(12) STANDARD PATENT (11) Application No. **AU 2007243252 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
An isolated DNA fragment of the SPARC human promoter and its use

(51) International Patent Classification(s)
A01N 63/00 (2006.01) ***A61K 39/12*** (2006.01)

(21) Application No: **2007243252** (22) Date of Filing: **2007.04.26**

(87) WIPO No: **WO07/127347**

(30) Priority Data

(31) Number	(32) Date	(33) Country
P060101724	**2006.04.28**	**AR**

(43) Publication Date: **2007.11.08**
(44) Accepted Journal Date: **2013.06.20**

(71) Applicant(s)
Fundacion Instituto Leloir;Consejo Nacional De Investigaciones Cientificas Y Tecnicas (CONICET)

(72) Inventor(s)
Viale, Diego Luis;Cafferata, Eduardo Gustavo Alfredo;Podhajcer, Osvaldo Luis;Lopez, Maria Veronica

(74) Agent / Attorney
FB Rice, Level 23 44 Market Street, SYDNEY, NSW, 2000

(56) Related Art
WO 2005/017183 A2 (JOHNS HOPKINS UNIVERSITY) 24 February 2005
WO 2000/072679 A1 (UNIVERSITY OF VIRGINIA PATENT FOUNDATION) 7 December 2000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 November 2007 (08.11.2007)

PCT

(10) International Publication Number
WO 2007/127347 A3

(51) International Patent Classification:
A01N 63/00 (2006.01) *A61K 39/12* (2006.01)

(21) International Application Number:
PCT/US2007/010196

(22) International Filing Date: 26 April 2007 (26.04.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P060101724 28 April 2006 (28.04.2006) AR

(71) Applicant *(for all designated States except US)*: **INIS BIOTECH LLC.** [US/US]; 203 NE Front Street, Suite 101, Milford, Kent County, DE 19963 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **PODHAJCER, Osvaldo, Luis** [AR/AR]; Eduardo Acevedo 338, Buenos Aires, 1405 (AR). **CAFFERATA, Eduardo, Gustavo, Alfredo** [AR/AR]; Neuquen 715, Buenos Aires, 1405 (AR). **LOPEZ, Maria, Veronica** [AR/AR]; Calle 528 Bis N 1092, 1900 Tolosa, La Plata, Province Of Buenos Aires (AR). **VIALE, Diego, Luis** [AR/AR]; Jose Otamendi 247 4D, Buenos Aires, 1405 (AR).

(74) Agent: **SHEKLETON, Gerald, T.**; Welsh & Katz, Ltd, 120 South Riverside Plaza, 22nd Floor, Chicago, IL 60606 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

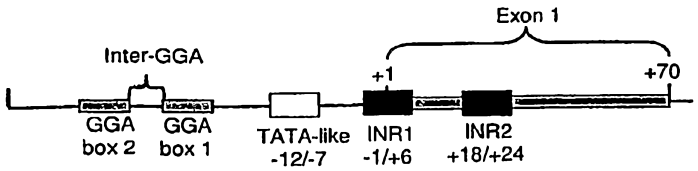
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
3 January 2008

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: AN ISOLATED DNA FRAGMENT OF THE SPARC HUMAN PROMOTER AND ITS USE



(57) Abstract: An isolated DNA sequence corresponding to a region of the SPARC gene human promoter from base pair -513 to base pair +3 5 capable of driving the expression of a heterologous gene of interest, that can be associated to any other promoter sequence, such as radiation responsive sequence, hypoxia responsive sequence and free-radical responsive sequence. The invention also provides constructs and DNA recombinant expression viral vectors, comprising said isolated sequence of the SPARC gene human promoter and at least one heterologous gene operably linked thereto, wherein said promoter sequence drives the expression of the at least one heterologous gene in tumor cells. Pharmaceutical compositions and a method for treating tumors are also provided.

WO 2007/127347 A3

AN ISOLATED DNA FRAGMENT OF THE SPARC HUMAN PROMOTER AND ITS USE FOR DRIVING THE EXPRESSION OF AN HETEROLOGOUS GENE IN TUMOR CELLS

Field of the invention

The present invention refers to the field of gene therapy. In particular, the present invention refers to an isolated DNA sequence having promoter activity, capable of driving the expression of a gene of interest, particularly in a tumor cell. More particularly, the present invention refers to vectors containing a DNA fragment isolated from the SPARC promoter associated to a gene of interest, to pharmaceutical compositions and its use in cancer therapy.

Background of the invention

The SPARC protein (secreted protein, acidic and cystein-rich protein) also known as osteonectin or BM40, is a secreted glycoprotein, highly distributed in human and non-human tissue, which functions and effects are wide and various. It has been found that it interacts with extracellular matrix components, with growth factors, with cytokines and with the expression of matrix metalloproteinases.

The SPARC protein has been initially described by Ledda M.F. et. al. (Ledda, M.F., Adris, S., Bravo, A.I., Kairiyama, C., Bover, L., Chernajovsky, Y., Mordoh, J., and Podhajcer, O.L., *Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells*. Nat Med, *3:* 171-176, 1997) as having a central role in the malignity of human melanoma. Subsequent

studies showed that SPARC over-expression is associated to the malignant progression of various tumor types (Porte, H., Triboulet, J.P., Kotelevets, L., Carrat, F., Prevot, S., Nordlinger, B., DiGioia, Y., Wurtz, A., Comoglio, P., Gespach, C., and Chastre, E. *Overexpression of stromelysin-3, BM-40/SPARC, and MET genes in human esophageal carcinoma: implications for prognosis.* Clin Cancer Res, *4:* 1375-1382, 1998). The SPARC protein is highly expressed both in endothelium and activated fibroblasts of *in vivo* tumors (Lane, T.F. and Sage, E.H. *The biology of SPARC, a protein that modulates cell-matrix interactions.* Faseb J, *8:* 163-173, 1994).

Human SPARC promoters (Hafner, M., Zimmermann, K., Pottgiesser, J., Krieg, T., and Nischt, R. *A purine-rich sequence in the human BM-40 gene promoter region is a prerequisite for maximum transcription.* Matrix Biol, *14:* 733-741, 1995), murine SPARC promoters (McVey, J.H., Nomura, S., Kelly, P., Mason, I.J., and Hogan, B.L. *Characterization of the mouse SPARC/osteonectin gene. Intron/exon organization and an unusual promoter region.* J Biol Chem, *263:* 11111-11116, 1988) and bovine SPARC promoters (Young, M. F., Findlay, D. M., Dominguez, P., Burbelo, P. D., McQuillan, C., Kopp, J. B., Robey, P. G., and Termine, J. D. *Osteonectin promoter. DNA sequence analysis and S1 endonuclease site potentially associated with transcriptional control in bone cells.* J Biol Chem, *264:* 450-456, 1989) have been cloned and characterized. The comparison between these promoters shows that, similarly to what it is observed at gene level, a high sequence homology occurs.

The structure of the human SPARC promoter is shown in Figure 1, wherein the first exon, the GGA1 and GGA2 boxes, the 10 nucleotide Inter-CGA region separating them and the

TATA non-consensus sequence are depicted. The human SPARC promoter lacks a TATA consensus box (Breathnach, R. *et al.*, *Organization and expression of eucaryotic split genes coding for proteins*, Annu Rev Biochem, *50:* 349-383, 1981) but contains a so-called TATA-like element that shares some bases with the conventional sequence. The promoter has two GCA1 and GCA2 boxes, of which the GCA1 box exhibits a great similarity between the human and bovine species.

Hafner *et al.* observed that the GCA1 box is necessary and sufficient for obtaining a maximum transcriptional activity, while the spacing element separating the two CGA boxes has a negative effect on its expression (Hafner M. *et al.*, 1995). It is important to note that this group has demonstrated that, in humans, the promoter region containing only the CGA boxes is not sufficient by itself to confer expression specificity in different cell lines. Domínguez et. al. described the region between bases -504 to +11 of the bovine promoter as a positive element for SPARC transcription in fetal bovine cells. This fragment also confers specific expression, showing higher activity in cells with a higher expression level of SPARC mRNA (Domínguez, P., Ibaraki, K., Robey, P.G., Hefferan, T.E., Termine, J.D., and Young, M.F. *Expression of the osteonectin gene potentially controlled by multiple cis- and trans-acting factors in cultured bone cells*. J Bone Miner Res, *6:* 1127-1136, 1991). They also observed that only the GC box (which is a common element in many promoter regions and their consensus sequence is GGGCGG, may be present in more than one copy. It is located between the -40 to -100 bp) and the GCA1 box are not sufficient for the maximum SPARC expression in bovine bone cells, and that the region located between the bases -927 to -504 produces a dramatic inhibition of transcription.

The gene therapy potentially represents one of the most important developments taking place in medicine. In order to modify a specific cell or tissue type, therapeutic genes have to be efficiently administered to the cell so that the gene expresses in the appropriate level and for a sufficient amount of time. Two types of strategy are being applied for DNA supply to cells, these are by viral and non-viral vectors. Even though a great number of virus destined to gene transfer have been developed, the major interest has been centered in retrovirus, adenovirus, adeno-associated virus and herpes simplex virus type 1. The first generation adenovirus are defective in E1A protein, hence they do not replicate. The early E1A protein is the first protein that produces the viral DNA inside the cell. E1A has many functions such as helping other viral proteins to be produced and stimulating the cell growth by bonding Rb and releasing E2F, facilitating viral transcription and replication. Even though those E1A protein defective adenovirus were successfully used as vectors in cancer pre-clinical models, the same results were not achieved when used in clinical trials, being its low *in vivo* transduction capacity one of the major problems (Vile, R. *Cancer gene therapy-new approaches to tumour cell killing*. J Gene Med, *2:* 141-143, 2000).

One way to overcome this drawback has been the creation of a new generation of vectors capable of conditionally replicating in the tumor environment; these vectors are called CRAd (Conditionally Replicative Adenovirus or Oncolytic adenovirus). CRAds are constructed by modifying the adenoviral genome in order to regulate the expression of E1A protein with a promoter that is specifically active in the required tissue or cell type, in such a way to prevent damage to surrounding tissues.

In the last years, several research groups have devoted to the recombinant adenovirus construction. This way, some of the viral genes that had been removed in the past are being re-inserted again given that they enhance viral replication. That is the case of the E3 region. E3 is a viral DNA fragment encoding 9 proteins, the main function of which is the inhibition of cell death induced by the host immune response. Among the 9 proteins, ADP (Adenoviral Death Protein) stands out, has a contradictory function when compared to their E3 mates, because it promotes the late cell lysis in the viral infection cycle to allow for the release of mature virions to the cell microenvironment. Cells infected with an non-expressing ADP adenovirus have been shown to remain viable for a longer time than cells infected with the wild type adenovirus (Tollefson, A.E. *et al. The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants,* Virology, *220:* 152-162, 1996; Tollefson, A.E *et al., The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells,* J Virol, 70: 2296-2306, 1996; Kruyt, F.A. *et al., A new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis,* Hum Gene Ther, 13: 485-495, 2002.).

One of the most attractive ways of gene therapy is the use of suicide genes. The basis of the system consists of introducing a gene encoding an enzyme with the capacity of metabolizing a non-toxic prodrug turning it into a toxic drug. One of the mostly used genes is the Herpes simplex virus thymidine kinase or HSV/TK, which codifies for an enzyme capable of phosphorilating the prodrug acyclovir/ganciclovir (commonly used antiviral for viral infections), a guanosine analogue. In its phosphorilated

form, the anti-herpetic agent is incorporated to the DNA molecule, avoiding its duplication and causing cell death (Moolten, F. L., *Drug sensitivity ("suicide") genes for selective cancer chemotherapy*, Cancer Gene Ther, *1:* 279-287, 1994). The neighbor non-transduced tumor cells may also be eliminated by the so-called bystander effect, which allows the toxic metabolites to be transferred from an affected cell to a non-affected cell.

A tumor is formed by tumor cells, fibroblasts and endothelial cells. This is why an effective therapy with viral vectors requires the virus to be able to replicate in these three cell types, which are responsible of tumor progression. Given that SPARC is over-expressed in all these cell types, it represents a good candidate for the construction of a CRAd, such that the SPARC promoter drives the gene of interest, such as an E1A gene and eventually another therapeutic gene. In this sense, the tumor cells would be eliminated by the replication of the virus itself or by the action of the toxic drug produced in the tumor environment.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Brief description of the invention

In a first aspect, the invention provides an isolated DNA of a nucleic acid promoter having a sequence consisting of SEQ ID NO:1.

In a second aspect, the invention provides an expression vector comprising the isolated DNA of the first aspect and a heterologous gene operably linked thereto.

In a third aspect, the invention provides a method for expressing foreign DNA in a host cell, the method comprising introducing into the host cell the expression vector of the second aspect.

In a fourth aspect, the invention provides a pharmaceutical composition comprising the expression vector of the second aspect in a pharmaceutically suitable carrier.

In a fifth aspect, the invention provides a method for treating a tumor in a patient suffering therefrom, the method comprising administering to the patient an effective amount of the pharmaceutical composition of the fourth aspect.

In a sixth aspect, the invention provides an expression vector comprising a nucleic acid promoter having a sequence consisting of SEQ ID NO: 1 according to the first aspect.

It is therefore a preferred aim of the present invention to provide an isolated DNA sequence, with promoter activity, capable of driving the expression of a gene of interest, particularly in a tumor cell.

Herein there is disclosed an isolated fragment, from the human SPARC gene promoter, capable of driving the expression of a gene of interest, particularly in a tumor cell.

More particularly, the invention provides an isolated DNA sequence comprising the polynucleotide sequence SEQ ID NO 1 that corresponds to a region of the human SPARC promoter from base pair -513 to base pair +35 or a fragment or variant of said polynucleotide sequence that has been modified by insertion, substitution or deletion of one or more nucleotides, and which has a substantially equivalent function.

According to an additional aspect, it is provided an isolated DNA recombinant expression construct that comprises the promoter sequence of the invention operably linked to a gene of interest.

According to another additional aspect, the polynucleotide sequence SEQ ID NO 1, that corresponds to a region of the human promoter of the SPARC gene according to the present invention may also be associated to any other promoter/regulating sequence, such as sequences responsive to radiation, hypoxia, free-radicals, etc.

According to yet another relevant aspect of the present invention, it is provided a viral recombinant expression vector, containing the previously defined DNA promoter sequence of the invention and/or the previously defined construct of the invention, wherein the DNA promoter sequence is operably linked to a therapeutic gene of interest.

The invention also provides a method for expressing foreign DNA in a host cell that comprises introducing in the host cell a DNA recombinant expression construct or a viral recombinant expression vector of the invention comprising the promoter DNA molecule of polynucleotide sequence SEQ ID NO 1 operably linked to a foreign DNA encoding a desired polypeptide or RNA, wherein said foreign DNA is expressed.

Furthermore, herein is disclosed a method for treating a tumor in a patient suffering therefrom, that comprises administering to the patient an effective amount of a pharmaceutical composition comprising a DNA recombinant expression construct or a viral recombinant expression vector, comprising the promoter sequence of the invention, capable of driving the viral replication and/or the expression of a therapeutic gene of interest, operably linked thereto.

Brief description of the drawings

Figure 1 shows a structure scheme of the human SPARC promoter, HSBM40DNA (Genbank # X88259)

Figure 2 shows a comparative assessment of the expression levels of SPARC mRNA in different tumor and normal lines.

Figure 3 shows two schemes of the SPARC promoter region where the different fragments derive from.

Figure 4A shows the promoter activity of 11 fragments of the SPARC promoter by measuring the enzymatic activity of the Luciferase reporter gene in A375N (melanoma), HeLa (cervix) and T-47D (breast) cell lines.

Figure 4B compares the promoter activity of the fragments -513/+35 (F512) and -1175/+71Δ10 (Spdel) of the SPARC promoter by measuring the enzymatic activity of the Luciferase reporter gene in various normal and tumor cell lines.

Figure 5A shows schemes of Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) adenoviruses, constructed according to the present invention.

Figure 5B shows the restriction profile of Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) adenoviruses of the present invention with HindIII enzyme.

Figure 6 shows the monolayer cytopathic effect in different tumor lines of Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) adenoviruses, constructed according to the present invention, using Ad5-wt as a control.

Figures 7A, 7B, 7C and 7D show the monolayer lytic effect (MTT assay) of Ad-F512 and Ad(I)-F512(E3) adenoviruses of the present invention, at different MOIs 10 days after infection; A- Melanoma, SB2 cells; B- Melanoma, Mel-J cells; C- Colon cells; D- Breast cells.

Figure 8 shows the production of Ad-F512 and Ad(I)-F512(E3) adenoviruses of the present invention in different human tumor lines.

Figure 9 shows the cooperative effect of the E1A gene given by Ad-F512 on the replication of the Ad-β-gal adenovirus.

Figures 10A to 10F show the *in vivo* assay results. Figure 10A shows the tumor growth curve for the assay with Ad(I)F512-TK. Figure 10B shows the tumor growth curve of the animals in the first assay with Ad-F512. Figure 10C shows tumors treated with Ad-β-gal and with Ad-F512. Figure 10D shows histological photographs of the regions shown in 10C, 14 days after. Figure 10E shows Kaplan-Meier curve for assay B. Figure 10F shows Kaplan-Meier curve for the second assay with Ad-F512.

Figure 11 shows the cytopathic effect in monolayer of Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) CRAds of the invention. The lytic effect (staining with violet crystal)

of the CRAds in cell monolayers at different concentrations after 10 days post-infection is shown.

Figures 12A to 12E show the cytopathic effect of Ad-F512, Ad(I)-F512-TK and AD(I)-F512(E3) CRAds of the invention, in normal cells; Figure 12A shows CRAds photographs taken after 10 days post-infection in normal melanocytes; photographs of AD-wt and no-treatment (PBS) controls are included; Figure 12B shows the cytopathic effect on normal CCD841 colon cells; Figure 12C shows the cytopathic effect on normal MCF12A breast cells; Figure 12D shows the lytic effect on microendothelial cells; Figure 12E shows the viral effect on keratinocytes and fibroblasts (CCD1140 and Malme-3).

Figures 13A to 13C show the monolayer cytopathic effect of Ad(I)-F512-TK; the lytic effect (staining with violet crystal or survival measured by MTT) of Ad(I)-F512-TK on monolayers of cells at different viral concentrations after 10 days post-infection in the presence or absence of GCV prodrug; Figure 13A shows the assay with SB2 melanoma cells; Figure 13B shows the survival of hMEC-1 cells in the presence of GCV and GCV + virus; Figure 13C shows the cytopathic effect on BAEC cells.

Figures 14A to 14D show *in vivo* assays with melanoma tumors; Figure 14A shows assays with SB2/WI-38/hMEC-1 tumors treated with Ad-F512; Figure 14B shows assays with SB2/WI-38 tumors treated with Ad-F512; Figure 14C shows SB2/hMEC-1 tumors treated with Ad-F512; Figure 14D shows SB2/WI-38 tumors treated with Ad(I)-F512-TK + GCV, n = 5 or PBS+GCV n = 4, where n is the number of treated animals.

Figures 15A to 15D show *in vivo* assays with colon and pancreas tumors; Figure 15A shows assays on LoVo tumors

treated with Ad-F512 (n = 7), Ad(I)-F512-TK (n = 6) or PBS (n = 7); Figure 15B shows the average of LoVo/hMEC-1 tumors treated with Ad-F512 (n = 6), Ad(I)-F512-TK + GCV (n = 6) or PBS + GCV (n = 6); Figure 15C shows the tumor growth of Mia-PaCa/hMEC-1 treated with Ad-F512 (n = 6), Ad(I)-F512-TK + GCV (n = 6), or PBS + GCV (n = 5). In all cases n indicates the number of treated animals.

Detailed description of the invention

As disclosed above, the present invention provides in a main aspect, a DNA isolated sequence comprising the polynucleotide sequence SEQ ID NO 1, which corresponds to a region of the human SPARC gene promoter from base pair -513 to base pair +35 or a fragment or variant of said polynucleotide sequence, which has been modified by insertion, substitution or deletion of one or more nucleotides, and which has a substantially equivalent function.

The term "isolated" as used herein, means substantially separated or purified with respect to contaminant sequences in the cell or organism in which the nucleic acid is naturally present and includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared either by recombinant technique or chemical synthesis.

The term "variant" as used herein, refers to a DNA molecule wherein the nucleotide sequence is substantially identical to the sequence established as SEQ ID NO 1. The variant may be achieved by means of modifications such as insertion, substitution or deletion of one or more nucleotides,

provided that those modifications are neutral mutations and they do not affect the performance of the DNA molecule.

A "fragment" of nucleic acid sequence according to the present invention is a nucleic acid sequence portion that is shorter in length than the complete sequence and comprises at least a minimum length capable of getting specifically hybridized with the nucleic acid sequence of the present invention under astringent conditions, said fragment keeping the biological conditions required in the present invention.

The present invention also provides a recombinant expression construct effective in driving transcription of a selected codifying sequence that comprises: (a) a DNA sequence corresponding to the human SPARC promoter; and (b) a sequence codifying for a gene of interest, operably linked to the promoter in (a) in such a way that the sequence of the gene of interest may be transcripted and translated in a host cell.

According to one embodiment, the preferred DNA sequence corresponding to the human SPARC promoter goes from base pair -513 base pair to +35 according to sequence shown in SEQ ID NO 1 of the Sequence Listing.

A "heterologous" gene as used herein, means a DNA sequence encoding an amino acid or protein sequence of interest, in association with another DNA sequence, such that said association is not present in nature.

In general, "a therapeutic gene" as used herein, means a DNA sequence encoding an amino acid or protein sequence, capable of eliciting a therapeutic effect on the host cells. Preferably, according to one embodiment of the present invention the host cells are tumor cells, more

particularly, the tumor cells are melanoma cells, breast cells, colon cells, cervix cells.

According to a preferred embodiment, the gene of interest may be selected from: the E1A gene, a suicide gene such as the hsv-TK gene, the adenoviral genomic region called E3, the gene of an interleukin such as IL-10, IL-12 or IL-23, etc.

According to particular embodiments, the polynucleotide sequence SEQ ID NO 1 that corresponds to a region of the human SPARC gene promoter, according to the present invention, may drive genes by itself or may be associated with sequences responsive to radiation, hypoxia, free-radicals, etc. These are defined DNA sequences, which are usually located upstream of said promoter. One characteristic example of this kind of combinations is the use therein of the hypoxia responsive element (HRE) which has already been used for potentiating the transcriptional activity of a promoter or responsive element under low oxygen pressure conditions. Hernandez-Alcoceba *et. al.* have used the hypoxia responsive elements (HRE) for potentiating the promoter response containing estrogen responsive elements (ERE) in breast tumors (Hernandez-Alcoceba R, Pihalja M, Nunez G, Clarke MF, *Evaluation of a new dual-specificity promoter for selective induction of apoptosis in breast cancer cells* Cancer Gene Ther. 2001 Apr; 8(4):298-307). Said responsive elements have been combined with radiation responsive elements (Greco O. *et al., Novel chimeric gene promoters responsive to hypoxia and ionizing radiation.* Gene Ther 2002; 9: 1403-1411). They may be a part of the replicative or non-replicative adenovirus (Ido A., Uto H., Moriuchi A., Nagata K., Onaga Y., Onaga M., Hori T., Hirono S., Hayashi K., Tamaoki T., Tsubouchi H., *Gene therapy targeting for hepatocellular carcinoma:*

selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter, Cancer Res. 2001 Apr 1; 61(7):3016-21; Park J.O., Lopez C.A., Gupta V.K., Brown C.K., Mauceri H.J., Darga T.E., Manan A., Hellman S., Posner M.C., Kufe D.W., Weichselbaum R.R., *Transcriptional control of viral gene therapy by cisplatin*. J Clin Invest. 2002 Aug; 110(3):403-10; Cowen R.L., Williams K.J., Chinje E.C., Jaffar M., Sheppard F.C., Telfer B.A., Wind N.S., Stratford I.J., *Hypoxia targeted gene therapy to increase the efficacy of tirapazamine as an adjuvant to radiotherapy: reversing tumor radioresistance and effecting cure*. Cancer Res. 2004 Feb 15; 64(4):1396-402).

According to the invention, it is also provided a method for expressing foreign DNA in a host cell, which comprises introducing in the host cell a DNA recombinant expression construct or a viral recombinant expression vector of the invention, comprising the promoter molecule of polynucleotide sequence SEQ ID NO 1 operably linked to a foreign DNA encoding a desired polypeptide or RNA, wherein said foreign DNA is expressed.

The introduction of DNA in the host cells may be carried out by means of any construct and includes plasmids, DNA virus, retrovirus, as well as isolated nucleotide molecules. Liposome mediated transfer may also be used.

Adenovirus are an example of said DNA virus that may be used in the present invention. More than 40 different serotypes of human adenovirus are well known, being the Ad5 adenovirus especially preferred as a viral vector in the present invention; however, modified capsid and/or fiber Ad5 adenovirus, such as with the adenovirus 3 capsid or the fiber modification with a RGD motif, are not discarded.

The construction of suitable vectors containing the promoter sequence and the sequence of the desired therapeutic gene may be performed by standard linking and restriction techniques, which are well known in the art. The DNA cleavages in a specific site were performed by treatment with the appropriate restriction enzymes, under conditions indicated by the manufacturer, for approximately 3-16 hs. In general, the restriction results may be verified by electrophoretic separation in agarose gels (0.8-1.6%) in TAE solution (40 mM triacetate, 2mM $Na_2EDTA.2H_2O$, pH 8.5), using ethidium bromide and visualized with UV light under trans-illuminator (Ultraviolet Products Inc., Upland, CA). Ligations are made by DNA ligase from bacteriophage T4, following the manufacturer's protocol (New Englands Biolabs Inc., Beverly, MA). Insert:vector ratios of from 1:1 to 3:1 were used, calculating the ratio between the fragments through the following formula:

$$\left[\frac{\text{ng vector} * \text{Kb insert}}{\text{Kb vector}}\right] * \left[\text{ratio } \frac{\text{insert}}{\text{vector}}\right] = \text{ng insert}$$

In the vector construction it is advantageous to be able to distinguish the vector incorporating foreign DNA from non-modified vectors by means of a quick assay. Marker systems are known that in general comprise a gene the expression of which confers an identifiable phenotype to the transformed cells when the cells are grown in an appropriate media. The β-galactosidase gene is for instance a detectable gene in clones exhibiting a blue phenotype in plaques with X-gal.

This invention involves driving a gene of interest towards a tumor cell so that the protein encoded by the gene is

expressed and said protein directly or indirectly enhances the patient state.

According to a particular embodiment of the invention, a CRAd or oncolytic vector (Conditionally Replicative or Oncolytic Adenovirus) is prepared upon the basis of an adenovirus, comprising a gene of the E1A protein, under the regulation of a DNA sequence fragment of the SPARC promoter. Advantageously, the CRAds of the invention drive the expression of E1A in different types of tumor cells (melanoma, breast, colon, cervix) causing their lysis and elimination through the replication of the virus itself. Also advantageously, the CRAds of the invention containing the E1A gene, such as exemplified below, have attenuated lytic activity in normal cells (mesenchymal, endothelial and fibroblasts) given that its expression is driven by a promoter that expresses mainly in tumor cells.

Also, according to another particular embodiment, CRAd vectors further comprising a suicide gene such as, e.g., Herpes simplex virus thimidine kinase (*hsv*-TK) codifying for an enzyme capable of phosphorilating the prodrugs acyclovir/ganciclovir. In its phosphorilated form, the anti-herpetic agent is incorporated to the DNA molecule, avoiding its duplication and causing cell death. The CRADs prepared according to this particular embodiment drive *hsv*-TK expression in different tumor cell types, completing the lytic action and at the same time having an attenuated lytic activity in normal cells given that its expression is driven by a promoter that expresses mainly in tumor cells

Also, according to another particular embodiment, CRAd vectors further comprising a genome adenoviral region called E3 codifying for 9 proteins, is prepared. Among them, the ADP (Adenoviral Death Protein) stands out, which

promotes the late cellular lysis within the viral infection cycle for allowing the release of mature virions to the cellular microenvironment. The CRAds containing the E3 region potentiate the lytic activity of E1A, at the same time it has an attenuated lytic activity in normal cells given that its expression is driven by a promoter that expresses mainly in tumor cells.

The constructs or vectors of the present invention may be administered to a patient in need thereof, by injection, oral or topic administration, vehiculized in a suitable carrier. Suitable carriers may be aqueous, lipidic, liposomal, etc.

For the data analysis of luciferase, spheroids and *in vivo* studies, ANOVA variance analysis was used, followed by Tukey's test. A P-value lower than 0.05 was considered significant. Also, the survival curves were performed according to the Kaplan-Meier method and the statistical comparisons between the different groups was performed applying the log-rank test.

The present invention is illustrated below by means of detailed experimental examples. Said examples are intended to provide for a better understanding of the invention, but they should not be deemed to limit the invention in any way, since the scope of the invention shall be established in the annexed claims.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

EXAMPLES

Example 1.- Evaluation of the expression of SPARC mRNA in different tumor and normal lines

The levels of produced messenger RNA (mRNA) from the SPARC protein were assessed by Real-Time PCR in tumor cell lines and normal cell lines.

The human lines HeLa (cervix cancer, ATCC No. CCL-2), T-47D (breast cancer, ATCC No. HTB-133), WI-38 (fetal pulmonary fibroblasts, ATCC No. CCL-75), WI-38 VA (transformed fetal pulmonary fibroblasts, ATCC No. CCL-75.1), HFL-1 (fibroblasts, ATCC No. CCL-153), 293 (embryo kidney, ATCC No. CRL-1573), LoVo (colon cancer, ATCC No. CCL-229), HCT-116 (colon cancer, ATCC No. CCL-247), CaCO2 (colon cancer, ATCC No. HTB-37), HT-29 (colon cancer, ATCC No. HTB-38), T84 (colon cancer, ATCC No. CCL-248) and aortic endothelial cells (Bovine Aortic Endothelial Cells, BAEC ATCC No. CRL1395) were obtained from ATCC (American Tissue Culture Collection, Rockville, MD, USA). The human melanoma cell lines IIB-MEL-LES and IIB-MEL-J-N were previously described by Ledda *et. al.*, 1997; the human melanoma lines SB2, A375N and MEL-888 were kindly provided by Dr. Estela Medrano (Houston, Texas). All of the cells were cultured in the recommended medium supplemented with 10% bovine fetal serum (provided by Natocor, Carlos Paz, Argentina), 2.5 U/ml de penicillin (Sigma-Aldrich Corp., St. Louis, MO) and 2.5 µg/ml streptomycin (Sigma-Aldrich Corp., St. Louis, MO) and kept at 37°C in an atmosphere with 5% CO_2. BAEC cells were supplemented with 5% BFS.

The relative quantification of the SPARC mRNA levels was performed according to Pfaffl, M.W. *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, *29:* e45, 2001. Total RNA was extracted by using Tri Reagent (Sigma-Aldrich Co., St. Louis, MO). 5 µg of RNA were retro-transcripted with 200U SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using 500 ng Oligo(dT) primers. The cDNA Real-Time PCR reaction was

performed in an iCycler iQ System (Bio-Rad Laboratories, Hercules, CA, USA) thermocycler. The reaction was carried out in a 25 µl volume containing 1 Platinum® Taq DNA polymerase (Invitrogen) unit, 1X PCR Reaction Buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM Mg_2Cl, 2.5 µg BSA, 0.01% glycerol, 0.4 µM of each specific primer: SPARC (SRTse; AACCGAAGAGGAGGTGGTG, SEQ ID NO 2 /SRTas; GCAAAGAAGTGGCAGGAAGA, SEQ ID NO 3) and β-actin (Acse; AGAAAATCTGGCACCACACC, SEQ ID NO 4 /Acas; CAGAGGCGTACAGGGATAGC, SEQ ID NO 5) 200 µM dNTPs and 0.3 X SYBR Green solution. The reaction conditions were: 90 seconds at 94°C and then 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C. Each reaction was performed in triplicate and the results obtained for SPARC were normalized with the results obtained simultaneously for β-actin.

The results are shown in Figure 2, wherein it can be seen that the A375N melanoma cells express the higher SPARC mRNA levels. The rest of the melanoma lines express low to moderate mRNA levels compared to A375N. It was observed that the breast (T-47D), cervix (HeLa) and colon (LoVo, HCT-116, CaCO2, HT-29 and T84) cancer lines express negligible SPARC levels. Low SPARC expression levels were observed for the kidney line, while the fibroblasts lines show moderate expression levels and one aortic bovine endothelium line (BAEC) shows a higher SPARC expression level than A375N.

Example 2.- Cloning of 11 fragments of the human SPARC promoter in the pGEM and TOPO plasmids.

A sequence analysis of the human SPARC promoter performed by the present inventors revealed a DPE sequence *(Downstream Promoter Elements)* present between the bases +29/+33 as well as the 2 possible transcription initiation sites which had already been described, INR1 and INR2 (see above cited Figure 1). The DPE sequence was described in *Drosophila* promoters and is considered to have a role in the transcriptosome assembly in those promoters not containing TATA sequences (Kadonaga, J.T. *The DPE, a core promoter element for transcription by RNA polymerase II.* Exp Mol Med, *34:* 259-264, 2002). As a result of this analysis, present inventors carried out a directed mutation of the promoter. For that purpose, they worked on the 5′ ends: -1175 (for cloning the complete promoter), -513 (by analogy with the bovine promoter) and -120 (which includes exactly the GGA1 sequence). The 3′ end mutations include the complete exon 1 and diverse deletions including, or not, the DPE sequence (+24, +28, +35, +71). Figure 3 shows two schemes of the SPARC promoter where the different fragments are derived from. The locations of the GGA boxes, the TATA-like sequence of the possible transcription initiation sites (INR) and the DPE sequence are indicated. Different limits are indicated that were used for constructing the SPARC promoter fragments. The 5′ end was cloned up to the bases -1175, -513 and -120; while from the 3′ end the bases were cloned up to +24, +28, +35 and +71. The deleted region between the GGA boxes is also indicated, this deletion gives origin to the fragment -1175/+71Δ10.

A fragment of 1246 bp from the human SPARC promoter region was amplified by PCR (-1175 to +71 bp relative to the transcription initiation site) from genomic DNA of human lymphocytes with the Spfse and SPP3′2 oligonucleotides (see Table 1). This PCR product, -1175/+71, was cloned in the

pGEM-T-easy vector (Promega Corp., Madison, WI) for obtaining pGEM (-1175/+71). This plasmid was used as a template of the promoter variants, the fragments of which were in turn amplified by PCR using the primers as detailed in the following Table 1.

Table 1

Fragment	Primer	Enzyme	T mel	5′-3′ Sequence	SEQ ID NO
-1175/+71	SPfSE	NheI	T=64.3	CTAGCTAGCAGCTGGGTGTTGTGGCAT	6
	SPP3′2	SalI	T=70.2	ACGCGTCGACCTCAGTGGCAGGCA	7
-1175/+71Δ10	SPfSE	NheI	T=64.3	CTAGCTAGCAGCTGGGTGTTGTGGCAT	6
	Δ10r	-	T=75.4	CGGCCTCCTCCTTCTCCCCTGTC-TCTGTCTTTCATTTC	8
	Δ10f	-	T=65.2	CTAGCTAGCGGGAGAAGGAGGAGGCC	9
	R71	BglII	T=59.8	GCAGATCTCCTCAGTGGCAGGC	10
-1175/+35	F1175	MluI	T=65.2	GCACGCGTAGCTGGGTGTTGTGG	11
	R35	BglII	T=60.8	CGAGATCTGCTCTCCGGGCAG	12
-1175/+28	F1175	MluI	T=65.2	GCACGCGTAGCTGGGTGTTGTGG	11
	R28	BglII	T=63.1	CGAGATCTGGGCAGTCTGAAGGACC	13
-513/+71	F512	MluI	T=63.7	CGACGCGTGCAGCTTGTCTTGTC	14
	R71	BglII	T=59.8	GCAGATCTCCTCAGTGGCAGGC	10
-513/+35	F512	MluI	T=63.7	CGACGCGTGCAGCTTGTCTTGTC	14
	R35	BglII	T=60.8	CGAGATCTGCTCTCCGGGCAG	12
-513/+28	F512	MluI	T=63.7	CGACGCGTGCAGCTTGTCTTGTC	14
	R28	BglII	T=63.1	CGAGATCTGGGCAGTCTGAAGGACC	13
-513/+24	F512	MluI	T=63.7	CGACGCGTGCAGCTTGTCTTGTC	14
	R24	BglII	T=60.2	GCAGATCTAGTCTGAAGGACCGCG	15
-120/+71	F120	MluI	T=60.9	GAACGCGTGGGAGAAGGAGGAG	16
	R71	BglII	T=59.8	GCAGATCTCCTCAGTGGCAGGC	10
-120/+35	F120	MluI	T=60.9	GAACGCGTGGGAGAAGGAGGAG	16
	R35	BglII	T=60.8	CGAGATCTGCTCTCCGGGCAG	12
-120/+28	F120	MluI	T=60.9	GAACGCGTGGGAGAAGGAGGAG	16

	R28	BglII	T=63.1	CGAGATCTGGGCAGTCTGAAGGACC	13

The amplification cycle corresponds to an initial denaturalization at 94°C for 3 minutes, followed by 35 amplification cycles: 94°C, 1 minute, annealing T (variable, depending on each product, Table 1), 1 minute and 72°C 2 minutes, followed by a final extension at 72°C for 10 minutes. The PCRs were performed in the PTC-200 thermocycler (MJ Research Inc., Whaltam, MA).

The PCR products were firstly cloned in the pGEM-t-easy vector (Promega Corp., Madison, WI) or TOPO-pCR4 vector (Invitrogen Corp., Carlsbad, CA) thus obtaining the vectors:

a- pGEM **(-1175/+71)**, (SEQ ID NO 17), complete promoter. The sequence +1/+70, includes the first exon, not translated.

b- pGEM **(-1175/+71Δ10)** (SEQ ID NO 18). This promoter is a modification of the complete SPARC promoter **-1175/+71**, wherein a sequence of 10 bp considered to inhibit transcription was deleted, located between the two GGA boxes (Hafner, M. *et al.*, 1995). For this purpose, the splicing by overlap extension PCR (PCR-SOE) was applied using two SPARC promoter fragments that had been previously cloned by the present inventors: Spdel1.1Kb of 1084 base pairs and GGA1 of 209 base pairs. The final PCR product was cloned in the pGEM vector and the deletion was confirmed by sequencing.

c- TOPO **(-1175/+35)**, (SEQ ID NO 19), includes the DPE sequence

d- TOPO **(-1175/+28)**, (SEQ ID NO 20), excludes the DPE sequence

e- TOPO **(-513/+71)**, (SEQ ID NO 21), includes the complete first exon.

f- TOPO **(-513/+35)**, (SEQ ID NO. 1), includes the DPE sequence

g- TOPO **(-513/+28)**, (SEQ ID NO. 22), excludes the DPE sequence

h- TOPO **(-513/+24)**, (SEQ ID NO 23), includes up to the INR2 sequence

i- TOPO **(-120/+71)**, (SEQ ID NO 24), includes the complete first exon.

j- TOPO **(-120/+35)**, (SEQ ID NO 25), includes the DPE sequence

k- TOPO **(-120/+28)**, (SEQ ID NO 26), excludes the DPE sequence

Example 3A.- Selection of a fragment of the human SPARC promoter

The promoters obtained according to Example 2 were subcloned in the *Mlu*I/BglII, *Mlu*I/*Xho*I or *Nhe*I/*Xho*I sites of the pGL3-Basic plasmid (Promega Corp., Madison, WI, USA) upstream of the luciferase reporter gene. All of the clonings was confirmed by restriction profiles and by sequencing the vectors (pGEM, TOPO and/or pGL3) with the universal primers T7 (TACGACTCACTATAGGG; SEQ ID NO 27); Sp6 (ATTTAGGTGACACTATAG; SEQ ID NO 28), T3 (ATTAACCCTCACTAAAGGGA; SEQ ID NO 29) or P2 (CTTTATGTTTTTGGCGTCTTCCA; SEQ ID NO 30) and P3 (CTAGCAAAATAGGCTGTCCCC; SEQ ID NO 31).

The pGL3-Basic plasmid contains the modified *Firefly* luciferase reporter gene (*luc+*) for preventing the binding of genic regulatory factors, removing the restriction

sites, preventing the protein transport to peroxisomas and it contains a Kozak sequence in the 5' end of the luciferase gene for optimizing the translation efficiency.

The presence of the luciferase reporter gene allowed for the quantification of SPARC promoter activity in the 11 fragments obtained in Example 2, by measuring the luciferase enzymatic activity. The assay was performed in three cell lines, using the (melanoma) A375N line as a model since it expresses high SPARC levels and the (cervix) HeLa and (breast) T-47D lines, not expressing SPARC, according to the teachings of Example 1 above.

The cells were seeded in 24-well plates with a density of 4 x 10^4 cells/well. After 24 hours they were transfected using Lipofectamine2000 (Invitrogen Corp., Carlsbad, CA) according to the conditions indicated by the manufacturer. Each treatment was carried out at least in duplicate for each cell line, incubating 0.8 µg of treatment plasmid with 0.1 µg of pRL-CMV plasmid for 5 minutes with 50 µl DMEM medium without antibiotic and simultaneously, 1 µl Lipofectamine2000 with 50 µl of the same medium. These two preparations were mixed and incubated for 20 minutes at room temperature. The medium with cell serum was removed, washed with PBS and 200 µl high glucose DMEM was added without serum and without antibiotics; subsequently, 100 µl medium containing the lipofection mixture was added and 800 µl of culture medium corresponding to each cell line, supplemented with BFS, was added 4 hours later. The cells used were kept in a stove for 46 hours at 37°C with 5% CO_2. The Dual Luciferase Reporter Assay System kit (Promega Corp., Madison, WI) was used for the luciferase assay. This system implies the simultaneous expression of two individual reporter enzymes in the same system,

allowing for the evaluation of the activity produced by the luciferase enzymes from the Firefly glow-worm (Photinus pyralis) and the Renilla coelenterate (Renilla reniformis) in only one sequential assay (Sherf, B.A. *et al., Dual Luciferase Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and Renilla Luciferase Assays.* Promega Notes Magazine: 2-9, 1996). The data were normalized in the following way:

$$\frac{\text{Firefly Luciferase Units}}{\text{Renilla Luciferase Units}} = \text{Relative Luciferase Units (RLU)}$$

The data are expressed as induction amount relative to the activity obtained with the pGL3-Basic control plasmid (without promoter). The results are shown in Figure 4A. The pGl3-promoter plasmid (pGL3-prom, Promega Corp., Madison, WI) wherein the viral promoter from SV40 drives the expression of the luciferase enzyme, was used as a transfection control.

The lower promoter activity in melanoma lines was observed with the complete SPARC promoter (-1175/+71). The higher unspecificity was observed with the fragment -120/+71 and the fragment showing the higher specificity and higher activity in the line expressing SPARC turned out to be the fragment -513/+35 (Figure 4A). The promoter -513/+35, from now onwards **F512**, was selected through this analysis in order to continue with its characterization in comparison to the promoter -1175/+71Δ10, called Spdel given that it had the same activity than F512, in the A375N melanoma line.

Example 3B.- Selection of a fragment from the human SPARC promoter

The two promoters F512 and Spdel, having similar promoter activity and as identified by the method of Example 3A, were assessed in different tumor and normal cell lines (see references in Example 1). The results are shown in Figure 4B.

From the comparative results, it can be noted that Spdel presents an average of 140 times higher activity, with respect to the empty vector pGL3-Basic, both in tumor and normal lines, with the exception of the melanoma line MEL888, where its activity is 430 times higher (Figure 4B). Hence, the Spdel activity results independent of SPARC expression in the cell line used.

The F512 promoter turned out to be active in the melanoma lines and in the endothelium BAEC line having the higher SPARC expression. The SB2 line has the lower promoter activity, approximately 1/3 of the activity observed in A375N, this result being coincident with the SPARC mRNA expression ratio (Figures 2 and 4B). Surprisingly, the activity in two melanoma lines, Mel888 and MelJ, is 3.5- to 4-fold higher than for A375N. These results show that the F512 fragment is active in the melanoma lines but its behavior is not always coincident with the SPARC mRNA expression. Unlike Spdel, which has the same activity in all lines, F512 presents lower activity in those lines with little or null SPARC expression such as colon (T84, LoVo), glioma (U87) or cervix (HeLa) (Figure 4B). However, this activity is not negligible but, quite on the contrary, in a few cases like T84, it reaches half the activity observed for the melanoma lines. In addition, the promoter is also active in the fibroblast line used (WI-38 VA).

These results allowed for the selection of the F512 promoter for driving the expression of genes of interest in an adenovector.

Example 4.- Construction of a *shuttle* plasmid containing F512 upstream of E1A gene.

The construction of a shuttle plasmid containing the genes of interest was carried out starting from pADPSY shuttle vector, containing the human adenovirus type 5 whose genes in regions E1 (necessary for its replication) and E3, had been deleted. Then, Rous sarcoma virus (RSV) promoter and the polyadenilation signal of SV40 (Mariano J. Alvarez, Federico Prada, Edgardo Salvatierra, Alicia I. Bravo, Viviana P. Lutzky, Cecilia Carbone, Fernando J. Pitossi, H. Eduardo Chuluyan and Osvaldo L. Podhajcer; *Secreted Protein Acidic and Rich in Cysteine Produced by Human Melanoma Cells Modulates Polymorphonuclear Leukocyte Recruitment and Antitumor Cytotoxic Capacity*; Cancer Research 65, 5123-5132, June 15, 2005) have been inserted in ΔE1 region. In order to improve this vector so as to increase the variety of unique cloning sites, a multiple cloning site (MCS) was designed and included, replacing RSV promoter, obtaining pAd-Xp plasmid (SEQ. ID. NO 32); this vector was sequenciated in order to verify the presence of the multiple cloning site.

In the last years many studies showed that primers that were specific for a given cell type, did not act as such when they were introduced in the viral genome (Steinwaerder, D.S. and Lieber, A. *Insulation from viral transcriptional regulatory elements improves inducible transgene expression from adenovirus vectors in vitro and in vivo.* Gene Ther, 7: 556-567, 2000.). This is due to the

fact that ITR sequences (inverted terminal repetitions) and the adenovirus genome encapsulation signal have *enhancers* influencing the promoters activity modifying their specificity (Hearing, P. and Shenk, T. *The adenovirus type 5 E1A enhancer contains two functionally distinct domains: one is specific for E1A and the other modulates all early units in cis.* Cell, *45:* 229-236, 1986.). This problem can be partially avoided by the use of *insulators*, i.e. sequences that isolate the promoter activity and allow it to develop its specificity (Steinwaerder, D. S. et al., 2000; Martin-Duque, P., Jezzard, S., Kaftansis, L., and Vassaux, G. *Direct comparison of the insulating properties of two genetic elements in an adenoviral vector containing two different expression cassettes.* Hum Gene Ther, *15:* 995-1002, 2004.). From these findings the present inventors decided to clone, in the pAd-Xp *shuttle* vector MCS, an *insulator* sequence (the stop signal of the bovine growth hormone) (Martin-Duque, P. et al., 2004)) yielding new pAd(I)-Xp *shuttle* vector (SEQ. ID. NO 33). The *insulator* sequence was amplified by PCR using the INSU-F-SpeI (CC**ACTAGT**GCTAGAGCTCGCTGATCAGC; SEQ. ID. NO 34) and INSU-R-KpnI (**CGGTACC**ATCCCCAGCATGCCTGC; SEQ. ID. NO 35) primers. The product of this reaction was cloned in TOPO-pCR4 (Invitrogen Corp., Carlsbad, CA) and subsequently subcloned in *SpeI* and *KpnI* sites of pAd-Xp, yielding pAd(I)-Xp.

The cDNA of E1A protein was cloned by PCR starting from the genomic DNA of 293 human embryo kidney cells (ATCC No. CRL-1573) that constitutively express it. A fragment corresponding to 560-1632 nucleotides of the virus genome was amplified by PCR. This fragment was cloned in TOPO-pCR4 (SEQ ID NO 36 and SEQ ID NO 37) vector and sequenced in order to verify the identity of the sequence. The constructs were subcloned in pcDNA3 expression vector and expressed in HeLa cells. A *Western Blot* of proteins from

the complete lysate was carried out and they could be identified with anti-E1A antibody (BD Pharmigen, #554155).

In a first step, E1A protein gene was cloned in pAd(I)-Xp and pAd-Xp vectors. For that purpose, the E1A protein cDNA was extracted from TOPO-pCR4-E1A vector (SEQ IND NO 36) with *Bgl*II and *BamH*I enzymes and was inserted in *Bgl*II site of pAd(I)-Xp and pAd-Xp yielding pAd(I)-Xp-E1A (SEQ. ID. NO 38) and pAd-Xp-E1A (SEQ. ID. NO 39). In a second step F512 was extracted from pGL3(-513/+35) with *Mlu*I and *Bgl*II and it was cloned in *Mlu*I and *Bgl*II sites of pAd(I)-Xp-E1A and pAd-Xp-E1A, leaving the E1A protein cDNA downstream of F512. Thus, **pAd(I)-F512** (SEQ. ID. NO 40) and **pAd-F512** (SEQ. ID. NO 41) vectors were obtained; both vector sequences were confirmed by sequenciation with pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ. ID. NO 42) primer.

Example 5.- Construction of a *shuttle* plasmid containing F512 upstream of *hsv*-TK suicide gene.

cDNA coding for *hsv*-TK was amplified by PCR using as template the DNA of pAGO plasmid (Berenstein, M., Adris, S., Ledda, F., Wolfmann, C., Medina, J., Bravo, A., Mordoh, J., Chernajovsky, Y., and Podhajcer, O. L. *Different efficacy of in vivo herpes simplex virus thymidine kinase gene transduction and ganciclovir treatment on the inhibition of tumor growth of murine and human melanoma cells and rat glioblastoma cells.* Cancer Gene Ther, *6:* 358-366, 1999) and FTK/RTK (GC**CCATGG**CTTCGTACCCCGGCC; SEQ. ID. NO 43 /GC**GTCGAC**TCAGTTAGCCTCCCCCATCTC; SEQ. ID. NO 44) primers. The product of this PCR reaction was cloned in TOPO-pCR4 vector. The obtained TK-TOPO-pCR4 plasmid (SEQ. ID. NO 45) was confirmed by digestion with restriction enzymes and by

sequenciation using T3 (ATTAACCCTCACTAAAGGGA; SEQ. ID. NO 29) and T7 (TAATACGACTCACTATAGGG; SEQ. ID. NO 27) universal primers.

The cDNA of *hsv*-TK enzyme was extracted from TK-TOPO-pCR4 plasmid (SEQ ID NO 45) by enzymatic digestion with *Nco*I and *Sal*I. This *Nco*I-TK-*Sal*I fragment was cloned in pCITE vector (Invitrogen, Carlsbad, CA) downstream of a internal ribosomal entry site (IRES) allowing for a greater translation efficiency without capping. The resulting pCITE-TK vector was confirmed by digestion with restriction enzymes. Subsequently, the IRES-TK fragment of the pCITE-TK vector was extracted by digesting with *EcoR*I and *Sal*I enzymes. *EcoR*I site was filled with Klenow enzyme and the IRES-TK-*Sal*I fragment was subcloned in pAd(I)-F512 vector obtained in Example 4, previously digested with *EcoRV* and *Sal*I enzymes. The obtaining of the resulting vector from said cloning, **pAd(I)-F512-TK** (SEQ ID NO 46 and SEQ ID NO 47), was confirmed by digestion with restriction enzymes and sequenciation with pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ. ID. NO 42) and pAd-antisense (CACAAATTTCACAAATAAAGCATTT; SEQ. ID. N° 48) primers.

Example 6.- Construction of a *shuttle* plasmid containing F512 upstream of the gene coding for EGFP green protein.

The EGFP protein cDNA linked to an IRES sequence at its 5' end was extracted from pDC315-iGFP plasmid (modified plasmid from pDC315 commercial plasmid from Microbix Biosystems Inc., Toronto, Ontario, Canada) by digestion with *EcoR*I and *Sal*I enzymes. The *EcoR*I site was filled with Klenow enzyme and the IRES-EGFP-*Sal*I fragment was subcloned in the pAd(I)-F512 vector obtained according to what is disclosed in Example 4, previously digested with

EcoRV and *SalI* enzymes. The obtaining of the vector resulting from this cloning, **pAd(I)-F512-EGFP** (SEQ ID NO 49 and SEQ ID NO 50), was confirmed by digestion with restriction enzymes and sequenciation with pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ ID NO 42) and pAd-antisense (CACAAATTTCACAAATAAAGCATTT; SEQ ID NO 48) primers.

Example 7.- Obtaining of Ad-F512 recombinant virus.

The pAd-F512 plasmid (SEQ ID NO 41) obtained according to what is disclosed in Example 4, was linearized with *FspI* enzyme and cotransfected together with a adenovirus type 5 fragment previously restricted with *ClaI* enzyme, including from mu 2.6 to mu 100 with deletion in E3 region. Cotransfection was carried out in 293 cells by means of calcium phosphate (Ferrari, C. C., Depino, A. M., Prada, F., Muraro, N., Campbell, S., Podhajcer, O., Perry, V. H., Anthony, D. C., and Pitossi, F. J. *Reversible demyelination, blood-brain barrier breakdown, and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain.* Am J Pathol, *165:* 1827-1837, 2004) and according to Nevins et al. protocol in 293 cells (Nevins, J. R. *Definition and mapping of adenovirus 2 nuclear transcription.* Methods Enzymol, *65:* 768-785, 1980). By homologous recombination between the adenoviral regions of the two transfected fragments Ad-F512 (SEQ ID NO 51 and SEQ ID NO 52) the recombinant adenovirus was obtained. An Ad-F512 constructed adenovirus scheme is shown in Figure 5A.

Once obtained, the Ad-F512 recombinant adenovirus was cloned and a stock purification was made by means of a cesium chloride double gradient (Lieber, A., He, C. Y., Kirillova, I., and Kay, M. A. *Recombinant adenoviruses*

with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. J Virol, *70:* 8944-8960, 1996). The adenovirus preparation was titrated by means of DICT50 (Lieber, A. et al., 1996) in 293 cells obtaining 10^{12} vp/ml for Ad-F512. Ad5-wt was included as a positive replication control in the assay, obtaining a preparation therefrom the title of which was 6,8 x 10^{11} vp/ml.

Besides, in order to confirm the identity of the viral stock, a viral DNA preparation was made, which was used to be digested with restriction enzymes and to be sequenciated using pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ ID NO 42) and pAd-antisense (CACAAATTTCACAAATAAAGCATTT; SEQ ID NO 48) internal primers, (See Figure 5B).

Example 8.- Obtaining of the Ad(I)-F512-TK recombinant virus.

In a similar way as described in Example 7, the pAd(I)-F512-TK (SEQ ID NO 46 and SEQ ID NO 47) plasmid obtained in Example 5, was linearized with *Fsp*I enzyme and cotransfected together with a Ad5 fragment, previously restricted with *Cla*I enzyme, including from mu 2.6 to mu 100, with deletion in region E3. Cotransfection was carried on in 293 cells by means of the calcium phosphate method (Ferrari, C.C. et al.; 2004) and according to Nevins' protocol in 293 cells (Nevins, J.R.; 1980). By homologous recombination between adenoviral regions of the two cotransfected fragments, the Ad(I)-F512-TK (SEQ ID NO 53 and SEQ ID NO 54) recombinant adenovirus was obtained. A scheme thereof is shown in Figure 5A.

Once obtained, the Ad(I)-F512-TK adenovirus was cloned and a stock purification was made by means of a cesium chloride double gradient (Lieber, A. et al.; 1996). The adenovirus preparation was titrated by means of DICT50 (Lieber, A. et al., 1996) in 293 cells yielding a titer of 10^{12} vp/ml. Ad5-wt was included as a positive replication control in the assay, obtaining a preparation therefrom the title of which was 6,8 x 10^{11} vp/ml.

Besides, in order to confirm the identity of the viral stock, a viral DNA preparation was made, which was used to be digested with restriction enzymes and to be sequenciated using pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ ID NO 42) and pAd-antisense (CACAAATTTCACAAATAAAGCATTT; SEQ ID NO 48) internal primers, (See Figure 5B).

Example 9.- Obtaining of the Ad(I)-F512(E3) recombinant virus.

The pAd(I)-F512-EGFP plasmid (SEQ ID NO 49 and SEQ ID NO 50) obtained in Example 6, was linearized with *FspI* enzyme, like in Examples 7 and 8, but in this case the cotransfection was carried out together with another plasmid, JM17 (Microbix Biosystems Inc., Toronto, Ontario, Canada) containing the whole Ad5 genome, except for the E1 region, but containing the E3 region. The cotransfection was carried out in 293 cells by means of calcium phosphate method (Ferrari, C.C. et al.; 2004) and according to the Nevins' protocol in 293 cells (Nevins, J.R.; 1980). By homologous recombination between the adenoviral regions of the two cotransfected fragments, the Ad(I)-F512(E3) (SEQ ID NO 55 and SEQ ID NO 56) recombinant adenovirus was obtained, the scheme of which is shown in Figure 5A, and that contains the E3 region of the original virus (Ad5).

Once obtained, the recombinant adenovirus containing the E3 region was cloned and the stock was purified by means of a double cesium chloride gradient (Lieber, A. et al., 1996). The adenovirus preparation was titrated by means of DICT50 (Lieber, A. et al., 1996) in 293 cells, obtaining a titer of 1,5 x 10^{12} vp/ml. As a positive replication control in the assay, Ad5-wt was included, obtaining a preparation therefrom the title of which was 6,8 x 10^{11} vp/ml.

Besides, in order to confirm the identity of the viral stock, a viral DNA preparation was carried out, which was used to be digested with restriction enzymes and to be sequenciated using pAd-sense (TGTTTTTCTCAGGTGTTTTCCG; SEQ ID NO 42) and pAd-antisense (CACAAATTTCACAAATAAAGCATTT; SEQ ID NO 48), internal primers (see Figure 5B).

Example 10.- *In vitro* assays with Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) adenoviruses for the determination of cell infectivity.

For *in vitro* assays the adenoviruses obtained in Examples 7, 8 and 9 were used. The cell lines used have already been disclosed in Example 1.

The viruses used in the present invention are based on Ad5, the entrance via of which is through CAR receptor. (*coxsackie-adenovirus receptor*) and integrins (Kanerva, A. and Hemminki, A. *Adenoviruses for treatment of cancer*. Ann Med, *37*: 33-43, 2005). Given that the CAR expression is heterogeneous, translation assays were made in different cell lines with a non-replicative adenovirus expressing β-galactosidase enzyme (Ad-β-gal).

The different tumor and normal lines were infected at different multiplicities of infection (MOI) with Ad-β-gal, and the infection percentage was registered (see Table 2). After 3 days an assay with X-gal was carried out in order to reveal β-galactosidase. Blue cells were counted (infected cell indicator) and the percentage regarding non-infected cells was calculated. At least three different fields were counted.

Table 2

	% infection							
MOI	A375N	HeLa	LoVo	T84	SB2	WI-38	WI-38 VA	BAEC
1	0.03125	0.25325	0.006	0.0215	5.833333	0.02	0.24	0.0266
10	3.05	7.5	0.0095	0.64	27.9	0.41	2.86	0.85
100	27.675	21.5	2.15	10	95.2	24.69	20.5	13.9
500	35.33333	77	32.5	80.5	NA	60.35	75.25	52.6
1000	53.33333	84.75	73.33334	85	NA	91	79.65	55.94

It was noted that, at a multiplicity of infection (MOI) of 1000, almost all of the lines are infected at least in a 75%, excepting those of BAEC and A375N cells, which are only infected in a 50%. At a MOI lower than 100 a low cell infectivity is observed (about 25%) excepting the SB2 melanoma line having an infectivity of more than 95%. At a MOI of 10 almost no infected cells can be seen in any line, but the SB2.

Summarizing, the infection capability of the tested cell lines would be SB2>T84>HeLa>WI-38VA>WI-38>LoVo>BAEC>A375N.

Example 11.- *In vitro* assays with Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) adenovirus for the assessment of oncolytic capability.

In order to test the oncolytic capability of the adenovirus (CRAds) constructed according to the present invention, they were used to infect *in vitro* cells having different levels of SPARC expression. Particularly, they were used SB2 melanoma, A375N and MelJ lines; WI-38, WI-38 VA and HFL-1 fibroblasts lines; T84 and LoVo colon lines; HeLa cervix line; BAEC endothelial line; and normal mesenchymal cells. The Ad5-wt (*wild type*) was used as control. The different tumor lines were infected with Ad-F512 (SEQ ID NO 51 and SEQ ID NO 52), Ad(I)-F512-TK (SEQ ID NO 53 and SEQ ID NO 54), Ad-(I)F512(E3) (SEQ ID NO 55 and SEQ ID NO 56) and Ad5-wt adenoviruses. The adenoviruses lytic effect was assessed by staining with violet crystal the cells that remained attached to the plate after the experiment and through the quantification of the metabolic activity by means of the MTT assay.

The lytic effect of adenoviruses was assessed by staining with violet crystal: Referring to Figure 6, the monolayer infection proceeding for the study of the cytopathic effect is as follows: the cells were seeded in 24-well plates at a density of 1 x 10^4 cells/well. The following day they were infected in 200 µl of high glucose DMEM 2% BFS during 3 hours at different multiplicities of infection (MOIs). After the infection 800 µl of the corresponding medium for each cell line were added. Ten days later the cells were stained with violet crystal (solution 0.75% in 40% methanol) or stained with β-galactosidase, and in this last case two or more fields

were counted in order to register the infection percentage. Figure 6 shows a photograph of the assay of staining with violet crystal in plate, for the different cell lines employed.

The lytic effect of the adenoviruses was assessed through quantification of metabolic activity by means of MTT assay: The MTT assay (cell viability) is based on the cleavage of the yellow tetrazolium salt in a purple compound (formazan). This reaction only takes place in metabolically active cells having succinate-tetrazolium reductase enzyme present in the mitochondria respiratory chain. The lytic effect measured as a function of metabolic activity (MTT assay) is shown in Figure 7. In this assay 96-well plates were seeded at a density of 5 x 10^3 cells/well and the following day they were infected during 2 hours in 25 µl (high glucose DMEM 2%). After 2 hours 100 µl of the corresponding medium for each cell line were added. Ten days later the cell viability was accounted by means of the MTT method (Mosmann, T. *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, *65:* 55-63, 1983).

The viability assays with MTT are shown in Figure 7. Generally, a lytic activity is noted for the various cell types similar to the one shown through violet crystal; but in this case a greater viability was noted for each MOI than those of the corresponding staining with violet crystal. In these assays three lines of breast cancer (578T, MCF7 and T-47D) were included, all of them with very low SPARC expression, and in the case of T-47D with low F512 activity. Surprisingly, two lines (MCF7 and T-47D) turned out to be susceptible to the CRAds at a MOI of 100.

Surprisingly, all of the tumor cells were lysed by the CRAds. However, the lytic activity of the CRAds in the tumor cells turned out to be independent of the SPARC mARN expression degree or of the promoter activity in the cell line. Thus, e.g., for melanoma cells, all of the viruses resulted more effective in SB2 than in MelJ or A375N. This result is coincident with the cell infectivity but not with the promoter activity or with the SPARC expression. Given that the viruses have different infection capability in each line, the comparison between the different viruses was made mainly within the same cell line. In the first place, SB2 is lysed more effectively with Ad(I)-F512(E3) > Ad-F512 ~ Ad(I)-F512-TK±GCV, while in MelJ (wherein the promoter showed a much higher activity) Ad(I)-F512(E3) ~ Ad-F512 > Ad(I)-F512-TK±GCV.

From this comparison it is clear that the less powerful virus is that which has got included the *insulator* sequence, and that the addition of the E3 region makes the lysis more effective. Besides, no increase of the lytic effect is noted upon adding GCV to the cell culture infected with Ad(I)-F512-TK.

On the other hand, it was noted that T84 colon and LoVo lines turned out to be very susceptible to Ad-F512. In the case of LoVo this effect is stronger than the one observed for SB2 melanoma cells. It can be noted that Ad-F512 was capable of lysing the WI-38 fibroblasts (which express SPARC protein in a moderate way), at a MOI of 500, while the two viruses having an *insulator* sequence between the virus ITR and the promoter, turned out to be lytic at a MOI of 1000. The addition of GCV slightly increases the lytic capability of Ad(I)-F512-TK. Two additional

fibroblast lines -WI-38VA and HFL-1- analyzed with Ad-F512 virus were poorly lysed at a MOI of 1000. In the three fibroblast lines, Ad5-wt was capable of lysing at a MOI of 1. The endothelial cells were not susceptible to Ad-F512, independently of their high SPARC expression. Primary mesenchymal cells derived from the marrow bone were not affected by Ad-F512 but by Ad5-wt, at a MOI of 100.

As a conclusion, F512 within three adenovectors turns out to be lytic in the most part of tumor lines, while it does not affect normal cells like mesenchymal and endothelial cells and some fibroblasts.

Example 12.- Virus yield in different cell lines

In order to assess the capability of replication of CRAds in different tumor lines, an assay on *virus yield* was carried out. The results thereof are shown in Figure 8.

The general proceeding for the virus production in different cell lines is disclosed below:

On day 0, 100000 cells were seeded in each well of a 6-well plate. On day 1 cells were infected at a 50 MOI in a volume of 300 µl per well in high glucose DMEM medium 2% BFS. The infection was carried out during 1 hour at 37°C with at least two stirring operations. Subsequently, the medium was removed, washed twice with PBS and 1 ml of 10% BFS medium was added. After three days, cells were kept at room temperature for 15 minutes and were picked up together with the medium. The supernatant was placed into an Eppendorf tube and three freezing/thawing cycles were made using liquid nitrogen. Subsequently, it was centrifuged at 4500 rpm and 1/10 serial dilutions of said supernatant were made

in high glucose DMEM medium 5% BFS and 0.01M HEPES. 100 µl of each dilution were seeded (sextuplicate) in a well of a 96-well plate that was seeded the day before with 293 cells at a rate of 20000 cells/well. Five days later the cells were fixed with 4% paraformaldehyde and stained with violet crystal solution.

Referring to Figure 8, the adenovirus yield in the different lines (melanoma, colon, breast, fibroblasts and endothelium) is expressed as viral particles per ml (vp/ml), and each bar represents the average of at least two independent assays.

In the first place, it can be pointed out that the adenovirus yield is independent of the SPARC mRNA expression. In the second place, all of the lines show greater Ad(I)-F512(E3) replication than that of Ad-F512 virus. In the third place, the Ad5-wt adenovirus yield differs among the different lines in a ratio of up to 10^5 (e.g., comparing 578T to LoVo), what in part can be due to the low infectivity of some lines. Besides, the lines producing greater amounts of CRAds are those having greater susceptibility to CRAds (colon lines).

Example 13.- Study on the cooperative effect between non-replicative viruses and replicative viruses

As it was stated above, a non-replicative adenovirus requires the provision of the E1A protein in trans, so as to initiate replication. When an non-replicative adenovirus is amplified, they are used 293 cells that have been modified in order to constitutively express the E1A protein, but this protein can also be provided by a replicative adenovirus. In order to study the cooperation

of Ad-F512 and a non-replicative adenovirus (Ad-β-gal), T84 colon cells were infected with a constant amount of Ad-β-gal (MOI 10) and increasing amounts of Ad-F512. Ad-F512 is capable of complementing the non-replicative adenovirus, allowing its replication and distribution through neighbour cells, since as the Ad-F512 increases, an increase in the blue-stained cells is observed, denoting a local increase in Ad-β-gal virus. Figure 9 shows the staining of βgal for the different assayed MOIs in T84 colon cells.

Example 14.- *In vivo* assays using Ad-F512 and Ad(I)-F512-TK CRAds

6-8 week-old Athymic male N:NIH(S)-nu mice (provided by the Veterinary College of the University of La Plata) were used for the assays. Three different *in vivo* assays were carried out using the recombinant adenoviruses obtained according to the present invention: two assays with Ad-F512 and one with Ad-(I)F512-TK.

The animals were subcutaneously injected with an inoculum of 4 x 10^6 SB2 melanoma cells. When tumors reached a mean volume of 100 mm^3 (about 20 days after injection), the intratumoral injection of adenovirus started at 10^{10} vp/30 µl per dose. Each animal received three doses on days 0, 2 and 8 counting from the beginning of the treatment. In one of the assays, when using the CRAd expressing simultaneously the *hsv*-TK herpetic enzyme, the treatment with adenovirus was alternated with the injection of ganciclovir prodrug (GCV, Cytovene®, Rotang), according to what is shown in Figure 10A, wherein the arrows indicate

CRAd injections as well as the treatment with GCV. This drug was administered once a day at 30 mg/kg.

The control group received injections of an non-replicative adenovirus (Ad-β-gal), also expressing a non-therapeutic gene, instead of one of the two CRAds used in the present assay.

Figure 10A shows the tumor growth curves in the assay using Ad-F512-TK CRAd, wherein the administered CRAd injections and the ganciclovir administration are also indicated. Figure 10B shows the curves of tumor growth in the mice used in the assay with Ad-F512. Figure 10C is a photograph of the tumoral zone in a control mouse (injected with Ad-β-gal) and in one treated with Ad-F512 CRAd. Figure 10D shows photographs of histological cuts made on some animals after 14 days from the beginning of the treatment. Finally, Figures 10E and 10F are Kaplan-Meier curves (survival %) for both assays realized with Ad-F512.

In both assays with Ad-F512 it was noted a total remission in most part of the treated tumors (Figures 10B, 10E and 10F) leaving a scar in the tumor site (shown by means of an arrow in Figure 10C). Referring again to Figures 10E and 10F, these curves indicate that most part of the animals treated with Ad-F512 are still alive after 90 days, either because their tumors do not grow, they totally regress or they grow at a lower rate, while the control animals (tumor + Ad-β-gal) are dying because their tumors keep on growing.

Histological studies were carried out in some animals, by fixing in formaldehyde 10% and subsequent processing. This way, a histological study of the scar zone showed

differences between days 14 and 90 counting from the beginning of the treatment: while on day 14 abundant macrophage infiltrate with pigments inside, with polymorphonuclear foci, nuclear remainings and granulomatose vascularization can be observed (see Figure 10D), on day 90 it can be noted that the tissue has been repaired completely. In both cases an autopsy was carried out in the whole animal, not finding metastasis; however, on day 90 in one of the animals, the spleen with a fibrosis focus and the liver with a hepatitis focus were observed. None of the analyzed controls showed these characteristics.

The survival curves (Figures 10E and 10F) in both assays with Ad-F512 are very similar to each other and they are significantly different from the control.

Besides, referring to Figure 10A, the assay with the CRAd expressing the suicide TK gene did not show statistically significant differences in the presence or in the absence of GCV, but the virus results equally effective compared to the control.

Example 15.- *In vitro* assays using Ad-F512, Ad(I)-F512-TK and Ad(I)-F512(E3) CRAds

a) Analysis of tumor lines

In this step, the previous analysis carried out as described in Example 11 was extended by testing other tumor lines of melanoma, colon, breast and pancreas cancers. The cytopathic effect of viruses on the cells was analyzed by staining with violet crystal the cells that remained attached to the well after 10 days post-infection with the adenovirus. The results are shown in Figure 11.

The Mel888 melanoma cells (kindly provided by Dr. Estela Medrano, Houston, TX) were lysed by the adenoviruses at same viral concentrations than for the assays with SB2 and IIB-Mel-J cells (the former kindly provided by Dr. Estela Medrano, Houston, TX and the latter by Ledda et al., *Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells*. Nat Med. 1997 Feb; 3(2):171-6) tested in Example 11 above. The two colon lines HT29 (ATTC No. HTB-38) and CaCO-2 (ATCC No. HTB-37) included in these new assays also show the susceptibility of colon cells to the constructed CRAds. It has been found that the MIA-PaCa-2 pancreas cancer line (ATCC No. CRL-1420) is sensitive to a 5 x 10^7 vp/ml adenovirus concentration, while the four analyzed 578T breast lines (ATCC No. HTB-126), T-47D (ATCC No. HTB-133), MCF-7 (ATCC No. HTB-22) and MDA-231 (ATCC No. HTB-26) are not affected by CRAds, (see Figure 11). In all cases, the wild type virus (AD5-wt) is capable of eliminating all tumor cells.

b) Analysis of normal lines

One of the requirements for using an oncolytic adenovirus in a clinical assay is that it should be active in tumor cells and inactive in normal cells. In order to establish the attenuation level in normal cells of the three constructed oncolytic viruses, their activity was analyzed in a panel of said cells. Normal melanocyte, colon and breast cell lines were included as well as fibroblasts, keratinocytes and human microendothelial cells. After 10 days post-infection with 5 x 10^6 viral particles/ml of Ad-F512, Ad(I)-F512-TK or Ad(I)-F512(E3) the viability of melanocytes was greater than 95% while 100% of cells were lysed by the Ad-wt (see Figure 12A). It was shown (see Figure 12B) that the oncolytic viruses also had no effect on normal CCD841 colon cells (ATCC No. CRL-1790) and

normal MCF-12A breast cells (ATCC No. CRL-10782) (see Figure 12C). These lines have no SPARC expression (see Table 3 below). hMEC-1 human microendothelial cells were also analyzed (kindly provided by Isaiah Fidler, Houston, TX) with SPARC expression and it was noted that they are lysed by the adenoviruses at a high MOI (see Figure 12D). Subsequently other cell components present in the skin, like keratinocytes or fibroblasts were analyzed. The former do not produce SPARC while the latter have an intermediate SPARC expression when compared to A375N tumor cell line (see Table 3 below). The viral effect on keratinocytes (HaCaT, kindly provided by Craveri Laboratory), on CCD1140 fibroblast cells (ATCC No. CRL-2714) and on Malme-3 (ATCC No. HTB-102) is shown in Figure 12E. It was noted that neither the keratinocytes nor the fibroblast lines were lysed by the adenoviruses.

Therefore, CRAds do not lyse normal cells which do not express SPARC (colon, breast, melanocytes, keratinocytes,), lysing, instead, hMEC-1 microendothelial cells which do express SPARC.

Table 3: Relative SPARC expression of the cell lines used in the study. The average SPARC mRNA expression is shown in relation to the A375N tumoral line expression. SD: Standard deviation.

Cell line	Origin	Average	SD
A375N	Melanoma	1	0
Mel888	Melanoma	0.1523333	0.00617342
HT-29	Colon	0.00083554	1.4217E-05
CaCO2	Colon	0.00194873	0.00047591
578T	Breast	1.585	0.345

T-47D	Breast	0.00152448	0.00045275
MCF-7	Breast	0	0
MDA-231	Breast	0	0
BxPC3	Pancreas	0	0
MiaPaca-2	Pancreas	0	0
NHM	Normal melanocytes	0.05	0
CCD841	Normal colon	0.04033333	0.01125956
MCF12-A	Normal breast	0	0
hMEC-1	microendothelial	0.20225	0.01271728
HACAT	keratinocytes	0.0005	0.0005
CCD1140	fibroblasts	0.417	0.0609836

c) Ad(I)-F512-TK lytic effect in cells expressing SPARC: tumoral and stromal cells (fibroblasts and endothelium) growing in monolayer.

In vitro assays with tumoral stromal cells (endothelial and fibroblasts) showed that even with high or moderated SPARC expression the adenoviruses did not have the same activity as in tumor cells. According to the present inventors' hypothesis, normal cells have more resistance to viral replication. In this case, adding a toxic gene would allow for the enhancement of the lytic capability in a specific manner in those cells wherein the SPARC promoter was active. Thus, *in vitro* assays with Ad(I)-F512-TK were carried out, in which the cells were also treated by adding ganciclovir (GCV) prodrug. In the first place, it was noted that adding GCV improves virus lysis in SB2 melanoma cells (see Figure 13A); this improvement

is more effective if the addition of GCV starts 72 hs after the beginning of the oncolytic virus action. Regarding endothelial cells, they are sensitive to the presence of the prodrug alone (see in Figure 13B the first three bars on the left). Adding the virus at a MOI of 750 and in presence of CGV 50 μM or 100 μM allows for the elimination of almost all of the cells (last two columns in Figure 13B). Other analyzed endothelial cells were those of bovine aorta (BAEC, kindly provided by Helene Sage, Seattle, USA). However, these cells which express higher SPARC values than A375N and in which the F512 human promoter is very active (see Examples 1 and 3B above), do not get properly infected with the Ad5 capsid. Similarly to hMEC-1, the cells are sensitive to the prodrug, but the presence of the GCV prodrug in addition to the virus allows for the elimination of cells at viral concentrations in which the virus itself has no effect (see Figure 13C).

Example 16.- *In vivo* assays using Ad-F512 and Ad(I)-F512-TK CRAds

In vivo assays were carried out in animals (6-8 week-old athymic male mice N:NIH(S)-nu), that were injected with mixed melanoma tumors comprising tumoral and stromal cells. Subsequently, these tumors were treated with the CRAds obtained according to the present invention, using the same protocol than that for tumors of only melanoma cells.

Figure 14A shows the results of an assay in which animals carried SB2/hMEC-1/WI-38 mixed tumors and were treated with Ad-F512. It was noted that treatment with the adenovirus produces a relevant delay in growth tumor, even

when the malignant cells were co-injected with endothelial cells (hMEC-1) and fibroblasts (WI-38), showing that this virus can be effective, in a similar situation occurring in a tumor.

The same result was obtained when repeating the assay (data not shown).

It was also assessed the efficacy of the adenoviral treatment on mixed tumors of tumoral cells and fibroblasts. It was noted that the treatment of SB2/WI38 tumors with Ad-F512 resulted partially effective (Figure 14B). When SB2/hMEC-1 cells were injected, it was noted that some of the animals responded much better to the treatment, even though there were no rejections (Figure 14C). Finally, SB2/WI-38 tumors were treated with Ad(I)-F512-TK+GCV, noting that 3 animals of a total of 5 animals completely rejected the tumor, indicating that the addition of the TK toxic gene significantly improves the virus effect (Figure 14D).

Besides, it has been disclosed (Yamazaki, M., Straus, F. H., Messina, M., Robinson, B. G., Takeda, T., Hashizume, K., and DeGroot, L. J. *Adenovirus-mediated tumor-specific combined gene therapy using Herpes simplex virus thymidine/ganciclovir system and murine interleukin-12 induces effective antitumor activity against medullary thyroid carcinoma*. Cancer Gene Ther, *11:* 8-15, 2004.) that many CRAds show greater unspecificity *in vitro* than *in vivo*. As disclosed in Example 11, the viruses of the present invention were able to eliminate colon cancer cells *in vitro*. However, previous studies (Yamazaki et al., 2004, above) suggested that this could be due to

unspecific effects. Therefore, *in vivo* assays with colon and pancreas cancer tumors were carried out.

A remarkable *in vivo* effect on colon tumors was noted. In fact, Ad-F512 and Ad(I)-F512-TK were not able to eliminate *in vivo* the LoVo cell tumor (Figure 15A); however, the addition of hMEC-1 endothelial cells to those tumors and in the presence of GCV allowed for the delay in the colon tumor growth, compared to the tumors of the control group, indicating that the presence of endothelial cells expressing SPARC could enhance the elimination of tumors the malignant cells of which do not express SPARC (Figure 15B).

On the same basis, in another group of experiments, Mia-PaCa-2 (ATCC No. CRL-1420) (malignant, pancreas)/hMEC-1 mixed tumors were treated with Ad-F512 and Ad(I)-F512-TK+GCV, showing, surprisingly, that these tumors are completely eliminated (Figure 15C).

While the illustrated embodiments of the invention disclosed herein, fulfill the preferred aims of the present invention, many modifications and other embodiments may be apparent for a person skilled in the art. Therefore, it shall be understood that the annexed claims are intended to encompass all of said modifications and embodiments, that will be within the spirit and scope of the present invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. An isolated DNA of a nucleic acid promoter having a sequence consisting of SEQ ID NO:1.

2. The isolated DNA of claim 1, wherein the promoter sequence is operably linked to a heterologous gene.

3. The isolated DNA of claim 1, further comprising a second nucleic acid promoter.

4. The isolated DNA of claim 3, further comprising a regulating sequence selected from a radiation responsive sequence, a hypoxia responsive sequence or a free-radical responsive sequence.

5. The isolated DNA of claim 2, wherein the heterologous gene is a therapeutic gene.

6. The isolated DNA of claim 2, wherein the heterologous gene is selected from the group consisting of—an E1A gene, a suicide gene, the adenoviral genomic E3 region and a gene encoding an interleukin.

7. An expression vector comprising the isolated DNA of claim 1 and a heterologous gene operably linked thereto.

8. The expression vector of claim 7, wherein the vector is a plasmid or a viral vector.

9. The expression vector of claim 8, wherein the viral vector is a recombinant adenovirus.

10. The expression vector of claim 8, wherein the viral vector is a Conditionally Replicative Oncolytic Adenovirus.

11. The expression vector of claim 7, wherein the heterologous gene is a therapeutic gene.

12. The expression vector of claim 11, wherein the therapeutic heterologous gene is an E1A protein gene.

13. The expression vector of claim 7, wherein the heterologous gene is a suicide gene.

14. The expression vector of claim 13, wherein the suicide gene is the thymidine kinase gene of a herpes virus (*hsv*TK).

15. The expression vector of claim 7, further comprising an adenoviral genomic E3 region.

16. A method for expressing foreign DNA in a host cell, the method comprising introducing into the host cell the expression vector of claim 7.

17. A pharmaceutical composition comprising the expression vector of claim 7 in a pharmaceutically suitable carrier.

18. A method for treating a tumor in a patient suffering therefrom, the method comprising administering to the

patient an effective amount of the pharmaceutical composition of claim 17.

19. An expression vector comprising a nucleic acid promoter having a sequence consisting of SEQ ID NO: 1 according to claim 1.

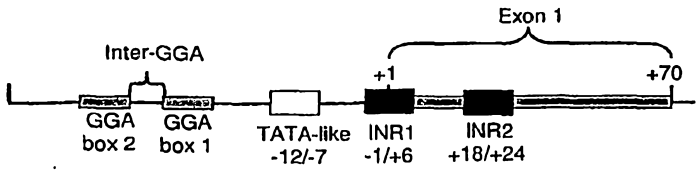


FIG. 1

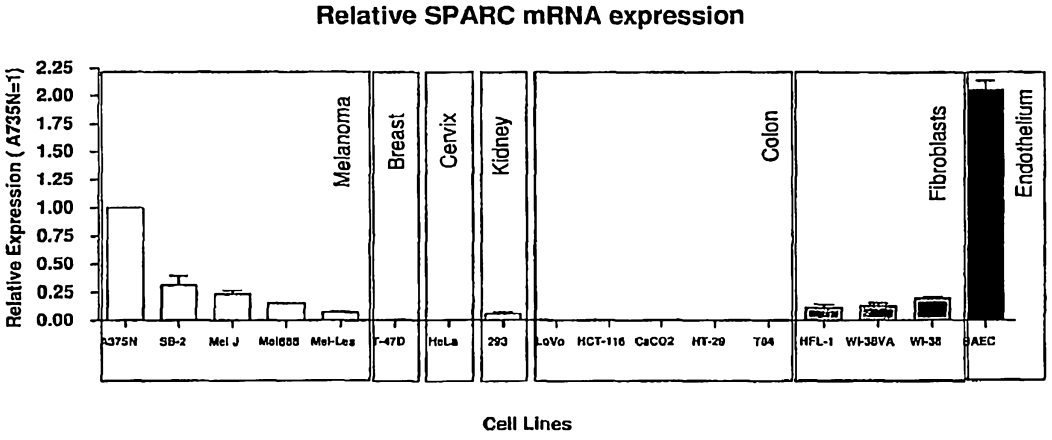


FIG. 2

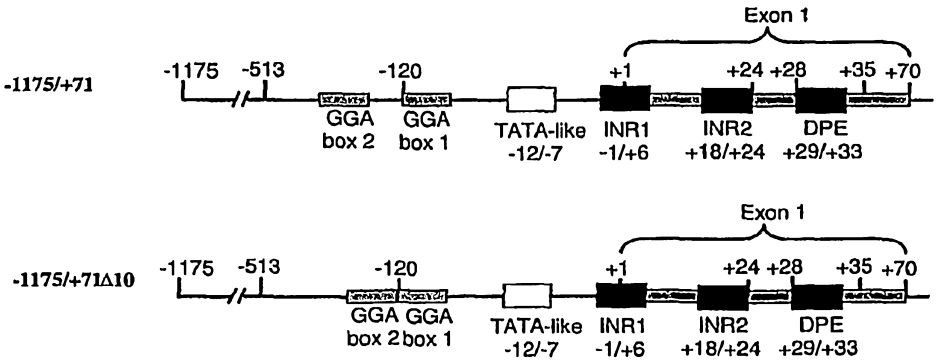


FIG. 3

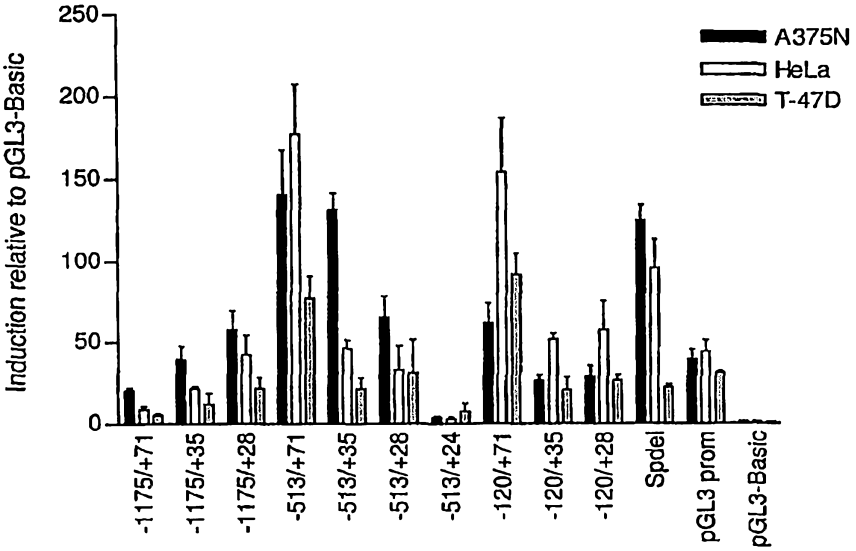

Induction relative to pGL3-Basic
250
200
150
100
50
0
A375N
HeLa
T-47D
-1175/+71
-1175/+35
-1175/+28
-513/+71
-513/+35
-513/+28
-513/+24
-120/+71
-120/+35
-120/+28
Spdel
pGL3 prom
pGL3-Basic

FIG. 4 A

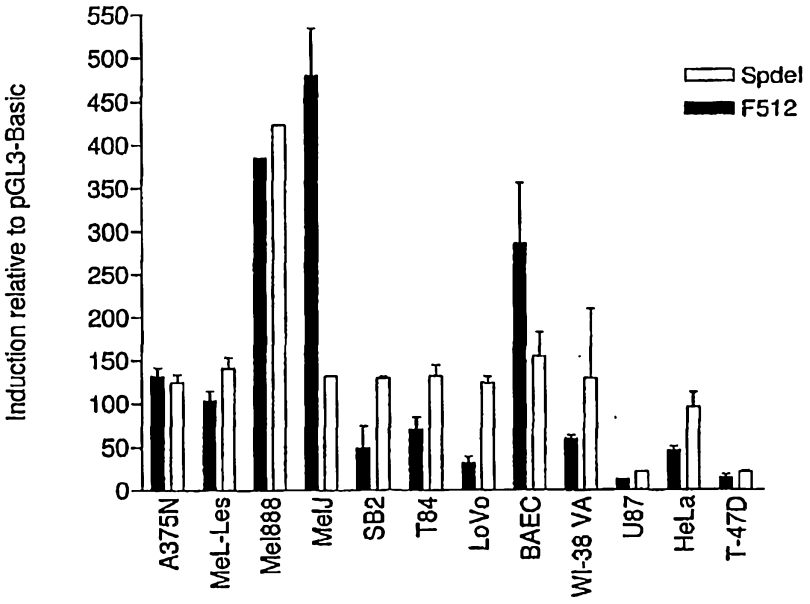

Induction relative to pGL3-Basic
550
500
450
400
350
300
250
200
150
100
50
0
Spdel
F512
A375N
MeL-Les
Mel888
MelJ
SB2
T84
LoVo
BAEC
WI-38 VA
U87
HeLa
T-47D

FIG. 4 B

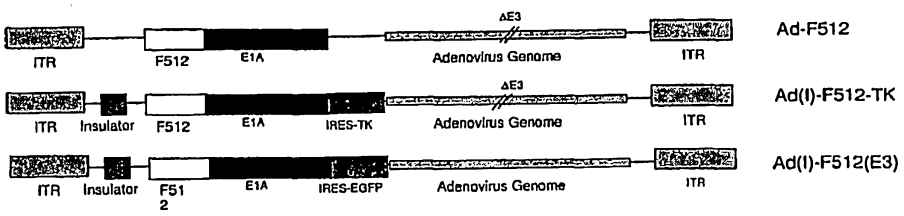

ΔE3
ITR
F512
E1A
Adenovirus Genome
ITR
Ad-F512
ΔE3
ITR
Insulator
F512
E1A
IRES-TK
Adenovirus Genome
ITR
Ad(I)-F512-TK
ITR
Insulator
F51
2
E1A
IRES-EGFP
Adenovirus Genome
ITR
Ad(I)-F512(E3)

FIG. 5 A

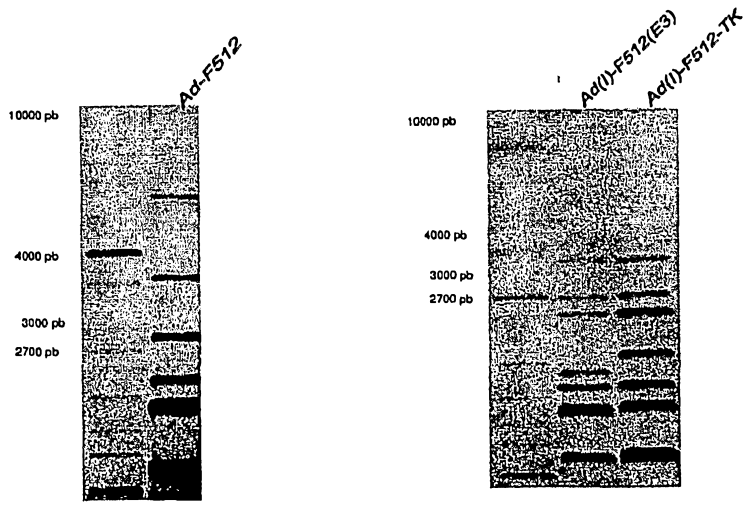

Ad-F512
10000 pb
4000 pb
3000 pb
2700 pb
Ad(I)-F512(E3)
Ad(I)-F512-TK
10000 pb
4000 pb
3000 pb
2700 pb

FIG. 5 B

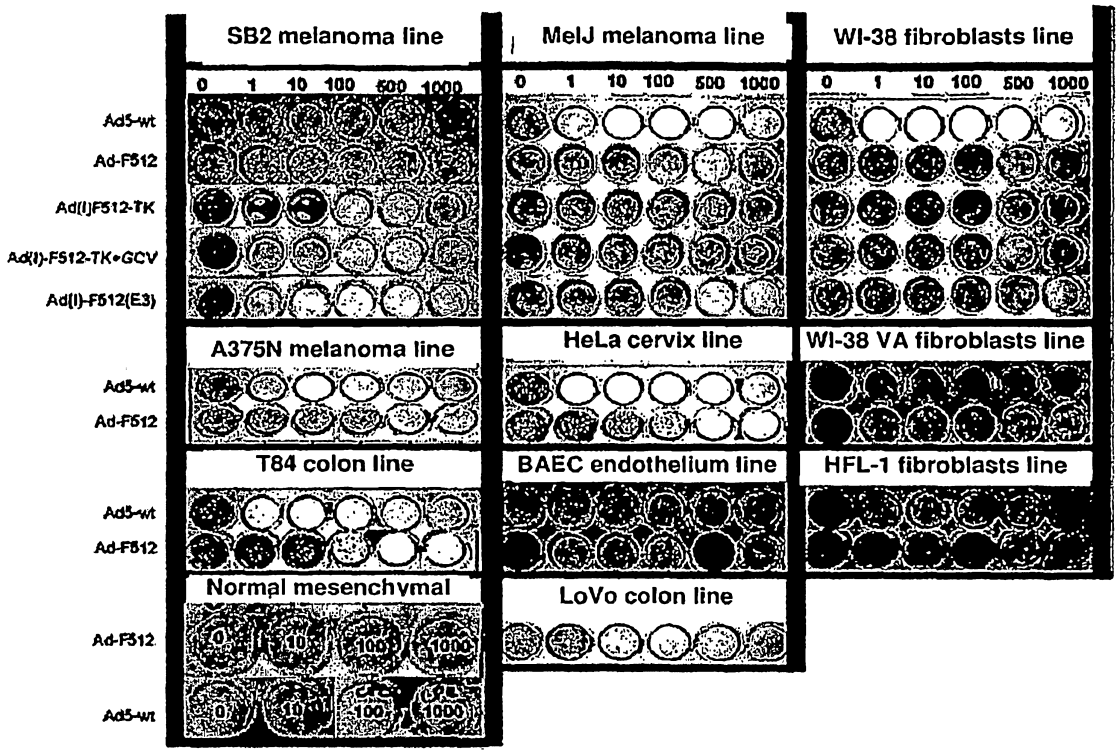

SB2 melanoma line
MelJ melanoma line
WI-38 fibroblasts line
0 1 10 100 500 1000
Ad5-wt
Ad-F512
Ad(I)F512-TK
Ad(I)-F512-TK+GCV
Ad(I)-F512(E3)
A375N melanoma line
HeLa cervix line
WI-38 VA fibroblasts line
Ad5-wt
Ad-F512
T84 colon line
BAEC endothelium line
HFL-1 fibroblasts line
Ad5-wt
Ad-F512
Normal mesenchymal
LoVo colon line
Ad-F512
Ad5-wt

FIG. 6

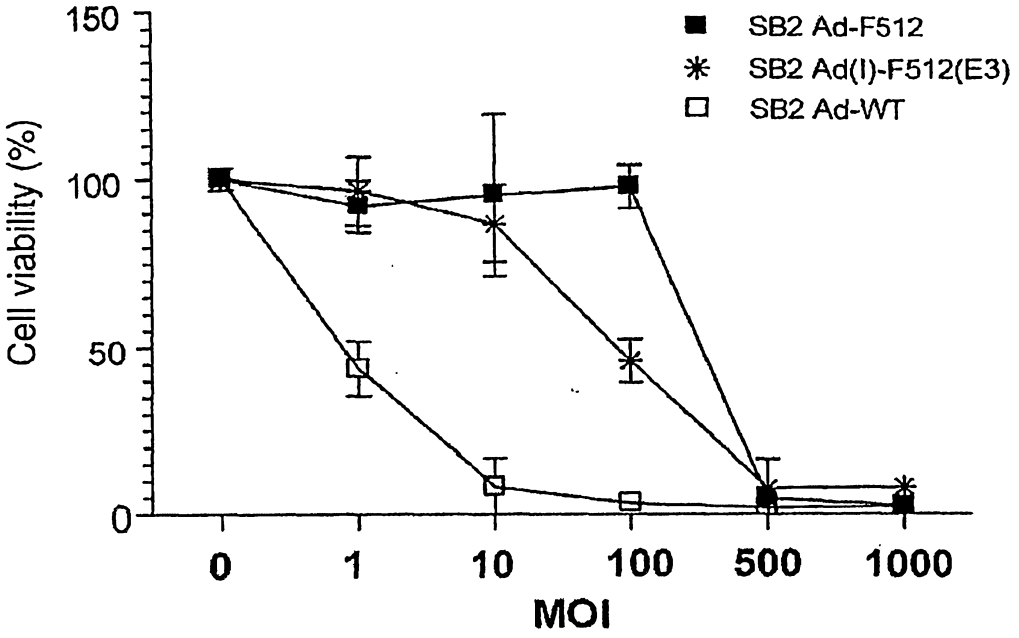

150
100
50
0
Cell viability (%)
SB2 Ad-F512
SB2 Ad(I)-F512(E3)
SB2 Ad-WT
0
1
10
100
500
1000
MOI

FIG. 7 A

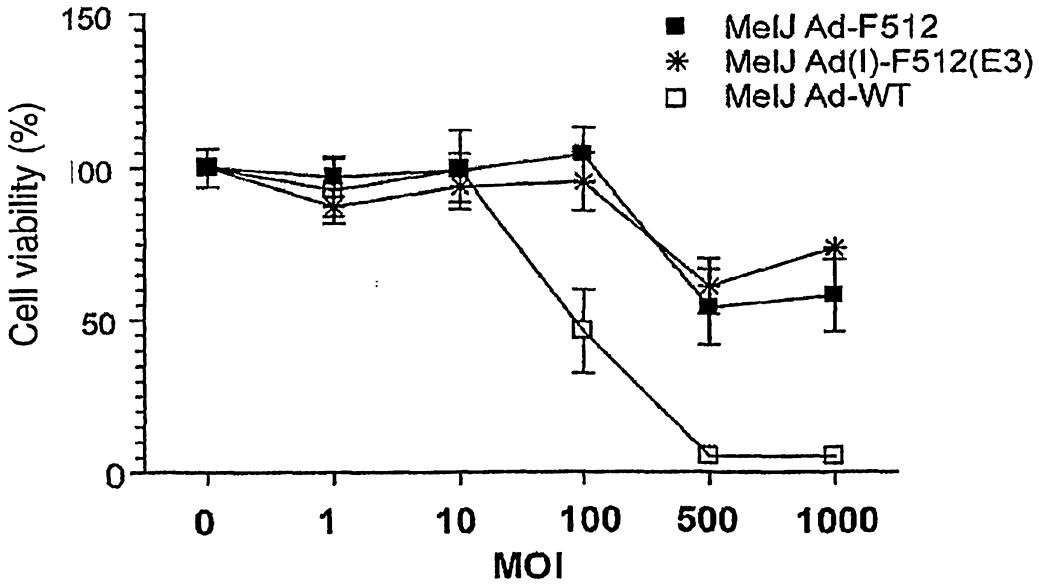

150
100
50
0
Cell viability (%)
MelJ Ad-F512
MelJ Ad(I)-F512(E3)
MelJ Ad-WT
0
1
10
100
500
1000
MOI

FIG. 7 B

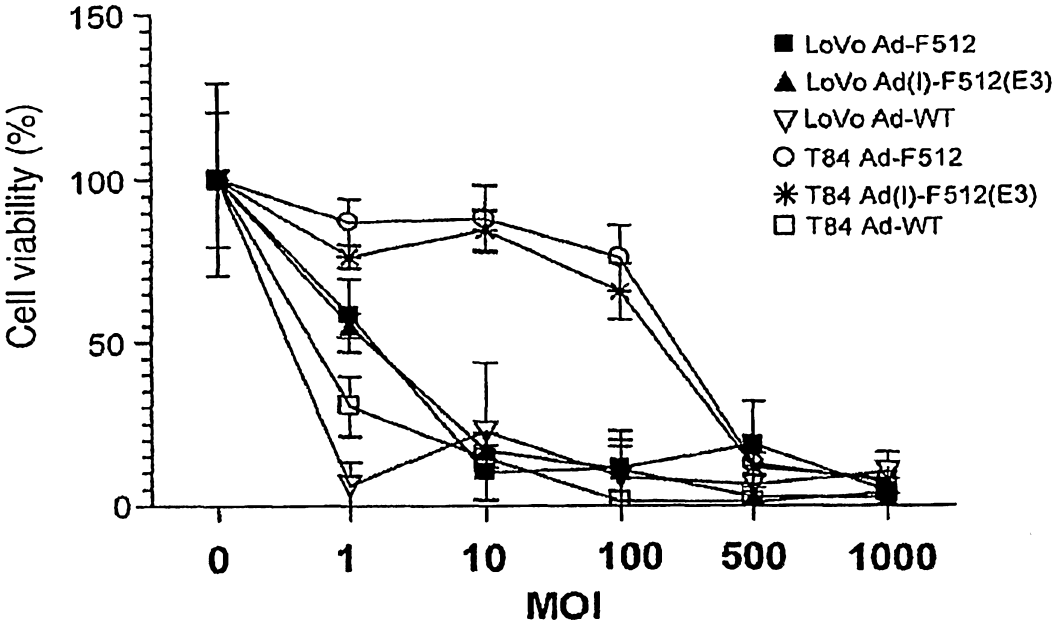

150
100
50
0
Cell viability (%)
0
1
10
100
500
1000
MOI
LoVo Ad-F512
LoVo Ad(I)-F512(E3)
LoVo Ad-WT
T84 Ad-F512
T84 Ad(I)-F512(E3)
T84 Ad-WT

FIG. 7 C

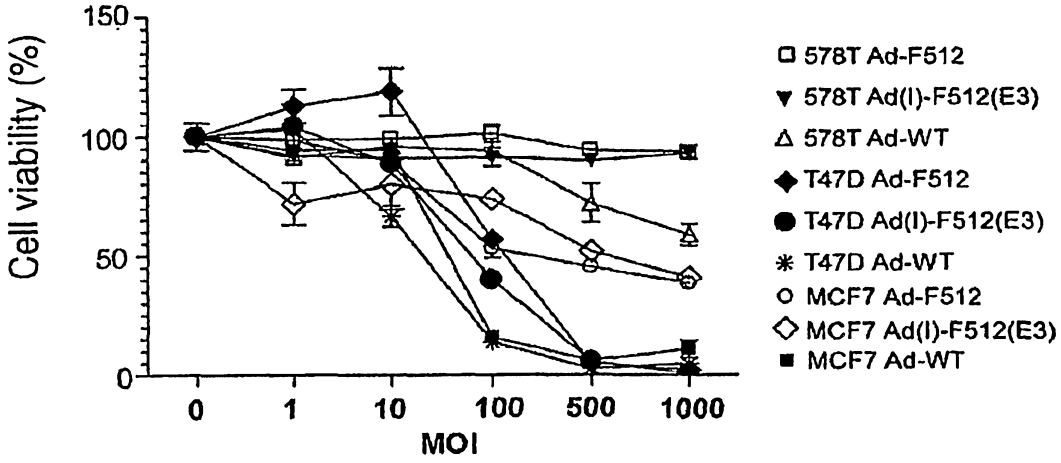

150
100
50
0
Cell viability (%)
0
1
10
100
500
1000
MOI
578T Ad-F512
578T Ad(I)-F512(E3)
578T Ad-WT
T47D Ad-F512
T47D Ad(I)-F512(E3)
T47D Ad-WT
MCF7 Ad-F512
MCF7 Ad(I)-F512(E3)
MCF7 Ad-WT

FIG. 7 D

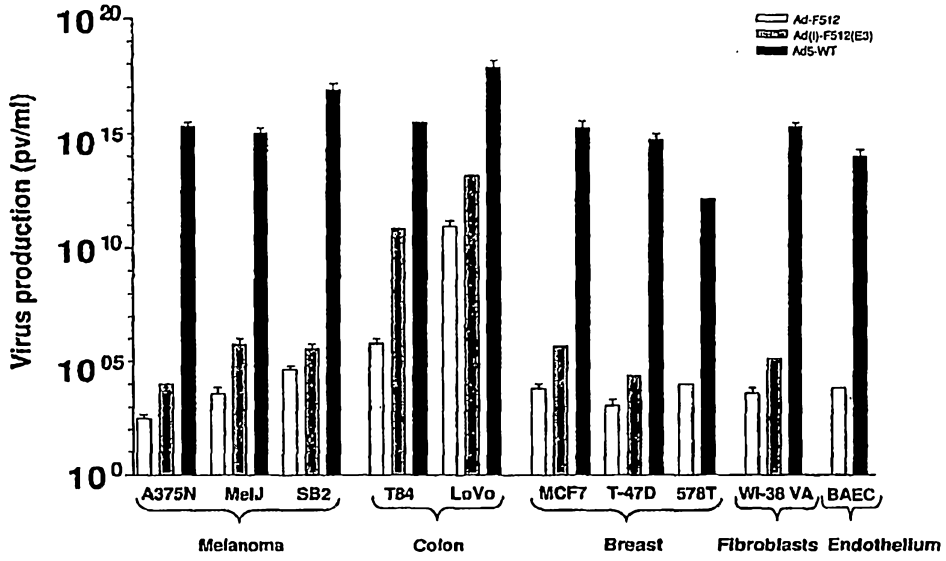

Ad-F512
Ad(I)-F512(E3)
Ad5-WT
Virus production (pv/ml)
10^20
10^15
10^10
10^05
10^0
A375N
MelJ
SB2
T84
LoVo
MCF7
T-47D
578T
WI-38 VA
BAEC
Melanoma
Colon
Breast
Fibroblasts
Endothelium

FIG. 8

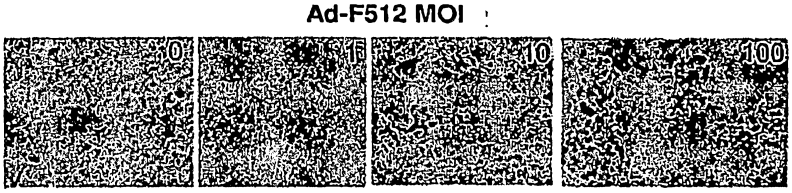

Ad-F512 MOI
0
1
10
100

FIG. 9

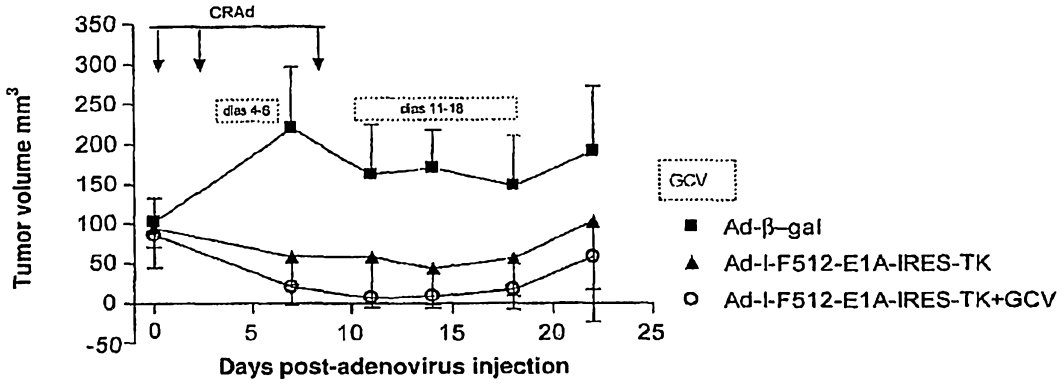


FIG. 10 A

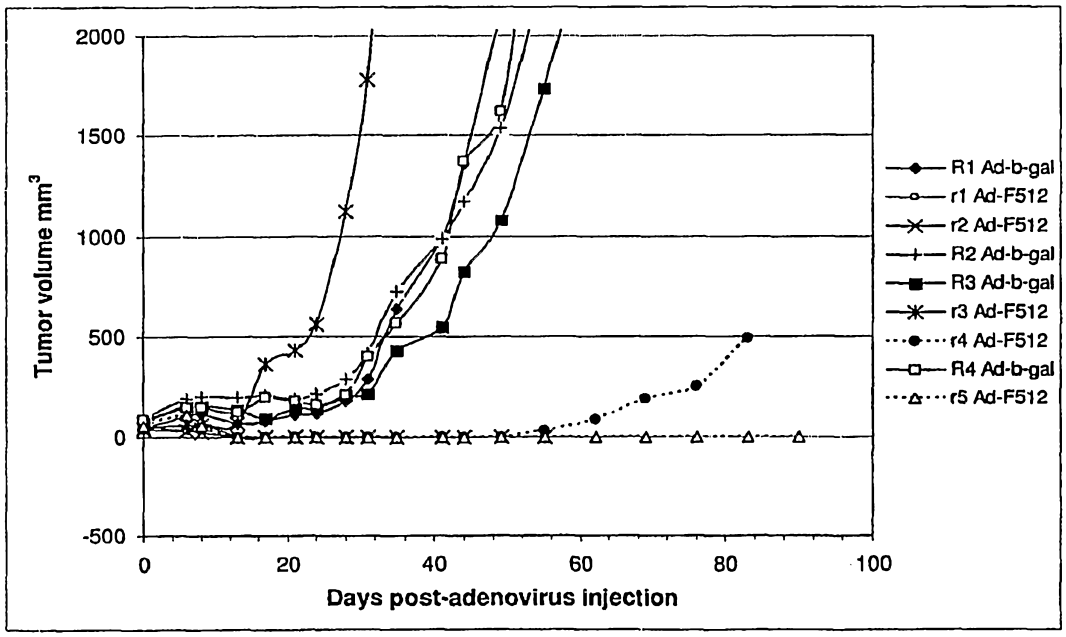


FIG. 10 B

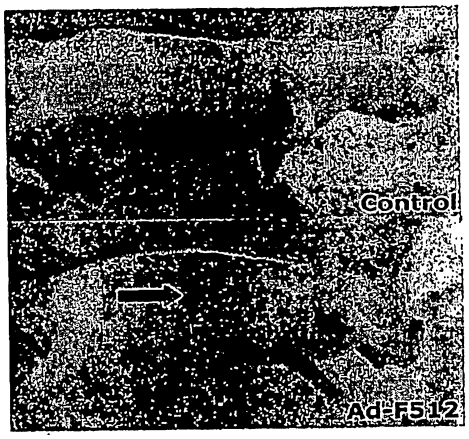


FIG. 10 C

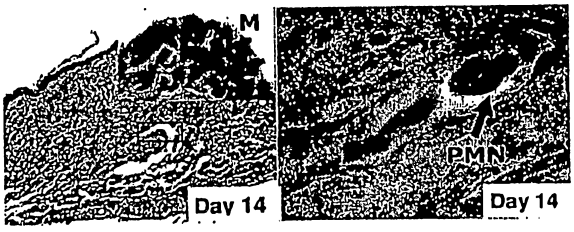


FIG. 10 D

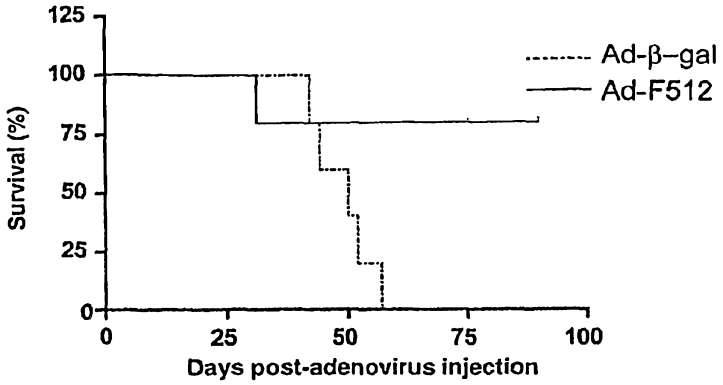


FIG. 10 E

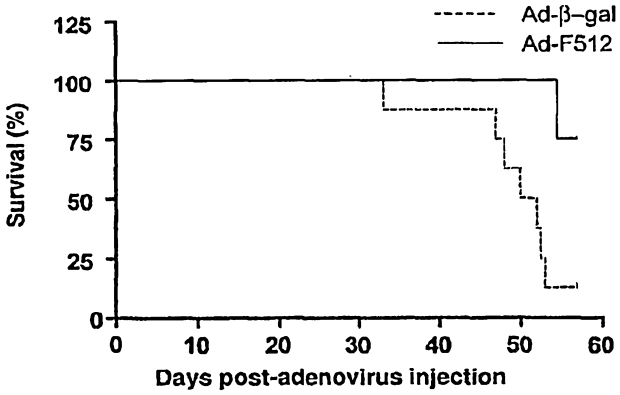


FIG. 10 F

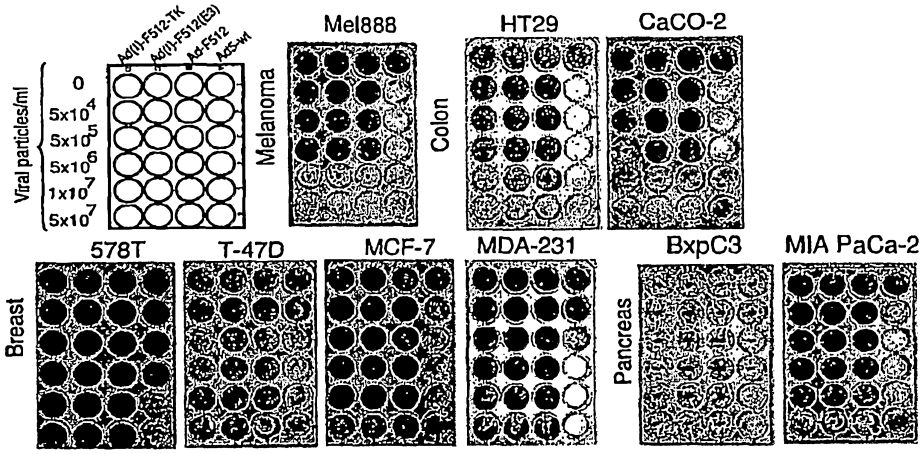

Ad(l)-F512-TK
Ad(l)-F512(E3)
Ad-F512
Ad5-wt
Viral particles/ml
0
5x10^4
5x10^5
5x10^6
1x10^7
5x10^7
Melanoma
Mel888
Colon
HT29
CaCO-2
Breast
578T
T-47D
MCF-7
MDA-231
Pancreas
BxpC3
MIA PaCa-2

FIG. 11

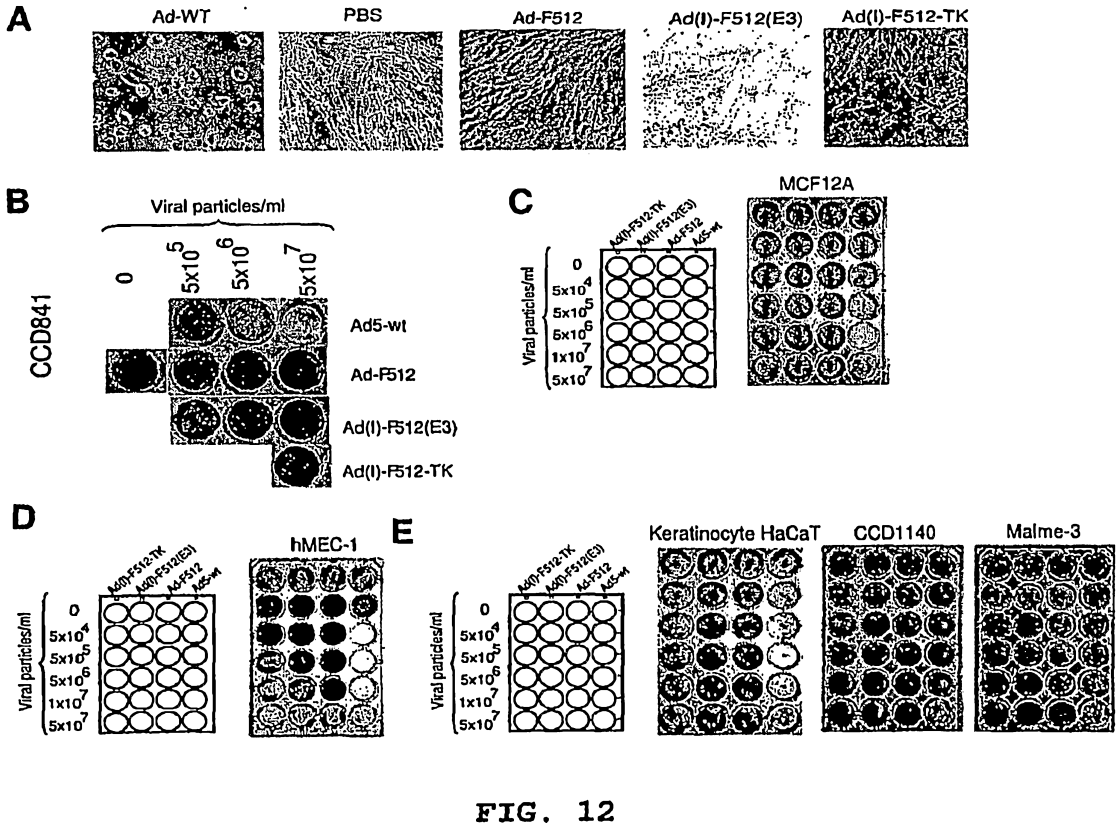

A
Ad-WT
PBS
Ad-F512
Ad(l)-F512(E3)
Ad(l)-F512-TK
B
Viral particles/ml
0
5x10^5
5x10^6
5x10^7
CCD841
Ad5-wt
Ad-F512
Ad(l)-F512(E3)
Ad(l)-F512-TK
C
MCF12A
D
hMEC-1
E
Keratinocyte HaCaT
CCD1140
Malme-3

FIG. 12

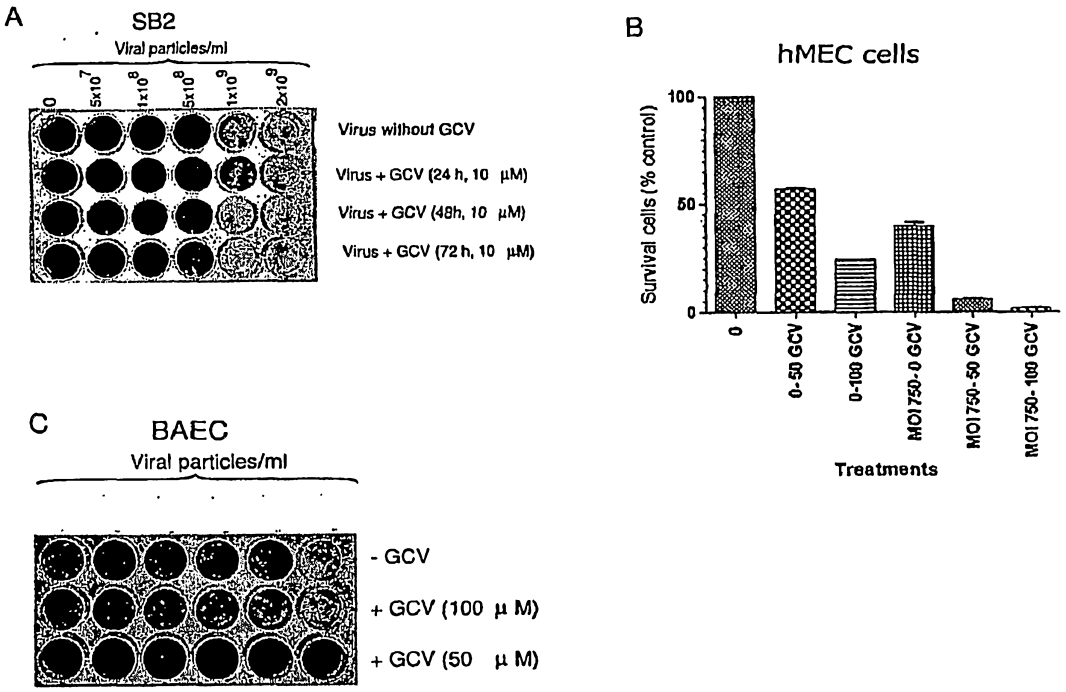

A
SB2
Viral particles/ml
0
5x10^7
1x10^8
5x10^8
1x10^9
2x10^9
Virus without GCV
Virus + GCV (24 h, 10 µM)
Virus + GCV (48h, 10 µM)
Virus + GCV (72 h, 10 µM)
B
hMEC cells
Survival cells (% control)
100
50
0
0
0-50 GCV
0-100 GCV
MOI 750-0 GCV
MOI 750-50 GCV
MOI 750-100 GCV
Treatments
C
BAEC
Viral particles/ml
- GCV
+ GCV (100 µ M)
+ GCV (50 µ M)

FIG. 13

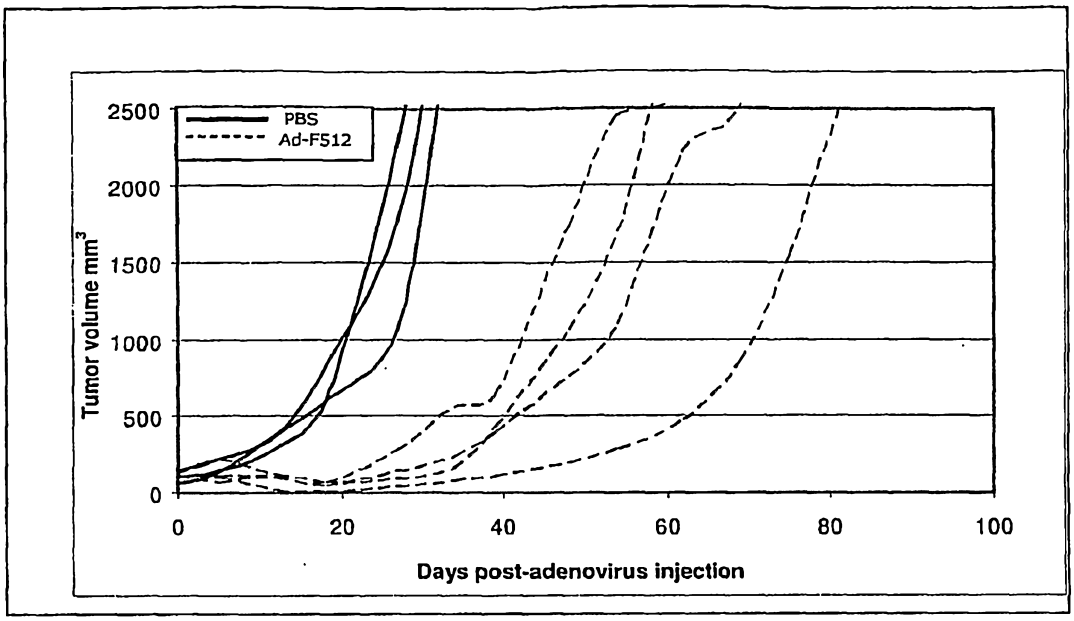


FIG. 14 A

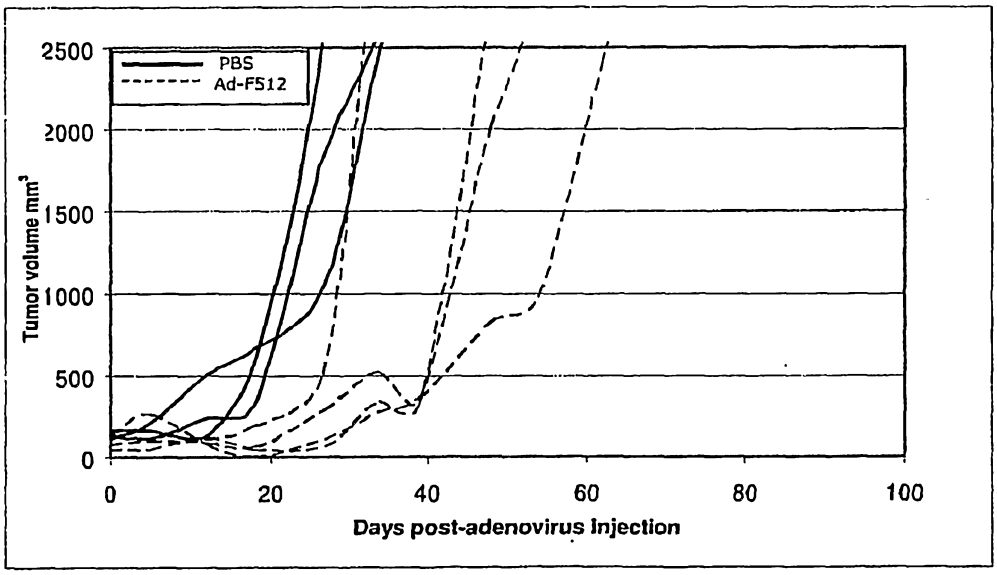


FIG. 14 B

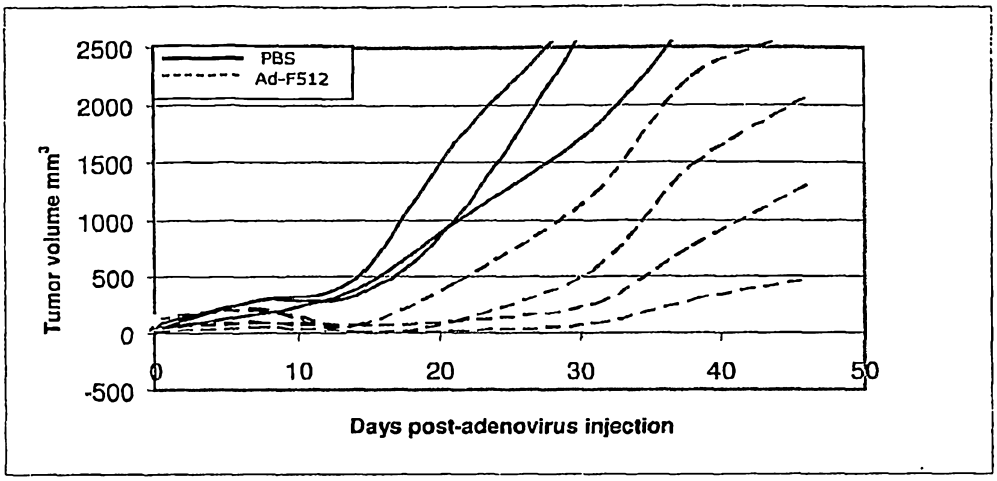


FIG. 14 C

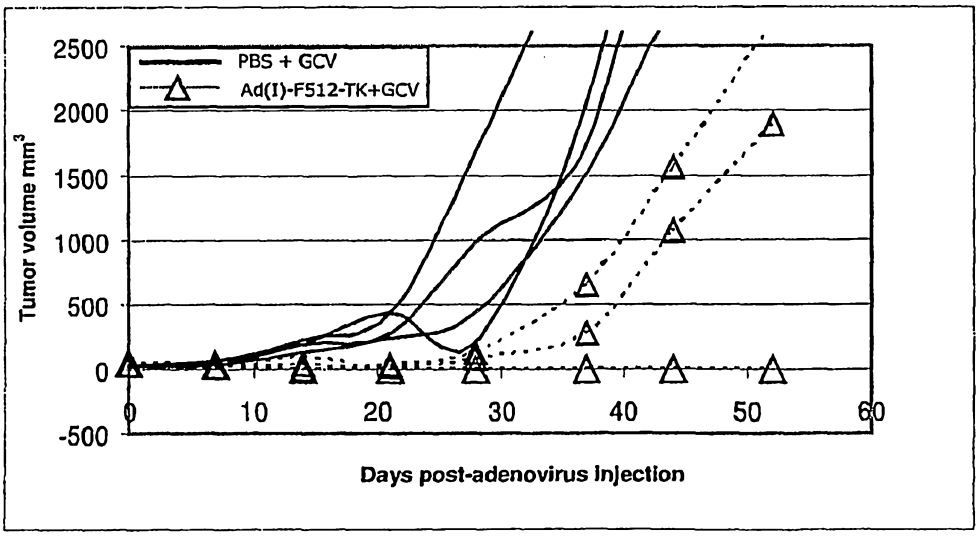


FIG. 14 D

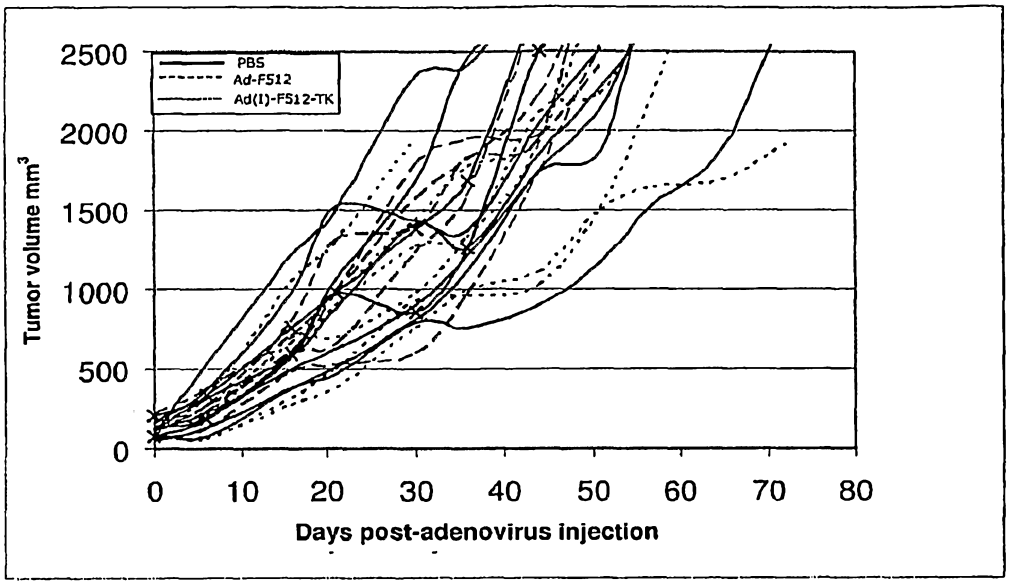


FIG. 15 A

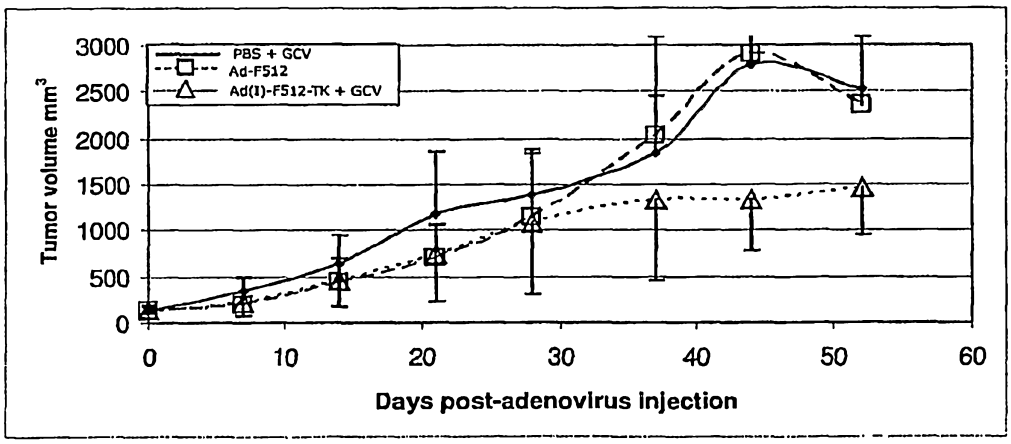


FIG. 15 B

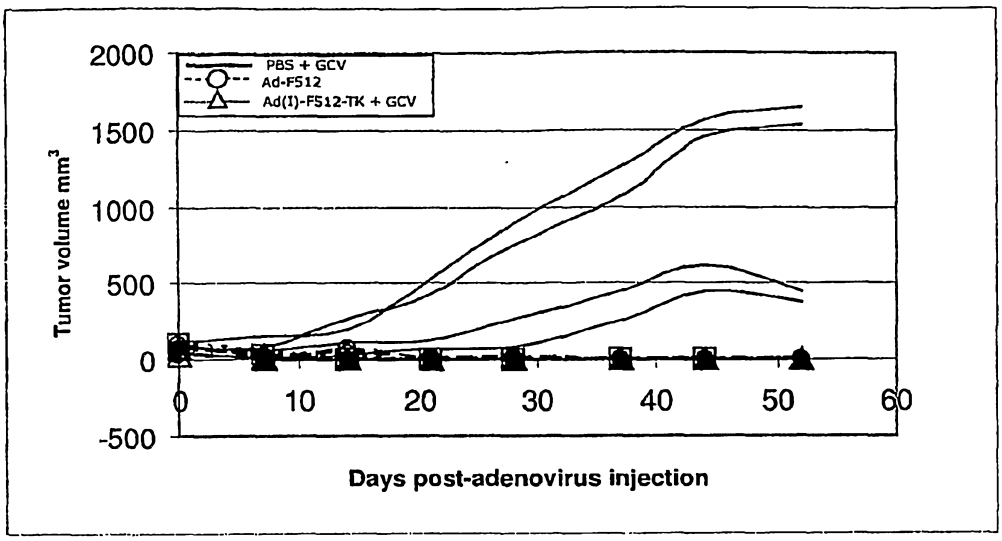

2000
1500
1000
500
0
-500
Tumor volume mm3
PBS + GCV
Ad-FS12
Ad(I)-FS12-TK + GCV
0
10
20
30
40
50
60
Days post-adenovirus injection

FIG. 15 C

SEQUENCE LISTING

<110> INIS Biotech LLC

<120> An Isolated DNA Fragment of the SPARC Human Promoter and Its Use for Driving the Expression of An Heterologous Gene in Tumor Cells

<130> SPARC

<160> 56

<170> PatentIn version 3.4

<210> 1
<211> 548
<212> DNA
<213> Artificial sequence

<220>
<223> Promoter sequence derived from the complete human promoter of the human SPARC protein

<400> 1
gcagcttgtc ttgtctgtac agtggtaagt cctggccttg cctttgtggc aaatacaacc 60

cccttgaatt gcttggccct tctcagcatt gcctaatatt agggaggact cctgtaaagc 120

tcactggtta gaagatcaag acacttgggc ctggttctgc ccctgggggc cattgggtaa 180

ttccttgcag tctccaggcc tcacttgccc tctgaacaag aaagaggctg ttctgggtca 240

tccctccagg cctgtccagc cctggcactc tgtgagtcgg tttaggcagc agccccggaa 300

cagatgaggc aggcagggtt gggacgtttg gtcaggacag cccaccgcaa aaagaggagg 360

aaagaaatga aagacagaga cagctttggc tatgggagaa ggaggaggcc gggggaagga 420

ggagacagga ggaggaggga ccacggggtg gaggggagat agacccagcc cagagctctg 480

agtggtttcc tgttgcctgt ctctaaaccc ctccacattc ccgcggtcct tcagactgcc 540

cggagagc 548

<210> 2
<211> 19
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 2
aaccgaagag gaggtggtg 19

<210> 3
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 3
gcaaagaagt ggcaggaaga 20

<210> 4
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 4
agaaaatctg gcaccacacc 20

<210> 5
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 5
cagaggcgta cagggatagc 20

<210> 6
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 6
ctagctagca gctgggtgtt gtggcat 27

<210> 7
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 7
acgcgtcgac ctcagtggca ggca 24

<210> 8
<211> 38
<212> DNA
<213> Artificial sequence

<220>

<223> Chemically synthesized primer

<400> 8
cggcctcctc cttctcccct gtctctgtct ttcatttc 38

<210> 9
<211> 26
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 9
ctagctagcg ggagaaggag gaggcc 26

<210> 10
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 10
gcagatctcc tcagtggcag gc 22

<210> 11
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 11
gcacgcgtag ctgggtgttg tgg 23

<210> 12
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 12
cgagatctgc tctccgggca g 21

<210> 13
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 13
cgagatctgg gcagtctgaa ggacc 25

<210> 14
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 14
cgacgcgtgc agcttgtctt gtc 23

<210> 15
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 15
gcagatctag tctgaaggac cgcg 24

<210> 16
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 16
gaacgcgtgg gagaaggagg ag 22

<210> 17
<211> 1246
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence of the human SPARC promoter corresponding to the bases -1175/+71 from the initiation of transcription

<400> 17
agctgggtgt tgtggcatgt gcgcctgtaa tcccagctac tctggaggct gaggcgcgat 60

aattgcttga acccgggagg cagaggttgc agtgagccga aatcatacca ctgcactcca 120

gcctgggcga cagagtgagt gagactctgt ctcaaaacaa aacaaaacaa acaaacaaaa 180

aaaccggaaa ccacaaaact ttttgaggac aaggaccagg tatttattaa ttctcatacc 240

tcccagagtg ttaggcacaa aataaacatt caaccaagac ctgttgcact gagcagttca 300

tatataacag gagtgaccca agttgaaacg tagaatcagc cctctcatac cacttttgc 360

caggtgatca taggcaagtt acttagcatc tatgtttcct tattattaaa atggtcataa 420

ttacaatgcc taagataagg ggttgctgtg aagattatta aatcctcagt aaactttggc 480

tattgttact cctatgatta tcatcaatat catcaattac cttatctgtt caatactggt 540

ggcacaggtc caccagctag atgtctaatc ccttatgtgt ctattagtgg tacaagtgga 600

gtttgagtgg gatttttttt tttaagacca gttccaaatc atcaaggatg ataccactag 660

tagcagcttg tcttgtctgt acagtggtaa gtcctggcct tgcctttgtg gcaaatacaa 720

ccccccttgaa ttgcttggcc cttctcagca ttgcctaata ttagggagga ctcctgtaaa 780

gctcactggt tagaagatca agacacttgg gcctggttct gcccctgggg gccattgggt 840

aattccttgc agtctccagg cctcacttgc cctctgaaca agaaagaggc tgttctgggt 900

catccctcca ggcctgtcca gccctggcac tctgtgagtc ggtttaggca gcagccccgg 960

aacagatgag gcaggcaggg ttgggacgtt tggtcaggac agcccaccgc aaaaagagga 1020

ggaaagaaat gaaagacaga gacagctttg gctatgggag aaggaggagg ccgggggaag 1080

gaggagacag gaggaggagg gaccacgggg tggaggggag atagacccag cccagagctc 1140

tgagtggttt cctgttgcct gtctctaaac ccctccacat tcccgcggtc cttcagactg 1200

cccggagagc gcgctctgcc tgccgcctgc ctgcctgcca ctgagg 1246

<210> 18
<211> 1236
<212> DNA
<213> Artificial sequence

<220>
<223> This promoter is a modification of the complete SPARC promoter -1175/+71, wherein a 10 pb sequence comprised between the two GGA boxes has been deleted

<400> 18
agctgggtgt tgtggcatgt gcgcctgtaa tcccagctac tctggaggct gaggcgcgat 60

aattgcttga acccgggagg cagaggttgc agtgagccga aatcatacca ctgcactcca 120

gcctgggcga cagagtgagt gagactctgt ctcaaaacaa aacaaaacaa acaaacaaaa 180

aaaccggaaa ccacaaaact ttttgaggac aaggaccagg tatttattaa ttctcatacc 240

tcccagagtg ttaggcacaa aataaacatt caaccaagac ctgttgcact gagcagttca 300

tatataacag gagtgaccca agttgaaacg tagaatcagc cctctcatac cacttttgc 360

caggtgatca taggcaagtt acttagcatc tatgtttcct tattattaaa atggtcataa 420

ttacaatgcc taagataagg ggttgctgtg aagattatta aatcctcagt aaatgttact 480

cctatgatta tcatcaatat catcaattac cttatctgtt caatactggt ggcacaggtc 540

caccagctag atgtctaatc ccttatgtgt ctattagtgg tacaagtgga gtttgagtgg 600

gatttttttt tttaagacca gttccaaatc atcaaggatg ataccactag tagcagcttg 660

tcttgtctgt acagtggtaa gtcctggcct tgcctttgtg gcaaatacaa ccccccttgaa 720

ttgcttggcc cttctcagca ttgcctaata ttagggagga ctcctgtaaa gctcactggt 780

tagaagatca agacacttgg gcctggttct gcccctgggg gccattgggt aattccttgc 840

agtctccagg cctcacttgc cctctgaaca agaaagaggc tgttctgggt catccctcca 900

ggcctgtcca gccctggcac tctgtgagtc ggtttaggca gcagccccgg aacagatgag 960

gcaggcaggg ttgggacgtt tggtcaggac agcccaccgc aaaaagagga ggaaagaaat 1020

gaaagacaga gacagctttg gctatgggag aaggaggagg ccgggggaag gaggagacag 1080

gaggaggagg gaccacgggg tggaggggag atagacccag cccagagctc tgagtggttt 1140

cctgttgcct gtctctaaac ccctccacat tcccgcggtc cttcagactg cccggagagc 1200

gcgctctgcc tgccgcctgc ctgcctgcca ctgagg 1236

<210> 19
<211> 1210
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 19
agctgggtgt tgtggcatgt gcgcctgtaa tcccagctac tctggaggct gaggcgcgat 60

aattgcttga acccgggagg cagaggttgc agtgagccga aatcatacca ctgcactcca 120

gcctgggcga cagagtgagt gagactctgt ctcaaaacaa aacaaaacaa acaaacaaaa 180

aaaccggaaa ccacaaaact ttttgaggac aaggaccagg tatttattaa ttctcatacc 240

tcccagagtg ttaggcacaa aataaacatt caaccaagac ctgttgcact gagcagttca 300

tatataacag gagtgaccca agttgaaacg tagaatcagc cctctcatac cacttttgc 360

caggtgatca taggcaagtt acttagcatc tatgtttcct tattattaaa atggtcataa 420

ttacaatgcc taagataagg ggttgctgtg aagattatta aatcctcagt aaactttggc 480

tattgttact cctatgatta tcatcaatat catcaattac cttatctgtt caatactggt 540

ggcacaggtc caccagctag atgtctaatc ccttatgtgt ctattagtgg tacaagtgga 600

gtttgagtgg gatttttttt tttaagacca gttccaaatc atcaaggatg ataccactag 660

tagcagcttg tcttgtctgt acagtggtaa gtcctggcct tgcctttgtg gcaaatacaa 720

ccccccttgaa ttgcttggcc cttctcagca ttgcctaata ttagggagga ctcctgtaaa 780

gctcactggt tagaagatca agacacttgg gcctggttct gcccctgggg gccattgggt 840

aattccttgc agtctccagg cctcacttgc cctctgaaca agaaagaggc tgttctgggt 900

catccctcca ggcctgtcca gccctggcac tctgtgagtc ggtttaggca gcagccccgg 960

aacagatgag gcaggcaggg ttgggacgtt tggtcaggac agcccaccgc aaaaagagga 1020

ggaaagaaat gaaagacaga gacagctttg gctatgggag aaggaggagg ccgggggaag 1080

gaggagacag gaggaggagg gaccacgggg tggaggggag atagacccag cccagagctc 1140

tgagtggttt cctgttgcct gtctctaaac ccctccacat tcccgcggtc cttcagactg 1200

cccggagagc 1210

<210> 20
<211> 1203
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 20
agctgggtgt tgtggcatgt gcgcctgtaa tcccagctac tctggaggct gaggcgcgat 60

aattgcttga acccgggagg cagaggttgc agtgagccga aatcatacca ctgcactcca 120

gcctgggcga cagagtgagt gagactctgt ctcaaaacaa aacaaaacaa acaaacaaaa 180

aaaccggaaa ccacaaaact ttttgaggac aaggaccagg tatttattaa ttctcatacc 240

tcccagagtg ttaggcacaa aataaacatt caaccaagac ctgttgcact gagcagttca 300

tatataacag gagtgaccca agttgaaacg tagaatcagc cctctcatac cacttttgc 360

caggtgatca taggcaagtt acttagcatc tatgtttcct tattattaaa atggtcataa 420

ttacaatgcc taagataagg ggttgctgtg aagattatta aatcctcagt aaactttggc 480

tattgttact cctatgatta tcatcaatat catcaattac cttatctgtt caatactggt 540

ggcacaggtc caccagctag atgtctaatc ccttatgtgt ctattagtgg tacaagtgga 600

gtttgagtgg gatttttttt tttaagacca gttccaaatc atcaaggatg ataccactag 660

tagcagcttg tcttgtctgt acagtggtaa gtcctggcct tgcctttgtg gcaaatacaa 720

ccccccttgaa ttgcttggcc cttctcagca ttgcctaata ttagggagga ctcctgtaaa 780

gctcactggt tagaagatca agacacttgg gcctggttct gcccctgggg gccattgggt 840

aattccttgc agtctccagg cctcacttgc cctctgaaca agaaagaggc tgttctgggt 900

catccctcca ggcctgtcca gccctggcac tctgtgagtc ggtttaggca gcagccccgg 960

aacagatgag gcaggcaggg ttgggacgtt tggtcaggac agcccaccgc aaaaagagga 1020

ggaaagaaat gaaagacaga gacagctttg gctatgggag aaggaggagg ccgggggaag 1080

gaggagacag gaggaggagg gaccacgggg tggaggggag atagacccag cccagagctc 1140

tgagtggttt cctgttgcct gtctctaaac ccctccacat tcccgcggtc cttcagactg 1200

ccc 1203

<210> 21
<211> 584
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 21
gcagcttgtc ttgtctgtac agtggtaagt cctggccttg cctttgtggc aaatacaacc 60

cccttgaatt gcttggccct tctcagcatt gcctaatatt agggaggact cctgtaaagc 120

tcactggtta gaagatcaag acacttgggc ctggttctgc ccctgggggc cattgggtaa 180

ttccttgcag tctccaggcc tcacttgccc tctgaacaag aaagaggctg ttctgggtca 240

tccctccagg cctgtccagc cctggcactc tgtgagtcgg tttaggcagc agccccggaa 300

cagatgaggc aggcagggtt gggacgtttg gtcaggacag cccaccgcaa aaagaggagg 360

aaagaaatga aagacagaga cagctttggc tatgggagaa ggaggaggcc gggggaagga 420

ggagacagga ggaggaggga ccacggggtg gaggggagat agacccagcc cagagctctg 480

agtggtttcc tgttgcctgt ctctaaaccc ctccacattc ccgcggtcct tcagactgcc 540

cggagagcgc gctctgcctg ccgcctgcct gcctgccact gagg 584

<210> 22
<211> 541
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 22
gcagcttgtc ttgtctgtac agtggtaagt cctggccttg cctttgtggc aaatacaacc 60

cccttgaatt gcttggccct tctcagcatt gcctaatatt agggaggact cctgtaaagc 120

tcactggtta gaagatcaag acacttgggc ctggttctgc ccctgggggc cattgggtaa 180

ttccttgcag tctccaggcc tcacttgccc tctgaacaag aaagaggctg ttctgggtca 240

tccctccagg cctgtccagc cctggcactc tgtgagtcgg tttaggcagc agccccggaa 300

cagatgaggc aggcagggtt gggacgtttg gtcaggacag cccaccgcaa aaagaggagg 360

aaagaaatga aagacagaga cagctttggc tatgggagaa ggaggaggcc gggggaagga 420

ggagacagga ggaggaggga ccacggggtg gaggggagat agacccagcc cagagctctg 480

agtggtttcc tgttgcctgt ctctaaaccc ctccacattc ccgcggtcct tcagactgcc 540

c 541

<210> 23
<211> 537
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 23
gcagcttgtc ttgtctgtac agtggtaagt cctggccttg cctttgtggc aaatacaacc 60

cccttgaatt gcttggccct tctcagcatt gcctaatatt agggaggact cctgtaaagc 120

tcactggtta gaagatcaag acacttgggc ctggttctgc ccctgggggc cattgggtaa 180

ttccttgcag tctccaggcc tcacttgccc tctgaacaag aaagaggctg ttctgggtca 240

tccctccagg cctgtccagc cctggcactc tgtgagtcgg tttaggcagc agccccggaa 300

cagatgaggc aggcagggtt gggacgtttg gtcaggacag cccaccgcaa aaagaggagg 360

aaagaaatga aagacagaga cagctttggc tatgggagaa ggaggaggcc gggggaagga 420

ggagacagga ggaggaggga ccacggggtg gaggggagat agacccagcc cagagctctg 480

agtggtttcc tgttgcctgt ctctaaaccc ctccacattc ccgcggtcct tcagact 537

<210> 24
<211> 191
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 24
gggagaagga ggaggccggg ggaaggagga gacaggagga ggagggacca cggggtggag 60

gggagataga cccagcccag agctctgagt ggtttcctgt tgcctgtctc taaacccctc 120

cacattcccg cggtccttca gactgcccgg agagcgcgct ctgcctgccg cctgcctgcc 180

tgccactgag g 191

<210> 25
<211> 155
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 25
gggagaagga ggaggccggg ggaaggagga gacaggagga ggagggacca cggggtggag 60

gggagataga cccagcccag agctctgagt ggtttcctgt tgcctgtctc taaacccctc 120

cacattcccg cggtccttca gactgcccgg agagc 155

<210> 26
<211> 148
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 26
gggagaagga ggaggccggg ggaaggagga gacaggagga ggagggacca cggggtggag 60

gggagataga cccagcccag agctctgagt ggtttcctgt tgcctgtctc taaacccctc 120

cacattcccg cggtccttca gactgccc 148

<210> 27
<211> 17
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 27
tacgactcac tataggg 17

<210> 28
<211> 18
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 28
atttaggtga cactatag 18

<210> 29
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 29

attaaccctc actaaaggga 20

<210> 30
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 30
ctttatgttt ttggcgtctt cca 23

<210> 31
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 31
ctagcaaaat aggctgtccc c 21

<210> 32
<211> 93
<212> DNA
<213> Artificial sequence

<220>
<223> Multiple cloning site, included in the pADPSY vector, chemically synthesized

<400> 32
ggtaccggag gcctcgctag ccacgcgtgg cggcagatct tgcagatatc cgcggatcga 60

tccgccagta cgtactttcc ttaggcagtc gac 93

<210> 33
<211> 7303
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 33
ttctcatgtt tgacagctta tcatcatcaa taatatacct tattttggat tgaagccaat 60

atgataatga gggggtggag tttgtgacgt ggcgcggggc gtgggaacgg ggcgggtgac 120

gtagtagtgt ggcggaagtg tgatgttgca agtgtggcgg aacacatgta agcgacggat 180

gtggcaaaag tgacgttttt ggtgtgcgcc ggtgtacaca ggaagtgaca attttcgcgc 240

ggttttaggc ggatgttgta gtaaatttgg gcgtaaccga gtaagatttg gccattttcg 300

cgggaaaact gaataagagg aagtgaaatc tgaataattt tgtgttactc atagcgcgta 360

atatttgtct agggccgcgg ggactttgac cgtttacgtg gagactcgcc caggtgtttt 420

tctcaggtgt tttccgcgtt ccgggtcaaa gttggcgttt tattattata gtcaggggga 480

tcctctagaa ctagtgctag agctcgctga tcagcctcga ctgtgccttc tagttgccag 540

ccatctgttg tttgcccctc ccccgtgcct tccttgaccc tggaaggtgc cactcccact 600

gtcctttcct aataaaatga ggaaattgca tcgcattgtc tgagtaggtg tcattctatt 660

ctgggggggtg gggtggggca ggacagcaag ggggaggatt gggaagacaa tagcaggcat 720

gctggggatg gtaccggagg cctcgctagc cacgcgtggc ggcagatctc cgggactgaa 780

aatgagacat attatctgcc acggaggtgt tattaccgaa gaaatggccg ccagtctttt 840

ggaccagctg atcgaagagg tactggctga taatcttcca cctcctagcc attttgaacc 900

acctaccctt cacgaactgt atgatttaga cgtgacggcc cccgaagatc ccaacgagga 960

ggcggtttcg cagatttttc ccgactctgt aatgttggcg gtgcaggaag ggattgactt 1020

actcactttt ccgccggcgc ccggttctcc ggagccgcct caccttcccc ggcagcccga 1080

gcagccggag cagagagcct tgggtccggt ttctatgcca aaccttgtac cggaggtgat 1140

cgatcttacc tgccacgagg ctggctttcc acccagtgac gacgaggatg aagagggtga 1200

ggagtttgtg ttagattatg tggagcaccc cgggcacggt tgcaggtctt gtcattatca 1260

ccggaggaat acgggggacc cagatattat gtgttcgctt tgctatatga ggacctgtgg 1320

catgtttgtc tacagtaagt gaaaattatg ggcagtgggt gatagagtgg tgggtttggt 1380

gtggtaattt ttttttaat ttttacagtt ttgtggttta aagaattttg tattgtgatt 1440

tttttaaaag gtcctgtgtc tgaacctgag cctgagcccg agccagaacc ggagcctgca 1500

agacctaccc gccgtcctaa aatggcgcct gctatcctga gacgcccgac atcacctgtg 1560

tctagagaat gcaatagtag tacggatagc tgtgactccg gtccttctaa cacacctcct 1620

gagatacacc cggtggtccc gctgtgcccc attaaaccag ttgccgtgag agttggtggg 1680

cgtcgccagg ctgtggaatg tatcgaggac ttgcttaacg agcctgggca acctttggac 1740

ttgagctgta aacgccccag gccataaggt gtaaacctgt gattgcgtgt gtggttaacg 1800

cctttgtttg ctgaatgagt tgatgtaagt ttaataaagg gtgagataat gtttggatct 1860

tgcagatatc cgcggatcga tccgccagta cgtactttcc ttaggcagtc gacctcgaga 1920

tccagacatg ataagatata ttgatgagtt tggacaaacc acaactagaa tgcagtgaaa 1980

aaaatgcttt atttgtgaaa tttgtgatgc tattgcttta tttgtaacca ttataagctg 2040

caataaacaa gttggggatc tcgaggggg gcccatcgaa ttcctgcagc ccgggggatc 2100

tggaaggtgc tgaggtacga tgagacccgc accaggtgca gaccctgcga gtgtggcggt 2160

aaacatatta ggaaccagcc tgtgatgctg gatgtgaccg aggagctgag gcccgatcac 2220

ttggtgctgg cctgcacccg cgctgagttt ggctctagcg atgaagatac agattgaggt 2280

actgaaatgt gtgggcgtgg cttaagggtg ggaaagaata tataaggtgg gggtcttatg 2340

tagttttgta tctgttttgc agcagccgcc gccgccatga gcaccaactc gtttgatgga 2400

agcattgtga gctcatattt gacaacgcgc atgccccat gggccggggt gcgtcagaat 2460

gtgatgggct ccagcattga tggtcgcccc gtcctgcccg caaactctac taccttgacc 2520

tacgagaccg tgtctggaac gccgttggag actgcagcct ccgccgccgc ttcagccgct 2580

gcagccaccg cccgcgggat tgtgactgac tttgctttcc tgagcccgct tgcaagcagt 2640

gcagcttccc gttcatccgc ccgcgatgac aagttgacgg ctcttttggc acaattggat 2700

tctttgaccc gggaacttaa tgtcgtttct cagcagctgt tggatctgcg ccagcaggtt 2760

tctgccctga aggcttcctc ccctcccaat gcggtttaaa acataaataa aaaaccagac 2820

tctgtttgga tttggatcaa gcaagtgtct tgctgtcttt atttaggggt tttgcgcgcg 2880

cggtaggccc gggaccagcg gtctcggtcg ttgagggtcc tgtgtatttt ttccaggacg 2940

tggtaaaggt gactctggat gttcagatac atgggcataa gcccgtctct ggggtggagg 3000

tagcaccact gcagagcttc atgctgcggg gtggtgttgt agatgatcca gtcgtagcag 3060

gagcgctggg cgtggtgcct aaaaatgtct ttcagtagca agctgattgc caggggcagg 3120

cccttggtgt aagtgtttac aaagcggtta agctgggatg ggtgcatacg tggggatatg 3180

agatgcatct tggactgtat ttttaggttg gctatgttcc cagccatatc cctccgggga 3240

ttcatgttgt gcagaaccac cagcacagtg tatccggtgc acttgggaaa tttgtcatgt 3300

agcttagaag gaaatgcgtg gaagaacttg gagacgccct tgtgacctcc aagattttcc 3360

atgcattcgt ccataatgat ggcaatgggc ccacgggcgg cggcctgggc gaagatattt 3420

ctgggatcac taacgtcata gttgtgttcc aggatgagat cgtcataggc catttttaca 3480

aagcgcgggc ggagggtgcc agactgcggt ataatggttc catccggccc aggggcgtag 3540

ttaccctcac agatttgcat ttcccacgct ttgagttcag atgggggat catgtctacc 3600

tgcggggcga tgaagaaaac ggtttccggg gtaggggaga tcagctggga agaaagcagg 3660

ttcctgagca gctgcgactt accgcagccg gtgggcccgt aaatcacacc tattaccggg 3720

tgcaactggt agttaagaga gctgcagctg ccgtcatccc tgagcagggg ggccacttcg 3780

ttaagcatgt ccctgactcg catgttttcc ctgaccaaat ccgccagaag gcgctcgccg 3840

cccagcgata gcagttcttg caaggaagca aagtttttca acggtttgag accgtccgcc 3900

gtaggcatgc ttttgagcgt ttgaccaagc agttccaggc ggtcccacag ctcggtcacc 3960

tgctctacgg catctcgatc cagcatatct cctcgtttcg cgggttgggg cggctttcgc 4020

tgtacggcag tagtcggtgc tcgtccagac gggccagggt catgtctttc cacgggcgca 4080

gggtcctcgt cagcgtagtc tgggtcacgg tgaaggggtg cgctccgggc tgcgcgctgg 4140

ccagggtgcg cttgaggctg gtcctgctgg tgctgaagcg ctgccggtct tcgccctgcg 4200

cgtcggccag gtagcatttg accatggtgt catagtccag cccctccgcg gcgtggccct 4260

tggcgcgcag cttgcccttg gaggaggcgc cgcacgaggg gcagtgcaga cttttgaggg 4320

cgtagagctt gggcgcgaga aataccgatt ccggggagta ggcatccgcg ccgcaggccc 4380

cgcagacggt ctcgcattcc acgagccagg tgagctctgg ccgttcgggg tcaaaaacca 4440

ggtttccccc atgcttttg atgcgtttct tacctctggt ttccatgagc cggtgtccac 4500

gctcggtgac gaaaaggctg tccgtgtccc cgtatacaga cttgagaggc ctgtcctcga 4560

gcggtgttcc gcggtcctcc tcgtatagaa actcggacca ctctgagaca aaggctcgcg 4620

tccaggccag cacgaaggag gctaagtggg aggggtagcg gtcgttgtcc actaggggt 4680

ccactcgctc cagggtgtga agacacatgt cgccctcttc ggcatcaagg aaggtgattg 4740

gtttgtaggt gtaggccacg tgaccgggtg ttcctgaagg ggggctataa aaggggtgg 4800

gggcgcgttc gtcctcactc tcttccgcat cgctgtctgc gagggccagc tgttggggtg 4860

agtactccct ctgaaaagcg ggcatgactt ctgcgctaag attgtcagtt tccaaaaacg 4920

aggaggattt gatattcacc tggcccgcgg tgatgccttt gagggtggcc gcatccatct 4980

ggtcagaaaa gacaatcttt ttgttgtcaa gcttggtggc aaacgacccg tagagggcgt 5040

tggacagcaa cttggcgatg gagcgcaggg tttggttttt gtcgcgatcg gcgcgctcct 5100

tggccgcgat gtttagctgc acgtattcgc gcgcaacgca ccgccattcg ggaaagacgg 5160

tggtgcgctc gtcgggcacc aggtgcacgc gccaaccgcg gttgtgcagg gtgacaaggt 5220

caacgctggt ggctacctct ccgcgtaggc gctcgttggt ccagcagagg ccggccgacg 5280

cgctgggcta cgtcttgctg gcgttcgcga cgcgaggctg gatggccttc cccattatga 5340

ttcttctcgc ttccggcggc atcgggatgc ccgcgttgca ggccatgctg tccaggcagg 5400

tagatgacga ccatcaggga cagcaaaggc cagcaaaagg ccaggaaccg taaaaaggcc 5460

gcgttgctgg cgtttttcca taggctccgc cccctgacg agcatcacaa aaatcgacgc 5520

tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga 5580

agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt 5640

ctcccttcgg gaagcgtggc gctttctcat agctcacgct gtaggtatct cagttcggtg 5700

taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc 5760

gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg 5820

gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc 5880

ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg 5940

ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc 6000

gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct 6060

caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 6120

taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa 6180

aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa 6240

tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc 6300

tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct 6360

gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca 6420

gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt 6480

aattgttgcc gggaagctag agtaagtagt tcgccagtta atagtttgcg caacgttgtt 6540

gccattgctg caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc attcagctcc 6600

ggttcccaac gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa agcggttagc 6660

tccttcggtc ctccgatcgt tgtcagaagt aagttggccg cagtgttatc actcatggtt 6720

atggcagcac tgcataattc tcttactgtc atgccatccg taagatgctt ttctgtgact 6780

ggtgagtact caaccaagtc attctgagaa tagtgtatgc ggcgaccgag ttgctcttgc 6840

ccggcgtcaa cacgggataa taccgcgcca catagcagaa ctttaaaagt gctcatcatt 6900

gggaaaacgt tcttcggggc gaaaactctc aaggatctta ccgctgttga gatccagttc 6960

gatgtaaccc actcgtgcac ccaactgatc ttcagcatct tttactttca ccagcgtttc 7020

tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg cgacacggaa 7080

atgttgaata ctcatactct tccttttttca atattattga agcatttatc agggttattg 7140

tctcatgagc ggatacatat ttgaatgtat ttagaaaaat aaacaaatag gggttccgcg 7200

cacatttccc cgaaaagtgc cacctgacgt ctaagaaacc attattatca tgacattaac 7260

ctataaaaat aggcgtatca cgaggccctt tcgtcttcaa gaa 7303

<210> 34
<211> 28
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 34

ccactagtgc tagagctcgc tgatcagc 28

<210> 35
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 35
cggtaccatc cccagcatgc ctgc 24

<210> 36
<211> 1020
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with T3 primer

<220>
<221> misc_feature
<222> (1009)..(1009)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (1013)..(1013)
<223> n is a, c, g, or t

<400> 36
gaatgccctt gatcactatc cggtgaattg aattagcggc cgcgaattcg cccttcgaga 60

tctccggact gaaaatgaga catattattt gccacggagg tgttattacc gaagaaatgg 120

ccgccagtct tttggaccag ctgatcgaag aggtactggc tgataatctt ccacctccta 180

gccattttga accacctacc cttcacgaac tgtatgattt agacgtgacg gcccccgaag 240

atcccaacga ggaggcggtt tcgcagattt ttcccgactc tgtaatgttg gcagtgcagg 300

aagggattga cttactcact tttccgccgg cgcccggttc tccggagccg cctcaccttt 360

cccggcagcc cgagcagccg gagcagagag ccttgggtcc ggtttctatg ccaaaccttg 420

taccggaggt gatcgatctt acctgccacg aggctggctt tccacccagt gacgacgagg 480

atgaagaggg tgaggagttt gtgttagatt atgtggagca ccccgggcac ggttgcaggt 540

cttgtcatta tcaccggagg aatacggggg acccagatat tatgtgttcg ctttgctata 600

tgaggacctg tggcatgttt gtctacagta agtgaaaatt atgggcagtg ggtgatagag 660

tggtgggttt ggtgtggtaa ttttttttt aatttttaca gttttgtggt ttaaagaatt 720

ttgtattgtg attttttttaa agggtcctgt gtctgaacct gagcctgagc ccgagccaga 780

accggagcct gcaagaccta cccgccgtcc taaaatggcg cctgctatcc tgagacgccc 840

gacatcacct gtgtctagag aatgcaatag tagtacggat agctgtgact ccggtccttc 900

taacacacct cctgagatac acccggtggt cccgctgtgc ccattaaacc agttgccgtg 960

agagttggtg ggcgtcgcca ggctgtggaa tgtatcgagg acttgcttna cgncctgggc 1020

<210> 37
<211> 1029
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with T7 primer

<220>
<221> misc_feature
<222> (15)..(15)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (32)..(32)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (57)..(57)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (75)..(75)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (87)..(87)
<223> n is a, c, g, or t

<400> 37
ccccatttta tacancatta cgccaagctc anaattaacc ctcactaaag ggactanctc 60

ctgcaggttt aaacnaattc gcccttncgg atccaaacat tatctcaccc tttattaaac 120

ttacatcaac tcattcagca aacaaaggcg ttaaccacac acgcaatcac aggtttacac 180

cttatggcct ggggcgttta cagctcaagt ccaaaggttg cccaggctcg ttaagcaagt 240

cctcgataca ttccacagcc tggcgacgcc caccaactct cacggcaact ggtttaatgg 300

ggcacagcgg gaccaccggg tgtatctcag gaggtgtgtt agaaggaccg gagtcacagc 360

tatccgtact actattgcat tctctagaca caggtgatgt cgggcgtctc aggatagcag 420

gcgccatttt aggacggcgg gtaggtcttg caggctccgg ttctggctcg ggctcaggct 480

caggttcaga cacaggaccc tttaaaaaaa tcacaataca aaattcttta aaccacaaaa 540

ctgtaaaaat taaaaaaaaa attaccacac caaacccacc actctatcac ccactgccca 600

taattttcac ttactgtaga caaacatgcc acaggtcctc atatagcaaa gcgaacacat 660

aatatctggg tcccccgtat tcctccggtg ataatgacaa gacctgcaac cgtgcccggg 720

gtgctccaca taatctaaca caaactcctc acccctcttca tcctcgtcgt cactgggtgg 780

aaagccagcc tcgtggcagg taagatcgat cacctccggt acaaggtttg gcatagaaac 840

cggacccaag gctctctgct ccggctgctc gggctgccgg gaaaggtgag gcggctccgg 900

agaaccgggc gccggcggaa aagtgagtaa gtcaatccct tcctgcactg ccaacattac 960

agagtcggga aaaatctgcg aaaccgcctc ctcgttggga tcttgggggc cgtcacgtct 1020

aatcataca 1029

<210> 38
<211> 1562
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 38
tctcaggtgt tttccgcgtt ccgggtcaaa gttggcgttt tattattata gtcaggggga 60

tcctctagaa ctagtgctag agctcgctga tcagcctcga ctgtgccttc tagttgccag 120

ccatctgttg tttgcccctc ccccgtgcct tccttgaccc tggaaggtgc cactcccact 180

gtcctttcct aataaaatga ggaaattgca tcgcattgtc tgagtaggtg tcattctatt 240

ctgggggggtg gggtggggca ggacagcaag ggggaggatt gggaagacaa tagcaggcat 300

gctggggatg gtaccggagg cctcgctagc cacgcgtggc ggcagatctc cgggactgaa 360

aatgagacat attatctgcc acggaggtgt tattaccgaa gaaatggccg ccagtctttt 420

ggaccagctg atcgaagagg tactggctga taatcttcca cctcctagcc attttgaacc 480

acctaccctt cacgaactgt atgatttaga cgtgacggcc cccgaagatc ccaacgagga 540

ggcggtttcg cagatttttc ccgactctgt aatgttggcg gtgcaggaag ggattgactt 600

actcactttt ccgccggcgc ccggttctcc ggagccgcct caccttcccc ggcagcccga 660

gcagccggag cagagagcct tgggtccggt ttctatgcca aaccttgtac cggaggtgat 720

cgatcttacc tgccacgagg ctggctttcc acccagtgac gacgaggatg aagagggtga 780

ggagtttgtg ttagattatg tggagcaccc cgggcacggt tgcaggtctt gtcattatca 840

ccggaggaat acgggggacc cagatattat gtgttcgctt tgctatatga ggacctgtgg 900

catgtttgtc tacagtaagt gaaaattatg ggcagtgggt gatagagtgg tgggtttggt 960

gtggtaattt ttttttaat ttttacagtt ttgtggttta aagaattttg tattgtgatt 1020

tttttaaaag gtcctgtgtc tgaacctgag cctgagcccg agccagaacc ggagcctgca 1080

agacctaccc gccgtcctaa aatggcgcct gctatcctga gacgcccgac atcacctgtg 1140

tctagagaat gcaatagtag tacggatagc tgtgactccg gtccttctaa cacacctcct 1200

gagatacacc cggtggtccc gctgtgcccc attaaaccag ttgccgtgag agttggtggg 1260

cgtcgccagg ctgtggaatg tatcgaggac ttgcttaacg agcctgggca acctttggac 1320

ttgagctgta aacgccccag gccataaggt gtaaacctgt gattgcgtgt gtggttaacg 1380

cctttgtttg ctgaatgagt tgatgtaagt ttaataaagg gtgagataat gtttggatct 1440

tgcagatatc cgcggatcga tccgccagta cgtactttcc ttaggcagtc gacctcgaga 1500

tccagacatg ataagatata ttgatgagtt tggacaaacc acaactagaa tgcagtgaaa 1560

aa 1562

<210> 39
<211> 1335
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<400> 39
tctcaggtgt tttccgcgtt ccgggtcaaa gttggcgttt tattattata gtcaggggga 60

tcctctagaa ctagtgtgat caggtaccgg aggcctcgct agccacgcgt ggcggcagat 120

ctccgggact gaaaatgaga catattatct gccacggagg tgttattacc gaagaaatgg 180

ccgccagtct tttggaccag ctgatcgaag aggtactggc tgataatctt ccacctccta 240

gccatttga accacctacc cttcacgaac tgtatgattt agacgtgacg gcccccgaag 300

atcccaacga ggaggcggtt tcgcagattt ttcccgactc tgtaatgttg gcggtgcagg 360

aagggattga cttactcact tttccgccgg cgcccggttc tccggagccg cctcaccttt 420

cccggcagcc cgagcagccg gagcagagag ccttgggtcc ggtttctatg ccaaaccttg 480

taccggaggt gatcgatctt acctgccacg aggctggctt tccacccagt gacgacgagg 540

atgaagaggg tgaggagttt gtgttagatt atgtggagca ccccgggcac ggttgcaggt 600

cttgtcatta tcaccggagg aatacggggg acccagatat tatgtgttcg ctttgctata 660

tgaggacctg tggcatgttt gtctacagta agtgaaaatt atgggcagtg ggtgatagag 720

tggtgggttt ggtgtggtaa ttttttttt aatttttaca gttttgtggt ttaaagaatt 780

ttgtattgtg attttttaa aaggtcctgt gtctgaacct gagcctgagc ccgagccaga 840

accggagcct gcaagaccta cccgccgtcc taaaatggcg cctgctatcc tgagacgccc 900

gacatcacct gtgtctagag aatgcaatag tagtacggat agctgtgact ccggtccttc 960

taacacacct cctgagatac acccggtggt cccgctgtgc cccattaaac cagttgccgt 1020

gagagttggt gggcgtcgcc aggctgtgga atgtatcgag gacttgctta acgagcctgg 1080

gcaacctttg gacttgagct gtaaacgccc caggccataa ggtgtaaacc tgtgattgcg 1140

tgtgtggtta acgcctttgt ttgctgaatg agttgatgta agtttaataa agggtgagat 1200

aatgtttgga tcttgcagat atccgcggat cgatccgcca gtacgtactt tccttaggca 1260

gtcgacctcg agatccagac atgataagat acattgatga gtttggacaa accacaacta 1320

gaatgcagtg aaaaa 1335

<210> 40
<211> 776
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized

<220>
<221> misc_feature
<222> (5)..(5)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (39)..(39)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (128)..(128)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (684)..(684)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (721)..(721)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (727)..(727)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (734)..(734)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (738)..(739)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (752)..(752)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (755)..(755)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (758)..(758)
<223> n is a, c, g, or t

<400> 40
ccctnggact gaagctggcg ttttcaatta tagtcaggng gatcctctag aactagtgct 60

agagctcgct gatcagcctc gactgtgcct tctagttgcc agccatctgt tgtttgcccc 120

tcccccgntg ccttccttga ccctggaagg tgccactccc actgtccttt cctaataaaa 180

tgaggaaatt gcatcgcatt gtctgggtag gtgtcattct attctggggg gtggggtggg 240

gcaggacagc aaggggggagg attgggaaga caatagcagg catgctgggg atggtaccgg 300

aggcctcgct agccacgcgt gcagcttgtc ttgtctgtac agtggtaagt cctggccttg 360

cctttgtggc aaatacaacc cccttgaatt gcttggccct tctcagcatt gcctaatatt 420

agggaggact cctgtaaagc tcactggtta gaagatcaag acacttgggc ctggttctgc 480

ccctgggggt cattgggtaa ttccttgcag tctccaggcc tcacttgccc tctgaacaag 540

aaagaggctg ttctgggtca tccctccagg cctgtccagc cctggcactc tgtgagtcgg 600

tttaggcagc agccccggaa cagatgaggc aggcagggtt gggacgtttg gtcaggacag 660

cccaccgcaa aaagaggagg aaanaaatga aagacagaga cagctttggc tatgggagaa 720

ngaggangcc gggngaannng atgacacagg anganganngg accaccgggt ggaggg 776

<210> 41
<211> 507
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense primer

<220>
<221> misc_feature
<222> (2)..(2)

<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (8)..(8)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (26)..(26)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (498)..(498)
<223> n is a, c, g, or t

<400> 41
cncccggntg aagctggcgt ttattnttat agtcaggggg atcctctaga actagtgatc 60

aggtaccgga ggcctcgcta gccacgcgtg cagcttggtc ttggtctgta cagtggtaag 120

tcctggcctt gcctttgtgg caaatacaac ccccttgaat tgcttggccc ttctcagcat 180

tgcctaatat tagggaggac tcctgtaaag ctcactggtt agaagatcaa gacacttggg 240

cctggttctg cccctggggg tcattgggta attccttgca gtctccaggc ctcacttgcc 300

ctctgaacaa gaaagaggct gttctgggtc atccctccag gcctgtccag ccctggcact 360

ctgtgagtcg gtttaggcag cagccccgga acagatgagg caggcagggt tgggacgttt 420

ggtcaggaca gcccaccgca aaaagaggag gaaagaaatg aaagacagag acagctttgg 480

ctatgggaga aggaggangg ccggggg 507

<210> 42
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 42
tgtttttctc aggtgttttc cg 22

<210> 43
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 43
gcccatggct tcgtaccccg gcc 23

<210> 44
<211> 29
<212> DNA
<213> Artificial sequence

<220>
<223> Chemically synthesized primer

<400> 44
gcgtcgactc agttagcctc ccccatctc 29

<210> 45
<211> 957
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with T3 primer

<400> 45
gcatctgcgg gggtttcggg gcgaagttac gattcgccct tgcgtcgact cagttagcct 60

cccccatctc ccgggcaaac gtgcgcgcca ggtcgcagat cgtcggtatg gagccggggg 120

tggtgacgtg ggtctggacc atcccggagg taagttgcag cagggcgtcc cggcagccgg 180

cgggcgattg gtcgtaatcc aggataaaga cgtgcatggg acggaggcgt ttggccaaga 240

cgtccaaggc ccaggcaaac acgttgtaca ggtcgccgtt gggggccagc aactcggggg 300

cccgaaacag ggtaaataac gtgtccccga tatggggtcg tgggcccgcg ttgctctggg 360

gctcggcacc ctggggcggc acggccgtcc ccgaaagctg tccccaatcc tcccgccacg 420

acccgccgcc ctgcagatac cgcaccgtat tggcaagcag cccgtaaacg cggcgaatcg 480

cggccagcat agccaggtca agccgctcgc cggggcgctg gcgtttggcc aggcggtcga 540

tgtgtctgtc ctccggaagg gcccccaaca cgatgtttgt gccgggcaag gtcggcggga 600

tgagggccac gaacgccagc acggcctggg gggtcatgct gcccataagg tatcgcgcgg 660

ccgggtagca caggagggcg gcgatgggat ggcggtcgaa gatgagggtg agggccgggg 720

gcggggcatg tgagctccca gcctcccccc cgatatgagg agccagaacg gcgtcggtca 780

cggcataaag gcatgcccat tgttatctgg gcgcttgtca ttaccaccgc cgcgtccccg 840

ggccgatatc tcaccctggt cgaggcggtg ttgtgtgggt gtagatgttc gcgattggtc 900

tcgcagcccc caagcaactg cagtaaagtc aatcgggcct cggggtaacc gtttagg 957

<210> 46
<211> 238
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense priner

<400> 46
ccggctttac gctggcgttt tattattata gtcaggggga tcctctagaa ctagtgctag 60

agctcgctga tcagcctcga ctgtgccttc tagttgccag ccatctgttg tttgcccctc 120

ccccgtgcct tccttgaccc tggaaggtgc cactcccact gtcctttcct aataaaatga 180

ggaaattgca tcgcattgtc tgggtaggtg tcattctatt ctgggggggtg gggtgggg 238

<210> 47
<211> 965
<212> DNA
<213> Artificial seqeunce

<220>
<223> Sequence obtained with pAd-antisense primer

<400> 47
acggtgttgt taggttctca ttctgttgtg gtttgtccaa actcatcaat gtatcttatc 60

atgtctggat ctcgaggtcg actcagttag cctcccccat ctcccgggca aacgtgcgcg 120

ccaggtcgca gatcgtcggt atggagccgg gggtggtgac gtgggtctgg accatcccgg 180

aggtaagttg cagcagggcg tcccggcagc cggcgggcga ttggtcgtaa tccaggataa 240

agacgtgcat gggacggagg cgtttggcca agacgtccaa ggcccaggca aacacgttgt 300

acaggtcgcc gttgggggcc agcaactcgg gggcccgaaa cagggtaaat aacgtgtccc 360

cgatatgggg tcgtgggccc gcgttgctct ggggctcggc accctggggc ggcacggccg 420

tccccgaaag ctgtccccaa tcctcccgcc acgacccgcc gccctgcaga taccgcaccg 480

tattggcaag cagcccgtaa acgcggcgaa tcgcggccag catagccagg tcaagccgct 540

cgccggggcg ctggcgtttg gccaggcggt cgatgtgtct gtcctccgga agggccccca 600

acacgatgtt tgtgccgggc aaggtcggcg ggatgagggc cacgaacgcc agcacggcct 660

gggggggtcat gctgcccata aggtatcgcg cggccgggta gcacaggagg gcggcgatgg 720

gatggcggtc gaagatggag ggtgagggcc gggggggcggg gcatgtgagc tcccagcctc 780

ccccccgata tgaggagcca gaacggcgtc ggtcacggca taaggcatgc ccattgttat 840

ctgggcgctt gtcattacca ccgcccgcgt ccccggccga tatctcaacc ctgatcgagg 900

cggatgtatg gtggtggtgt agaattgttt cgacgaattt gtctctcgcg aaagaggccc 960

cccca 965

<210> 48
<211> 25
<212> DNA
<213> Artificial sequence

<220>

<223> Chemically synthesized primer

<400> 48
cacaaatttc acaaataaag cattt 25

<210> 49
<211> 911
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense primer, partial sequence of the virus

<220>
<221> misc_feature
<222> (5)..(5)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (8)..(8)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (22)..(22)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (34)..(34)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (751)..(751)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (854)..(854)
<223> n is a, c, g, or t

<400> 49
catcngcnag cggcgctcta tnattatagt catnggatcc tctagaacta gtgctagagc 60

tcgctgatca gcctcgactg tgccttctag ttgccagcca tctgttgttt gcccctcccc 120

cgtgccttcc ttgaccctgg aaggtgccac tcccactgtc ctttcctaat aaaatgagga 180

aattgcatcg cattgtctgg gtaggtgtca ttctattctg gggggtgggg tggggcagga 240

cagcaagggg gaggattggg aagacaatag caggcatgct ggggatggta ccggaggcct 300

cgctagccac gcgtgcagct tgtcttgtct gtacagtggt aagtcctggc cttgcctttg 360

tggcaaatac aacccccttg aattgcttgg cccttctcag cattgcctaa tattagggag 420

gactcctgta aagctcactg gttagaagat caagacactt gggcctggtt ctgcccctgg 480

gggtcattgg gtaattcctt gcagtctcca ggcctcactt gccctctgaa caagaaagag 540

gctgttctgg gtcatccctc caggcctgtc cagccctggc actctgtgag tcggtttagg 600

cagcagcccc ggaacagatg aggcaggcag ggttgggacg tttggtcagg acagcccacc 660

gcaaaaagag gaggaaagaa atgaaagaca gagacagctt tggctatggg agaaggagga 720

ggccggggga aggaggagac aggaagagga nggaccacgg ggtggaaggg agatagaccc 780

agcccagagc tctgagtggt ttccctgttg cctgtctcta aacccctcca cattcccgcg 840

gtccttcaga ctgnccggag agcagatctc cggactgaaa atgagacata ttatttgcca 900

cggaaggtgt t 911

<210> 50
<211> 908
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-antisense primer, partial sequence of the virus

<220>
<221> misc_feature
<222> (11)..(11)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (18)..(18)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (30)..(31)
<223> n is a, c, g, or t

<400> 50
gcattctagt nctctaangg tccaaactcn ntcaatgtat cttatcatgt ctggatctcg 60

aggtcgacca ctgtgctggc ggccgcttta cttgtacagc ctcgtccatg ccgagagtga 120

tcccggcggc ggtcacgaac tccagcagga ccatgtggat cgcgcttctc gttggggtct 180

ttgctcaggg cggactgggt gctcaggtag tggttgtcgg gcagcagcac ggggccgtcg 240

ccgatggggg tgttctgctg gtagtggtcg gcgagctgca cgctgccgtc ctcgatgttg 300

tggcggatct tgaagttcac cttgatgccg ttcttctgct tgtcggccat gatatagacg 360

ttgtggctgt tgtagttgta ctccagcttg tgccccagga tgttgccgtc ctccttgaag 420

tcgatgccct tcagctcgat gcggttcacc agggtgtcgc cctcgaactt cacctcggcg 480

cgggtcttgt agttgccgtc gtccttgaag aagatggtgc gctcctggac gtagccttcg 540

ggcatggcgg acttgaagaa gtcgtgctgc ttcatgtggt cggggtagcg gctgaagcac 600

tgcacgccgt aggtcagggt ggtcacgagg gtgggccagg gcacgggcag cttgccggtg 660

gtgcagatga acttcagggt cagcttgccg taagtggcat cgccctcgcc ctcgccggac 720

acgctgaact tgtggccgtt tacgtcgccg tccagctcga ccaggatggg caccaccccg 780

gtgaacagct cctcgcccct gctcaccatg gttgtggcca tattatcatc gtgtttttca 840

aaggaaaacc acgtccccgt ggttcggggg gcctaagacg ttttttaac ctcgactaaa 900

acacatgt 908

<210> 51
<211> 842
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense primer, partial sequence of the virus

<220>
<221> misc_feature
<222> (4)..(5)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (14)..(15)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (19)..(19)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (24)..(25)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (37)..(39)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (61)..(61)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (159)..(159)

<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (795)..(795)
<223> n is a, c, g, or t

<400> 51
tccnngtaat cagnnagcng ctannaaggg tttgtcnnna gggatcctct agaactagtg 60

ntcaggtacc ggaggcctcg ctagccacgc gtgcagcttg tcttgtctgt acagtggtaa 120

gtcctggcct tgcctttgtg gcaaatacaa ccccccttgna attgcttggc ccttctcagc 180

attgcctaat attagggagg actcctgtaa agctcactgg ttagaagatc aaggacactt 240

gggcctggtt ctgcccctgg gggtcattgg gtaattcctt gcagtctcca ggcctcactt 300

gccctctgaa caagaaagag gctgttctgg gtcatccctc caggcctgtc cagccctggc 360

actctgtgga gtcggtttag gcagcagccc cgggaacaga tgaggcaggc agggttggga 420

cgtttggtca ggacagccca ccgcaaaaag aggaggaaag aaatgaaaga cagagacagc 480

tttggctatg ggagaaggag gaggccgggg gaaggaggag acaggaggag gagggaccac 540

ggggtggagg ggagatagac ccagcccaga gctctgagtg gtttcctgtt gcctgtctct 600

aaacccctcc acattcccgc ggtccttcag actgcccgga gagcagatct ccggactgaa 660

aatgagacat attatttgcc acggaggtgt tattaccgaa gaaatggccg ccagtctttt 720

ggaccagctg atcgaagagg tactggctga taatcttcca cctcctagcc attttgaacc 780

acctaccctt cacgnaactg tatgatttag acgtgacggg cccccgaaga tcccaacgag 840

ga 842

<210> 52
<211> 851
<212> DNA
<213> Artificial seqeunce

<220>
<223> Sequence obtained with pAd-antisense primer, partial sequence of the virus

<220>
<221> misc_feature
<222> (5)..(5)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (10)..(10)
<223> n is a, c, g, or t

<220>
<221> misc_feature

<222> (12)..(12)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (14)..(14)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (19)..(19)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (29)..(29)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (34)..(34)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (42)..(42)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (65)..(65)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (90)..(90)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (103)..(103)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (130)..(130)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (393)..(393)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (539)..(539)
<223> n is a, c, g, or t

<400> 52
aaaangacgn cngnaagcnc taaggggcna attncaaagg gnatcttatc atgtctggat 60

ctcgnaggct cggactgcct aaggtaaagn acgtactggc ggnatcgaat ccgcgggata 120

tctgctaagn atccaaacat tatctcaccc tttattaaac ttacatcaac tcattcagca 180

aacaaaggcg ttaaccacac acgcaatcac aggctttaca ccttatggcc tggggcgttt 240

acagctcaag tccaaaggtt gcccaggctc gttaagcaag tcctcgatac attccacagc 300

ctggcgacgc ccaccaactc tcacggcaac tggtttaatg gggcacagcg ggaccaccgg 360

gtgtatctca ggaggtgtgt tagaaggacc ggnagtcaca gctatccgta ctactattgc 420

attctctaga cacaggtgat gtcgggcgtc tcaggatagc aggcgccatt ttaggacggc 480

gggtaggtct tgcaggctcc ggttctggct cgggctcagg ctcaggttca gacacaggna 540

ccctttaaaa aaatcacaat acaaaattct ttaaaccaca aaactgtaaa aattaaaaaa 600

aaaattacca caccaaaccc accactctat cacccactgc ccataatttt cacttactgt 660

agacaaacat gccacaggtc ctcatatagc aaagcgaaca cataatatct gggtcccccg 720

tattcctccg ggtgataatg gacaagacct gcaaccgtgg cccggggtgc tccacataaa 780

tctaacacaa actcctcacc ctcttcatcc tcgtcgtcac tgggtggaaa gccagccctc 840

gtggcaggta a 851

<210> 53
<211> 854
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense primer, partial sequence of the virus

<400> 53
aaaaagaacc ttttaatgcg cgctcggtcg cgttttatta ttatagtcag ggggatcctc 60

tagaactagt gctagagctc gctgatcagc ctcgactgtg ccttctagtt gccagccatc 120

tgttgtttgc ccctcccccg tgccttcctt gaccctggaa ggtgccactc ccactgtcct 180

ttcctaataa aatgaggaaa ttgcatcgca ttgtctgggt aggtgtcatt ctattctggg 240

gggtggggtg gggcaggaca gcaaggggga ggattgggaa gacaatagca ggcatgctgg 300

ggatggtacc ggaggcctcg ctagccacgc gtgcagcttg tcttgtctgt acagtggtaa 360

gtcctggcct tgcctttgtg gcaaatacaa ccccccttgaa ttgcttggcc cttctcagca 420

ttgcctaata ttagggagga ctcctgtaaa gctcactggt tagaagatca agacacttgg 480

gcctggttct gcccctgggg gtcattgggt aattccttgc agtctccagg cctcacttgc 540

cctctgaaca agaaagaggc tgttctgggt catccctcca ggcctgtcca gccctggcac 600

tctgtgagtc ggtttaggca gcagccccgg aacagatgag gcaggcaggg ttgggacgtt 660

tggtcaggac agcccaccgc aaaaagagga ggaaagaaat gaaagacaga gacagctttg 720

gctatgggag aaggaggagg ccgggggaaa ggaggagaca ggaggaggag ggaccacggg 780

gtggaggggg agatagaccc agccagagct cctgagtgtt tcctgttggc ctgtctctaa 840

acccccctcc caca 854

<210> 54
<211> 840
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-antisense primer, partial sequence of the virus

<400> 54
ttcccatttt taaccttatg tccttgattg ttgattcact ctcatgttct atctgtctgg 60

tctcgaggcg cttaaaaaac tcccccatct cccgggcaaa cgtgcgcgcc cctgccgcaa 120

atcgtcggta tggagccggg ggtggtgacg tgggtctgga ccatcccgga ggtaagttgc 180

agcagggcgt cccggcagcc ggcgggcgat tggtcgtaat ccaggataaa gacgtgcatg 240

ggacggaggc gtttggccaa gacgtccaag gcccaggcaa acacgttgta caggtcgccg 300

ttgggggcca gcaactcggg ggcccgaaac agggtaaata acgtgtcccc gatatggggt 360

cgtgggcccg cgttgctctg gggctcggca ccctggggcg gcacggccgt ccccgaaagc 420

tgtccccaat cctcccgcca cgacccgccg ccctgcagat accgcaccgt attggcaagc 480

agcccgtaaa cgcggcgaat cgcggccagc atagccaggt caagccgctc gccggggcgc 540

tggcgtttgg ccaggcggtc gatgtgtctg tcctccggaa gggcccccaa cacgatgttt 600

gtgccgggca aggtcggcgg gatgagggcc acgaacgcca gcacggcctg gggggtcatg 660

ctgcccataa ggtatcgcgc ggccggggta gcacaggagg gcggcgatgg ggatggcggt 720

cgaagatgag ggtgagggcc gggggggcggg gcatgtgagc tcccagcctc ccccccgatt 780

tgaggagcca gaacggcgtc gggtcacggc taaggcatgc ccattggtaa tcggggccct 840

<210> 55
<211> 833
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-sense primer, partial sequence of the virus

<400> 55
cctggcatct ttttgaaagt cggatcttgt cgcgtttatt attatagtca gggggatcct 60

ctagaactag tgctagagct cgctgatcag cctcgactgt gccttctagt tgccagccat 120

ctgttgtttg cccctccccc gtgccttcct tgaccctgga aggtgccact cccactgtcc 180

tttcctaata aaatgaggaa attgcatcgc attgtctggg taggtgtcat tctattctgg 240

ggggtggggt ggggcaggac agcaaggggg aggattggga agacaatagc aggcatgctg 300

gggatggtac cggaggcctc gctagccacg cgtgcagctt gtcttgtctg tacagtggta 360

agtcctggcc ttgcctttgt ggcaaataca acccccttga attgcttggc ccttctcagc 420

attgcctaat attagggagg actcctgtaa agctcactgg ttagaagatc aagacacttg 480

ggcctggttc tgcccctggg ggtcattggg taattccttg cagtctccag gcctcacttg 540

ccctctgaac aagaaagagg ctgttctggg tcatccctcc aggcctgtcc agccctggca 600

ctctgtgagt cggtttaggc agcagccccg gaacagatga ggcaggcagg gttgggacgt 660

ttggtcagga cagcccaccg caaaaagagg aggaaagaaa tgaaagacag agacagcttt 720

ggctatggga gaaggaggag gccgggggaa ggaggagaca ggaggaggag ggaccacggg 780

gtggagggga gatagaccca gcccagagct ctgagtggtt tcctgtttgc ctg 833

<210> 56
<211> 816
<212> DNA
<213> Artificial sequence

<220>
<223> Sequence obtained with pAd-antisense primer, partial sequence of the virus

<400> 56
cctcatcccc taacgcatcc gcctctgaat gtgatgtcca actcatcatg tatcttatca 60

tgtctggatc tcgcggtctc cccctgtgct ggcggccgct ttacttgtac agctcgtcca 120

tgtttgagag tgatcccggc ggcggtcacg aactccagca ggaccatgtg atcgcgcttc 180

tcgttggggt ctttgctcag ggcggactgg gtgctcaggt agtggttgtc gggcagcagc 240

acggggccgt cgccgatggg ggtgttctgc tggtagtggt cggcgagctg cacgctgccg 300

tcctcgatgt tgtggcggat cttgaagttc accttgatgc cgttcttctg cttgtcggcc 360

atgatataga cgttgtggct gttgtagttg tactccagct tgtgccccag gatgttgccg 420

tcctccttga agtcgatgcc cttcagctcg atgcggttca ccagggtgtc gccctcgaac 480

ttcacctcgg cgcgggtctt gtagttgccg tcgtccttga agaagatggt gcgctcctgg 540

acgtagcctt cgggcatggc ggacttgaag aagtcgtgct gcttcatgtg gtcggggtag 600

cggctgaagc actgcacgcc gtaggtcagg gtggtcacga gggtgggcca gggcacgggc 660

agcttgccgg tggtgcagat gaacttcagg gtcagcttgc cgtaggtggc atcgccctcg 720

ccctcgccgg acacgctgaa cttgtggccg tttacgtcgc cgtccagctc gacagggatg 780

ggcaccaccc cggtgaacag ctcctcgccc ttgctc 816