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(54) **Polypeptide having an improved cytosine deaminase activity**

Polypeptid mit einer verbesserten Zytosindeaminase-Aktivität

Polypeptide ayant une activité désaminase améliorée de cytosine

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WO-A-99/54481 **US-A1- 2003 121 068**

- **WYBRANIETZ W A ET AL: "ENHANCED SUICIDE GENE EFFECT BY ADENOVIRAL TRANSDUCTION OF A VP22-CYTOSINE DEAMINASE (CD) FUSION GENE" GENE THERAPY, MACMILLAN PRESS LTD., BASINGSTOKE, GB, vol. 8, no. 21, November 2001 (2001-11), pages 1654-1664, XP001088128 ISSN: 0969-7128**
- **KERN L ET AL: "The FUR1 gene of Saccharomyces cerevisiae: cloning, structure and expression of wild-type and mutant alleles" GENE, ELSEVIER, AMSTERDAM, NL, vol. 88, no. 2, 1990, pages 149-157, XP002087000 ISSN: 0378-1119**
- **DATABASE UniProt [Online] 13 September 2005 (2005-09-13), "RecName: Full=Uracil phosphoribosyltransferase; EC=<A HREF="http: //srs.ebi.ac.uk/srsbin/cgi-bin/ wgetz?[enzyme-ECNumber:2.4.2.9]+-e">2.4.2. 9</A>; AltName: Full=UMP pyrophosphorylase; AltName: Full=UPRTase;" retrieved from EBI accession no. UNIPROT:Q48V26 Database accession no. Q48V26**

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Description

[0001] The present invention relates to the use as a negative selection marker of a nucleotide sequence encoding a polypeptide possessing a Cytosine deaminase (CDase) activity, wherein said polypeptide possessing a CDase activity is a yeast CDase to which is added an amino acid sequence of 100 to 400 amino acids having a degree of identity greater than 70 % with a polypeptide possessing an UPRTase activity, with the proviso that said polypeptide possessing a CDase activity has neither uracil phosphoribosyl transferase (UPRtase) nor thymidine kinase activity.

[0002] The present invention also relates to the use as a negative selection marker of a recombinant vector which carries said nucleotide sequence, placed under the control of the elements which are required for expressing it in a host cell.

[0003] Gene therapy is defined as being the transfer of genetic information into a host cell or organism. The first protocol applied to man was initiated in the United States, in September 1990, on a patient who was genetically immunodeficient on account of a mutation which affected the gene encoding Adenine Deaminase (ADA). The relative success of this first experiment encouraged the development of this approach for a variety of diseases, including both genetic diseases (with the aim of correcting the malfunction of a defective gene) and acquired diseases (cancers, infectious diseases, such as AIDS, etc.). This technology has experienced a large number of developments since then, including "suicide gene" therapy, which uses genes whose expression products are able to transform an inactive substance (prodrug) into a cytotoxic substance, thereby giving rise to cell death. In 1992, several groups demonstrated the relevance of this novel approach for treating tumors and inhibiting dissemination of the HIV virus, which is responsible for AIDS.

[0004] In this respect, the gene encoding the herpes simplex type 1 virus thymidine kinase (HSV-1 TK) constitutes the prototype of the suicide genes (Caruso et al., 1993, Proc. Nati. Acad. Sci. USA 90, 7024-7028; Culver et al., 1992, Science 256, 1550-1552; Ram et al., 1997, Nat. Med. 3, 1354-1361). While the TK polypeptide is not toxic as such, it catalyzes the transformation of nucleoside analogues such as acyclovir or ganciclovir (GCV). The modified nucleosides are incorporated into the DNA chains which are in the process of elongation, inhibiting cell division as a consequence. A large number of suicide gene/prodrug pairs are currently available. Those which may more specifically be mentioned are rat cytochrome p450 and cyclophosphophamide Human Gene Therapy 5, 969-978), *Escherichia coli* (E. *Coli)* purine nucleoside phosphorylase and 6-methylpurine deoxyribonucleoside (Sorscher et al., 1994, Gene Therapy 1, 223-238), *E. coli* guanine phosphoribosyl. transferase and 6-thioxanthine (Mzoz and Moolten, 1993, Human Gene Therapy 4, 589-595) and cytosine deaminase (CDase) and 5-fluorocytosine (5FC).

[0005] CDase is involved in the pyrimidine metabolic pathway by which exogenous cytosine is transformed into uracil by means of a hydrolytic deamination. While CDase activities have been demonstrated in prokaryotes and lower eukaryotes (Jund and Lacroute, 1970, J. Bacteriol. 102, 607-615; Beck et al., 1972, J. Bacteriol. 110, 219-228; De Haan et al., 1972, Antonie van Leeuwenhoek 38, 257-263; Hoeprich et al., 1974, J. Inf. Dis. 130, 112-118; Esders and Lynn, 1985, J. Biol. Chem. 260, 3915-3922), they are not present in mammals (Koechlin et al., 1966, Biochem Pharmacol. 15, 435-446; Polak et al., 1976, Chemotherapy 22, 137-153). The *Saccharomyces cerevisiae (S. cerevisiae) FCY1* and the *E. coli codA* genes, which respectively encode the CDase of these two organisms, are known and their sequences have been published (EP 402 108; Erbs et al., 1997, Curr. Genet. 31, 1-6; W093/01281).

[0006] CDase also deaminates an analogue of cytosine,i.e.5-fluorocytosine (5-FC), thereby forming 5-fluorouracil (5-FU), which is a compound which is highly cytotoxic when it is converted into 5-fluoro-UMP (5-FUMP). Cells which lack CDase activity, either because of a mutation which inactivates the gene encoding the enzyme or because they are naturally deficient in this enzyme, as are mammalian cells, .are resistant to 5-FC (Jund and Lacroute, 1970, J. Bacteriol, 102, 607-615; Kilstrup et al., 1989, J. Bacteriol. 1989 171, 2124-2127). By contrast, mammalian cells into which the sequences encoding CDase activity were transferred became sensitive to 5-FC (Huber et al., 1993, Cancer Res. 53, 4619-4626; Mullen et al., 1992, Proc. Nati. Acad. Sci USA 89, 33-37; WO93/01281). In addition, the neighboring, untransformed cells also become sensitive to 5-FC (Huber et al., 1994, Proc. Natl. Acad. Sci. USA 91, 8302-8306). This phenomenon, which is termed a bystander effect, is due to the cells which are expressing the CDase activity secreting 5-FU, which then intoxicates the neighboring cells by straightforward diffusion across the plasma membrane. This property of 5-FU in diffusing passively represents an advantage as compared with the tk/GCV reference system, where the bystander effect requires there to be contact with the cells which are expressing tk (Mesnil et al., 1996, Proc. Natl. Acad. Sci. USA 93, 1831-1835). All the advantage which CDase offers within the context of gene therapy, in particular anticancer gene therapy, can therefore be readily understood.

[0007] In order two improve the efficiency of methods using CDase activity, the prior art document WO-A-96/16183 recommends using a fusion protein which encodes a two-domain enzyme possessing CDase and UPRTase activities, and demonstrates *in vitro* that the transfer of a hybrid codA::upp or *FCY1::FUR1* gene, carried by an expression plasmid, increases the sensitization of transfected B16 cells to 5-FC. WO99/54481 provides an improvement of this invention by using a mutated *FUR1* gene which encodes a UPRTase which is deleted in its N-terminal part.

[0008] The present invention is art improvement of the earlier techniques in that it uses a polypeptide possessing a CDase activity, **characterized in that** it is derived from a native CDase addition of an amino acid sequence of 100 to

400 amino acids having a degree of identity greater than 70 % with a polypeptide possessing an UPRTase activity, with the proviso that said polypeptide possessing a CDase activity has no UPRTase or thymidine kinase activity.

[0009] The present invention provides a more efficient polypeptide, thereby making it possible to increase the sensitivity of cells to 5-FC or the bystander effect induced by the production of 5-FU. This mutant can be used for a large number of applications, in particular all applications which require cell death.

[0010] "Cytosine deaminase activity" (CDase activity) is understood as covering the deamination of cytosine or one of its analogs.

[0011] "derives from a native CDase" widely means that said polypeptide possessing a CDase activity comprises an amino acid sequence which has a degree of identity which is greater than 70%, advantageously greater than 80%, preferably greater than 90% and, very preferably greater than 95% with said native CDase. Still more preferably, the polypeptide possessing a CDase activity comprises the amino acid sequence of a native CDase.

[0012] Within the meaning of the present invention, a native CDase refers to a CDase of prokaryotic or eukaryotic origin. Preferably, the CDase is a yeast CDase, in particular that encoded by the *Saccharomyces cerevisiae FCY1* gene. The cloning and the sequence of the genes encoding the CDases of different origins are available in the literature and the specialized databases. For information, the sequence of the *FCY1* gene is disclosed in Erbs et al. (1997, Curr. Genet, 31, 1-6).

[0013] A preferred example is a native CDase which comprises an amino acid sequence which is substantially as depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Glu residue in position 158. The term "substantially" refers to a degree of identity with said SEQ ID NO: 1 sequence which is greater than 70%, advantageously greater than 80%, preferably greater than 90% and, very preferably greater than 95%, Still more preferably, the polypeptide comprises the amino acid sequence depicted in SEQ ID NO: 1 sequence identifier starting at the Met residue in position 1 and finishing at the Glu residue in position 158. A polypeptide having the amino acid sequence as depicted in SEQ ID NO: 1 sequence identifier starting at the Met residue in position 1 and finishing at the Glu residue in position 158 is very particularly appropriate for implementing the invention.

[0014] According to an advantageous embodiment, a polypeptide encoded by a nucleotide sequence used according to the invention exhibits a CDase activity which is appreciably higher than that of said native CDase. Thus, the examples which follow demonstrate that the addition of an amino acid sequence which has no UPRtase activity makes it possible to increase the sensitization of the target cells to 5-FC and/or the bystander effect induced in the treated animal. The factor by which the sensitization is increased is advantageously at least 2, preferably at least 5 and, very preferably, 10 or more.

[0015] "Polypeptide which has no UPRtase activity" encompasses polypeptide which are unable to convert 5-FU in 5-FUMP. The ability of an amino acid sequence to convert 5-FU in 5-FUMP can be assessed by using the method disclosed in the working example of the present application.

[0016] "polypeptide which has no thymidine kinase activity" encompasses polypeptide which are unable to to phosphorylate nucleosides (e.g., dT) and nucleoside analogues such as ganciclovir (9-{[2-hydroxy-1-(hydroxymethyl)ethoxyl methyl}guanosine), famciclovir, buciclovir, penciclovir, valciclovir, acyclovir (9-[2-hydroxy ethoxy)methyl]guanosine), trifluorothymidine, 1-[2-deoxy, 2-fluoro, beta-D-arabino furanosyl]-5-iodouracil, ara-A (adenosine arabinoside, vivarabine), 1-beta-D-arabinofuranoxyl thymine, 5-ethyl-2'-deoxyuridine, 5-iodo-5'-amino-2,5'-dideoxyuridine, idoxuridine (5-iodo-2'-deoxyuridine), AZT (3' azido-3' thymidine), ddC (dideoxycytidine), AIU (5-iodo-5' amino 2', 5'-dideoxyuridine) and AraC (cytidine arabinoside).

[0017] According to a preferred embodiment, the amino acid sequence added to the native CDase, is between 10 and 1000, more preferably between 100 and 400 and very preferably between 200 and 300 amino acid length.
Even though the addition can take place at any site in the native CDase, the N- or C-terminal ends are preferred, in particular the C-terminal end.

[0018] Advantageously, the amino acid sequence which added to the native CDase, is derived from a polypeptide having an UPRTase activity.

[0019] "Derived from a polypeptide having an UPRtase activity " widely means that said amino acid sequence has a degree of identity which is greater than 70%, advantageously greater than 80%, preferably greater than 90% and, very preferably greater than 95% with the amino acid sequence of a polypeptide having an UPRTase activity.

[0020] Within the meaning of the present invention, a polypeptide possessing a UPRTase activity refers to a polypeptide which is able to convert uracil, or one of its derivatives, into a monophosphate analog, in particular 5-FU into 5-FUMP. "Mutation" is to be understood as being the addition, deletion and/or substitution of one or more residues at any site in said polypeptide.

[0021] The polypeptide having an UPRtase activity from which the amino acid sequence encoded by a nucleotide sequence used according to the invention may derive can be of any origin, in particular of prokaryotic, fungal or yeast origin. By way of illustration, the UPRTases from *E. coli* (Anderson et al., 1992, Eur. J. Biochem 204, 51-56), from *Lactococcus lactis* (Martinussen and Hammer, 1994, J. Bacteriol. 176, 6457-6463), from *Mycobacterium bovis* (Kim et al., 1997, Biochem Mol. Biol. mt 41, 1117-1124) and front *Bacillus subtilis* (Martinussen et al., 1995, J. Bacteriol. 177,

271-274), can be used within the context of the invention. However, very particular preference is given to using a yeast UPRTase, in particular that encoded by the S. *cerevisiae FUR1* gene, whose sequence is disclosed in Kern et al. (1990, Gene 88, 149-157). More particularly, by way of information, the sequences of the genes, and those of the corresponding UPRTases, can be found in the literature and in specialized databases (SWISSPROT, EMBL, Genbank, Medline, etc.). More particularly, mutants encoded by the fur 1-7, fur 1-8 and fur 1-9 can be used in the present invention. In a preferred embodiment, the amino acid sequence which derives from a polypeptide having an UPRTase activity is the amino acid sequences encoded by the fur 1-8 allele (Kern et al., 1990, Gene 88, 149-157).

[0022] According to one particularly advantageous embodiment, the amino acid sequence, added to the native CDase, derives from a deletion mutant of a native UPRTase. The deletion is preferably located in the N-terminal region of the original UPRTase. The deletion can be total (affecting all the residues of said N-terminal region) or partial (affecting one or more residues which may or may not be continuous in the primary structure). In a general manner, a polypeptide consists of an N-terminal part, of a central part and of a C-terminal part, with each part representing approximately one third of the molecule. For example, in the case of the S. cerevisiae UPRTase, which contains 251 amino acids, the N-terminal part consists of the first 83 residues, starting with the so-called initiating methionine, which is located in the first position of the native form. In the case of the E. coli UPRTase, the N-terminal part covers positions 1 to 69.

[0023] It has been shown that the expression product of the mutant gene, designated FUR1Δ105, is able to complement an S. cerevisiae furl mutant, thereby demonstrating that it is functional.

[0024] Very preferably, polypeptide having an UPRTase activity is derived from a native UPRTase at least by deleting all or part of the N-terminal region upstream of the second ATG codon of said native UPRTase. The total deletion of the aforesaid region is preferred. For example, the UPRTase encoded by the *FURI* gene comprises a first ATG codon (initiating ATG codon) in position +1 followed by a second in position +36. Thus, it is possible to envisage deleting residues +1 to 35 within the context of the present invention, thereby giving a polypeptide which starts at the methionine which is normally found in position +36 of the native form. It has been shown that the expression product of the mutant gene, designated FUR1Δ105, is able to complement an S. cerevisiae furl mutant, thereby demonstrating that it is functional.

[0025] A preferred amino acid sequence, added to the native CDase, comprises an amino acid sequence which is substantially as depicted in SEQ ID NO: 2 sequence identifier, starting at the Ser residue in position 2 and finishing at the Val residue in position 216. The term "substantially" refers to a degree of identity with said sequence which is greater than 70%, advantageously greater than 80%, preferably greater than 90% and, very preferably greater than 95%. Still more preferably, the amino acid sequence, added to the native CDase, comprises the amino acid sequence depicted in SEQ ID NO:2 sequence identifier starting at the Ser residue in position 2 and finishing at the Val residue in position 216. A amino acid sequence, added to the native CDase, having the amino acid sequence as depicted in SEQ ID NO: 2 sequence identifier starting at the Ser residue in position 2 and finishing at the Val residue in position 216 is very particularly appropriate for implementing the invention. This amino acid sequence, added to the native CDase, can contain additional mutations. Substitution of the serine residue in position 2 of SEQ ID NO:2 sequence identifier with an Alanine residue may in particular be mentioned.

[0026] A preferred polypeptide possessing a CDase activity comprises an amino acid sequence which is substantially as depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Val residue in position 373. The term "substantially" refers to a degree of identity with said SEQ ID NO: 1 sequence which is greater than 70%, advantageously greater than 80%, preferably greater than 90% and, very preferably greater than 95%. Still more preferably, the polypeptide comprises the amino acid sequence depicted in SEQ ID NO: 1 sequence identifier starting at the Met residue in position 1 and finishing at the Val residue in position 373. A polypeptide having the amino acid sequence as depicted in SEQ ID NO: 1 sequence identifier starting at the Met residue in position 1 and finishing at the Val residue in position 373 is very particularly appropriate for implementing the invention.

[0027] The nucleotide sequence used according to the invention can be a cDNA or genomic sequence or be of a mixed type. It can, where appropriate, contain one or more introns, with these being of native, heterologous (for example the intron of the rabbit β-globin gene, etc.) or synthetic origin, in order to increase expression in the host cells. The sequences employed within the context of the preset invention can be obtained by the conventional techniques of molecular biology, for example by screening libraries with specific probes, by immunoscreening expression libraries or by PCR using suitable primers, or by chemical synthesis. The mutants can be generated from the native sequences by substituting, deleting and/or adding one or more nucleotides using the techniques of site-directed mutagenesis, of PCR, of digesting with restriction and legation enzymes, or else by chemical synthesis. The ability of the mutants and constructs to function can be verified by assaying the enzymatic activity or by measuring the sensitivity of target cells to 5-FC and/or 5-FU.

[0028] The present invention also relates to the use as a negative selection marker of a recombinant vector which carries a nucleotide sequence as described above which is placed under the control of the elements which are required for expressing it in a host cell. The recombinant vector can be of plasmid or viral origin and can, where appropriate, be combined with one or more substances which improve the transfectional efficiency and/or stability of the vector. These

substances are widely documented in the literature which is available two the skilled person (see, for example, Feigner et al., 1987, Proc. West. Pharmacol. Soc. 32, 115-121; Hodgson and Solaiman, 1996, Nature Biotechnology 14, 339-342; Remy et al., 1994, Bioconjugate Chemistry, 5, 647-654). By way of non-limiting illustration, the substances can be polymers, lipids, in particular cationic lipids, liposomes, nuclear protein or neutral lipids. These substances can be used alone or in combination. A combination which can be envisaged is that of a recombinant plasmid vector which is combined with cationic lipids (DOGS, DC-CHOL, spermine-chol, spermidine-chol, etc.), lysophospholipides (for example Hexadecylphosphocholine) and neutral lipids (DOPE).

[0029] According to a preferred embodiment, the cationic lipids which can be used in the present invention are the cationic lipids describes in EP901463B1 and more preferably pcTG90.

[0030] The choice of the plasmids which can be used within the context of the present invention is immense. They can be cloning vectors and/or expression vectors. In a general manner, they are known to the skilled person and, while a number of them are available commercially, it is also possible to construct them or to modify them using the techniques of genetic manipulation. Examples which may be mentioned are the plasmids which are derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogene) or p Poly (Lathe et al., 1987, Gene 57, 193-201). Preferably, a plasmid which is used in the context of the present invention contains an origin of replication which ensures that replication is initiated in a producer cell and/or a host cell (for example, the ColE1 origin will be chosen for a plasmid which is intended to be produced in *E. coli* and the oriP/EBNA1 system will be chosen if it desired that the plasmid should be self-replicating in a mammalian host cell, Lupton and Levine, 1985, Mol. Cell. Biol. 5, 2533-2542; Yates et al., Nature 313, 812-815). The plasmid can additionally comprise a selection gene which enables the transfected cells to be selected or identified (complementation of an auxotrophic mutation, gene encoding resistance to an antibiotic, etc.). Naturally, the plasmid can contain additional elements which improve its maintenance and/or its stability in a given cell *(cer* sequence, which promotes maintenance of a plasmid in monomeric form (Summers and Sherrat, 1984, Cell 36, 1097-1103, sequences for integration into the cell genome).

[0031] With regard to a viral vector, it is possible to envisage a vector which is derived from a poxvirus (vaccinia virus, in particular MVA, canarypoxvirus, etc.), from an adenovirus, from a retrovirus, from a herpesvirus, from an aiphavirus, from a foamy virus or from an adenovirus-associated virus. It is possible to use replication competent or replication deficient viral vectors. Preference will be given to using a vector which does not integrate. In this respect, adenoviral vectors and MVA are very particularly suitable for implementing the present invention.

[0032] According to a preferred embodiment, the viral vector derives from a Modified Vaccinia Virus Ankara (MVA).Mva vectors and methods to produce such vectors are fully described in European patents EP83286 and EP206920, as well as in Mayr et al. (1975, Infection 3, 6-14) and Sutter et Moss (1992, Proc. Natl. Acad. Sci. USA 89, 10847-10851). According to a more preferred embodiment, the nucleotide sequence may be inserted in deletion I, II, III, IV, V and VI of the MVA vector and even more preferably in deletion III (Meyer et al., 1991, J. Gen. Virol. 72, 1031-1038; Sutter et al., 1994, Vaccine 12, 1032-1040).

[0033] Retroviruses have the property of infecting, and in most cases integrating into, dividing cells and in this regard are particularly appropriate for use in relation to cancer. A recombinant retrovirus used in the invention generally contains the LTR sequences, an encapsidation region and the nucleotide sequence according to the invention, which is placed under the control of the retroviral LTR or of an internal promoter such as those described below. The recombinant retrovirus can be derived from a retrovirus of any origin (murine, primate, feline, human, etc.) and in particular from the MOMuLV (Moloney murine leukemia virus), MVS (Murine sarcoma virus) or Friend murine retrovirus (Fb29). It is propagated in an encapsidation cell line which is able to supply *in trans* the viral polypeptides gag, pol and/or env which are required for constituting a viral particle. Such cell lines are described in the literature (PA317, Psi CRIP GP +Am-12 etc.). The retroviral vector used in the invention can contain modifications, in particular in the LTRs (replacement of the promoter region with a eukaryotic promoter) or the encapsidation region (replacement with a heterologous encapsidation region, for example the VL3O type) (see French applications 94 08300 and 97 05203).

[0034] Preference will be also given to using an adenoviral vector which lacks all or part of at least one region which is essential for replication and which is selected from the E1, E2, E4 and L1-L5 regions in order to avoid the vector being propagated within the host organism or the environment. A deletion of the E1 region is preferred. However, it can be combined with (an)other modification(s)-/deletion(s) affecting, in particular, all or part of the E2, E4 and/or L1-L5 regions, to the extent that the defective essential functions are complemented in *trans* by means of a complementing cell line and/or a helper virus. In this respect, it is possible to use second-generation vectors of the state of the art (see, for example, international applications WO-A-94/28152 and WO-A-97/04119). By way of illustration, deletion of the major part of the E1 region and of the E4 transcription unit is very particularly advantageous. For the purpose of increasing the cloning capacities, the adenoviral vector can additionally lack all or part of the nonessential E3 region. According to another alternative, it is possible to make use of a minimal adenoviral vector which retains the sequences which are essential for encapsidation, namely the 5' and 3' ITRs (Inverted Terminal Repeat), and the encapsidation region. The various adenoviral vectors, and the techniques for preparing them, are known (see, for example, Graham and Prevect, 1991, in Methods in Molecular Biology, Vol 7, p 109-128; Ed: E.J. Murey, The Human Press mc).

[0035] Furthermore, the origin of the adenoviral vector used in the invention can vary both from the point of view of the species and from the point of view of the serotype. The vector can be derived from the genome of an adenovirus of human or animal (canine, avian, bovine, murine, ovine, porcine, simian, etc.) origin or from a hybrid which comprises adenoviral genome fragments of at least two different origins. More particular mention may be made of the CAV-1 or CAV-2 adenoviruses of canine origin, of the DAV adenovirus of avian origin or of the Bad type 3 adenovirus of bovine origin (Zakharchuk et al., Arch. Virol., 1993, 128: 171-176; Spibey and Cavanagh, J. Gen. Virol. 1989, 70: 165-172; Jouvenne et al., Gene, 1987, 60: 21-28; Mittal et al., J. Gen. Virol., 1995, 76: 93-102). However, preference will be given to an adenoviral vector of human origin which is preferably derived from a serotype C- adenovirus, in particular a type 2 or 5 serotype C adenovirus.

[0036] The term "replication-competent" as used herein refers to a viral vector capable of replicating in a host cell in the absence of any transcomplementation. In the context of the present invention, this term also encompasses replication-selective or conditionally-replicative adenoviral vectors which are engineered to replicate better or selectively in cancer or hyperproliferative host cells.

[0037] According to a preferred embodiment of the invention, the replication competent vector is a replication competent adenoviral vector. These replication competent adenoviral vectors are well known by the one skilled in the art. Among these, adenoviral vectors deleted in the Elb region coding the 55kD P53 inhibitor, as in the ONYX-015 virus (Bischoff et al, 1996; Heise et al., 2000; WO 94/18992), are particularly preferred. Accordingly, this virus can be used to selectively infect and kill p53-deficient neoplastic cells. A person of ordinary skill in the art can also mutate and disrupt the p53 inhibitor gene in adenovirus 5 or other viruses according to established techniques. Adenoviral vectors deleted in the E1A Rb binding region can also be used in the present invention. For example, Delta24 virus which is a mutant adenovirus carrying a 24 base pair deletion in the E1A region (Fueyo et al., 2000). Delta24 has a deletion in the Rb binding region and does not bind to Rb. Therefore, replication of the mutant virus is inhibited by Rb in a normal cell. However, if Rb is inactivated and the cell becomes neoplastic, Delta24 is no longer inhibited. Instead, the mutant virus replicates efficiently and lyses the Rb-deficient cell.

[0038] An adenoviral vector used in the present invention can be generated *in vitro* in *Escherichia coli (E. coli)* by ligation or homologous recombination (see, for example, international application WO-A-96/17070) or else by recombination in a complementing cell line.

[0039] The elements required for expression consist of all the elements which enable the nucleotide sequence to be transcribed into RNA and the mRNA to be translated into polypeptide. These elements comprise, in particular, a promoter which may be regulable or constitutive. Naturally, the promoter is suited to the chosen vector and the host cell. Examples which may be mentioned are the eukaryotic promoters of the PGK (phosphoglycerate kinase), MT (metallothionein; McIvor et al., 1987, Mol. Cell Biol. 7, 838-848), α-1 antitrypsin, CFTR, surfactant, immunoglobulin, β-actin (Tabin et al., 1982, Mol. Cell Biol. 2, 426-436) and SRa (Takebe et al., 1988, Mol. Cell Biol. 8, 466-472) genes, the early promoter of the SV4O virus (Simian virus), the LTR of RSV (Rous sarcoma virus), the HSV-1 TK promoter, the early promoter of the CMV virus (Cytomegalovirus)., the p7.5K pH5R, pK1L, p28 and p11 promoters of the vaccinia virus, and the E1A and MLP adenoviral promoters. The promoter can also be a promoter which stimulates expression in a tumor or cancer cell. Particular mention may be made of the promoters of the MUC-1 gene, which is overexpressed in breast and prostate cancers (Chen et al., 1995, J.. Clin. Invest. 96, 2775-2782), of the CEA (standing for carcinoma embryonic antigen) gene, which is overexpressed in colon cancers (Schrewe et al., 1990, Mol. Cell. Biol. 10, 2738-2748) of the tyrosinase gene, which is overexpressed in melanomas (Vile et al., 1993, Cancer Res. 53, 3860-3864), of the ERBB-2 gene, which is overexpressed in breast and pancreatic cancers (Harris et al., 1994, Gene Therapy 1, 170-175) and of the α-fetoprotein gene, which is overexpressed in liver cancers (Kanai et al., 1997, Cancer Res. 57, 461-465). The cytomegalovirus (CMV) early promoter is very particularly preferred.

[0040] However, when a vector deriving from a Vaccinia Virus (as for example an MVA vector) is used, the promoter of the thymidine kinase 7.5K gene is particularly preferred.

[0041] The necessary elements can furthermore include additional elements which improve the expression of the nucleotide sequence used in the invention or its maintenance in the host cell. Intron sequences, secretion signal sequences, nuclear localization sequences, internal sites for the reinitiation of translation of IRES type, transcription termination poly A sequences, tripartite leaders and origins of replication may in particular be mentioned. These elements are known to the skilled person.

[0042] The recombinant vector used in the invention can also comprise one or more additional genes of interest, with it being possible for these genes to be placed under the control of the same regulatory elements (polycistronic cassette) or of independent elements. Genes which may in particular be mentioned are the genes encoding interleukins IL-2, IL-4, IL-7, IL-10 and IL-12, interferons, tumor necrosis factor (TNF), colony stimulating factors (CSF), in particular GM-CSF, and factors acting on angiogenesis (for example PA1-1, standing for plasminogen activator inhibitor). In one particular embodiment, the recombinant vector used in the invention comprises the gene of interest encoding IL-2 or encoding interferon γ (INFγ). It is also possible to envisage combining the nucleotide sequence used in the invention with other suicide genes such as the HSV-1 TK gene, the ricin gene, the cholera toxin gene, etc.

[0043] The present invention thus relates to the use of the sequences or recombinant vectors according to the invention as negative selection markers. For example, the sequences or recombinant vectors according to the invention can be used to select non human embryonic stem cells or non human cells obtained after nuclear transfer in which genes are interrupted or modified (e.g. in methods for preparing transgenic animals) (see, for example, Capecchi, 1989, Science 244, 1288-1292; Reid et al., 1990, Proc. Nati. Acad. Sci. USA 87, 4299-4303). Such a use, in combination with the gene for resistance to neomycin, for example, can make it possible to select the cells which have undergone an homologous recombination event and which will alone be able to grow in the presence of Geneticin and the corresponding fluorinated pyrimidines (5-FC when a nucleotide sequence encoding a CDase activity is used). The cells which have undergone a non-targeted recombination event are able to grow in the presence of Geneticin but not in the presence of the fluorinated pyrimidines. Another potential use as a negative selection marker is to be found in the plant field since, just like mammalian cells, plants do not possess any endogenous CDase activity. They can be sensitized to 5-FC by transfecting a nucleotide sequence according to the invention which enables an exogenous CDase to be expressed (see, for example, Perera et al. 1993, Plant. Mol. Biol. 23, 797-799).

[0044] The sequences or recombinant vectors according to the invention may also be used as negative selection marker for bacteria or yeast. In this particular embodiment yeast or bacteria lacking a native CDase gene are preferably used. Among those Bacteria, those disclosed in EP0792369BB1 are particularly preferred. For example, the sequence according to the invention may be used for cloning sequences, by homologous recombination, in a plasmid comprising the sequence according to the invention. In these experiments, the sequence according to the invention is located at the site where the sequence to be cloned has to be inserted. Therefore, after homologous recombination, cells comprising the desired plasmid are able to grow in the presence of 5-FC.

[0045] Figure 1 shows the growth of LOVO tumor cells in CD1 nude mice treated with the vector according to the invention.

Example 1: Construction of Fcu1-8

[0046] FCU1-8 has been constructed by directed mutagenesis of FCU1, starting from pCI-neoFCU1 (pTG13046) Arg in position 183 has been replaced by a Ser with a oligo 5' -gtattcttattactgatgatgg-3 ' (in order to modify A549en T) to give the plasmide pCI-neoFCU1-8 (pTG15546).

Example 2: Expression of FCY1, FCU1 and Fcu1-8 in mammalian cells and determination of the activities CDase and UPRTase

[0047] The stock of mammal COS7 transitorily transfected by the plasmides pCIneo (Promega), pCI-neoFCY1 (pTG15916), pCI-neoFCU1 (pTG13046) and pCI-neoFCU1-8 (pTG15546) was tested for CDase and UPRTase activities.

[0048] The protocol was as follows: 5 105 COS7 cells were sown with 37°C 60 mm flasks containing 5ml of DMEM/10%FCS medium. After 24 hours, the cells were treated by 20μl of lipofectine (Gibco BRL) in absence or in the presence of 5μg of plasmid. After 20 hours at 37°C in 2 ml of medium DMEM, the cells were incubated in the presence of 5 ml of DMEM/10%FCS. After 48 hours, at 37°C, the cells were washed in PBS then suspended in 25μl of lysis buffer(Tris-HCl pH 7.5 50 mM/NaCl 150 mM/EDTA 5 mM/DTT 1 mM/triton 1%). After 30 minutes of lysis at 4°C followed by a centrifugation, the CDase and UPRTase activities was measured in the supernatant containing the cellular lysate.

[0049] For CDase activity: 4μl of cellular lysate are incubated, 20 minutes at 37°C, in the presence of 2μl of reactional CDase buffer (Tris-HCl pH 7.5 100 mM/[H3]5-FC 3 mM with 0.25μCi/μl).

[0050] For UPRTase activity: 4μl of cellular lysate are incubated, 20 minutes at 37°C in the presence of 2μl of reactional UPRTase buffer (Tris-HCl pH 7.5 100 mM/$MgCl_2$ 10 mM/5-PRPP 10 mM/[C14]5-FU 3 mM at 0.02μCi/μl).

[0051] The enzymatic reactions are stopped at 100°C during 1 minute. The aliquots of 1μl are deposited on TLC polyethylenimine-cellulose plates (Merck). 5-FU is separated from the 5-FC by using a mixture water/butanol-1 (14%/86%) as solvent and the separation of 5-FUMP and 5-FU is carried out by using water as solvent. After migration, TLC plate was scanned in PosphorImager (445SI; Molecular Dynamics). The protein concentration was measured by the method of Bradford.

[0052] The results of the enzymatic activities, which are calculated from three independent measurements, are given in the following table:

	CDase pmoles of 5-FC transformed/min/mg of protein	**UPRTase** pmoles of 5-FU transformed/min/mg of protein
not transfected	not detectable	not detectable
pCI-neo	not detectable	not detectable

(continued)

	CDase pmoles of 5-FC transformed/min/mg of protein	UPRTase pmoles of 5-FU transformed/min/mg of protein
pCI-neo FCY1	783 +/- 311	not detectable
pCI-neo FCU1	9268 +/- 628	3866 +/- 518
pCI-neo Fcu1-8	8263 +/- 782	not detectable

[0053] Compared to FCU1 gene, the Fcu1-8 mutant losses UPRTase activity with a conservation of CDase activity which is 10 times higher than CDase activity of native FCY1 gene. The Arg183 change in Ser in gene FCU1 leads to a loss in UPRTase activity without modifying CDase activity.

Example 3: Construction of an adenovirus expressing FCU1 (AdTG14800)

[0054] The XhoI-MluI fragment of pCI-neoFCU1 (pTG13046) containing FCU1 gene is isolated and introduced into the transfer vector pTG13387 cleaved by these same enzymes, to give the transfer vector pTG14799. The adenoviral vector AD-FCU1 (pTG14800) is reconstituted by homologous recombination in the BJ 5183 E.coli between the fragment PacI-BstEII of pTG14799 and the vector pTG6624 linearized by ClaI. Final construction AD-FCU1 contains the E1 and E3 deleted Ad5 genome (nucleotides 459 to 3510 and 28249 to 30758) and instead of E1, an expression cassette comprising FCU1 gene is placed under the control of the CMV promoter and β-globine/IgG and followed and the poly A sequences of bGH. The adenoviral particles expressing FCU1 (AdTG14800) are generated by transfection in a complementation cell line (for example line PERC6).

Example 4: Construction of an adenovirus expressing Fcu1-8 (AdTG15606).

[0055] The XhoI-MluI fragment of pCI-neoFCU1-8 (pTG15546) containing the Fcu1-8 gene is isolated and introduced into the transfer vector pTG13387 cleaved by these same enzymes, to give the transfer vector of pTG15547. The adenoviral vector AD-FCU1-8 (pTG15606) is reconstituted by homologous recombination in the BJ 5183 E.coli strain between the fragment PacI-BstEII of pTG15547 and the vector pTG6624 linearized by ClaI. Final construction AD-FCU1-8 contains the Ad5 genome deleted of the E1 (NT 459 to 3510) and E3 (NT 28249 to 30758) regions and instead of E1, a expression cassette comprising the Fcu1-8 gene placed under the control of the CMV early promoter hybrid β-globine/IgG and MVC and the followed by poly A sequences of bGH. The adenoviral particles expressing Fcu1-8 (AdTG15606) are generated by transfection in a cell line complementating the E1 function, for example PERC6.

Example 5: Infection by the adenovirus expressing FCU1 (AdTG14800) and Fcu1-8 (AdTG15606):

in vitro results

Determination of the activities CDase and UPRTase

[0056] The human tumoral cell line A549 (ATCC Ccl-185) was infected by an empty adenovirus (AdTG15149), an adenovirus expressing FCU1 (AdTG14800) and by an adenovirus expressing Fcu1-8 (AdTG15606). The infection protocol was as follows: 5 106 cells in suspension in 50μl of PBS-2%FCS -cations1% are incubated 30 minutes at 37°C in the presence of the adenovirus at MOI 5. The totality of the 5 106 cells is then incubated in 60 mm flasks in the presence of 5 ml of DMEM-10%FCS. After 24 hours at 37°C, the cells were washed in PBS then resuspended in 25μl of lysis buffer(Tris-HCl pH 7.5 50 mM/NaCl 150 mM/EDTA 5 mM/DTT 1 mM/triton 1%). After 30 minutes of lysis at 4°C followed by a centrifugation, the CDase and UPRTase activities was measured in the supernatant containing the cellular lysate (according to the previously described protocol).

[0057] The results of the enzymatic activities, which are calculated from three independent measurements, are given in the following table:

	CDase pmoles of 5-FC transformed/min/mg of protein	UPRTase pmoles of 5-fu transformed/min/mg of protein
not infected	not detectable	not detectable

(continued)

	CDase pmoles of 5-FC transformed/min/mg of protein	**UPRTase** pmoles of 5-fu transformed/min/mg of protein
Ad-empty (AdTG15149)	not detectable	not detectable
Ad-FCU1 (AdTG14800)	12000 +/- 1870	1110 +/- 190
Ad-FCU1-8 (AdTG15606)	12970 +/- 1350	not detectable

[0058] For the cells infected by an adenovirus expressing Fcu1-8, the results indicate a loss of UPRTase activity whereas CDase activity is preserved.

Example 6: Construction of MVA expressing FCU1 (MVATG15637)

[0059] The transfer vector Mva-fcu1 (pTG15637) is reconstituted by homologous recombination in the BJ 5183E.coli strain between the HindIII-KpnI fragment of pCI-neoFCU1 (pTG13046) and the vector pTG14269 linearized by XhoI. This transfer vector MVA-fcul (pTG15637) contains the expression cassette of the FCU1 gene placed under the control of the p11K7.5 promoter and framed by the sequences bordering deletion III of MVA. MVA Particles expressing FCU1 (MVA,TG15637 with FCU1 inserted in deletion III and under the control of the p11K7.5 promoter) are generated by homologous recombination between the MVAN33 and the plasmid pTG15637 in the embryonic chicken cells.

Example 7: Construction of Mva expressing Fcu1-8 (MVATG15638)

[0060] The transfer vector MVA-FCU1-8 (pTG15638) is reconstituted by homologous recombination in the BJ 5183 E.coli strain between the HindIII-KpnI fragment of pCI-neoFCU1-8 (pTG15546) and the vector pTG14269 linearized by XhoI. This transfer vector MVA-FCU1-8 (pTG15638) contains the expression cassette of the Fcu1-8 gene placed under the control of the p11K7.5 promoter and framed by the sequences bordering deletion III of MVA. MVA particles expressing Fcu1-8 (MVATG15638 with Fcu1-8 inserted in deletion III and under the control of the p11K7.5 promoter) are generated by homologous recombination between the MVAN33 and the plasmid pTG15638 in embryonic chicken cells.

Example 8: Infection by MVA expressing FCU1 (MVATG15637) and FCU1-8 (MVATG15638):

in vitro results

Determination of the activities CDase and UPRTase

[0061] The human tumoral cell line A549 (ATCC CCCL-185) was infected by an empty MVA (MVAN33), MVA expressing FCU1 (MVA15637) and of MVA expressing FCU1-8 (MVA15638). The infection protocol was as follows: 5 106 cells in suspension in 50μl of PBS-FCS 2%-cations1% are incubated 30 minutes at 37°C in the presence of the adenovirus at an MOI 0.05. The totality of the 5 106 cells is then incubated in a 60 mm flasks in the presence of 5 ml of DMEM-10%FCS. After 24 hours at 37°C, the cells were washed in PBS then resuspended in 25μl of lysis buffer (TRIS-HCl pH 7.5 50 mM/NaCl 150 mM/EDTA 5 mM/DTT 1 mM/triton 1%). After 30 minutes of lysis at 4°C followed by a centrifugation, the CDase and UPRTase activities was measured in the supernatant containing the cellular lysate (according to the previously described protocol).

[0062] The results of the enzymatic activities, which are calculated from three independent measurements, are given in the following table:

	CDase pmoles 5-FC of transformed/min/mg of protein	UPRTase pmoles of 5-FU transformed/min/mg of protein
not infected	not detectable	not detectable
MVA-empty (MVAN33)	not detectable	not detectable

(continued)

	CDase pmoles 5-FC of transformed/min/mg of protein	UPRTase pmoles of 5-FU transformed/min/mg of protein
MVA-FCU1 (MVATG15637)	177860 +/- 6650	43860 +/- 4230
MVAV-FCU1-8 (MVATG15638)	232220 +/- 15580	not detectable

[0063] For the cells infected by MVA expressing FCU1-8, the results indicate a loss of UPRTase activity whereas CDase activity is preserved.

Example 9: Experiment in vivo

[0064] 5.106 human cells LoVo (adenocarcinoma of colon) are injected by intracutaneous way in 8 groups of 15 CD1 nude mouse at J0. As soon as the tumours have a volume of 30-50 mm3 (J+10), MVA are injected by intratumoral administration with an amount of 5.10^6 PFU. From J+10, 0.5 ml water or 0.5 ml of a 0.5%5-FC solution are given per os, twice by day and during 14 days. Volumes of the tumours (Figure 1) highlight a better control of the size of the tumours with group MVA-fCU1-8/5-FC compared to group MVA-FCU1/5-FC.

SEQUENCE LISTING

[0065]

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<130> D21447

<150> US 60/508 274
<151> 2003-10-06

<150> EP 03/360 087
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         35                  40                  45
Gly His Asn Met Arg Phe Gln Lys Gly Ser Ala Thr Leu His Gly Glu
     50                  55                  60
Ile Ser Thr Leu Glu Asn Cys Gly Arg Leu Glu Gly Lys Val Tyr Lys
 65                  70                  75                  80
Asp Thr Thr Leu Tyr Thr Thr Leu Ser Pro Cys Asp Met Cys Thr Gly
                 85                  90                  95
Ala Ile Ile Met Tyr Gly Ile Pro Arg Cys Val Val Gly Glu Asn Val
            100                 105                 110
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        115                 120                 125
Val Val Val Val Asp Asp Glu Arg Cys Lys Lys Ile Met Lys Gln Phe
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Glu Pro Phe Lys Asn Val Tyr Leu Leu Pro Gln Thr Asn Gln Leu Leu
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Gly Leu Tyr Thr Ile Ile Ser Asn Lys Asn Thr Thr Arg Pro Asp Phe
            180                 185                 190
Ile Phe Tyr Ser Asp Arg Ile Ile Arg Leu Leu Val Glu Glu Gly Leu
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    210                 215                 220
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225                 230                 235                 240
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                245                 250                 255
Ser Val Arg Ile Gly Lys Ile Leu Ile Gln Arg Asp Glu Glu Thr Ala
            260                 265                 270
Leu Pro Lys Leu Phe Tyr Glu Lys Leu Pro Glu Asp Ile Ser Glu Arg
        275                 280                 285
Tyr Val Phe Leu Leu Asp Pro Met Leu Ala Thr Gly Gly Ser Ala Ile
    290                 295                 300
Met Ala Thr Glu Val Leu Ile Lys Arg Gly Val Lys Pro Glu Arg Ile
305                 310                 315                 320
Tyr Phe Leu Asn Leu Ile Cys Ser Lys Glu Gly Ile Glu Lys Tyr His
                325                 330                 335
Ala Ala Phe Pro Glu Val Arg Ile Val Thr Gly Ala Leu Asp Arg Gly
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Arg Tyr Tyr Cys Val
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            20                  25                  30
Pro Asp Phe Ile Phe Tyr Ser Asp Arg Ile Ile Arg Leu Leu Val Glu
        35                  40                  45
Glu Gly Leu Asn His Leu Pro Val Gln Lys Gln Ile Val Glu Thr Asp
    50                  55                  60
Thr Asn Glu Asn Phe Glu Gly Val Ser Phe Met Gly Lys Ile Cys Gly
65                  70                  75                  80
Val Ser Ile Val Arg Ala Gly Glu Ser Met Glu Gln Gly Leu Arg Asp
                85                  90                  95
Cys Cys Arg Ser Val Arg Ile Gly Lys Ile Leu Ile Gln Arg Asp Glu
            100                 105                 110
Glu Thr Ala Leu Pro Lys Leu Phe Tyr Glu Lys Leu Pro Glu Asp Ile
        115                 120                 125
Ser Glu Arg Tyr Val Phe Leu Leu Asp Pro Met Leu Ala Thr Gly Gly
    130                 135                 140
Ser Ala Ile Met Ala Thr Glu Val Leu Ile Lys Arg Gly Val Lys Pro
145                 150                 155                 160
Glu Arg Ile Tyr Phe Leu Asn Leu Ile Cys Ser Lys Glu Gly Ile Glu
                165                 170                 175

Lys Tyr His Ala Ala Phe Pro Glu Val Arg Ile Val Thr Gly Ala Leu
            180                 185                 190
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        195                 200                 205
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    210                 215
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Claims

1. Use as a negative selection marker of a nucleotide sequence encoding a polypeptide possessing a Cytosine deaminase (CDase) activity, wherein said polypeptide possessing a CDase activity is a yeast CDase to which is added an amino acid sequence of 100 to 400 amino acids having a degree of identity greater than 70 % with a polypeptide possessing an UPRTase activity, with the proviso that said polypeptide possessing a CDase activity has neither uracil phosphoribosyl transferase (UPRtase) nor thymidine kinase activity.

2. The use according to claim 1, wherein the amino acid sequence, added to the yeast CDase, is between 200 and 300 amino acids long.

3. The use according to claim 1 or claim 2, wherein the amino acid sequence, added to the yeast CDase, is linked to the C terminal end of the yeast CDase.

4. The use according to any of claims 1 to 3, **characterized in that** said polypeptide possessing an UPRTase activity has a degree of identity greater than 80% with a yeast UPRTase, in particular that encoded by the Saccharomyces cerevisiae FUR1 gene.

5. The use according to claim 4, **characterized in that** the amino acid sequence, added to the yeast CDase, has a degree of identity greater than 80% with the sequence depicted in SEQ ID NO: 2 sequence identifier, starting at the Ser residue in position 2 and finishing at the Val residue in position 216.

6. The use according to claim 5, **characterized in that** the amino acid sequence, added to the yeast CDase, is as depicted in SEQ ID NO: 2 sequence identifier, starting at the Ser residue in position 2 and finishing at the Val residue in position 216.

7. The use according to any of claims 1 to 6, **characterized in that** said yeast CDase is encoded by the Saccharomyces cerevisiae FCY1 gene.

8. The use according to anyone of claims 1 to 7, **characterized in that** the yeast CDase comprises an amino acid sequence having a degree of identity greater than 80% with the sequence depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Glu residue in position 158.

9. The use according to claim 8, **characterized in that** the yeast CDase comprises an amino acid sequence as depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Glu residue in position 158.

10. The use according to anyone of claims 1 to 7, **characterized in that** the yeast CDase comprises an amino acid sequence having a degree of identity greater than 80% with the sequence depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Val residue in position 373.

11. The use according to claim 10, **characterized in that** the yeast CDase comprises an amino acid sequence as depicted in SEQ ID NO: 1 sequence identifier, starting at the Met residue in position 1 and finishing at the Val residue in position 373.

12. Use as a negative selection marker of a recombinant vector which carries a nucleotide sequence as defined in any of claims 1 to 11, placed under the control of the elements which are required for expressing it in a host cell.

13. The use according to any of claims 1 to 12, wherein said selection marker is used as a negative selection marker in mammalian cells, provided that the use does not involve the use of human embryos for industrial or commercial purposes.

14. The use according to any of claims 1 to 12, wherein said selection marker is used as a negative selection marker in plant cells.

15. The use according to any of claims 1 to 12, wherein said selection marker is used as a negative selection marker in bacteria or yeast lacking a native CDase gene.

Patentansprüche

1. Verwendung als einen negative Selektionsmarker einer Nucleotidsequenz, die ein Polypeptid codiert, das Zytosindeaminase (CDase)-Aktivität besitzt, wobei das eine CDase-Aktivität besitzende Polypeptid eine Hefe-CDase ist, der unter der Bedingung, dass das eine CDase-Aktivität besitzende Polypeptid weder eine Uracilphosphoribosyl-Transferase (UPRtase) noch eine Thymidinkinase-Aktivität besitzt, eine Aminosäuresequenz von 100 bis 400 Aminosäuren mit einem Grad der Ähnlichkeit von über 70 % mit einem Polypeptid hinzugefügt wird, das eine UPRTase-Aktivität besitzt.

2. Verwendung nach Anspruch 1, wobei die der Hefe-CDase hinzugefügte Aminosäuresequenz eine Länge von 200 bis 300 Aminosäuren aufweist.

3. Verwendung nach Anspruch 1 oder 2, wobei die der Hefe-CDase hinzugefügte Aminosäuresequenz an das C-terminale Ende der Hefe-CDase gekoppelt ist.

4. Verwendung nach einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** das eine URPTase-Aktivität besitzende Polypeptid, insbesondere jenes, das durch das FUR1-Gen des Saccharomyces cerevisiae codiert ist, einen Grad der Ähnlichkeit von über 80 % mit einer Hefe-URPTase aufweist.

5. Verwendung nach Anspruch 4, **dadurch gekennzeichnet, dass** die der Hefe-CDase hinzugefügte Aminosäuresequenz einen Grad der Ähnlichkeit von über 80 % mit der in der Sequenzbeschreibung SEQ ID NR.: 2 angegebenen

Sequenz aufweist, die am Ser-Rest in Position 2 beginnt und am Val-Rest in Position 216 endet.

6. Verwendung nach Anspruch 5, **dadurch gekennzeichnet, dass** die der Hefe-CDase hinzugefügte Aminosäuresequenz wie in der Sequenzbeschreibung SEQ ID NR.: 2 angegeben ist und am Ser-Rest in Position 2 beginnt und am Val-Rest in Position 216 endet.

7. Verwendung nach einem der Ansprüche 1 bis 6, **dadurch gekennzeichnet, dass** die Hefe-CDase durch das FCY1-Gen des Saccharomyces cerevisiae codiert ist.

8. Verwendung nach einem der Ansprüche 1 bis 7, **dadurch gekennzeichnet, dass** die Hefe-CDase eine Aminosäuresequenz mit einem Grad der Ähnlichkeit von über 80 % mit der in der Sequenzbeschreibung SEQ ID NR.: 1 angegebenen Sequenz umfasst, die am Met-Rest in Position 1 beginnt und am Glu-Rest in Position 158 endet.

9. Verwendung nach Anspruch 8, **dadurch gekennzeichnet, dass** die Hefe-CDase eine Aminosäuresequenz wie in der Sequenzbeschreibung SEQ ID NR.: 1 angegeben umfasst, die am Met-Rest in Position 1 beginnt und am Glu-Rest in Position 158 endet.

10. Verwendung nach einem der Ansprüche 1 bis 7, **dadurch gekennzeichnet, dass** die Hefe-CDase eine Aminosäuresequenz mit einem Grad der Ähnlichkeit von über 80 % mit der in der Sequenzbeschreibung SEQ ID NR.: 1 angegebenen Sequenz umfasst, die am Met-Rest in Position 1 beginnt und am Val-Rest in Position 373 endet.

11. Verwendung nach Anspruch 10, **dadurch gekennzeichnet, dass** die Hefe-CDase eine Aminosäuresequenz wie in der Sequenzbeschreibung SEQ ID NR.: 1 angegeben umfasst, die am Met-Rest in Position 1 beginnt und am Val-Rest in Position 373 endet.

12. Verwendung eines negativen Selektionsmarker eines rekombinanten Vektors, der eine Nucleotidsequenz nach einem der Ansprüche 1 bis 11 trägt, gestellt unter die Kontrolle der Elemente, die erforderlich sind, um ihn in einer Wirtszelle zu exprimieren.

13. Verwendung nach einem der Ansprüche 1 bis 12, wobei der Selektionsmarker als ein negativer Selektionsmarker in Säugerzellen verwendet wird, vorausgesetzt, dass die Verwendung die Verwendung keine Verwendung von humanen Embryos für industrielle oder gewerbliche Zwecke einbezieht.

14. Verwendung nach einem der Ansprüche 1 bis 12, wobei der Selektionsmarker als ein negativer Selektionsmarker in Pflanzenzellen verwendet wird.

15. Verwendung nach einem der Ansprüche 1 bis 12, wobei der Selektionsmarker als ein negativer Selektionsmarker in Bakterien oder Hefe ohne natives CDase-Gen verwendet wird.

Revendications

1. Utilisation en tant que marqueur de sélection négatif d'une séquence nucléotidique codant pour un polypeptide possédant une activité cytosine désaminase (CDase), **caractérisée en ce que** ledit polypeptide possédant une activité CDase est une CDase de levure à laquelle est ajoutée une séquence d'acides aminés de 100 à 400 acides aminés ayant un degré d'identité supérieur à 70 % avec un polypeptide possédant une activité UPRTase, à condition que ledit polypeptide possédant une activité CDase n'ait ni une activité uracile phosphoribosyle transférase (UPRtase), ni une activité thymidine kinase.

2. Utilisation selon la revendication 1, **caractérisée en ce que** la séquence d'acides aminés, ajoutée à la CDase de levure, a une longueur d'entre 200 et 300 acides aminés.

3. Utilisation selon la revendication 1 ou la revendication 2, **caractérisée en ce que** la séquence d'acides aminés, ajoutée à la CDase de levure, est liée à l'extrémité C-terminale de la CDase de levure.

4. Utilisation selon l'une quelconque des revendications 1 à 3, **caractérisée en ce que** ledit polypeptide possédant une activité UPRTase a un degré d'identité supérieur à 80 % avec une UPRTase de levure, en particulier celle codée par le gène FUR1 de Saccharomyces cerevisiae.

5. Utilisation selon la revendication 4, **caractérisée en ce que** la séquence d'acides aminés, ajoutée à la CDase de levure, a un degré d'identité supérieur à 80 % avec la séquence décrite dans l'identifiant de séquence SEQ ID NO: 2, en commençant au résidu Ser à la position 2 et en terminant au résidu Val à la position 216.

6. Utilisation selon la revendication 5, **caractérisé en ce que** la séquence d'acides aminés, ajoutée à la CDase de levure, est comme décrit dans l'identifiant de séquence SEQ ID NO: 2, en commençant au résidu Ser à la position 2 et en terminant au résidu Val à la position 216.

7. Utilisation selon l'une quelconque des revendications 1 à 6, **caractérisée en ce que** ladite CDase de levure est codée par le gène FCY1 de Saccharomyces cerevisiae.

8. Utilisation selon l'une quelconque des revendications 1 à 7, **caractérisée en ce que** la CDase de levure comprend une séquence d'acides aminés ayant un degré d'identité supérieur à 80 % avec la séquence décrite dans l'identifiant de séquence SEQ ID NO: 1, en commençant au résidu Met à la position 1 et en terminant au résidu Glu à la position 158.

9. Utilisation selon la revendication 8, **caractérisée en ce que** la CDase de levure comprend une séquence d'acides aminés telle que décrite dans l'identifiant de séquence SEQ ID NO: 1, en commençant au résidu Met à la position 1 et en terminant au résidu Glu à la position 158.

10. Utilisation selon l'une quelconque des revendications 1 à 7, **caractérisée en ce que** la CDase de levure comprend une séquence d'acides aminés ayant un degré d'identité supérieur à 80 % avec la séquence décrite dans l'identifiant de séquence SEQ ID NO: 1, en commençant au résidu Met à la position 1 et en terminant au résidu Val à la position 373.

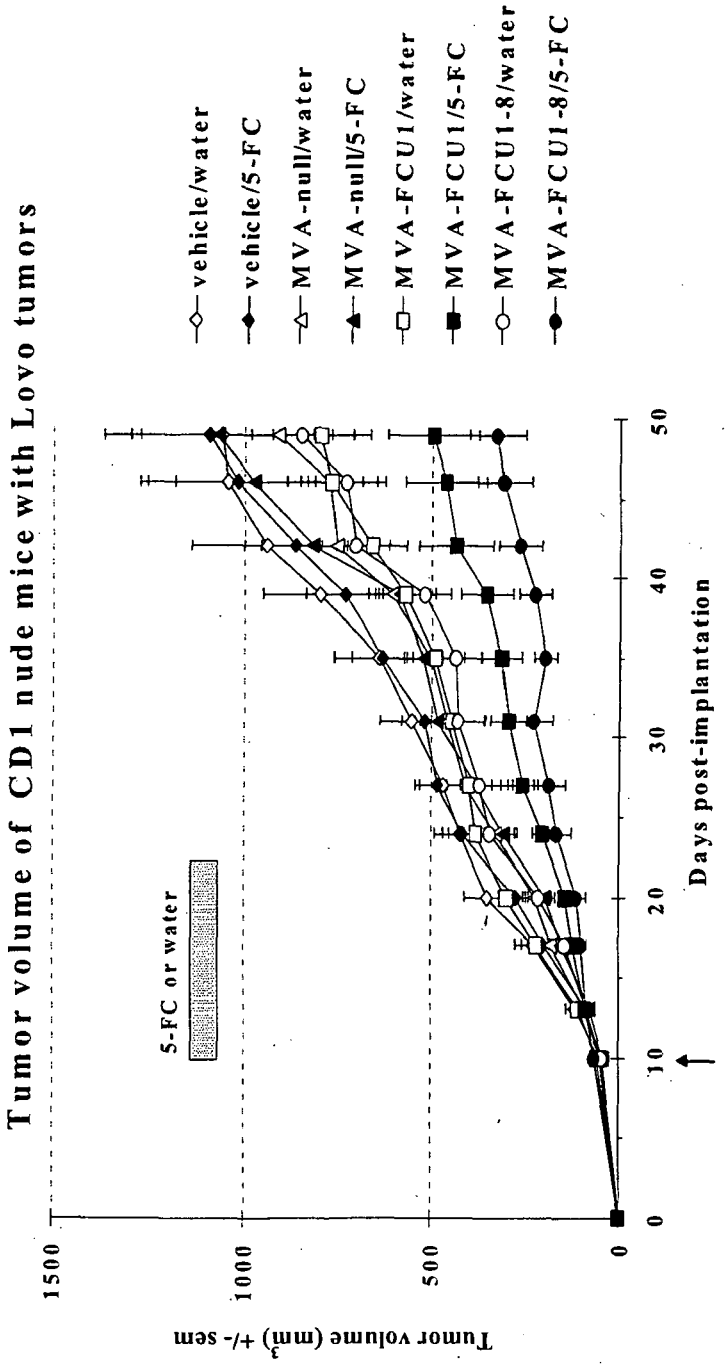
11. Utilisation selon la revendication 10, **caractérisée en ce que** la CDase de levure comprend une séquence d'acides aminés telle que décrite dans l'identifiant de séquence SEQ ID NO: 1, en commençant au résidu Met à la position 1 et en terminant au résidu Val à la position 373.

12. Utilisation en tant que marqueur de sélection négatif d'un vecteur recombinant qui comporte une séquence nucléotidique telle que définie dans l'une quelconque des revendications 1 à 11, placée sous le contrôle des éléments qui sont requis pour exprimer celle-ci dans une cellule hôte.

13. Utilisation selon l'une quelconque des revendications 1 à 12, **caractérisée en ce que** ledit marqueur de sélection est utilisé en tant que marqueur de sélection négatif dans des cellules de mammifères, à condition que l'utilisation n'implique l'utilisation d'embryons humains pour des applications industrielles ou commerciales.

14. Utilisation selon l'une quelconque des revendications 1 à 12, **caractérisée en ce que** ledit marqueur de sélection est utilisé en tant que marqueur de sélection négatif dans des cellules de plante.

15. Utilisation selon l'une quelconque des revendications 1 à 12, **caractérisée en ce que** ledit marqueur de sélection est utilisé en tant que marqueur de sélection négatif dans des bactéries ou une levure ne comportant pas de gène de CDase natif.


Tumor volume of CD1 nude mice with Lovo tumors
vehicle/water
vehicle/5-FC
MVA-null/water
MVA-null/5-FC
MVA-FCU1/water
MVA-FCU1/5-FC
MVA-FCU1-8/water
MVA-FCU1-8/5-FC
5-FC or water
Tumor volume (mm³) +/- sem
1500
1000
500
0
0
10
20
30
40
50
Days post-implantation

REFERENCES CITED IN THE DESCRIPTION

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