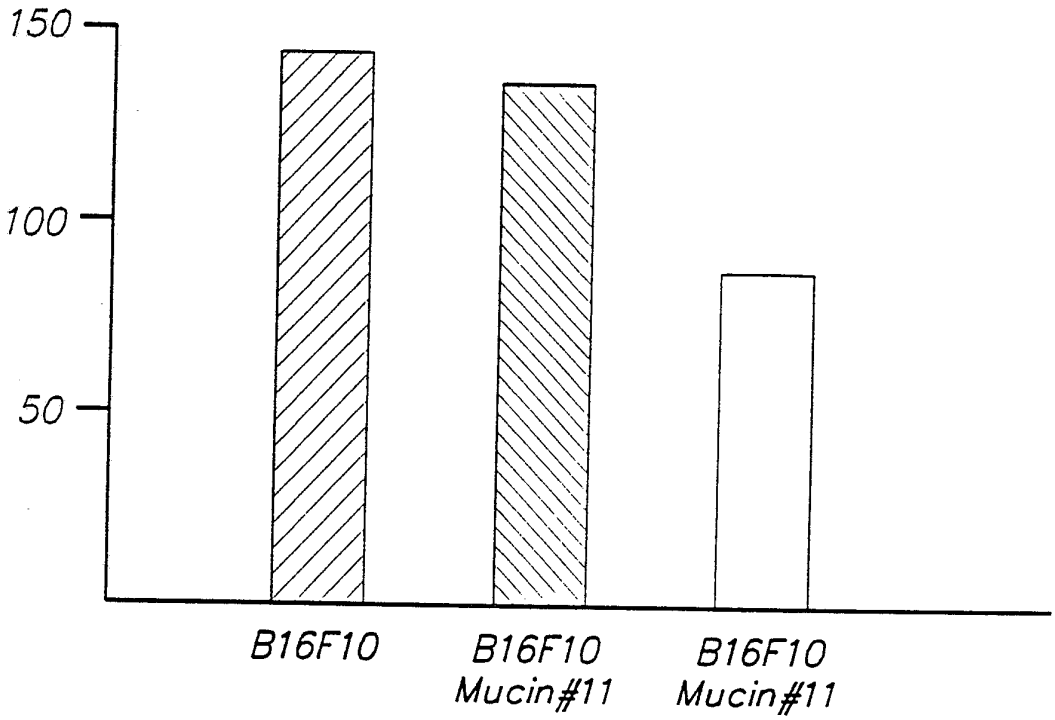

FIGURE 9

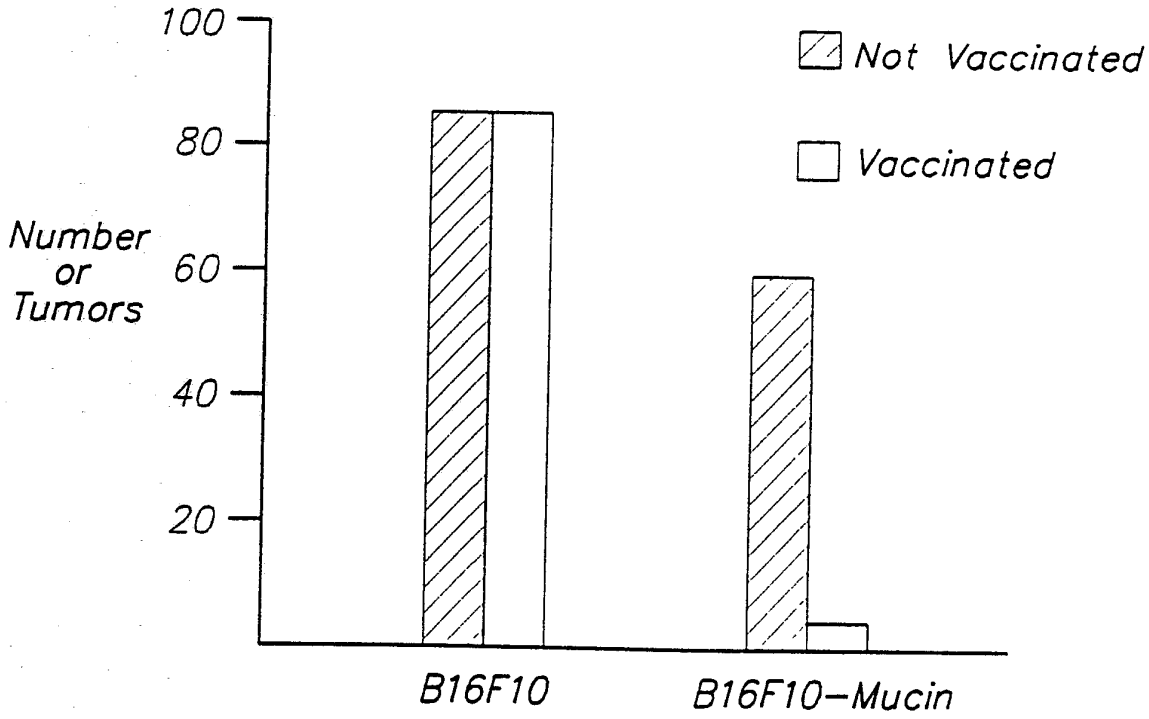

FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/10309

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 39/00, A61K 48/00, C12N 15/85, C12N 5/10
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)

IPC5: A61K, C12N

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)

CANCERLIT, WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A2, 8901973 (BIOTECHNOLOGY, INC. ET AL.), 9 March 1989 (09.03.89), pages 3-9, page 21, lines 1-6	1,4,5,11,14, 15,17-22
Y	--	1-22
X	Vaccines 88., 1988, Ginsberg H. et al. Eds., Cold Spring Harbor Laboratory, pages 19-23, Sara J. McKenzie et al: "Induction of Antitumor Immunity by Immunization with a Vaccinia Virus Vector Expressing an Oncogene-encoded Product", see page 20	1,4,5,11,14, 15,17-22
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 1993	13. 04. 93
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer CARL OLOF GUSTAFSSON

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/10309

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc. Natl. Acad. Sci. USA, Volume 87, December 1990, Mara Hareuveni et al, "Vaccination against tumor cells expressing breast cancer epithelial tumor antigen" page 9498 - page 9502	1,4,10,11, 14-22
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X	Dialog Information Services, File 159 Cancerlit., Dialog accession no.00693187, Lathe R et al: "Antitumor immunity: exploration of vaccination in breast cancer (meeting abstract)", & Biennal International Breast Cancer Research Conference, March 5-9, 1989, Tel Aviv, Tel Aviv University, 1-21, 1989.	1,5,10,16
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P,X	WO, A1, 9207000 (TRANSGENE S.A.), 30 April 1992 (30.04.92), see claims 7-14, page 6, line 18	1,2,4,11, 14-22
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X	Vaccine, Volume 9, Sept 1991, M. Hareuveni et al., "Vaccinia recombinants expressing secreted and transmembrane forms of breast cancer-associated epithelial tumour antigen (ETA)", page 618 - page 626, see esp. page 619, left column, page 623-625	1,4,5,10,11, 14-22
Y		1-22
	--	
X	WO, A1, 9014357 (GENENTECH, INC.), 29 November 1990 (29.11.90), see page 13, lines 22-25, pages 22-24 and claim 14	1,4,17,18, 20,21
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Y	Dialog Information Services, file 159, Cancerlit., Dialog accession no.00943106, Hill A. et al: "Mutant P53--A target for cytotoxic T lymphocytes? (meeting abstract)", Mutant Oncogenes: Targets for Therapy? September 9-10, 1991, London, UK.	1-7
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/10309

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., Volume 173, January 1991, Stefan Jung et al, "Human T Lymphocytes Recognize a Peptide of Single Point-mutated, Oncogenic ras Proteins" page 273 - page 276	2,6,7
A	CANCER CELLS, Volume 7, 1989, H. Rabin et al, "Expression of ras and neu Oncogene Proteins as Determined by Monoclonal Antibodies" page 157 - page 160	5,6
X	Vaccines 88, Ginsberg H et al Eds., Cold Spring Harbor Laboratory, pages 47-52, Shiu-Lok Hu et al: "Recombinant Vaccinia Virus Expressing the Human Melanoma-associated Antigen p97 as a Therapeutic Anti-tumor Vaccine",	1,17,18,20, 21
X	Dialog Information Services, file 159, Cancerlit., Dialog Accession no. 00846034, Salgaller M. et al: "Expression of a native (180 KD) and deleted from (70 KD) of thehuman carcinoembryonic antigen following transduction of a mouse adenocarcinoma cell line (meeting abstract)", & Proc Annu Meet Am Assoc Cancer Res., 32:A1622 1991	1,9
Y		9,12,13
A	Dialog Information Services, file 159, Cancerlit., Dialog Accession no. 00915583, Jolly D.J. et al: "Retroviral vectors as vaccines and immunotherapeutics", & Semin Immunol; 2(5):329-39, 1990	9

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/02/93

International application No.
PCT/US 92/10309

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A2- 8901973	09/03/89	NONE	
WO-A1- 9207000	30/04/92	FR-A- 2668064	24/04/92
WO-A1- 9014357	29/11/90	EP-A- 0474727	18/03/92